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AGAROSE GEL FILTRATION OF *TRITICUM VULGARE* PROTEINS IN DISSOCIATING SOLVENTS

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Abstract—Certain wheat proteins (glutenins) emerged from 4% agarose columns at the void volumes even in the presence of 6 M guanidine hydrochloride, 4 M urea with or without 1% sodium dodecyl sulphate, and 8 M urea. These proteins were of considerably greater molecular size than bovine thyroglobulin (subunit MW 335000). Urea plus sodium dodecyl sulphate was the most effective dissociating solvent. Low MW wheat flour proteins, which had been covalently labelled with a fluorescein derivative, were not incorporated through formation of new disulphide bonds into higher MW fractions during acidic extraction of flour. Limited incorporation through non-covalent association was observed. The results do not support the contention that glutenin is an artifact of extraction. It has been confirmed that all the protein of wheat flour is not extractable with water followed by 2 M urea.

INTRODUCTION

THE PROTEIN remaining after removal of the albumins, globulins and gliadins from wheat flour consists of glutenin, a very high MW fraction as shown by its failure to enter the pores of starch-gels during electrophoresis. In 1968, Lee[1] stated that practically all the gluten protein (i.e. that remaining after extraction of albumins and globulins) could be solubilized in 2 M urea, and that these extracts contained gliadin-like materials relatively free from high MW glutenin. This is contrary to the general belief that glutenin is present in flour to approximately the same extent as gliadin. He suggested that, under normal conditions of extraction of flour using acidic solvents, glutenin could arise through intermolecular (possibly disulphide) cross-linking of gliadin molecules. These observations were not incompatible with those of Woychik *et al.*[2] who found that starch-gel electrophoretic bands of glutenin after disulphide bond reduction corresponded to bands in reduced gliadin. Amino acid analyses of the two materials were also found to be similar.[2]

This paper describes experiments devised to determine whether glutenin is present in wheat flour, or is an artifact of extraction.

RESULTS AND DISCUSSION

General Column Properties

Inamine *et al.*[3] showed that agarose columns were capable of resolving glutenin proteins from proteins of lower MW. This observation was confirmed in the present work, although a return to baseline in between peaks was incomplete, particularly for highly viscous

[1] LEE, J. W. (1968) *J. Sci. Food Agr.* **19**, 153.
[2] WOYCHIK, J. H., HUEBNER, F. R. and DIMLER, R. J. (1964) *Arch. Biochem. Biophys.* **105**, 151.
[3] INAMINE, E. S., NOBLE, E. G. and MECHAM, D. K. (1967) *Cereal Chem.* **44**, 143.

solutions. Gliadins, albumins and globulins emerged in the same peak from columns equilibrated with urea solutions, as judged by starch-gel electrophoresis. On GuHCl columns (see Experimental) the latter two groups were held back and partial separation from gliadins observed. Dialysis did not remove all the sodium dodecyl sulphate (SDS) from proteins, and therefore identification of proteins in peaks from USDS columns (see Experimental) was not possible by electrophoresis.

Extraction of Flour with 2 M Urea

The observation by Lee[1] that 97·7% of the protein of wheat flour could be extracted by 2 aqueous and 8 2 M urea extractions could not be verified in the present study for both Cappelle–Desprez and Wichita flours. Also the 2 M urea extracts contained considerable proportions of glutenin. While this work was in progress Huebner and Rothfus[4] reported similar findings and recently Lee[5] has stated that he has not confirmed his earlier observations on Gabo wheat flour when working with other wheat flour samples. Therefore, experimental details and results are not quoted here. Instead, further experiments (using Cappelle–Desprez flour) designed to determine if glutenin is an artifact formed from lower MW wheat flour proteins during extraction are detailed.

Experiments with Fluorescein-conjugated Proteins

The stable, covalently bound, coloured derivatives of the water-soluble proteins (gliadins + albumins) of wheat flour contained only one fluorescein molecule per protein molecule on average and therefore general chemical properties would not have been radically affected. These derivatives were blended with flour under acidic conditions. If the colour due to fluorescein was subsequently found in the higher MW (glutenin) peak, this would be evidence either for association of the gliadins and albumins with glutenins, or the actual conversion of low to high MW proteins. Both possibilities could occur through the formation of new covalent bonds or strong secondary intermolecular forces. The only obvious mechanisms for the formation of new covalent bonds are those of sulphydryl–disulphide interchange or sulphydryl oxidation. Even these reactions are unlikely during acidic blending as they would be favoured by a high pH at which the sulphydryl group exists as the anion.

