ROLE OF DISULFIDE BONDS IN THE DEVELOPMENT OF THE RHEOLOGICAL PROPERTIES OF GLUTEN

I. REDUCTION AND REOXYDATION OF THE DISULFIDE BONDS

By

R. LÁSZTITY

Department of Food Chemistry Technical University, Budapest

(Received August 13, 1968)

Presented by Prof. Dr. L. TELEGDY-KOVÁTS

The knowledge of factors influencing and determining rheological properties of gluten proteins is an important aim of cereal chemistry and of research work carried out on grain processing. To know these relations would be of great value both from a theoretical and a practical point of view. Flour qualification would be possible on entirely objective grounds. There would be means to influence favourably, by determined physico-chemical and chemical treatment, the rheological properties of wheat proteins and a raw material of even quality could be assured for the baking industry at all events, this being one of the important conditions for automation of the technological processes. The obtained results would have also an important influence on the growing and improving of wheat.

The complex problem outlined above, has been discussed in detail in papers of summarising character [1, 2]. Here it is wanted only to stress that beside quantitative and qualitative conditions influencing the rheological properties of proteins forming gluten, primarily also their interactions, covalent and non covalent bonds have a part.

Many examples are known about the important role of disulfide bonds concerning the protein structure and its mechanical properties. The gluten proteins contain relatively small quantities of cystine and cysteine, respectively (in average 2 to 3%). Primarily their importance was called upon in relation with problems of flour improvers. In consequence, a very intensive research work was started in this domain, especially in the last two decades. Nevertheless, there are still many unsolved problems. To clear up the role of disulfide bonds involves further manysided investigations. Some useful data may be obtained by studying the changes occurring in the rheological properties due to decomposition of the disulfide bonds. Among the procedures used in protein chemistry to break down disulfide bonds, especially reduction seems to be promising, because of the possibility to reconvert the formed thiol groups into disulfide bonds, after removal of the reducing agent.

For many proteins and enzymes it has been proved experimentally that reoxydation of the thiol groups of reduced protein, re-establishes — under

determined conditions — the original biological activity of the protein. In this way e.g. reoxydation and re-establishment of the native properties could be successfully realized among others, for ribonuclease [3], α-amylase [4, 6] insulin [5]. ANFINSEN and HABER [6] suppose that the tertiary structure of proteins is determined, or rather fixed, by the amino acid sequence and secondary bonds are developing between the various side chains.

In case of gluten neither the biological functions nor its activity are known and the native properties are quantitatively still hardly outlined. However, the processes occurring during reduction and reoxydation might be highly interesting for knowing how both structure and rheological properties are forming.

Tests of BECKWITT et al. [7] on wheat gliadin showed gliadin reduction (break down of the S—S-bonds) involving no perceptible change in molecular weight. Though ferograms obtained by gelelectrophoresis show minor deviations in mobility, this can, however, be explained by changes in conformation. Reduced and alkylated gliadin has practically no parts of a helical structure, according to optical dispersion tests, whereas in active gliadin their ratio is about 15% based on computations.

In case of reoxydation in a diluted solution, investigations of the above mentioned authors show that practically the native gliadin is recovered. Their statement has been enhanced by their ultracentrifugal, electrophoresis and rotatory dispersion tests. No data are published about the rheological properties of the reoxydized product. The statement according to which reoxydation in a more concentrated solution (5%) involves development of products of a greater molecular weight and less soluble than the original gliadin is very interesting. It is supposed that intermolecular disulfide bonds are forming in this case.

Recently BECKWITT and WALL [8] reported of the reduction and reoxydation of the other classic gluten component. Tests carried out with similar technics and control methods as above, proved as in sulfitolysis, the decomposition of the disulfide bonds, resulting in disintegration of the large gluten component molecules. In case of reoxydation in solutions of higher concentration, a product with properties very near to those of native glutenin may be obtained. According to information of the authors, physical properties of the product formed in course of reoxydation are much influenced by the reoxydation conditions, primarily by pH and the carbamid concentration. No numerical data on the rheological properties of the glutenins produced under different conditions are given, probably no such measurements have been carried out.

