SOME OBSERVATIONS ON THE ELECTROPHORESIS OF GLIADIN

by

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Gluten, the principal protein of wheat, may be separated into two fractions by extraction with 70% alcohol. The soluble material was given the name gliadin by TADDEI[1] and has been the subject of numerous investigations. OSBORNE[2] believed that gliadin was a single protein, but HAUGAARD AND JOHNSON[3] showed that fractions of differing physical properties could be obtained from it. KREJCI AND SVEDBERG[4] demonstrated the presence of at least two components by means of the ultra-centrifuge, while LAMM AND POLSON[5] showed by diffusion measurements that gliadin did not behave in the manner expected of a single protein. Further evidence of heterogeneity was found in the osmotic pressure studies of BURK[6] and the sedimentation experiments of MCCALLA AND GRALEN[7].

Electrophoretic studies on gliadin have been made by SCHWERT, PUTNAM AND BRIGGS[8] who obtained evidence of at least two components. The electrophoretic behaviour was rather abnormal and this was ascribed to the presence of a mixture of proteins capable of forming reversibly dissociable complexes. More recently, LAWS AND FRANCE[9] have described further electrophoretic experiments, but their patterns give the impression of having many convective disturbances.

During recent studies on gliadin from a mixed flour a new method of fractionation has been used, and a preliminary electrophoretic examination of the products has already been discussed (BUTLER, CREETH AND MILLS[10]). This examination has now been extended to gliadin from a single variety of wheat, by the use of several buffer systems at different ionic strengths.

MATERIALS AND METHODS

Preparation of gliadin

Wet gluten freshly prepared from an unmixed Manitoba flour was extracted with twice its weight of 70% ethanol-water (v/v) for 24 hours at 37° C. The liquid was poured off and the residue extracted again in the same way. The two extracts were combined and centrifuged to remove suspended starch. After dilution with an equal volume of water the liquor was kept at 2° C overnight. The precipitated product is referred to below as "whole gliadin".

For the preparation of the gliadin fractions, the extract was first freed of starch and then centrifuged in open, wide mouthed cups, when some alcohol evaporated from the exposed surface. After about an hour a sticky syrup began to accumulate in the bottom of the cup and when this reached an arbitrarily chosen volume (about 5% of the original volume of solution) the supernatant liquor was poured off. The precipitated syrup was called Fraction 1. The supernatant liquor was cooled overnight to 2° C and the precipitated protein separated off. This was called Fraction 2. Both fractions were dried over sulphuric acid in a desiccator.

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Buffer solutions

The buffer combinations sodium acetate-acetic acid (NaAc-AcOH), potassium hydrogen phthalate-hydrochloric acid (KHP-HCl), sodium citrate-hydrochloric acid (Cit-HCl) and citric acid-disodium phosphate (Cit-PO_4) were made up according to CLARK[11] to the required pH and ionic strength. Sodium acetate-hydrochloric acid (NaAc-HCl) was made from the directions of WALPOLE[12]. The glycine-sodium chloride-hydrochloric acid (Gly-HCl) was made up in three concentrations as follows:

1. 1 litre of a solution 0.05 *M* in glycine and 0.01 *M* in NaCl added to 250 ml 0.05 *M* HCl.
2. 1 litre of 0.1 *M* glycine containing 0.01 *M* NaCl added to 250 ml 0.1 *M* HCl.
3. Made up as under (2) but with 0.02 *M* NaCl.

Another system was used in the experiments of BUTLER *et al.*[10] and this has also been used with the present gliadin samples. It was made up at two ionic strengths consisting of 0.2 *M* acetic acid containing 0.02 *M* and 0.04 *M* NaCl respectively (AcOH-NaCl).

The pH of the buffer solutions was measured at 20° C with a glass electrode.

Preparation of gliadin solutions

A weighed quantity of the protein was mixed with 25 ml of the buffer solution and gently stirred with a glass rod until it was fully hydrated. The solution and any suspended matter was then dialysed against the buffer for two days at 2° C, during which time the suspended material usually dissolved.

