Studies of the Role of Ascorbic Acid in Chemical Dough Development. II. Partial Purification and Characterization of an Enzyme Oxidizing Ascorbate in Flour

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ABSTRACT

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Less than 20% of the ascorbic acid oxidase in a bread flour could be extracted with water or dilute salt solution. Soluble preparations containing up to 70% of the total activity of a flour suspension were obtained by extraction at room temperature with 75% saturated Na_2SO_4 solution at pH 8.3. Such extracts had high specific activity and contained a dialyzable component that absorbed strongly at 258 nm. Dialysis against water caused large losses of activity. Concentrated Na_2SO_4 solution stabilized the interaction between the dialyzable and the nondialyzable components. Ultracentrifugal analysis of the latter indicated a molecular weight of 21,000 in neutral dilute saline solution. When chromatographed on Sephadex® G-100 columns in the presence of concentrated Na_2SO_4, the active species, which included the component absorbing at 258 nm, was eluted in the void volume. We postulate an oligomeric complex that dissociated into inactive monomeric species upon reduction of the ionic strength of the solution. Nitrate ion and halide ions, particularly fluoride, inhibited the enzyme. Calcium ion was stimulatory.

Widely differing hypotheses on the role of ascorbic acid in chemically developed doughs have been proposed. These include protection of flour lipids from oxidation, modification of the hydrogen bonding in dough, and oxidation of excess thiol groups after dough development has been completed (Grant 1974, Bloksma 1978). In flour-water suspensions and in doughs, added ascorbic acid is oxidized by an enzyme that is endogenous to wheat flour (Grant 1974, Kuninori and Matsumoto 1963). We have tentatively designated this enzyme as ascorbic acid oxidase. Oxidation is rapid enough to suggest that the reaction is important in the chemistry of dough development. Because of this, we have attempted to isolate and characterize this enzyme.

MATERIALS AND METHODS

Experiments were performed using untreated patent flour milled commercially from Canadian hard red spring wheat. Sample A had an ash content of 0.35% and a crude protein ($N \times 5.7$) of 11.8%. In a 1:3 w/v flour-water suspension, 8.2 μg of added ascorbic acid was oxidized per minute per gram of flour (23°C). Sample B was very similar; the respective analyses were 0.37%, 11.5%, and 7.8 μg/min per gram of flour.

All chemicals were reagent grade or better. Glass distilled water was used throughout.

Analysis of L-ascorbic acid and measurement of ascorbic acid oxidase activity were performed as described previously except that the pH of the enzyme reaction mixture was adjusted to the optimum value of 6.3 (Grant 1974). Enzyme reaction times were adjusted so that the extent of ascorbic acid oxidation was within the range of 10–80% except for extracts showing very low or no activity. Substrate concentration was 8.0 μg/ml.

Flour extracts were prepared by stirring flour suspensions in water or aqueous solution, usually in the ratio of 1:3 w/v, for 30 min at room temperature and removing the insoluble residue by centrifugation at 15,000 × *g*. Protein content of extracts was determined by the biuret method (Pinckney 1961) as described (Grant 1973).

Salt fractionation was by Osborne's method (1909).

The most purified enzyme preparation was obtained by twice extracting flour with five volumes of water at pH 5.9, followed by further extraction of the wet residue with three volumes[2] of 75% saturated[3] Na_2SO_4 solution at pH 8.3. The Na_2SO_4 solution extract was then adjusted to pH 6.3 with dilute H_2SO_4 solution.

Ultraviolet absorbance spectra were determined with a Carey Model 14 recording spectrometer.

Gel chromatography was performed on columns of Sephadex® G-100 by the reverse flow technique according to the procedure recommended by the manufacturer (Pharmacia Fine Chemicals, Uppsala, Sweden). Gel columns were prepared and eluted with 50% saturated Na_2SO_4 solution, pH 6.3, at room temperature. A 100 × 2.5 cm column was used for determining elution volumes, and a 26 × 5.0 cm column was used for measuring enzyme distribution in the eluant fractions.

