Vol. 93, No. 4, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1980 Pages 1189-1195

SUPEROXIDE INVOLVEMENT IN THE REDUCTION OF DISULFIDE BONDS OF WHEAT GEL PROTEINS

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Received March 14, 1980

Summary. The sodium dodecyl sulfate (SDS) insoluble gel proteins of wheat flour are converted under the influence of mixing energy and air or oxygen into SDS soluble proteins, which during the resting period after mixing reassociate into SDS insoluble gel proteins. These processes take place via cleavage and formation of disulfide (SS) bonds, respectively. During dough-mixing, O_2 is reduced to the superoxide anion ($O_2^{-\cdot}$); its electron is transferred via an Mn, Cu-protein system to an SS bond, which is then reduced to SH groups. An Mn^{3+}-protein compound is responsible for the formation of new SS bonds.

Introduction.

Disulfide reagents such as cysteine, glutathione and sulphite change the physical properties of a dough considerably. It is reasonable to assume that SS bonds play a significant role in the formation of the protein network of dough. Some researchers (1, 2, 3) have shown that protein solubility increases during dough-mixing as a result of the breaking down of the high-molecular proteins. Other researchers (4, 5) have tried to prove with the aid of certain additives like cysteine and glutathione that SH/SS interchange reactions take place while the dough is being mixed. However they failed to give convincing evidence.

The subject of the present investigation is the redox reactions that occur in the gel proteins during dough-mixing and during the resting period. For the investigation use was made of an extraction and fractionation method with a sodium dodecyl sulfate (SDS) solution as described in a previous publication (6).

Materials and methods

Materials

A Dark Northern Spring (DNS) wheat flour with a protein content of 13 % (14 % moisture basis) was used, the same flour as in the previous study (6). Cytochrome C (from horse heart), xanthine, xanthine oxidase (from butter

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milk), superoxide dismutase (from bovine blood) were purchased from Sigma Chemical Co. Ltd.

Mixing of dough. Doughs were prepared from 100 g flour, 2 % NaCl and water in a GRL mixer (7) at a speed of 66 revs/min under nitrogen, air or oxygen. After the appropriate mixing time the dough was divided into two parts, one was directly freeze-dried, the other was placed in a plastic box and stored for indicated times at a temperature of 30 °C and then freeze-dried. Before extraction of the proteins with a SDS solution the freeze-dried samples were pulverized in a coffee grinder.

Isolation of gel proteins. Flour or ground freeze-dried dough was gently suspended in a 1.5 % sodium dodecyl sulfate solution (SDS) (5 g/90 ml) and magnetically stirred for 5 min at 10 °C. The suspension was centrifuged at 80,000 x g for 30 min at 15 °C. The residue consisted of three layers, a starch layer at the bottom, a light brown layer, and a gel-layer on the top (6). The SDS soluble proteins were separated into an alcohol soluble and an alcohol insoluble fraction by adding ethanol to a concentration of 70 %.

Electron Paramagnetic Resonance (EPR) Spectroscopy. A varian E-3 spectrometer equipped with a multi-purpose EPR cavity was used. All measurements were made on 100 mg freeze-dried samples in standard 3 mm i.d. quartz tubes, at room temperature, at a microwave power of 20 mW and 100 kHz field modulation. Modulation amplitude was 10 G and 4 min scans were made of 2000 G with a 0.3 s time constant and a microwave frequency of 9.22 GHz.

Results.

Breakdown and Re-formation of the gel proteins.

The amount of the gel proteins determined in doughs after different mixing times and resting periods is given in Fig. 1. The results show that during mixing under air or oxygen the gel layer (gel proteins) was converted into SDS soluble material and during the resting period after dough-mixing a new gel layer was formed. This re-formation of the gel layer under air or oxygen during the resting period took place more slowly than the breakdown. After 10 min mixing under air, 80 % of the original gel layer re-formed and after 10 min mixing under oxygen 60 %. When mixing under nitrogen it was found that virtually no breakdown took place. The breakdown products of the