Electrophoresis

The technique used was based on the descriptions of TISELIUS[13], LONGSWORTH[14] and SVENSSON[15], the patterns being obtained by the optical scanning method of PHILPOT[16]. Owing to the fact that the solutions used were rather dilute, the patterns formed at the end of a run were often at a high magnification which made it difficult to obtain a clear photograph. The temperature of the cooling bath was 2.5° C.

RESULTS

Comparison experiments have shown that there is very little difference in the electrophoretic behaviour of "whole gliadin" and the fraction 2, and more attention has been paid to the latter. A series of runs was made in NaAc-HCl buffers of ionic strength (I) up to 0.05, which corresponds to the lower limit of useful solubility of gliadin. Experiments over a narrower range of conditions were made with the other buffers, which showed that the results were comparable with those for NaAc-HCl under similar conditions, except for the phthalate buffer. In this the solubility of the gliadin was totally inadequate.

Experience with the multi-component "whole gliadin" showed that the systems NaAc-HCl, Gly-HCl and AcOH-NaCl were the most satisfactory and as fraction 1 proved difficult to work with, it was examined in these buffers only.

As the solubility of gliadin is a critical function of pH, the buffers ranged only between pH 2.5 and 3.5 but no significant difference in the patterns was observed with this variation.

In all the figures given below, the ascending boundaries (migration from right to left) are shown unless otherwise indicated. The patterns for whole gliadin duplicate those of fraction 2 at all but the highest ionic strength. At $I = 0.04$ the diagram of Fig. 3b was obtained. In order to avoid duplication of figures separate patterns for whole gliadin have been omitted.

Sodium acetate-hydrochloric acid buffers

a. Whole gliadin and fraction 2. Fig. 1 shows the type of pattern found at values of I less than 0.03, it being 0.015 in the case shown. A number of rapidly moving,

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convective boundaries were produced in the cathode limb, apparently resulting from the formation of complexes and gravitationally unstable boundaries. The major part of the protein moved very slowly toward the anode. At this low value of I protein concentrations of 2 or 3% could be used.

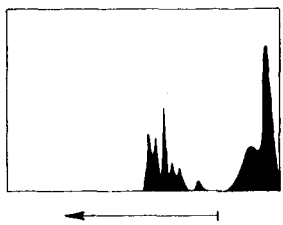

Fig. 1. Fraction 2. 3.3% in NaAc-HCl; pH = 2.89; I = 0.015; 30 mins at 5.5 volts/cm.

At ionic strengths between about 0.03 and 0.04 the pattern of Fig. 2 was obtained. Five distinct peaks could be discerned in the ascending limb, which have been called A_1, A, B, C, and D as shown. Migration was cathodic (as in all other buffers at comparable values of I) and the boundaries moved fairly rapidly under a potential gradient of the order of 4 volts/cm. The experiments at $I = 0.03$ were made at protein concentrations of 1.5%. 0.8% and 0.4%, the corresponding electrophoretic patterns are shown in Fig. 2 and were obtained under identical conditions. The proportion of the leading component relative to the others changed considerably as the protein concentration was reduced.

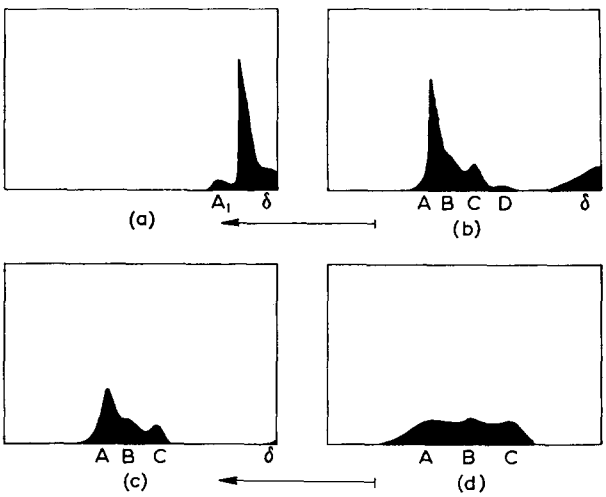

Fig. 2. Fraction 2 in NaAc-HCl; pH = 2.67; I = 0.035; a. 30 mins at 4.5 volts/cm. Protein concn. 0.8%; b. 170 mins at 3.9 volts/cm. Protein concn. 1.5%; c. Pattern (a) after 170 mins; d. 170 mins at 4.5 volts/cm. Protein concn. 0.4%.

