Protein Analysis of Wheat by Monoclonal Antibodies and Nuclear Magnetic Resonance

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1 Introduction

Wheat ranks first among our cultivated plants. Production in 1990 approached 600 million metric tons. Despite increasing industrial end uses, most wheat is used for food. In addition to providing a range of nutrients, it also possesses remarkable technological properties which allow the production of a variety of different processed foodstuffs such as bread, biscuit, and pasta. In addition, upon removal of the water-soluble components of a flour, *wheat proteins* have the unique property to form, with a few percent lipids, an insoluble and viscoelastic proteinaceous mass termed *gluten*, which forms the basis of the rheological properties of dough. Dry gluten, also, is increasingly used as improver or additive in flours and in various foods.

This great nutritional and functional importance of wheat proteins has stimulated investigation of the genetics and biosynthesis of wheat storage proteins, while the traditional interests of physical chemists in understanding the contributions of wheat protein components to milling, dough-forming, and baking properties of wheat have continued undiminished (Kasarda et al. 1976). The protein content of wheat grain is one of the basic measurements of its quality in marketing, while protein composition is primarily responsible for quality differences among different varieties. For instance, the presence or ratio of some allelic variants of gliadin or glutenin fractions are valuable indicators of the bread-making potential in wheat breeding programs.

There is some evidence that the conformations adopted by some specific protein components (e.g. HMW or LMW subunits of glutenin) play an important role in dictating the functional properties of wheat gluten. However, these proteins are highly heterogeneous and largely insoluble: their functionality appears in a weakly hydrated dough medium in which hundreds of constituents interact to determine cohesive, extensible, and elastic characteristics. The understanding of their functional properties cannot be derived from studies of protein solutions; and the detailed investigations of their basic components cannot be carried out while respecting the integrity of their native structure. These are the reasons why many conventional methods based on solubility, electrophoretic or chromatographic fractionations, and aimed at exploring gluten structure or detailed composition, have been only partially successful.

Despite many years of study, therefore, we do not yet have a detailed understanding at the molecular level of the basis for the unique properties of doughs and the way in which gliadins, glutenins, and other constituents contribute to the functional properties of different wheat flours.

Several recent advances provide the potential to make a significant step forward in a more complete understanding of the fundamental bases of quality as well as and in the development of improved wheat varieties and wheat products or dietary foods that come within legal requirements.

This chapter reviews the potential of two especially powerful techniques: *monoclonal antibodies* and *nuclear magnetic resonance*, which offer alternative means to study conformational aspects of cereal storage proteins, to yield information on the functionally important sites on proteins while approaching the quantification, composition, structure, and function of some important wheat protein fractions.

An understanding of the nomenclature and basic physical chemistry of wheat proteins is assumed; the reader is referred to the published reviews on cereal proteins (Shewry and Miflin 1984; Feillet 1988, Wrigley and Bietz 1988; Bushuk and MacRitchie 1989) and on physical (Tatham et al. 1990) and immunochemical (Skerritt 1988) aspects.

2 Monoclonal Antibodies

2.1 Immunochemistry in Wheat Proteins

The immunochemical studies of wheat proteins have been pursued by two main groups of researchers. Physical chemists have used immunochemical methods to study protein structures. Simultaneously, clinical immunochemists have investigated the serum antibody response to wheat and other cereal proteins in gluten intolerances such as celiac disease.

Approaches to immunochemical studies of wheat proteins and enzymes are numerous, and each of them can incompass various techniques. They include immunoprecipitation in solution or in gel, immunoabsorption, immunoaffinity chromatography, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), dot-binding and immunoblotting assays, and immunohistological techniques.

Early immunological studies of wheat relied on less informative techniques requiring precipitin lines to indicate antibody-antigene reaction and polyclonal antisera comprising a multiplicity of antibodies. Much more specific information can now be obtained with monoclonal antibodies, and with radio- or enzyme-linked assays together with direct testing of zones separated by gel electrophoresis. Fuller reviews of the principles of these techniques and their particular use in the study of cereal proteins and enzymes have been recently provided by Daussant and Bureau (1988) and Skerritt (1988).

2.2 General Principles of Monoclonal Antibody Production and Utilization

Immunochemistry makes use of special seric proteins (immunoglobulins), called antibodies, which appear in vertebrates in response to the injection of foreign constituents called antigens. During immunization, antigens induce the proliferation of specific clones of B-lymphocytes and the production of antibodies specific for various discrete parts of the antigen molecules. These parts consist of a small number of amino acids and are called antigenic determinants, or epitopes (Daussant and Bureau 1988). Because proteins generally have several structurally different epitopes, several B-cells with different receptors can be selected. They multiply and give birth to several clones of cells. All cells from the same clone synthesize antibody with the same specificity. But, as several clones are involved in this production, the antibodies of an immune serum are called *polyclonal antibodies.* As the population of antibody-producing cells is not constant, the antibody sets vary. Consequently (even if a single antigen is used for immunization), immune serums sampled at different times, or from different animals immunized with the same antigen, generally contain an antibody specific for the same epitope and others specific for different epitopes.

In contrast, antibodies specific for a single epitope can be proliferated in tissue culture by the selection and multiplication of a single-immune competent cell. Such antibodies produced by cells of the same clone and possessing therefore exactly the same specificity are called *monoclonal antibodies.* Presently, a clone can be obtained from a single cell by fusion of myeloma cells with B-lymphocytes taken from the spleen of an immunized mouse. The resulting hybridoma retains the properties of both mother cells: it secretes antibodies with the same specificity and multiplies indefinitely. Hybridomas are selected by culturing the cells in a medium in which the growth of either parental line is not possible. The hybridomas are then separated, and the antibodies produced by the resulting clones are tested for the desired specificity by ELISA and immunoblotting. In contrast to polyclonal antibodies, which constitute a collection of immunoglobulins with different specificities, monoclonal antibodies are constant in their specificity; they can be produced in unlimited quantities; their secreting cells can be stored in liquid nitrogen to be later expanded at will (Daussant and Bureau 1988).

However, monoclonal antibodies from one clone can only precipitate if this antigene possesses only two copies of the particular epitope that is recognized by the monoclonal antibodies. If only one copy is found per antigen molecule, only small, nonprecipitating complexes consisting of one antibody molecule combining with identical epitopes on two separate antigen molecules can be generally formed (Daussant and Bureau 1988). In contrast to the polyclonal antibodies, therefore, monoclonal antibodies from one clone alone cannot be used in immunochemical techniques based on the formation of large antigen-antibody complexes. They are, however, well suited for other analytical techniques, where detection of antibody-antigen recognition is based on other means. Such methods include the immunofluorescence method and the ELISA (Vaag and Munck 1987; Gallant et al. 1989).

2.3 Methodological Problems in the Immunochemical Study of Wheat Proteins and Use of Monoclonal Antibodies

One factor that has complicated the use of immunochemical techniques in cereal protein analysis was the difficulty to obtain good serum antibody responses to certain protein fractions such as prolamins. For instance, the titer of the hordein antiserum obtained by Asano et al. (1983) was 1/100 of that of an albumin-globulin antiserum, and, to produce rabbit antisera to gliadin, Ciclitira et al. (1985) performed over a dozen immunizations.

According to Skerritt (1988), the *poor imune response* to prolamins was due to immune tolerance or suppression resulting from antigen feeding rather than to poor immunogenicity of prolamins as such.

A second major difficulty resulted from the *insolubility* of storage proteins. Initial attempts based on diffusion-in-gel methods appear best suited for water- or salt-soluble extracts. Since they required long periods for diffusion and consume large amounts of antisera, they were unsuited to prolamins or glutelins. Fortunately, the enzyme-immunoassay methods developed over the last years with monoclonal antibodies proved adaptable to water-insoluble proteins, they are rapid (a few minutes), amenable to automation, sensitive, and suitable for analysis of large numbers of samples (Skerritt and Henry 1988).

However, because many of the common solvants for wheat storage proteins (urea, alcohols, acids, detergents) are protein denaturants, antigenic reactivity may still be lost or reduced. It has been often necessary, therefore, to optimize conventional procedures in order: (1) to retain native epitope structures; (2) to produce an acceptable signal; and (3) to have a good retention on the solid phase, making the monoclonal antibodies able to function with convenient materials (microwell plastic trays). In addition, the screening assays had to be chosen with greatest care, for instance, to select monoclonal antibodies able to recognize the spatial (as opposed to sequential) organization of residues and, in any case, to select antibodies designed to specific purposes (EIA, immunoblotting, immunocytochemistry, etc.).

