Commission of the European Communities

ECLAIR Programme

Contract n° AGRE 0052

Coordinator: IRTAC, 16 Rue Nicolas-Fortin 75013 Paris, France

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To Explore and Improve the Industrial Use of EC Wheats

Final Technical Report

from 01.01.1991 to 31.03.1995

Table of contents

	Page
Introduction,	3
Subprogramme A: Industrial Processes	12
Subprogramme B: Functional Components and their Interactions	46
Subprogramme C: Biochemical-Genetics and Physiology	84
Conclusions:	121
Annex: Publications by participants in the ECLAIR Programme	123

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INTRODUCTION

1. Origin and Objectives of the Project

The main objectives of the project were to explore and improve the industrial use of EC wheats.

The rationale behind these objectives was manifold. For instance:

- Despite the fact that wheat is an essential crop for European agriculture and for the wheat-processing industry (milling, breadmaking, biscuit-making and starch/gluten industries), EC wheats are not really adapted to this wide range of applications, especially to their future developments, because the various processes have not been clearly explained in terms of process requirements and wheat quality requirements.

- Whereas Europe is deficient in good quality strong wheat, the milling and baking industries require higher quality wheat because of modern developments in technology. In particular, the use of 'cold' methods in baking (refrigeration and deep-freezing of dough) makes it necessary to have available flours of higher protein content and greater and greater strength.

- On the other hand, the fact that current methods of breeding are predominantly focused on white-breadmaking and pasta production stands more and more in contrast to the current applications of wheat in wholemeal, biscuit manufacture, wheat/starch production, sweet leavened products and fermented products, and considering that quality is also related to flour extraction rate (the amount of white flour extractable from wheat), performance in flour blends and degree of sprout damage.

- The consistency of the quality of the greater part of existing wheat is insufficient because of too great a sensitivity to agronomic and climatic factors. In Southern Europe, the climate is often the factor limiting both yield and quality; in the coastal regions of Northern Europe, where the crop can be cultivated intensively, sprouting puts a severe strain on both yield and quality.

Based on these observations, the need to intensify research work aimed at exploring new outlets and developing new applications for wheat and at improving the quality of wheat was strongly emphasized during the conference organised by the Commission of the European Communities at San Angelo Lodigiano, Italy, in June 1987.

This gave rise to two different proposals aimed at improving quality of EC wheats that were presented at the 1988 call of the Commission of the European Communities in the frame of the Agro-Industrial Programmes:

- Proposal 0007, coordinated by IRTAC, France,

- Proposal 0103, coordinated by TNO, The Netherlands,

These proposals were evaluated in March 1989, but were placed in the second wave of

proposals under consideration. After the Commission suggested that the two wheat proposals be integrated into a single one, a joint project was resubmitted in October 1989 and was accepted in April 1990 as the *AGRE 0052 Programme*, that was carried out from January 1st, 1991 to March 31st, 1995.

The *specific objectives* of this joint project were formulated as follows:

- To stimulate breeding in order to tailor novel wheat varieties that combine good agronomic character and excellent technological qualities, which would satisfy simultaneously the farming and manufacturing industries and the export markets;

- To maximise EC grown wheat quality by providing tools to minimise sprout damage and maximise milling quality;

- To further improve the economy of EC wheats by relating current processing requirements to wheat characteristics, thereby enabling traders, millers and breeders to select on these characters;

- To open new outlets for wheat by investigating and developing new applications of wheat and wheat products (flour, starch, gluten).

2. Project Methodology (Figure 1)

Such a project represented a completely new strategy, not only innovative in this respect, but also in the advanced methodologies used to tackle the often complex problems. The expected economic benefit from this was evident. Of the about 75 million tons of wheat grown annually in the EC, 15-16 million tons were surplus. Decreasing this surplus with minimising the need to apply costly intervention regulations was likely to save the EC millions ECU. In the following, the objectives of the programme will be explained in more detail, using different research topics of the project.

Processing Requirements and their Application to Wheat Selection and Quality Definition

As stated earlier, most of the research in this area was focused on relations between protein content and composition and *white* breadmaking quality. Modern wheat breeding for example has exploited the relation between certain HMW glutenin subunits and breadmaking quality. However, data both from applied studies as from fundamental studies indicated that knowledge of the interaction between flour components was lacking. Therefore, the proposed project was focused on this approach using *advanced biochemical and physico-chemical methodology* (combination between Subprogrammes A and B). Furthermore, the proposed project had to try to fill the gap between wheat quality requirements and present wheat applications ('cold' methods in baking, wholemeal breadmaking, starch/gluten separation, biscuit-making, sweet leavened products, and fermented products) by studying the suitability of wheats for these applications on an applied level. This was likely to lead to a better understanding of different quality characters required, to rapid selection tools for use in breeding and trade and thereby to a better exploitation of EC

grown wheats. Furthermore, these studies would enable increased quality assurance, improved products and the development of new products and/or new processes. The economic benefits from this (eliminating the need for wheat imports, better use of EC wheats, improved quality through selection) were evident but not easy to quantify.

Milling Quality

Milling quality is an aspect of wheat which has been necessarily left out of selection programmes until the last stages. Nevertheless, taking the amount of wheat produced annually in the EC, *one percent increase in milling yield represents an advantage of 40 million ECU per year*. The approach followed to tackle this problem was innovative through the use of image analysis techniques in combination with sensitive chemical assays. Strong cost reduction of image analysis equipment would enable the development of rapid test based on this equipment offering both a technical advantage as a economical advantage (decrease in labour costs, ability to select wheats on milling quality on intake or in early stages of breeding programmes).

Starch/Gluten Separation

The application of wheat as a raw material for starch/gluten separation is relatively new. Using pilot scale equipment recently developed at the participating laboratories was likely to make possible the *improvement of process economy* by using enzyme methodology and by enabling the use of wholemeal flours in the new separation processes. The use of *enzymes* was planned in order to tackle the problem of variation in processing properties, allowing economical benefits in using locally grown cheaper wheats. The use of wholemeal would enable a higher yield of starch/ton of wheat. The use of new processing would also enable reduced losses in terms of wastage and costs of waste water treatment.

Sprout Damage

Prevention of sprout damage is an objective long yearned for in the EC. The average costs of sprout damage once in every five years (leading to 10 % loss in yield and reduction of the amount of breadmaking quality by 50 %) was evaluated to 50-60 million ECU per year. The approach envisaged in this project was entirely new in both concept as methodology. Instead of detecting levels of amylase work was focused on developing for example immunoassay-based fluorescence tests for factors related to dormancy. This was likely to enable rapid detection at an early stage (technical advantage), prevention (economic advantage) and selection of sprouting resistance in breeding programmes.

Several recent advances provided the potential to make a significant step forward in both more effective utilisation and in the development of better European wheat varieties for the future.

1. The availability of isogenic, aneuploid and translocation stocks which enable to pin-point the gene products that are important in functional performance.

2. The introduction of original approaches based on new concepts (*e.g.* intrinsic quality of wheat genotypes), or new protein fractions (*e.g.* friabilin, HMW-albumin, S-

protein,...), that stand out clearly against the old classical Osborne's scheme.

3. The acknowledgement that quality is not determined (and cannot be predicted) solely by protein composition, but also by interaction of the proteins with various flour components: starch, pentosans, lipids.

4. The development of modern physical and spectroscopic methods that can observe the behaviour of individual components (*e.g.* proteins, lipids) in a complex mixture (in situ NMR spectroscopy, rheological measurements).

5. The demonstration of the potential of monoclonal antibodies to quantify specific components in a mixture and to probe their dynamics and distribution within various systems (dough development, seed dormancy).

6. The development of a range of physico-chemical techniques that determine interfacial and aggregation behaviour.

Apart from these purely scientific and technical aspects, *a particularly innovative element of this project was the establishment of a multidisciplinary programme (bringing together physical chemists, biochemists, immuno-chemists, rheologists and geneticists) and involving different industries (millers, bakers, biscuit manufacturers, starch/gluten manufacturers and breeders)*. The large number of participants of this programme (see **Work Chart, Table I,** and **Organisational Structure, Figure 2**) was without doubt the price one had to pay in order to make progress on such a complex problem as satisfying, year after year, the industrial need for quality in wheat.

3. Management of the Research Programme

3.1 Organisation

The research programme was organised as three interdependent Subprogrammes, each of which being under the direction of a Subprogramme Manager :

A. Industrial Processes : (*Dr. R.J. Hamer*, TNO Biochemistry and Chemistry Institute, Zeist, The Netherlands).

B. Functional Components and their Interactions : (*Dr. J.J. Plijter*, Gist Brocades, Delft, The Netherlands).

C. Biochemical-Genetics and Physiology : (*Dr. N.E. Pogna*, Istituto Sperimentale per la Cerealicoltura, Rome, Italy)

Dr. J.C. Autran (INRA-IRTAC, Montpellier, France) was the Scientific Coordinator. *Ms M. Richard* (IRTAC, Paris, France) was in charge of the administrative aspects.

In addition, because it was essential that any scientific problem identified in one Subprogramme could benefit from the expertise of the other two, a *Scientific Management Committee* comprising Mrs M. Monique Richard (IRTAC, Paris), Drs. J.C. Autran (INRA-IRTAC, Montpellier), R.J. Hamer (TNO, Zeist), J.J. Plijter (Gist Brocades, Delft), N.E. Pogna (Istituto Sperimentale per la Cerealicoltura, Milano), was

set up to guarantee the cohesion of the whole programme.

3.2 <u>Reports and newsletters</u>

3.3.1 - Newsletters

Every year, half-way between annual reports, a newsletter was compiled by the Subprogramme Managers from individual contributions of the participants (one page per participant), then by the Scientific Coordinator. The newsletters contained: progress of the work to date; main results obtained; publications; meetings attended; any delays or deviations that occurred which required modifying the working chart.

3.3.2 - Progress Reports

Every year, an annual progress report was compiled by the Subprogramme Managers from individual contributions of the participants (two pages per participant, including 1-2 tables or figures), then by the Scientific Coordinator. The annual progress reports contained: a full description of the work carried out during the reporting period; the results obtained in relation to each task and subtask described in the charts; a critical evaluation of the progress (in relation to the objectives and deliverables); a short discussion of remaining problems and suggested modifications if required.

Progress reports and newsletters were prepared in a sufficient number of copies to be personally distributed to all people who actively participated in the work and to the Commission

3.3.3 - Meetings

The organisation of meetings was the responsibility of the Subprogramme Managers and of the Scientific Management Committee. The minutes of meetings were published in the newsletters.

a) All the three Subprogramme Managers organised two *Meetings of Subprogramme* per year in alternating the meeting places, which enabled to visits almost all laboratories or pilot plants of participants. These meetings were primarily aimed at promoting the widest possible interaction between the participants, stimulating cooperation, exchanging information, preparing reports and comparing methodologies.

b) *Technical meetings* or *cross groups meetings* were organised on topics whose progress was essential and for which collaboration between Subprogrammes had to be intensified. For instance:

- LMW subunits of glutenin (leader: Domenico Lafiandra)
- HMW subunits of glutenin (leader: Arthur S. Tatham)
- Pentosanes/pentosanases (leader: E. N. Clare Mills)
- Rheology (leader: Johan J. Plijter)
- Human Capital and Mobility (leader: Robert J. Hamer)

Some of these meetings were in fact workshops open to an international discussion involving specialists outside from the EC. For instance, a meeting on glutenin subunits was held the day after the "5th Workshop on Gluten Proteins", 10 June 1993, *Detmold*,

Germany. Another meeting on “Molecular Interactions in Dough and Gluten” was organised by Peter Shewry and Peter Belton the day before the Meeting on "Wheat Kernel Proteins - Molecular and Functional Aspects" (27 September 1994, *Viterbo*, Italy).

c) *Plenary Meetings of the Programme*. Two plenary meetings were organised. The first one was held in the course of the second year, the day after the 9th International Cereal and Bread Congress, 4 June 1992 in *Paris*. The second one was near the end of the programme (10 June 1994, after the ICC Meeting in *The Hague*, The Netherlands) to help in clearing the major results obtained and drawing the main conclusions in view to the final report.

3.3.4 - Book of Methods

An analytical methods book, covering all quality related methods used in the course of the programme was set up by participants to Subprogramme A. It was made available on both a bound version and a word processor form by Dr. van Lonkhuysen and distributed to all partners in November 1992.

3.3.5 - Profile Sheets of Participants

This book was prepared by the Scientific Coordinator to make clear which are the aims in the different partners of the programme and to make the collaborations easier. Based on the updated content of the technical annex, this set of record cards contained the address, phone, fax, languages spoken and picture of each participant, with a short description of the field of expertise (key words), so that everybody could easily know and contact the relevant person for any problem and could detect where the other Subprogrammes are the most supportive.

4. Presentation of the Final Technical Report

In complement to the four-page *Summary Report* and to the sixteen-page *Synthesis Report*, this *Final Technical Report* was compiled from the summary of activities prepared, for each task, by each participant in the programme :

It comprises the reports of the three Subprogrammes A, B and C:

A - Industrial Processes

B - Functional Components and their Interactions

C - Biochemical-Genetics and Physiology

This report is a true reflection of all our group's efforts to "Explore and Improve the Industrial Quality of EC Wheats", and especially of the following people who have actively participated in our four-year research programme:

Partner 01	IRTAC (Coordinator), Paris	Jean-Claude Autran Monique Richard
Partner 02	Produttori Sementi, Bologna	Enzo DeAmbrogio Luca Bersanetti Parivash Jenabzadeh Marilena Paolini Stefano Poluzzi Stefano Ravaglia
Partner 03	ISC, S. Angelo Lodigiano	Basilio Borghi Anna Biancardi Norberto Pogna Rita Redaelli
Partner 04	SME Ricerche, Caserta	Giancarlo Malgarini Aristide Angelillo Rita Calabria Egidio Fournier Robert Finsterer Massimo Saracino
Partner 05	Champagne Céréales, Reims	François Plénier Savine Brisson
Partner 06	Roquette Frères, Lestrem	Guy Flèche Jean-Jacques Caboche Monique Dumont
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Partner 08	Danione Branche Biscuit, Paris	Aliette Verel Anne-Catherine Villain Anne-Sophie Contamine Laëtitia Kugener C. Lamiche Agnès Meunier
Partner 09	ITCF, Paris	Michel Leuillet Christine Bar Marie-Hélène Bernicot
Partner 12	IATA, Valencia	Carmen Benedito Concepción Collar Encarnacion Ibañez Maria-Antonia Martínez-Anaya Claudia Martínez Caicedo Ofelia Rouzaud Elvira Seytre
Partner 13	Technical University, Berlin	Friedrich Meuser Norbert Pahne Claudia Rennau
Partner 14	CCFRA, Chorleywood	Tony Evers Philip Greenwell Peter Pritchard Brigitta Abel Dhan Bhandari Ged Oliver Sarabjit Sahi

		Douglas Smith
Partner 15	Gist-Brocades, Delft	Johan Plijter Mariette Uijen
Partner 16	AFRC-IFR, Norwich	Peter Belton Mike Morgan Ian Colquhoun Alex Grant Sara Holden Clare Mills Mary Parker Neil Rigby
Partner 17	TNO, Zeist	Robert J. Hamer Marcel Kelflkens W.J. Lichtendonk Roelof Orsel A.M. van de Pijpekamp H.P.M. van Laarhoven J.W. van Oosten Peter L. Weegels
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Partner 22	University of Padova	Angelo D.B. Peruffo Andrea Curioni Luco Furegon
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Partner 25	ENMP, Elvas	Francisco Bagulho Carla Moita Brites José Cutinho Benvindo Maçãs

SUBPROGRAMME A:

INDUSTRIAL PROCESSES

Summary

Subprogramme A was aimed at *improving the industrial use of EC wheats*. This aim was approached along two broad lines of research.

1. Tools were developed in order *to maximise EC grown wheat milling quality*. Using image analysis and sensitive biochemical assays tests were developed to predict milling quality.

2. A concerted effort was made by laboratories from six EC member countries *to fill the gap between current wheat selection in breeding programmes and trade on one hand and current applications of wheat on the other*. Applications of wheat in the wheat starch industry, in wholemeal breadmaking, flour blends, fermented products (sour dough) and biscuit manufacture were studied on an applied level (in connection with Subprogramme B which studied processes on a fundamental level). This included both the use of advanced biochemical and physico-chemical methodology as well as recently developed process technology. Studies were aimed at *understanding processing requirements and their underlying physico-chemical/biochemical causes*. This led to the identification of process customised *selection criteria*. This in turn enabled an improved use of EC wheats, improved guidelines and criteria for breeding and improved products and processing of wheat.

Better knowledge of the various applications of wheat (milling, white and wholemeal breadmaking, starch/gluten industry, flour blends, fermented products, and biscuit manufacture) was obtained, each main parameter of wheat quality being expressed in terms of functional properties and related to specific constituents and their interactions, resulting in improved wheat uses.

For instance, in *milling quality,* a comprehensive model was proposed, describing the relative influence of both chemical (potassium content) and morphological (bran friability and kernel width) parameters on milling quality.

In the *starch/gluten* project, a system was developed to separate gluten and starch directly from wholemeal flour. This has been hitherto proven to be impossible, due to the fact that gluten coagulation in the presence of bran particles leads to a gluten unsuitable for further use in the bakery. This problem was solved by a combination of low shear sieving and a unique miniaturised decanter centrifuge. The latter allows considerable reduction of residence time of gluten in the system, a factor of importance with respect to gluten properties. Also, a unique analytical instrument was developed. This instrument was developed in conjunction with a manufacturer of grain analytical instruments, sold world-wide. The tool resembles a miniaturised gluten-starch separation system enabling the evaluation of flour processing properties and yields of gluten and starch using as little as 5 grams of flour. With this instrument it was clearly demonstrated that pentosans and hemicellulose in flours have a strong effect on gluten yield. Another important finding was that flour processing properties

are strongly determined by the way flour milling fractions are blended.

The main findings in *baking studies* were the strong relation demonstrated between molecular structure (glutenin depolymerisation, gel protein elastic modulus) and dough behaviour or baking performance. In addition, flour blending studies focused on predicting dough properties from flour constituents confirmed the major role played by gel proteins (or GMP - gluten macropolymer). On the other hand, it was shown that the wholemeal loaf volumes could not be predicted from those in white flour breadmaking and that in wholemeal bread performance the protein content was more important than gluten strength.

In the work on identification of flour parameters determining the quality of *semi-sweet biscuits,* a main finding was the importance of the interaction between mixing conditions and flour protein quality related parameters (protein aggregation: gliadins/glutenins ratio; dough free water content; insoluble pentosans) which were also confirmed by experiments on an industrial scale.

In view to determine the relation between flour properties and the quality of *sweet bakery products* and rheological characterisation of flour samples, a test bake procedure was optimised in terms of reproducibility and reliability. Dynamic rheological studies with flour slurries have succeeded to discriminate flours in terms of Ge*, which relates to structural characters of the protein network. Both techniques, which were previously unavailable for this type of research, have helped to better describe flour requirements.

Interactions between selected microorganisms and wheat flour components were investigated and applied to improve breadmaking processes using sour dough from frozen and dehydrated bacterial starters. Data showed clearly the overall importance of flour parameters on suitability for sour dough breads, a specific criterion of flour quality being the level of proteolytic activity of the flour. A valuable basis was developed for an *expert system* on sour dough production and related flour selection. First results indicate a clear suitability of non Spanish flours for the Spanish type sour dough products and the use of darker flours to specifically modify sour dough sensorial properties and induce product innovation.

Individual Progress Reports

Task A.1.1 - Milling Quality

Partner 17 - TNO Food and Nutrition
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Partner 14 - CCFRA (former FMBRA)
(Camden and Chorleywood Food Research Association)
Chorleywood, Rickmansworth, Hertfordshire, WD3 5SH, UK

1. Key measures of achievement - Objectives

Predicting milling quality of wheat samples from morphological characteristics of representative grains.

2. Progress

The milling tasks were carried out in a closely cooperative programme between the two above named organisations, using a series of common sample sets drawn from three countries within the European Union, as the basis for comparing milling performance in the two institutes and evaluating predictive tests for milling performance.

This report covers the entire programme of activities in order to demonstrate the relationship between individual tasks and the overall plan. Individual tasks which were already described in detail in earlier reports are merely summarised here, while more recent activities are described in detail.

Milling techniques

Before establishing means of predicting milling performances, which would have value in a European context it was necessary to establish whether milling methods adopted in different institutes, in different countries, produce comparable results. This question was addressed at an early stage in the programme by milling a series of wheats drawn from the Netherlands, France and the UK, at TNO and at CCFRA. It emerged that TNO and CCFRA, using Buhler laboratory mills in the manners described in the methods book, for the respective institutes, produced significantly different results. Not only were extraction rates different for aliquots of the same sample but ranking of samples also differed between the institutes. TNO produced

their best extraction rates on Dutch-grown samples and CCFRA did likewise with UK-grown types. Performance of both institutes on the French-grown wheats was variable. The differences among sample sets originating in the different States were subtle but they were able to be detected by image analysis, using simple examination methods.

Prediction methods

While some predictive testing with universal applicability in mind has continued, the additional difficulties that this imposes were, to some extent, avoided by additional separate developments in the individual institutes. At CCFRA the developments have involved use of image analysis, to quantitatively describe grains and grain samples, while at TNO they were dependent on chemical analyses by both traditional and new rapid methods for measuring mineral contents in whole grains and their components.

At CCFRA detailed image analysis measurements on individually prepared grains were made, to provide an estimate of endosperm content - the factor that ultimately limits extraction rate and which notionally increases with grain size and plumpness. In a set of 20 samples varying in extraction rates between 74 % and 81 %, no image analysis parameters proved consistently capable of predicting extraction rate. These results prompted investigations by dissection into the validity of the notional relationship between grain size and endosperm content. They showed endosperm content to be independent of grain size and even effects of all but extreme shrivelling to be minimal. It was subsequently demonstrated that size and shrivelling can affect extraction rate, but this is now considered to result from different responses to milling by grains of different sizes, rather than inherent differences in endosperm content.

One factor influencing milling response is bran friability, and at TNO attempts were made to quantify the importance of this factor. The amount of bran present as specks in a white flour is measured in continental Europe by gravimetric determination of ash residues after burning. Ash values reflect mineral contents which are higher in bran components than in endosperm. The validity of the measurement as an indication of bran content of flour was however questioned by the demonstration at TNO of the variation in mineral contents of endosperm as a function of wheat cultivar and growing conditions. In consequence the millability of wheat samples can be predicted by measuring the mineral content of their endosperms. This can be effectively achieved, without the need of separation of endosperm by dissection, by determining ash residues on the whole grain, as it is in the endosperm where the greatest variation in minerals occurs. The objective of the Milling task of the programme was thus achieved and indeed it was shown that prediction can be improved if potassium contents are monitored rather than total mineral content.

In the UK, chalk is added to flours for nutritional purposes and this masks variations in their total mineral content. Should potassium rather than total ash be adopted as a criterion of flour purity in continental Europe this would facilitate conformation by the UK, assuming the potassium content of the added chalk to be negligible. Such a change may be welcomed as the method of assessing bran-speck content currently employed in the UK, grade colour measurement, was shown to be respond to

pigmentation of endosperm as well as fragments of coloured bran. However, to rely entirely on ash values or grade colour as indicators of bran content in white flours would disadvantage those European wheats with high mineral contents or darker pigmentation in their endosperms, and such a characteristic may be irrelevant to processing requirements. Thus a more fundamental analysis of the problem was favoured, whereby due consideration is given to the original purpose in mind when tests of flour purity were introduced. The intention was to distinguish material originating in the endosperm from that originating in the pericarp. It was desirable to do so for two reasons: 1. because the pericarp contains no components that contribute functionally to the making of good bread; and 2. because specks of the coloured pericarp darkens the crumb of bread, thus reducing comsumers' perception of its purity and hence its desirability. Because of the variations in endosperm characteristics described above, neither ash values nor grade colour measure pericarp content reliably on a range of wheat types. An alternative approach is to use image analysis to measure directly the relative areas that bran components contribute to total flour area at the face of a glass fronted cell into which dry flour is packed. At TNO experiments were made with a system depending on fluorescence to identify non endosperm components, notably ferulic acid. Ferulic acid was also determined spectroscopically in flours and, in collaboration with CCFRA, comparisons were made of results from this method with the conventional ash and grade colour values. In one comparison a series of machine flours, produced on a pilot mill, were analysed. Blends of the machine flours were made, starting with that comprising the purest starchy endosperm and adding flours with progressively increasing levels of non-starchy endosperm material. Curves representing the values for ash and ferulic acid respectively, recorded at each level of addition are shown in **Figure 3**. The same principle was adopted in producing curves from grade colour values and from results of image analysis performed with a visible light system developed at CCFRA for measuring bran particles distinguished from endosperm particles by their dark colour. These curves are shown in **Figure 4.** Greater sensitivity of ferulic acid measurements and image analysis measurements of bran specks is indicated by the more rapid rises in the curves based on these methods than in those associated with the more conventional methods. Further evaluation of these techniques is being undertaken with a view to adoption in commercial enterprises.

Summary

In summary, it can justifiably be claimed that the objective of this part of the programme was achieved on the basis of conventional milling standards. However the inadequacy of traditional conventions, when applied to modern European cultivars, has shown the need for a more creative attitude to quality evaluation, and this programme has permitted the early development of methods appropriate to this view.

3. Publications describing work carried out under Subprogramme A Task 1.1

Published papers

Evers A.D. (1992). Predicting milling quality of wheat samples from morphological characteristics of representative grains. Chorleywood Digest, 118, 70-73.

Evers A.D. (1993). On-line quantification of bran particles in white flour. Food

Science and Technology Today, 7, 23-26.

Evers A.D. (1993). Milling for breadmaking. In: Proceedings of an International Conference on Bread - Breeding to Baking", FMBRA, Chorleywood, 15-16 June, Chameleon Press Limited, London, pp. 20-30.

Evers A.D., Kelfkens M. and Whitworth M.G. (1994). Dusts and ashes. Chorleywood Digest, 134, 20-22.

Evers A.D. (1994). Does grain size matter. Chorleywood Digest, 138, 70-72.

Delivered papers

Evers A.D. (1993). Shape, size and flour powder. AACC 78th Annual Meeting, Miami Beach, October 3-7. Abstract published in Cereal Foods World, 38 (8), 618.

Evers A.D. (1992). Quantifying bran particles in white flour by image analysis. Cereal and Bread Congress ICC, Paris, June 1-5.

Task A.1.2.1 - Improved Separation of Gluten and starch through the Use of Enzymes

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3700 AJ, Zeist, The Netherlands

1. Key measures of achievement - Objectives

- To obtain a better understanding of the role of non starch polysaccharides (NSP) in starch/gluten separation.
- To obtain a better understanding of the mechanism of action of hemicellulases in starch/gluten separation.
- To evaluate the use of enzymes as processing aids.

2. Progress

The project on the improved separation of gluten and starch has enfaced substantial progress. Spectacular differences in processing properties were observed between the different milling streams. The differences were due to differences in protein content (break roll flours) and probably to differences in protein quality and in fibre/hemicellulose content, which is high in the last reduction roll fraction (**Figure 5**). The reduction roll fractions were more sensitive to overmixing, which causes a decrease in gluten yield.

Since non-starch polysaccharides are known to affect gluten aggregation, pentosans (soluble hemicellulose; 2.5 %) and hemicellulose (insoluble; 5 %) were added to the flour streams and gluten yields were determined. Depending on the milling stream either hemicellulose or pentosans or both decreased gluten yield (**Figure 6**). The results indicate that the gluten yield and therefore the processing properties of wheat can be seriously affected by pentosans and hemicellulose. Furthermore, improper blending of milling fractions can affect the processing properties of flour.

Task A.1.2.2 - Characterisation of Wheat Gluten Produced by New Separation Processes

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1. Key measures of achievement - Objectives

- To characterize the properties of the glutens obtained from different wheat varieties using a laboratory scale system and to establish why they developed.

- To create processing conditions which would enable the glutens extracted in the laboratory scale system and those extracted on an industrial scale to be compared.

- To derive correlations between the composition of the raw materials, the processing technique and the characteristic properties of the glutens.

2. Progress

Introduction

A newly developed laboratory-scale process to obtain starch, gluten and fibre from white and wholemeal wheat flour by extracting the soluble components before separating out the solids fraction from process water was examined for the efficiency of mass recovery and the composition and properties of the gluten obtained. The idea behind this process design is to increase the concentration of dissolved substances in the process water. This aims mainly at an economical recovery of these substances from the process water.

While yields of starches and glutens obtained using this process correspond to their percentages in the raw materials and the purity of the starches is also identical, the relative composition of the glutens and, above all, their characteristic properties, including stretching properties and colour, varied slightly. As the applications of gluten depend on these properties, putting the newly developed starch extraction process into practice on an industrial scale cannot be considered until it is known which parameters influence the properties of the glutens obtained from wholemeal flours.

The aim of this research project was therefore to characterize the properties of the glutens obtained from different wheat varieties using the laboratory scale system and to establish why they developed. The findings would then be used to derive correlations between the composition of the raw materials, the processing technique and the characteristic properties of the glutens. An essential precondition for this was to create processing conditions which would enable the glutens extracted in the

laboratory scale system and those extracted on an industrial scale to be compared.

Reconstruction of a laboratory scale extraction system

So that the necessary trials could be carried out, an existing laboratory system operating in accordance with the new processing concept first had to be redesigned in such a way that the residence time of the starch and protein material in the laboratory scale system were reduced so that residence times comparable with those of the known industrial process were obtained. This was necessary in order for the possible action of enzymes on the gluten properties, which depends on the reaction time in the test runs, to be placed on a comparable footing.