Above $I = 0.04$, the maximum solubility of the protein falls off rapidly and this value of I was the highest that could be used. The protein concentration under these conditions was 0.4% and Fig. 3 shows the patterns found. The results for whole gliadin and fraction 2 were no longer identical under these conditions, the pattern for whole gliadin showing a more pronounced B component than that for fraction 2. Both the A_1 and D components were observed under these conditions but, being present in only small proportion, the boundaries had become diffuse at the time the patterns of Fig. 3 were photographed.

b. Fraction 1. At an ionic strength of 0.02 this fraction gave a pattern which showed a single, rapidly moving boundary which partly resolved into two peaks after 170 mins at a field strength of 3.6 volts/cm (Fig. 4a). The boundaries formed at this ionic strength

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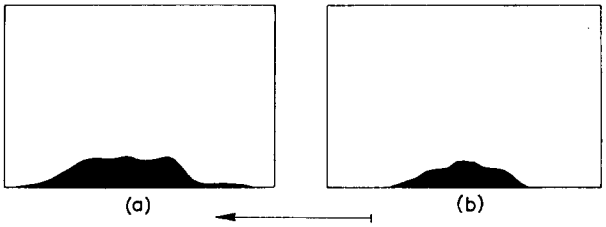

Fig. 3. a. Fraction 2. 0.4% in NaAc-HCl; pH = 2.9; I = 0.04; 170 mins at 4.51 volts/cm. b. Whole gliadin. 0.4% in NaAc-HCl; pH = 2.6; I = 0.04; 170 mins at 4.51 volts/cm.

always remained very sharp for the duration of the experiment. When I was increased to 0.04 it was difficult to get sufficient protein into solution and the boundaries became very diffuse at the end of the run. Fig. 4b shows the pattern after 90 mins at a field strength of 4.6 volts/cm. At least two components could be observed. This picture was not substantially changed after 170 mins.

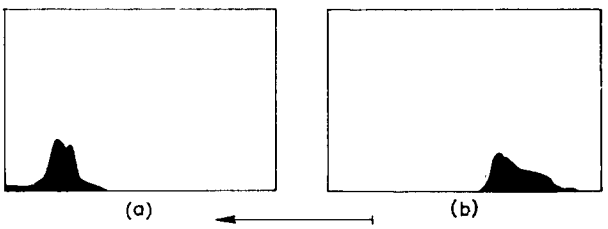

Fig. 4. a. Fraction 1. 0.4% in NaAc-HCl; pH = 2.9; I = 0.02; 170 mins at 3.6 volts/cm. b. Fraction 1. 0.4% in NaAc-HCl; pH = 2.8; I = 0.04; 90 mins at 4.6 volts/cm.

Glycine-sodium chloride-hydrochloric acid buffers

a. Whole gliadin and fraction 2. The dependence of the electrophoretic pattern on the value of I was similar to that in the previous system. At I = 0.02 there were marked gravitational anomalies and at ionic strengths of 0.03 and 0.04 the patterns duplicated those in NaAc-HCl (Figs. 5 and 6). It can be seen from Fig. 5, however, that change

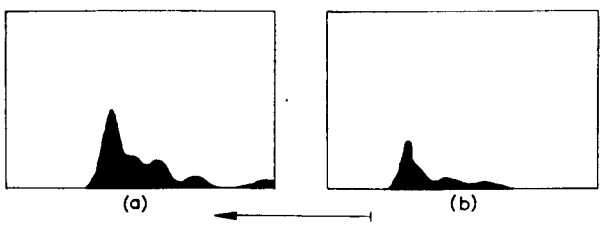

Fig. 5. a. Fraction 2. 0.7% in Gly-HCl; pH = 2.9; I = 0.03; 170 mins at 3.02 volts/cm. b. Fraction 2. 0.4% in Gly-HCl; pH = 2.92; I = 0.03; 170 mins at 3.1 volts/cm.

