

Purification and Characterization of a Hexose Oxidase with Excellent Strengthening Effects in Bread

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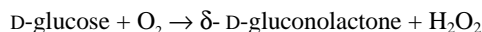
ABSTRACT

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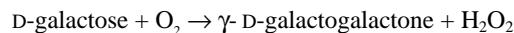
Hexose oxidase (EC 1.1.3.5) (HOX) was purified 51-fold from the red algae *Chondrus crispus*, by several chromatography methods, including hydrophobic interaction, chelating Sepharose, anion exchange, gel filtration, and chromatofocusing. Purified HOX was subjected to native PAGE and activity staining with nitroblue tetrazolium. For HOX electroeluted out of the gel and digested with endoproteinase Lys-C, the internal peptide sequence determined was: D-P-G-Y-I-V-I-D-V-N-A-G-T(V or P)-D-K-P-D-P-X. The molecular mass, determined by gel filtration, was 126 kDa, versus 65 kDa determined by SDS-PAGE. The pI was determined to 4.64 and 4.79 as a double band on an isoelectrofocusing gel. K_m was

determined to 2.7 mM for D-glucose, 3.6 mM for D-galactose, 20.2 mM for cellobiose, 43.7 mM for maltose, 90.3 mM for lactose, 102 mM for xylose, and 531 mM for arabinose. The oxidation of thiol groups in gluten was determined by using Ellman's reagent: 5,5'-dithiobis (2-nitrobenzoic acid). The effect of HOX was compared to that of glucose oxidase. Both enzymes caused a dose-responsive reduction in the free thiol groups. Extensigraph measurements and baking tests confirmed that HOX caused increased dough strength and increased bread volume more efficiently than glucose oxidase used in the same dosage.

Oxidizing agents have a beneficial effect on dough development and dough quality. Dough quality is crucial for the volume, texture, and crumb structure of the final baked product. Some of the chemical additives traditionally used for bread improvement have been prohibited. Also, consumers in North America and Western Europe increasingly prefer food products with a "clean label". As a result, the baking industry is looking for alternatives to chemical bread improvers. Different oxidative enzymes have been suggested, and glucose oxidase (GOX) (EC 1.1.3.4), particularly, has found commercial use. GOX catalyzes the conversion of glucose and oxygen into gluconolactone and hydrogen peroxide (H_2O_2). The H_2O_2 formed then acts by oxidizing the thiol groups in the gluten proteins forming disulfide bonds (Haarasilta and Pullinen 1992). This leads to increased dough strength. It would be advantageous to find a new enzyme which, unlike GOX, can utilize several monosaccharides and oligosaccharides. Such an enzyme would presumably be more efficient and have broader application possibilities than GOX. In this work, we have studied such an oxidative enzyme, hexose oxidase (EC 1.1.3.5) (HOX) which catalyzes the conversion of a number of mono- and oligosaccharides into corresponding lactones, with subsequent hydrolysis to the respective aldobionic acids, concomitant with the formation of H_2O_2 . The reaction catalyzed by HOX can be illustrated as:



or



HOX has been isolated from the two red algae species *Iridophycus flaccidum* (Bean and Hassid 1956) and *Chondrus crispus* (Sullivan and Ikawa 1973, Kerchensteiner 1978). Additionally, the algal species *Euthora cristata* (Sullivan and Ikawa 1973), the bacteria *Malleomyces pseudomallei* (Dowling and Levine 1956), and young citrus fruits (Bean et al 1961) have been shown to produce an enzyme capable of oxidizing several saccharides. In this work, we describe the purification and characterization of HOX from *C. crispus* and show that it has the same effects in dough and bread as GOX, but due to a much broader substrate specificity and a lower K_m value for glucose, it is a much more efficient enzyme than GOX.

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MATERIALS AND METHODS

Chemicals

GOX was obtained from *Aspergillus niger* (Grindamyl S 757, Danisco Ingredients, Denmark). Maltotriose and maltotetraose were obtained from Megazyme, Ireland. Peroxidase, *o*-dianisidine, nitroblue tetrazolium, phenazine methosulphate, and endoproteinase Lys-C were obtained from Sigma Chemical Co., St. Louis, MO. Phenyl Sepharose FF, chelating Sepharose FF, Source Q, Superdex 200, Mono P HR 5/5, isoelectrofocusing (IEF), and molecular weight standards were from Pharmacia, Sweden. The 8–16% tris-glycine gels used for electrophoresis and the precast 3–10 IEF gels were from Novex, San Diego, CA. The inorganic chemicals used were analytical grade. A Vydac C18 column was obtained from The Separation Group, CA. A Brownlee C2 column was obtained from Applied Biosystems. A Dionex PA2 column was from Dionex.