The effects of various solvents of wheat gliadins and glutenins, and the nature of the solid phase used for antigen immobilization were thoroughly studied by Skerritt and Martinuzzi (1986). Antigen solvents affected both protein retention on the solid phase and the sensitivity of detection: alcohol- and urea-based extractants were far superior to SDS solutions. A panel of 12 monoclonal antibodies also showed marked differences in their abilities to bind to antigen immobilized on nitrocellulose and on plastic. Despite better convenience of microwell plastic trays for screening large numbers of samples, the lower binding capacity of gluten proteins (some clones did not produce a signal with gliadin bound to microwell EIA plates, even at high concentrations) and the possible alteration of epitope structure made the use of nitrocellulose disks (prepared from a nitrocellulose solid phase soaked in food extracts) a more suitable solid phase for use with trace antigens in protein mixtures or with certain monoclonal antibodies (Skerritt and Martinuzzi 1986). Recent developments from Skerritt and Hill (1990a), however, including optimization of coat-

ing and washing polystyrene microwells and choice of solvent and extracting conditions, allowed the development of novel high-affinity monoclonal antibodies able to function for the first time in microwell sandwich EIA and to accurately quantitate gluten in all types of foods.

Mills et al. (1989a) confirmed that direct absorption of proteins onto the surface of microtitration plates may cause considerable loss of native structures. To retain absorbed gliadin without disrupting conformational epitopes, they developed another two-site, enzyme-linked immunosorbent assay for wheat gliadins in which chicken antibodies against gliadin are absorbed to the microtitration plate as capture antibodies.

2.4 Main Applications of Monoclonal Antibodies to the Study of Wheat Proteins

Interest in the application of monoclonal antibodies to wheat proteins has come from two main directions: investigation of structural relationships between protein fractions of wheat and other cereals by physical chemists and study on clinical sensitivity to cereals. Alternatively, other applications of these methods have given insights in various aspects of cereal knowledge:

- Genetic studies including gene expression, genome relationships, and varietal identification.
- Studies of grain development and localization of specific protein components.
- Aid in molecular biology studies.
- End-use quality studies.
- Immunological detection of gluten in foods.

The following sections evaluate the present impact of monoclonal antibody methods, pointing out the various applications, limitations, and trends.

2.4.1 Structural Homologies Between Wheat Proteins

Immunochemical techniques have great potential for studying antigenic homologies between proteins and thus structural homologies of regions on the molecular surface. Monoclonal antibodies raised to gluten proteins have been used to examine the extent of structural homology of gluten proteins within or between genotypes of bread wheat, and also between similar proteins in related cereal species.

In earliest studies, Skerritt et al. (1984) prepared monoclonal antibodies to a gliadin protein extract of *Triticum aestivum* and specific antibody-cereal protein interactions were detected using horseradish peroxidase-coupled second antibodies after transfer of proteins to nitrocellulose following electrophoresis. Many clones showed broad specificity, while several clones secreted antibodies selective for smaller families of gliadins such as ω-gliadins and bound neither

with HMW subunits of glutenin, albumins, globulins, nor a variety of other proteins.

However, certain related species such as durum wheat, barley, and rye contained endosperm proteins recognized by these monoclonal antibodies. This observation confirmed that *sequence homologies exist between prolamins from wheat and related cereal species* as well as between certain gliadins from hexaploid wheat (du Cros et al. 1984; Skerritt et al. 1984).

Skerritt and Underwood (1986) have used immunoblotting methods to classify the specificities of a library of monoclonal antibodies. While most anti-gliadin monoclonal antibodies bound to all gliadin bands separated by PAGE, several antibodies binding to smaller gliadins were identified. At higher concentrations, however, these specific antibodies bound to an increasing number of α-, β-, and γ-gliadins, indicating very high sequences homologies between groups of gliadins.

In recent investigations, Skerritt and Lew (1990) prepared a library of monoclonal antibodies to wheat gluten proteins and studied quantitatively the interactions with extracts of total seed storage proteins from related cereals using immunoblotting and EIA techniques. They found antibodies giving four cross-reaction types that were generally in agreement with structural homologies determined from DNA sequencing: (1) selective binding to wheat proteins; (2) similar specificity with gliadins with binding to prolamins from other species (rye, barley, oats); (3) specific binding to certain γ- and ω-gliadins and HMW subunits of glutenin, with strong binding to rye and barley proteins; and (4) anomalous cross-reactivities such as binding to wheat or maize proteins but no binding to rye or barley proteins. In these studies, however, the interpretation of quantitative data was limited by a number of constraints such as differences in binding according to the cereal variety within a species, solvent, assay format (indirect, competition, or sandwich ELISA), solid phase (plastic, nylon, or nitrocellulose membrane).

The effects on apparent antibody specificity and cross-reaction have been investigated in detail by Skerritt and Hill (1990b). Because specificity could be manipulated by variation of one of these parameters, these authors strongly recommended *to define cross-reaction of antibodies with respect to the assay format used* when reporting any immunological homology. Moreover, because cross-reactivity of monoclonal antibodies to cereal proteins showed considerable variation with either broad or narrow specificities, Freedman et al. (1988) stressed the point that, in any attempt to determine homologies or classification of cereal proteins, *a very careful selection of antibodies* (and of proteins or peptides used for their characterization) is needed.

Although *glutenins* have been the subject of fewer studies than gliadins, monoclonal antibodies to glutenins were also produced, with specificity for all major groups of glutenins, although at low concentration some bound selectively to a single subunit (Skerritt and Underwood 1986). Other anti-glutenin antibodies bound to a variety of proteins including γ-gliadins or HMW subunits of glutenin, suggesting the presence of identical or at least similar epitopes in these different groups (Mills et al. 1990) and weakening the conclu-

sion of Ewart (1977) that such homologies could largely result from mutual contamination. Moreover, Skerritt and Robson (1990) compared the immunological homologies of low molecular weight (LMW) subunits of glutenin with the other major gluten polypeptides (HMW and gliadins) by one-step, or two-step SDS-PAGE in concert with immunoblotting and EIA methods. Many antibodies raised to gliadins and HMW bound to LMW and antibodies with specificities for similar groups of gliadins bound to similar groups of glutenins (Table 1), supporting conclusions from gene sequencing studies that LMW may be responsible for many biochemical properties and quality effects previously attributed to gliadins. On the other hand, some antibodies bound to each of the major gliadins, LMW and HMW, but not to other grain proteins, suggesting the existence of "common gluten" amino acid sequences or conformations (Skerritt and Robson 1990).

Another class of wheat endosperm nongluten protein, which is increasingly considered, is *surface starch granule protein (SGP)*, a fraction (0.2% – 0.3% by

Table 1. Cross-reaction of monoclonal antibodies with gliadins and glutenins – immunoblotting results (Skerritt and Robson 1990)

Gliadin specificity	Glutenins[a]		
	HMW (A)	LMW	
		B	C
$\alpha\beta\gamma$ (high-mobility)-Gliadin binding			
221/23 ($\alpha>$)[b]	–	–	+ +
230/9 ($\alpha\beta$)	–	+	+
403/8 ($\alpha\beta>\gamma$)	–	–	+
227/22 (β)	–	–	–
404/6 ($\beta>\gamma$)	–	–	±
222/5 ($\alpha\beta\gamma$)	–	–	+ +
$\gamma\omega$-Gliadin binding			
218/17	±	+ +	+
236/9	+	+	±
237/24	+	+	±
246/21	+	+	±
401/16	+	+ +	+ +
ω-Gliadin binding			
122/24 (ω slow)[b]	+	+	+
304/13 (ω fast)	+ +	–	–
401/21 (ω slow)	+	+	+
405/7 (ω slow)	+ +	+ +	+ +
Broad specificity			

[a] No reaction (–), rather weak reaction (±), weak-moderate reaction (+), very strong reaction (+ +). HMW and LMW, high and low molecular weight, respectively.
[b] Specificity determined by acidic-buffer PAGE and immunoblotting.

weight) that is retained by washed wheat starch. Monoclonal antibodies have been raised to SGP by Ariss (1986), giving surprisingly cross-reactions with HMW subunits of glutenin and ω-gliadins. Recently, Skerritt et al. (1990a), using immunoblotting, ELISA, and immunocytochemical techniques, observed that antibodies with similar gliadin and glutenin specificities had similar SGP specificities. For instance, some antibodies to α-, β-, or γ-gliadins labelled both protein bodies and the periphery of starch granules in sections of immature grains. In addition, antibodies binding broadly to all major gluten protein classes also bound most SGPs, although the Mr 15000 fraction, which has been associated with endosperm softness, appeared immunologically distinct.

2.4.2 Genetic Studies: Genome or Variety Relationships and Gene Expression

Storage proteins show considerable polymorphism and prove useful as genetic markers. In connection with various immunochemical studies applied to taxonomy and genome-species relationships, monoclonal antibodies (especially through immunoblotting methods and quantitative immunoassays) have recently permitted further understanding of genetic linkages between gliadins genes on specific chromosomes (Skerritt 1988). Interestingly, removal of one chromosome pair and substitution of a homoeologous pair did not alter the binding of certain antibodies, so that genes on several chromosomes seemed to be necessary for full binding of (for instance) one β-gliadin-specific monoclonal antibody. This finding suggests that the corresponding epitopes were present on proteins coded by any of a large number of genes on different chromosomes and supports the theory that the large number of gliadin polypeptides in each wheat variety results from duplication and divergence of a few ancestral genes (Kasarda 1984). This possibility and additional information on the possible genome-species origin of various epitopes were reinforced by the study of the primitive wheats such as *Triticum monococcum* (A-genome donor) or *Triticum speltoides* (putative B-genome donor) (Skerritt 1988).