The process realized using the laboratory scale system is characterized by a wheat milling product being suspended with water in a stirrer tank in order to extract the soluble components (**Figure 7**). The suspension is then passed through a sieving machine in order to separate out the fibrous components, these being mainly bran from wholemeal flour produced by impact-grinding with an ultra-rotor.

The laboratory scale system (**Figure 8**) was equipped with two basket centrifuges operating both batch-wise and alternately for 20 minutes at a time in order to separate out the undersize material coming from the sieving machine. The material separated out in the basket centrifuges exhibited a solids content of around 50 %. After water was added to this material it was homogenised in a mixer (Braun MX 32) in order to agglomerate the gluten. The result was a dough with a solids content of 42 % which was then separated into two phases in the tubes of a centrifuge (Heraeus Minifuge RF). The phase containing the gluten, B-starch and pentosan fractions was washed out manually over a sieve with water, the gluten being obtained as a residue.

This procedure meant that the length of the time elapsing between suspending the flour and washing out the gluten was around 50 min. However, the process - starting with the gluten agglomeration in mixers, through the centrifugal separation of starch and gluten to washing out the gluten by sieving - takes only 20 to 30 min in an industrial system.

Essentially, the duration of the process in the laboratory scale system was reduced by using a decanter centrifuge to modify the procedure for concentrating the undersize material obtained by separating the bran from the suspended wholemeal flour. As the throughput to be dealt with by the decanter centrifuge to be used was only 40 kg/h, a centrifuge of this type with a suitably small throughput rate had to be designed especially for the laboratory scale system. This called for the bowl and screw of the decanter to be of small dimensions (**Figure 9**).

The throughput passing through the redesigned laboratory scale system comprised around 13 kg/h flour suspension with a dry matter content of 17.5 % and 27 kg/h recirculated process water. The doughy mass emerging from the decanter centrifuge had a solids content of 42 %. The gluten which it contained was already agglomerated by the action of force during separation and transport and distributed homogeneously throughout it. Portions of the material each weighing 600 g were separated into two phases in the tubes of a centrifuge (Heraeus Minifuge RF). The gluten in the phase

obtained as a supernatant *w*as extracted by washing out the B-starch and pentosan fractions which it contained by means of a purpose-built extraction system (**Figure 10**).

Concentration of the undersize material coming from the sieving machine by centrifugal separation in the decanter centrifuge and by mechanically washing out the gluten in the extraction system resulted in the required reduction in the residence time to around 30 min. The process engineering requirements were thus satisfied so that the planned investigations could be carried out in order to shed light upon the different gluten properties that was observed.

Material and Methods

In order to solve the problem, white and wholemeal flours were produced from pure samples of different wheat varieties (**Table II**). The wheat samples were ground to flour using either a Bühler mill (type: MLU 202) or an ultra-rotor (type: Ultra-Rotor IIIa). The extraction rate of the white flours ranged from 66.5 % to 73.4 %, with ash contents between 0.48 % and 0.74 %. Prior to impact-grinding in the ultra-rotor, the wheat samples were conditioned in such a way that the impact grinding resulted in ground products which contained a high percentage of coarse flocculent bran. In addition, wheat flours and commercial wheat glutens obtained from a German starch factory (Crespel & Deiters) and glutens from several other European starch factories (Amylum, Italgrani, Jäckering, Kröner, Roquette) were investigated.

The redesigned laboratory system was used to extract the glutens and the A- and B-starch fractions as well as the bran fractions and the fractions containing the soluble components from the flours. The isolated glutens and soluble components were freeze-dried. Both the A- and B-starches and the brans were dried in thin layers in a forced circulation drying cabinet at an air temperature of 50 °C. The starch and soluble α-glucose polymer content as well as the protein, ash, pentosan, dietary fibre and lipid contents in the fractions were determined by standard methods (ECLAIR Analytical Methods Book). To this end, the dry mass of both the starch and bran fractions was determined by drying them overnight at 105°C while that of the gluten fraction and the fraction containing the soluble components was established by titration by the Karl Fisher method. Materials balances were drawn up using the dry masses of the fractions and their starch and protein contents. The starch and gluten yields were then computed. The glutens were investigated in order to determine their chemical and physical properties. This was done by identifying the molecular composition of their proteins (SE-HPLC, RP-HPLC, AAA), their viscosity and viscoelasticity (Haake viscometer, Bohlin rheometer, Glutograph), their sedimentation value, their baking performance and their colour. In addition to this, their enzymatic status was determined (α-amylases, proteases).

Apart from performing germ counts of the lactic acid bacteria and yeasts, the metabolites formed (lactic and acetic acid) and the pH value were determined to establish the influence of the microflora proliferating in the process water during the

course of the trials.

Results and Discussion

Yield of starch and gluten

It was possible to process all milling products in the redesigned laboratory system in the way that was characteristic of the process. The intended content of soluble components in the process water of around 6 % was achieved for all the wholemeal flours. It would, in principle, were also possible to achieve the same content for the white flours. However, the laboratory system was operated at a concentration of solubles in the process water of around 5 % for the white flours in order to limit the duration of each trial.

The bran was separated from the wholemeal flour by wet-sieving without any difficulty. The bran and gluten yields corresponded approximately to those obtained using the original system under comparable trial conditions (**Table III**). The yield of A-starch and gluten in relation to the wheat variety used differed only marginally when wholemeal flours and white flours were processed.

In each case, the yields were slightly higher for the wholemeal flours than for the white flours. This can be explained by the fact that, owing to the wheat flour yield of only 66.5 % to 81.9 % obtained using the Bühler mill, part of the endosperm was separated out by dry-sieving with the bran, with the result that it was not available for wet extraction in the laboratory system.

However, the gluten and A-starch that is was possible to extract from milling products obtained from individual wheat samples varied depending on the respective gluten protein and starch contents of the latter. The yield of protein as gluten ranged from 42.8 % to 61.1 % in relation to the protein content of the wheat samples for all white and wholemeal flours. Consequently, the white and wholemeal flours obtained from the wheat samples differed with regard to the yield of protein as gluten. However, the yields depended only to a very small extent on the type of milling product used, being similar for white and wholemeal flours in each case. This indicates that the gluten yield is a characteristic of the wheat variety. The higher gluten yield obtained for the wholemeal flours in contrast to the white flours can also be explained if it is considered that the flour yield of between 66.5 % and 81.9 % was considerably less than the maximum possible yield. This is due to the fact that part of the gluten-forming proteins ended up as endosperm particles in the bran during dry milling and was therefore not available for wet extraction. In contrast to this, the gluten-forming proteins in the wholemeal flours processed in the laboratory system were fully available for agglomeration and thus for the gluten yield. The results also demonstrate that virtually none of the gluten proteins had become bound to the wet-sieved bran particles. Thus previous experience gathered with the original system was confirmed. Similar yield relationships were obtained for the starches, ranging from 59.2 % to 68.8 % for each of the raw materials used.

Analytical composition and properties of the glutens

The glutens extracted were investigated using known chemical and rheological methods in order to determine their chemical and physical properties. The investigation into the composition of the gluten samples showed that the glutens extracted from the white and wholemeal flours obtained from each of the processed wheat varieties were essentially similar in composition (**Table IV**). It is interesting to note that it was possible to extract the glutens from the milling products of one and the same wheat variety in such a way that they exhibited virtually identical protein contents and similar starch contents.

Considerable differences were only established in the lipid, ash, pentosan and dietary fibre contents although these were still within the range of values obtained for the commercial glutens with which they were compared. Generally speaking, higher contents of the substances referred to above were determined in the glutens extracted from wholemeal flours (G-WM) with the laboratory system than those obtained from the corresponding white flours (G-WF). This can be explained by the fact that it was not possible to separate out the bran components present in the wholemeal flours completely as bran fraction by wet-sieving over 355 μm, as was the case when the dry-sieving process was applied over 180 μm when producing the flours. These bran components, which had become part of the undersize material, became concentrated in the starch and gluten when these were subsequently separated. The same applies to the wheat germ, part of which was damaged to such an extent by impact-grinding during production of the wholemeal flours that its lipids content became mixed with the gluten fraction during the test runs.

The investigation into the functional properties of the gluten samples revealed that these also varied considerably in some cases. Greater differences were established above all in the viscoelastic properties but also in those analytical characteristic values normally used in testing the quality of commercial glutens and in estimating their properties to improve the baking quality of flours. Compared to the inherent capacity of a wheat flour to form a characteristic loaf volume the addition of dried gluten to flour results in an increase of the loaf volume of bakery products. This characteristic property of gluten is referred to as its baking property.

In addition to the sedimentation volume of the gluten proteins in a solution of SDS and lactic acid, the flour quality improving properties of gluten can be estimated from the intrinsic viscosity of the dissolved gluten and its turbidity in a lactic acid solution. When applying these methods of investigation it was established that the glutens extracted from wholemeal flours generally exhibited lower sedimentation volumes, viscosity levels and turbidity values than those extracted from white flours produced from the same wheat variety. In a recent investigation it was possible to demonstrate that there was a correlation between these analytical characteristic values and the baking properties of commercial glutens. Accordingly, there is a positive correlation between the baking properties and the sedimentation volume and a negative one between the baking properties and the turbidity value.

It was, however, not possible to confirm such correlations for the glutens extracted in the laboratory system using the new processing concept. This was demonstrated in baking trials in which the effect of adding dried gluten to flour on the loaf volume and

the sensory characteristics of the baking products made from such flour were investigated. To this end, a flour with a low protein content was used as a reference flour. The flour was fortified with 4 % of gluten extracted from the wheat varieties. Furthermore, doughs were made both with the flours obtained from the raw materials and from the fortified flours, and were characterized according to their rheological properties.

The white flours produced from the wheat varieties exhibited a typical rheological behaviour when made into dough, enabling them to be classified in order of quality (*e.g.* 'Fresco': extra strong quality, 'Camp-Remy': breadmaking quality, 'Kanzler': breadmaking quality, 'Obelisk': poor quality, etc.). However, the great differences in the rheological properties of the flours did not apply to the reference flour when fortified with glutens extracted from the milling products obtained from the various wheat varieties. In each case, fortification resulted in flours with a higher water

absorption and doughs of greater stability. Their stretching resistance and energy absorption were also higher than those of the reference flour (**Figure 11**). However, it was not possible to establish any differences that could be attributed to the type of milling product used to extract gluten.

In each case, fortifying the reference flour with gluten also resulted in a marked increase in the volume of the baked product which more or less followed the order of quality of the wheat varieties referred to above. The volume of the baked products generally increased in the same way when gluten from different milling products obtained from the same wheat variety was added. From this it follows that the differences observed in the functional properties of the glutens - which it was possible to attribute to the type of milled product used to extract gluten - were not reflected in the baking results as described by McDermott (1985). All in all, the increase measured in the volume of the baked products lay within the range of values determined in comparative trials using commercial glutens. An improvement in the sensory characteristics of the baked products was not achieved by fortifying the flours with gluten as adding gluten had a negative effect on the crumb characteristics in each experimental set-up.

Furthermore, there were obvious variations in the colour of the glutens. Thus the glutens extracted from wholemeal flours were darker than those extracted from white flour which, in turn, were darker in colour than the commercial glutens with which they were compared (**Table IV**). It was shown that these differences in colour were only attributable in part to the fibrous components, which were more highly concentrated in the G-WM. The darker colour was predominantly caused by enzymatic browning reactions. It was possible to demonstrate this in an experiment in which the browning reaction was suppressed by adding a condensed phosphate salt (Sporix) to wet gluten (**Figure 12**). The salt used specifically inhibited the polyphenol oxidases.

Finally, it was established that the most important technical and operational differences lay in the viscoelastic properties of the gluten extracted using the new process. The differences consisted in the wet glutens extracted from the wholemeal

flours generally being less elastic than those extracted from the white flours. The decrease in their stretching resistance during a test-run of the laboratory system lasting several hours was also greater than that of the glutens extracted from the wholemeal flours (**Figure 13**). All glutens exhibited a low degree of elasticity after freeze-drying, those extracted from wholemeal flours being less elastic than those extracted from white flours (**Table IV**).

This gave rise to the supposition that even the freeze-drying process which is supposed to exhibit in comparison to flash-drying a rather moderate impact on the structure of the gluten proteins exerted a considerable influence on the viscoelastic properties of the glutens. Two additional experiments were carried out to assess this influence. In one of the experiments, a gluten sample obtained from a starch factory as wet gluten was freeze-dried. Its viscoelastic properties were compared with a gluten sample that was taken from the process at virtually the same time and flash-dried. In the second experiment, six gluten samples of this kind originating from different starch factories were rehydrated and subsequently freeze-dried. The viscoelastic properties of the samples treated in this way were then compared with those of the initial samples.

With regard to the first experimental set-up, it was established that, as expected, the freeze-dried gluten retained more of its elasticity than that which was flash-dried. In the second experimental set-up, it was demonstrated that once the elasticity of the gluten samples was determined by flash-drying, it underwent virtually no further changes when the rehydrated gluten was freeze-dried (**Table V**). These investigations show that the large differences in the elasticity of the glutens extracted using the new process cannot only be attributed to the differences in the heat impact of the drying processes used.

As the uses of gluten in its most important fields of application depend above all on its viscoelastic properties, it had to be investigated which influencing parameters caused the differences that were described and the changes in the properties that occurred during the process. There were three possible causes. As the raw materials were processed under the same processing conditions, the differences observed could only be connected with either the chemical composition, the composition of the gluten proteins or the properties of the raw materials used. It was interesting to note that the raw materials were not only characterized by their different chemical compositions but that their enzymatic and microbiological status also varied. The aim of the subsequent work was thus to investigate the effect of the influencing parameters referred to on the viscoelastic properties of each type of gluten.

Amino acid and protein composition of the glutens

Although the glutens extracted using the new processing concept did not differ as far as their amino acid composition was concerned, they did differ with regard to the molecular composition of their proteins. It was possible to demonstrate this by RP-HPLC chromatograms of proteins extracted with ethanol/water (70 % v/v) from the gluten samples. The chromatograms revealed differences that corresponded to the wheat varieties tested. However, there was no correspondence with the types of flour

used (white or wholemeal flour). From this it follows that the composition of the glutens extracted from white and wholemeal flours obtained from a single wheat variety was the same as regards the proteins that were soluble in ethanol/water.

This also applied to the protein distribution investigated by means of SE-HPLC. This method enabled the proteins dissolved under careful extraction conditions to be separated according to their molecular weight by exclusion chromatography (Beckman TSK 4000-SW column, pore size: 450 Å). Although the disadvantage of the extractant chosen (0.1 *M* Na_2HPO_4 with 2 % SDS) was that it was not possible to bring the gluten proteins totally into solution, its advantage was that disaggregation of the proteins was virtually ruled out.

Elution profiles characterized by four peaks were obtained under the chromatography conditions. It was possible to assign molecular weight ranges to the peaks by comparing them with standards. Accordingly, the high-molecular substances (Mr > 650 kDa) corresponding to the HMW-glutenins were eluted first and recorded as Peak F1. Substances with an apparent molecular weight of between 115 and 650 kDa (LMW-glutenins) were recorded as Peak F2. Peaks F3 and F4 were assigned to monomeric protein molecules, the apparent molecular weight of which corresponds to that of the gliadins and salt-soluble proteins. By comparing the areas below the peaks it was possible to calculate the distribution of the dissolved gluten proteins over the fractions referred to above as a percentage.

The values obtained for the four protein fractions, which are compiled in **Table VI**, reveal that the percentages obtained for each of the fractions were spread over a wide range. Thus the percentage of the F1 fraction was between 14 and 19 %, that of the F2 fraction between 20 and 25 % and those of the F3 and F4 fractions were between 48 and 54 % and 8 and 12 % respectively. The distribution of the values thus corresponded essentially to the range obtained for the commercial glutens with which they were compared (**Table VII**).

Evaluation of the values obtained showed that it was not possible to relate the protein composition of the glutens to either the wheat varieties or the flours. Although the values seemed to indicate that the glutens extracted from wholemeal flours contained a higher proportion of the F2 fraction than those extracted from white flours, the variations were only slightly greater than those that had occurred during the test run. Thus the glutens extracted after a 4 h test-run of the laboratory system had revealed protein compositions that differed from those of the glutens extracted after a 2 h test-run. The statistical evaluation which was carried out taking all the results into account demonstrated that it was not possible to prove that there was any significant functional relationship between the distribution of the protein fractions and the rheological properties of the glutens.

To sum up, it can therefore be said that the differences in the functional properties of the glutens cannot be explained by the differences shown to exist in the molecular composition of the proteins.

Influence of the gluten composition on the viscoelastic properties

Starting from the result that the glutens obtained from the wholemeal flours were characterized by higher pentosan, ash and dietary fibre contents than those extracted from white flours, it had to be investigated whether the viscoelastic properties were possibly influenced by these secondary components.

The influence of fibre particles on the physical properties of the gluten was investigated in model experiments involving commercial wheat glutens in which the changes in the properties occurring after the addition of finely ground wheat bran were measured by means of a Glutograph. Measurements were carried out after the sample was prepared using a method that was modified specifically for these investigations and led to more easily reproducible test results (**Figure 14**). The method is characterized by a dough being prepared mechanically with the dry gluten in a Glutomatic specially adapted for this purpose after which the gluten mass is formed centrifugally in a centrifuge tube and then relaxed.

The addition of bran to the glutens was selected in such a way that the maximum ash content of each mix was comparable to that of the glutens extracted from wholemeal flour in the laboratory scale system. The ash content of the commercial glutens used in the experiments was similar to that of the glutens extracted from the white flours. Adding up to 8 % finely ground wheat bran resulted in a decrease in the stretching properties and an increase in the Glutograph time measured. This was 2.5 to 3 times higher than that for the rehydrated gluten without added fibre (**Figure 15**).

It is interesting to note here that this result corresponded with the differences in the stretching properties established for the gluten extracted in the laboratory scale system using the new processing concept. The Glutograph times of the wet and dried glutens obtained from wholemeal flours - which had an ash content similar to that of the bran-enriched glutens in the experiments - were also several times longer at the beginning of the relevant test-runs than those of the glutens containing less ash which was extracted from the corresponding white flours. This observation applied to the dried glutens both in general and irrespective of the test-run. Thus it was proved that the differences between the stretching properties of the glutens could be attributed at least partly to the percentage of fibres that they contained.

However, this did not apply to the decrease in the stretching properties of the wet glutens occurring after a test-run of the experimental system lasting several hours. The decrease was greater for the glutens extracted from wholemeal flours than for those extracted from the corresponding white flours so that the former were even softer than the latter after a 4 h test-run. The ash content of the glutens had, however, remained constant throughout each test-run. Furthermore, the decrease in the stretching properties of the wet glutens was cancelled out by subsequent drying. The stretching properties of the wet and dried glutens as a function of the different operating times of the laboratory system were compared in **Figure 16** in order to illustrate these results.

These changes in the characteristics of the wet glutens which took place during the laboratory process could therefore only be due to causes other than the fibres. The enzymatic and microbiological processes occurring under the processing conditions were considered a possible cause.

Influence of the enzymatic and microbiological status on the viscoelastic properties

The aim of subsequent work was, therefore, to investigate the influence of the microbiological and enzymatic activity occurring under the processing conditions on the physical properties of the glutens. To this end, the protease activity in the raw materials, in the glutens extracted from these and in the process water was determined during a test-run of the laboratory scale system lasting several hours.

It was shown that there were differences in the enzymatic status of the raw materials and the glutens obtained from them. Thus wholemeal flours exhibited a slightly higher protease activity than the white flours obtained from the same wheat sample (**Table VIII**). The same difference in protease activity was observed when comparing the glutens extracted from wholemeal and white flours. However, the proteolytic activity of the glutens and that of the other products extracted as solids was, on the whole, considerably less than that of the raw materials used. Most of the enzymes present in the raw materials entered the process water.

This was also demonstrated by the fact that the proteolytic activity in the recirculated process water increased during a test-run of the laboratory system lasting several hours. After an increase in concentration over the first few hours, a state of equilibrium was reached which settled at a constant level, just as it did for the constituents extracted from the flour. It was particularly interesting that the level of protease activity occurring during the processing of wholemeal flours was not greater than that which took place when white flours obtained from the same wheat variety were processed. It cannot therefore be assumed that the differences in the viscoelastic properties of G-WM and G-WF that was observed could be attributed to proteolytic processes occurring during the test runs. The investigations discussed above which was carried out using RP-HPLC or SE-HPLC also demonstrated this. The increase in proteolytic activity in the process water in line with the duration of the test run evidently did not have the effect of subjecting the gluten proteins to varying degrees of proteolysis.

This did not apply to the way in which the process water affected the properties of the glutens. It was shown that the pH value of the process water decreased in all experiments irrespective of the raw material used, approaching asymptotically a pH value of 5 (**Figure 17**). The titratable acids content of the process water increased linearly, in line with the drop in the pH value. The increase in the titratable acids content was significantly higher when wholemeal flours were processed instead of white flours. In this connection it is interesting to note that the microflora proliferating in the process water, and the composition thereof, were independent of the type of flour used. During a 30 h test run of the laboratory system, a lactic acid bacteria germ count of 10^8 CFU/ml (CFU = Colony Forming Units) was measured when processing both wholemeal and white flours, starting at a germ count of 10^6 CFU/ml (**Figure 18**). In each case, the maximum germ count was reached after an operating time of around 16 h.

The increase observed in the degree of acidity was essentially attributable to the formation of lactic acid. After a 30 h test run of the laboratory system, a lactic acid

content of 2.5 g/l in the process water was reached when processing wholemeal flours (**Figure 19**). The lactic acid content when processing white flours obtained from the same wheat variety under the same conditions was 1.8 g/l. From this it follows that the glutens were exposed to different lactic acid contents in the course of the test-runs.

It was therefore assumed that there was a direct correlation between the decrease in the stretching properties and the lactic acid content. Accordingly, an experiment was then performed in which a commercial gluten was rehydrated with lactic acid solutions in concentrations of 0 - 3 g/l. The stretching properties of the gluten decreased as the acid content rose (**Figure 20**). The reaction time intensified the decrease. It was possible to stop this process completely by neutralising the lactic acid solutions used to rehydrate the gluten with NaOH before using them. This proved that the lactic acid content of the process water in the test runs both with wholemeal and white flours had a considerable effect on the stretching properties of the glutens.

The model experiment was exactly in the range of the lactic acid content that had also occurred in the test run of the laboratory system. However, the results did not give any clear answer as to why the glutens had lost their previously soft consistency after drying and had virtually reassumed the consistency of the wet gluten that had existed at the beginning of the test-run. Consequently, it still needs to be investigated how the change in the ionic environment during freeze-drying - occurring as a result of the interaction of the minerals and the lactic acid - influences the stretching properties of the glutens.

In addition to the correlations demonstrated here, there were also indications that the stretching properties of the glutens are also affected by the reduction and oxidation processes to which the proteins were exposed during the test-runs, in particular during the extraction of the soluble components. The glutathione content was certainly a factor here. This was indicated by investigations in which oxidation processes were deliberately brought about or prevented during gluten extraction or the rehydration of dried glutens. It was not possible to complete and analyse such investigations as were required to shed light on these complex relationships within the scope of the work dealt with here. They will therefore be the subject of further investigations.

Summary

The gluten samples extracted from wholemeal flour (G-WM) and from white flour (G-WF) under the given process conditions had almost the same overall chemical and protein composition as well as amino acid composition. Slight differences were observed in the contents of lipids, ash and insoluble dietary fibre. But these were in the range of the composition of commercial gluten samples. Besides these similarities in part substantial deviations were found in the glutens properties. The SDS sedimentation volume, the percentage reduction of turbidity in lactic acid suspension and the intrinsic viscosity of G-WM were lower than that of G-WF. Their colour was also darker predominantly caused by enzymatic browning reactions. Moreover it became obvious that the viscoelasticity of G-WM was lower and their stretching resistance decreased more rapidly during process run. All glutens exhibited a low degree of elasticity after freeze-drying, those extracted from wholemeal flours being

less elastic than those extracted from white flours.

As the uses of gluten in its most important fields of application depend above all on its viscoelastic properties, it was investigated which influencing parameters caused the differences that was described and the changes in the properties that occurred during the process. As the raw materials were processed under the same processing conditions, the differences observed could only be connected with either the composition of the gluten proteins, the chemical composition or the properties of the raw materials used.

Investigations to identify the molecular composition of the gluten proteins showed that the differences in the functional properties of the glutens could not be explained by the differences shown to exist in the molecular composition of the proteins. The chromatograms revealed differences that corresponded to the wheat varieties tested. However, there was no correspondence with the types of flour used (white or wholemeal flour). From this it follows that the composition of the glutens extracted from white and wholemeal flours obtained from a single wheat variety was the same as regards the proteins that were identified by means of RP-HPLC and SE-HPLC.

The influence of the gluten composition on the physical properties of the gluten was investigated in model experiments. Involving commercial wheat glutens in which the changes in the properties occurring after the addition of finely ground wheat bran were measured by means of a Glutograph, it was proved that the differences between the stretching properties of the dried glutens could be attributed partly to the percentage of fibres that they contained.

The decrease in the stretching properties of the wet glutens could be attributed to the lactic acid formed by the microflora proliferating in the process water during the course of the trials. The lactic acids content of the process water increased linearly, in line with the drop in the pH value. The increase in the lactic acid content was significantly higher when wholemeal flours were processed instead of white flours and the stretching properties of the gluten decreased as the acid content rose. Finally indications were also found that the stretching properties of the glutens were also affected by the reduction and oxidation processes to which the proteins were exposed during the test-runs.

The investigation contributes to a better understanding of the properties of wheat gluten extracted from wholemeal flour which is an important precondition for the implementation of the newly developed process into industry.

3. Publications describing work carried out under Subprogramme A Task 1.2.2

Althoff F., and Meuser F. (1995). Extraction of starch, gluten and fibre from wholemeal wheat flour using a newly developed laboratory scale process. In: Progress in Plant Polymeric Carbohydrate Research. (F. Meuser, D.J. Manners and W. Seibel, eds). Behr's Verlag, Hamburg, Germany, pp 141-145.

Meuser F. (1993). Möglichkeiten konzeptioneller Veränderungen bei der Stärkegewinnung. In: Schriftenreihe des Bundesministers für Ernährung,

Landwirtschaft und Forsten, Reihe A, Sonderheft: Symposium Nachwachsende Rohstoffe - Perspektiven für die Chemie, Landwirtschaftverslag Münster, pp. 114-144.

Meuser F. (1994). Wheat utilisation for the production of starch, gluten and extruded products. In: Wheat: Production, properties and role in human nutrition (W. Bushuk and V.F. Rasper, eds). Chapman and Hall, London, England (1994) pp. 79-204.

Meuser F. und Pahne N. (1994). Prozeßtechnisch bedingte Eigenschaften von Weizenvitalkleber bei Anwendung eines neuen Verfahrens zur Weizenstärkegewinnung. Lecture held at: 45th Conference on Cereal Chemistry, Detmold, Germany, June 15-16. (in preparation).

Pahne N., Meuser F. und Althoff F. (1993). Verfahrenstechnische Aspekte zur Weiterentwicklung der Weizenstärkegewinnung. Lecture held at: 9th International Conference on Problems of Cereal Processing and Chemistry, Bergholz-Rehbrücke, October 18-20.

Task A.2.1/2 - The Characteristics and Processing Requirements of Wheat for Specific End-uses: White Bread and Wholemeal Bread

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1. Key measures of achievement - Objectives

- To assess the ability of these new 'strong' gluten type varieties to carry varieties with weaker gluten characteristics, and to determine the extent to which the work input requirements of such blends are compatible with contemporary baking equipment and breadmaking processes.
- To determine the underlying physico-chemical reasons for these differences in gluten strength and breadmaking quality and thus provide feedback that will focus to plant breeding programmes aimed at producing wheats with superior breadmaking quality.
- To develop small scale tests suitable for use in plant breeding or in grain trading that are capable of differentiating between wheats with different gluten properties.
- To define the factors that affect wholemeal breadmaking quality of flours, distinguishing between effects of milling procedures on flour performance and those resulting from the distribution and composition of anatomical components.

2. Progress

Two separate tasks, respectively on white bread (A.2. 1) and wholemeal bread (A.2.2) were merged early in the programme when it became apparent that there were many areas of overlap between the two tasks. Both tasks were aimed at a better understanding of the relationship between grain quality and technological performance in breadmaking processes For convenience, the principal achievements of the programme are cited separately for white and wholemeal bread.

Task A.2.1 - White bread

Much of the effort was directed at determining and understanding the mixing requirement of UK-grown wheat varieties **(Figure 21)**. It was shown that the work-input requirement ranged from 5 Wh/kg (18 kJ/kg) to 20 Wh/kg (73 kJ/kg). Samples with work-input requirements greater than 11 Wh/kg (40 kJ/kg) may not achieve their full potential in a breadmaking process based upon a fixed energy input during mixing such as the Chorleywood Bread Process (CBP). These high work-input varieties were shown to be suitable for blending with weaker varieties, for example a 50:50 blend of 'Fresco' (extra-strong) with 'Riband' (weak) resulted in a quality similar to that of 'Avalon', a popular breadmaking variety during the 1980s.

Work carried out on the gel-protein fraction of the glutenin proteins (originally identified by Graveland) showed that the extra-strong varieties had glutenin polymers of a greater size than those in normal breadmaking varieties. This study, also showed that the extra-strong character of 'Fresco'-type varieties and observed weakness in a variety called 'Pastiche' which were not detected by tests such as the SDS sedimentation volume test were identified by the elastic modulus of gel-protein, measured on a Bohlin VOR rheometer. This small-scale test of quality has now been introduced into the testing protocol for the UK National Recommended List trial samples as an additional parameter for the evaluation of new varieties.