Assay for HOX Activity

The assay of Sullivan and Ikawa (1973) was scaled down to be run in microtiter plates. The absorbance was read in a microplate reader. The HOX assay method was also used for the assay of GOX. One unit is defined as the amount of enzyme that catalyzes the production of 1 μ mole of H_2O_2 per minute at 25°C, pH 6.3, and at a substrate concentration of 50 mM D-glucose. The assay was used to determine the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) for D-glucose, D-galactose, cellobiose, maltose, lactose, xylose, and arabinose, respectively. The data were fitted by nonlinear regression to $v = V_{max}S/(K_m + S)$, where S is substrate concentration and K_m is the concentration giving half maximum velocity. The values were determined by a curve-fitting computer program, EZ-FIT (Perella 1988).

Determination of Protein and Carbohydrate

The protein concentration of the samples was measured in microtiter plates using the microassay method (Bradford 1976) according to Bio-Rad (Anonymous 1984). Bovine serum albumin was used as the standard in the protein determination. The carbohydrate concentration was determined according to the phenol-sulfuric acid method (DuBois et al 1956).

Purification of HOX

The *C. crispus* seaweed used in these purifications was obtained partly from Danisco Ingredients, Sobal, Landerneau Cedex, France, and partly from Hirsholmene, Denmark. The seaweed was transported to Danisco Ingredients under cold conditions and then stored at -20°C.

In a typical purification procedure, seaweed (France) (500 g) was rinsed in distilled water, dried with a towel, and frozen in liquid nitrogen. Afterward, the seaweed was blended in a Waring blender. Then 1,000 mL of 0.1M sodium phosphate buffer, 1M NaCl, pH 6.8, was added to the seaweed. The mixture was extracted under continuous stirring for four days at 5°C, followed by centrifugation at 30,000 × g for 30 min. The supernatant was filtered through a glass filter. The pellets were resuspended in 1,000 mL of H₂O and then centrifuged at 30,000 × g for 30 min. The resultant supernatant was pooled with the extract, giving a total volume of 1,850 mL. Ammonium sulfate was added to a final concentration of 1.2M. The sample was filtered through a glass filter, and half of the sample was applied to a 5- × 8.7-cm column with 170 mL of phenyl Sepharose FF equilibrated in a 25 mM sodium phosphate buffer, pH 6.3, with 1.2M (NH₄)₂SO₄. The column was washed with equilibration buffer followed by elution of bound proteins using a 1,800-mL linear gradient from 1.2M to 0.06M (NH₄)₂SO₄ in 25 mM sodium phosphate buffer, pH 6.3, at a flow rate of 15 mL/min. Fractions of 15 mL were collected. Fractions containing the HOX activity were pooled. This run was repeated for the second half of the extract. The two pools were combined after hydrophobic interaction (total volume 1,400 mL) and applied to a 5- × 8.7-cm column with 170 mL of chelating Sepharose FF loaded with Ni⁺⁺ according to the manufacturer instructions, and equilibrated in 50 mM sodium phosphate, 1M NaCl, pH 7.4. The column was washed with equilibration buffer and bound proteins were eluted using a 1,500-mL linear gradient of 0–35 mM imidazole, pH 7.4–4.7, in the equilibration buffer. The column was eluted at a flow rate of 15 mL/min, and fractions of 15 mL were collected. Fractions 18–35 (270 mL) were pooled. That pool was concentrated and desalted by ultrafiltration in an Amicon 8400 unit equipped with a 30-kDa membrane. The resultant 20-mL sample (now in 20 mM triethanolamine buffer, pH 7.3) was applied to a 1.6- × 10-cm column with 20 mL of Source Q30 equilibrated in 20 mM triethanolamine, pH 7.3. The column was washed with equilibration buffer, and bound proteins were eluted using a 150-mL linear gradient from 0 to 0.65M NaCl in the equilibration buffer. The column was eluted at a flow rate of 4 mL/min, and 4-mL fractions were collected. Fractions 15–35 (44 mL) were pooled. That pool was freeze-dried, resolubilized in 5 mL of H₂O and then loaded on to a column of 300-mL Superdex 200 PG (2.6 × 57 cm) equilibrated in 20 mM triethanolamine, 0.3M NaCl, pH 7.3. The column was eluted with a flow rate of 2 mL/min; 3-mL fractions were collected. HOX that eluted in fractions 14–22 (27 mL) was pooled and concentrated to 5 mL by ultrafiltration in an Amicon 8400 unit equipped with a 10-kDa membrane. The sample was then desalted on two PD10 desalting columns equilibrated in 25 mM bis-Tris buffer, pH 6.0, and then subjected to chromatofocusing on a Mono P HR 5/5 column in three runs. Mono P start buffer was 25 mM bis-Tris, pH 6.0, in which the column was equilibrated. The column was eluted with 10% Pollybuffer 74 in H₂O, adjusted to pH 3.5 with HCl. Flow rate was 1.0 mL/min, fractions of 0.5 mL were collected. A summary of the purification is provided in Table I.