Results and discussion

The conditions following reoxydation of the gluten reduction as well as the rheological properties of the products developed have been studied to provide the necessary information.

To reduce and reoxydize gluten samples, the methodology applied by BECKWITT et al. [7, 8] has been adopted, the rough outline of which is as follows: from the gluten sample a 5% solution in 6 mole carbamide has been prepared, then the reduction was carried out with β-merkaptoethanol during nitrogen circulation for 12 hours. Part of the reduced gluten was alkylated with acrylnitryl and after dialysis and lyophilization the S-cyanoethyl gluten derivate has been obtained. The reoxydation was carried out with different carbamide concentrations (solutions of 1 to 8 mole) and pH values (3.5—5.5—8.5). The gluten content of the solution ranged from 1 to 10% because preliminary tests showed this concentration range to be the most favourable for producing a product similar to native gluten.

Reoxydation followed oxygen circulation for 168 hours. By the end of the reoxydizing process the product was cleaned by dialysis, compacted in a centrifugal apparatus and finally free water has been evacuated from the agglomerating mass by hand kneading in a polyethylene bag. This sample was tested for stress relaxation by the above described method [9, 10].

Table 1 recapitulates the rheological properties of reoxydized glutens, produced under different conditions.

Data in Table 1 show that the rheological properties of the developing products are highly influenced by the reoxydation conditions. Products best approaching the properties of native gluten may be produced with a carbamide solution of 4 mole at pH 5.5 and with a carbamide solution of 6 mole at pH 3.5. In general, with a low carbamide concentration a cohesive product without elastic properties is obtained, whereas in case of a 8 mole carbamide concentration, the reoxydized gluten is tougher but has no appropriate elasticity. In alcaline media (pH 8.5) no product with properties similar to native gluten could be obtained in either carbamide concentration. It has to be noted that reoxydation was essentially more rapid in an alcaline medium. In general, the reoxydized product yielded a practically inelastic, cohesive mass when mixed with water. Its properties could be compared for the most part with gluten extensively denaturated by heat.

The present study of the rheological properties shows distinctly the important role of disulfide bonds in the development of the physical properties of gluten. At the same time it is distinctly apparent that the absolute number of disulfide bonds alone does not determine unambigously above properties. In this respect also the position of the disulfide bonds is essential.

The influence of pH and the carbamide concentration on the properties

Table I

No.	Carbamide conc. (mole)	pH	τ_h din/cm²	t_r (sec)	Remarks
1	1	3.5	150	immensurable	—
2	1	5.5	220		
3	1	8.5	350		
4	2	3.5	240	30	
5	2	5.5	280	42	—
6	2	8.5	1200	immensurable	
7	4	3.5	540	68	elastic
8	4	5.5	770	92	
9	4	8.5	1800	150	
10	6	3.5	300	42	slightly
11	6	5.5	270	29	elastic
12	6	8.5	1900	180	—
13	8	3.5	320	35	—
14	8	5.5	2700	immensurable	nonelastic
15	8	8.5	2800	immensurable	nonelastic
16	native gluten of control		590	83	gluten of average elasticity and extensibility

of the reoxydized products can be explained as follows: depending on the pH and the carbamide concentration, changes may occur in the conformation of the peptide chains, in the steric position of the individual groups, their dissociation conditions, their reactivity, defining the type and place of the forming disulfide bonds. The effect of carbamide, of the pH, and the ion concentration on the protein conformation is generally known. Recently Wu et al. [11] have published some positive data about gluten proteins. According to these by changing e.g. the ion-concentration, the ratio of the helix formation of the polypeptide chains of glutenin increases from 6 to 12%. In case of gliadin, the observed effect is essentially lower, the numerical values of the increase are 14 and 18%, respectively.

