CEREAL CHEMISTRY

VOL. 43 JULY, 1966 NO. 4

DISULFIDE-SULFHYDRYL INTERCHANGE STUDIES OF WHEAT FLOUR

I. The Improving Action of Formamidine Disulfide[1]

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ABSTRACT

Formamidine disulfide was found to have very good maturing action on flour — probably because of blocking its –SH groups. The compound also provided a useful tool for measuring the extent of disulfide-sulfhydryl interchange in flour extracts and doughs. Although, theoretically, disulfide-sulfhydryl interchange had seemed a logical explanation for the changes in the rheological properties of flour doughs effected by improvers, results have shown this reaction may not, in fact, take place to any significant degree in the normal pH range of doughs. Evidence of interchange with glutathione was obtained only in alkaline medium.

Formamidine disulfide hydrochloride, or 1,1′-dithioformamidine dihydrochloride (referred to hereafter as FDS), has the formula

$$H_2N-\overset{\overset{NH}{\|}}{C}-S\text{–}S-\overset{\overset{NH}{\|}}{C}-NH_2\cdot 2HCl$$

When this compound splits, it forms the free radical

$$H_2N-\overset{\overset{NH}{\|}}{C}-S\cdot$$

which can combine with RSH, as follows

$$NH_2(NH)CS\text{–}SC(NH)NH_2 \rightleftharpoons 2NH_2(NH)CS\cdot$$

$$2NH_2(NH)CS\cdot + RSH \rightarrow RSSC(NH)NH_2 + (NH_2)_2CS$$

An excess of RSH can exchange with the derivative

$$RSSC(NH)NH_2 + RSH \rightarrow RSSR + (NH_2)_2CS$$

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Certain transamidinases have been shown to be strongly inhibited by FDS; also, it has been found that FDS inhibits papain and urease (1). All of these enzymes are known to require –SH groups for their activity. One mechanism that has been suggested for inhibition by FDS (1) is as follows:

$$\text{EnzymeSH} + H_2N-\overset{\overset{NH}{\|}}{C}-S-S-\overset{\overset{NH}{\|}}{C}-NH_2 \rightarrow \text{EnzymeSS}-\overset{\overset{NH}{\|}}{C}-NH_2 + H_2N-\overset{\overset{S}{\|}}{C}-NH_2$$

Formamidine disulfide inhibition of papain and transamidinases can be reversed by a high stoichiometric excess of cysteine. Prior incubation of urease with *p*-chloromercuribenzoate protects this enzyme from inhibition by FDS (1).

It is well known that all commonly used flour-maturing agents owe their efficacy, in part, to reaction with the –SH groups. Every improver seems to have a different mechanism. Many disulfides, such as cystine, are believed to act as flour-maturing agents because of their capacity to oxidize the –SH groups (2). In recent years, most investigators have subscribed to the hypothesis that disulfide-sulfhydryl interchange is responsible for many of the rheological changes in flour doughs. McDermott and Pace (3) and Mauritsen and Stewart (4) have presented data which they interpreted as evidence of exchange. It was the purpose of the present work to study FDS as a flour improver, since it can be used as a model compound to react with available –SH groups and, consequently, the disulfide derivative[3] formed could exchange with the remaining sulfhydryl to form new protein disulfide linkages:

$$PrSH + PrSSCNH(NH_2) \rightarrow PrSSPr + (NH_2)_2CS$$

Materials and Methods

The flour was an untreated, spring-winter blend, analyzing 0.41% ash and 11.9% protein.

Formamidine disulfide dihydrochloride and thiourea were obtained from Eastman Kodak Co.

Gassing power was determined according to the standard AACC procedure (5).

Extensigraph data were determined by the AACC procedure (5).

Reaction of Thiourea with bis-p-*Nitrophenyl Disulfide.* The reaction of free glutathione (GSH) is rapid and the resulting color can be read immediately. Cysteine requires longer for color development. Since thiourea can be formed by the reaction of RSH with FDS, ex-

[3] The word "derivative" is used to signify the compound formed from the reaction of FDS with any sulfhydryl reactant.

periments were made to determine the extent of interference, if any, of thiourea with the measurement of GSH under conditions of the Ellman procedure (6). Thiourea can tautomerize slightly to form a thiol. The equilibrium is far to the left:

$$(NH_2)_2CS \rightleftharpoons NH_2(NH)CSH\cdot$$

A range of thiourea concentrations from $2 \times 10^{-5}M$ to $6 \times 10^{-5}M$ was employed. This range exceeds the glutathione concentrations that are measurable. The color developed by thiourea was read immediately and after 65 min.