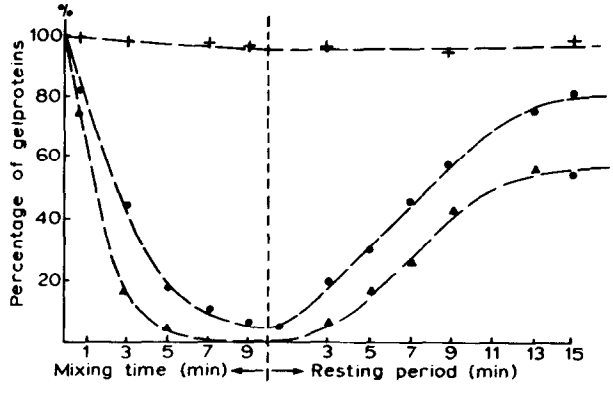


Fig. 1. Breakdown of the gel proteins during dough-mixing, and re-formation during the resting period. + dough mixed under nitrogen, ● under air, ▲ under oxygen.

Table I

Thiol (SH) and disulfide bonds (SS) in gel proteins + breakdown products (μmol per gram material)

Source of gel proteins + breakdown products	SH	SS
Flour	6	50
Dough, mixed (10 min) under oxygen	30	38
Dough, mixed (10 min) under air	26	40
Dough, mixed (10 min) under nitrogen	10	48
Dough, mixed (10 min) under air and rested for two hours	10	46

gel proteins, which were soluble in SDS could be isolated from the SDS supernatant by alcohol precipitation.

With the aid of the amperometric thiol and disulfide determination (8) the thiol group and disulfide bond content was determined in the gel proteins of flour and in the gel proteins plus breakdown products of doughs which had been mixed for 10 min under nitrogen, air and oxygen respectively. Also SH and SS were determined in the gel proteins of a dough mixed for 10 min under air and rested for 2 hours. The results are presented in Table I. During mixing under air and oxygen, 20 and 24 % of the SS bonds were cleaved respectively. It can be seen from the results that the SS bonds were reduced to SH groups and that these in turn were oxidized to SS bonds after the resting period. When mixing under nitrogen only 4 % of the SS bonds were reduced. On the basis of these results we concluded that the breakdown of the gel proteins during dough-mixing under air or oxygen took place via reduction of SS bonds and the re-formation of the gel proteins during the resting period took place via oxidation of SH groups.

In order to establish whether SH-containing components were involved in the reduction of SS bonds, flour was suspended in a 0.01 % N-ethylmaleimide (NEMI) solution (1 g/4 ml). After 48 hours of dialysis against distilled water to remove the excess NEMI, the suspension was freeze-dried and a dough mixed of the flour under air for different periods. The gel proteins broke down just as quickly as in the case of a dough of untreated flour. Hence no SH groups were involved in the breakdown. On the basis of these results we considered it improbable that SS bonds of the gel proteins are cleaved by means of SH/SS-interchange reactions.

Mn, Cu-Electron Transfer System.

In consequence of the results obtained by Evans et Windle (10) and Schroeder et Hoseney (11), who had suggested with the aid of physical measurements that dough proteins were modified via a free radical mechanism, we

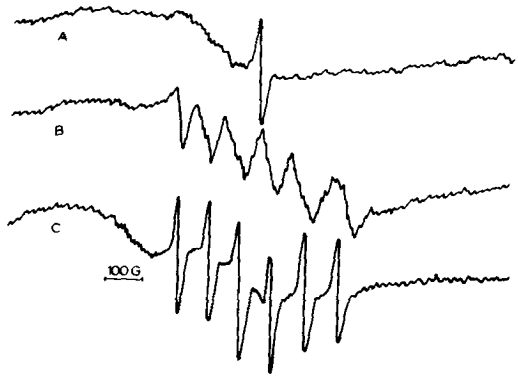


Fig. 2. EPR spectra of freeze-dried material. A, dough mixed under air; B, water extract of wheat flour (S_1); C, $CaCl_2$-dialisate of S_1.

assumed that the cleaving of SS bonds of the gel proteins might take place via a radical mechanism. This assumption was strengthened by EPR measurements, which did in fact demonstrate the presence of free radicals in freeze-dried dough (Fig. 2a). In flour no free radicals were found.

After extraction of the wheat flour with distilled water (100 g/600 ml H_2O, 10 min magnetic stirring at room temperature and centrifugation at 3000 x g) the flour residue was mixed for 10 min under air. In this case the gel proteins had only broken down 20 %. After a second water extraction no breakdown took place. Upon reconstitution of the freeze-dried water extract (S_1) and the flour residue (R), almost 100 % breakdown took place with mixing under air or oxygen.

