

Study on Barley and Malt Polyphenoloxidase. I. Chromatography of Barley, Steeped Barley, and Malt Polyphenoloxidase

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ABSTRACT

A comparative chromatography of polyphenoloxidase isolated from barley, steeped barley, and green malt was made. It consisted of extraction, precipitation, ion-exchange chromatography, gel filtration, and electrofocusing. Polyphenoloxidase was resolved into a high-molecular-weight enzyme and a low-molecular-weight one. The proportion of the two enzymes underwent variations during malting. An increase of polyphenoloxidase activity after steeping was confirmed. The mechanism involved in the enzymatic activation is discussed.

Key words: *Barley, Enzymatic activity, Malt, Oxidase, Polyphenols.*

As a result of a preliminary study on the barley and malt polyphenoloxidase, it appeared that the barley activity was always higher. The assumption was made that the weaker activity of malt could be caused by differences in varieties, malting conditions, and storage periods (19). In order to determine the main factors capable of modifying polyphenoloxidase activity, the evolution of activity during the three main steps of malting was observed. Polyphenoloxidase activity increased primarily during steeping and reached higher levels with recently harvested barleys. The different levels of increase depended also on the variety of barley: for instance, the activity of Aramir barley increased more than that of Gatinais barley during steeping. After studying the kinetics of polyphenoloxidase activation, the hypothesis was proposed that polyphenoloxidase existed partly as a latent form in barley and that it was converted into a more active form during steeping (20). During germination, the activity of malts from recently harvested barleys fluctuated and underwent a slight decrease from the 4th day; on the other hand, the activity of malts from barleys stored for a long time after harvest decreased more regularly and more obviously. The activity did not change with the addition of gibberellic acid. The activity fell slightly when the temperature of germination was raised. During kilning, the activity also decreased. This decrease was caused by blowing air to dry the malt, and not by raising temperature, because the enzyme was relatively thermostable. The decrease of activity could be caused by the inactivation of polyphenoloxidase during the catalysis of the oxidation of certain substrates. During storage, barleys and malts also lost polyphenoloxidase activity (47). Finally, following this study, polyphenoloxidase activity of a barley variety appears to be the result of an increase during steeping and a decrease during the following steps of malting and storage. If the increase during steeping is high (like the Aramir and Gatinais varieties) and the decrease during germination and kilning weak, the activity of malt will be superior to that of barley. If the situation is the opposite (as in the case of the Nordhal variety), the activity of malt will be weaker than that of barley. In order to better understand the different factors involved in the increase of activity during steeping (activation of a latent pre-existing enzyme or *de novo* biosynthesis) and the activity decrease during germination (inactivation reactions or proteolytic hydrolysis of the enzyme), polyphenoloxidase was purified during the steps where a change of activity appeared. In the present publication, we report the purification of polyphenoloxidase from barley, steeped barley, and green malt. The main characteristics of the purified enzyme are presented and discussed.

EXPERIMENTAL

Preparation of Polyphenoloxidase

Step 1. Preparation of Crude Enzymatic Extract. According to

the purpose of the experiment, the extract was prepared from barley, steeped barley (at 12° C for 20 hr), and green malt (6th day of germination). The barley used throughout was a sample of Gatinais from the 1975 harvest. Micromalting was carried out by the method described by De Clerck (9). Unless otherwise indicated, all preparative procedures were performed at 0–4° C.

One thousand kernels were extracted with 180 ml of chilled 0.1 M phosphate buffer pH 7.0 as described (19). After centrifuging at 6000 × g for 20 min, the supernatant was decanted and used as the crude enzymatic extract.

Step 2. Preparation of Final Enzymatic Extract. To the crude enzymatic extract, solid (NH₄)₂SO₄ was added in order to bring the solution to 20% saturation. After the mixture had stood for 30 min, the precipitate was separated by centrifugation at 6000 × g for 15 min and discarded. The concentration of the supernatant was raised to 70% saturation by a further addition of solid (NH₄)₂SO₄. After the mixture had stood for 2 hr, the resulting precipitate was collected by centrifugation at 6000 × g for 30 min. The pellet was resuspended in 0.1 M phosphate buffer pH 7.0 and dialyzed against 10⁻⁴ M phosphate buffer pH 7.0 and freeze-dried. The white powder obtained was designated the final enzymatic extract.