The forming disulfide bonds might be either intramolecular or intermolecular bonds. The ratio of inter- and intramolecular disulfide bonds is

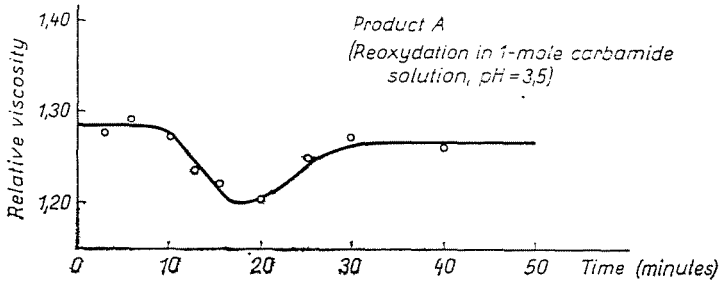

Fig. 1

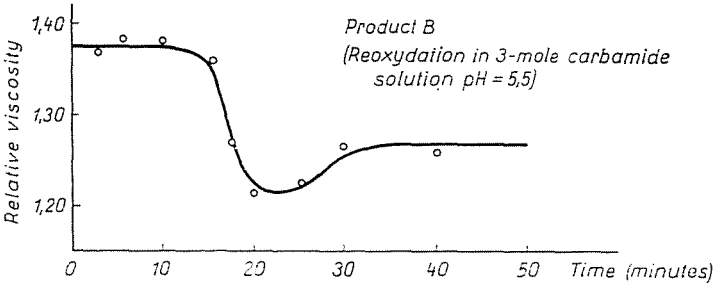

Fig. 2

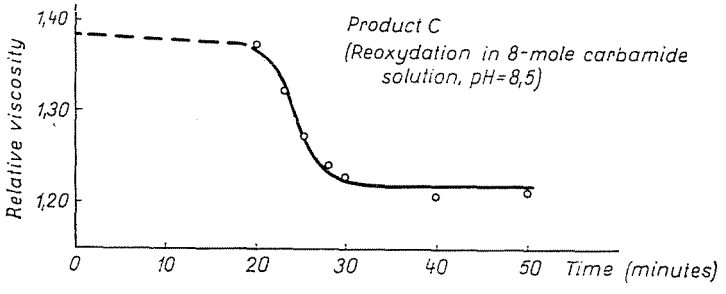

Fig. 3

supposed to be very important in the development of the rheological properties. To check this supposition, the change in viscosity of the solution in course of both performic acid decomposition and sulfitolysis of reoxydized glutens obtained in different ways has been investigated. The test method and the disintegration conditions were identical to those described above. The viscosity changes for solutions of different reoxydized glutens are shown in Figs. 1, 2, and 3.

It can be distinctly seen that the viscosity graphs versus time are different for reoxydized gluten products of different rheological properties. The simplest graph has been obtained for a reoxydized gluten in alcaline medium