Several important applications of this breakthrough in genome-species origin of wheat protein epitopes may be expected: measurement of the level of specific protein products of quality-related genes; determination of the gene copy number by immunological measurement of the level of a gene product; new insights in the regulation of gliadin gene expression by genes on distant chromosomes, whose study should be of high priority with regard to better control the expression of quality-related genes in wheat (Skerritt 1988).

Despite the availability of methods (immunoblotting, quantitative EIA, and RIA) for determining antibody specificities, fewer authors have been successful in preparing variety-specific antibodies to gluten proteins. Fritschy et al. (1985), using a "sandwich" EIA with antigen extracts of several *Triticum aestivum* and *Triticum durum* cultivars found large varietal differences in the binding of an antiserum to aggregable A-gliadin, but the varietal differences

of an antiserum to α-, β-, and γ-gliadins were lower. In connection with similar studies on various cereal varieties (Skerritt et al. 1986; Wrigley et al. 1987a), and supplementing electrophoresis, HPLC, or turbidity techniques, the binding of various antibodies (including an ω-gliadin-specific monoclonal antibody) was examined by du Cros et al. (1984) and Skerritt et al. (1988) among Australian cultivars, showing that a specific binding of this antibody to a pair of slow-moving ω-gliadins occurred in all varieties. Quantitative antigen-competition immunoassays, developed by Skerritt et al. (1987) to measure the effect of sulfur deficiency in wheats, were also reported by Skerritt (1988) with several monoclonal antibodies to assess varietal differences in binding.

Recently, with respect to discriminating cultivars and overcoming the high degree of homologous amino acid sequences between gliadins which result in a high degree of cross-reactivity when polyclonal or even monoclonal antibodies are prepared against whole gliadin, Dawood et al. (1989) prepared monoclonal antibodies against two purified gliadin components (γ-45 and α-74). While protein blotting of total gliadin separated by SDS-PAGE showed that monoclonal antibodies prepared against α-74 bound to a large region corresponding to α- and β-gliadins, those prepared against γ-45 bound to one discrete region corresponding to the location of the starting antigene, indicating that γ-45 had an unique epitope. According to Dawood et al. (1989), the higher specificity of the latter monoclonal antibody compared with the results of Skerritt et al. (1984) was presumably due to the use of a pure antigen, which gave the immunized animal a chance to develop antibodies against a unique epitope that may be present at only a low concentration in a total gliadin extract. Thus, such clones may have the potential to establish an immune-based test to differentiate wheat cultivars using a battery of monoclonal antibodies prepared against several purified gliadins, as well as to determine the proportions of wheat, rye, and other cereal grains in various food and feed products.

2.4.3 Identification of Translation Products of mRNAs and Characterization of cDNA Clones Expressing Specific Wheat Endosperm Proteins

The identification of in vitro translated proteins needs highly sensitive and specific techniques. When classical alcohol extraction and SDS-PAGE methodology are used to characterize gliadins, other proteins of similar molecular mass such as LMW-glutenins are also solubilized. On the other hand, highly aggregative and insoluble fractions such as HMW glutenins cannot be studied and prematurely terminated translation products or precursors cannot be recognized either.

Due to their high specificity, immunoprecipitation and immunoblotting are therefore increasingly used in concert with molecular biological techniques to identify the protein products of mRNAs, to study the relationship between mRNA levels and protein product level, to identify precursors, to detect post-translational processes, or to establish whether the synthesis of particular

Table 2. Specificities of monoclonal antibodies with blots from one-dimensional SDS-PAGE blots, double one-dimensional SDS-PAGE blots, and with starch granule proteins (Donovan et al. 1989)

Clone	Subclone	Isotype	SDS-blots (one-dimensional)	SDS blots (LMW glutenins double one-dimensional)	Starch granule proteins
218/17	7E8	IgG1	γ/Fast ω + HMW glutenin	+ +	+, Surface
221/23	8D11C8	IgG1	α-gliadin	+	+, Surface
222/5	9F9	IgG1	Gliadin, mainly α, β, γ	+ +	+, Surface and intrinsic
227/22	12H12	IgG1	β-Gliadin	−	+, Extrinsic
228/20		IgG1	α- and β-gliadin	+ +	−
236/9	13C6	IgM	γ, ω + HMW glutenin	+	+ +, Intrinsic
237/24	4H11	IgM	γ, ω + HMW glutenin	−	+, Intrinsic
246/21	18F2	IgM	γ, ω + HMW glutenin	+	+ +, Intrinsic
304/13	B2	IgG1	Fast ω, weak HMW glutenin	−	+, Intrinsic

endosperm proteins is under transcriptional or translational control (Reeves et al. 1986; Skerritt 1988).

In addition, molecular biology has provided potential tools for separating and characterizing both cDNA and genomic clones encoding wheat storage proteins and has allowed both the primary sequences of the proteins and the putative controlling regions of the genes to be deduced. The extensive homologies of gliadin or glutenin components, however, have resulted in a high frequency of cDNA cross-hybridization making the screening and characterization of the clones laborious and tedious. Following various studies on zein and other maize proteins, monoclonal antibodies have proved invaluable for the screening of *expression vector* libraries (such as in lambda gt11), i.e., in identifying hybrid proteins after their synthesis by bacterial colonies bearing recombinant DNA coding for wheat proteins (Donovan et al. 1989; Table 2).

2.4.4 Studies on Grain Development and Localization of Specific Protein Components by Immunocytochemical Methods

Immunological probes have been used to follow the developmental accumulation pattern of wheat storage proteins. The use of immunoblotting methods in concert with several monoclonal antibodies of different specificities allowed the recording of temporal differences in the deposition of various protein groups and showed that maturity patterns were altered by environmental stresses such as sulfur deficiency (Skerritt et al. 1988a).

Another application has been the localization of specific proteins on sections of grain, dough, or baked goods by immunocytochemical methods. These include tissue fixation, incubation with antisera labeled with either a fluorescent dye, an enzyme (peroxidase), or gold particles and examination by light or electron microscopy (Gallant et al. 1989). The use of monoclonal antibodies coupled directly or indirectly to colloidal gold has therefore enormous potential for locating individual proteins at the ultrastructural level and offers an alternative approach to conventional physical techniques for investigating the changes taking place during dough development and bread-making (Parker et al. 1990). In an early study, Ariss (1986) prepared monoclonal antibodies to various wheat proteins; some monoclonal antibodies to gluten proteins were protein body-specific, while some labeled both glutenins and starch granule proteins; certain antibodies labeled protein bodies unevenly, suggesting the presence of internal substructures. In addition, antibody staining showed concentric rings of integral starch granule proteins in A-type granules, but not in B-type granules.

On the other hand, because of the very well-defined structural specificity of monoclonal antibodies, structural changes could also be demonstrated using antibody cytochemical studies of doughs (Ariss 1986). In baked dough, this study confirmed the existence of a gluten network in which starch granules are embedded. In contrast, changes from a protein matrix in which starch granules are deposited to a starch matrix surrounding aggregates of proteins were observed in extruded products. In addition, a loss of antigenicity of gluten proteins (but not of starch granule proteins) at high extrusion temperatures was observed.

In a recent report, Parker et al. (1990) developed an indirect, two-step, immunolabeling procedure to locate gluten proteins in wheat and bread. In developing grain, storage protein bodies were immunolabeled and there was no cross-reactivity with aleurone proteins or nonstorage proteins associated with starch. In addition, some epitopes were not destroyed by the baking process: a specific antibody was bound strongly to the cut surface of bread crumbs but did not recognize the gluten-gas cell interface.

2.4.5 Study of Protein Structure and Interactions

Much of the challenge of the 1990s remains in obtaining a more accurate picture of the relationship between the presence or amount of specific proteins in wheat and end-use quality (baking strength, grain hardness etc.). Complementary to electrophoretic or HPLC techniques, it is possible to exploit the potential of immunochemical methods, especially those based on monoclonal antibodies, to recognize protein conformation, to yield information on the functionally important sites, or to quantitative specific endosperm polypeptides.

