DEHYDRO-L-ASCORBIC ACID REDUCING SYSTEM IN FLOUR[1]

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ABSTRACT

A dehydro-L-ascorbic acid (DHA) reducing system in flour was demonstrated by determination of the reduction rate of DHA in flour extract in the presence of glutathione (GSH). DHA added to flour-water extract (1:2) to the extent of 80 p.p.m. with excess of GSH was reduced about 90% at pH 5.9 at 35°C. within 30 min. The molar ratio of DHA to GSH, in this reaction, was 1:2. The catalytic system was found to be specific to DHA and GSH when compared with that of D-isoascorbic acid or cysteine and thioglycolic acid. The reaction rate was maximum at pH 7.0. It was thermolabile, since it was inactivated in 5 min., 72% at 60°C. and 100% at 70°C. From these results the system was presumed to be enzymatic, similar to that found in fresh potato juice, pea seedling, and cauliflower.

L-Ascorbic acid (L-AsA) has been considered as a dough improver which meets the present needs of the baking industry in its more rapid reaction than bromate.

Sandstedt and Hites (11) showed that L-AsA had a dough-improving effect like that of an oxidant, possibly in the form of dehydro-L-ascorbic acid (DHA). This assumption was confirmed to some extent by the results of Maltha (6), who showed that the reduction rates of DHA and oxidized analogs of L-AsA in the presence of aqueous flour extracts containing added glutathione (GSH) paralleled the dough-improving effects. The behavior of DHA and its relation to rheological properties of dough were also recently reported by Shimizu, Fukawa, and Ichiba (12).

The DHA reducing system found by Pfankuch in fresh potato juice catalyzed the reduction of DHA with cysteine (cys-SH); Pfankuch's observation was quoted by Crook and Hopkins (2). Crook and Hopkins (2), Crook (1), and Crook and Morgan (3) reported that cauliflower, broad bean, and various plant tissues contained dehydroascorbic acid reductase, requiring GSH as a hydrogen donor, and characterized this enzyme. Yamaguchi and Joslyn (13,14) studied its distribution and properties in pea seedlings. Mapson and Goddard (7) and Mapson and Moustafa (8) showed that L-AsA acted as a hydrogen donor in the respiratory chain in germinating pea seeds.

A similar system to that found in these plants may explain the mechanism of dough improvement by ascorbic acid.

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This paper follows the previous one which dealt with the L-AsA oxidizing system in dough (5).

Materials and Methods

Materials. The flour used in this study was an unbleached, improver-free, straight-grade flour, commercially milled from a blend of Canadian hard spring wheat, containing 13.1% protein (Kjeldahl nitrogen × 5.7) and 0.43% ash, on 14.3% moisture basis. It was stored in a refrigerator at 5°C. in a moistureproof container.

GSH was obtained from Kirin Brewery Co., cys-SH from Nutritional Biochemicals Co., D-isoascorbic acid from Fujisawa Pharm. Ind. Co., thioglycolic acid (TGA), L-AsA, bromine, and other reagents from Wako Pure Chemical Ind. Ltd.

DHA was prepared by the addition of bromine water drop by drop to L-AsA solution, 2 μmoles, until a faint orange color of bromine appeared. The excess bromine was removed by aeration. This process was carried out within 5 min. just before the addition of DHA to the reaction medium. L-AsA in the solution was found to be about 99.5% oxidized with this procedure.

Dehydro-D-isoascorbic acid was prepared from D-isoascorbic acid in the same way as DHA.

Methods. The DHA reducing activity of flour was estimated from a decrease in the DHA and GSH concentrations, and from formation of L-AsA in the presence of GSH, cys-SH, or TGA in the reaction medium of the flour extract.

The flour extract was prepared by mixing flour in a Waring Blendor with phosphate buffer or water for 1 min. before and after a 10-min. rest period, and centrifuging for 10 min. at 3,000 r.p.m.

The flour extractant and reaction medium are described below each table (Tables I to V).

The reaction was carried out under anaerobic conditions by bubbling nitrogen gas through the medium. To stop the reaction, an equal volume of 2% thiourea in 10% metaphosphoric acid was added. (Acetic acid, 0.5*N*, was used in the case of amperometric titration.) DHA was measured by a modification of Roe's method in which a 1-hr. reaction time was used at 50°C. (10). GSH and the total reducing activity of the medium were determined by amperometric titration with 0.002*N* mercuric chloride (9) and potassium iodate (4), respectively.

