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Biochem. J. (1963) **88**, 100

The Reaction of Glutathione with Serum Albumin, Gluten and Flour Proteins

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(Received 5 November 1962)

Previous work (Hird, 1962) has shown that the rate of reaction of GSH with the disulphide bonds of native serum albumin and insulin to form GSSG was slow, but that after digestion with trypsin or chymotrypsin or both the reaction was rapid. The formation of GSSG in such systems results from two thiol-disulphide-exchange reactions (1 and 2).

It is therefore possible that, with the native proteins, the first reaction leading to the formation of the mixed disulphide between GSH and the protein could occur and not be detected by the method used, namely oxidation of NADPH₂ by glutathione reductase. This assay method requires optical clarity and cannot be used in opaque systems; its application is also restricted to conditions that do not destroy enzymic activity. To overcome these difficulties, a polarographic method has been used to follow the disappearance of GSH in the systems studied.

In addition to the study of the reaction of GSH with serum albumin the method has been applied to a related study of the flour-water system in dough. It has been established that the removal of thiol groups markedly toughens dough and increases its viscosity (Goldstein, 1957; Bloksma, 1958; Frater, Hird, Moss & Yates, 1960; Meham, 1959; Bloksma & Hlynka, 1960); the addition of a small amount of a thiol has the opposite effects

(Frater *et al.* 1960). From these collected observations it has been concluded that thiol-disulphide-exchange reactions substantially determine the rheological properties of dough. The experimental facts, however, relate only to the removal and addition of thiol groups and not to participation of disulphide bonds as such. Accordingly we have attempted to obtain more experimental information on the interaction of GSH and GSSG with the disulphide bonds and thiol groups respectively of proteins in dough.

MATERIALS AND METHODS

Materials. Serum albumin (bovine) was purchased from the Commonwealth Serum Laboratories and had been crystallized 15 times. Trypsin and chymotrypsin were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and GSH from Hoemann-La Roche and Co. Ltd., Basle, Switzerland. Methylmercuric iodide was prepared by the method of Maynard (1932).

Gluten was a gift from Barret Bros., Melbourne, Australia, and had been prepared by the extraction of flour with acetic acid. Reduced gluten was prepared by the reduction of a gluten suspension (100 g.) in 10 l. of aq. 0.1 M-sodium borohydride. After reduction for 1 hr. at 50° the suspension was acidified to pH 6.5 to decompose the excess of sodium borohydride. The product, which is insoluble at pH 6.5, was collected by centrifuging and washed four times with water (1 l.), and the residue was freeze-dried. Before reduction the gluten contained 11.5 moles of disulphide groups/10⁵ g. and no thiol groups; after reduc-

tion it contained 7.2 moles of disulphide groups and 5.0 moles of thiol groups/10⁵ g.

'Thiolated gelatin' containing 12.9 moles of thiol/10⁵ g. was purchased from Schwartz Laboratories. Oxidized 'thiolated gelatin' was prepared by the addition of the exact amount of iodate to 3% (w/v) solutions of 'thiolated gelatin' so as to remove all thiol groups without having an excess of iodate. This amount of iodate was found experimentally to occur at a thiol:iodate molar ratio of 4.4. The flour was from a Victorian FAQ sample with a nitrogen content of 1.66 g./100 g. dry wt.

Assay of thiol and disulphide groups. These groups were determined amperometrically (Metrohm Polarecord E261) by titration with excess of methylmercuric iodide before and after reaction with sulphite, as recommended by Leach (1960). The sample of serum albumin used contained 1 mole of thiol groups/10⁵ g. dry wt. The gluten sample used did not contain detectable thiol groups. The serum albumin contained 25 moles and the gluten 8 moles of disulphide groups/10⁵ g. These analyses were made in the presence of 8 M-urea.

