

## Studies on the Glutathione-Dehydroascorbate Oxidoreductase (EC 1.8.5.1) from Wheat Flour

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### ABSTRACT

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Glutathione (GSH) dehydrogenase was partially purified from wheat flour after extraction, ammonium sulfate precipitation, and ionic-exchange chromatography on diethylaminoethyl (DEAE) Sepharose CL6B. Kinetic studies showed that the optimum pH was close to 7.5. The  $K_m$  values varied between 0.15 and 0.28 mM for dehydroascorbic acid (DHA) and between 1.8 and 0.62 mM for GSH when pH was varied from 5.5 to 7.5. The kinetic pattern was consistent with a sequential mechanism for the binding of GSH and DHA. NaCl is a competitive inhibitor with respect to GSH and is uncompetitive with respect to DHA, which suggests that the

enzyme combines with DHA before it does with GSH. IsoDHA can replace DHA as hydrogen acceptor but with a  $K_m$  of 1.2 mM.  $\gamma$ -Glu-cys was enzymically oxidized but much less efficiently than GSH ( $V_m = 47$  nkat/mL and  $K_m = 5.5$  mM compared to  $V_m = 362$  nkat/mL and  $K_m = 1.8$  mM for GSH), whereas cysteine and cys-gly were not substrates. In the presence of DHA, addition of cysteine and cys-gly to small amounts of GSH causes a large activation of the enzymatic formation of ascorbic acid suggesting coupled oxidation of these thiols.

The effect of ascorbic acid (AA) as a dough improver was first recognized by Jørgensen (1935). Since then, AA has been widely used in breadmaking, mainly in those countries where other chemical improvers are not permitted. On the basis of results obtained by numerous workers (Melville and Shattock 1938; Sandstedt and Hites 1945; Proskuryakov and Auerman 1959; Kuninori and Matsumoto 1963, 1964; Tsen 1964; Carter and Pace 1965), Tsen (1965) has suggested a mechanism where the AA-improving action is due to its effect on the sulfhydryl-disulfide exchange reactions. During the dough mixing, AA is rapidly oxidized to dehydroascorbic acid (DHA) (Elkassabany et al 1980, Nicolas et al 1980). The latter compound is the actual improver, promotes the oxidation of thiol groups to disulfide links in dough.

Among the different stereoisomers of AA, the *L-threo*-AA exhibits the highest improving effect in dough rheology and loaf volume (Sandstedt and Hites 1945, Mair and Grosch 1979, Kieffer et al 1990), showing that at least part of these two reactions are enzymically catalyzed. Although an AA oxidase has been characterized in wheat flour by numerous authors (Grant and Sood 1980; Pfeilsticker and Roewing 1980, 1982; Grant 1987; Every et al 1995, 1996), the possibility of AA oxidation to DHA by other enzymes such as lipoxxygenase (Cherdkiatgumchai and Grant 1986) or by nonenzymatic reactions (Proskuryakov and Auerman 1959, Grant 1974, Every et al 1995) cannot be ruled out. Moreover, Pfeilsticker and Roewing (1980) claimed that AA oxidase was highly specific for the *L*-AA, whereas recently, Every et al (1995) indicated that the enzyme had a low specificity for all the stereoisomers of AA. The latter result is more in agreement with the observations of Elkassabany et al (1980), who found that *L*-AA and *D*-AA were oxidized at the same rate in dough.

The reduction of DHA to AA, with the concomitant formation of disulfide bonds from thiol compounds, is undoubtedly catalyzed by an enzyme, at least when the sulfhydryl compound is the reduced form of glutathione (GSH) (Nicolas and Drapron 1983, Grosch 1986, Sarwin et al 1993). Since its first description in heat by Kuninori and Matsumoto (1964), little data on the GSH-

dehydroascorbate oxidoreductase (EC 1.8.5.1 and GSH dehydrogenase) has become available. Carter and Pace (1965) indicated that the enzyme is mainly concentrated in the germ. Kahnt et al (1975) had developed an indirect method for the assay of GSH dehydrogenase activity that was based on the fact that AA reacted much faster on 2,6-dichlorophenol than did GSH in 75% ethanol. They found that the enzyme was also largely present in flour and bran fractions of different wheat varieties. Using the same assay method, Boeck and Grosch (1976) have purified GSH dehydrogenase from wheat flour to homogeneity. The molecular weight was 24,000 Da (SDS-PAGE electrophoresis). According to these authors and Walther and Grosch (1987), the enzyme was specific for GSH as hydrogen donor and was inactive on cysteine and *L*-cysteinyl-glycine. Among the stereoisomers of DHA, *L-threo*-DHA was reduced faster than the *D*- and *L-erythro*-DHA, which in turn were reduced faster than the *D-threo*-DHA. Boeck and Grosch (1976) obtained an optimum pH >7, but they indicated that measurement at pH >7, was difficult because a significant nonenzymic reduction of DHA by GSH occurred. Lastly, the same authors found, at pH 7, a  $K_m$  value of 0.58 mM for *L*-DHA and indicated that a first-order kinetics was found for GSH concentrations up to 2.5 mM.

Using a direct spectrophotometric method for the assay of GSH dehydrogenase activity adapted from Maellaro et al (1994), the purpose of this study was, after a partial purification of the enzyme from wheat flour, to investigate further the kinetics of the wheat GSH-DHA oxidoreductase.