Sedimentation coefficients were determined with a Spinco Model E analytical ultracentrifuge, and molecular weights were determined according to Yphantis' method (1964).

Chloride concentration in solution was measured by the Mohr method (Skoog and West 1969).

RESULTS AND DISCUSSION

Purification of Wheat Flour Ascorbic Acid Oxidase

Very little ascorbic acid oxidase could be extracted from wheat

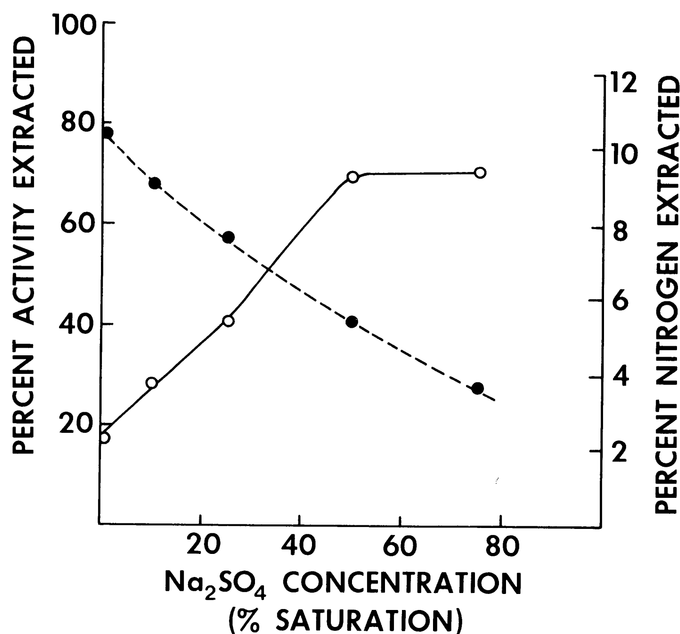

Fig. 1. Effect of Na_2SO_4 concentration on the solubililzation of ascorbic acid oxidase in flour B. — = % activity extracted; ---- = % N extracted.

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[2] The volume included the water present in the wet residue after aqueous extraction.
[3] The saturated Na_2SO_4 solution was 25 g of anhydrous Na_2SO_4 per 100 ml of H_2O. (Jacobs 1969).

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flour with pure water. After centrifugation of aqueous suspensions, 95% of the activity was recovered in the insoluble residue. The extent of solubilization depended on pH and was highest between 8 and 9. However, even at optimum pH, the percentage extraction averaged only 13 ± 3% (95% confidence limits). Observations during a three-month period indicated that the extractability tended to decrease with increasing age of the flour. Saturation of the extractant with $CaSO_4$ or addition of 1% w/v of Na_2SO_4 resulted in a slight further improvement in enzyme solubilization.

Although extraction efficiency with water was undesirably low, some success was achieved in fractionating the extracts by classical salting out procedures. Of the salts tested, Na_2SO_4 was the most suitable. At room temperature, the addition of solid Na_2SO_4 to 75% of saturation caused much of the inactive protein to precipitate from the extract. It also caused a dramatic stimulation of the enzyme that remained in the supernatent solution. The activity was nearly double that observed before the addition of Na_2SO_4.

Complete saturation of the water extract with Na_2SO_4 caused further protein precipitation, and very little activity remained in solution. However, only 50% of the activity that was presumed to be in the precipitate was recovered after reconstitution in 75% saturated Na_2SO_4 solution.

The stimulation of enzyme activity by Na_2SO_4 prompted an investigation to determine whether high concentrations of this salt would also promote improved solubilization. The results are illustrated in Fig. 1. Efficiency of enzyme extraction increased with increasing Na_2SO_4 concentration up to the 50% level of saturation. At the same time, the amount of total protein in solution declined continuously. The highest specific activity in an enzyme extract was attained by extracting flour with 75% saturated Na_2SO_4 solution after two preliminary extractions with water. Omission of the preliminary extractions resulted in somewhat higher recovery of total activity but also caused greater contamination by nonactive soluble protein. Data are summarized in Table I.