Assay of glutathione. This compound has a well-defined current-voltage wave with a half-wave potential at -0.25 v at pH 5.0 and -0.40 v at pH 9.0 with Ag/AgCl as the reference electrode. At a sensitivity of $2 \times 10^8 \mu\text{A}/\text{cm}$. on the recorder, 1 μmole of GSH in 20 ml. at pH 5.0 gave a pen-deflexion of 2.5 cm. At pH 9.0 the pen-deflexion was about half of this value. The current carried is sensitive to protein concentration and so this must not be a variable in any comparative experiment.

The method of following the reaction of GSH with the disulphide bonds of the proteins depends on the disappearance of the GSH that is diffusible, on the assumption that it reacts with the protein to form a mixed disulphide and a protein thiol which is non-diffusible:

The system consisted of albumin (400 mg., containing 100 μmoles of disulphide groups) and GSH (25 μmoles). The final volume of liquid in the polarograph cell was 20 ml.; it contained KCl (0.7 g.) as the carrier electrolyte and tris buffer (0.1 M), pH 7.5 or 9.0, or citrate buffer (0.1 M), pH 5.0 or 6.0. After a purging with nitrogen to remove oxygen the disappearance of GSH (added as a solid) was followed for periods up to 2 hr. Titration with methylmercuric iodide showed that, under the conditions of the experiment, there was no net loss of thiol groups; the disappearance of GSH was therefore due to combination with the protein. In the experiments with gluten a smaller amount of disulphide was used owing to the viscosity of the gluten solutions. The system used consisted of gluten (0.5 g., containing 40 μmoles of disulphide groups) and GSH (10 μmoles), thus maintaining the same disulphide:thiol ratio as in the experiments with serum albumin.

Reaction of glutathione with dough. Flour (50 g., containing 73 μmoles of thiol groups and 400 μmoles of disulphide groups) was mechanically mixed for 2 min. with 30 ml. of water containing GSH (25 μmoles). Samples (10 g.) of dough were taken at various times after the mixing and added to 10 ml. of citrate buffer (0.1 M), pH 5.0, in a polythene centrifuge tube and dispersed by mechanical shaking with a large glass marble for 2 min. The contents of the tube were then centrifuged at 3000g for 4 min., and a sample (5 ml.) of the supernatant fluid was transferred to the reaction vessel of the polarograph, which contained

0.2 g. of KCl as the carrier electrolyte. The current-voltage waves were then measured as described above. The experiments with GSSG were carried out in a similar manner with the same quantities of reactants.

Relaxation of strains in dough. The methods used to introduce circular strains and orientation into the dough and to follow their relaxation were those with the Brabender Extensograph described by Frater, Hird & Moss (1961). With this instrument a pin with a circular motion stirs a piece of dough (150 g.) and thereby introduces strains into the structure; the resulting ball of dough is then mechanically rolled into a 'sausage' shape and clamped in a cradle. After various times (20 sec. to 45 min.) these pieces are pulled by a hook, mechanically coupled with a recorder geared to movement of the hook. The resulting graph plots the resistance of the dough to pulling against the distance pulled. In the present experiments the maximum resistance reached has been used in plotting the decline of resistance with time, i.e. the relaxation of the maximum strains introduced into the dough by the moving pin. From the results obtained this sensitive rheological test most likely measures protein-protein interaction in dough systems.

Hydrolysis of serum albumin and gluten. Serum albumin as a 4% (w/v) solution was digested with trypsin (2% of serum albumin) at pH 8.0 in tris buffer (0.1 M) at 30° for 8 hr. and with chymotrypsin (1% of serum albumin) for 3 hr. Under these conditions (Hird, 1962) the hydrolysis by trypsin is completed with approx. 20 moles of H⁺ ion formed/mole of serum albumin, and the hydrolysis by chymotrypsin substantially completed with approx. 35 moles of H⁺ ion being formed/mole of serum albumin hydrolysed. The same conditions were used in the hydrolysis of gluten but in this case no measurement of the extent of the hydrolysis was made.