To prevent these sulphydryl-dependent reactions, the disulphide bonds of the conjugated materials were reduced and blocked before blending with flour. (The reaction was complete as judged by the absence of cysteine and cystine after hydrolysis and subsequent amino acid analyses.) The extracts were compared with those obtained when untreated conjugates were used. In order to minimize non-covalent intermolecular forces, extracted proteins were run on agarose columns in three different solvents known to possess powerful dissociating effects.

The results are in Table 1. The extracted materials were not always completely soluble in the column buffers but the approximate extent of insolubility may be judged by comparing total protein recoveries. Thus for sample B, the insoluble fraction in US buffer (see Experimental) constituted $[(62{\cdot}6-45{\cdot}9)/62{\cdot}6] \times 100\% = 27\%$ of the total.

In every case, small amounts of materials containing fluorescein were found in peak I (glutenin), whereas the purified conjugated materials had given no peak in this region before blending with flour. The results cannot be explained simply by overlap of colour from

[4] HUEBNER, F. R. and ROTHFUS, J. A. (1971) *Cereal Chem.* **48,** 469.
[5] LEE, J. W. and MACRITCHIE, F. (1971) *Cereal Chem.* **48,** 620.

peaks II into peaks I, as when individual tube fractions from the columns were monitored at 495 nm, definite peaks were observed associated with peaks I (and also peaks II). If glutenin was formed entirely from lower MW proteins during blending, the II:I ratio for protein should be the same as the II:I ratio for colour due to fluorescein; assuming that the conjugates were typical of the lower MW proteins of flour. The actual amounts of colour incorporated into peaks I were much lower than would be required, but the fact that limited incorporation was observed necessitates a more detailed investigation of Table 1.

TABLE 1. DISTRIBUTION OF TOTAL PROTEIN AND FLUORESCEIN CONJUGATES IN PEAKS FROM AGAROSE COLUMNS

Sample	Solvent	Protein recoveries (280 nm) I	Mid	II	Total	'Fluorescein' recoveries (495 nm) I	Mid	II	Total	Protein II:I	'Fluorescein' II I
A	US*	9 3	4 1	39 5	52 9	1 4	0 44	25·0	26 8	4 2	19
	USDS*	4 7	4 1	42 9	51 7	0·35	0 48	20 8	21 6	9 1	59
B	US*	8 5	4 2	33 2	45 9	1 8	0 66	14 9	17 4	3 9	8 3
	USDS	3 6	3 6	55 4	62 6	0 16	0 36	17 6	18 1	15	110
	GuHCl	14 2	7 0	41 4	62 6	1·3	1 7	19·7	22 7	2 9	15
C	US*	6 7	3 7	24 8	35 2	0 50	0 71	5 4	6 6	3 7	11
	USDS*	2 8	3 4	33 9	40 1	0 10	0 26	6 0	6·4	12	60
	GuHCl	15 4	†	32 1	47 5	0 48	†	4 8	5 3	2 1	10
D	US*	10 0	4·6	36 0	50 6	1 3	0 77	12 5	14 6	3 6	9 6
	USDS	6 2	4 6	48 9	59 7	0 24	0 22	15 9	16 4	7 9	66
	GuHCl	13 9	11 3	36 0	61 2	0 70	2 3	13 1	16 1	2 6	19

A and B were extracts obtained after blending flour in the presence of unblocked fluorescein conjugates. C and D resulted from the blending of flour with blocked conjugates. Peaks I and II represent 'high' and 'low' MW peaks respectively. 'Mid' is the region between these. Values given are in terms of optical density x volume of eluate, all figures having been adjusted to assume 200 mg material stirred in the appropriate solvent. Further details are given in the Experimental section.

* Incomplete solubility of sample in this solvent.

† In this case elution patterns were split into only two fractions as return to baseline between peaks was almost complete, due to only 64 mg being loaded.