Since thiourea is not measurable as free sulfhydryl under these conditions, any exchange of free GSH with its formamidine derivative, which would form thiourea, would be registered as loss of measurable free sulfhydryl.

Tests for Disulfide-Sulfhydryl Interchange

The marked maturing action of FDS results in a disulfide derivative which constitutes a blocked –SH group. If the derivative undergoes exchange with the remaining free –SH groups, new disulfide linkages would be formed within the protein:

$$RSS(NH)NH_2 + RSH \rightleftharpoons RSSR + (NH_2)_2CS$$

These tests involved systems containing free thiol in the presence of the formamidine derivative of the thiol. This was achieved by allowing formamidine disulfide to react in the presence of excess thiol. An exchange of free thiol with its formamidine derivative would be registered as a continuing loss of free thiol. These tests were applied to simple systems containing no flour as well as systems with flour.

Reaction of Glutathione and/or Cysteine with Formamidine Disulfide. Mixtures of GSH and its formamidine derivative were measured for free GSH at pH 5.0 at various time intervals; also, cysteine was used in place of GSH. Free sulfhydryl was measured by the bis-*p*-nitrophenyl disulfide (PNPD) method (6), using absorbance at 412 mμ and the following solutions: 5 ml. water, 4 ml. acetone, 1 ml. PNPD (30 mg. per 50 ml. acetone), 2 ml. phosphate buffer (pH 8.0), and 1 ml. of the supernatant liquid (or 1 ml. of water in the blank).

Glutathione also was determined in the presence of its formamidine derivative at pH 6.0 after 30 min. of incubation at 25°C.

Reaction of Flour, Formamidine Disulfide, and Glutathione. Flour (30 g.) was added to 100 ml. of 0.05*M* borax, pH 9.2, and mixed with 22.5 μmol. of FDS. After 15 min., 195 μmol. of GSH, 10 ml. of 0.05*M* borax, and 10 ml. of water were added to the slurry. At intervals, a

sample was removed and centrifuged. Supernatant liquid (8 ml.) was treated with 1 ml. of 10% sodium tungstate and 2 ml. of 0.67*N* sulfuric acid. This suspension was centrifuged and then analyzed for GSH by the method given above.

Reaction of Dough, Formamidine Disulfide, and Glutathione. Flour (50 g.), 30 ml. of water, and 37.5 μmol. of FDS were mixed in a farinograph for 5 min.; then 325 μmol. of GSH and 5 ml. water were added. The dough was mixed for another 5 min. The dough pH was 5.8. At intervals, a portion of the dough was taken from the mixer and stirred with four times its weight of water. The suspension was centrifuged, and to 8 ml. of the liquid were added 1 ml. of 10% sodium tungstate and 2 ml. of 0.67*N* sulfuric acid. This mixture was centrifuged and analyzed for GSH as previously outlined.

Baking Data

In all doughs, the chemical additives were dissolved in a small portion of the absorption water and mixed with the flour before any of the other ingredients were added.

Straight Doughs. Straight doughs were made, using the following formula: 100% flour, 59% water, 2% yeast, 2% salt, 8% sugar, and 5% nonfat dry milk. Mixing (optimum) was for 7 min. on a Hobart A-120 mixer, and dough temperature was 81°F. Fermentation was 1 hr. and 30 min. to the first punch, then 30 min. The dough was rounded and then rested 20 min. before being moulded. The loaves were proofed at 100°F. to 1 in. above the pan and baked for 28 min. at 450°F.

The effect of thiourea at various levels was observed in straight doughs because of the possibility of the formation of thiourea from the FDS.

