ON THE SOLUBILITY OF GLIADINLIKE PROTEINS

I. Solubility in Nonaqueous Media[1]

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ABSTRACT

The solubility of gliadinlike proteins in mixtures of methanol and chlorinated hydrocarbons has been determined. The requirements for a solvent include an active hydrogen atom and a hydroxyl group. Such mixtures are solvents only over a limited range of composition, and there is therefore a possibility of fractionating the protein mixture by alteration of solvent composition. Similar types of proteins are found in wheat, rye, oats, barley, rice, and corn, but the prolamins of wheat and corn may be differentiated on the basis of solubility in ethanol-chloroform mixtures. The amide groups of the protein are involved in the mechanism of solubility in methanol-chloroform in association with the cation history of the protein preparation.

Meredith *et al.* (1) showed that a considerable part of gluten was soluble in a mixture of methanol and chloroform. This bears some similarity to Osborne's definition (2) of gliadin by its solubility in aqueous ethanol. Other organic-aqueous mixtures have been investigated as gliadin solvents by Dill and Alsberg (3) and by De Deken and Mortier (4), but solubility of protein in a nonaqueous system is unusual. Singer (5) has recently reviewed the subject. Dimethylformamide has been used as a solvent for gluten (6), and chloroethanol has been used more recently for gluten (7) as well as for other proteins. It has

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been suggested that the solubility of prolamins in organic solvents may be due to the relatively nonionic character of these particular proteins (1,4). From the present results, some requirements of structure for the solvents and protein will be defined.

The difference between wheat and other cereal proteins with respect to the characteristic coherent properties may lie in the glutenin and not in the gliadin fraction (1). Evidence will be presented for the similarity of partial solubility of several cereal proteins in methanol-chloroform mixtures, in accord with the above suggestion, and supporting the statement by Geddes (8) that the proteins of cereal grains include alcohol-soluble proteins (prolamins or gliadins). Waldschmidt-Leitz and co-workers (see for example ref. 9) have carried out extensive work on the prolamins of several cereals.

Materials and Methods

The experiments have been carried out in England, New Zealand, and Canada with straight-grade bakers' flours of 75, 78, and 72% extraction respectively and without improvers or other additives. There has been sufficient repetition of experiments to show that the type of flour used has not significantly affected the nature of the results obtained. There is, however, a suggestion of quantitative differences in the behavior of different flours.

The major solvents were carefully redistilled after appropriate treatments to bring their oxidizing power to a low level. The series of chlorinated hydrocarbons were used as received from Eastman Organic Chemicals, Rochester, N.Y., with the exception of 1,2-dichloroethane which was redistilled to confirm what seemed at first an anomalous solubility effect. Hydrochloric acid was redistilled from analytical grade.

Flour was defatted by percolation with dry peroxide-free ether, followed by ethanol-ether (3:1), and then more ether, at room temperature; the residual solvent was removed under vacuum and by standing in air. Gluten was hand-washed from the lipid-free flour, generally after 5 min. of mechanical mixing of the dough, followed by 30 min. of rest, and freeze-dried. A further preparation was made by gently stirring powdered dry gluten into 0.01*N* formic acid solution and centrifuging after 30 min. By using only gentle stirring, not homogenizing, dissolution of gelatinous (glutenin) protein is avoided. Gliadin-like protein was precipitated from this acid solution by addition of sodium formate to 0.25*M* or by neutralization with sodium hydroxide. The settled precipitate was reacidified with formic acid to 0.1*N* and exhaustively dialyzed against distilled water, then freeze-dried.

A product of a higher degree of purity with respect to the particular proteins in which we are interested was obtained by extracting the dry acid-soluble preparation with 1:2 v/v methanol-chloroform (5 ml. per g.) and, after centrifuging, precipitating protein from the supernatant with 9 volumes of acetone.

Solubility of the gliadinlike preparation in organic mixtures was determined by shaking 2.5 g. protein with 50 ml. solvent for 60 min., filtering with precautions to avoid evaporation, then evaporating 20 ml. of the filtrate to constant dry weight at 105°C. In Figs. 1 to 4 the term "Relative Solubility" is used to indicate the percentage of the starting material which dissolved under these conditions.