Preparative Electrophoresis and Activity Staining

Samples of 15 µL per lane (in which 20% was sample buffer, 5×) of purified HOX were preparatively electrophoresed by native

PAGE using precast 8–16% Tris-glycine Novex gels according to manufacturer instructions. After electrophoresis, the first and the last lanes were stained for HOX activity by incubating the gel in a solution containing 50 mM sodium phosphate buffer, 100 mM glucose, 50 mg/L phenazine methosulphate and 250 mg/L nitroblue tetrazolium, pH 6.0 (Feinstein and Lindahl 1973, Michal et al 1983, Witteveen 1993). After ≈30 min, the HOX activity was visible as a double charcoal gray band. Equivalent areas in the rest of the lanes were cut out of the gel with a scalpel. The gel pieces were boiled in 25 mM Tris, 192 mM glycine, 2% SDS for 2 min, pH 8.5, to avoid proteolytic degradation during the electroelution.

Electroelution

The gel pieces were electroeluted for 5 hr in an electroeluter (model 422, Bio-Rad), according to the manufacturer instructions. Elution buffer was 25 mM Tris base, 192 mM glycine, 0.1% SDS, pH 8.5. The dialysis membrane in the electroeluter had a molecular weight cutoff of 12–15 kDa. The electroeluted pure HOX was concentrated in a centrifugal concentrator (Microsep, Filtron) equipped with a 10-kDa membrane to a final volume of ≈1.5 mL.

Determination of Molecular Mass

The subunit molecular mass was determined by SDS-PAGE of electroeluted hexose using a precast 8–16% Tris-glycine Novex gel. A low molecular weight (LMW) kit from Pharmacia (MW 14.4–94 kDa) was used as standard.

The native molecular mass was determined by gel filtration on a Superose 12 column (Pharmacia) equilibrated in 50 mM sodium phosphate buffer, pH 6.85, 0.2M NaCl, flow rate 0.65 mL/min, 0.22-mL fractions were collected and screened for HOX activity. Chymotrypsinogen A (25 kDa), bovine serum albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), and ferritin (440 kDa) were used as standards.

Determination of pI

A partially purified sample containing HOX activity was analyzed by IEF using a precast 3–10 IEF gel according to the manufacturer instructions. After electrophoresis, half of the gel was silver-stained. The other half of the gel was activity-stained with nitroblue tetrazolium as described earlier. A broad pI kit (pI 3.5–9.3) was used as a standard.

Protein Sequencing

The electroeluted freeze-dried enzyme was dissolved in 50 µL of 8M urea, 0.4M NH₄HCO₃, pH 8.1. To this sample was added 450 µL of 0.1% trifluoroacetic acid (TFA) and then the sample was run (10 runs) over a reverse-phase Brownlee C2 HPLC column (0.1% TFA gradient in acetonitrile) to remove SDS, glycine and Tris. The volume was reduced to 5–10 µL by freeze-drying. Then 50 µL of 8M urea, 0.4M NH₄HCO₃, pH 8.1, was added. After overlay with N₂ and addition of 5 µL of 45 mM dithiothreitol, the protein was denatured and reduced for 15 min at 50°C under N₂. After cooling to room temperature, 5 µL of 100 mM iodoacetamide was added for the cysteines to be derivatized for 15 min at room temperature in the dark under N₂. Subsequently, 135 µL of water and 5 µg of endoproteinase Lys-C in 5 µL of water was added and the digestion was conducted at 37°C under N₂ for 24 hr.

TABLE I
Summary of a Typical Purification from 500 g of *Chondrus crispus* Seaweed

Purification Stage	Volume (mL)	Total Protein (mg)	Total Activity (Units)	Recovery %	Specific Activity (Units/mg of Protein)	Purification (–fold)
Buffer extract	1,800	64.8	348.8	100	5.4	1.0
HiC	1,400	31.8	165.4	47	5.2	1.0
Chelation	270	0.98	57.9	17	59.1	11.0
Anion exchange	44	0.53	32.2	9.2	60.7	11.3
Gel filtration	27	0.33	22.9	6.6	69.4	12.9
Chromatofocusing	9.5	0.037	10.2	2.9	274.3	51.0

The resulting peptides were separated by reverse-phase HPLC on a Vydac C18 column (0.46 × 15 cm; 10 μm; Separation Group) using solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). The peptides were eluted by a 14-mL gradient from 10–80% B buffer. The largest peptides were loaded on to a 476A sequencer (Applied Biosystems) using pulsed-liquid fast cycles according to the manufacturer instructions.

Analysis of Dough Carbohydrates

Carbohydrate extraction. H₂O (40 mL) was added to 4 g of Danish flour and 4 g of dough from Danish rolls respectively. The suspensions were shaken for 1 hr in a waterbath at 40°C. Afterward, the suspensions were centrifuged for 20 min at 20,000 × g. The clear supernatants were analyzed for carbohydrate content.