Results

DHA Reducing Activity in Flour Extract. DHA reducing activity

in phosphate buffer at pH 5.9 was estimated with and without flour extract. The results are shown in Table I.

TABLE I

REDUCTION OF DHA WITH AND WITHOUT FLOUR EXTRACT AT pH 5.9

MEDIUM	DHA		
	Added	Reduced	Reduced
	p.p.m.	*p.p.m.*	%
With flour extract	19.7	17.7	89.9
Without flour extract	19.7	0.3	1.5

Reaction medium:
5 ml. of 22 μmoles GSH and 11 μmoles DHA solution in Sørensen's phosphate buffer (pH. 5.9).
5 ml. of extract of flour with the same buffer (flour:buffer = 1:2).
Reaction conditions: 35°C., 30 min.

From these experiments, flour extract was found to have strong catalytic activity for the reduction of DHA in the presence of GSH.

Molar Ratio of DHA to GSH. The molar ratio of DHA to GSH was studied in this catalytic reaction with more diluted extract and at higher pH than those used in experiment 1. GSH oxidized and DHA reduced in the medium are shown in Table II.

TABLE II

MOLAR RATIO OF DHA TO GSH IN THE DHA REDUCING SYSTEM IN FLOUR

EXPERIMENT	MEDIUM	GSH OXIDIZED [a]	DHA REDUCED [b]	DHA REDUCED [c]
		μmol.	*μmol.*	*μmol.*
a	With flour extract (a)	2.28	1.14	1.23
b	Without flour extract (b)	0.39	0.29	0.32
	a minus b	1.89	0.85	0.91

[a] Amperometric titration with mercuric chloride.
[b] Total reducing value with potassium iodate; value, see footnote a.
[c] Modified Roe's method.
Reaction medium:
5 ml. of 13.0 μmoles GSH and 3.0 μmoles DHA solution in Sørensen's phosphate buffer (pH 7.0).
5 ml. of extract of flour with the same buffer (flour:buffer = 1:500).
Reaction conditions: 30°C., 10 min.

From this table it is evident that the molar ratio of DHA reduced to GSH oxidized, as indicated in line 3 (a – b), is almost 1:2.

Substrate Specificity. Substrate specificity of the DHA reducing system in flour was tested, with three sulfhydryl compounds – GSH, cys-SH, and TGA – as hydrogen donors and with a DHA analog, dehydro-D-isoascorbic acid as a hydrogen acceptor. Table III indicates the effects of the hydrogen donors. These data show that GSH is most effective as a hydrogen donor to the DHA reducing system in flour.

TABLE III

COMPARISON OF GSH, CYS–SH, AND TGA AS HYDROGEN DONORS IN DHA REDUCING SYSTEM IN FLOUR

EXPERIMENT	HYDROGEN DONORS AND MEDIUM	DHA		
		Added	Reduced	Reduced
		p.p.m.	*p.p.m.*	%
a GSH	With flour extract	4.86	3.20	65.8
	Without flour extract	4.86	0.59	12.0
b cys–SH	With flour extract	4.86	1.66	34.1
	Without flour extract	4.86	1.61	33.1
c TGA	With flour extract	4.86	2.88	59.2
	Without flour extract	4.86	2.74	56.3

Reaction medium and conditions: The same as shown in Table II, except cys–SH and TGA were used in experiments b and c respectively at the same molar concentration as that of GSH.
The flour extract was flour: buffer 1:170.

cys-SH and TGA were found to be more active than GSH without the flour extract. No increase in the activity was detected by the addition of flour extract.

Dehydro-D-isoascorbic acid was added as a hydrogen acceptor, to compare it with DHA. From the results summarized in Table IV it is obvious that DHA was more reactive than dehydro-D-isoascorbic acid as a hydrogen acceptor.