Added Na_2SO_4 stimulated the enzyme activity in flour-water suspensions as well as in aqueous extracts, but the extent of stimulation was much less. The rate of ascorbic acid oxidation in a 1:3 w/v suspension of flour in 75% saturated Na_2SO_4 solution was only 14% higher than that observed when the same flour was suspended in water.

Properties of Wheat Flour Ascorbic Acid Oxidase

Extracts containing wheat flour ascorbic acid oxidase were moderately stable when stored under refrigeration. Approximately 20% of the activity was lost in one week at 5°C. Losses of the same magnitude occurred in 24 hr at room temperature or in 15 min at 45°C.

Enzyme activity also decreased with increasing age of the flour. Aqueous suspensions made with flour that had been stored for six months at room temperature contained 30% less activity than did similar suspensions of the freshly milled flour.

In aqueous solution, the most purified enzyme preparation absorbed strongly in the ultraviolet with a maximum at 258 nm. Some of the material absorbing at this wavelength was dialyzable. The spectra for both the extract and the dialyzate (material that passed through the membrane) are in Fig. 2. No ascorbic acid oxidase activity was detectable in the dialyzate. Activity losses in the extract after 24-hr dialysis against water at room temperature exceeded 50%, even when Na_2SO_4 was restored to optimum concentration. Recombining the lyophilized dialyzate with the dialyzed extract did not restore any of the lost activity.

Very different results were obtained by room temperature dialysis against 75% saturated Na_2SO_4 solution. The loss in activity after 24 hr was only 20%, similar to that observed during room temperature storage. The rate at which the UV absorbing material passed through the dialysis membrane into 75% saturated Na_2SO_4 was relatively slow, approximately one-half that observed during dialysis against water.

Further evidence of the importance of Na_2SO_4 concentration in maintaining enzyme activity was obtained by merely diluting the extracts with water. Decreases in activity considerably exceeded the degree of dilution. These results suggest that concentrated Na_2SO_4 either promotes or stabilizes an interaction with a low molecular weight species that is essential for the maintenance of the enzyme in an active form. Identification of this substance is an objective for future work.

Gel chromatography of the enzyme solution was performed on Sephadex G-100 in the presence of a high concentration of Na_2SO_4. The UV absorbing components emerged in a single peak corresponding to the void volume of the column. Experiments