The development of monoclonal antibodies has an important impact on the study of protein structure and interactions because *immunochemical analysis relates to the surface structure of proteins which are involved directly in inter-*

actions. Since they bind to single specific points of the antigen, monoclonal antibodies are especially valuable and may constitute surface markers of very high specificity. According to Feillet and Popineau (1990), raising monoclonal antibodies against various conformation states of a protein will yield *a series of different structural probes whose binding behavior can be related to the conformational state of the antigen.* Moreover, specific monoclonal antibodies of particular sequence domains can be prepared by raising them against peptides, natural or synthetic. Because the binding of monoclonal antibodies can be modified by conformational changes or protein-protein interactions that cause modifications in the access to antigenic sites, monoclonal antibodies (especially those raised against specific peptides) will allow a better identification of the interacting areas of wheat and dough proteins.

Because dough strength and baking properties are largely determined by both the subunit composition of glutenin polypeptides and the amounts of particular subunits, the effects of varying glutenin-subunit compositions on flour properties can be studied using genetic variants. With this objective, Skerritt and MacRitchie (1990) quantified certain glutenin subunits using a monoclonal antibody-based ELISA technique. With a number of sets of wheat varieties, both methods yielded positive correlations between the amount of glutenin aggregate or of subunits, and measures of dough strength. Studies, using lines lacking specific high and low molecular weight subunits, have shown that antibodies binding to D-genome HMW glutenin subunits (i.e., allelic types 5-10/2-12) yield the best correlations. In addition to providing valuable information on the structure of the gluten complex, these techniques can be applied to quality assessment in wheat breeding and by milling and bakery laboratories (Skerritt and MacRitchie 1990).

2.4.6 Breeding for Quality

As monoclonal antibodies can be utilized in very rapid (ca. 10 min) tests, which are easy to perform and allow efficient screening of large numbers of new lines for desirable characteristics, antibodies specific for proteins involved in quality may aid progeny selection in plant breeding programs. In addition, as single seeds could be used for source material in screening of breeders' samples for quality, the lengthy process of multiplication at present required for such characterization would be eliminated (Mills et al. 1989b).

Earlier attempts from Skerritt et al. (1987) were centered around the measurement of the detrimental effect of sulfur deficiency, which is associated with a change in relative proportions of gliadins, resulting in a decrease in dough extensibility. In this study, an antibody (clone 227/22) specific for a sulfur-rich fraction (β-gliadin) was identified whose binding in a competition enzyme-immunoassay showed significant correlation with flour sulfur and dough extensibility (Fig. 1).

In another study (Skerritt 1989) the screening of 71 wheat varieties from 26 countries with several gliadin- or HMW-glutenin-binding antibodies was de-

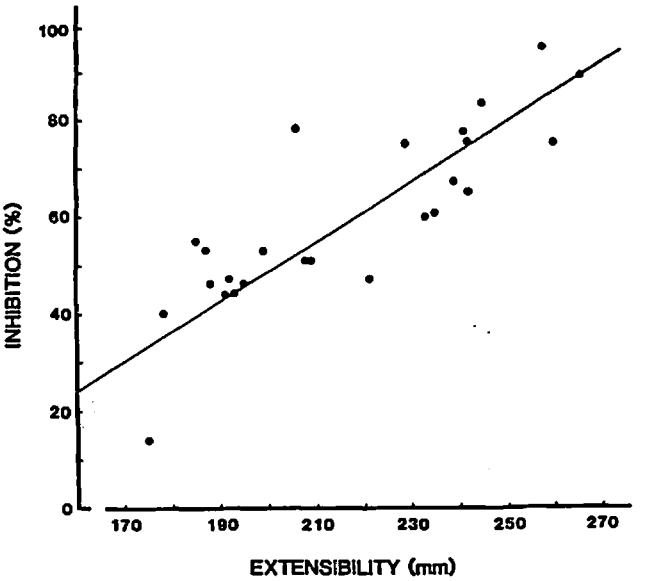

Fig. 1. Relationship between dough extensibility and inhibition of binding to antibody 227/22 (measured by competition immunoassay) for 25 samples of flour milled from field-grown Olympic grain (1980 trial). (Skerritt et al. 1987)

scribed, showing three types of antibody-binding patterns: (1) no varietal difference in binding other than that due to differences in total protein content; (2) varietal differences in binding noted, but no correlations found between antibody binding and quality parameters; and (3) varietal differences in binding, correlated with quality parameters. This final group of antibodies may be of use in quality testing. Of seven gliadin-specific antibodies tested by competition enzyme-immunoassay using flour extracts made with 1 M urea, two bound in a manner which correlated significantly ($P<0.02$) with dough resistance and dough-mixing work input. These were antibodies 230/9 and 404/6, with specificity to α/β- and β/γ-gliadins, respectively.

Another indication of the feasibility of using monoclonal antibody techniques to quantify specific proteins associated with quality traits was recently reported by Chan et al. (1990). Monoclonal antibodies were prepared to detect HMW-glutenin sequences that are assumed to indicate desired wheat characteristics such as the presence of a cystein residue at position 97 or of the amino acid sequence Thr-Cys-Pro, a characteristic of the HMW subunit No. 5. The use of monoclonal antibodies would therefore allow a rapid prediction of good baking quality and would be of benefit to millers wishing to control the quality of the flour which they buy, and also to cereal breeders who could readily determine the bread-making quality of a new wheat lines and thereby accelerate their cross-breeding programs.

2.4.7 Immunological Detection of Gluten in Foods – Adulteration of Wheat Products

Wheat gluten is used as an additive in foods, especially meat products or soups and desserts for economic reasons and for its functional properties, e.g.: viscoelasticity, thickening or binding capacity, and stabilization of oil-water emulsions. Consequently, there is a need to determine the amount of added gluten, which is strengthened by the existence of some individuals with gluten intolerances, including celiac disease (see Sect. 2.4.8).

Despite limitations due to the great variability of physical and chemical nature of foods and to the various mechanical or heat treatments that often result in a loss of solubility and antigenicity of proteins, immunochemical techniques have a great potential for gluten detection owing to their specificity and sensitivity.

Especially as conventional antisera used in food testing may fail to recognize antigens after cooking and because immunizing animals with heat-stable proteins may give rise to a large number of molecules of different specificity, Skerritt and Smith (1985) stressed the need to develop monoclonal antibodies, in particular to slow-moving ω-gliadins (which lack cysteine), the most heat-stable gluten fraction. Wheat and rye prolamins could thus be detected from a wide range of foods including baked goods and processed meats by simple transfer from PAGE patterns to nitrocellulose membranes, followed by treatment with enzyme-conjugated monoclonal antibody (EIA). Upon addition of the appropriate enzyme substrate, gluten-containing foods yield purple spots, while other prolamins from barley, oats, maize, or rice are weakly or not detected, and a wide range of nongluten common food proteins do not react (Skerritt and Smith 1985). This immunoassay was then improved to give quantitative results by soaking small disks of nitrocellulose in food extracts, incubating with an antibody and a horseradish-peroxidase substrate yielding a soluble product, and estimating the gluten content by photometric measurements using standard curves for gliadin (Skerritt 1985a, b;, Fig. 2). Further results and discussions on the factors governing the choice of suitable food and grain extractants, the methods for detecting a bound antibody, and the choice of primary or secondary antibodies were also reported by Skerritt et al. (1985), suggesting that artifact-free detection could be obtained using the peroxidase-antiperoxidase technique or by direct conjugation of horseradish peroxidase to the monoclonal antibodies. Because the assay based on handling of nitrocellulose disks proved tedious and lengthier than the use of microwells (which could not be used for quantitation of gluten in foods because of low affinities), novel types of high-affinity ω-gliadin-binding monoclonal antibodies that can be used in microwell sandwich EIA with an extremely high sensitivity have now been developed (Bony 1990; Skerritt and Hill 1990a).

Some other applications of monoclonal antibodies to detect adulteration of wheat products, including the detection of nonbaking varieties in baking wheats or of bread wheat in pasta, are of greatest relevance in countries with specific legislation prohibiting such additions (Skerritt 1988). Special attention

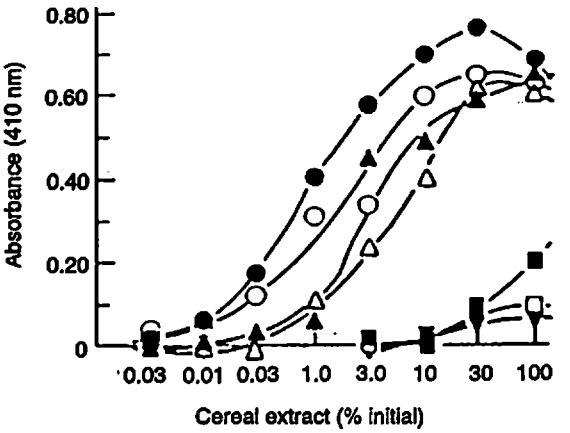

Fig. 2. Assessment of "gliadin-like immunoreactivity" in various cereals. Cereal extracts are indicated as follows: ● bread wheat, ○ durum wheat, ▲ rye, △ barley, ■ oats, □ maize, ▼ rice. The cereals were all assayed together. (Skerritt 1985a)

must be paid to the *determination of bread wheat in durum wheat pasta submitted to high temperature drying or precooking processes.* In such products, conventional electrophoretic methods (developed from low-temperature dried pasta and involving heat-sensitive albumins or enzymes) are useless. The difficulty may be overcome, however, by using antibodies against slow-moving ω-gliadins whose advantage is to bind to those proteins that are both highly resistant to heat treatments and encoded by specific genes on the D-genome of bread wheats. Alternatively, it could be considered to develop monoclonal antibodies against other – even heat-sensitive – proteins, which would recognize epitopes common to native and denatured structures. Should such antibodies be selected, accurate bread wheat determination will be possible in all types of pasta whether raw or processed.