Reduced and blocked conjugates (C′ and D′) were found to approximately the same extent as unblocked derivatives (A′ and B′) in peaks I, and therefore it seems unlikely that sulphydryl-disulphide interchange or sulphydryl oxidation were responsible for the apparent rise in molecular size.

Therefore it appears that a small proportion of the low MW conjugated proteins became associated through non-covalent bonds into the high molecular size fraction although dissociating solvents were used on the columns. Consideration should be given to the observation that although apparently clear ultracentrifuged solutions were loaded on the agarose columns, the first peaks possessed haze to varying extents. Although this was corrected for when measuring the optical densities of the eluates, the fact that precipitation took place could go far towards explaining why initially low MW conjugated proteins were found in the high MW peaks, bearing in mind the well-known tendency of proteins to co-precipitate with others. The aspects of non-covalent association and source of haze are dealt with in the next part of this section.

The Effect of Various Dissociating Solvents on Elution Patterns

Acidic extracts of wheat flour contained material eluted at the void volumes of agarose columns in all solvents used as judged by comparison with elution of Dextran Blue 2000. Thyroglobulin, which has a subunit MW of 335000[6] was mostly insoluble in US buffer but

[6] DARNALL, D. W. and KLOTZ, I. M. (1972) *Arch. Biochem. Biophys.* **149**, 1.

gave a clear solution in USDS and the major peak was eluted at 34 ml (peak maximum). Both the highest MW fractions of Dextran Blue and glutenin came off the same column at 19 ml. Therefore the molecular size of glutenin was much larger than that of thyroglobulin under the conditions used. Proteins with such high MWs in the presence of strong dissociating agents are rare.[6] Meredith and Wren,[7] using dextran gel filtration, found that very high molecular size wheat proteins were not dissociated in a solvent containing 3 M urea and a cationic detergent. However, Stanley *et al.*[8] claimed that all wheat proteins were dissociated in phenol–acetic acid–H_2O (PAW) (w/v/v, 1:1:1) mixtures to materials with average MWs. ranging from 14000 to 52000 for various flours, as measured in the ultracentrifuge. Evidence was given that such treatment does not split covalent bonds. However, the MW of bovine serum albumin was found to be 17000 in the same solvent,[8] whereas a value of approximately 69000[9] has been confirmed for this protein in the more commonly used protein dissociating agents GuHCl[10] and SDS[11] by gel filtration after disulphide bond reduction, and therefore the estimate for the wheat proteins may be low. Huebner and Rothfus[4] have confirmed that glutenins and gliadins appear unchanged after PAW treatment, but found that glutenin ran in gel filtration at the void volumes of dextran and polyacrylamide columns equilibrated with PAW (1·1:2) and estimated MWs greater than 10^5.

Comparison of the effects of various dissociating agents may be made by using results for protein contents of peaks from Table 1. Similar observations were made after blending flour without added conjugate.

Consideration of the values for sample D in Table 1, leads to the conclusion that in the presence of SDS, protein was transferred from the first (I) to the second (II) peak, when compared with US and GuHCl columns. (The 254 nm elution profiles supported this observation.) Total recoveries of the materials which were completely soluble in both USDS and GuHCl, were almost identical in these two systems. The lower total recovery for the US column is due to the partial insolubility, which in turn appears to reside in the glutenin fraction. The same trend is also seen for sample B and less obviously for A and C, where partial insolubility of two of the fractions leads to greater difficulty in interpretation. These observations may be explained either by assuming that more complete dissociation is achieved in SDS than in the other solvents, or that SDS affects agarose gel filtration to such an extent that certain larger molecular size materials are considerably retarded. The latter possibility seems unlikely in view of the fact that Fish *et al.*[11] found that the MWs of reduced polypeptide chains in SDS could be reliably estimated from their elution volumes on agarose gels, although this agreement did not hold so well for non-reduced proteins.