### MATERIALS AND METHODS

The flour used for the purification of the GSH dehydrogenase was an untreated, improver-free straight-grade flour, commercially milled by Les Moulins Soufflet (Nogent-sur Seine, France), containing 9.7% crude protein ( $N \times 5.7$ ), 0.61% ash on a 15.3% moisture basis. Activated charcoal (Norit) was from Prolabo, *L-threo*-AA, *D-erythro*-AA (isoascorbic acid), cysteine, GSH, GSSG, cys-gly,  $\gamma$ -glu-cys were obtained from Sigma Chemical Co. (St. Louis, MO). Diethylaminoethyl (DEAE) Sepharose CL6B was from Pharmacia and Bio-lyte 3/10 ampholytes were from Biorad SA (Yury-sur-Seine, France).

### Extraction and Purification Procedure

Unless otherwise stated, all steps were performed at 0–4°C. Flour (50 g) was suspended in 150 mL of cold distilled water for

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15 min and then centrifuged at  $10,000 \times g$  for 20 min. The supernatant constituted the crude extract. Powdered ammonium sulfate (291 g/L, 50% saturation) was slowly added to the crude extract, and the sample was kept for 2 hr. After centrifugation ( $10,000 \times g$ , 20 min), 194 g/L of ammonium sulfate were added to the supernatant ( $S_{50}$ ). After one night at  $4^\circ\text{C}$ , the extract was centrifuged ( $10,000 \times g$ , 20 min). The pellet was redissolved in 25 mL of 10 mM  $\text{NaH}_2\text{PO}_4$  buffer at pH 6.2 (buffer A) and dialyzed overnight against the same buffer. The dialyzed solution ( $C_{80d}$  extract) was loaded onto a  $15 \times 1.6$ -cm column of DEAE Sepharose CL6B (30 mL bed vol) equilibrated with buffer A at a flow rate of 80 mL/hr. After elution of unbound proteins by two bed volumes of buffer A, GSH dehydrogenase activity was eluted by a linear gradient of ammonium sulfate (0–0.2M) in five bed volumes of buffer A. The proteins still bound were washed out with buffer A containing 0.2M ammonium sulfate (one bed volume) and with buffer A containing 1M ammonium sulfate (two bed volumes). Each 6-mL fraction was estimated for GSH dehydrogenase activity and UV absorbance was measured at 280 nm. Active fractions were pooled and constituted the purified extract, which can be stored at  $-20^\circ\text{C}$  without loss of activity for at least three months.

Protein content of extracts was determined by the dye-binding method of Bradford (1976) using bovine serum albumin as a standard.

### Determination of pI

Isoelectrofocusing (IEF) in liquid-phase medium was performed using a Rotofor system (Biorad). The  $C_{80d}$  extract was dialyzed overnight at  $4^\circ\text{C}$  against water and then diluted to  $\approx 50$  mL with water. Ampholytes (pH 3–10) solution and glycerol were added to final concentrations of 2 and 10%, respectively. The solution was loaded onto the Rotofor cell without further treatment. Focusing was done at 12 W constant power for 4 hr at  $4^\circ\text{C}$ . Recorded voltages varied between 500 and 1,400 V. GSH dehydrogenase activity, pH value, and absorbance at 280 nm were measured on each of the 20 fractions collected.

### Enzyme Assay Conditions

*GSH dehydrogenase.* The GSH dehydrogenase activity was determined by the modified method of Maellaro et al (1994) based on the direct spectrophotometric assay of the formation of ascorbate at 266 nm. DHA (2.5 mM) and isoDHA (2.5 mM) were prepared immediately before the assay by bubbling 50 mL of water solution of L-AA (or isoAA) with air in the presence of 0.15 g of activated charcoal (Norit). After 15 min, the charcoal was removed by paper filtration (Whatman No. 1). The absence of AA in the filtrate was checked both by spectrophotometry at 266 nm and by HPLC. For routine assays, the test was performed in a final volume of 2.5 mL of 0.1M  $\text{NaH}_2\text{PO}_4$  buffer solution at pH 6.2 containing DHA (0.5 mM) and GSH (3 mM) at  $30^\circ\text{C}$ . When the pH was modified, the same buffer was used between pH 6 and 8, and acetate buffer was used between pH 4 and 6. For kinetic studies, DHA and GSH concentrations were varied from 0.02 and 0.5 mM and from 0.3 to 3 mM, respectively. For inhibition by NaCl (75

and 150 mM) at pH 6.2, the DHA concentration varied between 0.02 and 0.5 mM (GSH was kept constant at 3 mM) and the GSH concentration was varied between 0.3 and 3 mM (DHA was kept constant at 0.5 mM). In each case, nonenzymatic reduction brought about by GSH (or other thiol when present) alone was subtracted. Using a molar absorption of  $12,360\text{M}^{-1}\cdot\text{cm}^{-1}$  for AA, the GSH dehydrogenase activity is expressed as nkat (nmol of ascorbic acid formed/sec). All the determinations were performed in duplicate. Kinetic parameters were determined using a nonlinear regression data analysis software (Leatherbarrow 1987).

*AA oxidase activity.* The AA oxidase activity was measured by polarography at  $30^\circ\text{C}$  in a 1.5-mL reaction cell containing 2 mM AA in air-saturated  $\text{NaH}_2\text{PO}_4$  (0.1M) buffer solution at pH 6.2. The activity is expressed as nkat (nmol of  $\text{O}_2$  consumed/sec).