A collaboration with the Technical University of Berlin (Partner 13) resulted in the elastic modulus test being evaluated on German varieties in the Rapid Mix Test (RMT). Both the RMT and the CBP baking tests ranked the varieties in the same order and for both baking tests an elastic modulus greater than 40 Pa was shown to be deleterious for loaf volume (**Figure 22**). This work showed that the test developed for UK varieties and breadmaking process could be more generally applicable. Recent work has indicated that elastic modulus of gel-protein is influenced by fertiliser treatment which may explain differences between foliar applied urea-spray and soil-applied ammonium nitrate.

In a traditional bulk-fermentation baking process, several varieties spanning a wide range of baking quality showed a common lack of tolerance to increased fermentation time. This was attributed to a probable lack of fermentable sugars for the yeast.

Task A.2 2 - Wholemeal bread

At present, third country wheat (mainly from N. America) is imported by some sectors of the industry for wholemeal breadmaking. The advent of so-called extra-strong varieties raised the question as to whether they could be an alternative to imported high-protein wheat. Our studies showed that in wholemeal, the extra-strong character was lost, probably due to interactions between the glutenin polymer and components in the bran. These studies suggested that in wholemeal, baking performance is more influenced by protein content than is white bread. Gluten fortification did not alter the work-input requirement of the wholemeal flour from the varieties 'Fresco' and 'Mercia'.

Apart from the requirement for high-protein wheat for wholemeal and for other speciality products, the UK can be self-sufficient for breadmaking wheat except for the problem of *α-amylase*. Wet pre harvest conditions can result in premature sprouting in the ear and high levels of *α-amylase* which cause problems during breadmaking, particularly in mechanically developed dough systems where the problem may not be apparent until the slicing machine. A study of the effect of high levels of cereal *α-amylase (up* to 100 Farrand units-0.72 SKB units) showed that in terms of crumb density, resilience and stickiness. wholemeal bread was less affected than white bread. This could be due to destabilisation of the enzyme through lack of calcium, if the latter has been sequestered by the phytate in the bran layers.

It was not possible to predict wholemeal loaf volumes from those of white without making allowance for quality attributes such as hard or soft milling or extra-strong character. It was, however possible to predict (r=0.77) the wholemeal loaf volume of a test sample from measures of the baking performance of the endosperm and bran/offal components relative to those of a control sample of 'Mercia'. This study covering 11 varieties from several EU countries suggested that both the endosperm and bran/offal are influential in the baking performance of wholemeal flours.

In addition to the gel-protein test, a small-scale stress-relaxation test for yeasted bread doughs was developed. The relationship between initial relaxation and loaf volume of r = 0.82 included white, wholemeal and gluten fortified wholemeal flours (**Figure 23**). This test has potential for further development.

Conclusion

This ECLAIR programme has increased our ability to make best use of wheat varieties in both white and wholemeal breadmaking, The detailed work on 'Fresco'-type extra-strong varieties has identified problems with this trait and wheat breeding programmes are responding. The elastic modulus of gel-protein developed within this programme has now been adopted for routine testing of new varieties in the UK and has been shown to be applicable to material from other EU countries.

This study has concentrated upon varietal differences. The Baking Industry is, however, faced with the problem that wheat of a given variety does not perform consistently from season to season or indeed from site to site. The knowledge gained will be of value in addressing this problem and indeed our studies have already suggested that grain quality may be manipulated by plant husbandry, for example by nitrogen fertiliser regimes; thus further improving the balance between end-user and the quality of harvested grain.

3. Publications describing work carried out under Subprogramme A Task 2.1/2

Oliver G. and Pritchard P.E. (1993). Rheology of the gel protein fraction of wheat flour. Spec. Publ. - R. Soc. Chem., 113 (Food Colloids and Polymers: Stability and Mechanical Properties), 255-259.

Oliver G., Pritchard P.E. and Brock C.J. (1995). The glutenin fraction of wheat protein: Elastic modulus of gel-protein- a new measure of baking quality (in preparation).

Pritchard P.E., Collins T.H., Little K. and Sang B.E. (1992). The characteristics and processing requirements of wheat for breadmaking. Project Report No. 60, Home-Grown Cereals Authority, London.

Pritchard P.E. (1993). The glutenin fraction (gel-protein) of wheat protein - a new tool

in the prediction of baking quality. Aspects of Applied Biology, 36 (Cereal Quality III), pp. 75-84.

Pritchard P.E. and Oliver G. (1993). Assessment of wheat protein quality using advanced rheological techniques. In: "Proceedings of an International Conference on Bread - Breeding to Baking", FMBRA, Chorleywood, 15-16 June, Chameleon Press Limited, London, pp. 161.

Pritchard P.E. and Brock C.J. (1994). The glutenin fraction of wheat protein: The importance of genetic background on its quantity and quality. J. Sci. Food Agric., 65, 401-406.

Pritchard P., Oliver G. and Curtis P. (1994). Assessment of wheat protein quality using advanced rheological techniques. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp. 576-581.

Task A.2.3 - Evaluation of Technological Evaluation of Wheat Flours and Protein Fractions in Baked Products

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1. Key measures of achievement - Objectives

The aim of this task was to define an optimal biscuit flour to improve the knowledge of the biscuit dough.

2. Progress

The studies were focused on a semi-sweet biscuit (type *Petit Beurre*), for which a laboratory scale baking test (300 g of flour) was developed.

In a first park of the work, the set of samples included four flours coming from semi industrial milling of pure varieties harvested in France in 1992. The analytical characterisation of these flours was based on the method developed by INRA (Partner 07N) for the quantification of protein fractions. The gel protein determinations were carried out at CCFRA (Partner 14). An overview of the data obtained and the range of variation for each parameter is given in **Table IX**. Analytical data were statistically analysed to corroborate results obtained along the previous years. The results **(Table X)** are the following:

- It was confirmed that the alveograph parameter P/L increases with components which absorb water, like damaged starch or pentosanes. So, this indirect parameter, contrary to the literature, cannot really give an estimation of quality of proteins because it is based on constant hydration.

- The alveograph parameter W correlates to the gel protein (r = 0.87) and even to the breakdown rate (r = - 0.91). The two methods, alveograph and gel protein, which have a totally different principle, lead to the same type of results.
- The hydration determined by farinograph is difficult to predict with composition parameters and a multilinear regression with damaged starch, proteins, pentosanes, median diameter lead to an explanation of only 43 % of this absorption.
- There are some relationships between pentosanes, damaged starch and ash, and between quality of protein (either gliadin or glutenin rate) and length and thickness of end-products.

In order to improve the baking test, which was lacking of repeatability, a test was developed at a laboratory scale. New equipments were selected and installed. Operating conditions were redefined for each phase: mixing, sheeting, baking. The test was completely operational at the beginning of the year 1994, making possible the prediction of baking quality parameters by analytical parameters.

In a second step, technological analyses (characteristics of biscuits) were carried out on one hundred flours and related to physico-chemical characteristics by statistical analysis. Fifty of these flours were obtained by Brabender milling, from wheats provided by Subprogramme C partners: ITCF, INRA Clermont-Ferrand and Benoist (Club des 5). They corresponded to 15 varieties grown in 3 different French locations in 1992 and 1993.

The aptitude of these 15 varieties to biscuit-making production was determined and the flour parameters controlling dough and biscuits properties were identified.

The evaluation of the biscuit-making potential is based on dough machinability, density and surface aspect of biscuits. The scale of evaluation was established from industrial flours, and adapted to Brabender milled flours.

Among the available samples of flours and according to our test, only five varieties were identified as satisfactory for semi-sweet biscuit-making: 'Arminda', 'Beaver', 'Mercia', 'Sidéral' and 'Talent', because they produce biscuits of low density. Five other varieties appeared to be unsatisfactory for use in semi-sweet biscuits: 'Rossini', 'Récital', 'Baroudeur', 'Renan' and 'Rektor'.

Varieties such as 'Apollo', 'Viking', 'Thésée', 'Camp Rémy', 'Soissons' gave fluctuating results depending on the location and crop year.

The density (a determinant parameter for the quality of this type of biscuit) depends on the flour constituents which influence water absorption: damaged starch and pentosans, as well as particle size distribution.

A work in conjunction with INRA Montpellier (07M) was performed on the effect of mixing conditions on the rheological properties of dough. It highlighted the role of proteins on those properties. The expression of the proteins depends on the mixing conditions: mixing work (speed and time) and water content.

Task A.2.4 - Processing Properties of Flour Blends. Prediction and Improvement

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1. Key measures of achievement - Objectives

- To improve the knowledge of gluten composition in relation to dough functional characteristics in order:
- To improve the quality of EC flour blends in terms of dough (processing) properties;
- To compensate for year-to-year variations in flour quality.

2. Progress

In the project on the prediction of processing properties of flour blends good progress was made. During mixing the amount of glutenin macropolymer (GMP) decreased and during resting the amount increased again. The decrease in GMP could be predicted by an exponential decrease using a flour variable (GMP content of flour) and a process variable (mixing time; **Figure 24**). The relationship could explain 86 % of the variation in the GMP content of dough. The increase in GMP could be very well described by a function of the amount of macropolymer in flour and the resting time (87 % of the variation explained; **Figure 25**). With a similar function the quantity of the individual glutenin subunits in the polymer could be described (91 % of the variation explained). These findings indicate on the one hand that it is not so much the quality of the protein (subunit composition) which determines the reassembly of the protein during resting, but moreover the quantity. On the other hand the large amount of variation that can be explained indicates that it is possible to predict dough properties (GMP content of dough) on basis of a flour parameter (GMP content of flour) and a processing parameter (resting time). This is advantageous for the milling industry, which wants to predict dough properties prior to, *i.e.* without, processing, *e.g.* on basis of flour and processing parameters. Apart from the quantitative differences also qualitative differences occurred in the GMP. A large decrease (with one cultivar 10 times) in stiffness (G*) of the glutenin macropolymer was observed. The stiffness decreased exponentially with mixing time. Surprisingly, during resting no exponential increase in stiffness was observed, as is found normally in polymerisation reactions. Probably during mixing and resting the GMP is transformed from a linear polymer to a three dimensional gel structure. Even at mixing times where no change in amount was observed in the amount of polymer, large changes in stiffness were found. This indicates that rheological techniques are very well suited for establishing changes in the glutenin macropolymer, which cannot be detected by biochemical techniques.

3. Publications describing work carried out under Subprogramme A Task 2.4

Hamer R.J., Weegels P.L. and Orsel R. (1994). The polymerisation of glutenin in relation to end use quality. In: Wheat Kernel Proteins: Molecular and Functional Aspects, Proceedings of the Symposium at S. Martino al Cimino, Viterbo (Italy), September 28-30, pp. 139-144.

Weegels P.L., Flissebaalje Th. and Hamer R.J (1994). Factors affecting the extractability of the glutenin macropolymer. Cereal Chem. 71 (3), 308-309.

Weegels P.L., Hamer R.J. and Schofield J.D. (1994). Depolymerisation and polymerisation of individual glutenin subunits *in situ* in dough - Implications for the structure of gluten. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp. 57-66.

Weegels P.L., Orsel R., van de Pijpekamp A.M., Lichtendonk W.J., Hamer R.J. and Schofield J.D. (1995). Functional properties of low *M*r wheat proteins. II. Effects on dough properties. J. Cereal Sci., 21 (2), 117-126.

Thesis

Weegels P.L. (1994). Depolymerisation and repolymerisation of the glutenin macropolymer in dough and effects of low *M*r wheat proteins. King's College, University of London, 308 p.

Task A.2.5 - Rheological Characterisation of Wheat Samples and Identification of Specific Processing Requirements Related to Sweet Bakery Products with Sour Starters

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1. Key measures of achievement - Objectives

- Rheological measurement in order to obtain a better understanding of the properties of wheat; Small scale test.
- Development of a baking test to evaluate the baking quality of flour; Small scale test.

2. Progress

Rheological measurements

Dynamic measurements carried out with the Bohlin rheometer showed that it was possible to reach the region of linear viscoelasticity to characterize flour slurries.

Measurements carried out at a concentration of 40 % showed that there is a better discrimination of flours respect to the concentration of 35 % because of the further development of the structure.

Strain sweep tests show that there is a linear region until a strain of 0,5 %-1 % and that at higher values the curves decrease.

The lowest frequency does not allow to reach the well known slopes of 1 and 2 for G' and G" respectively, typically correct for a linear viscoelastic fluid. For this reason a calculation method to characterize flours was needed.

The rheological characterisation was done in terms of relaxation spectrum H(l) according to a viscoelastic analysis. As the frequencies range was limited, Ge represents not only the elastic (permanent) network but also the viscoelastic (temporary) network that has not the time to relax. For this reason we call Ge modulus, Ge*.

Only Ge* modulus allows the discrimination of flours. From this parameter we can obtain the quantity of structural unity that are formed and the kinetic of destructuration.

The obtained results indicate that flours have different values of the level of structuration and different values of the amplitude of deformation at which begin the rupture of the system. The deformation at the strain of 2,5 % is very high and all the flours were destructured. The dependence of Ge* slope from the angular velocity shows that flours have different behaviour of destructuration (**Figure 26**).

Rheological data were correlated with the value of W (**Table XI**), the mechanical energy required until the rupture of the dough.

We suppose that a flour that is much structured and with a low rate of destructuration will need more energy than a flour that is less structured and with higher rate of destructuration.

From the rheological data we observe that strong flours have an intermediate level of structuration and an intermediate rate of destructuration; alveographic values of W were the highest.

A flour presents a lower value of W because of the higher rate of destructuration, even if the level of structuration is higher. Other samples have a level of structuration comparable to strong flours but with lower rate of destructuration; so W values were lowest. At last, some samples show low rates of destructuration and low level of structuration.

Baking test

The research activities carried out during the period were the improvement of the reproducibility of the test and, in particular, of the rapeseed method for the evaluation of the volume, the correlation with other valuation methods, in particular, with alveographic data and the evaluation of the problem of water absorption.

We obtained good reproducibility using a new oven which allowed more homogeneous temperature distribution, a standardized yeast which determined more uniform leavening. Reproducibility of the rapeseed method for volume evaluation was tested and showed accurate results.

We have correlated the data obtained by the test (volume, specific volume and weight loss during baking (**Table XII**)) with the numbers obtained by the classification method and with the main alveographic parameters (W, P/L) (**Figure 27**).

The results show good agreement between the volume of the sample and the number of the classification method and between the volume of the sample and the protein content of the flour. The evaluation of the influence of the water absorption characteristics of flour samples was studied and again we found a correlation between water absorption and volume after baking, particularly for high water absorption flours.

Conclusion

The rheological characterisation allows to obtain a lot of information about the structure of the dough. The set in gear method permits to discriminate flours by means of the Ge* modulus. We think that future work will be to elaborate these data in order to obtain a quality judgement of the flours.

The results obtained with the baking test are accurate and show good reproducibility: the volume of the sample after baking can be considered as the result of the test.

Task A.2.6 - Interactions Between Selected Microorganisms and Wheat Components, and their Application to Improve Breadmaking Processes

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1. Key measures of achievement - Objectives

To increase the knowledge of the interactions between selected microorganisms and flour components and their application to improve breadmaking processes.

2. Progress

Changes in functional characteristics (Figure 28)

The objective was to study the effect of microorganisms on fermentative and rheological properties of doughs and bread characteristics in relation to process conditions.

Characterisation and selection of flours

A total of sixteen flours consisting of thirteen commercial flours (Spain) and three wheat cultivars ('Fresco', FR, United Kingdom; 'Obelisk', OB, Holland; 'Camp Rémy', CR, France) covering different extraction rates, proteolytic activity and breadmaking quality were analysed for average chemical composition, and rheological and fermentative properties using, when possible, ICC standard methods, anywhere else manufacturers' guidelines.

For commercial flours, percent of degradation (11-37 %) governed rheological and fermentative properties. During that period, no flours without proteolytic activity were available. Protein content (11-15 %) was the main chemical variable differentiating white flours. As expected, high protein levels resulted in greater flour strength (W, alveograph), water absorption (farinograph), resistance to extension (extensigraph) and dough level (maturograph). Increasing extraction rates obviously led to higher protein, fat and ash contents. In consequence, rheological and fermentative properties underwent deleterious effect as recorded in the extensogram, maturogram and impulsogram parameters; in addition, higher water absorption and degree of softening were observed. Flours from pure wheat varieties also showed variable proteolytic activity (0-45%), but lower degree of softening, dough level and final volume than commercial flours.

Changes in functional characteristics **(Figure 28)**

A selection of the analysed flours was made and the 10 chosen flours (pure cultivars: OB, FR; commercial blends: MBO, MCO, PBO, GB1, GB2 and B2) were used to prepare doughs and breads after inoculation with previously selected microorganisms *(Lactobacillus brevis* L-62, *L. plantarum* B 39). In addition to flour and microorganisms, processing conditions included different physical form of starter (frozen, dehydrated), and form of incorporation (sour dough, straight process). Functional characteristics evaluated were acidification properties of sour doughs, fermentative and rheological properties of bread doughs, and physico-chemical and sensory characteristics of breads. Methodological procedures used were included in a recently published paper (Collar *et al*., 1994). Results summarised below refer to the effects (single, interactions) of flour and microorganism by using frozen bacteria in a sourdough process. Additional effects of dehydrated starter or frozen bacteria in a straight dough process were respectively considered for commercial flours (see refereed published paper) and for biochemical approach respectively. A total of 50 variables were evaluated. The data handling procedures included the analysis of variance, to know the influence of significant interactions among process conditions, and multivariate data handling to classify the samples according to their functional characteristics, and to know the most significant grouping variables. Flour significantly influenced up to 34 variables. Some acidification properties, and rheological (maturograph) parameters depended on both type of flour and kind of starting microorganism. In factor analysis, three factors accounted for 70% of variability of data. The first factor (37% variance) was positively related to sensory attributes of breads and negatively to density and texture. Factor two (23% variance) was mainly related to energy in extensigraph, and the third factor (10% variance) was strongly correlated to lactic acid, pH and fermentation quotient of breads. The two first factors led to a distribution of samples in two well defined areas and a wider and general mixed group. Group 1 included samples made with white flour FR, and characterized by doughs of higher elasticity and dough level in maturogram, and energy and extensibility in extensogram, so as by breads of greater volume and most characteristic aroma. Group two included samples made with whole flours GB2 and B2, which reached the hardest texture and greatest density. The third general mixed group contained the remaining samples with very small separation among them; they had similar intermediate scorings in sensory analysis.

K-means clustering analysis of data led to similar classification of samples, but it provided information about trends in analytical variables within each cluster. First cluster grouped started samples prepared with lactobacilli and white flours except FR. The third cluster mainly included samples prepared with whole flours (with and without lactobacilli starters). Finally the second cluster consisted of a mixed group containing mainly FR and unsoured samples. Tendencies of analytical variables varied among clusters. Acidification properties and intensity of taste increased from cluster 1 to 3, whereas farinograph characteristics presented the opposite tendency. Maturogram and extensogram parameters showed variable trends depending on the parameter considered. Samples in cluster 1 and 2 had better and similar volume, density, width/height ratio and texture than samples in cluster 3. Sensory attributes reached the highest scores for cluster 2 and the lowest for samples in cluster 3.

Biochemical changes in flour components and fermentation metabolites

The objective was to investigate the effect of metabolic activities of starting microorganisms -yeast and lactic acid bacteria- from sour dough and bread doughs, on carbohydrate, nitrogen and lipid components.

The study of biochemical aspects related with the interactions of microorganisms in flour systems were focused on the evaluation of the effect of: 1) processing conditions, 2) processing step, and 3) storage time. For point 1 three flour samples (with varying degree of extraction) and one strain of *Lactobacillus plantarum* B-39 were used. Point 2 included 5 different flours (MBO, GB1, B2, FR0, FR3), two microorganisms (B-39 and *L. brevis* L-62) and two kind of frozen starters (added through a sourdough (SD) and a straight (ST) process). Finally point 3 was performed with FR and OB flours (70, 100 % extraction rate) started with frozen B-39 and L-62. Methodology used was reported in the following papers: Karkalas, 1985; Rouzaud and Martinez-Anaya, 1993; Martinez-Anaya *et al*., 1993; Barber *et al*., 1993; Békés *et al*., 1976; Collar *et al*., 1992, Collar *et al*., 1991.

Effect of processing conditions on biochemical components of sour dough.

A surface response design was used to study the effect of flour extraction rate, dough yield (g sour dough/100 g flour), fermentation temperature, and yeast addition. The effect of sourdough fermentation time was also considered. The most influencing process variables affecting sugars and oligosaccharide levels were the presence of yeast and in a lesser extension the extraction rate of flour. Dough yield and temperature played a minor role in controlling these parameters. The ash content of flour was the main factor with positive influence on the level of nitrogen metabolites in wheat sourdoughs, and the processing condition governing the extent in the accumulation of amino acids and peptides during fermentation. Fermentation temperature governed changes in prominent amino acids during SD fermentation and promoted the reduction of glycolipids. Dough yield increased the level of free lipid metabolites and induced variable influence on the amino acid profile of SD. The presence of yeast reduced the amount of nitrogen and lipidic components in fermented SD.

Influence of breadmaking stage on some fermentation metabolites.

Breadmaking step (sour dough SD, dough fermentation (unfermented dough UFD, fermented dough FD), and baking B) and flour showed the most significant single effects on total starch, sugars and low molecular weight dextrins (LMWD). Microorganisms only influenced sugar contents. Results of multivariate data analysis (factor, K-means clustering and discriminant analysis) showed that about 68 % variability of these components during the process was explained by levels of LMWD, and about 14 % by starch. Changes in LMWD were primarily affected by SD and dough fermentation steps; baking did not add further differences. SD and UFD had the

greatest sugar levels, and FD and B the highest of dextrins with degree of polymerisation (DP) over 5 **(Figure 29)**.

Flours of different extraction rate resulted in different pattern of some lipidic and nitrogen components along the breadmaking process. Metabolism of some amino acids during fermentation and their implication in baking reactions were an useful way of differentiating controls and SD samples and white from brown flours. In general, high extraction rate flours accounted for the highest values for non protein metabolites, whereas white flours were characterized by the maximum levels of glycolipids. Levels of total amino acids, neutral and polar lipids in doughs (UFD, FD) and breads depended on flour extraction rate; white flours showed the highest levels of polar lipids and the lowest of amino acids, whereas whole flours led to samples with higher quantities of neutral lipids. Doughs and breads also reached greater values for phospholipids, and intermediate values for predominant individual amino acids than SD. SD samples, on the other hand, clearly differentiate from doughs (UFD, FD) and B, and samples properly grouped according to flour extraction rate; sour doughs prepared with brown flours had the highest values for free amino acids and the lowest content of polar lipids, whereas white flours SD contained greater protein and lower neutral lipid levels.

Fatty acid profiles of free lipids were closely dependent on breadmaking process (sourdough, straight) used. Higher free lipid values were obtained for soured than for straight dough and bread samples. Oleic and linoleic acids concentration positively correlated with ash content of flours for both processes; in addition, SD characterized by low levels of saturated 16:0 and unsaturated 18 1 fatty acids, and straight bread doughs (controls and with starter) contained higher levels of minor 14:0 and 18:0. Changes in amino acids and polar lipids during fermentation and baking significantly correlated with both type of flour and type of process used Proliferation during fermentation (bonded lipids to free lipids) and depletion during baking (thermal degradation) of both glycolipids and phospholipids characterized low ash content samples, whereas the proliferation of some individual amino acids during fermentation, and their utilisation during baking properly characterized high ash content samples.

Effect of storage on some biochemical characteristics of breads

Breads were stored at 27°C until mould growth appeared. Storage variably influenced swelling power and soluble solids of breads, depending on flour and strain of microorganism used. Total starch and amylopectin retrogradation only underwent very slight changes. When data from chemical characterisation of lipidic and nitrogen components of breads stored up to 4 days were analysed together, two factors explained 100 % of the variability of data. Factor 1 (68 % VE) referred positively to amino acids, peptides and neutral lipids and negatively to polar lipids, and factor 2 (32 % VE) positively related to EtOH-soluble protein (ESP) content. Four groups of stored breads were clearly defined after plot of scores of factor 1 *vs*. factor 2. Factor I separated bread samples according to the extraction rate of flours but in factor 2 bread

sample distribution depend on the starter inoculated. Samples started with *L. brevis,* L-62 characterized by high protein content; storage time increased the level of ESP. Breads containing *L. L. plantarum,* B-39 showed low levels of ESP and its content lowered during storage.

Relationships between functional characteristics and biochemical changes

The objective was to evaluate the possible relationships between biochemical changes and functional properties of breads.

To investigate these relationships, data from functional characteristics and biochemical composition of bread samples were analysed by chemometrics methods. Two set of analysis were performed each including bread characteristics and carbohydrate related parameters, and nitrogen and lipidic components respectively. Results are discussed below.

The main factor explaining variability of data, when bread quality and carbohydrate related parameters were considered, depended on sensory attributes and physical characteristics of breads. The second factor was related with LMWD, and the third one with acidity and lactic acid contents of breads. First factor separated breads in two groups on the basis of flour extraction rate, in which, the greatest organoleptic quality corresponded to white flours breads, whereas LMWD allowed to differentiate white breads made with SD or straight processes due to their lower content in those components in the latter samples. Cluster analysis included acidity and lactic acid as a separate factor grouping unstarted together with straight process breads because their low amount of acids. The best canonical correlation was established between physico-chemical characteristics of breads (volume, texture, density and height/width ratio in the first set) and carbohydrates, soluble solids and swelling power in the second set. The first pair of canonical variables showed a squared correlation coefficient of 0.986, so linear combinations of experimental variables could be determined. Inclusion of variables related to acidity or sensory scores in any set of variables did not improve the correlation coefficient of regression **(Figures 30 and 31)**.

Levels of amino acids, peptides, glycolipids, total titratable acidity and acetic acid values correlated with the intensity of taste of breads; whereas, main physico-chemical and sensory attributes of breads were connected to the amount of neutral lipids. Better scores for sensory attributes (typical flavor, overall acceptance, external appearance, crumb edibility, and bread volume) and higher physico-chemical quality (low density and soft texture) of breads corresponded to samples performed with low and medium ash content of flour showing low amounts of neutral lipids. In addition, high intensity of taste of breads related to high levels of free amino acids and peptides and low amounts of acetic acid and glycolipids, and corresponded to 'Fresco' whole sour dough samples. Intensity of flavour of breads was positively correlated with the extent in the proliferation of amino acids after proofing and negatively related to the level of glycolipids and phospholipids in fermented doughs.

Specific enzyme activities (Figure 32)

Proteolytic and amylolytic activities of pure cultivars flours used above (OB, FR, CR, at 70 and 100 % extraction rate) and five lactobacilli strains *(Lactobacillus plantarum* B-39, L-73; *L. brevis* 25a, L-62 and *L. sanfrancisco* L-99), as well as dynamics of by-products from enzymatic degrading action in mixed flour-lactobacilli systems were investigated. Methodological aspects were reported in the following papers: Collar *et al*., 1990, Rouzaud and Martinez-Anaya, 1993; McCleary and Sheehan, 1987; Kruger, 1973 and AACC 1982.

Flour extraction rate did not influence endoproteolytic activity, but whole flours had more exoproteolytic activity. pH influenced exoproteases, which were more active at neutral (7.0) than at acidic (4.7) pH. Differences in endoproteolytic activities of lactobacilli strains were small. Exoprotease action depended on pH, corresponding to L-99 the highest exoproteolytic activity. Whole flours contained higher amounts of soluble nitrogen compounds (SSNC) (soluble protein nitrogen, SPN, and free peptides FPP and amino acids FAA) than white flours. FR flour had the greatest SSNC and CR the lowest. In mixed systems, SPN increased up to 24 h and decreased afterwards, whereas non protein fractions followed variable trends because their participation in metabolic pathways of lactobacilli. Dynamics of SSNC could be adjusted to a log-log-inverse function. Regression coefficients were significant for inoculated flours, the model fitting better FAA than FPP or SPN, due to the later components being able to further splitting to shorter size fragments. Protein and polypeptides included in SPN mainly corresponded to subfractions with molecular weight (*M*r) ranging from 21,000 to 67,000 Da. Proteins of *M*r from 43,000 to 67,000 Da were cleaved in the last stages to smaller size units, resulting in an increase of subfractions of *M*r from 20,000 to 43,000 Da. Subunits with lower (> 14,000) and higher (up to 232,000) *M*r were less abundant and also underwent variable changes depending on flour and strain. α-amylase activity of flours depended on ash content; whole flours had similar values, which were of the order or three to ten times that of white flours. L-99 had the lowest α-amylase activity and *L. plantarum (L-73,* B-39) the highest. Evolution of monosaccharides followed variable trends, depending on extraction rate of flour and strain. In white flours they accumulated in the beginning and were assimilated in the last stages. For whole flours a sharp increase was detected from 24 to 48 h. Dynamics of monosaccharides in flour-lactobacilli mixtures could not be adjusted to any of available regression functions. Maltose levels increased during fermentation, although an effective incorporation in lactobacilli metabolism was observed. *L. brevis* 25a was the strain with the most pronounced ability to incorporate this sugar. A second order polynomy could be adjusted to describe dynamics of maltose during fermentation. Low molecular weight dextrins (LMWD) with degree of polymerisation (DP) 3 to 7 only appeared and accumulated in flour-microorganism systems during the last stages of fermentation. DP3 was the only oligosaccharide able to be incorporated by lactobacilli, and only by 25a. The greatest size dextrins were apparently involved in further degrading enzymatic reactions to give smaller size fragments. Dynamics of LMWD followed exponential curves, reaching regression coefficients, in general, high values. Variability explained by regression surpassed in general 50 %, and decreased

as DP increased. Under conditions used in practice, flour resulted the main contributor to final enzymatic activity of flour-lactobacilli systems (**Figure 33**).

Conclusions

Flour extraction rate is the main factor influencing breadmaking performance and biochemical patterns. High degree of variability found within white flours influence quality and biochemical characteristics. Some cultivars have very distinctive behaviour (*e.g.* 'Fresco'), but, in general, with commercial flours, a good performance can be obtained.