Analysis of mono- and disaccharides. After 25× dilution, the carbohydrates were analyzed on a Dionex 4000 ion chromatograph with a PA1 column (4 × 250 mm) and a pulsed amperometric detector. The detector was fitted with an Au-working electrode and a pH reference electrode. The flow rate was 1 mL/min. The monosaccharides were eluted with 27 mM NaOH and the disaccharides were eluted with 150 mM NaOH. Quantification was done by comparison with external standards.

Measurement of Thiol Group and Disulfide Content in a Dough System

The dough model system is described elsewhere (Bak et al, *in press*). The methods of determining the reactive thiol group and disulfide content are described elsewhere (Chan and Wasserman 1993; Bak et al, *in press*).

Rheology and Baking Tests

These tests used partially purified HOX, which means enzyme from the different purification sequences that were tried in order to develop the final purification sequence reported here. The samples were tested for disturbing side activities that could possibly influence the dough strength. The enzymes tested were: protease (protease spot test on casein agar plates), xylanase (amount of carbohydrate solubilized by xylanase action on water-insoluble wheat arabinoxylan was determined by the phenol-sulfuric acid method [DuBois et al 1956]), lipase (determined according to Food Chemical Codex [1981] modified for sunflower oil instead of olive oil, and pH 5.5 instead of pH 6.5), catalase (degradation of H₂O₂ into H₂O and O₂ was followed spectrophotometrically at 240 nm), α-amylase (measured by the starch iodine method) and β-amylase

(measured by the Betamyl method, Megazyme, Ireland).

The partially purified samples of HOX used for baking and rheology tests were all devoid of these possibly interfering activities. The flour used for these tests was a Danish flour with a protein content of ≈12.6%. Extensigraph measurements were performed according to ICC standard no. 114/1 (ICC 1980).

For the baking tests, the recipe was: 1,500 g of flour, 90 g of yeast, 24 g of salt, 24 g of sugar, and water to 400 BU (2%). Mixing took place in a Hobart mixer with hook (2 min, slow; 9 min, fast). The dough temperature was 26°C. After resting at 30°C in a heating cabinet for 10 min, the dough was molded in a Fortuna 3/17/7. The dough was proofed for 45 min at 34°C and 85% rh. The dough was baked for 17 min at 220°C with 12-sec steam (Bago-oven).

RESULTS AND DISCUSSION

Purification of HOX

The crude extract had a specific activity of 5.4 U of HOX/mg of protein. This specific activity is 150× higher than the specific activity reported by Sullivan and Ikawa (1973) using the same assay conditions. This could be due to differences in the seaweed (Sullivan and Ikawa used air-dried *C. crispus*) or to differences in extraction conditions.

The carbohydrate content of the crude extract was very high (≈5–6 g carbohydrate/100 g of *C. crispus* is extracted), and this was a disturbing factor for the purification. This is a well-known phenomenon for seaweed and is probably mainly caused by carrageenan. The extract was bright, fluorescent red-orange in color due to the presence of photosynthetic pigments (phycoerythrin).

The fractionation on phenyl Sepharose did not increase the specific activity, but it was very effective in removing carbohydrate and colored pigments. Otherwise, those two contaminants severely reduced the capacities on the columns. The next step used metal chelating Sepharose FF loaded with Ni²⁺. HOX eluted as a sharp peak at ≈20 mM imidazole, pH 6, (Fig. 1). This step gave very good purification (11-fold). After concentration, the pool obtained

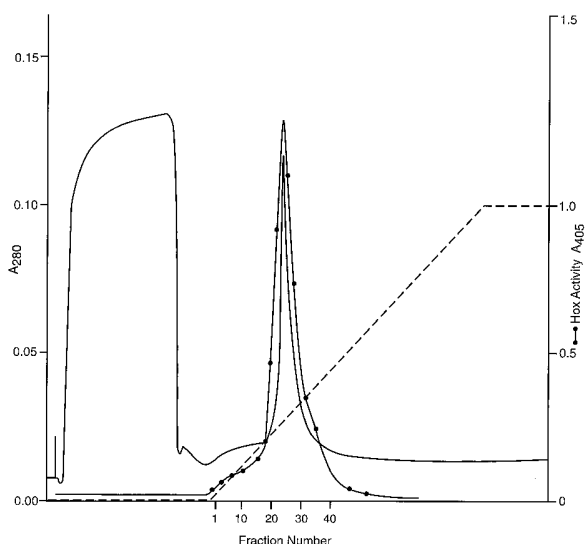


Fig. 1. Ni²⁺ chelating chromatography of hexose oxidase (HOX) after hydrophobic interaction chromatography. Fractions 18–35 were pooled.