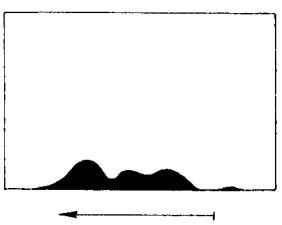

Fig. 6. Fraction 2. 0.4% in Gly-HCl; pH = 2.8; I = 0.04; 170 mins at 4.6 volts/cm.

of protein concentration without an accompanying change in ionic strength of the solvent does not cause the alteration in the pattern found in the previous case. The protein concentrations in Fig. 5 were 0.7% and 0.4%.

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b. Fraction 1. In this case similar patterns were observed at both $I = 0.03$ and 0.04, a single rapidly moving boundary becoming partially resolved into two after 170 mins. The final patterns are shown in Fig. 7.

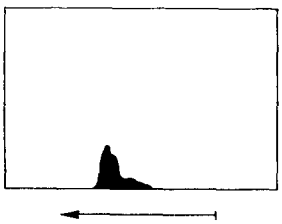

Fig. 7. Fraction 1. 0.4% in Gly-HCl; pH = 2.98; I = 0.04; 170 mins at 4.6 volts/cm.

Acetic acid-sodium chloride buffers

a. Whole gliadin and fraction 2. In this system also, convective boundaries were formed when the concentration of sodium chloride was below 0.02 M. When the salt concentration was made 0.02 M an ascending pattern similar to those already described in Figs. 2 and 5 was obtained (Fig. 8) with the exception that a new boundary was observed to move rapidly towards the anode. It was not seen in the descending limb. On raising the sodium chloride concentration to 0.04 M the patterns were exactly comparable with those found in the other buffers at $I = 0.04$ (Fig. 9). No anodic component was detected under these conditions. It is believed that the latter is a buffer anomaly of the type discussed by SVENSSON[15] but this question is still undecided. On dilution, the patterns showed the same change in the proportions of the components that was observed for NaAc-HCl buffers.

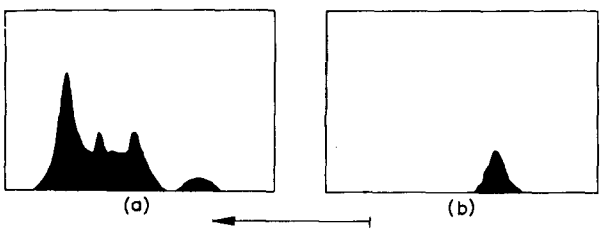

Fig. 8. Fraction 2. 0.8% in AcOH-NaCl; pH = 2.8; I = 0.02; 120 mins at 4.5 volts/cm. a. cathodic components; b. anodic components.

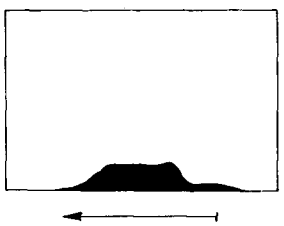

Fig. 9. Fraction 2. 0.4% in AcOH-NaCl; pH = 2.74; I = 0.04; 170 mins at 3.35 volts/cm.

b. Fraction 1. The effect of ionic strength on the appearance of the patterns was far less marked in this buffer than in the previous two cases. Fig. 10 shows the ascending boundaries at sodium chloride concentrations of 0.02 and 0.04 M; they are virtually indistinguishable, each having a large fast moving boundary with traces of slower components. The principal boundary remained exceedingly sharp throughout the run.