There is not a single parameter defining bread quality, but many different class (physical, sensory, chemical, biochemical) properties interacting, so more sophisticated techniques of data analysis are necessary. Microorganisms add further differences to those observed when using different flours, but their overall effects are smaller than those resulting from flours.

Microorganism performance relays in their form of addition. Differences in breadmaking quality attributed to the strain of microorganism used are less important than those due to flour of way of addition (sourdough or straight process). Sourdough processes are most effective than straight processes. Fermentation time during the sourdough step influence dynamics of components of flour and metabolites from fermentation than does not appear when using unstarted or straight process samples.

Dough fermentation results in differences in components and metabolites when compared with unfermented doughs, but these are lower than those created during the sourdough step. Baking does not add further differences to the fermentation step, in regarding starch degrading, nitrogen and lipidic compounds. Low molecular weight dextrins seem to play a role in detecting similarities among samples. Sourdough breads contain higher amounts of these dextrins than control or straight processed breads, and confirm the possible role of sour dough addition in improving the keeping characteristics of breads.

Flour is the main quantitative contributor to proteolytic and amylolytic activities in flour lactobacilli mixtures. Increasing ash content of flour leads to higher enzymatic activity. Lactobacilli qualitatively influence degradation of nitrogen and carbon compounds during fermentation. Effects are strain and species dependent. Metabolic and enzymatic reactions modify dynamics of changes in those components. Significant regression equations can be described for soluble nitrogen fractions, maltose and dextrins. Monosaccharides undergo the most complex changes due to their active participation in metabolism.

Results from this work furnish new information on the interactions between flours and starting microorganisms in breadmaking processes, but it should be noted that they relate to some EU flours samples subjected to particular processing conditions. In view of the conflicting data in the current literature on relationships between quality and metabolite content of wheat or flour, caution should be applied in extrapolating conclusions to a wider range of wheats and baking methods.

3. Publications describing work carried out under Subprogramme A Task 2.6

Collar C., and Martínez C.S. (1993). Amino acid profile of fermenting wheat sour doughs performed under different processing conditions. In: Proceedings of Euro Food Chem VII, Progress in Food Fermentation, Vol. 1. Ed.: C. Benedito, C. Collar, M. A. Martínez-Anaya, and R. Morell. IATA (CSIC) ISBN: 84-604-7038-5. Valencia, Spain, September 20-22, pp. 290-295.

Collar C., and Martínez C.S. (1993). Amino acid profile of fermenting wheat sour doughs. J. Food Sci., 58 (6), 1324-1328.

Collar C., and Martínez C.S. (1995). Effect of processing conditions on lipidic and nitrogen metabolism of wheat sour doughs. Int. J. Food Sci. Technol. (in press).

Collar C., Martínez-Anaya M.A. and Benedito de Barber C. (1993). Interactive effects between microbial breadmaking starters and wheat flours. In: Proceedings of Euro Food Chem VII, Progress in Food Fermentation, Vol. 1. Ed.: C. Benedito, C. Collar, M. A. Martínez-Anaya, and J. Morell. IATA (CSIC) ISBN: 84-604-7038-5. Valencia, Spain, September 20-22. p. 75-80.

Collar C., Martínez-Anaya M.A. and Benedito de Barber C. (1995). Interactive effects between microbial breadmaking starters and wheat flours. Rev. Esp. Cienc. Tecnol. Aliment. (in press).

Martínez-Anaya M.A. (1994). Factors influencing the quality of wheat sourdough processes, and the use of chemometrics in quality assessment. Rev. Esp. Cienc. Tecnol. Aliment., 34 (5), 469-493.

Martínez-Anaya M.A., Collar C. and Benedito de Barber C. (1995). Comparative study on functionality on Spanish and other EC flours when used in microbiologically started breadmaking processes. J. Food Sci. (in press).

Martínez-Anaya M.A., Collar C., and Benedito de Barber C. (1993). Comparative study on functionality of Spanish and other EC flours when used in microbiologically started breadmaking processes. In: Proceedings of Euro Food Chem VII, Progress in Food Fermentation, Vol. I. Ed.: C. Benedito, C. Collar, M. A. Martínez-Anaya, and J. Morell. IATA (CSIC) ISBN: 84-604-7038-5. Valencia Spain, September 20-22, p. 253-258.

Martínez-Anaya M.A. and Rouzaud O. (1995). Influence of flour, bacterial starter and breadmaking stage on total starch, sugars and low molecular weight dextrins. Z. Lebensm. Unters. Forsch. (in press).

Rouzaud O. and Martínez-Anaya M.A. (1993). Effect of processing conditions on sugar and oligosaccharide profile of wheat sourdoughs. Z. Lebensm. Unters. Forsch., 197, 434-439.

Rouzaud O., and Martínez-Anaya M.A. (1993). Effect of processing conditions on sugar and oligosaccharide profiles of wheat sourdoughs. In: Proceedings of Euro Food Chem VII, Progress in Food Fermentation, Vol. 1. Ed.: C.

Benedito, C. Collar, M. A. Martínez-Anaya and J. Morell. IATA (CSIC) ISBN: 84-604-7038-5. Valencia, Spain, September 20-22, p 308-313.

SUBPROGRAMME B:

FUNCTIONAL COMPONENTS AND THEIR INTERACTIONS

Summary

The study of *the interactions and the development of dough* formed the objectives of **Subprogramme B**, which had the following two main themes:

1. *Component interactions*: Proteins from glutenin and gliadin fractions which are linked to performance attributes had to be prepared in sufficient quantities to study their water-binding by NMR), their aggregation with each other or with other components by NMR, by equilibrium sedimentation, ultracentrifugation, turbidimetry, SE-HPLC, etc.) and their hydrophobicity by RP-HPLC and TNS binding). These properties were linked to performance tests in dough development and to associated indices of rheology. Study in lipids focused on the polar and protein-binding fractions using phosphorus NMR and fluorescence spectroscopy). The role of protein and lipid fractions in stabilising the dough-gas bubble) interface was determined by static and dynamic interfacial techniques. The minor protein components associated with starch granules were also investigated to establish their role(s) in relation to functional properties of wheat, flour and isolated starch, to extend research on the role of starch granule protein *Friabilin* in controlling endosperm texture in wheat and to devise a predictive test of endosperm texture for use in plant breeding as a selection tool with single seeds (in connection with Subprogramme C) and as a quality test at flour mill intake (Subprogramme A).

2. *Dynamics of dough development*: The effect of heat and mechanical treatment on the distribution and mobility of protein components were studied by NMR spectroscopy and linked with changes in dough rheology. Monoclonal antibodies were used to label specific proteins and hemicelluloses to determine the dynamics of their distribution by immuno-gold labelling within the developing dough particularly in relation to swelling and the formation of the biopolymer-gas interface. New oscillatory measurement techniques were used to distinguish between two fundamental liquid and elastic contributions to the overall viscoelastic response.

Relevant results in this Subprogramme include: purification and characterisation of glutenin subunits and of native gluten subfractions, reconstitution studies with purified components and subsequent rheological studies, NMR characterisation of subunits, homologies between starch granule proteins and lipid binding proteins, a better understanding of the dynamics of dough development based on monoclonal antibodies directed to arabinoxylans and on immunolocalisation in microscopic studies.

New ways of characterisation of *HMW and LMW subunits of glutenin* corresponding to specific alleles were improved further using still more sophisticated tools and use of chromosomal substitution lines and null lines. The development of a simple

procedure of determination of the number of cysteine residues directly from electrophoretic bands allowed to develop further the hypothesis on the relation between the number of cysteine residues of a subunit and its potential role in the determination of dough extensibility.

The effect of HMW and LMW glutenin subunits on glutenin polymer properties and on *rheological behaviour* of gluten was explored using lines with deletions of various gliadin or glutenin loci while mobility and rigidity of polypeptide chains was investigated through *electron spin resonance* of the gluten subfractions.

In the study of *minor components associated with starch granules*, a considerable progress concerning the biochemical nature of friabilin and puroindolin proteins was obtained. It is now clear that *in situ* friabilins have to be considered as lipoproteins which are involved in some way with endosperm texture. Moreover, puroindoline-a would be mainly located in the aleurone layer while puroindoline-b would not. Puroindoline was also shown to be mainly located in the starchy endosperm and to interact strongly with anionic phospholipids and to exhibit an important structural flexibility which controls lipid binding specificity and foaming properties. Such a behaviour might be important at the air-water interface during the gas phase expansion of bread doughs.

In the work on *interfacial behaviour of dough* during mixing it was demonstrated that the breakdown of macropolymers during mixing can be clearly seen in the surface active behaviour of dough samples, that added lipids have a strong influence on the surface behaviour, but that no difference is observed between soft and hard wheat types.

In the project on *dynamics of dough development*, a great success was obtained with the production of monoclonal antibodies to arabinoxylans using both water-insoluble arabinoxylans from bees wing bran and arabinoxylans conjugated to BSA as a protein carrier. These antibodies are now available for analysis of arabinoxylans in flour as well as for immunolocalisation in microscopic studies.

Individual Progress Reports

Task B.1.1. - Purification and Characterisation of Gluten Subfractions

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1. Key measures of achievement - Objectives

- Purification and characterisation of whole gluten, gluten subfractions and protein subunits of different genotypes;
- Study on the conformational and functional properties of the individual gluten proteins and the interaction of the different proteins and other wheat components.

2. Progress

Purification of glutenin subunits

First, a method for the preparation of low-molecular-weight subunits of glutenin of bread wheat without contamination by high-molecular-weight glutenin subunits or by gliadins was investigated. Using a simple protocol based on the selective precipitation by 40 % acetone, two fractions were obtained corresponding to HMW and LMW subunits respectively (**Figure 34**). The protein fractions can be obtained either reduced or reduced and alkylated. The effects of temperature, contact time, and of successively increasing concentrations of acetone were tested, indicating that the protocol could be scaled-up to obtain large quantities of LMW subunits and thus be used as a pre-purification step (Melas *et al.*, 1994).

Then, the procedure of separation of glutenin subunits according to their charge was refined. This was facilitated by the better control of the conditions of protein precipitation by acetone. The new protocol included the following steps: (i) extraction of glutenins according to Singh *et al.* (1991), with reduction and alkylation of subunits, (ii) fractionation of the latter extract on a FPLC cation exchange Mono-S column into 14 fractions, (iii) SDS-PAGE analysis and densitometric scanning of the patterns in order to determine the proportions of the various LMW and HMW subunits of glutenin in each fraction and therefore to determine the charge distribution profile of any wheat genotype.

Interestingly, different cultivars gave very different (and reproducible) charge distribution profiles. The study was extended to 50 French cultivars to assess the potential of this cation exchange FPLC method in view to better explain the physicochemical bases of baking quality.

By combining IE-FPLC and SDS-PAGE, two new HMW glutenin subunits (1Ax1** and 1Bx6*) were discovered. LMW glutenin subunits were characterized on the basis of their charge distribution in IE-FPLC enabling to infer a charge index (IC) for every variety. IC was found associated to the allelic composition of common wheat varieties and to the dough characteristics. Positive IC values reflected a favourable effect of the *Glu-A3 o* allele on dough extensibility (G), whereas negative IC values reflected a favourable effect of the *Glu-A3 m* allele an baking strength. These relationships allowed consideration of LMW glutenin subunits as functional elements of gluten and not as simple genetic markers. The hypothetical model according which LMW glutenin subunits could interact in the gluten protein network was discussed (Mélas and Autran, 1995).

On the other hand, using null forms (isolated in the Italian common wheat cultivars and supplied by ISC, S. Angelo Lodigiano, Partner 03) lacking *Gli-1*-encoded gliadins and LMW subunits, as well as 1B/1R translocated cultivars, several subunits (*e.g.* some of those encoded at the *Glu-3A* locus) could be obtained in a pure state (Melas, 1994).

Characterisation of glutenin subunits

A simple procedure of determination of the number of cysteine residues was adapted for wheat glutenin subunits. For instance, partially purified subunits were alkylated with appropriate mixtures of anionic and cationic reagents (iodoacetic acid and 4-vinyl-pyridine) and then analysed by acid-PAGE in the presence of 2 *M* urea. For instance, from a single HMW component containing N cysteines, a pattern containing N+1 bands was generated **(Figure 35)**. The procedure was used to check the number of cysteine residues of several already known HMW subunits from *T. durum* and *T. aestivum* cultivars and will be applied to the determination of the number of cysteine residues from unknown LMW subunits (Morel and Bonicel, 1994, 1996).

The 3 proteins which are the main respective products of 3 different *Glu-A1* alleles were found to possess 8 cysteine residues. So, in the peculiar case of these subunits, which were related to dough extensibility, the total cysteine number cannot account for different technological properties.

From the sequences of LMW glutenin subunits already reported by Okita *et al.* (1985), Cassidy and Dvorak (1990) or Colot *et al.* (1989), we know that the few LMW-GS which had a cysteine at the 5th residue from the N-terminal (*mc5*) have 8 cysteine residues.

In the case of 'Apollo' and 'Carlos', a same protein which behave like an ω-gliadin in SDS-PAGE was found to possess an apparent number of 1 cysteine residue. This protein is likely to be a true ω-gliadin as 'Apollo' and 'Carlos' have the same *Glu-D1* allele as 'Cheyenne', a cultivar found to be devoid of D LMW-GS by Masci *et al.* (1991).

3. <u>Publications describing work carried out under Subprogramme B Task B.1.1</u>

Published papers

Melas V., Morel M.H., Autran J.C. and Feillet P. (1994). Simple and rapid method for

purifying low-molecular-weight subunits of glutenin from wheat. Cereal Chem. 71 (3), 234-237.

Melas V. and Autran J.C. (1995). Caractérisation biochimique et fonctionnelle des gluténines du blé tendre par chromatographie d'échanges d'ions. Relation avec les caractéristiques technologiques de la pâte. Sciences des Aliments (submitted).

Morel M.H. (1994). Acid-PAGE of wheat glutenins: a new tool for the separation of high and low molecular weight subunits. Cereal Chem. 71 (3), 238-242.

Morel, M.H., Bonicel J., Melas V. and Autran, J.C. (1994). Multiple approach (IEF, SDS-PAGE and A-PAGE) of the composition of LMW subunits of glutenin and its effect on dough properties. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp. 244-254.

Morel M.H. and Bonicel J. (1994). Charge-density variations of glutenin subunits upon alkylation. A tool for cysteine determination. In: Wheat Kernel Proteins: Molecular and Functional Aspects, Proceedings of the Symposium at S. Martino al Cimino, Viterbo (Italy), September 28-30, pp. 183-188.

Morel M.H. and Bonicel J. (1996). Charge-density variations of glutenin subunits upon alkylation. A tool for cysteine determination. Electrophoresis (in press).

Thesis

Melas V. (1994). Sous-unités gluténines de faible poids moléculaire du blé tendre. Variabilité génétique, caractérisation biochimique, relation avec la qualité technologique. Thèse de Doctorat, Université des Sciences et Techniques du Languedoc, 92p.

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1. Key measures of achievement - Objectives

- Purification and characterisation of whole gluten, gluten subfractions and protein subunits of different genotypes;
- Study on the conformational and functional properties of the individual gluten proteins and the interaction of the different proteins and other wheat components.

2. Progress

Because gluten proteins are very insoluble it is difficult to prepare gluten subfractions without destroying more or less their original viscoelastic properties. However a technique preserving the functionality was introduced recently by an Australian group. It consists of extracting gluten with increasing concentrations of very dilute hydrochloric acid. This technique was adopted and applied with success to series of wheat genotypes differing widely in their prolamin compositions and technological properties. The biochemical and rheological analyses of the fractions obtained by this procedure demonstrated that it did not alter their properties. The determination of the size distribution of the proteins in the gluten and its fractions was a crucial point for understanding the variations of the functional properties. For this reason, we optimised a procedure involving size exclusion high performance liquid chromatography (SE-HPLC) of samples extracted with a sonication treatment. A complementary method, the quasi elastic light scattering (QELS), was also used for the first time to determine the sizes of gluten proteins. The conditions of the rheological dynamic analysis were optimised in order to obtain reliable and reproducible results.

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1. Key measures of achievement - Objectives

- Purification and characterisation of whole gluten, gluten subfractions and protein subunits of different genotypes;
- Study on the conformational and functional properties of the individual gluten proteins and the interaction of the different proteins and other wheat components.

2. Progress

Characterisation of gluten aggregates by free-flow isoelectric focusing

Wheat gluten contains a mixture of protein aggregates resulting from covalent as well as non covalent interactions among a number of polypeptides including gliadins, High Molecular Weight (HMW-) and Low Molecular Weight Glutenin Subunits (LMW-GS). At present a clear picture of the gluten structure does not exist, and several models based on the presence of protein aggregates were proposed to date. However the polypeptide composition of the aggregates remains obscure. In order to add a contribute to this point we have developed an electrophoretic system which allowed the separation of unreduced gluten proteins according to their isoelectric point (I.P.) in free solution, thus avoiding the problems related to molecular sieving effects. Native (unreduced) proteins were extracted from wheat flour or gluten in 50% propan-l-ol and fractionated in the Rotofor cell (Bio Rad) in the same solvent, in the presence of 2% Biolyte (pH 3 10) and 0.1% CHAPS. Focusing was performed at 20 W for 9 h. The 20 collected fractions were then analyzed by SDS-PAGE in reducing and unreducing conditions. The overall results from the comparison of the SDS-PAGE patterns obtained in different conditions (reduced *vs* unreduced) indicated that flour and gluten contain an extremely disperse population of relatively small polymeric proteins of molecular mass lower than 300 kDa. These polymers are heterogeneous in composition and I.. P. (from pH 4.5 to pH 11) and are formed by disulphide-linked HMW- and LMW-GS, as assessed by two-step one-dimensional SDS-PAGE. Most polymeric proteins comprised both x-type and y-type HMW-GS and some polymers contained new polypeptides (made detectable by the concentration effect of the system) with a *M*r similar to that of the HMW-GS and also other proteins with a *M*r of about 60 kDa. Small quantities of HMW-GS in single polypeptide form were also detected in unreduced SDS-PAGE patterns. Moreover the LMW subunits B and C of glutenin were found in molecules that comprised HMW-GS.

Adsorption chromatography on controlled-pore glass beads of acetic acid-soluble wheat gluten proteins

The availability of gluten polymers in a pure, native (unreduced) form is necessary for the study of the relationship between their structural features and their functional properties in dough-making. Glutenin polymers are difficult to purify because of their insolubility in the buffers normally used for protein separation and analysis. This is

due to their tendency to aggregate through hydrophobic interactions and hydrogen bonding. The use of detergents and/or dissociating agents prevents the possibility to investigate the functional properties of the aggregates, since these chemicals alter the native structure of the proteins. A new method for the obtaining of glutenin polymers free from monomeric proteins and retaining functionality was developed, based on adsorption chromatography on Controlled Pore Glass (CPG) beads in the absence of any denaturing agent. Moreover by the controlled use of different solvents and pHs, various fractions, differing in protein composition and behaviour at the liquid/solid interface, can be easily recovered (Peruffo et al, 1993, 1994).

Characterisation of the fractions obtained by frontal analysis

The 0.1 M acetic acid-soluble proteins from wheat gluten were fractionated on a column (1× 60 cm) of CPG beads (2,000 Å pore size, 100-200 mesh, surface area 12.1 m^2 / g) equilibrated with 0.1 *M* acetic acid. Frontal analysis was performed by loading subsequently increasing amounts of protein. The first loading (16 mg of protein) was completely adsorbed on the beads, whereas the following ones gave two distinct peaks of increasing area. Of the two, the first peaks (P I), deriving from each chromatographic run, contained purified glutenin aggregates of different size, which seemed to be dependent on the HMW/LMW ratio, as showed by SDS-PAGE. The second peaks (P II), examined by electrophoresis in unreduced conditions contained monomeric proteins, whose number increased with the number of the chromatographic step, along with some glutenin aggregates of molecular size smaller than that of the polymers present in P Is. A-PAGE fractionation showed that monomeric proteins were displaced from the column in order of increasing *M*r (S proteins fraction III, α- ,β- and finally γ-gliadins) indicating a stronger adsorption for the higher *M*r proteins. Glutenin polymers in the second peaks showed a similar elution behaviour, i. e., were displaced in order of increasing molecular mass.

After addition of salt, all the fractions obtained in this way were able to coagulate in an elastic mass, which resembled the original gluten, indicating that the functional properties of the gluten proteins were preserved.

The comparison of our results with those obtained by hydrophobic interaction chromatography indicated that the competitive process of protein adsorption/ desorption was controlled to a great extent by interactions with the glass surface that are hydrophobic in nature. Total recovery calculations indicated that, at the glass surface saturation point, 14.7 mg of protein remained adsorbed on 1 g of CPG beads.

Characterisation of adsorbed proteins

Adsorbed proteins were eluted by washing the column sequentially with different solutions in both reducing and unreducing conditions. Proteins deadsorbed by acetic acid plus 2 *M*, 4 *M* and 6 *M* urea were found to be γ-gliadins encoded by the group 1 chromosomes along with glutenin polymers. The following peak, obtained by adding SDS to the acetic acid-6M urea solution contained glutenin polymers free from monomeric proteins.

The protein still adsorbed onto the glass surface after washing with acidic eluents

could be selectively eluted by treating the matrix subsequently with sodium phosphate buffer pH 7.4 containing 6M urea plus 50 m*M* 2-mercaptoethanol (eluent 1) and with the same buffer made 0.5% in SDS (eluent 2). Eluent 1 gave rise to one protein peak (ME fraction), whose ascending part was constituted by HMW-GS, whereas its descending part contained some LMW-GS. However, the bulk of the LMW-GS (SDS fraction) was released only by eluent 2. Moreover the last eluting fractions of this latter peak contained two proteins, that, by A-PAGE; could be identified as the ω-gliadins encoded by chromosome 1D.

Since the adsorption to the glass surface is dependent, at least in part, on the hydrophobic character of the gluten proteins, the described results indicated that LMW-GS are the major responsible of the hydrophobicity of the gluten polymers. Moreover, the 1D encoded ω-gliadins, which were eluted only at the end of the experiment, seem to be, among the gluten proteins, those showing the higher affinity for the glass. This may be due to the lack of intramolecular disulphide bonds, which would allow a high degree of molecular spreading onto the glass surface. In conclusion we have demonstrated that the complex mixture of the acetic acid-soluble gluten proteins can be fractionated in several clearly resolved groups by the sequential use of different eluents. Moreover it appears that the gluten aggregates are present in a wide range states of different hydrophobicity.

The reported fractionation procedure can be exploited for both practical purposes (*e.g.* to prepare pure functional glutenin aggregates or large quantities of single protein groups for reconstitution experiments...) and theoretical studies.

For example, the protein at 60 kDa, which appears when the purified glutenin aggregated are reduced, was partially characterized (Curioni *et al.*, 1994). This protein was unambiguously identified as a β-amylase by immunoblotting and enzymatic staining in non-denaturing gels. The overall results indicate that the β-amylase would be linked to HMW- and LMW-GS by S-S bonds, and thus would be a part of glutenin aggregates.

Purification of glutenin subunits by preparative acid and 2-dimensional electrophoresis (Curioni *et al.*, 1995)

Glutenin subunits (HMW- and LMW-GS) are the most widely studied proteins of wheat endosperm because they play a key role in determining gluten functionality. The methods reported for their purification are often cumbersome and time-consuming, and, moreover, the purification of more than few subunits in one step is rarely achieved. Recently a new method for the electrophoretic analysis of glutenin subunits at acid pH (A-PAGE) was developed (Morel, 1994). With this system the HMW-GS are very well separated, as well as the majority of the LMW GS. However a complete separation of these latter subunits can be achieved only after a second dimension electrophoresis in the presence of SDS. This system was transferred to a preparative application, using the Prep Cell Mod. 591 (Bio Rad) apparatus for the A-PAGE and the ELFE system for the electroendosmotic preparative SDS-PAGE (Curioni *et al.*, 1989). After preparative A-PAGE of the reduced alkylated glutenin subunits it was possible to obtain a large number of fractions some of which, after

analytical electrophoresis, showed to contain purified protein bands. Proteins with the same *M*r and then not separable by SDS-PAGE, are eluted at different times from the preparative A-PAGE. On the other hand, protein that comigrate in A PAGE, and then found in the same fraction from the preparative electrophoresis could be purified in a second step by electroendosmotic preparative electrophoresis in the presence of SDS. The obtained results demonstrated that it was possible to recover purified LMW-GS in electrophoretically pure form, getting to an aim that is commonly considered very difficult to reach.

The HMW-GS can be easily purified by preparative A-PAGE, although the recovered quantity for each separated band is dependent on its distance from the nearest migrating subunit, *i.e.* on the HMW-GS composition of a given wheat variety. The extreme case are subunits 2 and 7 that, when present together, can not be separated with the described A-PAGE system. Hundreds of μg for each HMW-GS were recovered pure with this system, and, moreover also minor HMW bands of unknown origin were purified. Finally it is important to note that only 0.25% acetic acid was present in the collected fractions, accordingly the problems deriving from the presence of denaturant (SDS, urea) in the protein preparations were avoided.

The system described here overcomes most of the difficulties encountered in glutenin subunits purification. The HMW- and some of the LMW-GS can be recovered pure by preparative A-PAGE, that is a relatively inexpensive, simple, and rapid one-step procedure. Moreover, the LMW-GS bands that comigrate in A-PAGE could be purified by a second electrophoresis in the presence of SDS, resulting in a sort of two-dimensional preparative PAGE. The availability of purified glutenin subunits will improve the biochemical characterisation of the single components and, then, will be of help in the elucidation of their involvement in wheat gluten functionality.

3. Publications describing work carried out under Subprogramme B Task B.1.1

Curioni A., Furegon L., Peruffo A.D.B., Pogna N.E. and Zamorani A. (1994). A 60 kDa protein is a component of glutenin aggregates. Poster, in: Proceedings of the International Meeting "Wheat Kernel Proteins: Molecular and Functional Aspects", September 28-30, Viterbo (Italy), pp. 345-348.

Curioni A., Morel M.H., Furegon L., Redaelli R. and Peruffo A.D.B. (1995). Purification of wheat glutenin subunits by preparative acid and two-dimensional electrophoresis. Electrophoresis, 16, 1005-1009.

Peruffo A.D.B., Curioni A., Pressi G., Pogna N.E. and Zamorani A. (1994). Adsorption chromatography on controlled pore glass beads of acetic acid-soluble wheat gluten proteins. Cereal Chem. 71 (2), 122-129.

Peruffo A.D.B., Curioni A., Pressi G., Zamorani A. and Pogna N.E. (1994). Adsorption chromatography on controlled pore glass beads of unreduced acetic acid-soluble wheat gluten proteins. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop (Poster), June 7-9, 1993, Association of Cereal Research, Detmold (Germany), pp 661-667.

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1. Key measures of achievement - Objectives

- Purification and characterisation of whole gluten, gluten subfractions and protein subunits of different genotypes;
- Study on the conformational and functional properties of the individual gluten proteins and the interaction of the different proteins and other wheat components.

2. Progress

Variation for storage protein components and their relationship with flour technological properties.

Several bread wheats (lines, cultivars and different subspecies of the aestivum group) were electrophoretically analysed to assess their variation in gluten components. Two-dimensional electrophoretic analyses of gliadin components and low molecular weight glutenin subunits have indicated limited variation at the *Gli-D1* and *Glu-D3* loci. Two main electrophoretic patterns were in fact found at each of the above mentioned loci which resemble those found in the bread wheat cultivars 'Cheyenne' (CNN) and 'Chinese Spring' (CS). In particular it was observed that D subunits of low molecular weight glutenins, encoded at the *Glu-D3* locus are present only in those bread wheats with 1D-coded ω-gliadins similar to those of 'Chinese Spring', whereas they are absent in all the cultivars with ω-gliadins similar to those of 'Cheyenne'. The fact that CNN and the CNN-type cultivars do not possess D LMW-GS, along with the observation that the latter have isoelectric points and molecular weights very similar to those of ω-gliadins, seemed to indicate that they are modified ω-gliadins. In regard to the role of D subunits in determining quality characteristics, it was noted that quality evaluation by the SDS sedimentation test of the two biotypes present in the cultivar 'Newton', differing only in the presence or absence of D subunits 1D encoded indicated better quality for the biotype with no D subunits. These observations provided indirect evidences that the presence of D glutenin subunits in a variety makes a negative contribution to wheat technological properties.

Chromatographic analyses of HMW-GS were carried out on reduced and reduced and pyridylethylated (PE) subunits, using 4-vinyl pyridine. Shorter retention times were observed for PE-subunits, compared to subunits only reduced, this is a direct consequence of surface hydrophobicity modification following the introduction of a new chemical group; in fact PE-subunits result positively charged under the acidic conditions used and eluted earlier compared to non pyridylethylated subunits. Reduction in elution times observed, when the same subunit was analysed as reduced and reduced alkylated, was found to be associated with number of cysteine residues. For subunit 20 it was found that its elution time was reduced least upon alkylation, this is a direct consequence of a minor number of cysteine residue possessed by this subunit compared to other Bx type subunits (2 versus 4). In such analyses subunits

were detected which possessed similar apparent molecular weights, when separated in SDS-PAGE but different values of surface hydrophobicities when separated by using RP-HPLC. At the *Glu-A1* locus new alleles were detected which differ from subunit 2* and 1 in surface hydrophobicities and isoelectric points.