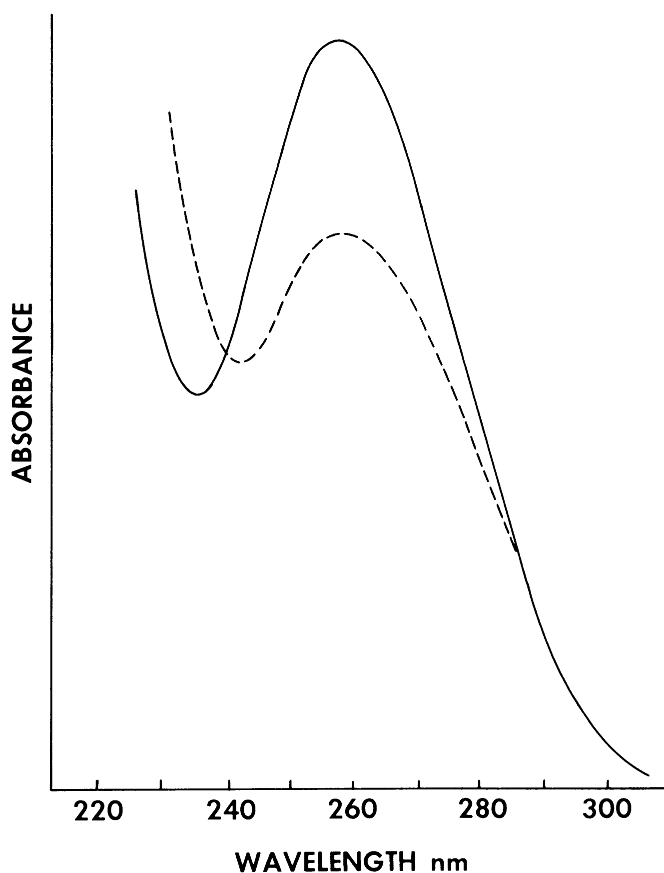

Fig. 2. Ultraviolet absorption spectra of ascorbic acid oxidase preparation (– – –) and its dialyzate (—) (flour B).

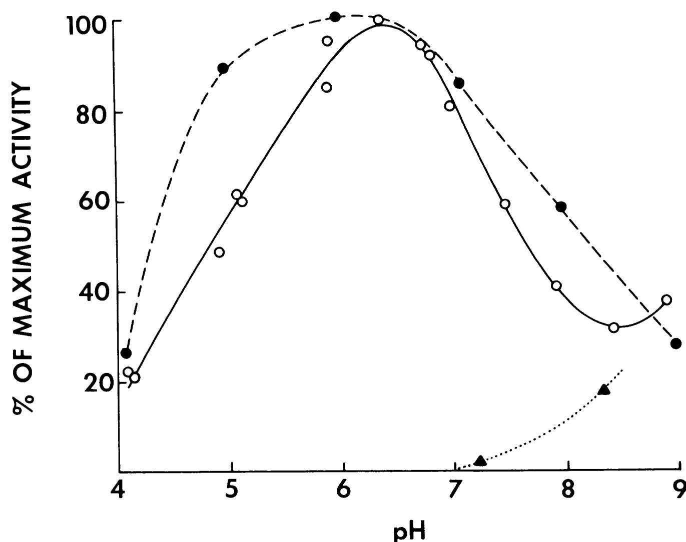

Fig. 3. Effect of pH on activity of ascorbic acid oxidase (flour B). — = flour water suspension; – – – = purified preparation in 75% saturated Na_2SO_4 solution; ··· = nonenzymatic oxidation.

using the short, large-diameter column showed that the only fractions that contained detectable ascorbic acid oxidase activity were those that also showed absorbance at 258 nm. These results indicated that the active species had a molecular weight well in excess of 100,000.

The enzyme solution showed two peaks in the analytical ultracentrifuge after dialysis against 1% aqueous NaCl. The major component had an S_{20w} of 2.3 and a molecular weight of 21,000. The minor component, approximately 10% of the sample, had an S_{20w} of 7.5 and a molecular weight of approximately 120,000. Preliminary attempts to determine molecular weight in the presence of high concentration of Na_2SO_4 gave inconclusive results.

Figure 3 shows the pH-activity profile of the enzyme extract in 75% saturated Na_2SO_4 solution compared with that reported earlier for flour-water suspensions (Grant 1974). The more purified preparation exhibits high activity over a broader pH-range.

Salt Inhibition Studies

Because NaCl inhibits ascorbic acid oxidase in flour-water suspension (Grant 1974), we examined the effect of adding various other salts to similar suspensions. Results are shown in Table II. At equimolar concentration, NaF was much more inhibiting than NaCl; similar concentrations of Na_2SO_4 had no significant effect. Calcium salts caused mild stimulation of the enzyme. The inhibitory effects, therefore, were those of specific ions rather than a general effect of salt. Inhibition by NaCl was clearly due to CL^-. Divalent cations other than CA^{++} frequently stimulate enzymes; therefore salts of Mg^{++} and Mn^{++} were also tested. These stimulated nonenzymic oxidation of ascorbic acid but appeared to have little effect on the enzymatic reaction.

The extent of inhibition of the purified enzyme preparation by fluoride ion was not as great as that observed in flour-water suspensions. At 0.72% w/v of added NaF, 82% inhibition was observed in extracts that were 75% saturated with Na_2SO_4, compared with 96% inhibition in flour suspensions containing no Na_2SO_4.