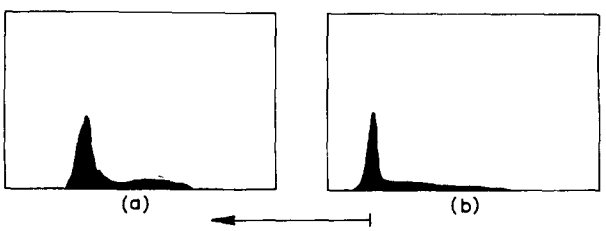

Fig. 10. Fraction 1. 0.4% in AcOH-NaCl; pH = 2.7; a. I = 0.02; 170 mins at 4.48 volts/cm; b. I = 0.04; 170 mins at 4.51 volts/cm.

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The results just described indicate that the electrophoretic examination of gliadin is best performed in buffers of $I = 0.04$ at a protein concentration of 0.4%. At lower ionic strengths the descending boundary became very diffuse and in some cases precipitation of protein took place. At $I = 0.04$ the "enantiography" of the patterns in the two limbs was greatly improved, although the descending limb became too diffuse to observe towards the end of the run. Fig. 11 shows the pattern of the two limbs after 90 mins in a typical run in Gly-HCl.

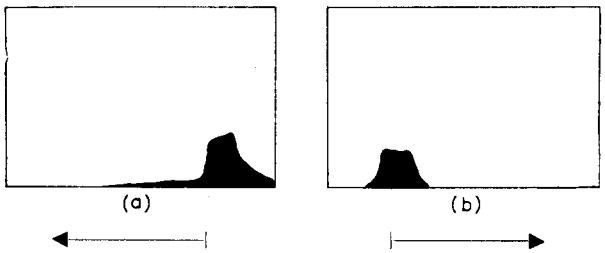

Fig. 11. Fraction 2. 0.4% in Gly-HCl; pH = 2.93; $I = 0.04$; 90 mins at 2.5 volts/cm. a. ascending limb.; b. descending limb.

These conditions having been established for the above three buffers, the remaining three were examined to discover whether the same conditions were satisfactory with them also. It will be seen from Fig. 12 that, although the patterns were broadly similar to those already described, they differed in certain details, especially in the case of NaAc-AcOH buffer. Here, the leading component separated more completely from the slower pair, which were somewhat distorted. After prolonged electrophoresis the leading peak became asymmetrical. It was doubtful whether in this case the D component was resolved.

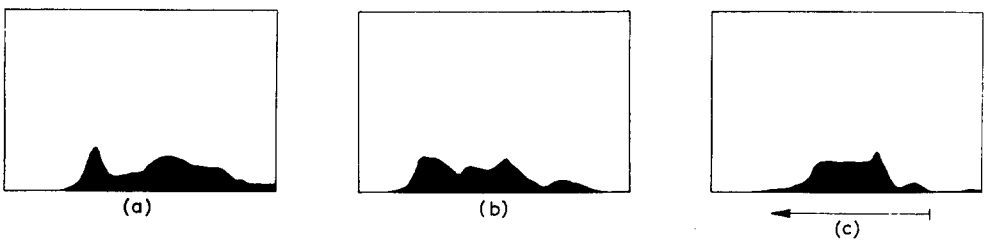

Fig. 12. Fraction 2. a. 0.4% in NaAc-AcOH; pH = 3.4; $I = 0.04$; 170 mins at 4.47 volts/cm. b. 0.4% in Cit-HCl; pH = 2.91; $I = 0.04$; 170 mins at 4.58 volts/cm. c. 0.4% in Cit-PO_4; pH = 3.02; $I = 0.04$; 80 mins at 4.3 volts/cm.

All the five components named above were observed in citrate buffers but certain anomalies were also noted. In Cit-HCl the A boundary showed a small shoulder on the leading edge, the presence of which has been confirmed in several experiments. In Cit-PO_4 buffer a similar, though less marked distortion of the A boundary was observed, together with an unusually large peak at the C boundary.

DISCUSSION

It is clear from the results just described that the fractionation process separated the original gliadin into two parts having different composition and properties. It is

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equally evident that gliadin comprises a mixture of several component proteins which are capable of forming complexes between themselves and with several common buffer ions.