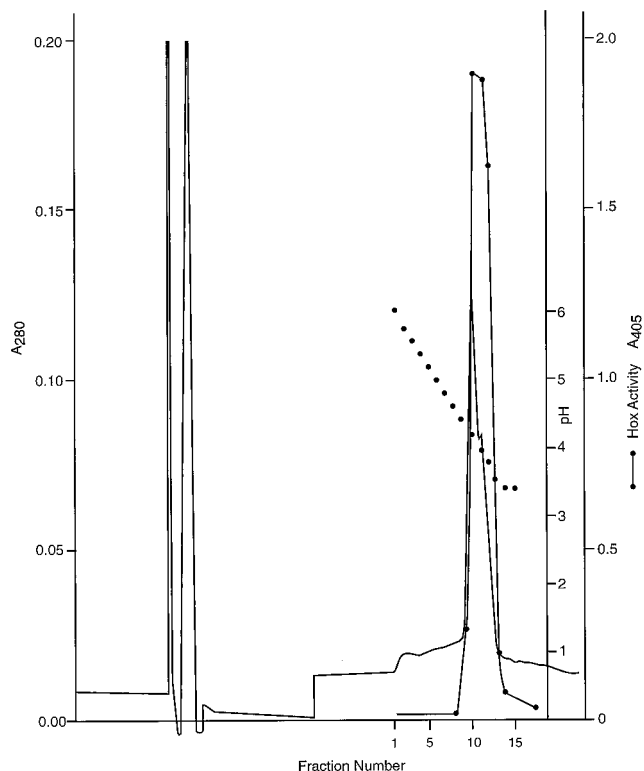


Fig. 2. Mono P chromatofocusing of hexose oxidase (HOX). Dots indicate measured pH gradient.

from metal chelating chromatography was loaded on to an ion-exchange column. Due to a loss in activity, this step did not give any increase in specific activity, but it did remove some contaminants. The same can be said about the subsequent gel-filtration step. The sample from the gel filtration was subjected to chromatofocusing on a Mono P column, from which HOX eluted between pH 4 and 4.2, (Fig. 2). This step gave good purification, resulting in a fourfold purification (Table I). Finally, a recovery of 2.9% and a 51-fold purification was obtained. The specific activity obtained in this work is 274 U/mg. Comparing this to the specific activity of 4.1 U/mg obtained by Sullivan and Ikawa (1973), the result is that HOX in this work is purified ≈ 67 -fold more.

SDS-PAGE of the purified enzyme revealed four major bands after silver-staining (MW 25.5, 34.9, 41.6, and 64.9 kDa, respectively) (gel not shown).

From this it was clear that HOX was still not pure or that it had been broken down into several degradation products. Therefore, native-PAGE followed by activity-staining of HOX was performed, and HOX was cut out of the gel. After silver-staining, HOX was seen as a double band free of contaminating proteins.

The electroeluted pure HOX was checked on SDS-PAGE. It showed one band at 65 kDa plus a broad band at 14–20 kDa (Fig. 3), accounting for the major part of the protein on the gel. We believe this broad band at low molecular mass to be the result of degradation of HOX during electroelution.

Properties of HOX

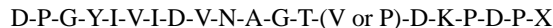
According to results of gel filtration on a Superose 12 column, the molecular mass of HOX is 126 kDa. SDS-PAGE of pure HOX electroeluted from a native gel resulted in a HOX band of 65 kDa. This suggests, that the enzyme consists of two subunits of 65 kDa each. This agrees with Kerschensteiner (1978), who determined the subunit to be 70.8 kDa by SDS-PAGE.

The pI of HOX was determined by IEF. The enzyme showed a double band with pI values of 4.79 and 4.64, respectively. These values are slightly higher than the pI value 4.40 determined by Kerchensteiner (1978).

Protein Sequencing

Several attempts to obtain an N-terminal sequence for the purified HOX were unsuccessful. An effort was made to treat the protein with different concentrations of HCl to remove a possible formyl blocking group on the N-terminal residue, but this still did

not result in any N-terminal data. Amino acid sequence data were, however, obtained from one of the peptides after endoproteinase Lys-C digestion of electroeluted HOX. The peptide had the sequence:



In the fourteenth position, both valine and proline were detected in equal amounts. This could arise from heterogeneity within the two subunits present in HOX.

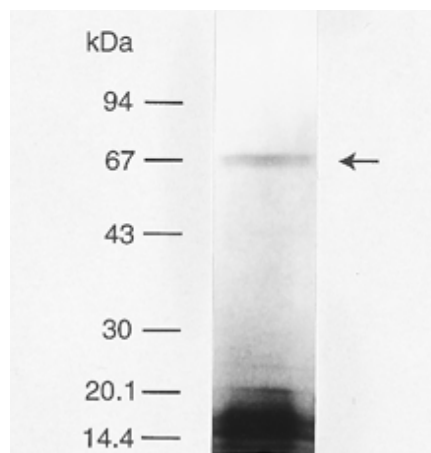


Fig. 3. Reduced sample of electroeluted hexose oxidase analyzed by SDS-PAGE and silver-stained. Band corresponding to hexose oxidase is indicated by arrow. Molecular masses of standard proteins are on the left.