The possibility of aggregation in US buffer was examined further. When the contents of peaks I from US columns were subjected to ultracentrifugation at 145000 *g* for 45 min, most of the protein was found in the precipitates. On re-running the supernatant liquors on larger (53 × 2·3 cm) US columns (to avoid the need for concentration), only glutenin peaks were observed. A portion of the initial ultracentrifuged solution which was allowed to stand for 24 hr instead of being loaded on the first column did not develop ultracentrifugable material. These observations are evidence for interaction between glutenin and lower MW materials even in US buffer. This interaction is insufficient to prevent separation on the

[7] MEREDITH, O. B. and WREN, J. J. (1966) *Cereal Chem.* **43,** 169.
[8] STANLEY, P. E., JENNINGS, A. C. and NICHOLAS, D. J. D. (1968) *Phytochemistry* **7,** 1109.
[9] REITHEL, F. J. (1963) *Advan. Protein Chem.* **18,** 123.
[10] FISH, W. W., MANN, K. G. and TANFORD, C. (1969) *J. Biol. Chem.* **244,** 4989.
[11] FISH, W. W., REYNOLDS, J. A. and TANFORD, C. (1970) *J. Biol. Chem.* **245,** 5166.

columns and separation leads to insolubility presumably due to association between released glutenin molecules. Certain low MW proteins may still be present in the insolubilized materials due to co-precipitation. It was shown in part (b) of this section that a small proportion of low MW fluorescein conjugated proteins were always found in the glutenin peaks (peaks I) after blending with flour and running on agarose columns. The significance of haze was studied in a similar manner to that described above. The glutenin peak from a US column was ultracentrifuged. The sediment was yellow and the supernatant was applied to the larger US column. Only a glutenin peak was observed and this did not absorb at 495 nm. Therefore, the small amounts of colour found in peaks I were possibly a direct consequence of the insolubilization occurring during separation on the columns.

When a non-ultracentrifuged peak I from a US column was made 1% in SDS, the haze disappeared. On running the solution on a large USDS column, approx. 40% of the protein was eluted in the gliadin region. Therefore some material in peaks I from US columns is eluted later on USDS columns, and an explanation of this may be sought by examining the known behaviour of proteins in the dissociating media used in this study.

Tanford,[12] in a review on protein denaturation, pointed out that a reagent which destroys all non-covalent structure will necessarily disrupt all non-covalent bonds between polypeptide chains. Completeness of transition to the denatured state in urea solutions has only been demonstrated rarely.[12] Ronalds and Winzor,[13] from a study of gel filtration elution profiles, detected interaction between glutenin and gliadin in 2 M urea at ionic strength 0·03. Winzor[14] had earlier found that interaction occurred in 3 M urea, $I = 0{\cdot}01$, but not in 3 M urea, $I = 0{\cdot}03$. This difference probably reflects a diminution of intermolecular ionic forces at the increased ionic strength. The US buffer in the present study contained 0·2 M sodium chloride, and therefore electrostatic interactions between polypeptide chains would be expected to be very weak.[12] Cluskey and Wu[15] deduced from optical rotatory dispersion measurements that glutenin and gliadin possessed little or no α-helix in 8 M urea. However, Crow and Rothfus[16] presented results which indicated that some association of glutenin proteins still occurred in 8 M urea.

Criddle *et al.*[17] showed that 6 M urea did not cause dissociation of the hexamer of cytochrome C_1, whereas SDS did so. SDS dissociates reduced proteins to their constituent polypeptide chains, although random coils are not produced. However, when the disulphide bonds are left intact, as was necessary in the present study, binding of SDS is less[18] and sufficient data are not available to ascertain whether complete dissociation occurs for all proteins.

Concentrated GuHCl solutions are stronger denaturing agents than urea[12] but some model polypeptides are able to retain regions of helical structure even in these.[19,20] The presence of disulphide bonds probably does not prevent the conversion of proteins to the random coil state in GuHCl.[12]

From such considerations it is unlikely that the glutenin (I) peaks consisted entirely of aggregation products of II proteins through non-covalent bonds but the possibility of some

[12] Tanford, C. (1968) *Advan. Protein Chem.* **23,** 121.
[13] Ronalds, J. A. and Winzor, D. J. (1969) *Arch. Biochem. Biophys.* **129,** 456.
[14] Winzor, D. J. (1966) *Arch. Biochem. Biophys.* **113,** 421.
[15] Cluskey, J. E. and Wu, Y. V. (1971) *Cereal Chem.* **48,** 203.
[16] Crow, M. J. A. and Rothfus, J. A. (1968) *Cereal Chem.* **45,** 413.
[17] Criddle, R. S., Bock, R. M., Green, D. E. and Tisdale, H. (1962) *Biochemistry* **1,** 827.
[18] Pitt-Rivers, R. and Impiombato, F. S. A. (1968) *Biochem. J.* **109,** 825.
[19] Sage, H. J. and Fasman, G. D. (1966) *Biochemistry* **5,** 286.
[20] Auer, H. E. and Doty, P. (1966) *Biochemistry* **5,** 1716.

association has not been eliminated. On one occasion 8 M urea was included in the US buffer instead of 4 M. This did not cause further dissociation of peak I.