2.4.8 Immunological Responses to Gluten in Humans: Celiac Disease

Celiac disease (gluten-sensitive enteropathy) is characterized by malabsorption of nutrients as a consequence of small intestinal mucosal injury. The incidence of celiac disease is thought to range between 1 in 300 and 1 in 1000 according to age group and country. Although further studies are still needed to understand the basic mechanisms of toxicity and to identify the toxic molecular structures, it is well accepted that these abnormalities are induced by *specific amino acid sequences contained in the gliadin fraction of wheat gluten* and, to a lesser extent, in the prolamin fraction of rye, triticale, and barley. Since as little as 100 mg of gliadin appeared to cause changes in the absorptive epithelial cells, the disease is treated by strict exclusion of dietary gluten; this treatment is lifelong. In order *to ensure the gluten-free nature of food* prescribed for such patients, or to monitor the legal limit of gluten that may be added in foods for economic or technological reasons, a rapid, specific, and reliable method for gluten detection is required (Ayob et al. 1988).

Because techniques such as electrophoresis, HPLC, or microscopy are slow or not really quantitative, and often unreliable with cooked or processed foods, immunochemical techniques are potentially the best to pick up traces of specific proteins among a large excess of others. Following several studies using polyclonal antisera against α-gliadin or total gliadin that failed to yield quantitative results in processed foods, rapid and more reliable immunoassays using monoclonal antibodies in two-site immunoassays have been developed by Skerritt (1985a, b) and Mills et al. (1989a). Recently, Skerritt and Hill (1990a) have obtained quantitative results by using a 40% ethanol extraction over a wide range of gluten contents (0.015% – 10%) and two simple monoclonal antibody-based test kits are now available for the quantitation of gluten. The first one is able to quantify gluten in all types of uncooked, cooked, and processed foods and is intended for laboratory use by the industry or government regulatory bodies, while the second provides rapid or semiquantitative results and is suitable for either home use or in-process quality control by manufacturers (Skerritt et al. 1990b). For these tests, antibodies to heat-stable ω-gliadins have been selected, so that binding is not influenced by food processing or cooking. In addition, these antibodies have been selected for binding to certain wheat, rye, triticale, or barley prolamins, with little cultivar variation, but not to proteins from maize or rice.

Panels of monoclonal antibodies have been developed to recognize the putative epitope that exacerbates celiac disease in individual cereal proteins. Through studies on the differential binding of the various fractions to specific antibodies, several research groups have found that α- and β-gliadins were more active than γ- or ω-gliadins in exacerbating the disease (Freedman et al. 1988). Moreover, by raising antibodies to synthetic peptides, some active sequences (e.g. PQPFPSQQPYLD) have been recently mapped to particular domains within the α-gliadin sequence (Ellis et al. 1989; Skerritt et al. 1990b). However, the enigma stands because closely homologous sequences were not found in barley or rye samples that were still celiac-toxic.

Only a few methods for accurate measurement of gliadin in food matrices have been proposed, however. RIAs using a polyclonal gliadin antibody showed some reactivity against barley, rye, and oat prolamins (Ciclitira et al. 1985). Monoclonal gliadin antibodies raised by Freedman et al. (1987a, b) also displayed reactivity against glutenins, indicating that the antigenic epitope may be present in several proteins but that it may not represent the polypeptide structure that is toxic to celiacs. Moreover, celiac disease may not be associated with a single characteristic pattern or amino acid sequence and distinct epitopes exacerbating the disease may exist in different regions. According to Friis (1988), the use of monoclonal antibodies to test the suitability of food products for the celiac diet may be insufficient, since they may be unable to measure all toxic polypeptides, so that a polyclonal prolamin-antibody reacting with various gliadin-like proteins might be preferred.

3 Nuclear Magnetic Resonance

3.1 What is NMR? Principles and Techniques

3.1.1 Why "Nuclear" and "Magnetic"?

As indicated by the qualifier "Nuclear", NMR concerns the atomic nucleus. A nucleus comprises uncharged neutrons and positively charged protons. Nuclei of certain isotopes with odd charge and mass numbers (^{1}H, ^{13}C, ^{31}P, etc.) possess a nonzero magnetic momentum μ, so that they behave as micromagnets. They are characterized by a spin quantum number and they have the capacity to interact with a magnetic field. See also Linskens and Jackson (1986).

If, for instance, hydrogen nuclei are placed in an external and uniform magnetic field, B_0, it is found that protons have only two allowable situations in the magnetic field (e.g. $+1/2$ and $-1/2$). All the nuclei will therefore distribute into these energy levels, which will result in the magnetization, M_0. *NMR is based on the population difference between these energy states.* It must be noted, however, that this difference is always very weak: a few units per millions of nuclei.

On the other hand, because the magnetic momentum results from the nucleus spinning, the momenta of the nuclei placed in B_0 will be in line with the field direction and, as in a top or a gyroscope motion, when pulled aside from its rotational axis, it slowly draws out a precessional orbit around B_0 with a frequency ν_0 called the Larmor frequency.

3.1.2 How to Attain the Resonance Condition

When a second oscillatory magnetic field B_1 (with a radiofrequency ν) is applied over a short time at 90° from the first one, an energy $E = h.\nu$ may be transferred to the system. By sweeping the frequency ν_0, the nuclear magnetic resonance condition can be attained and the energy transferred to the system can induce transitions between the energy states. Thus, for instance, many protons having the lower energy level ($+1/2$) will reach the higher energy level ($-1/2$) due to this net absorption of energy.

On stop of the radiofrequency field B_1, nuclei from the upper spin state will progressively return to the lower state according to various types of transitions called *relaxation processes.* These transitions will involve a release of energy as an emission wave that will be exchanged between close spins or between spins and the lattice or molecular nuclei. This emission wave may be detected in a perpendicular coil and it will correspond to the NMR signal.

3.1.3 What Information Can Be Deduced from the NMR Signal?

According to Guillou-Charpin et al. (1988), the versatility of the NMR signal is the result of its three main characteristic parameters: (1) initial intensity;

(2) spin-lattice relaxation, associated with time T1 (longitudinal spin relaxation time); and (3) spin-spin relaxation, associated with time T2 (transveral spin relaxation time). The first parameter depends on the density of a given type of nuclei in the field-excited region of the sample, and it can be exploited for quantitative determinations. On the other hand, *relaxation parameters are intimately related to molecular motions in the environment of the nuclei* (viscosity delays the return to equilibrium), and on *chemical structure and conformation of the microscopic surroundings of nuclei and molecules* (energy exchanges between precessing nuclei in close proximity shorten the lifetime of the spins in the higher energy state).

For instance, chemical shifts (usually reported in parts per million, ppm) of the resonance frequency of the nucleus may be observed. These arise because a given nucleus is shielded from the externally applied magnetic field to a different extent in different molecules (Wüthrich 1976).

The utility of the NMR method results therefore from the sensitive variation of the resonance frequency with chemical, microdynamic, and functional properties of polymers and their environments. In addition, a reconstitution of patterns, indicating the density and energy decay of a given type of nucleus, can be regarded as a photograph of the cell biochemistry.

NMR has recently gained a new dimension with the advent of various improvements such as computer-assisted processing of the signals, introduction of the Fourier transform method (FT-NMR) that turns the time-domain signal into the more familiar spectrum, use of color pictures of signals coming from several directions that allow one to draw two- or three-dimensional pictures, and the development of high-resolution methods using much higher frequencies (several tens or hundreds of MHz). Because a broadening of NMR lines was often observed, various technical improvements have been proposed. For instance, interactions with neighboring protons can be eliminated by irradiating the protons with high-power radio frequency (*dipolar decoupling*). On the other hand, chemical shift anisotropy can be eliminated by the process of "*magic angle spinning*" in which the sample is packed into a rotor and rotated at an angle of 54° 44′ in the direction of the magnetic field, yielding a narrower line provided the spinning rate is fast enough (Tatham et al. 1990).

Although nuclei with zero spin such as ^{12}C, ^{14}N, or ^{16}O are not accessible to NMR analysis, nonzero spin nuclei are omnipresent in biological materials. However, because the most sensitive signal is obtained with protons and small nuclei (resonance phenomena being more difficult to obtain with heavy nuclei), typical nuclei of interest in biology or agriculture and food industry are: ^{1}H, ^{13}C, ^{15}N, or ^{31}P. These have been used, for instance, to determine the amount of moisture, to investigate water movements and free/bound water, to control the origin and quality of food products by quantitation of the natural isotopes, and to carry out nondestructive studies on various biochemical mechanisms, or on the structure and dynamics of proteins.