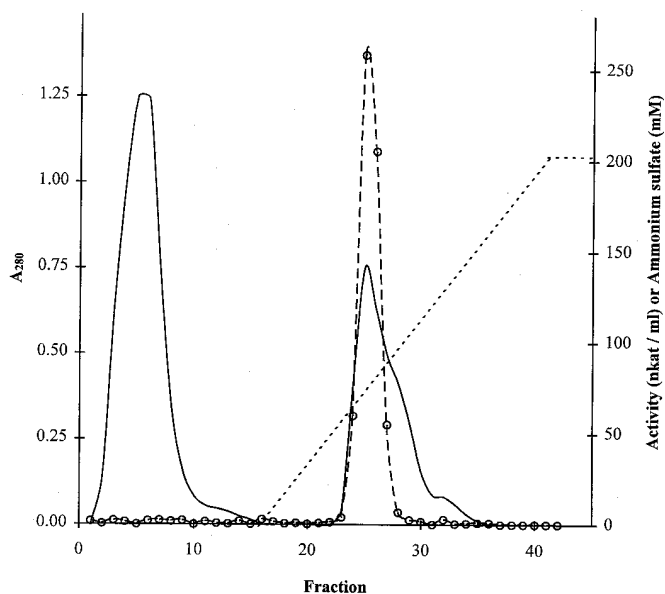
### Simultaneous Measurement of AA, IsoAA, Cysteine, and GSH

AA, isoAA, cysteine, and GSH were separated and quantified by HPLC using a 9012 pump driven by a 9020 workstation (Varian), a Valco C6W injector with a 10  $\mu\text{L}$  sample loop and an electrochemical detector equipped with a dual glassy carbon working electrode and an Ag/AgCl reference electrode (model Eldec 201, Chromatofield, France). Optimal separation was achieved using a 5- $\mu\text{m}$  Kromasil C18 column (250- $\times$ 4-mm i.d.) (AIT, France) with a mobile phase of  $\text{NH}_4\text{H}_2\text{PO}_4$  (0.1M) adjusted to a final pH of 2.8 with *o*-phosphoric acid. Samples were run isocratically at a flow rate of 0.8 mL/min. The potential of the first working electrode was fixed at + 0.60 V (vs. Ag/AgCl reference electrode) for the quantification of AA and isoAA. The potential of the second working electrode used for the quantification of cysteine and GSH was fixed at + 1.05 V. Linearity of the detector response was verified by injecting pure standard solutions and detector response for each compound was daily assessed from standard curves. In these conditions, the retention time and the minimum detectable quantity were 2.8 min and 5 pmol for cysteine, 4.9 min and 1 pmol for AA, 5.4 min and 1 pmol for isoAA, and 7 min and 10 pmol for GSH.

## RESULTS AND DISCUSSION

### Preliminary Studies

Our first aim was to obtain a partially purified preparation of GSH dehydrogenase devoid of AA oxidase activity. In this respect,



**Fig. 1.** Ion-exchange chromatography on diethylaminoethyl (DEAE) Sepharose CL6B of  $C_{80d}$  extract. Absorbance at 280 nm (—), glutathione-dehydrogenase activity (○), and  $(\text{NH}_4)_2\text{SO}_4$  concentration (---).

**TABLE I**

**Purification of Glutathione Dehydrogenase from Wheat Flour (50 g)**

Step <sup>a</sup>	Total Volume (mL)	Total Protein (mg)	Total Activity ( $\mu\text{kat}$ )	Specific Activity (nkat/mg)	Yield (%)	Purification Factor
Crude extract	120	240	6	25	100	...
$S_{50}$	125	118	5.63	47.7	94	1.9
$C_{80d}$	25	57.5	4.63	80.5	77	3.2
DEAE	12	8.5	2.52	296	42	12

<sup>a</sup>  $S_{50}$  = supernatant,  $C_{80d}$  = dialyzed solution, DEAE = diethylaminoethyl, Sepharose CL6B.

we checked both activities (GSH dehydrogenase and AA oxidase) in flour extracts obtained with different buffer solutions (between pH 4.5 and 8) and distilled water. The GSH dehydrogenase activity increased from 70 nkat/g (flour basis) at pH 4 to a maximum of 200 nkat/g at pH 7.5 and then decreased at pH 8 (190 nkat/g). The water extract was selected although the GSH dehydrogenase activity was slightly lower (170 nkat/g) because the amount of proteins was only 50% of that of the pH 7.5 extract. Moreover, the AA oxidase activity of the water extract was largely  $<0.1$  nkat/g, whereas the activity of the pH 7.5 extract was 0.35 nkat/g.

### Purification and IEF Experiments

Typical results of the purification procedure from 50 g of wheat flour are given in Table I. After extraction, the ammonium sulfate treatments yielded a 3.2-fold purification with a yield close to 80%. Further purification was accomplished by ion-exchange chromatography on DEAE Sepharose CL6B (Fig. 1). The GSH dehydrogenase activity was eluted in a single peak containing  $>75\%$  of the loaded activity. After pooling, the two most active fractions exhibited a specific activity close to 300 nkat/g, corresponding to an overall purification factor of 12 and a global yield  $>40\%$ . In these fractions, the AA activity was undetectable, whereas it was slightly higher than 1 nkat/mL in the  $C_{80d}$  extract loaded on the ion-exchange column. The active purified fractions can be stored frozen ( $-20^{\circ}\text{C}$ ) without activity loss for at least three months.

The only purification procedure for GSH dehydrogenase from wheat flour was proposed by Boeck and Grosch (1976). After a buffer extraction from wheat flour, ammonium sulfate treatments and two-step ion-exchange chromatography, they obtained a 460-fold purified fraction with a global yield  $<8\%$ . Compared to their results, the activity level of our crude water extract was similar, but the amount of proteins estimated by the Bradford's procedure was much smaller than the value given by Boeck and Grosch (1976) estimated by the absorbance at 280 nm. Both activity and absorbance at 280 nm of our DEAE Sepharose extract are comparable to those obtained by these authors after the first ion-exchange chromatography procedure. Because our partially purified extract obtained with a 42% yield was devoid of AA oxidase activity, we used it without further purification for the kinetic studies.

