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New Investigations about Wheat Gliadin Components by Means of Isoelectric Focusing

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Hi! Let me apologize for my french accent. I hope you will be able to understand my talk in spite of that...

I am from France. I work in the INRA (Institut National de la Recherche Agronomique) but I am working for 9 monthes in this building as a NATO posdoctoral fellow, in the Food Proteins Unit.

My research area is the study of some wheat proteins. At the present time, my particular job is to contribute, through the study of the gliadin fraction, to improve our knowledge about relationships between the common wheats, the bread wheats, and their supposed diploid ancestors such as T. monococcum, A. squarrosa, T. urartu, which are more or less supposed to have contributed to the three different genomes of the bread wheats.

So, in this purpose, I am trying to isolate, as far as possible, some pure gliadin fractions, comparatively from a bread wheat variety and from different wild species, in order to make possible their further comparison by means of the N-terminal amino acid sequencing.

The particular method choosed for this isolation, and of which I have now to speak, is called Isoelectric Focusing.

The Isoelectric Focusing is a relatively new method of separation of proteins. It represents a major advance in the field of high resolutiom separations of proteins. Although it would be carried out with a similar equipment than the regular electrophoresis, the principle of Isoelectric Focusing is quite different from that one of electrophoresis.

In a conventional gel electrophoresis, there is a constant pH and, with the application of an electric field, charged molecules such as proteins, move

in a gel medium towards the anode or towards the cathode according to their own net charge at the pH of the gel.

On the contrary, IEF is carried in a pH gradient. It is an equilibrium method in which proteins move in the pH gradient and are segregated according to their isoelectric points. The pH gradient is formed by electrolysis of amphoteric buffer substances,commercially available, known as carrier ampholytes, the most common trade name of which is Ampholines. The nature of theese Ampholines is not very well known, but it is likely that the trade preparations are made up by a mixture of a great number of polyamino-polycarboxylic acids, synthesized by a reaction between acrylic acid and pentaethylenehexamine or something like that. Their molecular weights are ranging from 3oo and 7oo.

If a protein is introduced in a such pH gradient (slide 1), at a pH lower than its pI, it will migrate towards the cathode and therefore in an environment of successive higher pHs which in turn will influence its ionisation. So, the protein will move untill it reaches its pI at which it exhibits a zero net charge. So, it stops at this place and, since the focusing effect works against diffusion, the separated fractions can be concentrated in very sharp bands, with a resolution that cannot normally be achieved through regular electrophoresis. Further, because it is an equilibrium method, insofar as the gradient is well settled and stable, the system is self correcting and therefore less demanding in terms of experimental technique.

It is possible to perform this IEF in different media;
- in liquid columns with sucrose density gradients
- in acrylamide gels (analytical IEF)
- in thin layers of dextrane

The latter procedure is a new preparative one because it allows to fractionate relatively high loads of proteins and to recover easily the focused fractions.

Now, I shall try to explain what I am doing with this IEF and how I have tried to apply it to wheat proteins. Of course, you can imagine that the perfection of a method of fractionation is generally not so easy in the case of wheat endosperm proteins than in the case of pure animal or human proteins (slide 2) such as ovalbumine, myoglobine or cytochrome c. The reason is that gliadins
1) exhibit very special properties of solubility and may precipitate at certain pHs
2) have relatively few charges on their macromolecules, so that they move very slowly in the electric field and, on the other hand, owing to this lack of charges, they do not stain easily with the common protein dyes.
3) their pIs are near from the neutrality, just in the pH range where the chances of getting artefacts and troubles in IEF are the most likely.

We have however achieved some separations of gliadins by means of both analytical and preparative procedures, using a LKB apparatus.

Concerning the analytical one (slide 3), here is an example of the patterns obtained from whole gliadin sample of different varieties. We have used polyacrylamide gel slabs of 10 x 5 inches, containing 2 % of Ampholines mixed previous to the polymerisation. The separation is completed in 2-3 hours when running across the width but it needs overnight when running across the length.

The experiment is relatively easy to carry out. For example, gliadin samples are deposited by means of small pieces of filter paper just put onto the gel (and not in a slot, like in regular electrophoresis). One tenth of milligram is enough to get a pattern.

It is possible to check the pH of any region of the gel using a surface pH electrode and therefore to discover the pI of any electrofocused band. For example, the most important bands of the whole gliadin pattern are ranging betwwen pH 6.8 and 7.5.