Step 3. DEAE-Cellulose Chromatography. Two hundred sixty mg of the final enzymatic extract was applied to a column (21 × 2.2 cm; total volume = 78 ml) of diethylaminoethyl-cellulose (Whatman DEAE-cellulose DE 52) previously equilibrated with 0.01 M tris-HCl buffer pH 8.6. The same buffer with a linear gradient of NaCl was used as the eluting solvent. Fractions (6 ml) were collected at an elution rate of 22 ml/hr. The active fractions were pooled, dialyzed against 10⁻⁴ M phosphate buffer pH 7.0, freeze-dried and stored at -20° C.

Step 4. Sephadex G-200 Chromatography. An aliquot (between 20 and 60 mg) of polyphenoloxidase from Step 3 was chromatographed on a column (60 × 1 cm; volume = 48.5 ml) of Sephadex G-200 (Pharmacia). The column was eluted with 0.1 M phosphate buffer pH 7.0 at a flow rate of 4 ml/hr. Fractions of 3 ml were collected and assayed for catecholase activity. The fractions corresponding to the two active peaks were collected separately, dialyzed against 10⁻⁴ M phosphate buffer pH 7.0, freeze-dried and kept at -20° C.

Enzyme Assay

Enzyme activity was determined by measuring oxygen consumption with a Clark polarographic electrode fitted into a water-jacketed (37° C) cell equipped with a magnetic stirrer (YSI Model 53) (19). The assay mixture containing 300 μmol of citrate phosphate buffer pH 6.0 and 10–50 units of enzyme was preincubated for 5 min at 37° C, before starting the reaction by the addition of 30 μmol of substrate (catechol or p-cresol). The final volume of the assay solution was 3 ml. The enzyme activity was calculated from the initial slope of the curve in the case of catecholase activity, and from the slope of the curve after the end of the lag period in the case of cresolase activity. One unit of enzyme activity is the amount which catalyzes the uptake of 1 nmol (10⁻⁹ mol) of O₂/min. The specific activity is the number of units/mg of protein.

Protein Determination

Protein concentrations were measured by the method of Lowry *et al.* (29). Bovine serum albumin was used as a standard.

Ribonucleic Acids (RNA) Determination

RNA were determined by the orcinol reaction of Schneider (40)

with yeast RNA (BDH) as a standard.

Deoxyribonucleic Acids (DNA) Determination

DNA were measured by the diphenylamine method (40).

Polyacrylamide Gel Electrofocusing

Electrofocusing was carried out on long gel rods, employing a Shandon apparatus. The gels (100×5 mm) contained 4.85% acrylamide, 0.15% NN'-methylenebisacrylamide, 2% "Ampholine" carrier ampholytes (L.K.B.) in the pH range 3.5–10, and $3 \times 10^{-4}\%$ riboflavin, used for photopolymerization. Two per cent monoethanolamine solution was used for the cathode compartment and 1% phosphoric acid for the anode compartment (on top). The pH gradient was first formed by applying a current of 1.5 mA per tube for 60 min. Afterwards, 0.5 mg of protein dissolved in 0.05 ml of a solution containing 10% sucrose and 2% Ampholine was loaded onto the top of each gel. A protective solution containing 5% sucrose and 2% Ampholine was carefully layered above the protein solution. During the electrofocusing, always performed at 2°C , the voltage was gradually increased to a maximum of 700 V, maintaining a maximum current of 1.5 mA per tube. The total time required for electrofocusing was 4 hr. On completion of electrofocusing, the gels were removed from the tubes. The course of pH gradient in gels was determined with a combined-contact glass electrode (Ingold). Proteins were detected by immersing the gels in 10% trichloroacetic acid solution. The gels were scanned at 445 nm in a Densitochord Photovolt Densitometer 552.

RESULTS

The polyphenoloxidase activity of barley, steeped barley, and green malt was extracted in soluble form in 0.1M phosphate buffer pH 7.0. Polyphenoloxidase could be precipitated at between 21 and 70% ammonium sulfate. Freezing and thawing had no effect on polyphenoloxidase activity. *Tris*-HCl buffer at pH 8.6 did not inhibit the polyphenoloxidase activity.