The experiments reported here were performed on acidic extracts of flour as direct extraction with the solvents led to solutions which were too viscous to load on columns. The unextracted materials were probably mainly of very high molecular size.

Glutenin was still present in acidic extracts of flour which had been previously treated with acrylonitrile in order to block sulphydryl groups, confirming conclusions in section (b) drawn from the experiments with conjugated materials; i.e. at least the major part of glutenin is not formed from other proteins as a consequence of sulphydryl dependent reactions during extraction. Meredith and Wren[7] reported similar findings.

It may be concluded that wheat flour contains proteins of very large molecular size (glutenins) which are not dissociated in solvents known to disrupt non-covalent bonds. These components are apparently present in flour and are not artifacts of extraction. Glutenin, as prepared in a typical fashion by acidic blending of gluten after a triple extraction with 70% ethanol, still contained a considerable proportion of lower molecular size proteins, judged by elution patterns on agarose columns. Therefore any experiments on glutenin should be accompanied by precise details of its preparation.

EXPERIMENTAL

Materials. Cappelle–Desprez wheat flour contained 9·2% protein. Sepharose 4B, Sephadex G25, and Blue Dextran 2000 were from Pharmacia Ltd. Fluorescein isothiocyanate (isomer 1, 10% on Celite) was from B.D.H Chemicals Ltd. 6 M guanidine hydrochloride was prepared from the carbonate.[21] Bovine thyroglobulin, type 1, was from Sigma Chemical Company, U.S.A.

Agarose columns. Sepharose 4B, as supplied in aqueous suspension, was packed to a height of 36 cm in columns of internal diameter 1·6 cm. At least 150 ml of the appropriate solvent were passed through the column during equilibration. The preparation of solvents and abbreviations used to describe them were: US—4 M urea, 0·2 M NaCl, 0·02% sodium azide, HOAc to pH 4·1; USDS—4 M urea, 1% sodium dodecyl sulphate (SDS), 0·01 M HOAc, pH 4·3; GuHCl—6 M guanidine hydrochloride at pH 6·7. Flow rates were approximately 10 and 7 ml/hr for US and USDS columns, respectively. Usually 3 ml/hr were eluted from GuHCl columns, but this could drop to 1·5 ml/hr when highly viscous samples were loaded. Eluates were monitored at 254 nm with an L.K.B. 'Uvicord'.

Amino acid analyses. Reduced and blocked samples were hydrolysed at 105° for 24 hr *in vacuo*, sealing with 'Quickfit' 'Rotaflo' stopcocks.[22]

Preparation of fluorescein-conjugated proteins. Flour (100 g) was added slowly to H_2O (400 ml) with vigorous stirring, which was continued for a further 10 min after addition. The mixture was centrifuged at 59 000 *g* for 1 hr and the residue cut into small pieces before re-extraction with 400 ml H_2O for 20 min. After centrifuging as before, the liquors were combined and freeze-dried to give *ca.* 4·5 g material. The conjugation procedure was essentially that described by Chadwick and Fothergill.[23] The aq. extract (3 g) was stirred for 10 min in H_2O (50 ml) before cooling at 0°. A mixture of $NaHCO_3$ (2·47 g) and anhyd Na_2CO_3 (0·40 g) was made up to 50 ml with H_2O and, after cooling in ice, was added to the extract. Fluorescein isothiocyanate on Celite (0·50 g) was added in small portions to the mixture, and stirring continued for 7 hr with cooling at 0° The mixture was left for a further 16 hr at *ca.* 2°, stirred for a further 1 hr at 0°, and then dialysed for 5 hr with 10 changes against 0·05 M HOAc with the addition of ice. The last dialysis was against pre-cooled 0·05 M HOAc. After centrifugation at 59000 *g* the liquor was freeze-dried. Residual reagents were removed from the conjugated proteins by stirring one-third of the product (*ca.* 0·7 g) in US buffer (25 ml) for 10 min centrifuging at 3000 *g* for 10 min and loading the liquor on Sephadex G25 columns (100 × 2·4 cm) equilibrated with US buffer. After dialysing against H_2O, the protein peaks were freeze-dried (total yield *ca.* 1·3 g). When desired, reduction and subsequent blocking of the conjugates were carried out at this stage by a modification of the method of Weil and Seibles.[24] Conjugate (0·50 g) was dissolved in 11·5 ml 0·2 M Tris