In the bread wheat cultivar 'Fiorello', which was reported to possess the recombinant pair of subunits 5+12 at the *Glu-D1* locus, the use of combined electrophoretic and RP-HPLC separations allowed to demonstrate that subunit 5 is different from other subunits 5 present in other bread wheat cultivars and normally associated with subunit 10. In particular, the RP-HPLC behaviour of subunit 5 of 'Fiorello' was more similar to that observed for subunit 2. The sequence of a cloned DNA fragment isolated from bread wheat cultivar 'Fiorello', corresponding to the N-terminal part of the protein, indicated the absence of the additional cysteine residue at the beginning of the repetitive domain, flanking the N-terminal region, typical of subunit 5.

Use of null lines in qualitative studies

SDS sedimentation test was performed on progeny obtained from a cross between the bread wheat cultivar 'Darius', lacking the *Gli-D1/Glu-D3* components, and the bread wheat landrace 'Nap Hal' lacking HMW-GS encoded at the *Glu-D1* locus. Results indicated that the absence of D-type LMW-GS affected gluten properties. On the contrary, studying the quality characteristics of flours from F4 progeny of a cross between the bread wheat cultivar 'Cheyenne' and a line lacking proteins encoded at the *Gli-D1/Glu-D3* loci, it was observed a large detrimental effect due to the lack of the above protein components. These results can be ascribed to the two different *Glu-D3* alleles (CS-type and CNN-type) compared with the null allele. As explained the most relevant difference between the LMW glutenin pattern of the two alleles is the presence of two D subunits in the CS type which are absent in the CNN-type. As these subunits could have formed as a consequence of a minor mutation of an ω-gliadin gene(s) that give rise to a codon for a cysteine residue, if only one cysteine is present in each D subunit, this can form only one intermolecular disulfide bond, with the consequence that the D subunits could determine a decrement of the size of glutenin polymers similarly to that suggested for other α- and γ-type glutenin subunits.

Purification and characterisation of glutenin components

The two 1D coded D subunits, present in 'Chinese Spring' were purified, using preparative isoelectric focusing and RP-HPLC. Their N-terminal amino acid sequences corresponded to those found for the two 1D ω-gliadins, which they resemble also for *M*r and isoelectric points:

ω-1	KELQSPQQSF
D1	KELQSPQQSF
ω-2	ARELNPSNKELQSPQQSF
D2	ARQLNPSNKE

To show the possible presence of cysteine residues in the D subunits, which should

account for their presence in glutenin, the reduced subunits were reacted with ABD-F, a fluorogenic reagent specific for sulphydryl groups. This was carried out on mixtures with the 1D controlled ω-gliadins, which were assumed to have no cysteine residues. When the mixture was separated on a RP-HPLC system equipped with UV absorbance and fluorescence detector in series, only the D subunit peaks exhibited fluorescence, but not the ω-gliadin peaks. The latter were detected, however, by their UV absorbance (**Figure 36**).

In order to detect number and position of cysteine residues the two 1D-coded D subunits of Chinese Spring were purified and alkylated with ABD-F. Each of the two subunits, made fluorescent with ABD-F, was digested with chymotrypsin and then peptides separated by RP-HPLC. Chromatograms of the chymotryptic digest of ABD-F alkylated subunits D1 and D2 are shown in **Figure 37**.

The upper half shows that the UV absorbance signal at 210 nm and the lower half shows the fluorescence signal. Use of an extended acetonitrile gradient and fluorescence detection involved multiple peaks in each pattern for the D1 and D2 subunits.

These peaks had identical elution times whether derived from D1 or D2, indicating that they corresponded to identical or highly similar peptides. N-terminal sequencing was carried out on the peptides from one subunits (D1).

Peak 1

PQQPQFSQQSFPQPQQSQQ

Peak 2

QSQQPFPQQPQQP

Peak 3

QSQQPFP

Because of the small amounts used for sequencing and the very low recovery of the ABD-F cysteine derivative during the Edman degradation, ABD-F cysteine gives rise to breakdown products, including a major one that elutes at the position of serine; so it is difficult to exclude serine as a possibility when the peak for ABD-F cysteine is weak. This is the reason why serine is indicated as an alternative to cysteine in the sequences reported. We considered the cysteine identification at position 2 in peak 3 as the most reliable. All the three peptides have identical sequences around the apparent cysteines. The three different peptides may have resulted from incomplete chymotryptic cleavages at several sites around the same cysteine residue. Presumably, this is also true of subunit D2 as well. The data obtained, although not completely conclusive, provide supporting evidences of only one cysteine residue per polypeptide chain in the D subunits of glutenin.

3. Publications describing work carried out under Subprogramme B Task B.1.1

Benedettelli S., Margiotta B., Porceddu E., Ciaffi M. and Lafiandra D. (1992). Effects

of the lack of proteins controlled by genes at the *Gli-D1/Glu-D3* loci on the breadmaking quality of wheat. J. Cereal Sci., 16 (1), 69-79.

Buonocore F., Lafiandra D., Caporale C., Porceddu E., Hickman D., Tatham A.S. and Shewry P.R. (1994). Structural studies of a high molecular weight glutenin subunit encoded at the *Glu-D1x* locus in bread wheat. In: Wheat Kernel Proteins: Molecular and Functional Aspects, Proceedings of the Symposium at S. Martino al Cimino, Viterbo (Italy), September 28-30, pp. 203-205.

Lafiandra D., Ciaffi M., Colaprico G. and Margiotta B. (1994). Comparative effect of null lines at the *Glu-D1* and *Glu-D3* loci on wheat qualitative properties. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp. 255-261.

Lafiandra D., D'Ovidio R., Porceddu E., Margiotta B. and Colaprico G. (1993). New data supporting high *M*r glutenin subunit 5 as the determinant of qualitative differences in the pairs 5+10 *vs*. 5+12. J. Cereal Sci., 18 (2), 197-205.

Masci S., Lafiandra D., Porceddu E., Lew E. J.-L., Tao P. and Kasarda D.D. (1993). D-glutenin subunits: N-terminal sequences and evidence for the presence of cysteine. Cereal Chem., 70 (5), 581-585.

Masci S., Lafiandra D., Porceddu E., Lew E. J.-L., Tao P. and Kasarda D.D. (1994). The 1D-coded glutenin subunits from Chinese Spring show 1D-coded ω-type N-terminal sequences. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp. 227-233.

Masci S., Porceddu E. and Lafiandra D. (1991). Two-dimensional electrophoresis of 1D-encoded B and D glutenin subunits in common wheats with similar omega gliadins. Biochem. Genet., 29 (7-8), 403-413.

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1. Key measures of achievement - Objectives

- Purification and characterisation of whole gluten, gluten subfractions and protein subunits of different genotypes;
- Study on the conformational and functional properties of the individual gluten proteins and the interaction of the different proteins and other wheat components.

2. Progress

The project was divided into two parts: the purification and development of purification methods for groups and individual proteins, and the biochemical characterisation of the proteins. The second part of the project was, in part, carried out in collaboration with Partner 23 (University of Viterbo) and Partner 16 (IFR Norwich).

The HMW subunits of glutenin have proved problematical to purify as single subunits in sufficient quantities for physico-chemical characterisation. A procedure was developed to purify HMW subunits in multi-mg quantities. Firstly the production of an enriched HMW subunit preparation using the method described by Marchylo *et al.* (1989), followed by fractionation by ion-exchange chromatography, using 10 *mM* glycine/acetate pH 4.6 buffers on carboxymethyl cellulose, with elution of HMW subunits with NaCl gradients. Subunits in which the cysteine residues were covalently modified with an alkylating group were relatively easy to separate using this technique, the unalkylated subunits, however were more difficult, preparations often being heterogeneous. Further fractionation by ion-exchange chromatography was often required. The final step was purification by reversed-phase HPLC, using gradients of acetonitrile and water, with semi-preparative columns (25× 1 cm) multi-mg quantities could be obtained, from single HPLC runs. In certain cases, resolution of all HMW subunits was not possible, the use of genetic stocks (isogenic, near-isogenic and null lines) facilitated separations, so that 'good' and 'poor' quality associated subunits could be purified in sufficient quantities to enable physico-chemical characterisation. To determine whether the subunits produced by these methods were functionally active, material Was provided to a CSIRO group in Australia to analyse on a prototype 2g Mixograph, the effects of incorporation into gluten polymers in base flours was compared with simple addition of HMW subunits. The flours resulting from simple addition of the HMW subunits were weaker than the base flours, whereas those where the HMW were incorporated into polymers showed an increase in mixing performance, enhancing the mixing properties of base flours. The proteins produced were considered to be functionally active and used for further studies (Békés *et al*., 1994).

A range of biophysical techniques were used to determine if there were differences in properties between HMW subunits that may account for the quality differences associated with different subunits. During the course of the ECLAIR project a paper

was published (Tilley *et al*., 1993) that indicated high levels of glycosylation of the HMW subunits. It was thought important to study glycosylation, an extensive glycosylation would markedly affect the physical properties of the proteins. The methods we were using were confined to protein and not glycoprotein structure. It was decided, therefore, to determine levels of glycosylation using mass-spectroscopy, which would give an approximate value of carbohydrate present. No extensive glycosylation was found within experimental error of the technique employed (matrix assisted laser desorption mass spectroscopy), indicating at most, one or two carbohydrate residues per HMW subunit (Hickman *et al*., 1995). This level of glycosylation, if present, was not considered to have a marked effect on the physical behaviour of the HMW subunits. This study did not lead to a marked deviation from the work plan, material already being prepared for other studies was used.

Fluorescence measurements were used to follow HMW subunit denaturation with urea and guanidinium-.HCl. However, initial studies indicated that the tryptophan residues were exposed to the solvent and would not give detailed information on the pathway of protein denaturation as they were already solvent exposed and not becoming solvent exposed during denaturation. Circular dichroism measurements were made as a function of denaturant concentration to determine secondary structure changes, and all subunits showed a gradual change in secondary structure content increasing denaturant concentration. No sudden structural transitions were found, as are associated with the denaturation of globular proteins or protein domains. Differences between x- and y-type subunits in susceptibility to urea denaturation were found using transverse urea gradient SDS-PAGE. We did not find significant differences between 'good' and 'poor' quality associated subunits. The difference in denaturation susceptibility between x- and y-type subunits was thought to be due to the different repeat motifs of the central repetitive domains, the x-types containing an additional GQQ tripeptide motif that makes the proteins less stable to denaturants.

The techniques used in the second part of the study did not indicate major structural differences/changes in denaturation between subunits which would account for the quality differences associated with different subunits. The differences may require more sensitive techniques than those employed in this study. They may also related to the size and degree of cross-linking of polymers that they are involved in. This aspect was not studied by our group.

HMW subunits of glutenin (both alkylated and non alkylated) were provided to Partner 16 for study by NMR and FT-IR, for comparison w-gliadins and C-hordeins (S-poor prolamins) were also provided. Details of results will be provided by Partner 16 (Belton *et al*., 1994 a, 1994 b, 1995).

Flours from isogenic and near isogenic stocks were provided by Partner 7 (INRA-Nantes)

3. <u>Publications describing work carried out under Subprogramme B Task B.1.1</u>

Békés F., Gras P.W., Gupta R.B., Hickman D.R. and Tatham A.S. (1994). Effects of a high *M*r glutenin subunit (1Bx20) on the dough mixing properties of wheat flour. J. Cereal Sci., 19 (1), 3-7.

Hickman D.R., Roepstorff P., Clarke A.R., Tatham A.S. and Shewry P.R. (1994). Physico-chemical characterisation of high molecular weight subunits of wheat glutenin. In: Wheat Kernel Proteins: Molecular and Functional Aspects, Proceedings of the Symposium at S. Martino al Cimino, Viterbo (Italy), September 28-30, pp. 75-80.

Hickman D.R., Roepstorff P., Shewry P.R. and Tatham A.S. (1995). Molecular weights of the high molecular weight (HMW) subunits of glutenin determined by mass spectrometry. J. Cereal Sci. (in press).

Hickman D.R., Shewry P.R. and Tatham A.S. (1994). The purification of alkylated and unalkylated HMW subunits. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp 460-467.

Tatham A., Hickman D.R. and Shewry P.R. (1994). High molecular weight subunits: a reassessment. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp. 38-46.

Task B.1.2. - Physicochemistry and Functionality of Wheat Proteins

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1. Key measures of achievement - Objectives

- Study of the physico-chemical properties of gluten and subfractions of gluten from wheat of different genotypes;
- Correlation of those properties with the functional properties of wheat to find a possible relation between wheat protein components and wheat functionality in *e.g.* starch/gluten separation industry.

2. Progress

Strategy

The objective of the project was to study how the protein composition of gluten, and especially the presence of polymeric proteins, would influence its rheological properties and to study protein interactions in the viscoelastic network. To reach this goal, it was first necessary to have the suitable tools, *i.e.* the right methods to achieve theses studies. A part of our activity has consisted to acquire such methods as the preparation of functional gluten fractions, the analysis of their rheological properties, the determination of protein sizes and of protein chain mobility.

After the methodology was well established, we studied the influence of several parameters on the rheological properties, the size distribution and the interactions of gluten proteins. We chose to study particular wheat genotypes, that could enable us to identify causal relationships between the compositions of glutens and their properties. These genotypes were essentially near-isogenic lines, substitution lines and translocation lines, which differed by a small number of identified prolamin components. A number of these lines were provided by partners working in the Subprogramme C. In these studies the effects of the HMW and LMW glutenin subunits were analysed.

Role of the size of prolamins in determining the viscoelastic behaviour of gluten.

The gluten subfractions prepared by sequential extraction in dilute hydrochloric acid differed widely in their gliadin/glutenin ratios, in the size of the glutenin polymers and also in their subunit compositions. The more extractable fractions were considerably enriched in monomeric prolamins (gliadins) whereas the content in glutenin polymers

increased in the less extractable fractions (**Figure 38**). Furthermore SE-HPLC allowed to distinguish two groups of glutenin polymers: the medium size polymers and the large size polymers. The rheological behaviour of the isolated fractions was similar to that of whole gluten, *i.e.* that of a transient network, but covered a large range of storage and loss moduli. It appears that gliadins act essentially as a plasticizer in gluten, since the gliadin-rich fractions exhibited a very low viscoelasticity and storage/loss moduli ratio below 1. The viscoelasticity of the subfractions was strongly correlated with the proportion of the large size glutenin polymers (**Figure 39**). Therefore it is assumed that the network connectivity is governed in gluten by the large glutenin polymers. These polymers are the richest in HMW subunits. This could explain the quantitative relationship observed between this type of subunits and the properties of the doughs. Gluten viscoelasticity is based on transient interactions of large concatenations formed by the association of LMW and HMW glutenin subunits.

Gluten network organisation and protein interactions

The physicochemical properties of glutens prepared from normal, defatted and non ionic detergent (removing lipids and non-prolamin proteins, NPP) extracted flours were examined by dynamic rheological analysis and ESR spectroscopy. All the glutens exhibited the same type of viscoelastic behaviour, whatever the conditions of preparation, *i.e.* that of a transient network. Only very slight variations were observed in the mechanical spectra when the lipids were removed, showing that they play a negligible role in the rheology of hydrated gluten (**Figure 40**). They account indeed for a very small fraction of the liquid phase therein. The NPP do not interfere in the organisation of the gluten polymers, for their removal did not change the rheological properties of the gluten. ESR spin probing revealed that two distinct liquid phases exist in gluten: the one is the aqueous phase (hydration water), the other one is the lipid phase (intrinsic lipids organised in vesicles). Using probes with different sizes, it was possible to show that the aqueous phase is distributed between two "compartments": the smaller is possibly corresponding to intra-prolamin aggregate aqueous phase, the larger to inter-aggregate water pockets. ESR analyses showed that labels specific of cysteine residues were binding primarily, if not only, to prolamins. Extraction of the lipids and NPP did not modify the mobility of the prolamin chains. This confirms that the viscoelastic protein network is constituted by the prolamins. The other proteins and the lipids do not interact with this network and do not contribute significantly to the rheological functionality of hydrated gluten.

The same type of network organisation was found in the gluten fractions as in whole gluten. However, the mobility of the protein chains was depending on the concentration in large glutenin polymers. The rigidity of the labelled protein segments was positively correlated to the content of the fractions in large glutenin polymers and also to the network connectivity and viscoelasticity (**Figure 41**). Less mobile side chains corresponded to an increased rigidity of the network. This "immobilisation" of the proteins could be due to the involvement of the polypeptide chains in weak interactions that are more numerous in larger polymers. The effects of heat treatments on gluten were investigated in order to gain information on the role of the covalent and non-covalent bonds in determining the aggregative and functional properties of gluten

proteins. Dynamic rheological analyses showed that complex changes occur in gluten when it is heated at temperatures comprised between 10 and 80°C. In the absence of thiol-blocking agent, a chemorheological ageing was observed. At equilibrium, the frequency of the maximum of the loss compliance peak was shifted towards higher frequencies when the temperature increased, but the mechanical spectra retained the same shape of transient network on the whole range of the temperatures. Gelatinisation of residual starch was clearly not involved in these processes. On the other hand, the variation of the height of the rubbery plateau, G_N^0, which measures the degree of network connectivity, presented two phases. G_N^0 decreased from 10 to 50°C, but it increased above 50°C. Finally at 80°C the value of G_N^0 was more than 100 times that at 50°C (**Figure 42**). Above 50°C, the amount of the large glutenin polymers increased also, because SH-SS interchanges resulted in additional intermolecular cross-linking. ESR spectra of glutens treated in the same conditions have shown that the mobility of the labelled protein segments increased continuously when the temperature increased. This should correspond to the disruption of hydrogen bonds by heating, as indicated by the value of the activation energy of the process (**Figure 43**). In relation with these data, we can interpret the evolution of the rheological behaviour between 10 and 80°C as due to two antagonistic processes. From 10 to 50°C, heating causes the weakening of intermolecular hydrogen bonds, thus that of network connectivity and viscoelasticity. No disruption of covalent bond seems to occur then. Above 50°C, SH-SS exchanges take place and result in additional cross-linking. This increases the size of the prolamin polymers, thus gluten viscoelasticity. This effect counter-balances and even overreaches that of the weakening of H-bonds. In the presence of a thiol-blocking agent, heating has practically no effect on gluten rheology. These data indicate that intermolecular H-bonds play a major role in the interactions of the prolamins in the gluten network, but also that the extent of covalent cross-linking influences strongly gluten rheology.

Effects of glutenin subunit composition on the physicochemical and rheological properties of gluten

The effects of quantitative as well as qualitative variations were examined. It was demonstrated, by analysing near-isogenic lines deleted of 1 to 5 HMW glutenin subunits, that these subunits play a specific role in the formation polymers and confer to them unique properties, although they account for only about 10% of the total gluten proteins. When the concentration of the HMW subunits is decreased, that of large glutenin polymers is reduced, as is gluten viscoelasticity. This effect is very drastic, since variations of 1 to 30 were noted in the values of storage moduli. When all the HMW subunits are suppressed (triple null lines) gluten becomes quasily unextractable, because prolamins do not aggregate sufficiently. Besides, differences in gluten viscoelasticity due to HMW subunit substitutions were confirmed. Wheat lines with the chromosome 1D encoded subunits 5 and 10 showed a higher gluten viscoelasticity than lines with the alleles 2 and 12 (**Figure 44**). In this case, no difference in HMW subunit contents was observed. With subunits 5 and 10 greater amounts of large glutenin polymers were present than with subunits 2 and 12. Differences in the structure of the subunits should explain their different ability to form large and highly aggregative polymers.

The results obtained with the lines differing in their LMW subunit compositions indicated that they influence also gluten rheology, even if in a lesser extent than HMW subunits. Their effect was unclear in the case of the translocated lines in which arms of rye chromosomes were introduced. On the other hand, a comparison of near-isogenic lines have shown that the null allele at the *Glu-D3* locus was related to larger glutenin polymers and higher gluten viscoelasticity. It is postulated that this effect is due to the suppression in the *Glu-D3* null line of D-type glutenin subunits, considered as terminating subunits in the glutenin polymers. Again, polymerization of glutenins appear as a key factor of gluten functionality.

Conclusion

The structure of hydrated gluten is that of a transient network constituted of glutenin polymers, distributed on a wide range of size, and of gliadins acting as a plasticizer. The other proteins (non-prolamin proteins) and the intrinsic four lipids do not interact with the polypeptide chains of the prolamins and do not contribute significantly to the viscoelastic behaviour of gluten. The larger glutenin polymers are the components that contribute predominantly to the viscoelasticity. They are associated through junction zones involving intermolecular hydrogen bonds. Although they account generally for not more than 10% of total gluten proteins, the HMW glutenin subunits play a specific role in the formation of glutenin polymers and confer to them unique properties. The size distribution of the polymers and their aggregative properties depend on their content in HMW subunits and on the structure of the subunits. Polymerisation of glutenins is also influenced by the presence of D-type subunits, acting as possible terminating elements in the polymers. Key factors of gluten functionality are glutenin polymerisation, involving inter-subunit disulfide bonds, and non-covalent association of prolamins through hydrogen bonds.

3. Publications describing work carried out under Subprogramme B Task B.1.2

Published papers

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Cornec M., Roger P. and Popineau Y. (1994). Determination of prolamin sizes by dynamic light scattering. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp 483-486.

Hargreaves J., Le Meste M. and Popineau Y. (1994). ESR studies of gluten-lipid systems. J. Cereal Sci., 19 (2), 107-113.

Hargreaves J., Le Meste M., Cornec M. and Popineau Y. (1994). Electron spin resonance studies of wheat protein fractions. J. Agric. Food Chem., 42 (12), 2698-2702.

Hargreaves J., Popineau Y. and Le Meste M. (1994). ESR spectroscopy, its application to gluten systems. In: Gluten Proteins 1993, Proceedings of the 5th

International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp. 114-123.

Hargreaves J., Popineau Y., Cornec M. and Lefebvre J. (1995). Relationships between aggregative, viscoelastic and molecular properties in gluten from genetic variants of bread wheat. Internat. J. of Biological Macromolecules (accepted).

Hargreaves J., Popineau Y., Le Meste M. and Hemminga M.A. (1994). Molecular flexibility in wheat gluten proteins submitted to heating. FEBS Letters (accepted).

Hargreaves J., Popineau Y., Marion D., Lefebvre J. and Le Meste M. (1995). Gluten viscoelasticity is not lipid-mediated. A rheological and molecular flexibility study on lipid and non-prolamin protein depleted glutens. J. Agric. Food Chem., 43 (5), 1170-1176.

Lefebvre J., Popineau Y. and Cornec M. (1994). Viscoelastic properties of gluten proteins: influence of prolamin composition and of temperature. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp. 180-189.

Lefebvre J., Cornec M., Pézolet M. and Popineau Y. (1994). The network structure and viscoelasticity of gluten. In: Wheat Kernel Proteins: Molecular and Functional Aspects, Proceedings of the Symposium at S. Martino al Cimino, Viterbo (Italy), September 28-30, pp. 227-231.

Popineau Y., Cornec M., Lefebvre J. and Marchylo B. (1994). Influence of HMW glutenin subunits on glutenin polymers and rheological properties of glutens and gluten subfractions of near-isogenic lines of wheat Sicco. J. Cereal Sci., 19 (3), 231-241.

Popineau Y., Pogna N. and Lefebvre J. (1994). Rheological properties of glutens differing by their glutenin subunit compositions. In: Wheat Kernel Proteins: Molecular and Functional Aspects, Proceedings of the Symposium at S. Martino al Cimino, Viterbo (Italy), September 28-30, pp. 129-134.

Delivered papers

Hargreaves J., Douillard R. and Popineau Y. (1994). Application of the polymer scaling concept to purified gliadins at air-water interface. Poster presented at the Conference "Food, Macromolecules and Colloids", Dijon (France).

Hargreaves J., Popineau Y., Le Meste M. and Hemminga M.A. (1994). The effect of heat on gluten as seen by ESR spectroscopy. Proceedings of the Conference on Application of Magnetic Resonance in Food Science (Poster), Aveiro (Portugal).

Lefebvre J., Cornec M. and Popineau Y. (1992). Rheological properties of wheat gluten proteins: influence of HMW glutenin subunit composition and molecular size. Presented at the 9th International Congress of Cereals and Bread, Paris, June.

Lefebvre J., Cornec M. and Popineau Y. (1992). Viscoelasticity of fully hydrated wheat gluten and the effect of temperature. Poster presented at the 11th International Congress on Rheology, Brussels.

Lefebvre J., Popineau Y. and Cornec M. (1994). The network structure and viscoelasticity of gluten. Poster presented at the Symposium "Wheat Kernel Proteins: Molecular and Functional Aspects", September 28-30, Viterbo (Italy).

Thesis

Cornec M. (1994). Comportement rhéologique du gluten : influence de la composition en prolamines et de leur état d'association. Thèse de Doctorat, Université de Nantes (France).

Hargreaves J. (1995). Etude physico-chimiques de l'organisation du réseau protéique du gluten de blé. Thèse de Doctorat avec Label Européen, Université de Bourgogne, Dijon (France), 20 Avril.

Task B.1.3. - Gluten Hydration and Interactions of Gluten Proteins with Other Components

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1. Key measures of achievement - Objectives

- Elucidation of the mechanism of gluten hydration.
- Study of the mechanisms which play a role in the interaction processes between gluten and other components.

2. Progress

It is generally accepted that differences in the baking quality of wheat flours are attributable to the viscoelastic properties of the hydrated gluten proteins, which depend in turn on the ability of a proportion of the proteins to form high molecular weight polymers. There is little consensus on the significance of non-covalent interactions which undoubtedly occur in both the hydrated gluten mass and in the wheat grain. Questions concerning the relative importance of hydrogen bonding and hydrophobic interactions are still controversial. In this work we have used nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) spectroscopy to investigate the relationship between these interactions and the level of protein hydration.

1. Initially we sought to determine whether the ^1H NMR relaxation time (T_2) of water in doughs could be related to the flour used (good/poor breadmaking quality) or to the mixing time. Little dependence on these variables was found and it was concluded that relaxation was determined by exchange of water protons with sites on the abundant starch molecules. Even in glutens no correlation could be found between T_2 and the origin of the gluten.

2. ^2H relaxation time (T_2) measurements of water in D_2O hydrated glutens were analysed as a sum of two or three exponentially decaying components. Results were typical of systems undergoing combined chemical exchange (of labile water and protein ^2H atoms) and diffusion (of water molecules between regions of different protein content). The component with the longest relaxation time, identified with the excess free water, decreased in proportion as the temperature was raised. This indicates that gluten is fundamentally hydrophilic despite being water insoluble.

3. The same NMR experiment showed even greater absorption of water by starch free gluten, proving that the protein, and not the residual starch, was responsible for the water loss. Extraction of lipids prior to hydration had little effect. The procedure required for complete starch removal appeared to cause irreversible changes to the gluten structure (as confirmed by FTIR spectroscopy).

4. Reduction of the bulk water fraction with increasing temperature was also noted in experiments on a high *M*r subunit, prepared in both an unalkylated form with intermolecular disulphide bonds, and in a reduced and alkylated (unpolymerised) form. This cast doubt on the proposal that the occurrence of repetitive β-turns in the structures of elastin and high *M*r subunits could provide a link between the mechanism of elasticity in the two proteins. Elastin/water mixtures, however, exclude water as the temperature is raised and the hydrophobicity of elastin is fundamental to explanations of the protein's elasticity.

5. A variety of NMR experiments (^{1}H, ^{13}C) showed that hydration produces a marked increase in the chain mobility of prolamins. This is in contrast to the behaviour of globular proteins at similar hydration levels. HMW subunits of glutenin, ω-gliadins and C-hordein (of barley), purified at IACR, Long Ashton, were examined in these experiments. T_l and T_{lr} measurements showed that the rotational freedom of the amide groups of glutamine side-chains was enhanced by addition of water. T_2^*, an empirical parameter characterising the decay time of the protein ^{1}H NMR signal is related to chain mobility and increases dramatically above a threshold water content (**Figure 45**). Parts of the protein chain are maintained in a less mobile state, even in excess water.

6. The FTIR spectra of protein films (high *M*r subunits, ω-gliadins) and model compounds (deamidated gliadins, polyglutamine, polyproline), hydrated to different levels, were interpreted (**Figure 46**). In dry proteins, secondary structure assignment is complicated by the contribution of glutamine side-chains to the amide I band. In the hydrated state the different contributions to the prolamin spectra become separable and were unequivocally assigned for the first time by comparison with model compounds.

7. The FTIR results indicated that internal hydrogen bonding (involving glutamine residues and the peptide backbone) gives the dry proteins a rather compact, rigid structure. Progressive hydration weakens these interactions, leading to a balance between protein-protein and protein-water interactions, and allowing changes in favour of more extended chain and β-sheet type structures to occur. These changes of secondary structure are very noticeable at water contents (30 - 40%) in the region of the NMR mobility threshold. β-sheet structure is absent in either the dry or fully hydrated (sol or gel) states of the protein.

8. It is proposed that addition of water creates mobile protein segments with freedom to associate with, and dissociate from, similar segments. The association can be inter- or intramolecular (in view of the repetitive nature of the sequences) and gives rise to new secondary structures. The association probably extends over short sequences only but the number of potential association sites makes complete dissociation of any chain statistically unlikely. These features were incorporated in a new model of gluten elasticity in which the consequences of applying external forces to such a system were explored.

3. Publications describing work carried out under Subprogramme B Task B.1.3

Belton P.S. (1994). A hypothesis concerning the elasticity of high molecular weight

subunits. In: Wheat Kernel Proteins: Molecular and Functional Aspects, Proceedings of the Symposium at S. Martino al Cimino, Viterbo (Italy), September 28-30, pp. 159-165.

Belton P.S. and Gil A.M. (1993). Proton nuclear magnetic resonance lineshapes and transverse relaxation in a hydrated barley protein. J. Chem. Soc. Faraday Trans., 89 (23), 4203-4206.

Belton P.S., Gil A.M. and Tatham A.S. (1994). ^{1}H NMR relaxation time studies of the hydration of the barley protein C-hordein. J. Chem. Soc. Faraday Trans., 90 (8) 1099-1103.