The DEAE-cellulose elution patterns of final extracts [obtained by $(\text{NH}_4)_2\text{SO}_4$ precipitation] from barley (Gatinais variety), steeped barley, and green malt are presented in Figs. 1, 2, and 3, respectively. Polyphenoloxidase, as an acidic protein, was strongly adsorbed on the anion exchanger. The enzyme was completely separated from peroxidases eluted at the beginning of the NaCl gradient (fractions 9 to 14). Polyphenoloxidase from barley and steeped barley had the same net charge at pH 8.6 and was eluted

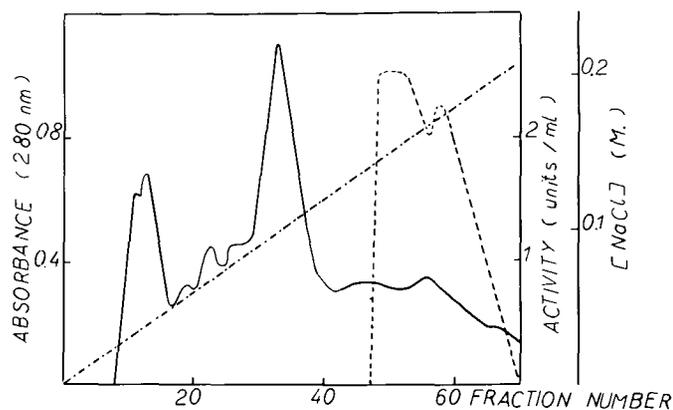


Fig. 1. DEAE-cellulose DE-52 chromatography of the final extract (260 mg) from barley. Column dimensions: 22×2.2 cm, 78 ml; eluting solvent: 10 mM *tris*-HCl buffer pH 8.6 with a linear gradient of NaCl at a flow rate of 22 ml/hr. Abscissa: elution volume indicated in fraction number (each fraction = 6 ml). Ordinate: (—) absorbance at 280 nm, 1-cm light path, (- - - -) catecholase activity, and (·····) NaCl gradient.

between fractions 51 and 67. On the other hand, polyphenoloxidase from green malt was less negatively charged: the enzyme was eluted sooner (fractions 45 to 56). The specific activity of the enzyme from steeped barley (Fig. 2) was three-fold higher than the specific activity of barley (Fig. 1) and two-fold higher than that of green malt (Fig. 3).

The active freeze-dried fractions obtained by chromatography on DEAE-cellulose column were purified by gel filtration. The Sephadex G-200 elution diagrams of polyphenoloxidase are shown in Figs. 4–6. Polyphenoloxidase was separated into two peaks of activity of which elution volume (v_e)/void volume (v_0) ratios were 2.17 for the L-enzyme (lower molecular-weight form) and 1.33 for the H-enzyme (higher molecular-weight form). The L- and H-enzymes were separated from inactive compounds eluted between fractions 16 and 21 and having an A_{280}/A_{260} ratio equal to 0.7–1.0. In barley, the total activity of the H-enzyme was higher than that of the L-enzyme (Fig. 4). After steeping, the L-enzyme had a higher activity (Fig. 5) and this tendency was more marked after 5 days germination (Fig. 6). During steeping and germination, there was no appearance of different forms or new isoenzymes of polyphenoloxidase. The specific activity of the L-enzyme isolated from steeped barley and green malt was superior to that of barley. As a whole, the activities of both H- and L-enzymes varied accordingly to the sequence: steeped barley > green malt > barley, as we have already observed during the DEAE-cellulose chromatography of different final extracts.

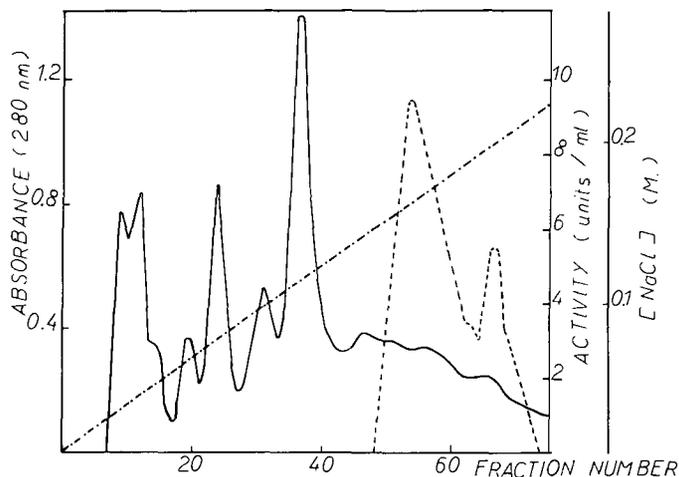


Fig. 2. DEAE-cellulose DE-52 chromatography of the final extract (260 mg) from steeped barley under the conditions described in Fig. 1.