Deamidation of protein was performed by incubation with 2*N* hydrochloric acid at 40°C. for times varying from 0.5 to 24 hr. The protein was dialyzed for 2 days and freeze-dried. The extent of deamidation was determined approximately by measuring the ammonia released after boiling with 6*N* hydrochloric acid for 1 hr. and distilling off rapidly from alkaline solution. No assessment has been made of the extent of peptide cleavage under these conditions.

Results

Extraction of Gluten by Methanol-Chloroform. The effects of acid treatment and of homogenizing during the purification of gluten were compared by determining the yields of methanol-chloroform-soluble material.

Dry gluten (100 g.) was stirred for 2 min. with 1 liter 0.01*N* formic acid solution and the slurry allowed to stand overnight at 2°C. It was then homogenized in a Waring Blendor and centrifuged. The solid was homogenized with a further 500 ml. dilute acid and the slurry centrifuged. The two acid extracts were combined. A second 100-g. portion of gluten was treated similarly, but gentle stirring was substituted for the homogenizing stages. A third 100-g. portion of gluten was treated similarly to the second, but water was substituted for the dilute acid.

Each of the three extracts was precipitated by addition of sodium formate to 0.25*M* concentration. The precipitate was homogenized into 2 liters of 1:2 v/v methanol-chloroform mixture and refluxed overnight. After filtration the solid residue was extracted twice more with 1 liter of solvent. Each combined filtrate was evaporated and redissolved in 0.01*N* formic acid. The solutions were dialyzed against distilled water and freeze-dried. The yields of methanol-chloroform-soluble material were corrected to a common nitrogen content.

The yields from the three extractions – 29.7% after homogenizing

with acid, 29.8% after stirring with acid, and 19.0% after stirring with water — suggested that treatment with acid increased the yield of solvent-soluble material but that homogenizing did not further increase the yield.

Solubility in Various Organic Solvent Mixtures. The mixture 1:2 v/v methanol-chloroform was originally used following the work of Folch *et al.* (10) on animal lipids. The solubility curve for gliadinlike protein in the complete range of methanol-chloroform mixtures was determined (Fig. 1) and showed that maximum solubility occurred at about 65 molar % methanol, but it also showed a second lesser solubility maximum at about 15–20 molar % methanol. Mixtures of

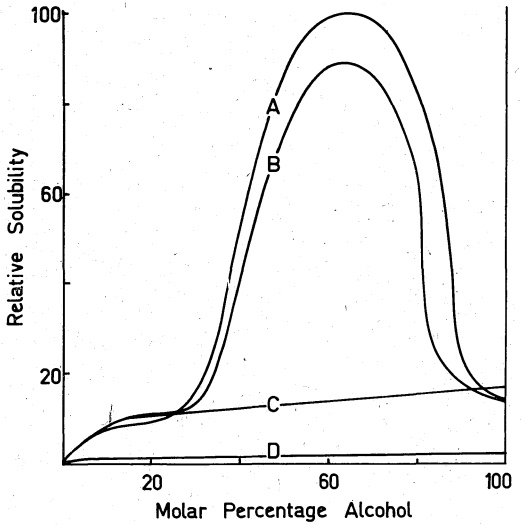

Fig. 1. Solubility curves for gliadinlike protein in mixed solvents: A, methanol-chloroform; B, methanol-methylene chloride; C, methanol-carbon tetrachloride; D, ethanol-chloroform.

methylene chloride and methanol gave a similar curve, but mixtures of carbon tetrachloride and methanol had scarcely any solvent property. Likewise, mixtures of chloroform and ethanol were not solvents for this protein system.

On the basis of these results, certain structural requirements for solvent action were formulated and tested on a wider range of compounds in admixture with 0, 60, 70, and 80 molar % methanol. The mixtures, shown in Table I, have for clarity been divided into those with negligible activity, those with moderate activity, and those which are good solvents. Methanol alone dissolved 5–15% of the protein.