Sponge Doughs. The sponge was made with 65% of the flour, 60% water, 2.5% yeast, 0.67% yeast food, and 3% lard. It was mixed for 1.5 min. at second speed on a Hobart A-120 mixer and set at 78°F. for 4.25 hr. when the temperature had risen to 86°F. The additional 35% of the flour was added at the dough stage, with water to 58% total absorption, 2% salt, 8% sugar, and 5% nonfat dry milk. Each dough was divided into two parts, one portion being mixed to the optimum (5 min.) and the other for 7 min. The dough temperature was 80°F. ±1°. The doughs were scaled at 18 oz. and baked at 435°F. for 23 min.

Continuous-System Doughs. A Dō-Maker, laboratory model, was used. The brew, containing all the yeast (2.75%), 8% sugar, 2.25% salt, and 0.5% yeast food in 3,000 ml. of water, was held 2 hr. at

30°C. Then the brew (pH 4.7), flour, oxidizing agents (bromate 60 p.p.m. and iodate 8 p.p.m.), and 3% shortening were premixed 1 min. (absorption total 65%) and fed to the continuous unit operating at 177 r.p.m. The average dough temperature was 100°F. The loaves were scaled by time (20 sec.) and baked at 500°F. for 17 min. Loaf volume was reported as specific volume – the average volume in cc. divided by the average weight in g. of four loaves. The speed of this process is such that this method of reporting gives greater accuracy.

Results and Discussion

Effect of FDS on Gassing Power. Measurements of gassing power showed that FDS does not affect the amount or rate of gas production at the levels used in the baking tests. Sixth-hour results (average of duplicates) were as follows: control (no treatment), 299 mm. mercury; 0.3 μmol. FDS, 296 mm.; and 0.6 μmol. FDS, 299 mm.

Maturing Action of FDS as Shown by Extensigrams. Extensigraph data comparing the action of FDS with iodate are shown in Table I.

TABLE I
EXTENSIGRAPH DATA: COMPARISON OF FORMAMIDINE DISULFIDE WITH POTASSIUM IODATE

	45 MINUTES			180 MINUTES		
	H[a]	E[a]	A[a]	H	E	A
	B.U.	*cm.*		*B.U.*	*cm.*	
Control	335	180	85.0	172	275	66.5
Control + 0.15 μmol. FDS	630	134	105.5	515	170	111.5
Control + 0.15 μeq. potassium iodate	380	165	85.0	330	180	81.0
Control + 0.30 μmol. FDS	860	80	73.5	630	120	93.0
Control + 0.30 μeq. potassium iodate	500	140	92.0	410	169	91.5
Control + 0.60 μmol. FDS	820	72	63.5	760	70	59.0
Control + 0.60 μeq. potassium iodate	720	77	63.5	700	76	60.5

[a] H = resistance to extension; E = extensibility; A = area.

The figures for resistance to extension (H values) as well as extensibility (E values) and area measurements show that FDS reacts even more rapidly than iodate with the flour proteins.

*Reaction of Thiourea with bis-*p*-Nitrophenyl Disulfide.* In order to be sure that thiourea did not interfere with the determination, it was measured in a range of concentrations under conditions of the Ellman method. Table II shows that thiourea had no appreciable effect on absorbance, even after 65 min.

A concentration of GSH corresponding to $4 \times 10^{-5}M$ gave an

TABLE II

EXTENT OF REACTION OF THIOUREA WITH BIS-*p*-NITROPHENYL DISULFIDE UNDER CONDITIONS OF THE ELLMAN PROCEDURE

THIOUREA CONCENTRATION IN TUBE	ABSORBANCE	
	Immediate	After 65 Minutes
$2 \times 10^{-5}M$	0.004	0.006
$3 \times 10^{-5}M$	0.007	0.013
$4 \times 10^{-5}M$	0.007	0.012
$5 \times 10^{-5}M$	0.007	0.010
$6 \times 10^{-5}M$	0.010	0.013

absorbance of 0.55. A range of concentrations of equimolar mixtures of GSH and thiourea were measured immediately and after 50 min. The presence of thiourea did not interfere with color development of GSH or upset the linear relationship of color with GSH concentration.

Tests for Disulfide-Sulfhydryl Interchange. Mixtures of glutathione and its formamidine derivative were assayed for free GSH at various time intervals, according to the following equation:

$$GSSC(NH)NH_2 + GSH \rightleftharpoons GSSG + (NH_2)_2CS$$

Mixtures of cysteine and its FDS derivative also were checked. These data are shown in Table III.