The 6-line EPR spectrum of S_1 (Fig. 2b) clearly shows the presence of Mn^{2+}, resulting from the hyperfine interaction of the unpaired electron with the 5/2 spin of the ^{55}Mn nucleus. The fact that the peaks are broad and more or less split up shows that Mn is somehow associated with the protein. After a night of dialysing S_1 against 0.1 M $CaCl_2$ all the Mn^{2+} was expelled from the protein by Ca^{2+} ions and had passed through the membrane. An EPR spectrum was made of the freeze-dried fraction that had passed through the membrane (fig. 2c). This spectrum clearly shows the presence of free Mn^{2+} in the dialisate. S_1 treated with $CaCl_2$ solution was no longer able to breakdown the gel proteins. After dialysis against $MnCl_2$ the activity was partially recovered. From these results we may conclude that protein-bonded Mn ions were involved in the break down of the gel proteins.

S_1 suspended in 90 % isopropanol (0.5 g/100 ml) at room temperature and stirred for 30 min could be separated after 2 min centrifugation at 3000 x g into an isopropanol soluble fraction ($S_{1\ sup}$) and a residue ($S_{1\ res}$). Each fraction added to the flour residue (R) was only able to break down the gel

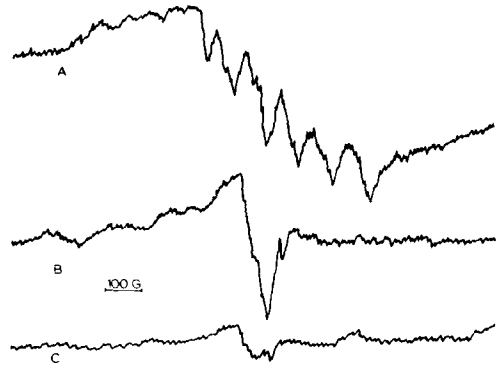


Fig. 3. EPR spectra of freeze-dried material.
A, 90 % isopropanol soluble material of S_1 ($S_{1\ sup}$);
B, 90 % isopropanol insoluble material of S_1 ($S_{1\ sup}$);
C, 70 % isopropanol soluble material of S_1 ($S'_{1\ sup}$).

proteins about 10 %, but addition of the combined fractions to the flour residue (R) completely restored the breakdown.

The EPR spectrum of $S_{1\ res}$ (Fig. 3a) was identical with the original S_1 spectrum, and thus also characteristic of a Mn^{2+} protein compound. The $S_{1\ sup}$ spectrum (Fig. 3b) however was characteristic of a 2 Cu^{2+} protein compound (12). These results gave rise to the assumption that also a 2 Cu^{2+}-protein compound was involved in the break down of the gel proteins.

It was possible to verify this assumption by suspending S_1 in 70 % isopropanol, and heating for 10 min at 70 °C. After 2 min centrifugation at 1000 x g $S'_{1\ sup}$ alone was capable of breaking down the gel proteins. The EPR spectra of $S_{1\ res}$ and $S'_{1\ sup}$ (Fig. 3c) showed no signal. Apparently Mn^{2+} had been oxidized to a higher valency (Mn^{3+}) and Cu^{2+} reduced to Cu^{+}. This 2 Cu^{+}-protein compound ($S'_{1\ sup}$) added to a flour residue (R) also broke down the gel proteins during mixing under nitrogen. With the aid of atomic absorption spectrophotometry (9) it was shown that S_1 contained 40 ppm Mn and 13 ppm Cu.

From this it was possible to conclude that during dough-mixing under oxygen the 2 Cu^{2+}-protein compound is reduced via Mn-protein compound to 2 Cu^{+}-protein compound, and that this in turn is capable of reducing an SS bond to SH.

Superoxide anions ($O_2^{-\cdot}$).