Belton P.S., Colquhoun I.J., Field J.M., Grant A., Shewry P.R. and Tatham A.S. (1994). ^{1}H and ^{2}H NMR relaxation studies of the high M_r subunits of glutenin and comparison with elastin. J. Cereal Sci., 19 (2), 115-121.

Belton P.S., Colquhoun I.J., Grant A., Wellner N., Shewry P.R. and Tatham A.S. (1994). NMR and FTIR studies on the hydration of a high M_r subunit of glutenin. In: Wheat Kernel Proteins: Molecular and Functional Aspects, Proceedings of the Symposium at S. Martino al Cimino, Viterbo (Italy), September 28-30, pp. 210-212.

Belton P.S., Colquhoun I.J., Field J.M., Grant A., Shewry P.R., Tatham A.S. and Wellner N. (1995). FTIR and NMR studies on the hydration of a high M_r subunit of glutenin. Intern. J. Biol. Macromol., 17 (2), 74-80.

Task B.1.4. - The Role of Minor Protein Components Associated with Starch Granules

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1. Key measures of achievement - Objectives

- To establish the role(s) of starch granule protein in relation to functional properties of wheat.
- To devise a predictive test for endosperm texture on the basis of starch granule surface components for use in wheat breeding and at mill intake.

2. Progress

Previous studies by Schofield and Greenwell (FMBRA) showed an association of the 15K surface protein and friable endosperm, but the role of starch granule protein in relation to functional properties of wheat, and the relation of this protein to the hard and soft alleles of the Hardness gene had still to be established.

In fact, FMBRA showed that anti-friabilin F7F antibody could not provide a predictive test on the endosperm texture in bread wheat, but a useful application for it was found in a durum wheat purity test (Durotest).

Major progress was achieved by comparison of the basic friabilin components (through capillary electrophoresis, NEPHGE, N-terminal sequences, immunoblotting) to the lipoproteins extracted by the detergent Triton X-114 by Marion at INRA (Nantes, France).

The results obtained have considerably advanced our knowledge of the biochemical nature of friabilin, and have began to clarify the status of friabilin as lipid-binding proteins. For instance a strong homology was demonstrated between some starch granule proteins (friabilin basic 2-3) and the main lipid binding protein named puroindoline *b* in regard to its unique tryptophan-rich domain.

So, friabilins are involved in some way with endosperm texture, but not in a way that has so far enabled us to use them in a rapid, sensitive diagnostic test for this important quality parameter of bread wheat. Presence of friabilin on starch might occur during starch purification and further work is needed to explain the true molecular basis of friabilin starch interaction. Moreover, bases of hardness are more likely to involve a lipid-like factor binding friabilin to starch on the surface of the granules.

3. Publications describing work carried out under Subprogramme B Task B.1.4

Delivered papers

Greenwell P. (1993). Flour proteins related to endosperm texture of bread wheat. Poster presented at the 5th International Gluten Protein Workshop, Detmold, Germany, 7-9 June.

Greenwell P. (1993). Flour proteins related to endosperm texture of bread wheat. Poster presented at the International Conference on Bread- from Breeding to Baking, FMBRA, Chorleywood, U.K., 15-16 June.

Greenwell P. and Brock C.J. (1993). Lecture entitled "Identity of starch-granule-surface proteins (friabilins) of bread wheat with detergent-soluble lipid-binding proteins from flour", presented at the AACC Annual Meeting, Miami, Florida, USA, 3-7 October. Abstract published in Cereal Foods World, 1993, 38, 615-616.

Sulaiman B.D., Brennan C.S., Greenwell P. and Schofield J.D. (1993). Lecture entitled "Isolation of friabilin and the use of polyclonal antisera for immunolocation studies", presented at the AACC Annual Meeting, Miami, Florida, USA, 3-7 October. Abstract published in Cereal Foods World, 1993, 38, 616.

Task B.1.5. - Lipid Interactions

<u>Partner 07N - INRA-Nantes</u>

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1. Key measures of achievement - Objectives

- Study of the wheat lipid composition and behaviour in dough with non-invasive methods;
- Study on the mechanisms which play a role at the interaction processes between wheat lipids and other components.

2. Progress

Strategy

Lipids play an important role in cereal technology. Wheat flour polar lipids or added surfactants are involved in the formation and stabilisation of the gas phase into bread doughs. These phenomena have positive effects on bread volume and crumb texture, two important criteria for bread quality (Marion, 1992).

However, lipids are not single and isolated components in bread doughs but form strong lipid-lipid and lipid-protein associations. Especially specific lipid binding proteins are able to interact with polar lipids. The aim of the project was to isolate and characterize these proteins, their interactions with lipids and their functional properties. During the course of this project an unexpected relationship between lipid binding proteins and proteins associated with starch granules (Task B.1.4) was highlighted. This discovery has stimulated a collaborative work with Partner 14 to study the localisation of these proteins in wheat seeds and to study the variability of lipid binding protein contents.

Isolation, purification of new wheat lipid binding proteins

The major problem encountered in the study of lipid binding protein is their extraction and specific isolation. Previous works on wheat lipid binding proteins have always led to the isolation of low molecular weight proteins and generally, of cysteine-rich proteins (for example thionins, CM-proteins, ligolin, S-proteins...). It was therefore obvious that the major lipid-binding proteins from wheat seeds should belong to the albumin fraction as defined by Osborne at the beginning of this century. Furthermore, looking back at the literature, it was also obvious that these proteins share common structural principles with most membranotoxins from bacteria and animal venoms and with the plant phospholipid transfer proteins. This statement means that two main group of proteins have to be considered in the soluble wheat protein fraction: transport and membrane active proteins. For the first type, an *in vitro* transport fluorescence

assays were used (Désormeaux *et al.*, 1992) and for the second type, we have developed a phase partitioning procedure in TX114 which is generally used to isolate transmembrane proteins (Blochet *et al.*, 1993). TX114 phase partitioning of wheat proteins has also permitted to improve the overall fractionation of wheat proteins, especially gliadins and glutenins, and to produce lipid-free and still functional glutens (showing that wheat lipids are not essential in the rheological properties of gluten) (Marion *et al.*, 1994; Hargreaves *et al.*, 1995). These methods have selected different new wheat proteins and among them, two abundant proteins were chosen as models for structure-function studies: a 9 kDa lipid transfer protein and puroindolines.

The main lipid transfer protein (LTP) isolated using the transfer assay has 90 amino acid residues for a molecular weight of 9,607. This LTP is basic and is composed of 8 cysteines forming four disulphide bridges. Its primary sequence exhibits high homology with the LTPs isolated from different plant sources. By TX114 phase partitioning a new family of proteins was isolated (Blochet *et al.*, 1993; Gautier *et al.*, 1994). The main component which was characterized was composed of 115 residues for a molecular weight of 12,759. This basic protein contains 10 cysteines forming 5 disulphide bridges and exhibits an unique tryptophan-rich domain (WRWWKWWK) which has led us to name this wheat protein, puroindoline. Subsequently, an other isoform was discovered, but with a truncated tryptophan-rich domain (WPTKWWK). Therefore, the former isoform was named puroindoline-a and the latter puroindoline-b (Gautier *et al.*, 1994).

The structure of major wheat lipid binding proteins

Puroindolines does not exhibit sequence homologies with any other known cystine-rich wheat protein but it is interesting to note that it is possible to find a relatively good alignment of the sequences of LTP and puroindolines except in the zone corresponding to the tryptophan-rich domain (**Figure 47**). The assignment of the disulphide bridges strengthens this structural homology. 4 out of 5 disulphide links are strictly identical between LTP and puroindolines: the fifth disulphide bridges links cysteines at the beginning and at the end of the non homologous zone containing the tryptophan-rich domain (**Figure 48**)(Guy *et al.*, 1994). Finally FTIR spectroscopy shows that the helix content of both proteins are quite similar (**Table XIII**). These results suggest that LTP and puroindolines exhibit probably a similar fold (Marion *et al.*, 1994).

The 3D structure of the wheat LTP was determined by multidimensional ^{1}H NMR in collaboration with the Centre de Biophysique Moléculaire (CNRS, Orléans, France)(Gincel *et al.*, 1994). The polypeptide backbone folds into a simple and original right handed winding. It is composed of a bundle of 4 helices linked by flexible loops which is packed against a C-terminal fragment having a non standard "saxophone" shape (**Figure 49**) (Gincel *et al.*, 1994). A similar fold could be modelled for puroindolines in which the tryptophan-rich domain would be included in a loop corresponding to the enlarged loop L1 of LTP (**Figure 50**)(Marion *et al.*, 1994). It is interesting to note that this modelling of the structure of puroindolines led to the polarization of the aromatic side chains (tryptophan and phenylalanine residues) present in these proteins. This is important because aromatic residue are often

involved in the interaction of proteins with membrane lipids.

The functional properties of puroindolines

Lipid binding

The lipid binding properties of LTP and puroindolines are quite different as expected from the TX114 phase partitioning behavior of these proteins. After phase partitioning LTP is found in the detergent poor phase and the puroindolines in the detergent rich phase. This behavior is respectively typical of extrinsic and intrinsic (transmembrane) membrane proteins. This means that LTP should interact only with the polar interface of lipid bilayer membranes while puroindoline should interact with both the polar interface and the hydrophobic core. This is clearly demonstrated by following the dynamic of fatty acyl chain during the gel-fluid liquid-crystalline (LC) phase transition of phospholipids by fluorescence polarization of a lipophilic probe embedded in the lipid bilayer. LTP induces a slight increase of transition temperature of DMPG liposomes from 23°C to 25°C without changing the cooperativity of the transition. This indicates that the protein is adsorbed on the surface of the bilayer liposomes and stabilises slightly the intermolecular interaction between the polar head groups retarding the transition temperature. On the contrary, puroindolines induces a decrease of the cooperativity of the transition until it is suppressed at protein saturation (Ri = 5) (**Figure 51**). This means that the protein penetrates sufficiently in the hydrophobic core to prevent fatty acid chains melting during the gel-fluid L.C. phase transition.

Comparison of the 3D structure of LTP and puroindolines suggest that the tryptophan-rich domain could be involved in the strong anchoring of puroindolines in bilayer membranes. It is well known that tryptophan plays a major role in the interaction of some membranotoxins with membranes and in the orientation of transmembrane hydrophobic helices in the lipid bilayer.

The weak interaction between LTP and liposomes is in agreement with the lipid transfer activity of such proteins which supposes obviously the formation of a transient complex between donor and acceptor membranes. However, this transfer activity supposes also that a lipid binding site is suited for receiving a lipid molecule. This complex cannot be observed with diacyl phospholipids forming liposomes but it is easily observed with monoacyl lipids which form micelles and behave as a normal ligand under their CMC. These complexes were investigated by FTIR and fluorescence spectroscopies (Désormeaux *et al*., 1992; Marion *et al*., 1994). One site was identified with a Kd of about 70μM, a value agreeing with a binding-release mechanism necessary to the transfer activity of LTP. With diacyl phospholipids, monolayer studies show that the binding occur at low surface pressure (10 mN/m) whereas an increase of this pressure (15 mN/m) leads to the transfer of the protein under the lipid film. However the fatty acyl chain is still bound to the protein and an increase of the surface pressure above 25 mN/m induces the release in the subphase (water) of a lipid-protein complex. Since LTP structure reveals that the hydrophobic tunnel can bind only one chain it is suggested that the second fatty chain could be bound to the lipid layer at 15 mN/m while above 20 mN/m it could be bound to another protein. Such models were already proposed for some extrinsic membrane

bound proteins (Subirade *et al*., 1995).

Antifungal activities

When a membrane active protein is isolated, it is generally interesting to explore its antimicrobial properties. Such activities can have important involvement in the defence mechanism of plant and consequently in their resistance to microbial pathogens. The antifungal activities of the wheat lipid binding proteins were tested (**Table XIV**). LTP does not exhibit such activities on different fungi. However, puroindolines are more efficient depending of the fungal strains. It is interesting to note that an important synergistic effect is observed when both purothionins and puroindolines are present in the medium (**Figure 52**). It is therefore obvious that the antimicrobial activity is related to the more or less strong association formed between proteins and membrane lipids (Marion *et al*., 1994).

Foaming properties

In a product such as bread the gas retention and expansion into dough during mixing proofing and baking are the key mechanisms to determine the quality of the end product. Polar lipids are known to play a role in forming a monolayer film at air-water interfaces and the effect of lipid binding proteins can be important in the stability of such films (Marion, 1992). This was investigated in the case of puroindolines in studying the foaming properties of puroindoline-phospholipid mixtures. A model system was chosen, composed of a phospholipid analogue, lysoPC and puroindoline a. The most surprising result was the improvement of foam stability when increasing amounts of phospholipid is added to the protein (Wilde *et al*., 1994). This occurs at lipid concentrations largely below concentration needed to form a foam from lysoPC alone. This synergistic effect is probably due to the formation of interaction between the lipid and protein components. With non lipid binding protein this generally led to a competitive displacement of the protein by the surfactant. On the contrary with puroindolines, this phenomenon is only observed for high concentration of the lysoPC (**Figure 53**).

In the case of lipid transfer proteins a group in Carlsberg Institute has demonstrated that barley LTP is an abundant proteins in beer foams and that such a protein exhibit in vitro good foaming properties. In the case of the homologous wheat protein we have shown using Langmuir techniques that its surface properties are quite good (Subirade *et al*., 1995).

Relationships with starch granule proteins, friabilins

During the course of this EEC program an unexpected results were obtained: puroindolines and basic friabilin belongs to the same protein family. Friabilin are proteins found on the surface of starch granule of soft wheat cultivar and it was suggested that they are involved in grain softness (Task B.1.4.). In fact the N-terminal amino acid sequence of the main basic friabilin is identical to that of puroindoline-b. The localisation of puroindoline in wheat grain using specific antibodies raised against puroindoline-a and puroindoline-b revealed that these proteins are mainly located in the aleurone layer and at the periphery of starchy endosperm (Dubreil *et al*. 1994).

This localisation precludes that puroindoline-basic friabilins- are functional marker of grain softness. The main question is their use as genetic markers of grain softness.

Conclusion

Through the isolation and structure-function characterisation of new lipid binding proteins (puroindolines and lipid transfer proteins) important progress was realized on the lipid-protein interactions in wheat dough systems. Although the functional properties of these proteins was only approached *in vitro*, the results obtained open new exciting perspective to improve the functionality of wheat lipids through the lipid binding proteins. In the future, this new strategy should be considered by wheat breeders to improve the quality of EC wheats. It is also interesting to note that lipid binding proteins are not only interesting for their surface activities but also for their antimicrobial properties, a result which should have important consequences in the defence mechanisms of plant against microbial pathogens and for the preservation of cereal containing food.

3. Publications describing work carried out under Subprogramme B Task B.1.5

Published papers

Blochet J.E., Chevalier C., Forest E., Pebay-Peroula E., Gautier M.F., Joudrier P., Pézolet M. and Marion D. (1993). Complete amino acid sequence of puroindoline, a new basic protein with a unique tryptophan-rich domain, isolated from wheat endosperm by Triton X-114 phase partitioning. FEBS Letters, 329 (3), 336-340.

Désormeaux A., Blochet J.E., Pézolet M. and Marion D. (1992). Amino acid sequence and conformation from Infrared and Raman spectroscopy of a wheat non-specific phospholipid transfer protein. Role of disulfide bridges and phospholipids in the stabilisation of the alpha helix structure. Biochim. Biophys. Acta, 1121 (1-2), 137-152.

Gautier M.F., Aleman M.E., Guirao A., Marion D. and Joudrier P. (1994). *Triticum aestivum* puroindolines, two basic cystine-rich proteins: cDNA sequence analysis and developmental gene expression. Plant Mol. Biol. 25 (1), 43-57.

Gincel E., Simorre J.P., Caille A., Marion D., Ptak M. and Vovelle F. (1994) Three-dimensional structure in solution of a wheat lipid transfer protein from multidimensional ^{1}H-NMR data- a new folding for lipid carriers. Eur. J. Biochem. 226, 413-422.

Hargreaves J., Popineau Y., Marion D., Lefebvre J. and Le Meste M. (1995) Gluten viscoelasticity is not lipid-mediated. A rheological and molecular flexibility study on lipid and non-prolamin protein depleted glutens. J. Agric. Food Chem. (in press).

Marion D. (1992). Functionality of wheat lipids in baking industry. Presented at the 9th International Cereal and Bread Congress "Cereal Chemistry and Technology: a Long Past and a Bright Future" (P. Feillet, ed.), Paris, 1-5 June

1992, pp. 57-62.

Subirade M., Salesse C., Marion D. and Pézolet M. (1995) Interaction of a non-specific wheat lipid transfer protein with phospholipid monolayers imaged by fluorescence microscopy and studied by infrared spectroscopy. Biophys. J. (in press).

Wilde P.J., Clark D.C. and Marion D. (1993). The influence of competitive adsorption of lysopalmitoyl phosphatidylcholine on the functional properties of puroindoline, a lipid binding protein isolated from wheat flour. J. Agric. Food Chem., 41, 1570-1576.

Delivered papers

Blochet J.E., Gautier M.F., Joudrier P., Pézolet M. and Marion D. (1993). Structure and lipid-binding properties of puroindoline, a new basic and cystine rich protein isolated from wheat endosperm. 22nd FEBS Meeting, Stockholm, Sweden, July 4.

Blochet J.E., Marion D. and Pézolet M. (1993). Secondary structure and conformation of puroindoline: a new cystein-rich and amphiphilic wheat protein with a unique tryptophan-rich domain. Biophysical Society Annual Meeting, Washington, USA, February 14-18.

Compoint G., Clark D.C. and Marion D. (1994). Role of aromatic side chains in protein-phospholipid interactions: an illustration with puroindoline-a and b two related lipid binding proteins from wheat flour. Food Macromolecules and Colloids Conference, Royal Society of Chemistry, Dijon, France, March 23-25.

Compoint G., Marion D., Narahisoa H. and Haertlé T. (1994). Role of tryptophan side chains in the interaction of puroindolines and their related synthetic tryptophan-rich peptides with phospholipids. Poster, in: Wheat Kernel Proteins: Molecular and Functional Aspects, Proceedings of the Symposium at S. Martino al Cimino, Viterbo (Italy), September 28-30, p. 201.

Dubreil L., Quillien L. and Marion D. (1994). Wheat lipid binding proteins probed by polyclonal antibodies. 14th ICC Congress, The Hague, The Netherlands, June 5-9.

Dubreil L., Quillien L., Compoint J.P. and Marion D. (1994). Variability and location of wheat kernel indolines and lipid transfer proteins. Poster, in: Wheat Kernel Proteins: Molecular and Functional Aspects, Proceedings of the Symposium at S. Martino al Cimino, Viterbo (Italy), September 28-30, pp. 331-333.

Gautier M.F., Aleman M.E., Marion D. and Joudrier P. (1993). Characterisation of cDNA clones encoding Triticum aestivum cystine-rich proteins. 22nd FEBS Meeting, Stockholm, Sweden, July 4.

Guy P., Ulrich J., Forest E., Scherrer N., Gagnon J. and Marion D. (1994). Assignment of the disulphide bridges in puroindoline, a new basic and cystine-rich protein isolated from wheat endosperm. Third International Symposium of Mass

Spectrometry in the Health and Life Sciences. San Francisco, USA, September 13-18.

Marion D., Compoint J.P., Branlard G. and Landry J. (1994). Non ionic detergents: a tool to improve the fractionation of wheat proteins and to produce a lipoprotein-free gluten. 14th ICC Congress, The Hague, The Netherlands, June 5-9.

Marion D., Gautier M.F., Joudrier P., Ptak M., Pézolet M., Forest E., Clark D.C. and Broekaert W. (1994). Structure and function of wheat lipid-binding proteins. In: Proceedings of the International Meeting "Wheat Kernel Proteins: Molecular and Functional Aspects", September 28-30, Viterbo (Italy), pp. 175-180.

Subirade M., Pézolet M. and Marion D. (1994). Influence of a lipid-binding protein on the conformation and orientation of phospholipid monolayers studied by polarized attenuated total reflectance infrared spectroscopy. Biophysical Society Annual Meeting, New-Orleans, USA, March 6-10.

Subirade M., Salesse C., Pézolet M. and Marion D. (1994). Interaction of a plant lipid transfer protein with phospholipid monolayers: a fluorescence microscopy and ATR study. Food Macromolecules and Colloids Conference, Royal Society of Chemistry, Dijon, France, March 23-25.

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1. Key measures of achievement - Objectives

Study of the surface active behaviour in dough. The influence of lipids, mixing and wheat type on this behaviour.

2. Progress

Work was carried out on the interfacial behaviour of dough during mixing. Dough was prepared from a commercial flour, with no other additives than salt, sugar, yeast and water. Samples were taken at different mixing times. The interfacial behaviour was studied with the aid of an overflowing cylinder. During mixing the components which determine the surface behaviour changed from a high molecular character to a low molecular character. The influence of lipids on the surface behaviour is an increase of it. This effect disappears during the fermentation process.

Besides, research was done on the interfacial behaviour of doughs prepared from different flour types (hard and soft wheats), provided by Philip Greenwell (Partner 14). Doughs were prepared from with no other additives than salt, sugar, yeasts and water, just like the former studies. Also the influence of the mixing time on the interfacial behaviour was studied, with the aid of an overflowing cylinder.

The flours tested were for the soft wheats; 'Admiral', 'Hunter', the 'Maris Hobbit' sib parent lines MHs-1-1 and MHs-3-1 and the chromosome-engineered 'Maris Hobbit' sib lines MHs (Bez5D)-1-1 and MHs (Bez5D)-3-2. For the hard wheats the following lines were tested; 'Mercia', 'Hereward' and the chromosome-engineered 'Maris Hobbit' sib lines MHs (Bez5D)-1-1 and MHs (Bez5D) -3-1.

With the aid of this technique no differences in surface behaviour could be observed between the flours from hard and soft wheats.

Conclusions

The breakdown of the gluten macropolymers during mixing can be clearly seen in the surface active behaviour of dough samples taken during this action. Also the influence of added lipids on the surface behaviour can be clearly demonstrated.

As started above one cannot observe differences in the surface active behaviour of the different soft and hard wheat types, which show clear differences in the amount of friabilin present.

3. Publications describing work carried out under Subprogramme B Task B.1.5

Plijter J.J. (1994). The surface behaviour of dough. In: Wheat Kernel Proteins:

Molecular and Functional Aspects, Proceedings of the Symposium at S. Martino al Cimino, Viterbo (Italy), September 28-30, p. 181.

Task B.2. - Dynamics of Dough Development

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1. Key measures of achievement - Objectives

- To study the behaviour and interactions of wheat components in doughs and baked goods with the aid of antibodies against the different components and microscopic techniques.

2. Progress

Subtask B.2.1. - Development of microscopic techniques for examining bread doughs

A **Developing of labelling techniques for scanning electron microscopy** (SEM):methodology was developed using the SEM which has advantages in terms of sample throughput, speed, and facilitates interpretation of labelling patterns in relation to sample topography. After light fixation and blocking with bovine serum albumin (BSA) samples were incubated with specific antibody, followed by anti-mouse IgG/IgM labelled with 1 nm colloidal gold. By using a silver enhancement procedure these particles were increased in size to 60 - 90 nm, rendering them clearly visible in the SEM.

B **Application to detection of gluten proteins in wheat and bread:** A range of anti gluten Mabs of increasing specificity were employed to explore the effectiveness of the silver-enhanced immunogold labelling technique. These were:

IFRN 0033 - γ-gliadin reactive Mab

IFRN 0610 - broadly reactive Mab recognising part of the repetitive domain sequence PQQFSH

IFRN 1602 - specific for lA and 1Dx-type HMW subunits

IFRN 0067 - specific for a 1D encoded LMW subunit of glutenin

All the Mabs recognised prolamins in both wheat and bread. Binding to cut-surfaces in bread was much heavier than around the gas-cells, and labelling of the latter was patchy. This confirmed the findings of a previous transmission EM study employing IFRN 0033. It therefore appears that (1) prolamin protein epitopes are not uniformly available on the gas cell surface, possibly due to the distribution of lipid, (2) epitopes of all anti-gluten Mabs tested were intact after baking, confirming biophysical studies that these proteins do not undergo denaturation in the way that globular

proteins do, and (3) that LMW and HMW subunits of gluten are distributed evenly throughout the protein matrix of wheat and bread. Due to the lack of Mab probes sensitive to heat denaturation of gluten proteins no investigations were made on doughs or part-baked breads, efforts being focused on other tasks.

Subtask B.2.2 - Characterisation of polyclonal and monoclonal antibodies to wheat pentosans (arabinoxylans)

Development of ELISA formats

Two strategies were employed to immobilise the hydrophilic arabinoxylans to microtitration plates. One utilised a lectin from red marine algae, which binds the terminal galactose on arabinoxylans, to coat microtitration plates and capture the polysaccharide. The second made use of feruloylated fragments (ferulic acid - arabinose (xylose)3, FAX3) coupled to a carrier protein, the FAX3-conjugate produced then being used to provide the ELISA solid phase.

Production of polyclonal antibodies

Two polyclonal anti-arabinoxylan antisera were produced to arabinoxylan from bees wing bran. Their usefulness was limited due to the presence of anti-gluten protein antibodies present in the antiserum from dietary exposure of animals to cereals.

Production of Mabs

Either a water insoluble arabinoxylan preparation from bees wing bran, or a FAX3-BSA conjugate was employed as the immunogen, Mabs being selected with the ELISA formats described above. Two Mabs were characterized in detail, which were raised to each of the immunogens, IFRN 0418 (to bees wing bran arabinoxylan) and 0420 (to FAX3-BSA).

Characterisation of Mabs

Dose-response curves with each Mab were obtained using both ELISA formats. IFRN 0418 was found to be totally specific for wheat arabinoxylans, recognising both soluble and insoluble forms (**Figure 54**) (provided by TNO, Partner 17). IFRN 0420 has yet to be characterized in detail.

Subtasks B.2.3 - Role of pentosans (arabinoxylans) in the structure of dough and baked products

Subtask B.2.4 - Study of interactions between proteins, pentosans and lipids in doughs and baked products.

Samples of white and wholemeal bread were obtained from CCFRA (Partner 14) and Gist Brocades (Partner 15) which was baked without improvers, with fungal amylase (crude, FMBA; pure, Gist Brocades) or with xylanase. CCFRA samples were prepared using the Chorleywood bread process, whilst those prepared by Gist Brocades had undergone a multi step fermentation process. Immunolabelling was performed using

the anti-arabinoxylan Mab IFRN 0418 and anti-gliadin Mab 0033. IFRN 0418 did not bind to the surfaces of cell walls present in bran or residual cell wall material originating from wheat endosperm (**Figure 55**). Labelling of bran *per se* indicated that the bulk of 0418 reactive sites were to be found at the edges where the bran layer was cross-sectioned. Clearly arabinoxylans are not accessible for Mab binding at cell wall surfaces. However, 0418 did bind to the protein matrix of wheat and bread indicating that soluble arabinoxylans may become attached to gluten protein, possibly during the labelling process (wheat) and/or during bread manufacture.

Addition of carbohydrases increased the size of silver - enhanced gold labelling obtained, indicating that such treatments altered the distribution of immunoreactive arabinoxylans in bread. This was particularly marked in xylanase treated breads, or those where a crude α-amylase preparation was employed which probably contained hemicellulases. The larger silver particles observed, result from a greater degree of clustering of 0418 binding sites, the individual silver particles being so close that they merge into a single, large, particle. Enzyme treatments had no effect on the recognition pattern of the anti-gliadin Mab 0033 during enhancement.

3. Publications describing work carried out under Subprogramme B Task 2.1-4

Holden S., Mills E.N.C., Parker M.L. and Morgan M.R.A. (1994). An investigation into the role of gluten components in the development of bread microstructure during baking using immuno-electron microscopy. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp 510-518.

Mills E.N.C., Brett G.M., Kauffman J.A., Tatton M.J., Tatham A.S., Shewry P.R. and Morgan M.R.A. (1994). Antibody probes for gluten protein structure and conformation - Tools for investigating structure/function relationships. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp 67-78.

Mills E.N.C., Brett G.M., Kauffman J.A., Holden S., Tatton M.J., Morgan M.R.A., Tatham A.S. and Shewry P.R. (1993). Antibodies and cereal quality. Aspects of Applied Biology, 36, 85-92.

Morgan M.R.A, Mills E.N.C., Brett G.M., Kauffman J.A., Tatton M.J., Alcocer M.J.C., Holden S. and Parker M.L. (1992). Structure, localisation and quality of gluten proteins in baked goods using antibody probes. Industries des Céréales, 77, 41.

SUBPROGRAMME C:

BIOCHEMICAL-GENETICS AND PHYSIOLOGY

Summary

Subprogramme C has sought *to analyse the biochemical, genetic and physiological bases of technological quality*. It was organised around the following topics:

1. Production of wheat samples in controlled conditions that were necessary to carry out studies of Subprogrammes A and B. Evaluation of these wheats in various environments for yield potential and quality attributes.

2. Determination of the agronomic, physiological, genetic and biochemical factors affecting technological quality and its stability of expression. They included predictive values of biochemical tests.

3. Allelic composition, chromosomal location and genetic links of genes coding for the storage proteins subunits of HMW and LMW glutenins and gliadins, for certain albumins and S-proteins by analysing the lineage and chromosomal substitution lines between varieties. This study was both qualitative (presence or absence of constituents) and quantitative contribution of LMW and HMW glutenin subunits to the total pool of wheat proteins).

4. Statistical analysis of a large collection of wheat cultivars in view to determine the relationships between allelic composition and baking quality. The protein fractions which appeared to be correlated with qualitative characteristics (notably in view of trials carried out in industrial laboratories) were subsequently purified so that their physico-chemical character could be determined within the framework of Subprogramme B.