TABLE I

PROPORTION OF PROTEIN PREPARATION DISSOLVED BY MIXTURES OF METHANOL WITH OTHER SOLVENTS

	Molar % Methanol			
	0	60	70	80
	%	%	%	%
Tetrachloromethane	1	0	1	2
Chloroethane	0	1	1	2
1,1,1-Trichlorethane	0	1	1	2
Ethanol	0	4	4	5
Trichlorethylene	0	2	3	4
(2-chloroethyl) Benzene	0	1	2	7
1,1-Dichloroethane	0	4	5	6
Acetylacetone	0	2	4	7
Acetonylacetone	1	1	2	6
Nitroethane	0	9	25	33
1,2-Dichloroethane	0	36	45	34
Nitromethane	0	68	87	61
Dichloromethane	1	88	85	67
Trichloromethane	1	100	79	29
1,1,2-Trichloroethane	0	98	100	100
1,1,2,2-Tetrachloroethane	0	98	98	98
1,1,2,2-Tetrabromoethane	0	100	100	100

Other solvent pairs which proved to be immiscible and therefore could not be tested were methanol-hexachloroethane, methanol-chlorodiphenylmethane, and ethylene glycol-chloroform.

The effect of water on the solubility was determined by adding 4% of water to each methanol-chloroform mixture. As shown in Fig. 2, the

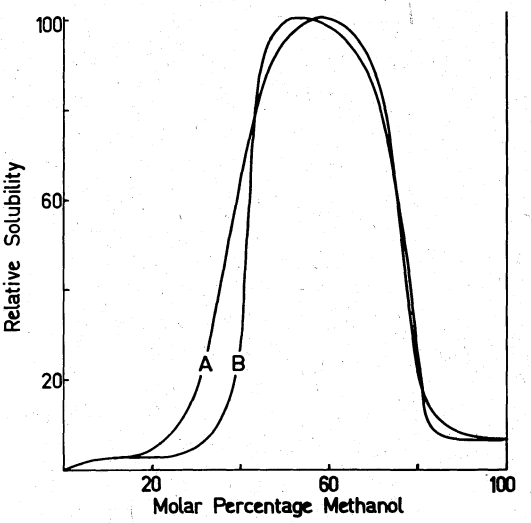

Fig. 2. Solubility curves for gliadinlike protein in methanol-chloroform mixtures: A, dry (0.28% water); B, wet (4% water).

essential form of the solubility curve was the same with and without water.

A similar experiment was carried out with ethanol-water mixtures (Fig. 3), paralleling the classic gliadin solubility.

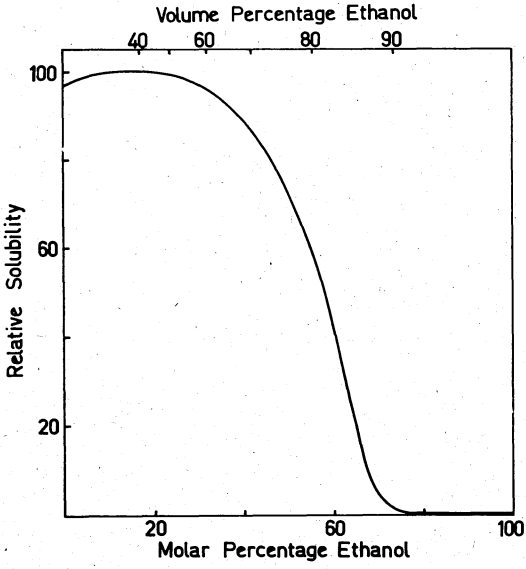

Fig. 3. Solubility curve for gliadinlike protein in ethanol-water mixtures.

Solubility and Protein Structure. It had been shown previously (1) that oxidation rendered the gliadinlike proteins insoluble in methanol-chloroform, and it was presumed that this was due to the production of strongly ionizable $-SO_3H$ groups from –SH and S–S groups of the protein. It follows, therefore, that partial deamidation of the protein should also cause insolubility in the nonaqueous system. Holme and Briggs (11) have shown that partial deamidation drastically affects the aqueous solubility of gliadin.