Table III shows that there is a stoichiometric relation between FDS and GSH even after 30 min. of reaction time. This relationship would not have been observed if there had been interchange between free GSH and the formamidine derivative of GSH.

Table IV gives data on the measurement of GSH in the presence of its formamidine derivative at pH 6.0 after 30 min. of incubation at 25°C. The table shows that there is a stoichiometric relation between FDS and GSH, even after 30 min. of reaction time. This relationship would not have been observed if there had been interchange between free GSH and the formamidine derivative of GSH.

TABLE III

MEASUREMENT OF GLUTATHIONE AND CYSTEINE IN THE PRESENCE OF FORMAMIDINE DERIVATIVE[a]

TIME	ABSORBANCE AT 412 mμ	
	Glutathione	Cysteine
min.		
5	1.01	0.88
21	0.97	...
32	0.98	0.88
45	0.99	0.90
61	0.99	0.89

[a] At pH 5.0 and 25°C. Free sulfhydryl, $1.75 \times 10^{-4}M$; FDS derivative, $0.75 \times 10^{-4}M$.

TABLE IV

STOICHIOMETRY OF REACTION OF GLUTATHIONE AND FORMAMIDINE DISULFIDE

FORMAMIDINE DISULFIDE ADDITION	GLUTATHIONE REACTED[a]
μmol./μmol. GSH	*μmol./μmol. GSH*
0.24	0.29
.36	.37
.48	.47
.60	.58
0.72	0.74

[a] Calculated from GSH loss. Conditions of reaction: 2.53 × $10^{-3}M$ GSH, pH 6.0, 25°C., 30 min. reaction time.

In a separate experiment, a mixture of GSH and FDS (0.36 μmol. FDS per μmol. GSH) was observed to give the same absorbance over an incubation-time range of 5 to 60 min. An equivalent amount of thiourea gave negligible absorbance under these conditions, as already indicated in Table II.

A flour slurry was reacted with FDS (1 μmol. per g.) at pH 5.8. The amount was in slight excess of available sulfhydryl, thus oxidizing part of the GSH when it was added, but causing no further loss with time, as shown in Table V. If there had been interchange between free GSH

TABLE V

MEASUREMENT OF GLUTATHIONE IN SLURRIES OF UNTREATED FLOUR AND FDS-REACTED FLOUR[a]

FORMAMIDINE DISULFIDE	TIME	GLUTATHIONE: ABSORBANCE AT 412 mμ
μmol./μmol. GSH	*min.*	
1.0	15	0.455
1.0	60	0.475
None	15	0.545
None	60	0.575

[a] Flour (20 g.) was slurried into water containing FDS and allowed to react for 15 min., and then 40 mg. of GSH and water were added to give a volume of 80 ml.; pH 5.8. After an incubation period, the slurry was assayed for GSH.

and the formamidine derivative of protein sulfhydryl, a loss of GSH would have been observed.

Figure 1 shows the disappearance, with time, of GSH in a flour slurry at pH 9.2. The rate of disappearance of GSH absorbance per min. was 0.0033 in the untreated flour slurry and 0.0042 in the FDS slurry. This increased rate in the presence of the formamidine derivative gives an indication of slight exchange of free GSH with its formamidine derivative at pH 9.2. The major loss of GSH in these systems at high pH (9.2) may be attributed to its oxidation or to its exchange with protein disulfide.

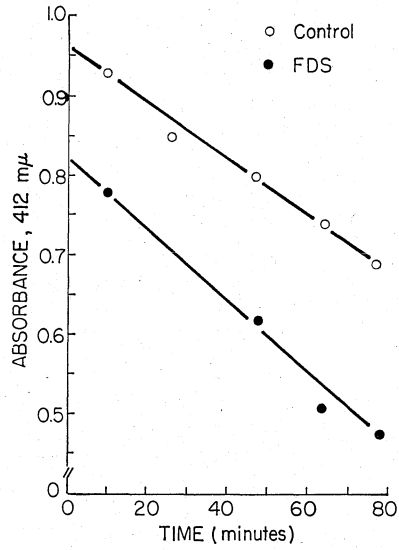

Fig. 1. FDS-GSH exchange in flour slurry at pH 9.2. FDS, 0.36 μmol. per g. of flour; GSH, 6.5 μmol. per g. of flour.