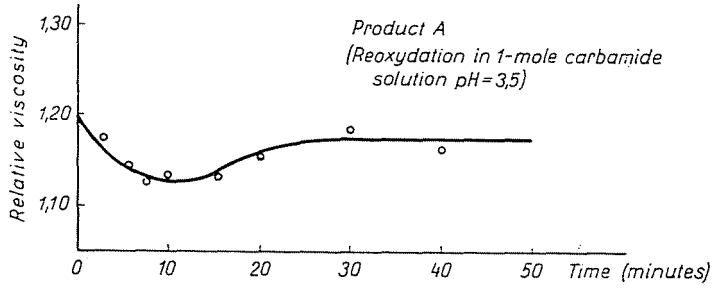

Fig. 4

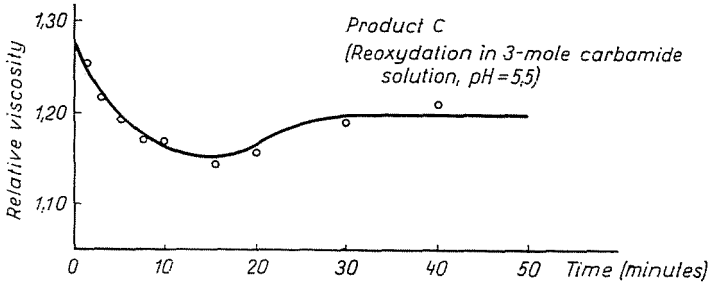

Fig. 5

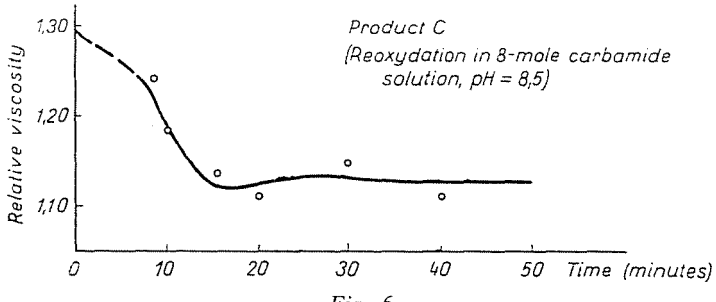

Fig. 6

at a higher carbamide concentration. After a relatively rapid viscosity decrease, it remains at an approximately identical value during further performic acid oxydation. The reoxydized product with properties approximating those of the native gluten shows different features. After rapid viscosity decrease, a minimum is reached in function of time, after which it is rising again towards a limit value. The solution of a reoxydized gluten product prepared at a small carbamide concentration with acid pH is subject to a smaller viscosity decrease during performic acid oxydation, the viscosity graph swhos a minimum, the increase in viscosity after the minimum is manifest and relatively of the highest value.

Disulfide bonds of reoxydized glutens have also been subjected to decomposition by sulfitolysis. Results are summarized in Fig. 4, 5, 6. The character of the curves is nearly identical, as seen in the figures.

The curves of both series of experiments clearly show differences between the disulfide bond systems of the reoxydized glutens with different rheological properties. The viscosity decrease occurring always at the beginning of the disintegration of the disulfide bridges indicates that because of the rupture of intermolecular disulfide bonds, larger protein molecules break down to smaller units. Subsequent plotting of the viscosity graph depends on whether the created smaller units possess at all, or to what extent, intramolecular disulfide bonds. Namely the disintegration of these intramolecular disulfide bonds entrains the change of the conformation of the molecule. The molecule, "opening" to a certain extent, increases the viscosity. When disintegration of the intramolecular disulfide bonds comes to an end, both the molecule conformation and the viscosity show constant values. Studying the curves from this point of view, it is seen that the reoxydized gluten prepared in an alcaline medium at a high carbamide concentration contains practically intramolecular disulfide bonds only. In a preparation close to native gluten, the number of intramolecular disulfide bonds is high and the same applies also to reoxydized gluten of acid pH in a low carbamide concentration.

Principially, the viscosity recovery after a minimum could be ascribed also to new aggregates entering secondary bonds, after disulfide bonds broke down. This supposition is, however, improbable, because no such increase is registered for reoxydized gluten prepared with alcaline pH at a high carbamide concentration, whereas the sub-units developing after decomposition are identical with the decomposition products obtained with the other two gluten varieties as concerns their amino acid composition and active groups.

This statement is supported by the rather extensive tests on reactivity of disulfide bonds of proteins. In general, disulfide bonds can be attacked best by nucleophil reagents (e.g. SO_3^{2-}, CN^-, RS^-, HS^-, etc.). Among the factors influencing reactivity of a given disulfide group, the most important are charge conditions around the disulfide bond, the steric inhibition and existence of stabile rings in consequence of the intramolecular disulfide bond.

The simplest way to define the effect of charge conditions is to state that groups with a charge in the vicinity of a disulfide bond have either an affinity to the ions of the reagent or are repelling them as an effect of Coulomb forces. Accordingly, the reaction is accelerated by groups of opposite charges. Therefore in disintegration by sulfolitolysis, close presence of positive side chains increases the reaction whereas vicinity of negative charged side chains is slowing it down.

Theoretically in steric inhibition every side chain may have some role, adjacent in a given peptide chain or situated at an appropriate place of an other sterically close peptide chain.