RESULTS

Reaction of glutathione with serum albumin.

Fig. 1 (a) shows that untreated serum albumin does not react with GSH at pH 5 or 6 but that at pH 7.5 or 9 there is a slow reaction. Figs. 1 (b), 1 (c) and 1 (d), on the other hand, show that, when the protein structure is opened up by urea or proteolytic enzymes, reaction with GSH occurs, trypsin being more effective than chymotrypsin. These figures show that, at the higher pH values, the reaction is not only extensive (with 75% of the GSH being removed) but is also extremely rapid. It therefore seems likely that the reactive species of glutathione is GS⁻ and not GSH. In the presence of sulphite, the proteolytic digests of serum albumin were found to contain thiol peptides small enough to diffuse and react with the dropping-mercury electrode. These thiols have the same half-wave potential as has GSH. It is therefore likely that when GSH reacts with the disulphide bonds of the hydrolysed protein some diffusible thiols will be liberated and be assayed as GSH. Consequently the extent of the reaction of GSH in these experiments may, in fact, be somewhat greater than

Fig. 1. Reaction of GSH (25 μmoles) with preparations of serum albumin (400 mg., containing 100 μmoles of disulphide groups). (a) Untreated serum albumin; (b) serum albumin in 8M-urea; (c) serum albumin hydrolysed by trypsin; (d) serum albumin hydrolysed by chymotrypsin. $\square$, pH 5.0; $\circ$, pH 6.0; $\triangle$, pH 7.5; $\bullet$, pH 9.0. Other conditions are given in the text.

Fig. 2. Reaction of GSH (10 μmoles) with preparations of gluten (0.5 g., containing 40 μmoles of disulphide groups). (a) Untreated gluten; (b) gluten in 8M-urea; (c) gluten hydrolysed by trypsin; (d) gluten hydrolysed by chymotrypsin. $\square$, pH 5.0; $\circ$, pH 6.0; $\triangle$, pH 7.5; $\bullet$, pH 9.0. Other conditions are given in the text.

observed. It is also likely that the reaction goes beyond the stage of the formation of mixed disulphides and that some GSSG is formed.

Reaction of glutathione with gluten. Fig. 2 (a) shows that untreated gluten has some reactivity with GSH, the reaction increasing in rate and extent with increase in pH. This rate is, in fact, only a little lower than that found with gluten in the presence of 8M-urea. As with serum albumin, hydrolysis by the two proteolytic enzymes (Figs. 2b, 2c and 2d) markedly increases the rate of the reaction, but, in this case, chymotrypsin and trypsin are very similar in the effects they produce.

Reaction of glutathione and oxidized glutathione with dough. Fig. 3 shows that on being mixed with dough and left to stand there is a disappearance of GSH. As the total amount of thiol in the system remained the same, the disappearance of GSH is assumed to be by reaction with the protein and not by oxidation. Sodium chloride in the amounts added are known to toughen markedly the rheological properties of dough, and Fig. 3 shows that it inhibits the reaction of GSH with the protein of dough. The reaction of GSH with digests of serum albumin is unaffected by sodium chloride and it is therefore likely that with dough the effect is on the solubility properties of gluten.

Table 1 shows that when GSSG is mixed with dough there is an increase in the wave height in the region of the current-voltage wave due to GSH. In connexion with other evidence presented below, this is taken to imply that GSSG has reacted with the protein thiol groups of the dough to form GSH and a mixed disulphide with the protein. It was noted that dough contains diffusible thiols, as shown by the control wave heights and the disappearance of this wave on the addition of methylmercuric iodide. In the presence of sulphite there

is a marked increase in the amount of diffusible thiol. By assuming, for the purpose of rough calculation, that the diffusion characteristics of this compound(s) are the same as those of GSH, amounts ranging from 33 to 67 μ moles/100 g. of flour have been found in the 20 flours so far examined. The disulphide from which the thiol is derived is not GSSG as it is not reduced by glutathione reductase and NADPH₂. However, by the glutathione-reductase method (Hird, 1962), diffusates have been shown to react with GSH to form GSSG. It therefore possesses accessible disulphide bonds.