Figure 2 illustrates the loss of GSH in flour-water doughs at pH 5.8, without FDS and with 0.75 μmol. of FDS per g. of flour. There was no difference in the rate of loss of GSH in the untreated and FDS-treated doughs. Loss of GSH absorbance per min. was 0.0012 for both doughs.

In no experiment, either on slurries or doughs, was there any evidence of exchange of free sulfhydryl with its formamidine derivative in the pH range of 5.0 to 6.0. In a yeasted dough, the pH would be lower than the 5.8 used in some of these experiments, and this would tend even more to eliminate the likelihood of exchange. Ryle and Sanger (7) found that dilute acid minimized disulfide interchange.

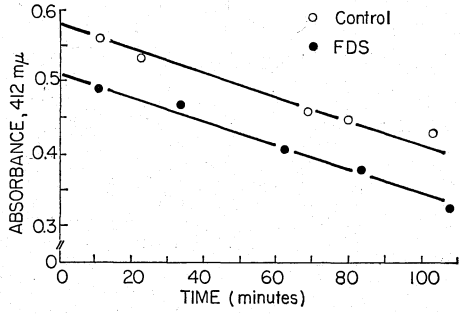

Fig. 2. Loss of GSH in flour-water doughs at pH 5.8, without and with FDS. GSH, 6.5 μmol. per g. of flour; FDS, 0.75 μmol. per g. of flour.

Spackman *et al.* (8), using a model reaction system of cystine and oxidized glutathione (GSSG), found the disulfide bonds to be most stable at pH 2.0. Both cystine and GSSG were stable at 40°C. for 30 hr. in the pH range of 2.0 to 6.5 in the presence and absence of guanidinium chloride. Above pH 7.0 in the absence of the guanidinium ion, there was a slight loss of both disulfides, with a corresponding increase in mixed disulfides. The exchange increased as the pH increased.

The evidence from the experiments on flour reported here does not indicate any appreciable exchange of formamidine derivatives of flour –SH groups with remaining free –SH groups as had been postulated previously with the following equation:

$$RSSC(NH)NH_2 + RSH \rightarrow RSSR + (NH_2)_2CS$$

Thus, the effect of FDS must be mainly that of a blocking agent. This was shown also by its effect on the mixing time recorded on the farinograph. Figure 3 shows curves of the original flour, the flour treated with 0.5 μmol. of N-ethylmaleimide (NEMI), and the flour treated with 0.5 μmol. of FDS. Both compounds shortened the mixing time, but the effect of the FDS was not as drastic as that of the NEMI.

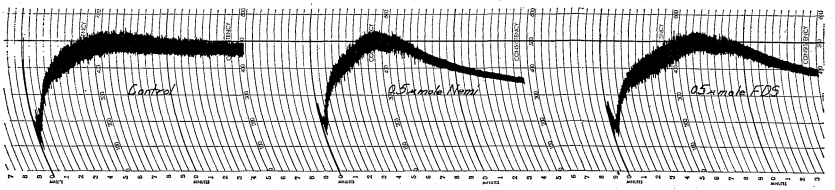

Fig. 3. Farinograms of control flour and the same flour treated with 0.5 μmol. of NEMI and with 0.5 μmol. of FDS.

Baking Data. The baking data on FDS are given for straight doughs, sponge doughs, and continuous-system doughs in Tables VI, VII, and VIII. Since thiourea could be a product of the reaction, baking data on this compound are given in Table IX.

TABLE VI

EFFECT OF FORMAMIDINE DISULFIDE ON BAKING PROPERTIES: STRAIGHT DOUGHS

FORMAMIDINE DISULFIDE	GRAIN	VOLUME
μmol./g. flour		*cc.*
0	98	100
0.15	100	105
0.30	100	107
0.45	99	103
0.60	99	102

TABLE VII

EFFECT OF FORMAMIDINE DISULFIDE ON BAKING PROPERTIES: SPONGE DOUGHS[a]

FORMAMIDINE DISULFIDE	MIXING TIME	GRAIN	VOLUME
μmol./g. flour	*min.*		*cc.*
0	5	96	100
	7	95	95
0.15	5	97	100
	7	97	102
0.30	5	99	104
	7	98–	104
0.45	5	99–	106
	7	98–	103
0.60	5	99	102
	7	97	105

[a] Treatment produced dough-handling properties superior to those of the control, with the exception of the highest level of treatment at the long mixing time; this dough became somewhat soft and sticky, similar to that of the untreated control.