An IEF experiment was done on a  $C_{80d}$  extract (Fig. 2). A single peak of GSH dehydrogenase activity was observed at pH 5 together with a shoulder at a pH close to 5.4. In their experiment, Boeck and Grosch (1976) obtained a  $pH_i$  of 4.69. They used a flatter pH gradient (pH 4–6), which might explain the difference.

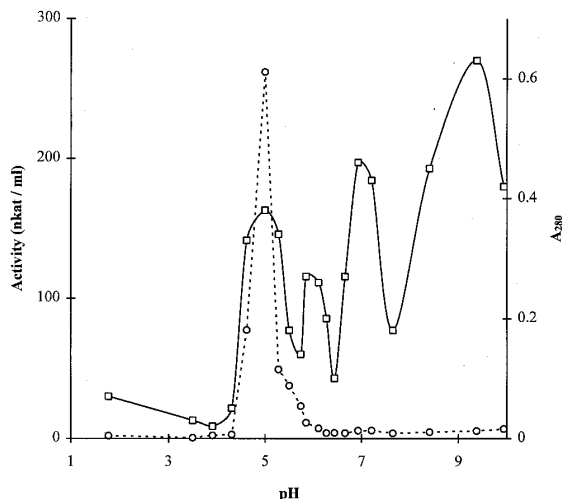


Fig. 2. Isoelectrofocusing of  $C_{80d}$  extract. Absorbance at 280 nm ( $\square$ ) and glutathione-dehydrogenase activity ( $\circ$ ).

### Effect of pH.

The pH dependence of dehydroascorbate reduction by GSH both in the presence and in the absence of GSH dehydrogenase is illustrated in Figure 3. The pH optimum of the enzymatic reaction obtained by the difference is close to pH 7.5. This value is in agreement with those obtained by previous workers for the wheat (Kuninori and Matsumoto 1964, Boeck and Grosch 1976), turnip (Gero and Candido 1964), pea (Yamaguchi and Joslyn 1952), human cell (Bigley et al 1981), and rat liver (Maellaro et al 1994) enzymes (in the pH 7–8 range).

The effect of pH was also studied on the kinetic constants ( $K_m$  and  $V_m$ ) of the enzyme towards its substrates GSH and DHA. The enzyme kinetics were always of the Michaelian type, whatever the pH tested for both substrates. The values of  $K_m$  and  $V_m$  obtained after a correction of nonenzymatic reaction are given in Table II. It appears that the affinity of the enzyme was always higher for the oxidant (DHA) than for the reducing substrate (GSH). However for DHA, the  $K_m$  values remained almost constant between pH 5.5 and 6.2 and doubled between pH 6.2 and 7.5, whereas for GSH, the  $K_m$  value was maximum at pH 6.2 and decreased when pH decreased to pH 5.5 or increased to pH 7.5. For both substrates, the apparent  $V_m$  (obtained at GSH = 3 mM for DHA and at DHA = 0.5 mM for GSH) increased from pH 5.5 to 7.5. For each pH, the theoretical  $V_m$  values can be calculated for the enzyme fully saturated by both substrates. The values obtained, obviously close (Table II), indicated that the maximum velocity was approximately twice as high at pH 7.5 than at pH 6.2. Nevertheless, further kinetic experiments have been made at pH 6.2, where the spontaneous reduction of DHA by GSH was almost nil, whereas at pH 7.5, it was far from negligible (Fig. 3).

At pH 7, Boeck and Grosch (1976) found a  $K_m$  value of 0.58 mM for L-DHA and indicated that a first-order kinetics was found for GSH concentrations up to 2.5 mM. At the same pH, our  $K_m$  value for DHA is lower (0.24 mM), whereas the  $K_m$  value for GSH was close to 1 mM. This discrepancy can be due to the different methods used for the assay of GSH dehydrogenase activity. Boeck and Grosch (1976) used an indirect method developed by Kahnt et al (1975) that was based on the fact that AA reacted much faster on 2,6-dichlorophenol than did GSH in 75% ethanol.

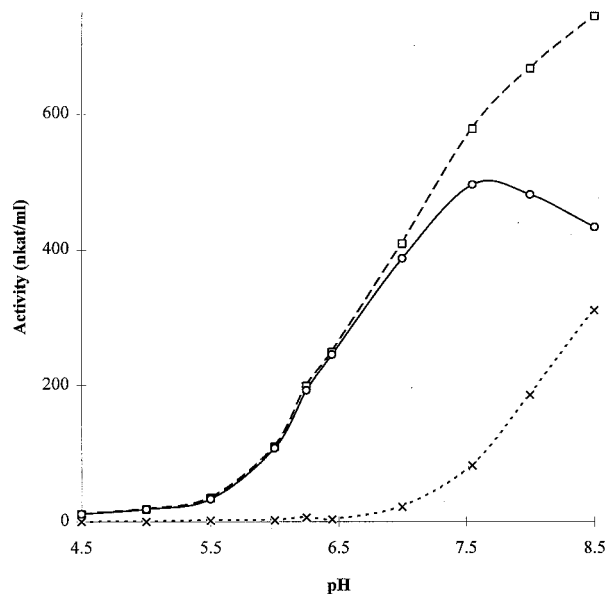


Fig. 3. Effect of pH on the velocity of dehydroascorbic acid (DHA) reduction by glutathione (GSH) dehydrogenase. Velocity with GSH dehydrogenase ( $\square$ ), velocity without GSH dehydrogenase ( $\times$ ), enzymatic reaction ( $\circ$ ).