Relaxations of strains in dough. Fig. 4 shows that in the presence of GSH and GSSG the resistance of the dough to pulling is much less than the control

Table 1. *Reaction of oxidized glutathione with dough*

GSSG (25 μ moles) and dough (50 g. of flour plus 30 ml. of water) were mixed in an atmosphere of nitrogen. Other conditions are given in the text. At 5 min. the increase in wave height is equivalent to approx. 2 μ moles of GSH.

Time after mixing (min.)	Wave height (cm.)	
	Control dough	Dough mixed with GSSG
5	3.3	5.8
30	3.9	5.9
60	4.7	6.1
90	4.2	6.3
120	4.0	5.0

Fig. 3. Reaction of GSH (25 μ moles) with dough (50 g. of flour plus 30 ml. of water, containing 400 μ moles of disulphide groups). □, NaCl absent; ○, 1 g. of NaCl present; ●, 2 g. of NaCl present. Doughs were mixed for 2 min. at 30° in an atmosphere of nitrogen. Other conditions are given in the text.

Fig. 4. Effect of GSSG and GSH on the relaxation of strains introduced into dough. Doughs were made from flour (300 g.), water (163 ml.) and NaCl (6.0 g.) with the following treatments: ■, control; ○, 40 μ moles of GSH present; □, 45 μ moles of GSH present; △, 60 μ moles of GSH present; ●, 150 μ moles of GSSG present. The doughs were mixed for 2 min. in air at 30°. Other conditions are given in the text. Brabender units are arbitrary units.

Fig. 5. Effect of the reduced and oxidized forms of gluten and 'thiolated gelatin' on the relaxation of strains introduced into dough. The doughs were made as in Fig. 4 and with the following treatments: (a) ●, control; ○, reduced gluten (3.5 g., containing 200 μ moles of thiol groups); □, gluten in oxidized form (3.5 g., containing 400 μ moles of disulphide groups); (b) ●, control; ○, 'thiolated gelatin' (3.0 g., containing 450 μ moles of thiol groups); □, oxidized 'thiolated gelatin' (3.0 g., containing 225 μ moles of disulphide groups). Other conditions are given in the text. Brabender units are arbitrary units.

and that the early rates of relaxation are faster. Fig. 4 also shows that GSH is over three times as effective as GSSG. Prior treatment of the dough with iodate completely abolishes the effect of GSSG. Iodate oxidizes thiol groups rapidly in such systems (Frater *et al.* 1960; Hird & Yates, 1961), and so the effect of GSSG is most likely due to its conversion into GSH by reaction with the thiol groups of the flour proteins. Similar results were obtained whether the dough was mixed in nitrogen or in air.

Fig. 5 shows that the addition of the thiol-containing proteins, reduced gluten and 'thiolated gelatin', increased the rate and extent of relaxation of the strains introduced into dough. The disulphide forms of these proteins on the other hand had the reverse effects, indicating the importance of protein thiol groups in determining the rheological properties.

DISCUSSION

It is difficult to explain the disappearance of GSH in solutions of modified serum albumin other than by thiol-disulphide-exchange reactions. There is little disappearance of GSH in the presence of the native protein and in a similar system GSSG has been shown to be a product of the interaction (Hird, 1962). Adsorption is thus most unlikely to be the explanation with serum albumin, but with gluten its exclusion is more difficult. However, the increase in the rate of GSH disappearance, after modification of the gluten, follows the same general pattern as with serum albumin. It is therefore

likely that the same type of exchange reactions are involved.