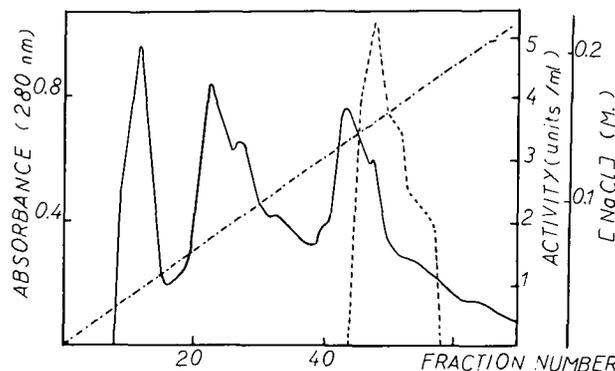


Fig. 3. DEAE-cellulose DE-52 chromatography of the final extract (260 mg) from green malt under the conditions described in Fig. 1.

A summary of the over-all purification obtained in a representative experiment with the four steps outlined above is shown in Table I. Starting from a crude extract of barley (Step 1), the L- and H-fractions eluted from Sephadex G-200 (Step 4) were purified 12.4- and 17.9-fold, respectively. At all stages of purification, enzyme fractions showed both dehydrogenative (catecholase) and hydroxylative (cresolase) activities. The last column of Table I indicates that little change in ratios of the two activities occurred during purification. After the last step of purification, two enzymes were obtained: L, with a catecholase specific activity of 12.4 units/mg, and H, with 17.9 units/mg. Purification data for polyphenoloxidase from steeped barley are given in Table II. The specific activity was equal to 26.2 units/mg for the L-enzyme and 15.2 units/mg for the H-enzyme. In barley, the H-enzyme represented 53.6% of the total activity; after steeping, this proportion decreased to 34.4%. Data for the purification of the green-malt polyphenoloxidase are not presented here because the results were almost the same as those obtained for the steeped barley enzyme, except that the specific activities were weaker.

As the A_{280}/A_{260} ratio was equal to 1.0, the RNA content of the L- and H-enzymes was determined. About 2.2% of RNA was found in the L-enzyme and 4.4% in the H-enzyme. Attempts to hydrolyze the RNA by ribonuclease (Boehringer) were tried. Twenty

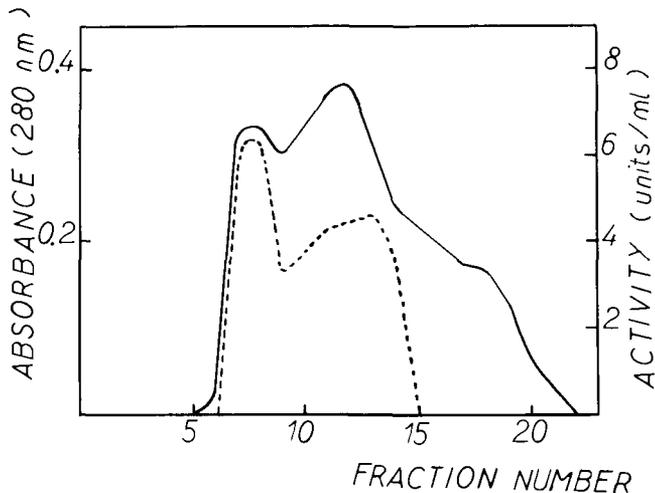


Fig. 4. Sephadex G-200 elution pattern of pooled-barley polyphenoloxidase obtained by ion-exchange chromatography (Step 3 purification). Enzyme (20 mg) was applied to a column (60×1 cm, 48.5 ml) of gel equilibrated with 100 mM potassium phosphate buffer pH 7.0. Elution was done with a buffer of similar composition at a flow rate of 4 ml/hr. Abscissa: fraction number (each fraction = 3 ml). Ordinate: (—) absorbance at 280 nm, 1-cm light path, (- - - - -) catecholase activity.