### Substrate Specificity

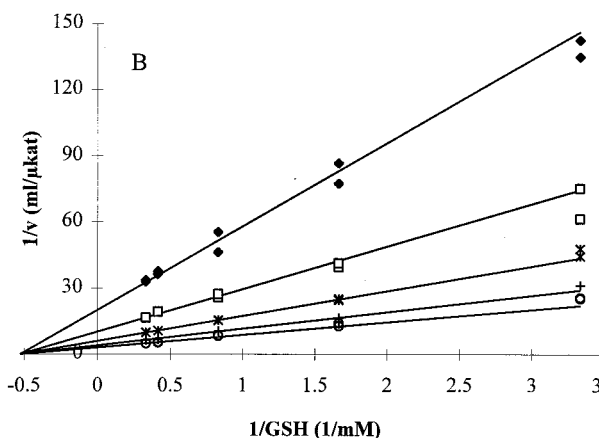
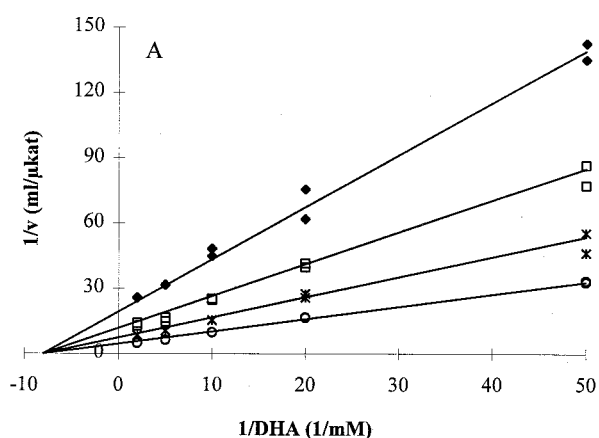
The effects of DHA and GSH concentrations on the initial velocity are shown in Figure 4. The results given in double-reciprocal form lead to a series of lines that intersect to the left of the vertical axis on the horizontal axis. Therefore,  $K_m$  for DHA is not affected by GSH concentration (Fig. 4A) nor is  $K_m$  for GSH affected by DHA concentration (Fig. 4B), whereas both apparent  $V_m$  for DHA and GSH increase as GSH and DHA concentrations increase. These experimental results suggest a sequential mechanism for the binding of GSH and DHA to the GSH dehydrogenase. In such a mechanism, the enzyme can bind the substrates either in a compulsory order or randomly. However, in

**TABLE II**  
Effect of pH on the Kinetic Constants of Glutathione-Dehydroascorbic Acid (GSH-DHA) Oxidoreductase

pH	DHA			GSH		
	$K_m$	$V_m^a$	$V_{mth}^b$	$K_m$	$V_m^a$	$V_{mth}^b$
5.5	0.15	81	113	1.2	89	116
6	0.13	241	370	1.6	268	338
6.2	0.14	275	442	1.8	362	463
6.5	0.19	377	571	1.5	440	607
7	0.24	567	738	0.90	496	734
7.5	0.28	792	956	0.62	603	941

<sup>a</sup> Apparent values obtained at GSH = 3 mM for DHA and at DHA = 0.5 mM for GSH.

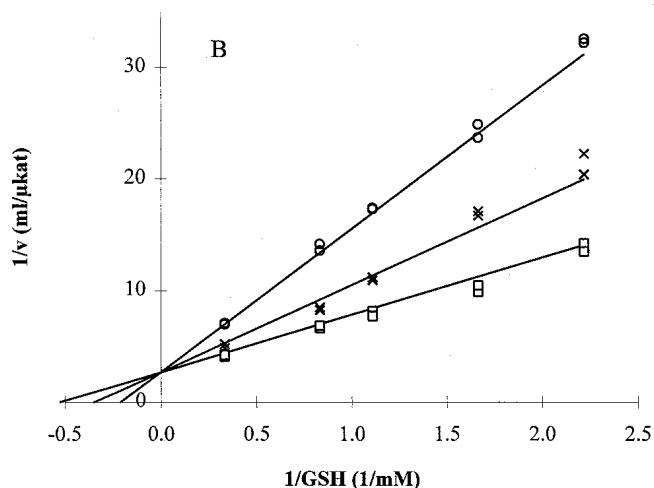
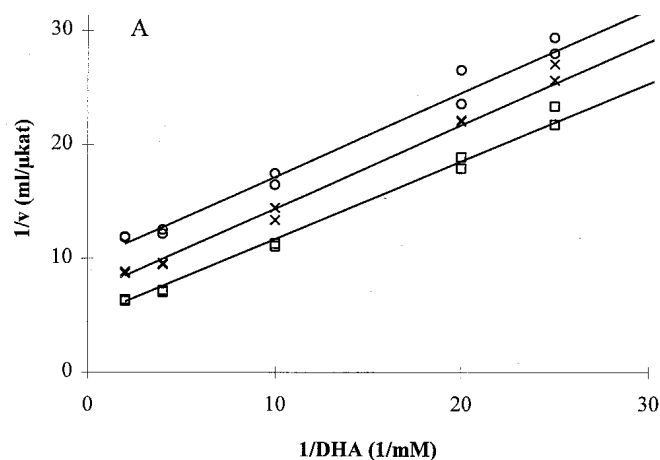
<sup>b</sup> Theoretical values (nkat/mL) of  $V_m$  and  $V_{mth}$  calculated when the enzyme is fully saturated by both substrates.  $K$  values expressed in mM.