Taking the above mentioned conclusion that the reduction of the 2 Cu^{2+}-protein compound is only possible in the presence of oxygen, it is reasonable to assume that this is only possible via the superoxide anion ($O_2^{-\cdot}$). This anion supposedly reduces Mn^{3+} to Mn^{2+} which in turn reduces Cu^{2+} to Cu^{+}. In doughs to which ascorbic acid or ferricytochrome C had been added

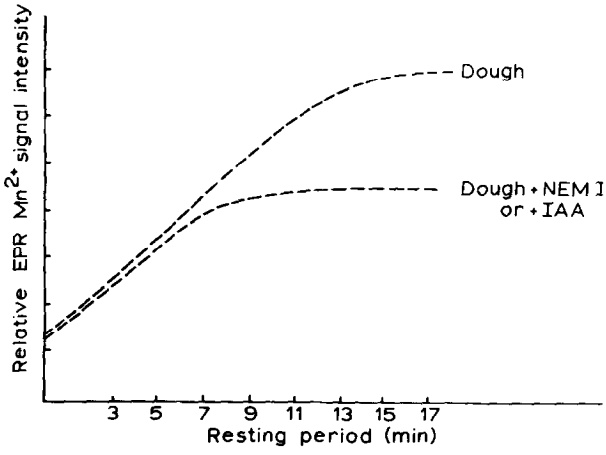


Fig. 4. Increase of Mn^{2+} during the resting period of a dough as affected by NEMI and IAA.

in quantities of 2 mg per 10 g flour, no breakdown of the gel proteins took place during mixing under air, i.e. these substances reacted with $O_2^{-\cdot}$ so that no $O_2^{-\cdot}$ remained to reduce the Mn^{3+}. Additional verification of the assumption that $O_2^{-\cdot}$ is needed for the reduction of SS bonds was obtained by adding superoxide dismutase (SOD) (10 mg/10 g flour) to the dough. The removal of the superoxide anions by SOD did not result in a breakdown of gel proteins. Addition of xanthine plus xanthine oxidase (both 10 mg/10 g flour) to a dough caused the gel proteins to be broken down more quickly than in a controle dough. This experiment once again showed that superoxide anions were needed for the breakdown of gel proteins.

Mn^{3+}-protein oxidizing agent.

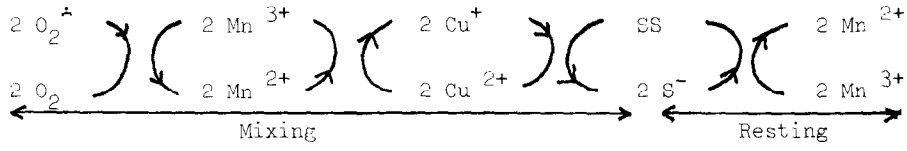
To study the re-formation of gel proteins during the resting period after dough-mixing parts of the dough were freeze dried after different resting periods. The manganese was extracted from the freeze-dried material with 0.01 M $CaCl_2$ solution (1 g/100 ml) at 5 °C and shaken for 5 min. After centrifugation the supernatant was freeze-dried and the Mn^{2+} and Cu^{2+} determined with EPR. Fig. 4 shows the results as far as Mn^{2+} is concerned.

In the first 15 min there is a marked increase in Mn^{2+}. With doughs to which NEMI or iodo-acetic acid (IAA) (2 µmol/g flour) had been added, the increase in the Mn^{2+} signal was much less marked; no new gel layer was formed, because the reagents had blocked all the SH groups. It was observed that the Cu^{2+} signal did not change during the resting period. On the basis of these results we concluded that the oxidation of SH groups is brought about by Mn^{3+} ions.

Discussion.

How the superoxide anions are formed in a dough during mixing is a debatable point. There are, however, indications that the substance which is responsible for the formation of $O_2^{-\cdot}$ is soluble in methanol. Further study is necessary to trace this substance which creates superoxide anion in wheat flour dough.

On the basis of the results obtained the transfer of the electron from $O_2^{-\cdot}$ to a SS bond can be represented as follows:



From the extraction experiments with isopropanol at 70 ^{o}C and the EPR measurements we suspect that we have here a protein complex which is built up of different sub-units. Besides a Mn-protein sub-unit and a 2 Cu-protein sub-unit the complex probably contains a compound which is soluble in methanol and forms $O_2^{-\cdot}$.

Acknowledgement.

We gratefully acknowledge the technical assistance of the department of molecular physics of Agricultural University, Wageningen for the EPR measurements.

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