5. Development of rapid tests for dormancy and for initial stages of sprouting related to kernel constituents which were aimed at producing wheat with a higher degree of sprouting resistance and early detection of sprouting damage in the field.

Laboratories and breeding companies involved in *biochemical-genetics and physiology (North-Western- and Southern-Europe Networks)* have carried out technological analyses leading to significant results in terms of: (i) potential yield of the top cultivars in several European locations, (ii) quality characteristics, (iii) correlation between quality traits and agronomic factors, (iv) effect of nitrogen fertilization, and (v) characterisation of growth environments. These results demonstrate that potential exists for breeding bread wheat in terms of improving adaptation and consistency of agronomic and quality traits.

A multiplicative model for genotype × environment interaction was developed which enabled evaluation of stability of quality and to identify cultivars on the basis of their response to environment.

Taking advantage of the availability of new genetic stocks such as near-isogenic lines, investigations on *gliadins* and *LMW glutenin subunits* provided breeders with a

genetic approach to describe allelic composition at the *Gli* and *Glu-3* as well as one- and two-dimensional techniques to identify the gliadin or glutenin components encoded by the different alleles at those loci. These allowed to make recommendations to breeders in view to better predict dough characteristics through consideration of specific HMW or LMW subunits of glutenin as well as of certain ω-gliadin components.

The work on genetic and technological aspects of *HMW glutenin subunits and HMW albumins* has added new information about: (i) effects of HMW subunit 2 on gluten quality, (ii) DNA sequence of unexpressed subunit 2 gene in the A6 line, and (iii) allelic variation for HMW albumins.

The work on *somaclonal variation* was especially relevant. It was clearly demonstrated that regeneration of adult plants from wheat embryo or anther culture was affecting breadmaking quality and could provide novel variation that is not readily obtained with conventional breeding methods. Most of this variation is interesting for study purposes only, but some of it can be satisfactorily exploited in a breeding programme.

Finally, the basic knowledge on *sprouting resistance* has made significant progress. Progenies showing a broad variation in dormancy were provided, while a bioassay was developed to monitor inhibitors of germination and fractions containing inhibitors were characterized biochemically.

Task C.1 - Multilocal Experiments of Advanced Lines and Varieties, and Production of Samples in Controlled Conditions

Subtask C.1.1 - Network 1: Southern Europe (SEN)

Partner 02 - Produttori Sementi S.p.A.
Via Macero, 1
40050 Argelato (Bologna), Italy

1. Key measures of achievement - Objectives

To assess by means of replicated yield trials the quality and production of a set of cultivars grown in different locations of Southern Europe and to produce seed samples to be used in Subprogrammes A and B.

2. Progress

In the Argelato location, three trials were carried out according to the best agronomic practices used in this area (Po Valley, Northern Italy).

The data obtained concerned quality characters such as test weight, SDS micro sedimentation test, grain hardness, protein content and agronomic characters: yield, plant height, lodging, disease score for powdery mildew and brown rust.

These data gave a fairly complete idea about the performance of the tested varieties in our area in different years. In order to take the maximum advantage from these data they had to be analysed in connection with all the other data obtained by the network of cooperators. In this way it was possible to assess quality and yield stability of the tested cultivars across environments and years of cultivation and to know better both varieties and environments where the trials were carried out.

It must be pointed out that fungicides or growth regulators were never used in the Argelato location. Considering the data obtained, this indicate that good results can be achieved with a low input of chemicals.

The trials carried out during the first two years allowed to also evaluate the effect on quality of a supplementary dose of nitrogen (40 kg/ha) applied at heading time. Altogether we did not notice a clear beneficial effect of this late application of nitrogen either because it was on top of the ordinary nitrogen application used in this area, that might have been sufficient to obtain the best quality, or because it was too late. (As a matter of fact, in our environment, the period of time between heading and harvest is very short).

As far as the production of seed samples for partners of Subprogramme A and B is concerned, we supplied samples to Danone, CCFRA, TNO and University of Viterbo.

Obviously, the exchange of samples between cooperators of Subprogramme C was frequent, especially in 1993 when different laboratories were acting as a reference laboratory for the various tests of quality evaluation. We were in charge of the determination of protein content by Near Infrared Reflectance Analysis. Correlations between different laboratories using NIRA and between NIRA data and Kjeldahl analyses were worked out allowing each laboratory to know the correlations between its own data and the data obtained by the reference method (Kjeldahl) and by the other laboratories.

The information obtained from the network of the SEN trials and from the partners of Subprogramme A and B, who analysed the seed samples produced in those trials, was useful for our breeding work aimed at the production of wheat varieties better suited to meet the needs of the industry.

3. Publication describing work carried out under Subprogramme C Task 1.1

DeAmbrogio E., Di Fonzo N., Rocchetti G. and Borghi B. (1992). I grani di forza in Europa. L'informatore Agrario, 48, 33-37.

Partner 03 - Istituto Sperimentale per la Cerealicoltura
Sezione Operativa di S. Angelo Lodigiano
Via Mulino, 3 20079 S. Angelo Lodigiano (Milano), Italy

1. Key measures of achievement - Objectives

To produce seed/flour samples to be used in Subprogrammes A and B, to study the stability parameters of quality expression, to identify environments and agronomic practices favourable for enhancing quality expression.

2. Progress

Introduction

The environment (soil, climate, agronomic practices, diseases, etc.) and the interaction genotype × environment exert a strong influence on the expression of technological quality.

The consistency of quality for most existing European wheat cultivars is insufficient because it is affected by agronomic and climatic factors.

Variation in protein content and rheological properties among seed samples of the same wheat cultivar from different environments can be as large as that among cultivars grown in one environment.

The evaluation of yield potential and expression of quality attributes (technological characteristics) of wheats in different environments were regarded as the necessary premise to all other activities envisaged in the Programme. In order to gather such information, a network of variety trials carried out with a rigorous and uniform methodology in wheat growing areas was established.

A set of varieties from Italy, France, Spain and Portugal was grown in trials for three years in order to evaluate their potential and stability in terms of production, quality and response to late nitrogen application and in order to produce seed/flour samples to be used in Subprogrammes A and B, to study the stability parameters of quality expression, to identify environments and agronomic practices favourable for enhancing quality expression.

Experimental approach

The Southern Europe Network involved Italy, Spain, Portugal, France. This sub network was especially devoted to wheats with high breadmaking quality, well adapted to the growing conditions prevailing in Southern Europe.

- *Locations*: 2-5 locations for each country for a total of 8-20 locations/year;
- *Varieties or advanced lines*: about 25 per year;
- *Experimental design*: balanced lattice with 3 replications;
- *Agronomic practices*: the best agronomic practices for the conditions of Southern Europe with minor modifications according to specific environmental requirements (two levels of nitrogen application). The final aim was to drastically reduced and in

many cases to eliminate the use of pesticides and growth regulators;
- *Statistical elaboration of the results is centralised*: the data were available at the end of each year;
- *Grain quality*: evaluated with the following analyses:

On each plot:
- protein content (%)
- SDS sedimentation volume
- hectolitre weight

Sample from a mix of the replications:
- rheological tests (alveograph, farinograph)
- baking tests
- other tests required by the industry

Results

Cultivars tested

During the 3 years of testing, 34 cultivars from Italy, France, Spain and Portugal, were evaluated in 33 testing sites (year/location). The cultivars and the testing years are listed in **Table XV** where is also presented the Country of origin and the pedigree.

Testing sites

The first SEN trial (1990-91) was sown at 12 locations, the second SEN trial (1991-92) was sown at 16 locations. For the third trial were available only the data from 5 locations in Italy related to the year 1993-94 as indicated in **Table XVI** and **Figure 56**.

Agronomic results

- First SEN trial (1990-91)

In **Table XVII** are summarized the results obtained in 12 locations expressed in $t \times ha^{-1}$ and in **Table XVIII** the same results are expressed in percent of field mean.

- Second SEN trial (1991-92)

In **Table XIX** are summarized the results obtained in 16 locations expressed in $t \times ha^{-1}$ and in **Table XX** the same results are expressed in percent of field mean.

- Third SEN trial (1992-93 and 1993-94)

The third SEN trial was sown in some locations during the year 1992-93 and in other locations in 1993-94 because in several places the seed samples were received too late to be planted. In this report are presented only the data obtained from the Italian locations in the year 1993-94.

In **Table XXI** are summarized the results obtained in 5 locations expressed in $t \times ha^{-1}$ and in **Table XXII** the same results are expressed in percent of field mean.

The performance of the most interesting cultivars in the three SEN trials is reported in **Figure 57**.

Qualitative results

In **Table XXIII** are synthetically reported the results of the qualitative test (Chopin Alveograph, protein content) and the classification of the samples in the following three categories:

1 = hard wheat (W higher than 280 $J \times 10^{-4}$),
2 = direct bread wheat (W higher than 180 $J \times 10^{-4}$),
3 = ordinary wheat (W in the range 120-180 $J \times 10^{-4}$).

3. <u>Publication describing work carried out under Subprogramme C Task 1.1</u>

DeAmbrogio E., Di Fonzo N., Rocchetti G. and Borghi B. (1992). I grani di forza in Europa. L'informatore Agrario, 48, 33-37.

<u>Partner 25 - Estação Nacional de Melheramento de Plantas - Elvas</u>
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P.O. Box 6, 7351 Elvas, Portugal

1. <u>Key measures of achievement - Objectives</u>

To produce seed/flour samples to be used in Subprogrammes A and B, to study the stability parameters of quality expression, to identify environments and agronomic practices favourable for enhancing quality expression.

2. <u>Progress</u>

Twenty five bread wheat cultivars from four EC countries were grouped to be tested in different locations in Portugal, Spain, Italy and France. These cultivars differed widely in duration of growth cycle, plant height, reaction to diseases and technological quality parameters. Trials were organised in RCBD with 3 replications and 2 nitrogen treatments. Results presented in this report were obtained at Elvas and, due to the climate pattern observed during 1990/91 to 1993/94 seasons, no nitrogen effect was observed. Data were treated as one single treatment. As a matter of fact, rainfall and temperature reported in **Figure 58**, were extremely irregular with negative effects on yield and quality.

One of the objectives was to evaluate the agronomic value of the wheat germplasm. Defining agronomic adaptation as the ability of a genotype to produce consistent and high yields over a range of environments (locations and years), a joint regression analysis procedure for grain yield was performed according to Gusmão (1985) and Ferreira *et al*. (1992). Other agronomic characteristics, that can help the evaluation of yield results, as heading date, 1000-kernel weight, and test weight are also presented.

Quality parameters are presented on the basis of the assumption that the milling and baking industries desire cultivars that have high probabilities of meeting defined quality standards. This quality standards are usually specified by upper and lower limits of acceptability, for multiple quality traits when wheat grown over an array of environments (Eskridge *et al*. 1 994).

Agronomic adaptation

Big differences were observed among genotypes and years concerning grain yield (**Table XXIV**). Heading time, test weight and thousand kernel weight, also showed a wide range of values (**Tables XXV and XXVI**). The extremely irregular weather conditions (rainfall distribution and temperature) that were observed during the four years of tests had marked influence on the behaviour of genotypes. Late genotypes like 'Salmon', 'Courtot', 'Pegaso', 'Soissons' and 'Amazons' showed low agronomic adaptability. These genotypes are winter types which flowering time occurs when temperature and evaporation rise, and when soil is becoming depleted of water.

On the other hand, very early spring types like 'Cajeme', 'Yecora', 'Rinconada', 'Festa' and 'Prinqual' are also of low agronomic interest, because they flower during a period when late frosts occur with high probability. In some of these genotypes, although,

with high regression coefficients (b>1), their annual means are always below general year mean. These are characterized as genotypes with genetic yield potential but not with good adaptability. The most suitable cultivar, for this Southern Europe region, fall within the group of late spring or alternative germplasm with 'Vital', 'MP477', 'Mondego' and 'Maestra', with the best performance, showing adaptation and yield stability (b>1) and cultivar mean > year mean).

Quality parameters

Several good quality genotypes could be identified. Allelic variation of high molecular weight glutenins (HMW) encoded by genes at *Glu-1* loci, was detected on this group using electrophoretic separation. These grain constituents have been considered as having great influence on breadmaking quality of wheat, and good correlation was demonstrated between HMW and gluten properties. Considering the very high values of protein content and rheological properties of dough (**Table XXVII**), 'Cajeme', 'Yecora', 'Rinconada', 'Prinqual', 'Soissons' and 'Salmon' produced flour suitable for very high strength doughs. However, these mentioned cultivars are of low agronomic interest because they do not have a fitted growth cycle for this region. In the group of agronomic suitable germplasm there are also wheats that can produce grain with strong gluten as 'Almansor', 'Mira', 'Mondego', 'MP 477', 'Golia', 'Pandas' and 'Tua'. In this group it is possible to find cultivars that combine good agronomic characters and technological value which can satisfy simultaneously farmers and milling and baking industries

Discussion

Despite the climatic constraints observed in this Mediterranean region, good quality wheats (strong gluten) can be obtained. However, significant variation among cultivars was found as response of agronomic and quality characteristics to environment, indicating the need to conduct adaptation local studies. For example, the Italian variety 'Salmone' has good genetic potential for quality but is not adapted to this region.

Potential exists for breeding bread wheat in terms of improving adaptation and consistency of agronomic and quality traits.

3. Publications describing work carried out under Subprogramme C Task 1.1

Bagulho F., Maçãs B., Brites C. and Coutinho J. (1995). Studies on yield stability and quality parameters on European bread wheats. Brotéria Genética, Lisboa, 16 (41), 35-41.

Brites C., Maçãs B., Coutinho J. and Bagulho F. (1992). Estabilidade na expressão das caracteristicas que determinam a qualidade tecnologica em 4 cultivares de trigo mole. Melheramento, 33 (2), 431-440.

Maçãs B., Coutinho J. and Coco J. (1992). Bread wheat for high technological values in Portugal. 9th International Cereal and Bread Congress, Paris, 1-5 June 1992.

Brites C., Maçãs B., Coutinho J. and Bagulho F. (1994). Quality characteristics of Portuguese bread wheat varieties: stability in technological parameters/gluten protein composition. In: Proceedings of the International Meeting "Wheat Kernel Proteins: Molecular and Functional Aspects", September 28-30, Viterbo (Italy).

Brites C., Maçãs B., Coutinho J. and Bagulho F. (1994). Quality environmental effect of 11 bread wheat advanced lines. Poster presented at ECLAIR Meeting, 9

June, The Hague, The Netherlands.

Maçãs B., Coutinho J., Brites C. and Bagulho F. (1994). Bread wheat quality evaluation: Yield potential *vs* quality expression. Symposium on "Future Prospectives of Cereal Breeding in Europe", 4-7 September, Plantahof, Landquart, Switzerland.

Subtask C.1.2 - Network 2: North Western Europe (NWEN)

Partner 07N - INRA - Clermont-Ferrand
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Domaine de Crouelle
63039 Clermont-Ferrand Cedex

1. Key measures of achievement - Objectives

Besides the study of stability parameters of quality expression, the aim of this experimentation was the definition of selection criteria to identify stable genotypes for quality expression in highly diversified growing conditions and the evaluation of yield potential.

2. Progress

This task had a twofold objective: production of samples in multilocal trials for partners of subprogrammes A and B, study of stability of quality. This report focuses on the second point.

Several genotypes grown in the same set of environments exhibited differential responses to the various growing conditions, for yield but also for quality. These differential responses are called stability of the trait considered. Study of quality is only possible if varieties are assessed in a multilocation trial. SEN (C1.1) and NWEN (C1.2) networks were thus well adapted to this task. Considering that all genotypes grown in such a trial were submitted to the same environment effects, we defined stability of quality according to the agronomic or dynamic concept: we considered that the variation of environments induced a variation of quality common to all genotypes tested. Thus, environmental variation of quality observed for one genotype was jointly due to location and genotype environment (G × E) interaction effects, but stability was only based on G × E interactions.

Cautions taken before analysing stability of quality.

Assessment of quality was made using technological tests correlated to final product. We first investigated the possible "laboratory effect" between laboratories performing the same test. Indeed, if such an effect was present, there would be a confounding effect between laboratory and environment effects and data could not be correctly interpreted. Our results confirmed that hypothesis for SDS sedimentation test and protein content assessed by NIR. To avoid statistical problem, analysis of stability for quality characteristics in a collaborative programme implies that technological skills must be distributed among laboratories. In one laboratory, all samples obtained on the network should be analysed for one particular test. Such a procedure was made, but was possible only on a smaller varietal list.

A second particularity of technological data, compared to yield data, is the low output of some technological tests (for example, alveograph currently performed in South

Europe). In some cases, tests were performed on only one composite sample made by blending grain of all replicates harvested in one environment, making it impossible to test variance of interaction by an ANOVA. So we had to use a variance of repeatability, considered as a measure of pure error variance, to detect G × E interactions.

Correlation between quality tests

The set of technological data allowed studies of correlation between the different tests used. In particular, we got gel protein data performed on some SEN samples from Partner 14 (CCFRA). Because the number of genotypes (7) was low, genetic correlations had a low meaning and should not be considered, whereas environmental correlations (18 environments) were reliable. Weight of protein gel was correlated to protein content, SDS sedimentation volume and Pelshenke tests, but breakdown rate and elastic modulus showed no environmental correlation with those tests. These results suggested that environmental factors acting on the two parameters of gel protein were different from those acting on the classical breeding tests used, or, that environments acted on several unrelated components of quality, each assessed by different tests. They suggested the interest of gel protein as a complementary test to usual tests to evaluate variation of quality due to environments.

Study of stability of quality

Following the work done during the programme, we have proposed a procedure to assess stability of quality.

First, we defined a stable genotype as one exhibiting low G × E interactions. We measured stability by ecovalence, *i.e.* the sum of square of interactions of one genotype. Stable cultivars had low ecovalences, whereas unstable cultivars presented high ecovalences. As ecovalence is a sum of squares, it does not consider signs of interactions. In order to better characterize genotypes for their stability, we modelled interaction using two statistical models. First, a linear regression model (in which the environment effect was used as the regression variable) that was frequently used for yield stability analysis in previous studies dealing with quality stability. Second, an Additive Main Effect and Multiplicative Interaction model with two axes (AMMI2), that is less common. The quality of a model for modelling purposes, was measured as the part of total ecovalence (sum of all ecovalences) explained by the model. Comparisons between the two models were made for SEN and NWEN varieties assessed using small-scale tests, and for NWEN varieties assessed with alveograph test. An environment was defined as the combination site × cultural practice × year, except for alveograph for which only experiments with use of fungicides were considered (**Table XXVIII**). Whatever the varietal set, the environment set and the technological tests considered, the AMMI2 model explained a considerably higher part of the total ecovalence. The low explanation by linear regression was expected, but this method was advocated for providing a measure of the responsiveness of each genotype to environments through the slope of the regression. In contrast, multiplicative scores affected to each genotype were usually difficult to interpret. Using a graphical method (**Figure 59**) and taking into account the part of ecovalence

explained by AMMI2, we showed that some pairs of genotypes with opposite or similar responses to environments could be identified. Thus a multiplicative model for interaction seemed adapted to evaluate stability of quality since it explained a greater part of ecovalence than the linear regression model and provided information on the response to environments for the different varieties studied. For example, considering the alveograph W, 'Renan' and 'Thésée' responded differently, whereas 'Renan' and 'Soissons' gave similar responses, even if their ecovalences were quite different. For the four parameters of alveograph, 'Camp-Rémy' was identified as a check for stability and 'Récital 'as a check for unstability. However, some cultivars were more stable or more unstable than these two varieties when only one parameter was considered. Of course, it should be kept in mind that results are dependent of the sets of genotypes and environments studied.

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1. Key measures of achievement - Objectives

Besides the study of stability parameters of quality expression, the aim of this experimentation was the definition of selection criteria to identify stable genotypes for quality expression in highly diversified growing conditions and the evaluation of yield potential.

2. Progress

Strategy

1. Producing samples in different environmental conditions during 3 years (1991, 1992 and 1993 harvests).
2. Analysing the quality of these samples.
3. Identifying stable genotypes for quality expression in highly diversified growing conditions.

This report focuses on the results of the 2 first points. The third point is developed in the report of subcontractor 07C (INRA Clermont Ferrand).

Material and methods

- The North Western Europe Network (NWEN) included 8 locations: ITCF conducted 2 of these trials and received the samples of the 6 others conducted by Club des 5 and INRA. Each trial included 16 varieties and 2 treatments (with and without fungicides). ITCF also sowed a trial for the South Europe Network (SEN) with 25 varieties and 2 nitrogen levels. Crops notations (diseases notations, yields, ...) were performed and analysed.

- The samples were analysed with different quality tests after being cleaned following a conventional procedure (2 mm sieve):

  - Hagberg falling number and protein content (ITCF) as a screening of the samples. On this basis, choice of samples in order to continue the technological evaluation of the 3 harvests.
  - SDS sedimentation value and Pelshenke test (INRA)
  - Alveograph test (Grands Moulins de Reims)

- Some samples were also analysed by participants of Subprogrammes A and B: Danone (biscuit making test) and CCFRA (gel protein test).

Main results

In the NWEN, the choice of varieties represented the French production with superior breadmaking varieties ('Soissons', 'Camp Rémy', 'Récital'), standard breadmaking varieties and also non breadmaking varieties ('Apollo', 'Beaver'). They represented also a large scale of earliness from 'Récital' to 'Beaver'. The varietal list sown in the trials has changed within the three years of experimentation.

The South network was conceived on an other basis and included exclusively improver wheats.

The behaviour of varieties was different within the harvests. The 1992 harvest was specific and the late varieties were penalised by the drought of May ('Apollo', 'Renan', 'Camp Rémy'). Some varieties were more steady ('Baroudeur', 'Soissons'). The average behaviours of varieties observed in this network were close to those observed at the national level even if the genotype × environment interaction was always important.

To conclude, the agronomic network allowed to produce a large range of environmental conditions and qualities (**Tables XXIX and XXX**). This diversity was used to study the ability of varieties to be stable in variable conditions (see report of Partner 07C).

On another point of view, the distribution of the quality analyses between different laboratories allowed interesting exchanges.

Table XXX. Means and variances values of quality tests performed on the samples of the SEN.

Varieties	Protein content	Pelshenke	SDS volume
Almonsor	12.7 (1.2)	164 (2412)	50 (41)
Golia	13.4 (2.5	194 (2134)	56 (112)
Maestria	13.1 (2.5)	176 (1948)	46 (63)
Mec	13.8 (1.5)	73 (469)	50 (91)
Mondego	12.7 (1.8	116 (787)	53 (49)
Pegaso	12.9 (2.1)	141 (1714)	60 (89)
Rinconada	13.7 (2.3)	246 (2366)	61 (75)

Partner 18 - Club des 5
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1. Key measures of achievement - Objectives

Besides the study of stability parameters of quality expression, the aim of this experimentation was the definition of selection criteria to identify stable genotypes for quality expression in highly diversified growing conditions and the evaluation of yield potential.

2. Progress

The last year of the ECLAIR wheat Subprogramme C ended with 1994, but the cooperation is now well established among national and European partners. For the industrial and seeds firms, the good connections existing now with the public labs and institutes involved in cereals research is especially evaluated as opening perspectives to new methodologies and applications.

The 1994 year was mainly devoted to synthesis of collective results presented by the contractor INRA, Clermont-Ferrand (Partner 07C), as it had been planned in the objectives of the programme. Several synthesis meetings between French partners and also participation in European general meetings were good opportunities to exchange and complete scientific information and experiences, which were regularly enlarged to GIERGC breeders.

A large information from the primary conclusions of the 4 years ECLAIR wheat programme was brought by GIERGC inside the international congress "DÉFI BLÉ 1994" organised by AGPB/UNIGRAINS in Paris 20-21 October 1994 (more than 1000 participants). In such an opportunity, GIERGC sponsored and chaired the section presenting the recent progress in cereals qualities and technologies, and asked to INRA, TNO Wageningen, breeders and industrial specialists to state on new technologies and to preview the year 2000 (Haslé, 1994)

The contribution of GIERGC (Partner 18) was a part of collective results in the Subprogramme C, GIERGC having the charge to realise multilocal and precise trials, to make scientific and agronomic observations in vegetation and to harvest, measure and supply the ECLAIR partners laboratories with seed trial samples:

- for Task C1 Network results
  . from the Mediterranean net, through Dr. Norberto Pogna (SEN trials)
  . from central West Europe net, through Dr. Nathalie Robert (NWEN trials)
- for task C2 Network results, in cooperation in France with Nathalie Robert (INRA) and Marie-Hélène Bernicot (ITCF).

Application for plant breeding in wheat

The French wheat breeders of GIERGC made results evaluation as follows:

A. *Progress on quality understanding* in breadmaking technology, in dough extensibility and rheology (macropolymeric net, glutenins), in function of small proteins and of pentosans, in milling quality, in grain enzymatic activity (germination and dormancy), wheat grain hardness evaluation in relation with dough rheology.

B. *Progress in genetic information* of grain proteins and electrophoresis description, with possible application in plant breeding and better understanding of environmental interactions.

C. *Potential of new lab tests* for better and/or quicker evaluation of wheat qualities according to the different industrial uses, with a chance for breeders to apply, if practicable and economical, on large series of varieties seed samples (routine tests).

Breeders are interested to know all the genetic keys able to help them to select more adapted wheat varieties in regard of industrial requirements and practices and to have new lab tests recognized by industrials and transferable to plant breeding labs. Such ECLAIR cooperation was a good opportunity to establish dialogue between scientists, breeders and industrials, and today the background of confidence and of experiences exchange is positive. Such spirit in partnership must be continued in anyway for the profit of the European research and industry.

3. Publications describing work carried out under Subprogramme C Task 1.2

Haslé H. (1994). Céréales : de la variété à la qualité alimentaire. Agro-Performances, 45, 53-55.

Task C.2- Genotype × Environment Interaction

Partner 07N - INRA - Clermont-Ferrand
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Domaine de Crouelle
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1. Key measures of achievement - Objectives

- To determine the environmental factors affecting yield and breadmaking quality, to study the accumulation kinetics of endosperm protein fraction in relation to the environmental variations and to obtain a better understanding of the variations in quality expression, the methodology was based on the study of mechanisms involved in dry matter yield elaboration. This approach was likely to reveal the environmental factors which are responsible for yield and quality variations.

- To determine if the genotypic potential is expressed and in the case of a negative answer which are the limiting factors in yield and quality expression.

- To complete the multilocal experiment it was worthwhile, in order to obtain a better understanding of the quality expression, to grow wheat genotypes in environments where the growth factors were controlled more precisely. Nitrogen dynamics was studied using ^{15}N-labelled fertilizers and a range of fertilization methods was developed.

2. Progress

Four varieties were studied during grain filling. Kinetics of accumulation of dry matter and nitrogen in the grain were studied. There were differences between the genotypes for accumulation of dry matter, total nitrogen and nitrogen fractions. Final quantities of nitrogen fractions seemed to be partly determined by final kernel weight. But an other mechanism occurred: the allocation of total nitrogen to storage nitrogen differed between cultivars. Kinetics of dry matter and nitrogen losses from vegetative organs were studied in order to explain varietal differences observed for growth of one grain and nitrogen accumulation in the kernel. Some differences between varieties existed for the different rates: in particular, 'Capitole', which had the highest rates for accumulation of dry matter and total nitrogen and which allocated more nitrogen to storage nitrogen fraction, had also the highest rates of translocation for all vegetative organs studied. There was no possible connection with task C1, as we were not able to obtain two environments (no difference for yield and quality between the two nitrogen fertilisations made) and thus we could not observe genotype × environment interactions for yield and quality and then relate them to possible variations of kinetics. But this work raised several questions. Is there any relationship between environmental variation of kernel weight and protein composition of grain and thus

quality ? Are quantitative variations of protein fractions in mature kernel, observed between environments, related to variations in kinetics of protein fractions ? In other words, is protein synthesis modified by environments ? All these questions are connected to understanding of mechanisms involved in stability of quality.

General conclusion. Perspectives.

The statistical procedure proposed for analysis of quality stability was original. However, the AMMI model is an explanatory model, because no biological interpretation of multiplicative scores was possible or easy. However, identification of genotypes showing opposite responses to various environments is an important point since these varieties are good candidates for further studies on stability of quality. Experiments planned to identify covariables related to stability of quality are already undertaken. Possible covariables are agronomic traits, yield, kernel weight and other yield components, since a same genotype elaborates its yield differently in different sites. But the covariables will be also biochemical traits: rates of accumulation of protein fractions, final protein composition of kernel. Depiction of final composition would take in consideration results yielded by programmes A and B (gel protein, macropolymers...). Such studies should identify predictor traits for stability of quality, which would be useful tools for breeding varieties combining productivity, quality and stability.

3. Publications describing work carried out under Subprogramme C Task 2

Robert N., Le Blevennec L. and Triboï E. (1993). Accumulation of protein fraction during grain filling: comparison of four bread wheat varieties. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp. 105-113.

Robert N., Le Blevennec L. and Triboï E. (1995). Protein composition during grain filling: comparison of four bread varieties. Cereal Chem. (submitted).

Task C.3- Experimentation on Populations for Breeding

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1. Key measures of achievement - Objectives

To increase the concentration of alleles determining good agronomic and qualitative characters in synthetic populations established by the Istituto Sperimentale per la Cerealicoltura (S. Angelo).

2. Progress

Three trials were carried out in order to assess the performance of a set of synthetic populations.

According to the results obtained after the first trials in two locations (Argelato and S. Angelo), the most interesting populations were chosen for a new cycle of intermating.

Individual plants were also selected from rows of the best populations artificially inoculated with powdery mildew and brown rust. The progenies of the plants, having the best quality in terms of SDS micro sedimentation test were introduced into our selection nursery.