**Fig. 4.** Effect of dehydroascorbic acid (DHA) and glutathione (GSH) concentrations (mM) on the initial activity of GSH dehydrogenase at pH 6.2. **A**, Concentrations in GSH of 0.3 (◆), 0.6 (□), 1.2 (\*), 3 (○). **B**, Concentrations in DHA of 0.02 (◆), 0.05 (□), 0.1 (\*), 0.2 (+), 0.5 (○).

the latter case (random mechanism), straight lines are obtained only if the rapid equilibrium assumptions are satisfied, that is, all binding and dissociation steps are very rapid compared to the catalytic step. Otherwise, the double-reciprocal plots are hyperbolic (Segel 1975). According to this author, the rapid equilibrium approach is not valid for most enzymes that catalyze ordered reaction sequences. Thus, one can think that GSH dehydrogenase catalyzes an ordered mechanism where one of the substrates is required to bind to the enzyme before the second substrate can bind. Since the velocity equation of this mechanism is symmetrical for the two substrates, no conclusion about the order of addition of GSH and DHA to GSH dehydrogenase can be made from the above experiments, and additional data are required.

Therefore, the inhibition mode of sodium chloride was studied by considering first GSH (Fig. 5B) and then DHA (Fig. 5A) as the variable substrate. NaCl is competitive with GSH because it does not affect the apparent  $V_m$  and increased the apparent  $K_m$  (Fig. 5B). The apparent  $K_i$  value was close to 0.17M. Thus, NaCl can bind only with enzymatic forms of GSH dehydrogenase that are free of GSH, that is, E or E-DHA (if the latter exists). The parallel pattern (Fig. 5A) obtained when DHA was the variable substrate indicates that NaCl is uncompetitive with DHA and the apparent  $K_i$  was close to 0.16M. In that case, the inhibitor can bind only enzymatic forms that contain DHA (E-DHA or E-DHA-GSH).



**Fig. 5.** Inhibition of glutathione (GSH) dehydrogenase by NaCl (mM) at pH 6.2 with dehydroascorbic acid (DHA) (**A**) and with GSH (**B**). 0 (□), 75 (×), 150 (○).

Therefore, these two propositions are only realized if the E-DHA form is present, which implies that DHA is the first substrate to be bound by GSH dehydrogenase.

Different amino acids and peptides derived from GSH have been tested as hydrogen donors for GSH dehydrogenase (Table III). In agreement with Kuninori and Matsumoto (1963) and Boeck and Grosch (1976), we found that cysteine is not a substrate for the enzyme. A very small activity is observed with the dipeptide cys-gly (close to 0.5% of the value found with GSH in the same conditions of concentration). Conversely, a significant activity is found with the other dipeptide  $\gamma$ -glu-cys because the  $V_m$  represents 13% of the value obtained with GSH, whereas the  $K_m$  was approximately three times higher. Therefore, GSH dehydrogenase is able to act on the dipeptide  $\gamma$ -glu-cys but much less efficiently than it does with GSH. In their study with the dipeptide cys-gly, Walther and Grosch (1987) did not find any formation of AA after 1 min of enzymatic reaction in a solution containing 0.28 mM of DHA and 2.5 mM of cys-gly, and they concluded that this peptide was not a substrate of the enzyme. To our knowledge, the dipeptide  $\gamma$ -glu-cys has never been tested before.

When isoDHA, a stereoisomer of DHA, was tested as hydrogen acceptor, it appeared that GSH dehydrogenase was also able to catalyze the transfer of hydrogen from GSH to isoDHA (Table III). However, this transfer was not as efficient as it was to DHA, due to a large decrease in affinity without an important modification of  $V_m$ . The ability of GSH dehydrogenase to use isoDHA as a hydrogen acceptor substrate has already been observed by previous authors (Yamaguchi and Joslyn 1952, Kuninori and Matsumoto 1964, Boeck and Grosch 1976, Walther and Grosch 1987).

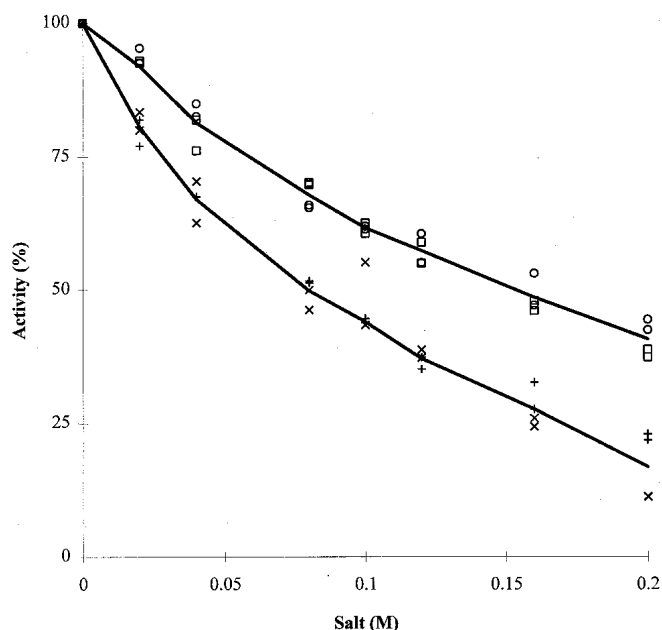
**TABLE III**  
Substrate Specificity of Glutathione (GSH) Dehydrogenase

	$V_m$ (nkat/mL)	$K_m$ (mM)
GSH	362 <sup>a</sup>	1.8
Cysteine	0	...
Cys-gly	1.3 <sup>b</sup>	...
$\gamma$ -Glu-cys	47 <sup>a</sup>	5.5
Dehydroascorbic acid (DHA)	275 <sup>c</sup>	0.14
isoDHA	305 <sup>c</sup>	1.2

<sup>a</sup> Values calculated from experiments with DHA = 0.5 mM

<sup>b</sup> Experimental velocity found with DHA = 0.5 mM and cys-gly = 3 mM

<sup>c</sup> Values calculated from experiments with GSH = 3 mM.