The reaction of GSH with the disulphide bonds of native serum albumin to form a mixed disulphide is very slow and limited in extent, but is increased markedly when configurational changes are brought about by proteolytic enzymes or urea. The present results are similar to those obtained by a method that depends on a second and subsequent thiol-disulphide-exchange reaction that leads to the formation of GSSG (Hird, 1962). With serum albumin, therefore, it appears that configurational changes that permit GSH to react with a disulphide bond (reaction 1) also permit the second thiol-disulphide-exchange reaction to form GSSG (reaction 2).

Evidence for the occurrence of thiol-disulphide-exchange reactions in dough is less direct but comes from several procedures that are different in principle: (1) The disappearance of GSH when it is mixed with dough under conditions where there was no loss of thiol in the system, and the appearance of extra diffusible thiol in the system when GSSG was mixed with dough. (2) The similarity of the rheological effects of GSH and GSSG when added to dough and the abolition of the effect of GSSG when the thiol groups of the flour proteins were removed. (3) Reduced gluten and 'thiolated gelatin' weakened the structure of dough whereas their disulphide forms have the reverse effects. (4) The incorporation of 'thiolated gelatin', as measured by the incorporation of hydroxyproline (McDermott & Pace, 1962), into the gluten fraction when 'thiolated gelatin' was mixed with dough.

GSSG has the opposite rheological effect of oxidized proteins whereas GSH is considerably more effective in weakening dough than are a corresponding number of thiol groups in the form of reduced gluten and 'thiolated gelatin'. Both these observations have a common explanation. Protein disulphides and thiols, if more than one group/molecule is present, participate in a three-dimensional network of disulphide bonds throughout the dough. GSH and GSSG (which gives rise to GSH) bring about an interruption of this network when they react with intermolecular disulphide bonds. A loss of strength would result. The effect of the addition of monothiols such as glutathione would thus be twofold: they would increase the rate of the thiol-disulphide-exchange reactions and they would interrupt the disulphide network in the dough. The latter effect is illustrated diagrammatically in Scheme 1.

The presence of a small amount of diffusible thiol and of relatively large amounts of diffusible disulphide (thiol precursors) indicates that flour naturally contains compounds that would resemble GSH in this effect on the structure of dough. The

Scheme 1. Diagrammatic representation of the reaction of GSH with intermolecular disulphide bonds of the protein system in dough to form a discontinuous network. The large circles represent gluten molecules.

diffusibility of these endogenous compounds would give them additional significance. They therefore must make an important contribution to the properties of dough.

The present results clearly indicate that thiol-disulphide-exchange reactions can, in fact, occur in dough. As the solubility properties of the gluten fraction are known to be markedly altered by mixing (Bloksma, 1959; Mecham, Sokol & Pence, 1962), there are likely to be configurational changes in the dough proteins caused by mixing. Such changes may well lead to an increase in the number of accessible disulphide bonds and so further increase the possibility of exchange reactions with thiol groups. Mixing thus may be

analogous to proteolysis and denaturation by urea in increasing the rate of thiol-disulphide-exchange reactions.

SUMMARY

1. The reaction of GSH with serum albumin and gluten treated in various ways has been studied by a polarographic method for the determination of GSH.

2. The reaction of GSH with native serum albumin is very slow between pH 5 and 9, but, after the protein structure has been opened up by treatment with urea and proteolytic enzymes, the reaction is greatly accelerated, particularly at the higher pH values. The same is true of gluten except that there is some reaction with untreated gluten. It is assumed that this reaction is by thiol-disulphide-exchange reactions.

3. Evidence is presented that is consistent with the reaction of GSH and GSSG with the disulphide and thiol groups respectively of the proteins of dough.

4. The relaxation of strains introduced into dough has been shown to be increased by GSH, GSSG and protein thiols, and to be decreased by protein disulphides.

5. A low-molecular-weight disulphide compound(s) in dough has been shown to be present in flour.

6. The results are discussed in connexion with the significance of thiol-disulphide-exchange reactions in determining the rheological behaviour of dough.

The authors acknowledge financial support from the Wheat Industry Research Council.

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