3.2 General Interest of NMR in the Study of Wheat Proteins

In contrast to the considerable information available on genetics and biochemistry of wheat proteins, much less is known about their physical properties such as conformation and structural dynamics. This lack of information essentially results from the fact that most wheat proteins are soluble only under relatively severe conditions that affect their native structure. According to Belton et al. (1987a), wheat gluten represents a very intractable material from the spectroscopic point of view. The great complexity of wheat proteins has accounted for the delay in the characterization of their physical nature, and the residual heterogeneity of purified protein fractions may have also hampered progress in their crystallization and characterization in the solid state by X-ray diffraction techniques.

Physical studies of wheat proteins in solution have been undertaken using circular dichroism and optical rotatory dispersion, however, whether these proteins exhibit the same properties in solution as in the solid state is a question that has not yet been answered (Schofield and Baianu 1982). The use of other physical techniques which can provide additional information about the molecular properties of wheat proteins in relation with functionality are therefore needed. *NMR is one of the few techniques which can be used to investigate the whole gluten viscoelastic mass because it has the potential to provide information on both molecular dynamics and chemical environment and may determine the chemical configurations associated with certain dynamic properties* (Belton et al. 1987a). In addition, the resolved resonances can be used as probes for local environmental conditions since the parameters extracted from NMR spectra depend greatly upon the conformational and dynamic characteristics of the compound, as well as upon its interaction with the surroundings (Lecomte et al. 1982).

Two main methods have been used: ^{13}C-high-resolution NMR and ^{1}H relaxation time measurements. ^{13}C solid-state NMR can provide direct evidence for the chemical identity and mobility of the components (Belton et al. 1989). In addition, two alternative schemes have been experienced: the single-pulse excitation (SPE) that discriminates in favor of mobile components of the sample, while cross-polarization magic-angle spinning (CP-MAS) deals with signals arising from solid-like regions. On the other hand, ^{1}H relaxation time measurements allow the quantitation of the number and degree of mobility of protons in each environment.

3.3 Main Applications of NMR to the Study of Wheat Proteins

3.3.1 Physical Characterization of Gluten Proteins

3.3.1.1 Assignment of NMR Signals

In the first NMR studies on wheat proteins, the assignments of the proton peaks were difficult due to the sample heterogeneity and the superimposition

of proton lines in a narrow range of chemical shifts, so that only partial assignments were proposed. Further studies with respect to physically characterizing gluten and glutenin- or gliadin-enriched subfractions of gluten were carried out by Schofield and Baianu (1982) using solid-state CP-MAS ^{13}C NMR. It was found that the glutenin-enriched fraction gave sharp resonances corresponding to aliphatic and aromatic amino acids, whereas the gliadin-enriched fraction gave broad peaks in these regions. This feature suggested that a much greater level of hydrophobic interaction existed in the gliadin-enriched fraction than in the glutenin-enriched fraction, indicating that these two populations had different degrees of mobility and different levels of interaction.

Under similar CP-NMR conditions, however, Moonen et al. (1985) could not observe sharp resonances. This was confirmed by Belton et al. (1985), who could only observe sharp resonances when SPE was used. It was suggested, therefore, that these sharp resonances (very unlikely to exist in the solid state) might have arisen rather from small organic contaminants or from mobile side chains. The exact contribution of lipids to gluten NMR signals has been problematic for several years. In the beginning, signals from lipids were discounted

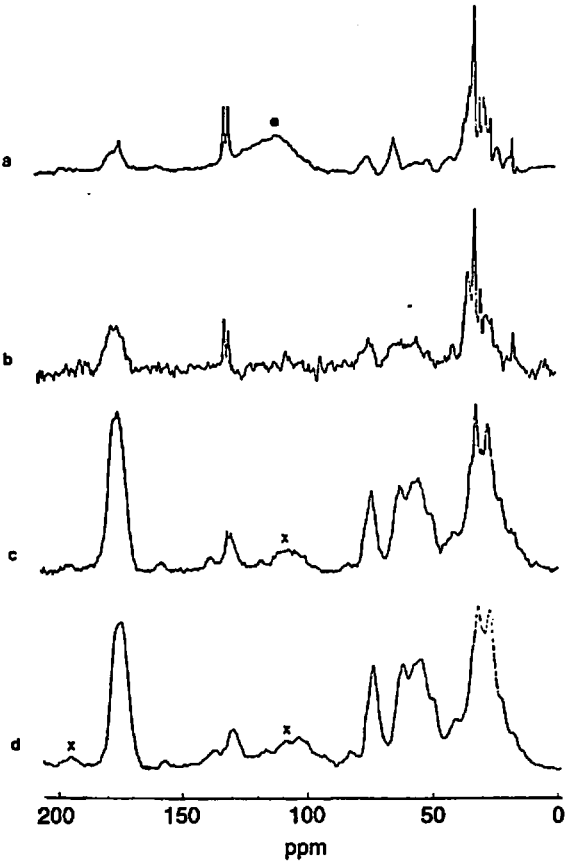

Fig. 3. Solid-state ^{13}C single-pulse excitation spectrum of gluten (a) and cross-polarization spectra with contact times of 20 (b), 5 (c), and 1 (d) μs. The peak marked with a *black dot* is a background signal; those marked with an *x* are spinning side bands. (Belton et al. 1988b)

because chloroform-methanol fractions gave broader bands than those observed by Schofield and Baianu (1982) from gluten. However, in another study (Belton et al. 1988a), the contribution of lipids was confirmed since sharp signals were completely lost upon total lipid extraction.

Belton et al. (1988b) and Tatham et al. (1990) interpreted the different results in terms of the *relative immobility in the proteins and mobility in the lipids.* They reported that the two sets of signals observed consist of a protein CP spectrum at short contact times and of a mobile lipid SPE spectrum or CP spectrum at long contact times (Fig. 3). Thus, whether gluten is studied as solution-state or solid-state, a signal from lipids may or not become visible.

3.3.1.2 Functional Properties of Dough and Gluten

In pioneer studies, Baianu (1981) and Schofield and Baianu (1982) reported high-field and high-resolution proton and ^{13}C NMR of wheat proteins (gliadins) in solution. Because a weighted summation of the spectra of the two fractions could not give a spectrum equivalent to that for the whole gluten, the authors suggested that hydrophobic interactions between gliadins and glutenins may be important in the gluten complex. In another study, Baianu et al. (1982) investigated changes in the NMR spectra of two cultivars and observed marked changes in gliadin spectra when temperature or concentration was increased. This suggested the possibility that ^{13}C (or ^{15}N) NMR spectra could provide a direct approach to *wheat variety identification, gluten microrheology,* and *nondestructive analysis of wheat proteins.*

However, ^{13}C solid-state methods could not give quantitative estimates of either mobility or the amounts of material in the different environments, making it necessary to use ^{1}H NMR relaxation methods and to interpret both transverse relaxation and spin-lattice relaxation. For instance, Le Grys et al. (1981) used ^{1}H-NMR and recognized the importance of exchange mechanisms in gluten and the possible contribution of mobile proteins to the relaxation process. More recently, Belton et al. (1987a, b, 1988a, b) could identify at least three motional regimes in transverse relaxation of dry gluten samples:

1) Component I (75% – 95% of the protons present) is characteristic of protons in very slow motions and originates from a rigid lattice condition.

2) Component III (3% – 23% of the protons present) indicates a mobile environment within the gluten and is assumed to arise from mobile protein.

3) Component IV, which disappears upon lipid extraction, is likely to represent the lipid content.