**Fig. 6.** Effect of different salts on the glutathione dehydrogenase activity.  $\text{Na}_2\text{SO}_4$  (+),  $\text{K}_2\text{SO}_4$  (x), KCl (O), NaCl (□).

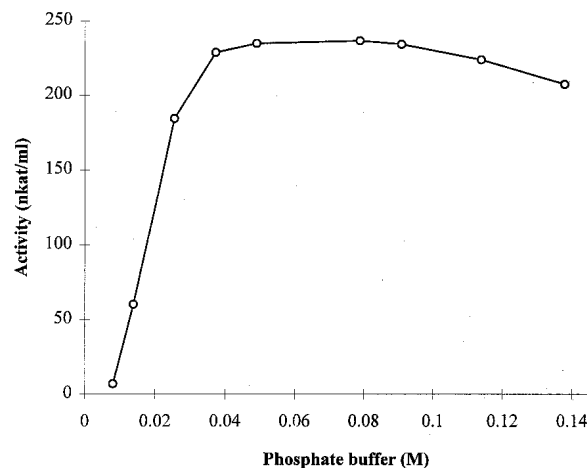
### Effect of Salts

Because sodium chloride was inhibitory for the GSH dehydrogenase activity, we have compared the effect of four salts: NaCl, KCl,  $\text{Na}_2\text{SO}_4$ , and  $\text{K}_2\text{SO}_4$  (Fig. 6). The sodium and potassium chloride salts have equivalent inhibitory properties. The same result is found when sodium sulfate and potassium sulfate are compared, but they are more efficient at the same concentration than the corresponding chloride salt. In the assay conditions, the monovalent salts (NaCl and KCl) have an  $\text{IC}_{50}$  of 0.14M compared to 0.08M for the divalent salts ( $\text{Na}_2\text{SO}_4$  and  $\text{K}_2\text{SO}_4$ ). From these experiments, the inhibition of GSH dehydrogenase appears more related to the concentration in cation than to those in anion.

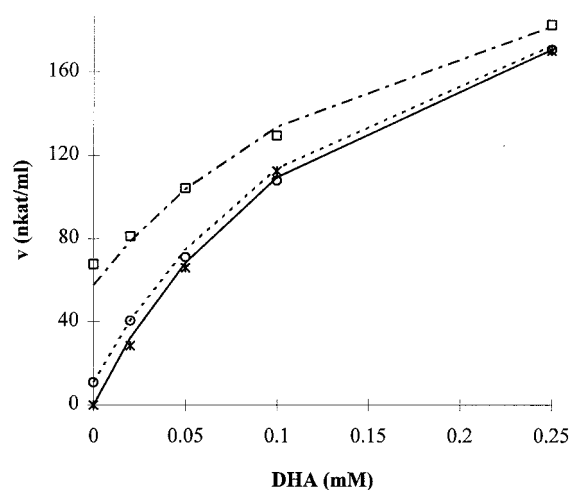
Because the GSH dehydrogenase activity is sensitive to ionic strength, we have checked the effect of the buffer salt concentration in the assay medium (Fig. 7). For low concentrations of phosphate (10 mM), the GSH dehydrogenase activity is very low and increases rapidly to a plateau between 50 and 100 mM of phosphate buffer. For concentrations >0.1M, increasing salt concentration results in a slow decrease of the enzymatic activity.

### Effect of isoDHA and Other Thiols in DHA and GSH

The effect of adding isoDHA to various amounts of DHA in 3 mM GSH was tested on GSH dehydrogenase activity (Fig. 8). Activation increases when isoDHA increases (at constant DHA)



**Fig. 7.** Effect of phosphate buffer concentration on the glutathione dehydrogenase activity.



**Fig. 8.** Effect of isodehydroascorbic acid (isoDHA) addition on the glutathione (GSH) dehydrogenase activity at pH 6.2 and at different concentrations (mM) of DHA. 0 (\*), 0.04 (O), 0.25 (□). Lines are curves calculated by using the equation in text with  $V_m$  and  $K_m$  values as given in Table III.

and decreases when DHA increases (at constant isoDHA). Because AA and isoAA have similar spectral properties, the theoretical velocity can be calculated from kinetic parameters  $V_m$  and  $K_m$  previously found for these two substrates (Table III):

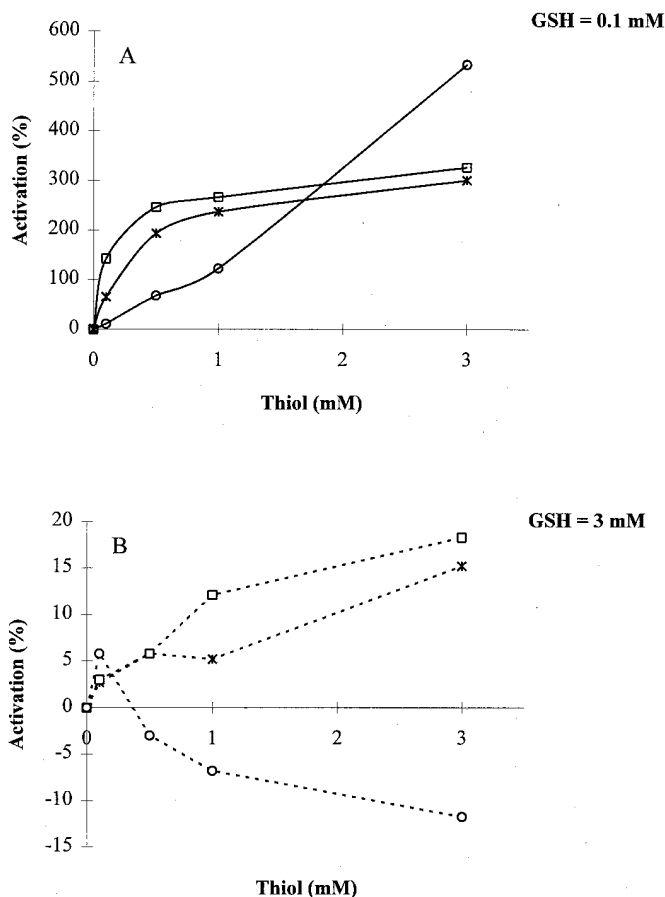
$$V = \frac{(V_{mDHA} \times S_{DHA}/K_{mDHA} + V_{misoDHA} \times S_{isoDHA}/K_{misoDHA})}{(1 + S_{DHA}/K_{mDHA} + S_{isoDHA}/K_{misoDHA})}$$