Trials carried out in the following two years in the same two locations allowed us to evaluate the stability of the agronomic and qualitative traits of the synthetic populations chosen after the trials of the first year.

Individual plants are now going to be selected from the populations having the most stable performance because of its interest for our breeding work.

Task C.4- Genetics of LMW Glutenin Subunits

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1. Key measures of achievement - Objectives

The research will determine (i) the genetic variability for B, C, and D groups of LMW glutenin subunits, (ii) the genetic linkage between loci coding for gliadins and glutenin on group 1 chromosomes and (iii) the effects of both LMW and HMW glutenin subunits on gluten properties.

2. Progress

Methods

The diversity of the HMW and LMW glutenin subunits was analysed using the one step method proposed by Singh *et al*., 1991. The ω−gliadins were separated by SDS PAGE and the nomenclature of the allele found in the material studied was that proposed by Khelifi *et al*., 1992. The identification of the different LMW GS patterns was achieved: 1) by pooling the sample of similar mobility phenotype on the second gel run out for that purpose, and 2) by using the cultivars 'Cappelle-Desprez', 'Courtot' and 'Chinese Spring' as checks.

In each population studied the quality of cultivars was assessed through the following technological tests: grain protein content, Chopin Alveograph and Pelshenke swelling time.

Material

Two main populations were used to study which was the part of the quality attributed to HMW glutenin subunits, LMW glutenin subunits and ω-gliadins:

- Population I which was constituted of 132 French bread wheat cultivars released between 1949 and 1984. These cultivars were grown at the INRA plant breeding station of Clermont-Ferrand.

- Population II, represented by a set of 61 cultivars originated from more than 15 countries, was also cultivated at Clermont-Ferrand for several years.

These two populations were very different for their genetic origin. Population I represented the diversity of the French wheat cultivars and the main genetic source of the actual varieties grown in France, whereas population II gathered numerous strains of good to very good quality from very diverse geographical origins. In addition

wheats from bad to medium quality were also present in population II.

Results

Both populations I and II exhibited numerous allelic variants at *Glu-A1, Glu-B1* and *Glu-D1* loci. The *Gli-1* alleles encountered in these two populations as revealed by SDS-PAGE also were numerous **(Table XXXI)**.

Few cultivars of identical protein composition were encountered in each population making the analysis of variance more difficult when the quality means attributed to each pattern were compared. The effects attributed to the *Glu-1*, *Glu-3* and *Gli-1* pattern diversity on the quality parameters were particularly studied **(Table XXXII)**.

Both populations I and II raised similar conclusion about the limit of variation explained by the three groups of chromosomes I encoded proteins.

The best estimated alveograph parameter was the W, whereas both P and G were the worst. For all the tested parameters tested the part attributed to the D-zone ω-gliadin was higher than that attributed to LMW GS. For P and G these two protein families encoded at *Glu-3* and *Gli-1* loci had total effects almost equivalent to those attributed to Glu-1 major loci.

Other analyses were carried out to assess the part of the LMW glutenin subunits and the D-zone ω−gliadins on quality. Several progeny were analysed, particularly from crosses including the French cultivar 'Darius'. Preliminary conclusions evidences that some very good alleles for rheological dough quality may be nevertheless found in the *Glu-3*/*Gli-1* loci.

3. Publications describing work carried out under Subprogramme C Task 4

Branlard G. and Dardevet M. (1994). A null *Gli-D1* allele with a positive effect on bread wheat quality. J. Cereal Sci., 20 (3), 235-244.

Branlard G., Dardevet M., Nieto-Taladriz M.T. and Khelifi D. (1994). Allelic diversity of the ω−gliadins as revealed by SDS-PAGE: Their possible implication in quality variation. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp. 234-243.

Khelifi D. and Branlard G. (1992). The effects of HMW and LMW subunits of glutenin and of gliadin on the technological quality of progeny from four crosses between poor breadmaking quality and strong wheat cultivars. J. Cereal Sci., 16 (3), 195-209.

Khelifi D., Branlard G. and Bourgoin-Greneche M. (1992). Diversity of some D-zone ω−gliadins of bread wheat as revealed by 2 step A-PAGE/SDS-PAGE technique. J. Genet. Breed., 46 (4), 351-357.

Nieto-Taladriz M.T., Branlard G. and Dardevet M. (1994). Polymorphism of ω-gliadins in durum wheat as revealed by the two-step A-PAGE/SDS-PAGE

technique. Theor. Appl. Genet., 87, 1001-1005.

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1. Key measures of achievement - Objectives

The research will determine (i) the genetic variability for B, C, and D groups of LMW glutenin subunits, (ii) the genetic linkage between loci coding for gliadins and glutenin on group 1 chromosomes and (iii) the effects of both LMW and HMW glutenin subunits on gluten properties.

2. Progress

Because of the difficulty to completely describe the LMW alleles with one single system *e.g.* SDS-PAGE, a new native polyacrylamide gel electrophoresis system buffered by acetic acid (A-PAGE) was developed and carried out on reduced and alkylated subunits of glutenin in combination with SDS-PAGE and IEF separations. These techniques were applied to a set of 10 intervarietal chromosome substitution lines (supplied by INRA-Clermont-Ferrand, Partner 07C) and to 22 Italian cultivars (supplied by ISC, S. Angelo Lodigiano, Partner 03) in which the allelic variation at the *Gli-1* locus (ω-gliadins) was previously determined by N.E. Pogna on the basis of Metakovsky's nomenclature. In a set of forty-two French bread wheat cultivars, respectively 4, 5 and 4 allelic variants were observed at the loci *Glu-A3*, *Glu-B3* and *Glu-D3*. The specific effect of LMW subunits (whose variation was shown to respectively explains 35 % and 25 % of the variations in baking strength and dough extensibility among the same set of French bread wheats), as well as the interactions between glutenin components encoded at the *Glu-1* and *Glu-3* loci might explain some major discrepancies observed in the relation between HMW composition and dough properties. When aiming at breeding, for instance, breeding-type wheats, it was therefore recommended to screen for genotypes containing the *Glu-B3 III* (≈ *Gli-B1 e, f*) allele associated to the *Glu-D1* allele encoding subunits 2+12. On the other hand, the amount of protein expressed by the various *Glu-B3* alleles might be related to their effect on dough extensibility.

On the other hand, several F_7 recombinant lines, obtained from the cross between the two bread wheat spring cultivars 'Neepawa' and 'Costantino', and characterized by different alleles at the three *Gli-1* loci, were analysed by two-dimensional electrophoresis to determine allelic composition at *Glu-3* loci. Reduced and alkylated glutenin subunits were fractionated in acid PAGE as described by Morel (1994) in the first dimension and by 15%, pH 8.4 SDS-PAGE (Dachkevitch *et al.* 1993) for the second dimension.

By comparison between the two-dimensional maps of parental cultivars and recombinant lines, the main polypeptides coded at each *Glu-3* locus were identified. In 'Costantino', 1, 5 and 5 subunits are coded, respectively, by *Glu-A3*, *Glu-B3* and *Glu-D3* whereas, in Neepawa, the three loci code for 1, 6 and 4 subunits, respectively **(Figure 60)** (Redaelli *et al.*, 1994).

3. Publications describing work carried out under Subprogramme C Task 4

Published papers

Morel M.H. (1994). Acid-PAGE of wheat glutenins: a new tool for the separation of high and low molecular weight subunits. Cereal Chem. 71 (3), 238-242.

Morel M.H., Bonicel J., Mélas V. and Autran, J.C. (1994). Multiple approach (IEF, SDS-PAGE and A-PAGE) of the composition of LMW subunits of glutenin and its effect on dough properties. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp. 244-254.

Redaelli R., Morel M.H., Autran J.C. and Pogna N.E. (1994). Two-dimensional (A-PAGE × SDS-PAGE) electrophoresis of *Glu-A3* alleles in some cultivars carrying different *Gli-A1* alleles. In: Wheat Kernel Proteins: Molecular and Functional Aspects, Proceedings of the Symposium at S. Martino al Cimino, Viterbo (Italy), September 28-30, pp. 115-120.

Redaelli R., Morel M.H., Autran J.C. and Pogna N.E. (1994). Genetic analysis of low *M*r glutenin subunits fractionated by two-dimensional electrophoresis A-PAGE × SDS-PAGE. J. Cereal Sci., 21 (1), 5-13.

Poster

Redaelli R., Morel M.H., Pogna N.E. and Autran J.C. (1993). Genetic analysis of low-molecular-weight glutenin subunits of wheat by two-dimensional electrophoresis. Poster presented at "Alpes-Phorèse", 11th Symposium of the French Society of Electrophoresis, December 1-3, Chambéry (France).

Task C.5 - Genetic and Technological Aspects of HMW Glutenin Subunits, HMW-Albumins and S-Proteins

Partner 03 - Istituto Sperimentale per la Cerealicoltura

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Sezione Operativa di S. Angelo Lodigiano
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1. Key measures of achievement - Objectives

The genetic variability for HMW glutenin subunit composition, the genetic structure of HMW-albumins and S-proteins, and the relationship with gluten viscoelastic properties are the objectives of this research.

2. Progress

Biotype 1 (*Glu-D1* encoded HMW subunits 2+12) and biotype 2 (only HMW subunit 12) were characterized at the molecular level (PCR analysis), confirming that the *Glu-D1*-1 gene coding for HMW subunit 2 is present but it is not expressed in biotype 2. The DNA sequence of this gene is currently being determined. The SDS sedimentation test carried out on flour from both biotypes grown in replicated plots showed that biotype 1 has superior breadmaking quality compared to biotype 2, suggesting a positive effect of HMW subunit 2 on gluten quality. Both biotypes were sown in replicated plots at S. Angelo Lodigiano. Several allelic variants of HMW albumins encoded by the group 4 chromosomes were identified in the F2 segregating progenies from seven crosses between Italian and French cultivars. The F3 progenies were sown in head-rows at S. Angelo Lodigiano.

3. Publications describing work carried out under Subprogramme C Task 5

Dachkevitch T., Redaelli R., Biancardi A.M., Metakovsky E.V. and Pogna N.E. (1993). Genetics of gliadins coded by the group 1 chromosomes in the high-quality bread wheat cultivar 'Neepawa'. Theor Appl. Genet., 86 (2-3), 389-399.

Pogna N.E., Metakovsky E.V., Redaelli R., Raineri F. and Dachkevitch T. (1993). Recombination mapping of Gli-5, a new gliadin-coding locus on chromosomes 1A and 1B in common wheat. Theor Appl. Genet., 87, 113-121.

Pogna N.E., Metakovsky E.V., Redaelli R., Dachkevitch T. and Chernakov V.M. (1994). The group 1 chromosomes of wheat contain several loci coding for gliadins. Proc. 8th Int. Wheat Genet. Symp., Beijing, China (in press).

Redaelli R., Pogna N.E., Dachkevitch T., Cacciatori P., Biancardi A.M. and Metakovsky E.V. (1992). Inheritance studies of the 1AS/1DS chromosome

translocation in the bread wheat variety 'Perzivan-1'. J. Genet. Breed., 46, 253-262.

Redaelli R., Metakovsky E.V., Davidov S.D. and Pogna N.E. (1995). Two-dimensional mapping of gliadins using biotypes and null mutants of common wheat cultivar 'Saratovskaya 29'. Hereditas (in press).

Task C.6 - Production of Lines and Near-Isogenic Lines with Different Glutenin Subunit Composition and Null-Forms

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1. Key measures of achievement - Objectives

The aims of these researches were (i) to study the effects of storage protein composition on breadmaking quality using near-isogenic lines grown in replicated plots and (ii) to introduce new alleles coding for LMW glutenin subunits and gliadins in European wheat germplasm.

2. Progress

Fourteen near-isogenic lines (NILs) of cv. 'Alpe' were characterized using one- and two-dimensional electrophoreses of storage proteins. These lines were grown in replicated plots in two locations and submitted to rheological analyses (alveograph and farinograph). Gluten form seven NILs lacking the *Glu-D1*/*Glu-D3* locus showed high elasticity and low extensibility compared to NILs possessing that locus. A strong interaction for gluten viscoelastic properties occurred between *Glu- B1*, *Glu-D1*, *Glu-B3* and *Glu-D3* loci. About 20 NILs isolated from the cross Neepawa × Costantino were sown in replicated plots at S. Angelo Lodigiano these lines are currently being analysed using the SDS sedimentation test.

3. Publications describing work carried out under Subprogramme C Task 6

Dachkevitch T., Redaelli R., Biancardi A.M., Metakovsky E.V. and Pogna N.E. (1993). Genetics of gliadins coded by the group 1 chromosomes in the high-quality bread wheat cultivar 'Neepawa'. Theor Appl. Genet., 86 (2-3), 389-399.

Ng P.K.W., Redaelli R., Vaccino P., Accerbi M., Pogna N.E. and Bushuk W. (1993). Biochemical and genetical characterisation of novel HMW glutenin subunits and their effects on breadmaking quality. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp. 161-169.

Pogna N.E., Redaelli R. and Biancardi A. (1993). Production and genetic characterisation of near-isogenic lines in the bread wheat variety 'Alpe'. Theor. Appl. Genet. (in press).

Redaelli R., Metakovsky E.V., Davidov S.D. and Pogna N.E. (1995). Two-dimensional mapping of gliadins using biotypes and null mutants of common wheat cultivar 'Saratovskaya 29'. Hereditas (in press).

Task C.7. - Chromosomal Location of Storage Protein Genes, Chromosome Interaction on Protein Synthesis and Development of New Germplasm

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1. Key measures of achievement - Objectives

The objectives of this task are (i) to learn more about variability and expression of genes coding for storage proteins in wheat, (ii) to develop new genetic material, and (iii) to study the relationship between protein composition and gluten quality.

2. Progress

In order to study the genetics of technological characters, 36 intervarietal substitution lines of bread wheat were investigated. The recipient was a good breadmaking variety: 'Courtot'. The substituted chromosomes were those of homoeologous groups 1 and 6 from 6 varieties ('Azteca', 'Cappelle-Desprez', 'Magdalena', 'Magnif 27', 'Prinqual' et 'Vilmorin 23'), with very different technological values. These lines were produced with 4 backcrosses and were checked with biochemical markers during production. Duplications were available for 13 substitution lines.

Parental varieties and substitution lines were characterized with several markers (morphological, biochemical, cytological and molecular). This permitted verification that the substitution had occurred: integration of donor chromosome (no occurrence of switch donor/recipient) without recombination (no occurrence of univalent-shift). Verification of the recovery of recipient background for the other marked loci was also possible. According to these verifications, 27 substitutions were successful and these lines had no donor allele on the other marked loci, for at least one of the duplications. Moreover, this work also contributed to the knowledge of some markers (chromosomal locations).

Based on these lines, 3 complementary studies were carried out: firth, the substitution lines were used to identify the important chromosomes; second, additivity, dominance and interaction effects of these chromosomes were evaluated in studies of F1 from diallel crosses of substitution lines; third, inbred recombinant lines for only 1 or 2 chromosomes were produced to specify respective effects of chromosome segments.

Parental varieties and substitution lines were studied in the nursery (1989-90) and in 2 trials of 3 replicates each (at Clermont-Ferrand and at Verneuil-L'Étang, in 1990-91) for some agronomic characters and the following technological characters: protein content, hardness, SDS sedimentation test, Pelshenke test and Chopin alveograph. The 2 substitution lines which differ the most from 'Courtot' were those of chromosomes

1A of 'Vilmorin 23' (poor quality) and 1D of 'Magdalena' (good quality but poor extensibility of dough). Each chromosome of homoeologous groups 1 and 6 may affect, more or less, protein content, a complex character greatly influenced by environmental conditions. Hardness is only slightly modified by these substitutions (only 1D of 'Magdalena' changed it slightly), according to the knowledge of this character (mainly controlled by chromosome 5D). The main effects on the other characters were principally due to homoeologous group 1 (coding for glutenin subunits). However, other chromosomes (group 6) may also have an effect. The polymorphism of HMW subunits of glutenin accounts for most of the variation in the Pelshenke test. This confirms, in a homogeneous background, the results of many studies made with a wide range of plant material. The results of SDS sedimentation test cannot be simply explained by HMW subunits of glutenin. The effect of chromosome 1B is very important on the alveograph parameters, elasticity and extensibility (negatively correlated) and consequently on strength (mainly correlated to elasticity). The lack of extensibility of 'Magdalena' and 'Prinqual' is due to chromosome 1D. The role of HMW subunits of glutenin is less clear for these characters and it may be necessary to use other factors such as LMW subunits of glutenin or some gliadins to explain them further.

Two types of diallel cross were made: on the one hand between substitution lines from diverse chromosomes of the same donor variety and the parental varieties, and on the other hand, between lines substituted for the same chromosome originated from different donors varieties. Some of these diallels (F1 and parents) were sown in the nursery (2 replicates, in 1990-91) and studied for the same characters as earlier (except alveograph). For this material, few interactions between non homologous chromosomes were noted and the main effects were additive rather than dominant, for technological characters, unlike for agronomic ones (1st diallel type). In the second type of diallel, own effects of each chromosome were more important than specific interaction between homologous chromosome too.

For the study of chromosome segments, 3 sets of inbred recombinant lines for only 1 or 2 chromosomes were produced by crossing substitution lines. The study of these lines had just begun during this work, but is continuing.

3. <u>Publication describing work carried out under Subprogramme C Task 7</u>

Bouguennec A., Bernard M., Branlard G., Rousset M., Gay G., Dardevet M., Cadalen T. and Bœuf C. (1995). Characterisation of a new set of intervarietal substitution lines of wheat with 'Courtot' as recipient variety. Agronomie (in press).

Task C.8. - Prediction of Quality from Protein Diversity

Partner 07N - INRA - Clermont-Ferrand
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1. Key measures of achievement - Objectives

The aim of this research was to develop a quality score based on the relationship between some LMW subunits of glutenin and quality as determined by biochemical and rheological tests.

2. Progress

(see report at Task C.4 above)

3. Publications describing work carried out under Subprogramme C Task 8

Branlard G. and Dardevet M. (1994). A null *Gli-D1* allele with a positive effect on bread wheat quality. J. Cereal Sci., 20 (3), 235-244.

Branlard G., Dardevet M., Nieto-Taladriz M.T. and Khelifi D. (1994). Allelic diversity of the ω–gliadins as revealed by SDS-PAGE: Their possible implication in quality variation. In: Gluten Proteins 1993, Proceedings of the 5th International Gluten Workshop, June 7-9, Association of Cereal Research, Detmold (Germany), pp. 234-243.

Khelifi D. and Branlard G. (1992). The effects of HMW and LMW subunits of glutenin and of gliadin on the technological quality of progeny from four crosses between poor breadmaking quality and strong wheat cultivars. J. Cereal Sci., 16 (3), 195-209.

Task C.9. - Somaclonal Variations for Factors Affecting Breadmaking Quality

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1. Key measures of achievement - Objectives

To study the effect of somaclonal variation upon qualitative and agronomic traits.

2. Progress

Six hundred progenies of regenerated plants were screened in the field. Those plants differing in morphology from their standard variety ("variants") were harvested and their seeds were analysed using Near Infrared Reflectance spectroscopy (NIRA) for protein content and grain hardness, and SDS micro sedimentation test for gluten quality. The same analyses were performed on seeds derived from plants belonging to the standard varieties grown as a control in the field.

All the variants showing large differences from the controls were studied in more detail using A-PAGE and SDS-PAGE electrophoresis and cytological analysis. More complex analyses were performed by other ECLAIR partners. Dr. Greenwell (CCFRA, Partner 14) measured starch friabilin amount and Particle Size Index. Prof. Lafiandra (University of Viterbo, Partner 23) performed 2-dimensional electrophoretic separation of gliadins and RP-HPLC separation of LMW glutenin subunits.

The agronomic potential of the most interesting variants and of the corresponding standard varieties were assessed in trials carried out on two different years.

The most common variations observed in the field were variations in plant height and earliness, whereas changes in shape and colour of leaves and spikes were less frequent, and changes to reaction to fungal diseases were very rare.

Deviations from the control varieties for the values of the SDS micro sedimentation test and of the grain hardness measured by NIRA were fairly common and in many cases were present in plants showing electrophoretic patterns of gliadins and/or glutenins different from those of the corresponding standard varieties. Usually, these differences consisted in the lack of the same components (the lack of glutenin components was detected only once). or differences in the amount of individual components.

The most common cytological abnormality observed in plants able to give normal progeny was the lack of a chromosome satellite.

The trials showed that, usually, the variants were producing less than the corresponding varieties, but we found rare exceptions that will be mentioned later.

In most cases, the above mentioned variations were stably inherited, the most common

exceptions being plants carrying gross cytological abnormalities that were usually sterile.

The variants we studied in more detail derived from the cultivar 'Salmone', that is carrying the two markers: Hairy glumes (*Hg*) on chromosome 1A, linked to the *Gli-A1* locus, and Red glumes (*Rg1*), on the satellite of chromosome 1B, linked to *Gli-B1* and *Glu-B3* loci.

Plants lacking the *Rg1* gene are very easy detected in the field because their glumes instead of red are white. The cytological analyses of these variants showed that in all of them only two satellites were present instead of the four carried by normal 'Salmone' plants. The two-dimensional electrophoretic analyses showed that three components were missing in the variants, two in the ω region and one in the γ region, these components being controlled by genes at the *Gli-B1* complex locus. The RP-HPLC analysis of LMW glutenins showed that a major peak of the LMW glutenin subunits was absent in the variants, these subunits being coded at the *Glu-B3* complex locus. From these observations, we deduced that *RG1*, *Gli-B1* and *Glu-B3* were lost through the deletion of the chromosome 1B satellite.

Cytological analyses and electrophoretic gliadin patterns of putative variants derived from the white glume variety 'Oderzo', suggesting that the lack of chromosome 1B satellite and therefore of the *Gli-B1* and *Glu-B3* loci, is relatively common.

The interesting thing about all the variants lacking the chromosome 1B satellite we studied, is that all of them showed a reduced grain hardness according to NIRA data. This is not easy to explain because it is known that a major gene controlling grain hardness (*Ha*) is located on chromosome 5D. On the other hand, we found a naturally occurring mutant and somaclonal variants of the cultivar 'Gemini' having reduced grain hardness but not lacking the 1B satellite.

The particle size index (PSI) and starch friabilin content of some of the variants showing reduced grain hardness when compared with the original variety were measured. In general, there was a good correlation between PSI and hardness estimated by NIRA. The softer variants of 'Gemini' and 'Oderzo' showed also an increase in starch friabilin content as the cultivar 'Salmone'.

A rare variant was found among the above-mentioned white glume variants of the cultivar 'Salmone'. In this variant also the HMW glutenin subunits coded at the Glu-B1 locus were missing.

Interesting information was obtained comparing the alveograph data of Salmone to those of the *Gli-B1*$^-$ *Glu-B3*$^-$, and also the *Gli-B1*$^-$ *Glu-B3*$^-$ *Glu-B1*$^-$ variants.

The *Gli-B1*$^-$ *Glu-B3*$^-$ variant showed more or less the same extensibility of Salmone (G, L), but a decrease of tenacity (P) and therefore a decrease in W. On the other hand, the *Gli-B1*$^-$ *Glu-B3*$^-$ *Glu-B1*$^-$ variant had a sharp reduction in extensibility but also a sharp increase in tenacity and, as a consequence, the W was only slightly lower than the one of 'Salmone'.

Other variants were found that we could not study in detail were glabrous glume

variants of Salmone lacking the gliadin components coded by genes at *Gli-A1* locus and variants of the cultivar 'Oderzo' showing the same ω-gliadin components of 'Oderzo', but with different intensity.

As it was already mentioned in the trials, the yield of the variants was usually lower than that of their control variety. Only in the last trial we found two variants of 'Salmone' (one of them with glabrous glumes) that outyielded significantly the control variety. The explanation of this unusual result is the much higher resistance to diseases (powdery mildew particularly) of these two variants, compared to 'Salmone'.

In conclusion, somaclonal variation is affecting breadmaking quality and can provide novel variation that is not readily obtained with conventional breeding methods. Most of this variation is interesting for study purposes only, but some of it can be satisfactorily exploited in a breeding programme.

Task C.10. - Sprouting Resistance

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1. Key measures of achievement - Objectives

Sprout damage is one of the main factors causing loss of quality in wheat thereby severely affecting farming profitability. Current methods to detect sprouting (Falling number test) are unsuitable for breeders and farmers.

2. Progress

Prevention of sprout damage is an objective long yearned for in the EU. The average costs of sprout damage once in every five years (leading to 10 % loss in yield and reduction of the amount of breadmaking quality by 50 %) is 50-60 million ECU per year. The approach envisaged by TNO in this project was entirely new in both concept and methodology. Instead of detecting levels of amylase, work has focused on:

- developing a bioassay to monitor inhibitors of germination

- purifying a fraction containing a germination inhibitor (which proved to be distinct from abscisic acid) in view to a rapid detection at an early stage and perhaps prevention In addition, the determination of the broadness of the genetic basis for dormancy should allow to select for sprouting resistance in breeding programmes.

Development of germplasm

Material from parent varieties has been characterized and indicate that there is a broad variation in dormancy, although the level of dormancy was rather low, due to the weather conditions before harvest. Progenies have been selected and are multiplied by the breeders according to plan. Harvest of the final material took place in August 1993. This material was highly variable in dormancy. Although a complete statistical analysis still has to be performed, a first analysis showed that the variation in dormancy exceeds the variation between parents. The statistical analysis could indicate how broad the genetic basis for dormancy is, and the degree of progress that can be made by selection.

Isolation of markers

Several promising purified fractions from six varieties are available and ready to test for inhibitory action in the bioassay. However problems have been risen in the aleurone test used as a testing system for endogenous germination inhibitors. Several possible causes have been evaluated, but non of them has been found as a final cause. This part of the project was therefore temporarily stopped.

A biochemical approach was chosen by purifying possible candidate inhibitors. The

fractions that were already purified and known from the bioassay as containing inhibitors were tested for abscisic acid and methyl esters of abscisic acid. From earlier experiments (gel filtration) was known that ABA would possibly not be the inhibitor. The molecule was expected to be larger (*M*r: 1000 - 2000).

Several monoclonal antibodies raised against pure (+)-ABA isotope and ABA methyl esters were used as a detection system.

Gel filtration

Figure 61 shows briefly the purification methods that were used. In the gel filtration fractions that were purified and still contain inhibitors, ABA esters were found (not shown).

Reversed phase and ion chromatography

The next steps in purification, ion exchange and reversed phase chromatography showed a further concentration and purification **(Figure 62a)**. The collected fractions were tested for ABA and methyl-ABA **(Figure 62b)**. The peak concentration of methyl-ABA has been determined by reversed phase chromatography. There is a clear peak in fraction 34 that contains a possible ABA methyl ester. ABA elutes under the same conditions a few fractions later. A known ABA methyl ester was also used as a reference. The concentration of different methyl esters will be measured in six varieties. Preparative columns are used to obtain sufficient material for identification of the ABA derivative. Further purification is in progress in order to elucidate the structure with NMR and Mass Spectrometry.

3. Publications describing work carried out under Subprogramme C Task 10

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CONCLUSIONS

As a whole, this ECLAIR project was a truly integrated programme. It has largely contributed to wheat improvement at a precompetitive level, witness the 140 papers already published by the participants, by improving the linkage of agriculture and industry through research, more especially by:

(i) filling the gap between process development and its understanding in terms of processing requirements and wheat quality requirements,

(ii) stimulating breeding and development of wheats capable of satisfying the present and future demands of European industry.

Its main results consisted of:

1. A better understanding on physico-chemical bases of the industrial processing of wheat and flour (milling, white and wholemeal breadmaking, starch/gluten industry, flour blends, fermented products and biscuit manufacture) which will now allows each participant to apply the newly acquired knowledge in his own industry.

2. An expression of the main wheat industrial processes and applications in terms of wheat quality requirements.

3. A development of improved methods for the rapid and efficient analysis and characterisation of lines in early stages of breeding and of wheat samples in trade.

4. A genetic base of strong-type lines which breeders can now utilise in view to introduce, in a longer term, and well beyond the limited framework of the four-year programme, new varieties of wheat with all the desired agronomic and technological characteristics, particularly the stability of the expression of quality in various environmental conditions of development of the plant and with the minimum use of chemical treatments.

5. A better identification of quality determinants whose genes should be identified, cloned, sequenced and possibly transferred.

Moreover, due to the tremendous exchanges of knowledge between participants and to the great success of the programme in a social sense, a European network with huge scientific power and excellent degree of communication was developed during the last four years, enabling to be optimistic about future research and development programmes on wheat science and technology.

PUBLICATIONS BY PARTICIPANTS IN THE ECLAIR PROGRAMME

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Abbreviations

A-PAGE	
SDS-PAGE	
NMR	
FTIR	
LMW	
HMW	
SE-HPLC	
RP-HPLC	
FPLC	
FTIR	Fourier transform infrared spectroscopy
NMR	Nuclear magnetic resonance
HPLC	
QELS	Quasi Elastic Light Scattering
NPP	Non Prolamin Proteins
ESR	Electron Spin Resonance
EC	European Community
EU	European Union
TNO-CIVO	
INRA	
ISC	
IATA	
EERM	
ENMP	
ITCF	
SDS	Sodium Dodecyl Sulfate
IEF	Iso Electric Focusing
CCFRA	
NSP	Non-Starch Polysaccharides
LMWD	Low Molecular Weight Dextrin
FD	Fermented Dough
UFD	Unfermented Dough
SD	Sour Dough

ST	Straight Process
SSNC	Soluble Nitrogen Compounds
ESP	Ethanol-Soluble Proteins
DP	Degree of Polymerisation
SPN	Soluble Protein Nitrogen
FPP	Free peptides
FAA	Free amino acids
BSA	Bovine serumalbumin
NIRA	Near infrared reflectance spectroscopy analysis