The transverse relaxation rate component III is of primary importance in protein dynamics because it may represent some part of the protein which is plasticized by interaction with the lipids (Tatham et al. 1990): lipids would interact with the hydrophobic side chains of the proteins, and act as a solubilizing medium, which would result in an increased mobility. In addition, the subset of protons that give rise to component III depends on the origin of the gluten. As shown in Fig. 4, component III significantly differs according to the

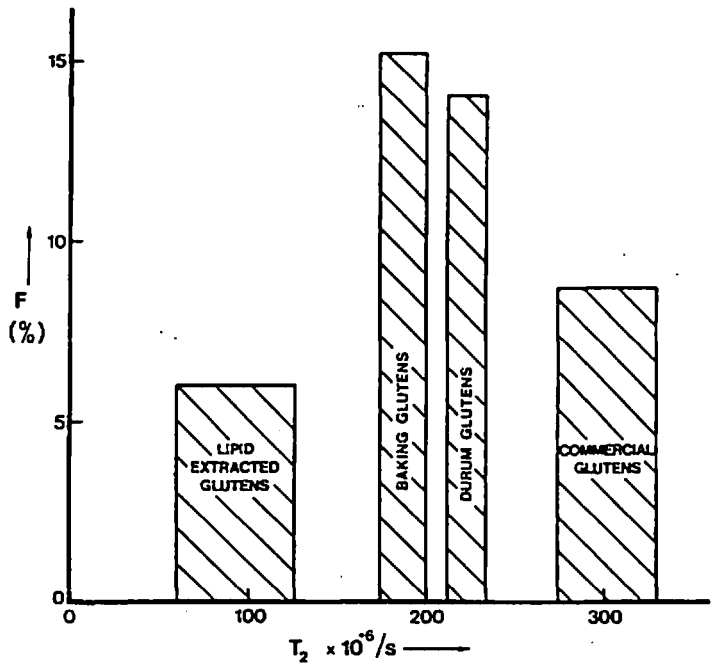

Fig. 4. The transverse relaxation rate component III observed in different glutens. F is the normalized population of the component with relaxation time T_2. (Belton et al. 1988a)

type of gluten (commercial, durum, baking, or lipid-extracted). Whether this difference represents the effects of heat or mechanical work or whether it can be used to assess or predict industrial gluten quality is not known, however.

On the other hand, spin-lattice relaxation has been investigated by Belton et al. (1988a, b) through both the rotating and laboratory frame. Whereas in the rotating frame, the slow- and fast-decaying signals have been assigned to lipids and proteins, respectively, the laboratory frame showed components insensitive to lipid content and gluten origin, in a domain of about 10 nm (in the order of the size of a small protein molecule).

Although it is still difficult to make general statements on the dynamics of the gluten system as a whole, a discussion of these phenomena has been reported by Tatham et al. (1990). These authors stress that *comments on mobility must be viewed in the context of the motional regime to which measurement responds.* They confirm the anisotropy of molecular motions in the gluten system, as motions of the gluten polymer chain are very slow in some directions but quite rapid in other directions. Whereas the *lipids affect the slow motions* of the polymers, leaving the faster motions unchanged, *disulfide bonds leave the slower motions unchanged but restrict the faster motions.*

Another approach has been attempted by Ablett et al. (1988) in the area of polymer dynamics based on relaxation data from ^{1}H-NMR with gluten hy-

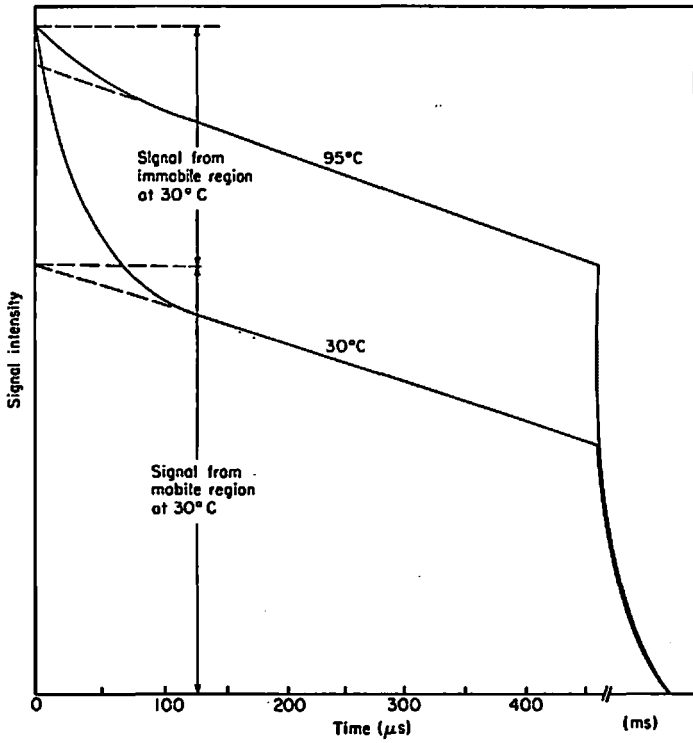

Fig. 5. Temperature dependence of the proton NMR-free induction decay signal for 40% gluten in D_2O. (Ablett et al. 1988)

drated with D_2O. (D_2O was used so that the NMR signal arises solely from the nonexchangeable protons of the protein, with no interfering water resonance). From the overall decay pattern, as shown in Fig. 5, they could separate an "immobile" component (fast-decaying) and a "mobile" component (slower-decaying). Furthermore, an approach to the changes that occur upon heating was possible since the mobile region covered about 95% of the protons at 90 °C instead of 65% at 30 °C. On this basis, Ablett et al. (1988) assumed that the changes in NMR spectra that occur upon heating are consistent with rheological changes that would be caused by the replacement of labile, noncovalent interactions by permanent disulfide bonds. Furthermore, both ^1H- and ^{13}C-NMR results would indicate that wheat proteins have open structures with a high degree of mobility, both at ambient and after heat setting, even though the formation of new disulfide bonds dramatically changes the rheological properties.

According to Wrigley and Bietz (1988), the potential of NMR in the study of hydrated dough systems must be pointed out because it can provide results likely to relate more directly to the conditions of actual dough mixing and baking. However, it still proved extremely difficult to show any consistent differ-

ence between molecular structures related directly to baking quality. For instance, in contrast to certain studies based on electron-spin resonance (ESR), Moonen et al. (1985) could not demonstrate any difference between glutenin subunits, as cultivars belonging to extremely different allelic acid types, such as Arminda (2–7–12) and Sicco (1–5–7–9–10), had identical CP-MAS spectra.

NMR spectroscopy is suitable in many other respects since it is theoretically possible, in the spectra of proteins, to determine that many signals arise from given amino types which can often be assigned to specific residues in the polypeptide chain. For instance, in an attempt to relate amino acid sequence and specificity of action in toxicity to phytopathogenic bacteria and insect larvae, the analysis of the NMR spectra of wheat and barley thionins enabled Lecomte et al. (1982) to identify all of the 84 methyl resonances by amino acid type and to assign 51 to specific sites.

3.3.2 Lipid-Protein Interactions in Wheat Gluten

It has been suggested that gluten is not composed of discrete molecules but of water-insoluble aggregates, including both proteins and lipids, so that a lipid-protein complex is formed during gluten preparation and dough formation. Lipid-protein interactions have been considered to play important roles in the physical and rheological properties of doughs since Grosskreutz (1961) postulated a gluten model involving the Danielli-type membrane to account for its extensibility. To give new insights in the dynamic properties of protein-lipid interactions in wheat gluten, nonintrusive physical techniques such as phosphorus NMR are the most desirable since the sensitive nucleus of phosphorus is naturally 100% abundant and no extrinsic probes are needed (Marion et al. 1987).

By applying ^{13}P-NMR spectroscopy in association with freeze-fracture electron microscopy, Marion et al. (1987) demonstrated that:

1) In aqueous gluten, extracted by hand-washing, phosphorus accounted for 160–190 mg/100 g dry matter and that more than 90% of the phosphorus NMR signal in gluten was due to phospholipids.

2) One of the features of ^{31}P-NMR spectra was the symmetrical shape of the signal, reflecting an isotropic motional averaging of phospholipids in gluten, which was retained during a heating-cooling cycle.

3) The line shape and broadening of the NMR signal was quite different when wheat flour lipids were extracted before gluten washing and the missing intensity in defatted samples was postulated to be due to phospholipids immobilized in the gluten protein framework as in biological membranes.

Furthermore, the interpretation of the line shape of NMR spectra, in concert with freeze-fracture electronic microscopy observations, gave further information on the macromolecular organization of gluten constituents. For instance, Le Roux (1987) and Akoka et al. (1988) suggested (1) that phospholipids in dough and gluten were organized in bilayer vesicles, whose radii ranged

from 30 to 150 nm; (2) that these vesicles were formed during flour hydration from hexagonal phospholipid structures (membrane remnants) of the starting flour; and (3) that the hexagonal phases themselves derived from a reversed-phase transition that occurred during the drying phase of grain development.

Moreover, the suppression of dipolar interactions and/or the decrease in the residual chemical anisotropy, which are indicated by the resonance broadening in ^{31}P-NMR, may be accounted for by the expulsion of lipid vesicles into the protein matrix upon gluten heating (Marion et al. 1987, 1989). Because heating also affects wheat glutenins (by promoting sulfhydryl-disulfide interchange reactions), the phospholipid rearrangements that are suggested by the increase in the resonance line from 25 °C to 70 °C may be concomitant to changes in gluten protein aggregates. Interestingly, the fact that vesicles would influence gluten protein aggregation may not necessarily involve interactions between classical gliadins or glutenins and lipids. In contrast to previous models, that of Marion et al. (1989) discounts the role (other than mechanical trapping) of Osborne's classical (1907) protein fractions in the lipoprotein interactions, and emphasizes rather the involvement of smaller (16.5 kD) and highly interactive molecules that showed homology with interface-stabilizer membrane proteins.