But it is very difficult to make an assignement between theese electrofocused bands and the electrophoresed bamds of the same sample because many bands revealed in acidic electrophoresis may have similar isoelectric points and in turn, several bands revealed in electrofocusing may correspond to a same gliadin present at different degrees of amidation and therefore giving a single band in acidic electrophoresis.

You can notice that the method affords the resolution of the whole gliadin in relatively sharp bands (more than 35 are visible on the best patterns). So, it allows to discover more components in the gliadin fraction, and probably some new components, compared to the electrophoresed ones, because the criterium of the separation is not the same. Until now, WRIGLEY was allmost the only one to run wheat gliadins through the use of IEF. But the difference is that WRIGLEY is running in gel rods while we are running in gel slabs.

So, analytical IEF 1) affords new possibilities and increases the chances of differenciation between samples which look identical with other methods of separation.
2) may give a better criterium of purity of a sample, owing to the fact that very slight differences (about 1/100 of pH unit) will lead here to significant different bands, while, for example, different components which differ only in degree of amidation are not displayed through electrophoresis in acidic pH.

Concerning now the preparative procedure, we have tried, in this second step, to extend the IEF to a preparative scale in order to isolate some pure components. So ,instead of polyacrylamide gel, the medium is here a thin layer of dextrane G 75 Sephadex. Under theese conditions, it is possible to fractionate samples of 100 mg of gliadin and even more. (Slide 4).

After focusing, a surface print is taken with a filter paper which is stained immediatly. Then the thin layer is fractionated with a grid and, after checking the pH of each compartment, we can scrape off the thin layer in the interesting compartments as judged by the pH values and by the paper print. Then we can elute the protein from the dextrane. Several mg of proteins which have focused in a pH range of less than 1/10 pH unit can be easily recovered like this.

But, of course, there are too many components in the whole gliadin to get pure components after this only step of fractionation. In order to isolate some pure components, it is probably necessary (that is what I am trying to do at the present time), to prepurify certain fractions by means of ion exchange and also, to spread out a little more the pH gradient.

IEF seems therefore to be a very nice technique, but it is necessary to be aware of the possibility of a certain amount of troubles or artefacts. For example, the pH gradient is supposed to be indefinitely stable. Actually,

that is not true. We have noticed everytime a slow shift of the gradient towards the cathode, so that after reaching its pI , any protein band does move slowly towards the cathodic end and can get out from the gel after 30 or 40 hours (slide 5). This drawback is particularly trouble-some in the case of gliadins,which are slow proteins, so that it may happem, under certain conditions, that they keep moving in the gel, never reaching their equilibrium.

Other example (slide 6), the electrofocused patterns are supposed to be the same whereever the starting sample is placed onto the gel. Unlike the case of some animal proteins, this is no more true with gliadins. Different locations of the sample lead to different or even very different patterns. The reason of this is for us still difficult to understand. We made several hypothesis such as:

- a conformational modification of the gliadin in basic medium,
- or perhaps the shift of the gradient prevents components put on the basic side to reach their own pI, against the stream,
- or may be it is more likely that there is a sort of binding occuring between gliadins and some basic ampholines, binding which gives a complex with a wrong apparent pI.

To sum up this talk, it can be said that:

At least the main fractions of the electrofocused patterns seem not to be artefacts, considering that a rerun of them leads to the same location. But, of course, the interpretation of the very complicated patterns obtained by the analytical procedure will require for a long time to be very careful and to use caution.

On the other hand, as the preparative procedure seems to work OK, we have hopes to get very soon some pure components in order to afford their N-terminal amino acid sequencing.

I have finished and I thank you very much.

h. c. face and exp.