TABLE IV

COMPARISON OF DHA AND DEHYDRO-D-ISOASCORBIC ACID AS HYDROGEN ACCEPTORS IN DHA REDUCING SYSTEM IN FLOUR

EXPERIMENT	HYDROGEN ACCEPTORS AND MEDIUM	DHA OR DEHYDRO-D-ISOASCORBIC ACID		
		Added	Reduced	Reduced
		p.p.m.	*p.p.m.*	%
a DHA	With flour extract	5.10	3.51	68.9
	Without flour extract	5.10	0.42	8.2
b Dehydro-D-isoascorbic acid	With flour extract	5.75	1.68	29.2
	Without flour extract	5.75	0.62	10.8

Reaction medium and conditions: The same as shown in Table II, except dehydro-D-isoascorbic acid was used in experiment A as hydrogen acceptor at the same molar concentration as DHA.
The flour extract was flour:buffer = 1:250.

Effect of pH. Effect of pH on the DHA reducing activity was studied and results are shown in Fig. 1. The activity indicated as (a) – (b) almost disappeared at pH's lower than 5.5. At pH's higher than 6.6 the catalytic reduction of DHA took place rapidly; at pH 7.0 it reached a maximum, and above, it decreased. Control values on line (b) increase progressively with increased pH.

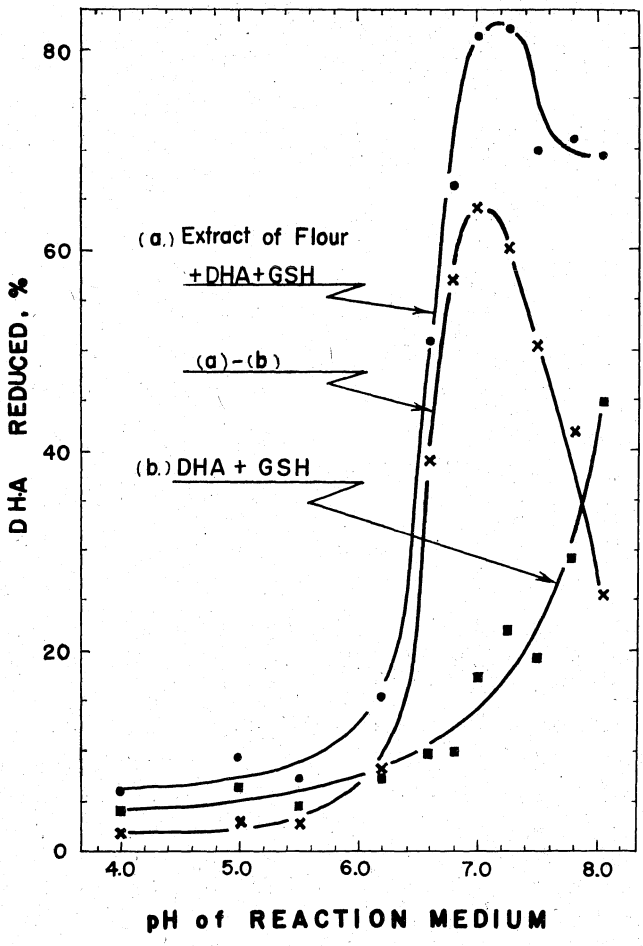

Fig. 1. Effect of pH for DHA reducing activity of flour extract. Reaction medium: 5 ml. of 13.0 μmoles GSH and 3.0 μmoles DHA solution in buffer; 5 ml. of extract of flour with water (flour:water = 1:170). McIlvaine's citrate-phosphate buffer below pH 6.5, Sørensen's phosphate buffer above pH 6.5.

Effect of Heat-Treatment. The DHA reducing activity of flour extract after heat-treatment was estimated in the same medium used in experiment 3. The heat-treatments were done at 55°, 60°, 65°, and 70°C. for 5 min., with the flour-water extract itself. The data summarized in Table V show that the catalyst is very unstable at the higher temperatures, that is, 41.3% being inactivated at 55°C., 71.8% at 60°C., 92.0% at 65°C., and about 100% at 70°C.

Effect of Dialysis. Flour extract with pH 6.0 phosphate buffer was dialyzed against the same buffer for 3 days at 5°C. in a Visking tube.

TABLE V

EFFECT OF HEAT-TREATMENT FOR DHA REDUCING ACTIVITY OF FLOUR EXTRACT

TEMPERATURE OF HEAT-TREATMENT	DHA				DHA REDUCING ACTIVITY (a — b)
	Added	Reduced	Reduced (a)	Reduced, without Flour Extract (b)	
°C.	*p.p.m.*	*p.p.m.*	%	%	
55	4.60	2.10	45.7	15.7	30.0
60	4.12	1.66	28.2	13.8	14.4
65	4.76	0.55	11.6	7.5	3.1
70	4.53	0.38	8.3	8.3	0.0
No treatment	4.60	3.03	66.8	15.7	51.1

Reaction medium and conditions: The same as shown in Table II, except 3 ml. of flour extract with water (flour:water = 1:100) were used after heat-treatment for 5 min. and dilution to 5 ml. with the buffer.