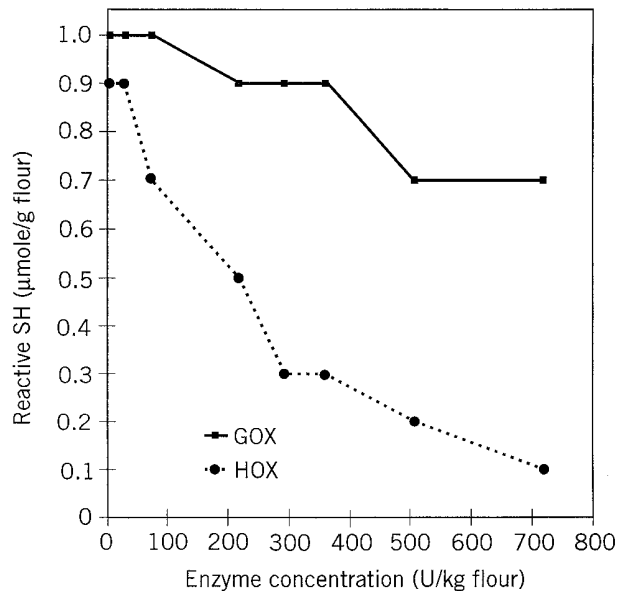


Fig. 4. Reactive thiol groups (SH) as a function of added hexose oxidase (HOX) and glucose oxidase (GOX), respectively.

TABLE II
Michaelis-Menten Constant (K_m) and Maximum Velocity (V_{max})
for Seven Different Sugars

Sugar	K_m (mM)	V_{max} (mUnits)	V_{max}/K_m
D-Glucose	2.7 ± 0.7	225.8 ± 7.2	83.6
D-Galactose	3.6 ± 1.0	169.2 ± 6.1	47.0
Cellobiose	20.2 ± 7.8	129.7 ± 12.6	6.4
Maltose	43.7 ± 5.6	255.8 ± 5.1	5.9
Lactose	90.3 ± 20.6	141.6 ± 12.7	1.6
Xylose	102 ± 26.0	117.1 ± 24.4	1.2
Arabinose	531 ± 158	276.1 ± 28.7	0.5

TABLE III
Reactive Thiol and Disulfide Groups as a Function of Addition of Hexose Oxidase (HOX) and Glucose Oxidase (GOX)

Concentration of HOX (U/kg of flour)	Reactive Thiol (μmole/g of flour)	Reactive Disulfide (μmole/g of flour)	Concentration of GOX (U/kg of flour)	Reactive Thiol (μmole/g of flour)	Reactive Disulfide (μmole/g of flour)
0	0.9	3.2	0	1.0	3.8
25	0.9	3.0	25	1.0	3.1
72	0.7	3.3	72	1.0	3.9
216	0.5	3.4	216	0.9	3.2
288	0.3	3.2	288	0.9	2.7
360	0.3	3.4	360	0.9	3.6
504	0.2	3.3	504	0.7	3.5
720	0.1	3.2	720	0.7	2.3

Substrate Specificity and Kinetics of HOX

HOX could utilize D-glucose, D-galactose, xylose, arabinose, cellobiose, lactose, maltose, maltotriose, and maltotetraose. The V_{max} and K_m values were determined for the sugars tested, except maltotriose and maltotetraose (Table II). HOX had the highest affinities for the monosaccharides D-glucose and D-galactose and for the disaccharides cellobiose and maltose. The affinity for the disaccharide lactose and for the pentoses xylose and arabinose is low. This is in agreement with the theory by Bean and Hassid (1956) that the enzyme has specificity with regard to the C-2 configuration and is nonspecific with regard to C-4 in the hexose. Previous studies found corresponding K_m values of 4 mM for glucose and 8 mM for galactose (Sullivan and Ikawa 1973). Kerschensteiner (1978) reported a somewhat higher value of 10.4 mM for glucose, but he used a different assay which might explain the higher value.

The ratio between V_{max} and K_m shows that the most optimal substrates are D-glucose and D-galactose.

Carbohydrate Content of Dough

To evaluate the level of substrates for HOX and GOX in dough, the carbohydrate content of a dough extracted directly after dough mixing was determined. The concentration in the dough extract (4 g of dough extracted with 40 mL of H₂O) was 4.1 mM maltose, 4.0 mM D-glucose, 3.72 mM fructose, 0.06 mM D-galactose, and below detection limit (0.03 mM) for sucrose, lactose, arabinose, and xylose, respectively. In a flour extract, however, we found the maltose content to be 5.5× higher than the glucose content. In the dough, the sucrose that was added is cleaved to glucose and fructose by yeast invertase. This accounts for the high content of glucose and fructose found here (Linko and Linko 1985). During dough fermentation, yeast will preferentially use glucose. Maltose, on the other hand, is not utilized by yeast. The ability of HOX to utilize maltose should, therefore, be very advantageous during dough development. Additionally, the maltose content could even increase during fermentation because of the activity of starch-degrading enzymes (e.g., β -amylase) that are endogenous in wheat flour or that could be added.