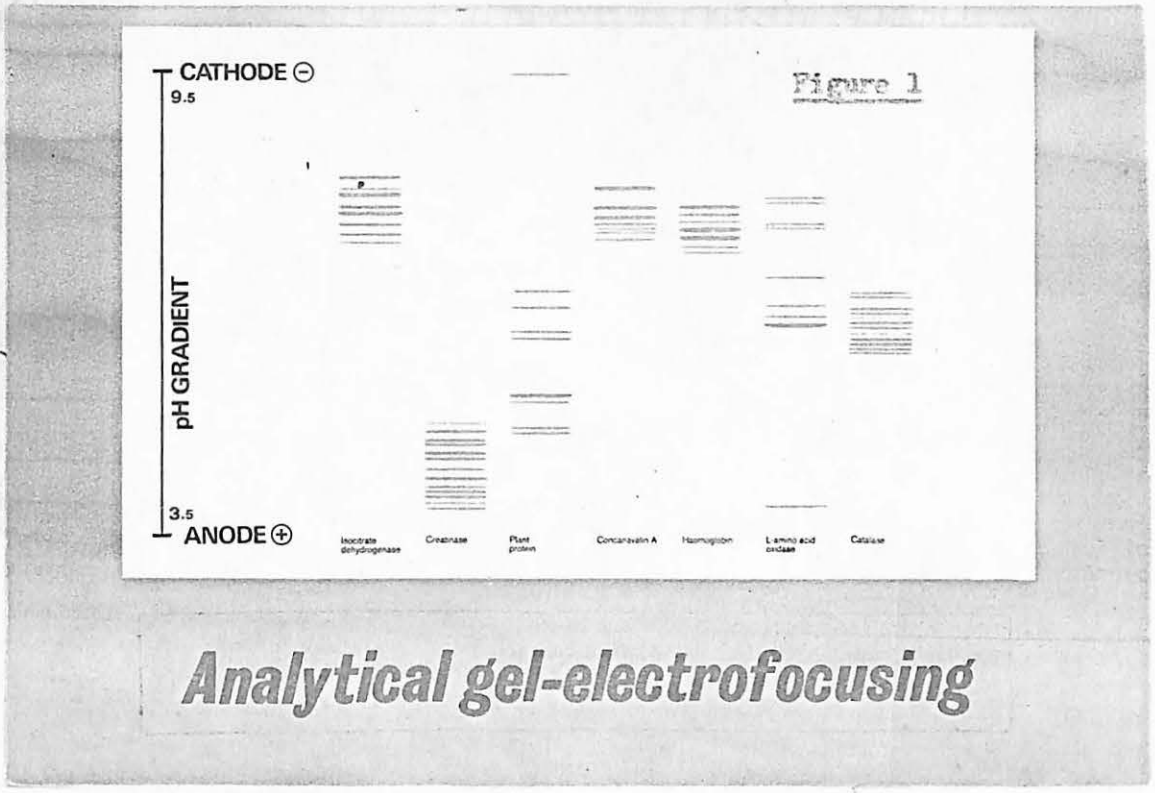


Figure 1

Analytical gel-electrofocusing

Preparative Flat-Bed Electrofocusing in a Granulated Gel

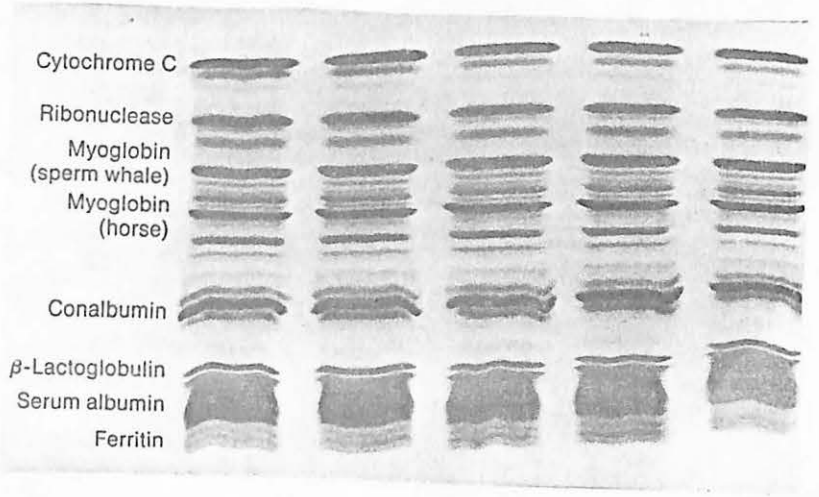


Figure 2

ELECTROFOCUSING OF WHEAT GRAIN PROTEINS

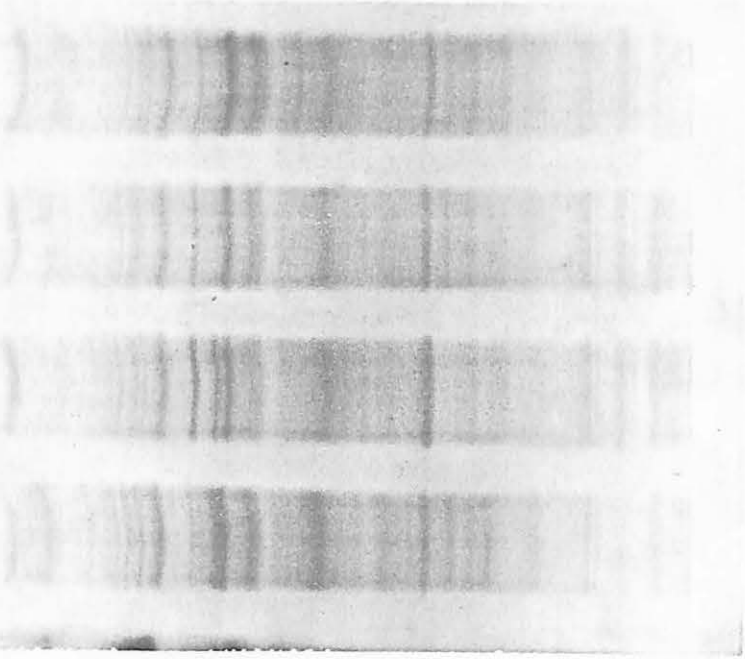
Variety :