The dependence of the interprotein reaction on the nature of the solvent buffer is clearly demonstrated by the results for fraction 1. Even at the highest usable ionic strength this fraction migrated as a single boundary in AcOH-NaCl. In both Gly-HCl and NaAc-HCl, however, two boundaries could be resolved. The resolution was slightly better in the latter system and the appearance of the pattern at $I = 0.04$ was quite unlike that in Gly-HCl at a comparable ionic strength. This seems to indicate a pronounced interaction with the buffer ions.

The interprotein interaction would probably be minimized by working at low concentrations and high ionic strengths. But the peculiar solubility characteristics of gliadin result in a rapid fall in protein concentration as the ionic strength is raised, and the highest workable value of the latter is about 0.04 corresponding to a protein concentration of 0.4%. The patterns of Fig. 2 show clearly the characteristic decrease in size of the A peak as the protein concentration was lowered, and the simultaneous increase in the proportion of the B and C components. This shows that A is a complex containing B and C. Unfortunately it has not been possible to decide whether there is a single protein corresponding to the peak A as lower dilutions cannot be used. An analysis of the areas under the peaks of Figs. 2a and 2b shows that the components A, B and C are in the approximate proportion 64:18:18 and 55:25:20, respectively. In Fig. 2c the proportions have become still more nearly equal.

It is noteworthy that components A_1 and D were observed in "whole gliadin" and fraction 2 in all buffers except NaAc-AcOH. These components apparently do not take part in complex formation so easily as A, B and C.

These results indicate that in certain solvent systems a complex is formed with at least two of the individual gliadin proteins and some of the components of the solvent. In 70% ethanol-water a complex is formed which is sufficiently stable to be isolated and one of similar stability is formed in AcOH-NaCl. The formation of long lived complexes of this kind offers a plausible explanation of the confusion of estimates available for the molecular weight of gliadin.

SCHWERT, PUTNAM AND BRIGGS[8] concluded from their results that gliadin formed a large number of intermolecular complexes but their experiments were done at very low ionic strength in NaAc-AcOH buffers. In the experiments reported here, all the buffers used gave similar patterns at ionic strengths of about 0.01, with gravitational anomalies and visible streaming in the U-tube. Some of the boundaries reported by SCHWERT *et al.*[8] must therefore be ascribed to this cause.

There must remain some doubt as to the exact number of individual gliadin proteins although the almost ubiquitous presence of A_1 and D, together with their characteristically different mobilities, suggests that they are separate components. The fact that A, B and C are never completely resolved makes it difficult to decide whether they correspond to individuals or to varying forms of a complex. At the same time, fraction 1 contains no C and unfractionated gliadin has more B than fraction 2; facts which argue the individuality of these components.

Accurate mobilities could not be estimated from these experiments as the descending boundaries became too diffuse for measurement and in any case some of the boundaries did not correspond to definite protein components. However, measurements on the B

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component gave mean values of 4.8, 4.8, and $5.0 \cdot 10^{-5}$ cm^2 sec^2 $volt^{-1}$ in AcOH-NaCl, Gly-HCl and NaAc-HCl respectively. For the C component an average value of $3.8 \cdot 10^{-5}$ cm^2 sec^2 $volt^{-1}$ was found in each case. This suggests that each component appears as a characteristic boundary in these buffers.

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SUMMARY

Gliadin has been separated into two fractions by a new method and both fractions have been subjected to electrophoretic analysis in different buffers. It has been concluded that gliadin is a mixture of at least four electrophoretically distinct proteins.

RÉSUMÉ

Le gliadin a été séparé en deux fractions par une nouvelle méthode et tous les deux fractions ont été analysées dans les tampons différente par l'électrophorèse. On a conclu que le gliadin est un mélange d'au moins quatre protéines qu'on peut distinguer par l'électrophorèse.

ZUSAMMENFASSUNG

Gliadin ist mittels einer neuen Methode in zwei Fraktionen gespalten worden und beide Fraktionen sind in verschiedenen Pufferlösungen einer elektrophoretischen Analyse unterworfen worden. Man ist zu dem Schluss gekommen, dass Gliadin eine Mischung von mindestens vier elektrophoretisch erkennbaren Proteinen ist.

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