The activity on the reduction of DHA of dialyzed extract was 50% of that of the stored extract under the same condition. The loss of activity on dialysis may be brought about by the instability, rather than by the factors dialyzed out. Data are not shown as a table.

Discussion

It has been suggested that DHA is involved in the oxidation of some reductant such as a sulfhydryl compound. If the reaction mechanism of the DHA reducing system found in other plants such as potato (juice), pea seedlings, and cauliflower were applicable to that in dough, the reaction occurring in dough could then be characterized. However, this might be questionable in that GSH was generally the other substrate in the system in these plants. To the authors' knowledge at that time the GSH in patent flour had not been detected.

The experimental results, that 90% of DHA was reduced to L-AsA within 30 min. in the presence of GSH and flour extract and that only 1.1 to 1.7% of it was reduced without the flour extract, indicate the presence of a catalytic system in flour and dough, although the pH conditions were not optimum at 5.9.

This finding also suggests a possible impurity in the DHA prepared by the indicated method, namely diketo-L-gulonic acid, a further oxidation product of L-AsA. This material could not be reduced by this system with GSH, and was found to be less than 10%.

The reaction ratio of DHA:GSH in Table II, estimated by Roe's colorimetric method and by amperometric titration with mercuric chloride respectively, was about 1:2.2. The medium appears to remain completely anaerobic during the reaction, as the total reducing values estimated with potassium iodate were the same before and after reaction.

Thus, it is reasonable to postulate the following reaction scheme (1):

$$\text{DHA} + 2\text{GSH} \rightleftharpoons \text{L-AsA} + \text{GSSG} \quad \ldots\ldots\ldots\ldots (1)$$

As is borne out by the results in Table III, the catalytic system of DHA reduction in flour extract is specific to GSH. However, it should be noted that the other hydrogen donors, cys-SH and TGA, gave higher blank test values without flour extract than that of GSH. These results differ to some extent from those obtained by Crook (1) with cauliflower and broccoli juice, while a finding similar to those of the authors was shown by Yamaguchi and Joslyn (14) with peas.

As to the specificity of the hydrogen acceptor, dehydro-D-isoascorbic acid was tested as an analog of DHA. In this case the rate of reaction was one-third of that with DHA with flour extract and GSH. This result was in good agreement with Maltha's data (6). The system is more specific to the hydrogen donor than to the hydrogen acceptor.

For the determination of optimum pH two buffers were used: McIlvaine's citrate-phosphate buffer below pH 6.5, and Sørensen's phosphate buffer above pH 6.5. In our experiment, the DHA reducing system had a similar activity at pH 6.5 with each of these two buffers, though DHA reductase from pea juice was more reactive in phosphate buffer than citric, metaphosphoric, or acetic acid at pH 6.3 by Yamaguchi and Joslyn (13).

Figure 1 shows that the optimum pH value was 7.0, which was similar to that found by Yamaguchi and Joslyn (13) with pea juice.

Heat-labile properties of this system, which was inactivated completely by heating at 70°C. for 5 min., were also quite similar to those shown by Maltha (6), Crook (1), and Yamaguchi and Joslyn (13) in other plants.

Those data obtained with the DHA reducing system in flour and dough show that the system is possibly an enzymatic one.

The problems left unsolved in this paper are: 1) purification of this enzyme under stable conditions, and 2) identification of a relationship between dough improvement and this system.

To find the latter relationship, the effects of DHA on extensigrams at pH 6.95 (the optimum pH of the reductase) and at pH 4.95 (at which there remained only low activity) were compared, but no pronounced difference was found between them. Direct effects of hydrogen ion presumably canceled the different effects of DHA at two pH environments, in these experiments.

Attempts were also made to estimate total sulfhydryl groups in flour under DHA effect, but no change was observed. The oxidation of a small amount of GSH, which could not be detected by amperometric

titration in this study may be related to dough improvement. Further discussion on these points will be made in a paper to follow.

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