TABLE VIII

EFFECT OF FORMAMIDINE DISULFIDE ON BAKING PROPERTIES: CONTINUOUS-SYSTEM DOUGHS

FORMAMIDINE DISULFIDE	SPEED	SPECIFIC VOLUME[a]	GRAIN
μmol./g. flour	*r.p.m.*		
0	188	4.49	90
0.05	188	5.06	93
0.10	188	5.06	97
0.15	188	5.00	100
0.20	188	5.33	100
0.30	188	5.00	100

[a] Specific volume equals the average volume in cc. divided by average weight in g. of four loaves. Levels higher than 0.3 μmol. (0.7 and 1.5) gave doughs with extrusion properties characteristic of extreme undermixing. This was indicated also by very heavy laminations of the bread crumb.

TABLE IX

EFFECT OF THIOUREA ON BAKING PROPERTIES: STRAIGHT DOUGHS

THIOUREA	VOLUME	GRAIN
μmol./g. flour	*cc.*	
0	100	100
0.15	96	100
0.30	101	101
0.45	99	101
0.60	100	101

Thiourea had a slight improving effect. Formamidine disulfide produced a marked maturing effect regardless of the baking procedure employed. In the straight doughs, the optimum level of FDS was between 0.15 and 0.3 μmol. per g. of flour; in the sponge doughs, it was about 0.3 μmol. per g. In continuous-dough production, the

optimum level of FDS for the flour used was found to be between 0.15 and 0.3 μmol. per g. Higher levels of FDS (0.7 to 1.5 μmol.) caused too rapid extrusion and, consequently, the doughs were extremely undermixed — similar to doughs obtained when excessive levels of iodate are used. Such doughs extrude rapidly as a result of greater softness and, therefore, show signs of underdevelopment and the need for more mixing.

As can be seen in Tables VI, VII, and VIII, FDS showed excellent improver action over a comparatively wide range whereas, in previous work, it had been observed that only carefully controlled, very low levels of NEMI (0.1 μmol.) effected any improvement. This may be attributable to the fact that FDS, containing a formamidine function, is more basic than NEMI. The mechanism by which FDS exerts its improver action needs further study.

Conclusion

Although disulfide interchange has provided a plausible explanation for the action of improvers, these experiments cast doubt on the theory that the –SH and –SS– groups of flour proteins interchange appreciably in the normal pH range of doughs. Currently, extensive studies are being conducted in this laboratory to attempt to establish more evidence on this question.

Acknowledgment

The authors are grateful to Robert S. Hinz, now a graduate student at the University of Wisconsin, for conducting a number of the experiments.

Literature Cited

1. WALKER, J. B., and WALKER, MARGARET. Inhibition of sulfhydryl enzymes by formamidine disulfide. Arch. Biochem. Bophys. **86:** 80–84 (1960).
2. SULLIVAN, BETTY. The mechanism of the oxidation and reduction of flour. Cereal Chem. **25** (6): Suppl. (1948).
3. MCDERMOTT, E. E., and PACE, J. Modification of the properties of flour protein by thiolated gelatin. Nature **192:** 657 (1961).
4. MAURITSEN, C. A. M., and STEWART, P. Disulfide-sulfhydryl exchange in dough. Nature **197:** 48–49 (1963).
5. AMERICAN ASSOCIATION OF CEREAL CHEMISTS. Cereal laboratory methods (7th ed.). The Association: St. Paul, Minn. (1962).
6. ELLMAN, G. L. A colorimetric method for determining low concentrations of mercaptans. Arch. Biochem. Biophys. **74:** 443–450 (1958).
7. RYLE, A. P., and SANGER, F. Disulfide interchange reactions. Biochem. J. **60:** 535–546 (1960).
8. SPACKMAN, D. H., STEIN, W. H., and MOORE, S. The disulfide bonds of ribonuclease. J. Biol. Chem. **235:** 648–659 (1960).