The K_m value of 2.7 mM for glucose for HOX reported here is very much lower than the K_m (glucose) values reported for the functionally related enzyme GOX from *A. niger* (values of 110 mM reported by Gibson et al [1964], 30 mM by Kalisz et al [1991], and 70 mM by van Stroe-Biezen et al [1994]). Despite the large differences in the reported K_m (glucose) values determined for GOX, this indicates that HOX has a much higher affinity for glucose than does GOX. Comparing the K_m values for O₂, HOX is 0.6 mM (Kerschensteiner 1978) and GOX is reported to be 0.80 mM (van Stroe-Biezen et al 1994) and 0.48 mM (Gibson et al 1964). This means that the two enzymes have almost the same affinity for oxygen.

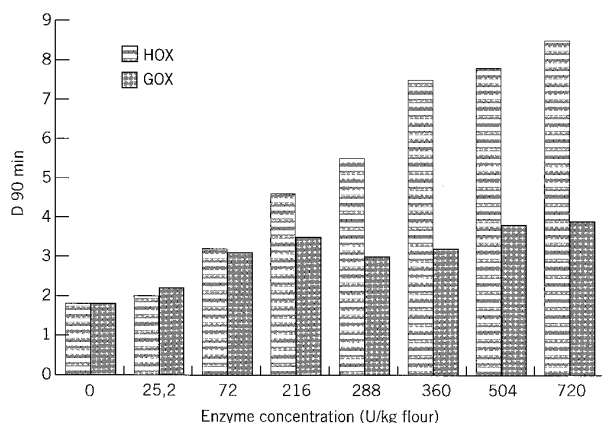


Fig. 5. Results of the D value (an index for dough strength) from extensigraph tests on hexose oxidase (HOX) and glucose oxidase (GOX) after 90 min of resting time.

Based on the substrate kinetics and substrate availability, it can be concluded that HOX has a very good potential as a dough improving additive.

We found that GOX has a positive effect in noodles where no sucrose and yeast is added. In this system, the endogenous flour β -amylase forms maltose, whereas the glucose level is low because of the lack of yeast and sucrose. HOX should therefore be a better, more efficient, improving agent than GOX in systems like noodles.

Thiol and Disulfide Group Content

The change in rheological active thiol and disulfide groups was determined in a dough model system after addition of HOX or GOX, respectively (Table III and Fig. 4). The effect of HOX on the thiol groups shows a strong decrease in the thiol content, which is presumably caused by oxidation by the H₂O₂ formed during the enzyme reaction. Comparing HOX to GOX reveals that GOX, as previously shown by Haarasilta and Pullinen (1992), also causes a reduction in the thiol group content, but the effect of GOX is less pronounced than that of HOX in the same dosages. This may be explained by the ability of HOX to utilize different substrates or by the fact that HOX has a higher affinity for glucose than does GOX.

The level of the thiol and disulfide is in accordance with the values quoted in the literature (Haarasilta and Pullinen 1992, Chan and Wasserman 1993). Direct comparisons cannot be made because of differences in flour and methods.

TABLE IV
Extensigraph Measurements^a for Three Different Resting Times as a Function of Hexose Oxidase (HOX) Addition

Test ^b	<i>B</i>	<i>C</i>	<i>D</i>
	45-90-135 min	45-90-135 min	45-90-135 min
Control (1)	290-300-310	165-162-160	1.8-1.9-1.9
Control (2)	250-290-330	158-164-156	1.6-1.8-2.1
Control (3)	210-275-280	171-182-175	1.2-1.5-1.6
HOX ^c			
25.2 (1)	270-330-380	160-165-146	1.7-2.0-2.6
72 (2)	330-470-540	156-145-129	2.1-3.2-4.2
216 (2)	460-650-750	153-142-125	3.0-4.6-6.0
360 (3)	580-870-880	130-116-117	4.5-7.5-7.5
288 (2)	490-710-745	139-130-102	3.5-5.5-7.3
504 (3)	640-825-920	122-106-94	5.2-7.8-?
720 (3)	730-905-?	109-107-80	6.7-8.5-?

^a *B* = measure of the resistance of the dough; *C* = an expression of the extensibility of the dough; *D* = index for dough strength (calculated as *B/C*).

^b Numbers in parentheses refer to different days of experiments. Results with the same number were made on the same day.

^c Units/kg of flour.

TABLE V
Extensigraph Measurements^a for Three Different Resting Times as a Function of Glucose Oxidase (GOX) Addition

Test ^b	<i>B</i>	<i>C</i>	<i>D</i>
	45-90-135 min	45-90-135 min	45-90-135 min
Control (1)	290-300-310	165-162-160	1.8-1.9-1.9
Control (2)	250-290-330	158-164-156	1.6-1.8-2.1
Control (4)	200-250-275	169-167-168	1.2-1.5-1.6
GOX ^c			
25.2 (1)	290-350-430	161-162-143	1.8-2.2-3.0
72 (1)	350-450-510	159-147-136	2.2-3.1-3.8
216 (2)	340-480-550	148-138-122	2.3-3.5-4.5
360 (4)	480-500-560	157-152-121	3.1-3.2-4.6
288 (2)	325-465-500	169-156-131	1.9-3.0-3.8
504 (4)	350-490-520	155-130-118	2.3-3.8-4.4
720 (4)	435-540-630	148-140-121	2.9-3.9-5.2

^a *B* = measure of the resistance of the dough; *C* = an expression of the extensibility of the dough; *D* = index for dough strength (calculated as *B/C*).