[21] CECIL, R. and WEITZMANN, P. D. J. (1964) *Biochem. J.* **93,** 1.
[22] DARBRE, A. (1971) *Lab Pract.* **20,** 726.
[23] CHADWICK, C. S. and FOTHERGILL, J. E. (1962) *Fluorescent Protein Tracing* (NAIRN, R. C., ed.), p. 21, E & S Livingstone, Edinburgh.
[24] WEIL, L. and SEIBLES, T. S. (1961) *Arch. Biochem. Biophys.* **95,** 470.

buffer, 8 M urea, pH 7·4. After the addition of 2-mercaptoethanol (1·2 ml), the tube was flushed with N_2, stoppered, and left at room temp. for 24 hr with occasional shaking. Acrylonitrile (2·3 ml) was dissolved in the mixture and, after 1 hr at room temp., the pH was adjusted from 7·4 to 3·5 with HOAc. The solution was dialysed (30 hr) against 0·01 M HOAc, until Cl^- was absent from the diffusate, before freeze-drying. Unblocked (hereafter named samples A′ and B′), and blocked material (sample C′) were freed of small amounts of glutenin-like material by dissolving portions (200 mg) in US buffer and loading on the normal US Sepharose columns. The main peaks were dialysed against H_2O and freeze-dried. The other blocked sample (D′) was submitted to a similar procedure using GuHCl columns. The extent of conjugation was measured as described by Fothergill.[25]

Blending of flour with conjugated materials. Conjugate was stirred in 0·05 M HOAc (50 ml) for 15 min and the pH adjusted to 3·1 with a few drops of HOAc. Flour was blended at full speed for 4 min with this mixture. (The flour to conjugate ratio was always 20:1, the actual amounts of flour blended being 3, 10, 3 and 4 g for samples A′, B′, C′ and D′ respectively.) After magnetically stirring for 30 min and centrifuging at 108000 *g* for 20 min, the liquors were freeze-dried to yield extracts A, B, C and D. In general, 0·20–0·24 g of extract (A, B, C or D) was stirred for 1 hr in 5 ml of the appropriate solvent for column loading. When necessary, insoluble material was removed by centrifugation at 145000 *g* for 45 min. 4 ml fractions were collected from the column. 1 ml M Tris buffer pH 8·0, 4 M urea, was added to each tube and the optical density of the conjugate read at 495 nm. Tube contents were then combined on the basis of the original 254 nm trace, and the optical densities read in 1 cm cuvettes at 280, 320, 340, 495, 600 and 700 nm against a blank (4 ml solvent plus 1 ml Tris buffer). Haze corrections at 280[26] and 495 nm were made by linearly extrapolating the values at 320 and 340 nm, and 600 and 700 nm, respectively.

Blocking sulphydryl groups of flour proteins. Acrylonitrile (1 ml) was mixed with H_2O (15 ml), and 1 ml of the solution was added to 100 ml 6 M GuHCl at pH 8·0 which had been purged with N_2. Flour (1 g) was added and the mixture stirred for 1 hr at room temp. in a stoppered flask. After adjustment of the pH from 7·9 to 4·0 with M HOAc, the solution was dialysed against H_2O and freeze-dried. (A 1000-fold excess of blocking reagent was used, assuming a value of *ca.* 1 μmol SH/g flour.)

Acknowledgements—The author thanks Dr. J. A. D. Ewart for advice and Mr. D. Smith for experimental assistance.

[25] FOTHERGILL, J. E. as in Ref. 23, p. 36.
[26] BEAVEN, G. H. and HOLIDAY, E. R. (1952) *Advan. Protein Chem.* 7, 319.