Further studies on NaCl inhibition in aqueous flour suspensions showed that the flour-water ratio and the salt concentration in solution both affect the extent of inhibition (Table III). A given level of dissolved NaCl inhibited the dilute suspension more than it did the concentrated one. In the absence of added salt, enzyme activity varied in direct proportion to the concentration of flour in suspension. With either 1 or 3% of added NaCl in solution, the more dilute flour suspension had disproportionately lower activity.

These results suggest that Cl^- may be adsorbed on the solid particles in which the enzyme activity resides. Other experimental results contradicted such a hypothesis, however. When flour was extracted with NaCl solution and aliquots of both the extract and the extracting solution were analyzed quantitatively for Cl^-, no detectable difference in Cl^- concentration was observed.

Our data suggest that the enzyme that oxidizes added L-ascorbic acid in aqueous flour systems is an oligomeric complex consisting of more than two subunits. In aqueous suspensions it exists and functions in the insoluble part of the mixture. A high concentration of Na_2SO_4 promotes solubilization and also stabilizes the active species in solution. At lower concentrations of this salt, the complex tends to dissociate into subunits. This coincides with the release of a low molecular weight component that absorbs strongly at 258 nm. Dissociation is accompanied by the loss of all or nearly all of the activity.

The enzyme that we have partially purified may not use ascorbic acid directly as a substrate, and therefore it may be improperly identified as ascorbic acid oxidase. Other enzymes can cause indirect oxidation of this compound, particularly those that result in formation of the superoxide anion (Morimitsu 1975). In the future, we intend to test our preparation for the presence of other enzymes and particularly for peroxidase activity.

TABLE I
Enzyme Purification

Sample	Percent of Total N	Percent of Total Activity	Specific Activity[a]
Flour-water suspension[b]	100	100	77
H_2O extract[c]	10.7	2.5	18
Na_2SO_4 extract I[d]	3.5	72	1580
Na_2SO_4 extract II[e]	1.7	55	2500

[a]Microgram of ascorbic acid oxidized per minute per gram of protein at 23° C.
[b]Flour B in 1:3 w/v suspension of 75% saturated Na_2SO_4 solution, pH 6.3.
[c]Water extract of flour B (1:5 w/v, pH 5.9).
[d]Extract of flour B with 75% saturated Na_2SO_4 solution (1:3 w/v, pH 8.3).
[e]Same as Na_2SO_4 extract I except flour was twice extracted with water at pH 5.9 before extraction with Na_2SO_4 solution.

TABLE II
Inhibition of Ascorbic Acid Oxidase by Various Salts

Salt	Salt Concentration[a] (% w/v)	Inhibition[b]
NaCl	1.00	19 ± 3
NaBr	1.48	32 ± 6
NaF	0.72	96 ± 10
$NaNO_3$	1.45	22 ± 9
Na_2SO_4	0.76	3 ± 9
$CaCl_2$[c]	0.63	−16 ± 9
$CaSO_4$[c]	0.20	−14 ± 5

[a]The concentrations of all salts gave the same ionic strength as that of 1% NaCl, except for $CaSO_4$, which was saturated.
[b]Data are for 1:3 w/v suspensions of flour A. Results based on three or more replicates are expressed within the 95% confidence limits.
[c]Ca salts stimulated enzyme activity.

TABLE III
Effects of Salt Concentration and Suspension Concentration on the Inhibition of Ascorbic Acid Oxidase by NaCl[a]

NaCl Concentration (% w/v)	Flour-Water Ratio (w/v)	Percent Inhibition[b]
1.0	1:3	19 ± 3
1.0	1:7.5	33 ± 4
3.0	1.3	41 ± 2
3.0	1:7.5	54 ± 3

[a]Data are for flour A.
[b]95% confidence limits, seven or more replicates.

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