3.3.3 Nondestructive Protein Content Determinations in Wheat Seeds

There are various methods to measure the cereal protein content and several NMR techniques have been described. When the proteins can be solubilized, classical NMR methods measure the relaxation of water protons submitted to two (uniform and oscillatory) magnetic fields. Because proteins can complex metals such as Cu or Fe, resulting in different relaxation kinetics, it is possible to correlate NMR signals with the protein concentration. However, because wheat samples are sometimes needed for further analyses, the determination of the protein content may have to be nondestructive and possible from single kernels, which is far more difficult.

In contrast to lipids or water, which constitute a highly mobile phase, the more rigid constituents of the seed, such as proteins or starch, cannot contribute quite as well to the spectrum obtained by liquid-state techniques because of the long ^{13}C spin-lattice relaxation times and the broadening influences of ^{1}H-^{13}C dipole-dipole interactions (O'Donnell et al. 1981).

^{13}C-NMR spectra of rigid bioorganic materials have been obtained, however, which contain only signals from carbons in structures of low mobility owing to suppression of the intense liquid signals. Such spectra consist of two groups of resonances centered at $\sim$75 and $\sim$25 ppm, representing respectively, carbohydrates and proteins, so that the protein content may be estimated by comparing these peak intensities. Further improvement in the quantitative analysis was obtained by using proton-enhanced MAS, making the resonance lines narrower and resolving the spectrum better (Rutar 1982). Under these conditions, ^{13}C-NMR is of great potential value for the nondestructive determination of

the protein content in viable seeds. It may promote, therefore, the rapid breeding of new varieties with higher protein content.

Similar studies based on the amplitude of signal relaxation were also reported in an attempt to determine the gluten content in individual seeds and hybrids (Bebyakin and Lutsishina 1987a, b), indicating a possible checking of the gluten content (and perhaps gluten quality) in breeding programs.

Serious limitations and technical problems, however, are still to be faced in the development of CP-MAS as a routine technique. Results might be influenced by the size and shape of the seed, making the use of an outstanding instrument and high spinning speeds (3000 rotations/s) necessary. Another limitation would be the length of the analysis: a minimum of 15–30 min/seed seemed to be required.

4 Conclusion and Future Research

The practical importance of wheat proteins is well recognized. In contrast to the rapid technological development and diversification in the application of wheat in various foods, the precise molecular basis of the functional properties of wheat proteins is not clearly understood. Several recent advances, however, provide the possibility to explore in more detail the wheat protein structure, thus permitting a more effective quality control of wheat products.

4.1 Monoclonal Antibodies

With the absolute reproducibility of specificity or affinity, and with ability to be produced (in theory) in unlimited quantities, monoclonal antibodies have a tremendous potential for studies on wheat proteins.

Despite the unusual solubility and alleged poor immune response of gluten proteins, high-resolution techniques, such as immunoblotting and ELISA, are now available, providing an alternative and most useful adjunct to conventional protein chemistry for structural and functional studies on wheat gluten proteins and for rapid and simple approaches to their role in grain technology and human nutrition.

Monoclonal antibodies make it possible to overcome many of the difficulties arising from the use of polyclonal antibodies with wheat proteins: variations in the responses of individual animals, previous dietary exposure of animals in which antisera have been raised, poor specificity, and cross-reactions that confused the interpretation of results. Although polyclonal antibodies may still be recommended for some routine determinations or perhaps for overall quantitation of various antigenic determinants during food processing, monoclonal antibodies are replacing conventional antisera in many fields of immunological research and diagnostic testing.

Immunochemical methods using monoclonal antibodies proved attractive in a number of applications:

1) They have allowed a better identification of toxic components of gluten in celiac disease and a better understanding of the mechanism of toxicity.
2) They proved valuable in the localization of protein components by immunocytochemistry and the screening of expression vector cDNA libraries.
3) Because of the well-defined structural specificity of monoclonal antibodies, some studies on the homologies that exist between different gluten polypeptides and on the configuration of native or denatured antigens have been made possible.

Although much work is still needed to develop tests for specific assays in industry, many indications (including various patent applications) suggest that the 1990s will see the availability of many standardized reagent kits, thus assisting dietary management for celiac patients, quality prediction by breeders, varietal identification or quality assessment by millers and bakers, and monitoring of the composition of various foods (Wrigley et al. 1987b; Skerritt and Hill 1989; Chan et al. 1990; Skerritt et al. 1990c).

Advantages of monoclonal antibodies techniques include: sensitivity (possibility to detect as little as 0.0016% gluten proteins), well-defined structural specificity, speed (superior to electrophoresis or chromatography, longer, however, than NIR reflectance), small-scale and possible screening of a large series of samples, economy, and simplicity (less specialized and costly equipment than HPLC or NIR). However, for the production of monoclonal antibodies, more skilled personnel and much heavier equipment are needed, since cell cultures are involved. The recognition of antibody-producing clones is also a time-consuming task, the cost of which must be considered.

A decisive point in the successful use of monoclonal antibodies depends on the screening of clones. Clones must be selected not only for a specific type of antigen but also for the type of end-product (raw or processed), level of binding affinity and broadness of specificity, assay format (e.g. indirect, competition, or sandwich ELISA; immunocytochemistry), solid phase for antigen or antibody immobilization (e.g. plastic, nylon, or nitrocellulose membrane) as well as the solvant used to extract proteins. Accordingly, nothing is ever gained beforehand when using monoclonal antibodies, however, due to the considerable number of possible epitopes, hope remains, provided the selection of the clone is extremely careful and judicious.

On the other hand, future research into the use of monoclonal antibodies should consider the following aspects:

1) To improve further the understanding of wheat protein structure through the use of antibodies raised against specific peptides, chemically modified proteins, or proteins modified by directed mutagenesis.
2) To investigate the basis of functional properties in situ, by developing antibodies able to discriminate the aggregated/nonaggregated states of wheat

storage proteins or to follow site integrity during aggregate formation in the developing grain.
3) To develop nondestructive seed tests by applying monoclonal antibodies to the screening of thousands of plastic-embedded and abraded half-seeds.

4.2 Nuclear Magnetic Resonance

Developed in the early 1960s in chemistry laboratories, nuclear magnetic resonance has emerged as one of the few and most powerful spectroscopic techniques capable of obtaining useful information on conformation, tridimensional structure, chemical environment, functionality, thermodynamic parameters, and molecular dynamics of proteins and peptides in solution, or on intact complex systems such as wheat gluten. Because the application of high-resolution NMR is not limited to solutions or to crystallized proteins, it may be complementary to circular dichroism, optical rotatory dispersion, crystallography, or X-ray diffraction.

Whereas until very recently, NMR called to mind high cost and outstanding techniques, new generations of commercial apparatuses are now available and can be used for nondestructive routine analyses of various agriculture and food products.

In this chapter on wheat proteins, it has been shown that investigations based on the various NMR methods, especially ^{1}N and ^{13}C relaxation studies, proved attractive in a number of applications:

- Identification of the main motional regimes in dry gluten, in relation to the behavior of individual components (e.g. proteins, lipids).
- Respective effect of lipids and disulfide bonds in the slow and fast molecular motions of the gluten system.
- Description of the changes in rheological properties of hydrated gluten in terms of replacement of labile, nonconvalent protein interactions by permanent disulfide bonds.
- Development of a new model of lipid-protein interactions in wheat gluten and dough, involving rearrangements of phospholipidic membrane structures.
- Development of nondestructive determinations of the protein content in seeds.

Despite their high potential value in exploring the molecular dynamics of protein systems, approaches based on NMR, however, have not been as yet really successful in identifying specific sequences associated with dough rheology or baking quality, or in devising microtests to predict the technological quality in breeding programs.

On the other hand, interpreting NMR experiments still remains an extremely difficult task, as illustrated by the controversial conclusions on the respective contributions of lipids and proteins to gluten NMR signals. Because the differ-

ent authors have been using various NMR schemes (CP, MAS, SPE, with short or long contact times) on various nuclei (^{13}C high-resolution NMR, ^{1}H relaxation time measurements, etc.), it is always difficult to make general statements on the reported results. Clearly, comments on mobility must be viewed only in the context of each NMR experience.

Because the elucidation of the exact molecular bases of wheat gluten functionality remains the most important challenge to cereal biophysicists in the coming years, future research in the NMR field, in concert with other physicochemical and biochemical studies, should tackle the following aspects:

- The identification of the structural elements which impart dough elasticity or extensibility.
- The determination of the fractions in which the mobility lies and an attempt to account for the differences observed between different cultivars or different gluten origins.
- The elucidation of protein hydration and the further characterization of the protein groups in this respect from comparative studies on dry or hydrated glutens and on purified gliadin or glutenin fractions.
- The elucidation of the dynamics of dough development and the effect of heat and mechanical treatment on the distribution and mobility of protein components through in situ NMR spectroscopy.

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