^b Numbers in parentheses refer to different days of experiments. Results with the same number were made on the same day.

^c Units/kg of flour.

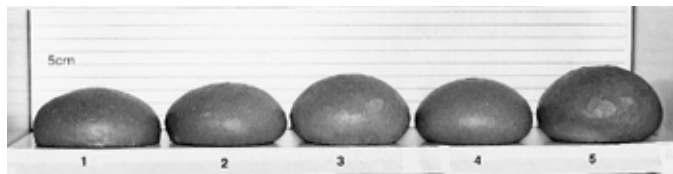


Fig. 6. Danish rolls baked with hexose oxidase (HOX) and glucose oxidase (GOX). 1) Control without enzymes, 2) 108 U of GOX 3) 108 U of HOX, 4) 216 U of GOX, and 5) 216 U of HOX/kg of flour, respectively.

The disulfide group determinations showed no clear trend. In these experiments, there was no correlation between the disappearance of thiol groups and the formation of disulfide groups.

Previous results with GOX (Bak et al, *in press*) showed a strong correlation between the disappearance of thiol groups and the formation of disulfide groups. A possible explanation for the lack of correlation found in the present experiments could be that, during oxidation of the thiols, some are oxidized to disulfides and some are oxidized further to higher oxidation products. Hird and Yates (1961) detected the higher oxidation products cysteine sulfinic and cysteine sulfonic acid after oxidation of thiol with iodate and bromate. This could also be the case here.

The results presented here support the theory that the functional mechanism of HOX is similar to that of GOX.

Rheology Tests

The partially purified preparations of HOX used for the rheology and baking tests were all without side activities that would influence the dough properties. The effect of HOX was compared with that of GOX used in the same dosages (both enzymes assayed on glucose in the standard assay). The results of the extensigraph trials are presented in Tables IV and V and in Fig. 5.

The *B* value is a measure of the resistance of the dough, whereas the *C* value is an expression of the extensibility of the dough. *D* is an index for dough strength and is calculated as *B/C*. The *D* value has to be in the 2.8–3.5 range to be optimum. In this range, the dough has suitable resistance and extensibility. Lower *D* values indicate that the dough is too weak, and higher *D* values that the dough is too strong.

HOX showed a clear strengthening effect. HOX already reached the optimal *D* range at the low dosages and at the short resting times. At the high dosages of HOX, the dough was getting too strong (the values could not even be measured). There was a clear correlation between enzyme dosage and extensigraph response.

In the case of GOX, a strengthening effect was also seen, but it was less pronounced. Higher dosages than in HOX had to be added to obtain high *D* values, and the correlation between dosage and response was less distinct than for HOX.

Baking Test

HOX was also evaluated and compared to GOX in baking tests. Both HOX and GOX had an improving effect on the dough properties and gave an increased specific volume compared to a control without enzyme (Fig. 6 and Table VI). Again, HOX had a stronger effect than GOX when applied in the same dosage.

CONCLUSION

The oxidative enzyme HOX was purified from *C. crispus*, and one peptide amino acid sequence was obtained. When HOX was tested in a dough model system, it caused a fall in the concentration of free thiol groups, presumably because of formation of disulfide bridges between proteins in the gluten fraction. In this experiment, this was not seen as an increase in S-S bonds, but previous experiments have supported this hypothesis. There was good correlation between the reduction in thiol group content and the increased dough strength as measured by the extensigraph. The

TABLE VI
Specific Volume Results for Baking Tests Comparing Glucose Oxidase (GOX) and Hexose Oxidase (HOX)

Test	Volume (cm ³)	Weight (g)	Specific Volume (cm ³ /g)
Control	5,325	1,027	5.18
GOX 108 U/kg of flour	6,075	1,030	5.89
HOX 108 U/kg of flour	6,650	1,036	6.41

results were confirmed by baking tests. Further studies are needed to discover whether the formation of disulfide bonds is the only explanation for the functionality. The beneficial effects might also be due to pentosan gelation or protein-pentosan coupling. The relative importance of these different oxidative events are not known.

HOX was compared with GOX, which is the currently preferred enzymatic alternative to chemical oxidizing agents for bread improvement. It is assumed that the functional mechanism of HOX acting in a dough system is the same as for GOX, but HOX is more effective in the same dosage because of a broader substrate usage and a higher affinity for glucose (lower *K_m* value).

An analysis of mono- and disaccharides from flour and dough indicates that the ability of HOX to utilize maltose makes it especially useful as an oxidative bread improver.

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