CAPPELLE

CAPITOLE

JOSS

CHEYENNE



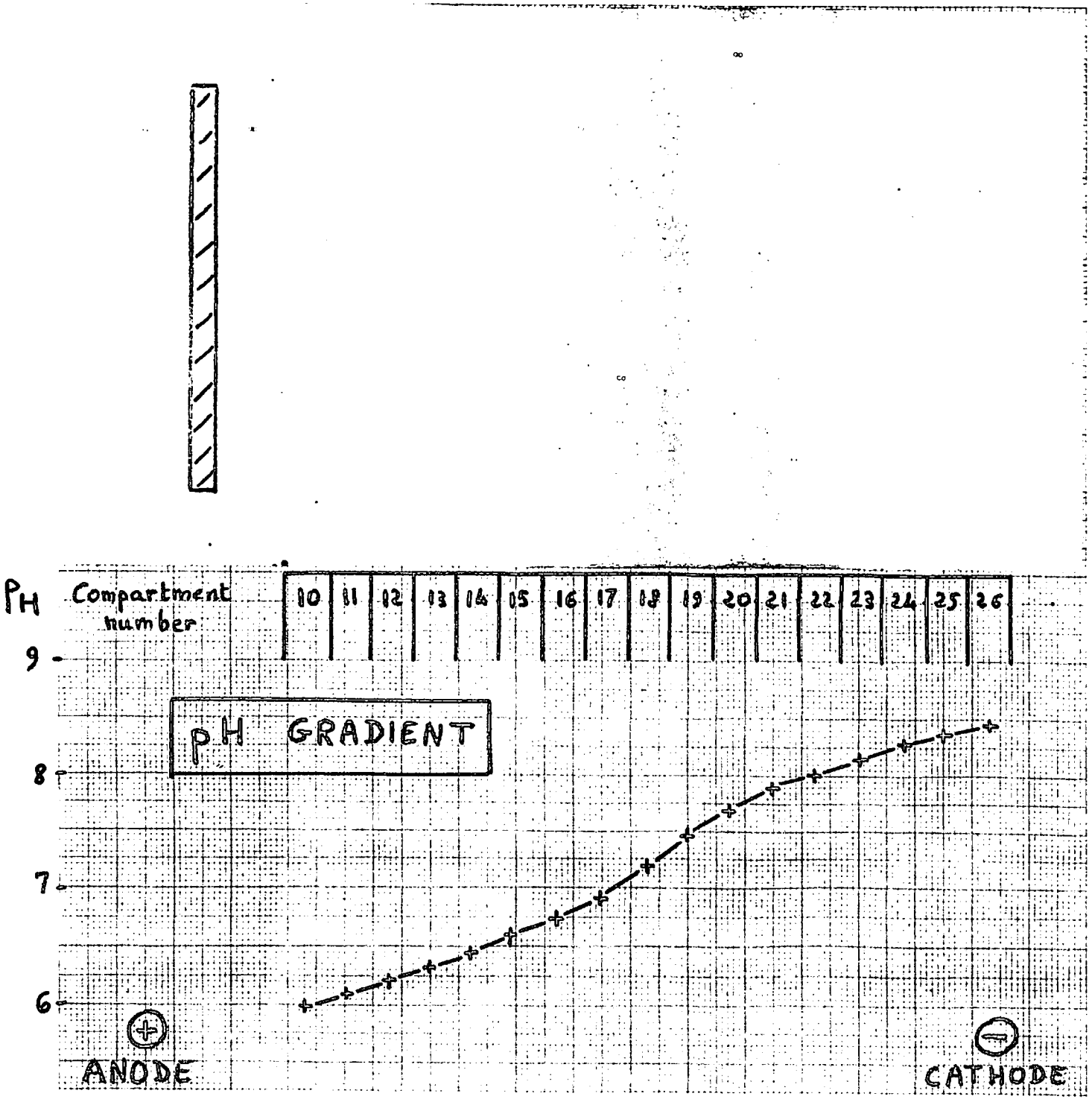
PH 6 ——— PH 9

Figure 3

Preparative Flat-Bed Electrofocusing in a Granulated Gel

Figure 4