The former equation developed for one enzyme acting on two substrates (Janovitz-Klapp et al 1990) assumes that GSH dehydrogenase acts independently on both DHA isomers and that the amount of GSH consumed per mole of DHA is the same for the

**TABLE IV**  
Comparison of the Consumption of Glutathione (GSH) and the Formation of Ascorbic Acid (AA) (or isoAA) Catalyzed by GSH Dehydrogenase in a Medium Containing Various Amounts ( $\mu M$ ) of GSH, Dehydroascorbic Acid (DHA), and isoDHA<sup>a</sup>

Initial Amounts			Consumed		Formed		Ratio
GSH	DHA	isoDHA	GSH	AA	isoAA	GSH/(AA+isoAA)	
500	100	0	160	82	0	1.95	
100	500	0	78	37	0	2.11	
1,000	500	0	379	191	0	1.98	
500	0	100	33	0	16	2.06	
1,000	0	500	212	0	108	1.96	
500	100	250	278	79	54	2.09	

<sup>a</sup> All reactions occurred at pH 6.2 and 30°C in the presence of 5 nkat of purified GSH dehydrogenase for a total volume of 2.5 mL and followed by spectrophotometry at 266 nm. At various times, 100  $\mu L$  of solution were withdrawn, conveniently diluted by the mobile phase, and immediately analyzed by HPLC.



**Fig. 9.** Effect of different concentrations of cysteine ( $\square$ ), cys-gly ( $*$ ), and  $\gamma$ -glu-cys ( $\circ$ ) at two concentrations (mM) of glutathione (GSH) on GSH dehydrogenase activity at pH 6.2. Dehydroascorbic acid (DHA) = 0.5 mM. A, GSH = 0.1 mM. B, GSH = 3 mM.

two isomers. By HPLC, we confirm that approximately two moles of GSH are needed for the consumption of one mole of DHA or isoDHA either alone or when both are present (Table IV). One can see in Fig. 8 that experimental values were in good agreement with the curves calculated by using the equation above.

The effect of various amounts of cysteine and the two dipeptides derived from GSH (cys-gly and  $\gamma$ -glu-cys) tested on the GSH dehydrogenase activity in the presence of DHA (0.5 mM) and GSH (0.1 or 3 mM) is shown in Fig. 9. At GSH = 0.1 mM (Fig. 9A), addition of cysteine and cys-gly results in a strong activation that increases rapidly as the thiol concentration increases until 1 mM and then stabilizes at 300% for 3 mM in thiol. At GSH = 3 mM, addition of the same amounts of these thiols causes a small activation that represents <20% at 3 mM (Fig. 9B). Conversely, addition of  $\gamma$ -glu-cys results in an activation that is roughly proportional to the amount of thiol at GSH = 0.1 mM and gives a slight inhibition at GSH = 3 mM in the same conditions.

The latter result can be explained because  $\gamma$ -glu-cys is a poorer substrate for GSH dehydrogenase than GSH. At GSH = 0.1 mM, the enzyme is far from saturated by thiol and any addition of  $\gamma$ -glu-cys results in a quasi-proportional increase in the activity of AA formation because the  $K_m$  of  $\gamma$ -glu-cys is close to 5 mM (Table III). At GSH = 3 mM, increasing amounts of  $\gamma$ -glu-cys cause an increasing fraction of the enzyme acting on this substrate but much less efficiently than with GSH.

This explanation does not hold for cysteine and cys-gly, which are not substrates of the enzyme. In this case, the activation could be due to a regeneration of reduced GSH by cysteine and cys-gly acting on the oxidized GSH with formation of a pure disulfide, cysteine or gly-cys-SS-cys-gly or a mixed disulfide GSH + cysteine or GSH + cys-gly. The latter proposition (formation of a mixed disulfide G-SS-cys) has been already given by Sarwin et al (1994). Moreover, the activation of GSH dehydrogenase by cysteine has already been reported by Kieffer et al (1990), who have proposed a nonenzymatic reduction of oxidized GSH by cysteine. Lastly, side reactions leading to the consumption of thiol without a concomitant formation of AA cannot be ruled out (Finley et al 1981).

In conclusion, we confirm the specificity of GSH dehydrogenase toward the H acceptors and the H donors and suggest a reaction mechanism. The enzyme appears highly sensitive to ionic strength. Therefore, the moment at which salt is added to the dough could greatly affect the GSH dehydrogenase activity during mixing. Lastly, we have shown that the enzyme is able to catalyze the consumption of other thiols in the presence of GSH and DHA. However, the nature of the products formed (mixed disulfide or oxidized form of thiols) remains to be elucidated.

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