Samples of protein were deamidated to varying extents, and approximate amide content and solubility in methanol-chloroform (1:2 v/v) were determined for each. Variation between replicate experiments was found, and it has been shown that the extent of dialysis greatly affects the amide content at which the marked fall in solubility occurs. Examples of experiments with varying dialysis conditions are shown in Fig. 4.

Proteins of Other Cereals. Protein preparations were made by the methods of Cunningham *et al.* (12,13) from several ground cereals, and

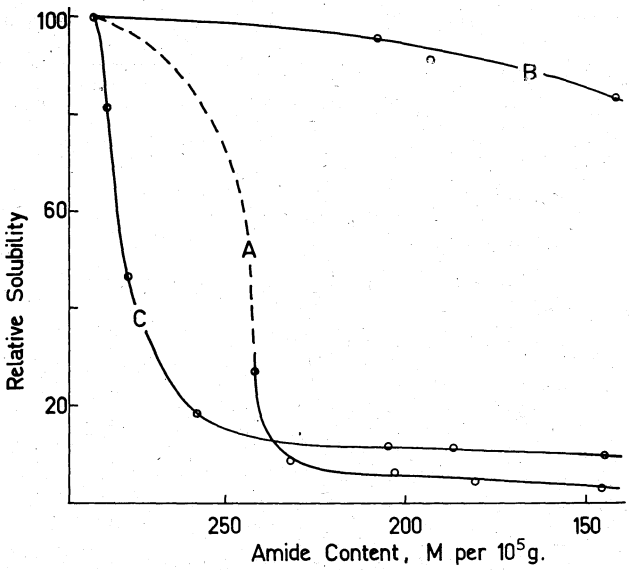

Fig. 4. Variation of solubility of gliadinlike protein in methanol-chloroform with amide content and dialysis conditions. A, dialyzed against water; B, dialyzed against 1 mM hydrochloric acid; C, dialyzed against 1 mM hydrochloric acid, 0.1 mM sodium chloride solution.

each freeze-dried preparation was assayed for solubility in 1:2 v/v methanol-chloroform, with the following results: oats 10% soluble, barley 24%, rye 19%, and rice 25%. Similar extractions were made with ethanol-chloroform to show that it was not lipid that was being extracted: oats 1% soluble, barley 1%. A sample of commercial zein was completely soluble in both methanol-chloroform and ethanol-chloroform.

Thus an appreciable amount of the proteins of each of these five cereals was soluble in the nonaqueous solvent.

Discussion

The first experiment concerning the extraction of gluten by methanol-chloroform under various conditions was initially interpreted to support the suggestion made earlier (14) that a complex structure of gluten is partly dissociated by treatment with acid. However, the variation in the relationship between amide content and nonaqueous solubility due to differing ionic treatments suggests that these first results may be interpreted more simply as due to differing cation content of the protein. Tschoegl (7) also found that the proportion of gluten extracted into chloroethanol depended on the presence of acid. It is indeed very evident that the solubility properties of this class

of protein both in aqueous and nonaqueous media depend markedly on the presence of traces of hydrion and other cations.

The insolubility of the protein in methanol-carbon tetrachloride and in ethanol-chloroform mixtures suggested two requirements for a solvent. These are 1) an active hydrogen atom and 2) a hydroxyl group, either on a small molecule or at a high activity relative to the size of the molecule. It has not been possible to determine which of these latter criteria are true, for small polyhydric alcohols were immiscible with the chlorinated hydrocarbons. Methanol remains the only alcohol which is active in this regard. It may be that electron-repelling groups other than hydroxyl are effective.

The two requirements are fulfilled not only by methanol-chloroform mixtures but also by aqueous acid which is likewise a good solvent for this class of proteins. And of course chloroethanol bears some similarity to the requirements, though the hydrogen is not very highly activated. It would seem that 2,2-dichloroethanol and 2-cyanoethanol would be even better solvents for gliadinlike proteins; 2-chloroethanol and 2-cyanoethanol were solvents for these protein preparations. Dichloroethanol has not been tried.