The best example for slower reaction of the intramolecular disulfide bonds is presented by the rather intensive tests on insuline. The tests of

FRAENKEL-CONRAT and FRAENKEL-CONRAT [12] showed that of the 3 present disulfide bonds only one could be reduced by thioglycolate. CECIL and LOENING [13] could disintegrate two disulfide bonds with sodium sulfite but could completely disintegrate only in presence of carbamide, guanidine or phenylmercuric hydroxyde the disulfide bridges. More intensive tests revealed that the intramolecular disulfide bond was the slowest and most difficult of all bonds to react.

Though there is no knowledge available about more complicated proteins and about e.g. the system of the disulfide bonds of gluten proteins by taking into consideration some general regularities and analogies, it may be stated that at least in case of the larger gluten protein molecules, the intramolecular disulfide bonds react slower because of the steric inhibition and are accessible only after a certain loosening up.

The quantity of developing thiol groups was examined simultaneously with the change in viscosity during sulfitolysis. On samples taken at intervals during sulfitolysis, amperometric thiol group determination has been performed as well. Comparing the changes of the thiol group and of the viscosity curve, it can be seen that at the viscosity minimum the disintegration of all disulfide bridges is not yet finished.

Finally the observation of BECKWITT and WALL [8] must be mentioned, according to which the amperometrically determined disulfide content of glutenin obtained by sulfite break down, is not more than about two thirds of the actual quantity present up to a carbamide concentration of 3 moles. The total disulfide content can be determined only in a carbamide solution of 6 moles or still higher concentration.

Tests on reduction and reoxydation of gluten indicate the importance of the disulfide bonds in the structure of gluten molecules and also in the development of the rheological properties of gluten. Accordingly, the gluten protein fractions of great molecular weight consist of polypeptide chains, connected by disulfide bridges. Beside the disulfide bonds connecting the polypeptide chains, also the number of disulfide bonds within the molecule is of importance. During reduction of the gluten, all the disulfide bonds break down, the obtained product lacks the rheological properties of the original gluten. When reoxydized, the reversion is practically quantitative. The position of the newly forming bonds, the propertion between inter- and intramolecular disulfide bonds depend on reoxydizing conditions. This fact is also reflected by the rheological properties of the reoxydized product.

It follows from the above that the absolute number of the disulfide bonds alone does not determine unambiguously the structure and the rheological properties. It becomes clear why in general no closer correlation could be established between the disulfide content and the rheological properties. It explains also why the correlation is stronger in gluten of the same wheat

variety. Namely, it may be assumed that in the given wheat varieties, the protein biosynthesis is a similar process, and so is the distribution of the disulfide bonds. This anyhow not very close correlation, is likely to be attributed to the many other factors involved in the development of the rheological properties.