Gliadin Proteins



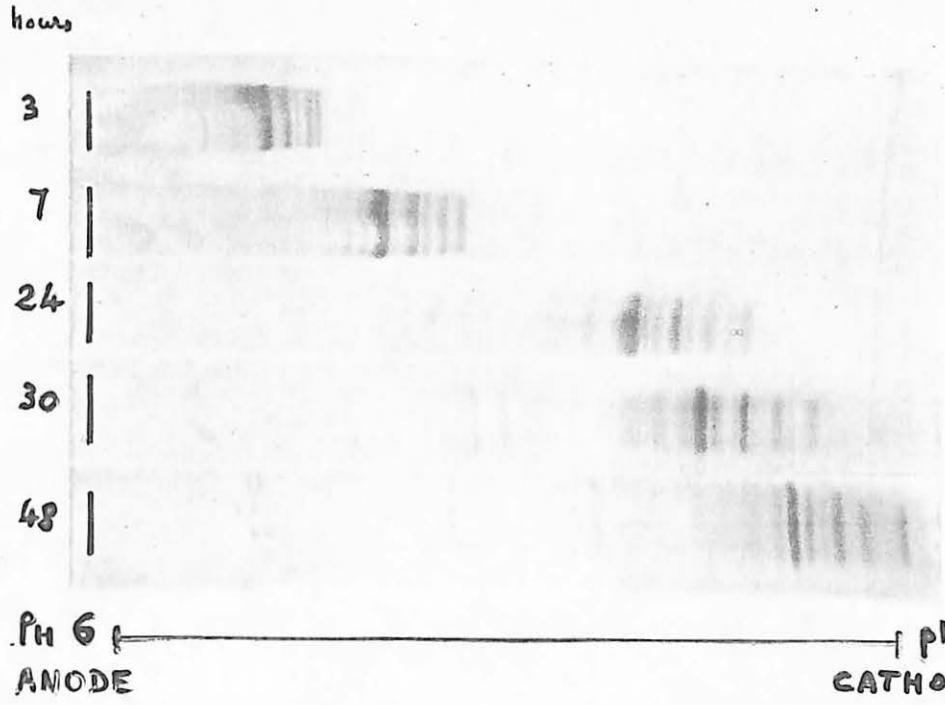


Figure 5

ELECTROFOCUSING OF WHEAT GRAIN PROTEINS

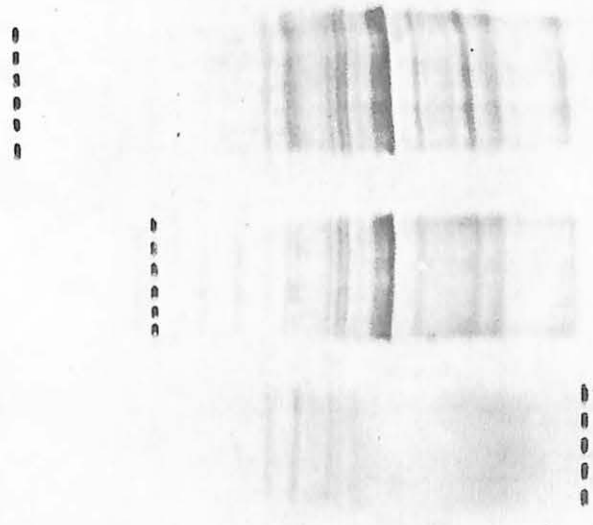


Figure 6