The results for other substituted hydrocarbons are in agreement with the inductomeric effects expected (15) to activate one or more hydrogen atoms. This is particularly well portrayed by the effectiveness of nitroethane compared with chloroethane. The lack of solvent effect by 1,1-dichloroethane would seem to be due to the electron-repelling effect of the methyl group countering the electron attraction of the two chlorine atoms, since dichloromethane is an effective solvent. To speculate a little, it would seem that any *small* molecule containing strong electron-attracting groups such as halogen, nitro, nitrile, or acetyl should be capable of functioning with methanol as a solvent for the gliadinlike proteins. *Small* molecule is emphasized since acetylacetone and acetonylacetone with methanol were not solvents for these protein preparations, though they are for zein.

One has only to compare the solubilities in the series methanol with methylene chloride, chloroform, and carbon tetrachloride to see that the dielectric constant of the mixture is not related to solubility in this instance.

The shape of the solubility curve, with maxima in the regions of 20 and 65 molar % methanol, indicates at least two components in the protein mixture. Even were this not so, it should still be possible to base a fractionation method on the variation of solubility with solvent composition. This fractionation will be discussed in Part III of the series.

The variables one considers in investigating the solubility of protein in mixed solvents are temperature, *t*, protein concentration, *p*, solvent composition, *c*, and proportion of protein dissolved, *s*. Most earlier workers on the prolamins were concerned with their solubility in aqueous alcoholic mixtures, and plotted curves of critical peptization temperatures against solvent composition: i.e., *c,t* diagrams, *p* being fixed and *s* either finite or infinite. In the present work likewise *p* has been fixed, but *c,s* curves have been determined at a fixed temperature. Sufficient is known of the behavior for us to be confident that as the temperature is raised *s* will become infinite over a wider and wider range of *c* until the boiling point of the solvent is reached. In fact this is the same situation that will be considered for aqueous acid solutions of these proteins in Part II.

When comparing the *c,t* diagram of Dill (16) with the present results (Fig. 3), one must remember that Dill's preparations were from a salt precipitation whereas the present preparations had been dialyzed against water after solution in acid. This difference greatly affects the solubility in salt-free water and hence affects the form of the low-alcohol end of the curves. Dill's results show insolubility below 15 molar % ethanol in water at 20°C., whereas in the present work the preparation was almost completely soluble in water and low alcohol concentrations.

Manley and Evans, in a most comprehensive work (17), considered many mixed solvents for the analogous prolamin, zein, but again it was the *c,t* curve which was determined. They tried all the solvent mixtures of the present work, and a distinction between the wheat and corn preparations is at once apparent, since their zein preparation was soluble in ethanol-chloroform as well as in methanol-chloroform, and showed a more limited but nevertheless considerable solubility in methanol-carbon tetrachloride. Mixtures of alcohols with nitro paraffins were found to be good solvents for zein, and the same was true for acetonylacetone in mixture with alcohols or water.

A reviewer of this paper has suggested that the effectiveness of the less polar solvents for these proteins demontrates the importance of hydrophobic forces in their structure. This is, of course, in addition to the well-established importance of electrostatic, hydrogen, and covalent disulfide bonds in the gluten proteins. No doubt all these forces act simultaneously, with their relative importance dependent on the ambient conditions.

Manley and Evans interpreted the suitability of solvents for zein in terms of their capacity for forming hydrogen bonds by virtue of electron donor and electron acceptor powers. They concluded that

both donor and acceptor functions were necessary to dissolve zein, though this could be fulfilled either by a pair of solvents or within a single amphoteric solvent. It appears that similar considerations apply to gliadinlike proteins, though it is a matter of speculation to interpret the findings in terms of any particular sort of bond. Indeed, it is not only gliadin and zein that are soluble in these conditions, since the present work has shown that other cereals possess proteins soluble in methanol-chloroform mixture, and Tschoegl (18) has shown that several cereal proteins are soluble in acid chloroethanol. Such a resemblance between the cereals is to be expected, and Blagoveshchenskii has recently suggested (19) a phylogenetic classification of the different types of plant proteins, including the prolamins.

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