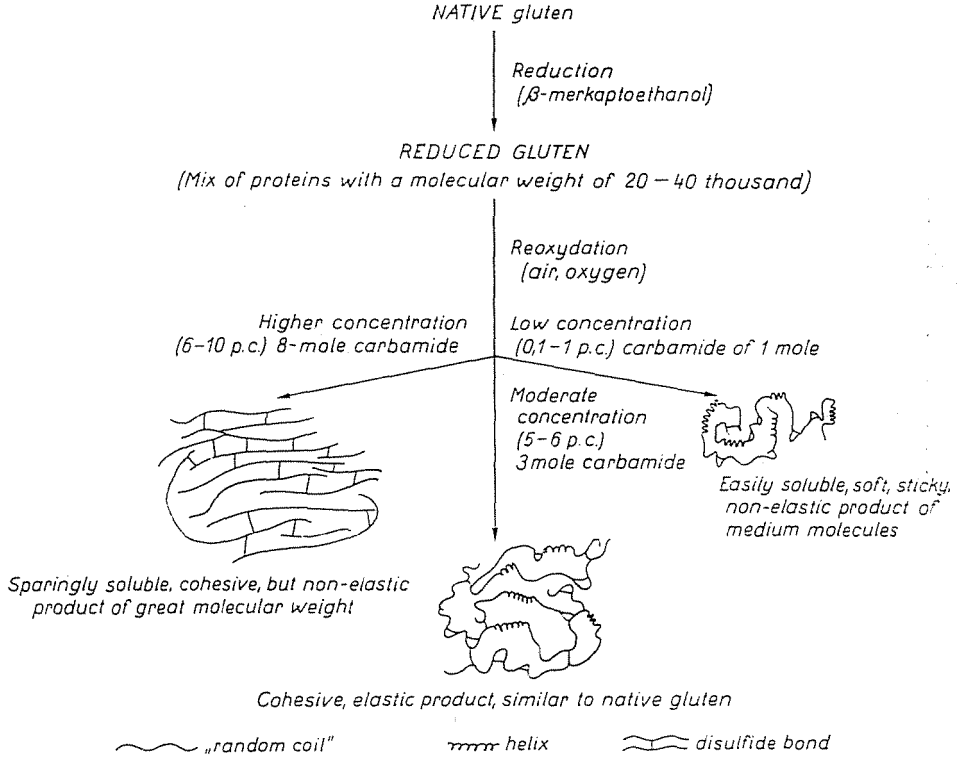

Fig. 7

The statement that products with variable rheological properties can be obtained, depending on the conditions of reoxydation after reducing a given gluten, may be of a paramount practical importance. Namely it implies the possibility to obtain with a given gluten preparations of differing rheological properties. Has an economical enough method of reduction and reoxydation been found, possibilities arise to break down the native glutens to units consisting of the fundamental polypeptide chains and by chosing appropriate conditions of reoxydation, a gluten with rheological properties best suiting the given grain processing technology can be produced.

The processing principle for a gluten with so determined rheological properties is illustrated by the sketch in Fig. 7.

Summary

The break down of the disulfide bonds of gluten by reduction fundamentally changes the rheological properties of the gluten. The obtained product is not cohesive and has no elastic properties.

Through reoxydation of the reduced gluten, the disulfide bonds can be re-established. The rheological properties of the reoxydated product change considerably in function of the reoxydation conditions (gluten concentration, pH, carbamide concentration). A product with properties approaching those of native gluten is obtained with a moderate carbamide concentration (3 mole) 5 to 8% gluten concentration and a weak acid pH (3.5 to 5.5).

The described results present a possibility to produce glutens of "directed" rheological properties.

References

1. LÁSZTITY, R.: Kémiai Közlemények **28,** 67 (1967).
2. LÁSZTITY, R.: Die Nahrung **12,** 1 (1968).
3. WHITE, F. H. JR.: J. Biol. Chem. **236,** 1353 (1961).
4. ISEMURA, T., TAGAKI, T.. MAEDA, Y., YUTANI, K.: J. Biochem. **53,** 155 (1963)
5. KATSOYANNIS, P. G., FUKUDA, K., TOMETSKO, A., SUZUKI, K., PILAK, M.: J. Am. Chem. Soc. **86,** 970 (1964).
6. HABER, E.—ANFINSEN, C. B.: J. Biol. Chem. **237,** 1839 (1962).
7. BECKWITT, A. C.—WALL, J. S.—JORDAN, R. W.: Arch. Biochem. Biophys. **112,** 16 (1965).
8. BECKWITT, A. C., WALL, J. S.: Biochem. Biophys. Acta **130,** 155 (1966).
9. LÁSZTITY, R.: Acta Chimica **53,** 169 (1967)
10. LÁSZTITY, R.: Élelmiszervizsg. Közl. 170 (1960)
11. WU, Y. V., CLUSKEY, J. E., SEXKSON, K. A.: Biochim. Biophys. Acta **113,** 83 (1967).
12. FRAENKEL CONRAT, J., FRAENKEL CONRAT, H.: Biochem. Biophys. Acta **5,** 89 (1950).
13. CECIL, R., LOENING, V. E.: Biochem. J. **66,** 189 (1957).

Dr. Radomir LÁSZTITY, Budapest XI., Műegyetem rkp. 3, Hungary