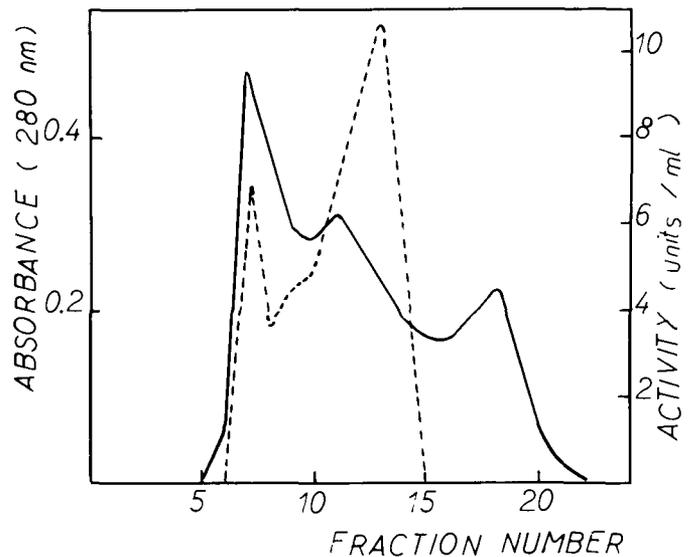


Fig. 5. Fractionation on Sephadex G-200 of steeped-barley polyphenoloxidase from 20 mg of pooled-active fractions obtained by ion-exchange chromatography (step 3 purification). The gel filtration experiment was performed under the conditions described in Fig. 4.

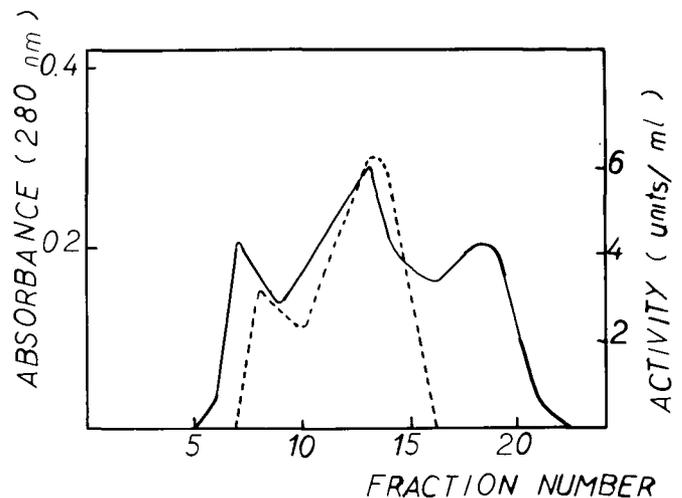


Fig. 6. Fractionation on Sephadex G-200 of green-malt polyphenoloxidase from 20 mg of pooled-active fractions obtained by ion-exchange chromatography (Step 3 purification). The gel-filtration experiment was performed under the conditions described in Fig. 4.

TABLE I
Purification of Barley Polyphenoloxidase

Steps	Proteins mg	Total Activity units	Specific Activity units/mg	Purification	Yield %	Catecholase	
						Cresolase	
I. Crude extract from 1000 kernels	600	600	1.0	1.0	100.0	1.00	
II. Final extract	512	543	1.1	1.1	90.5	1.00	
III. DEAE cellulose	50	443	8.9	8.9	73.8	1.00	
IV. G-200							
H-enzyme	12	215	17.9	17.9	35.8	1.20	
L-enzyme	15	186	12.4	12.4	31.0	1.30	

milligrams of steeped barley polyphenoloxidase obtained by ion-exchange chromatography (Step 3 purification) were incubated with 4 mg of RNase in 1 ml of 0.1M acetate buffer pH 6.0 for 5 hr at 20°C. After incubation, the mixture was submitted to Sephadex G-200 chromatography. The elution pattern, the RNA content, and the total enzymatic activity remained unchanged after the treatment. Increasing the incubation time up to 17 hr did not give any variations. In another experiment, 10 mg of yeast RNA (BDH) was added to 2.6 mg of steeped-barley polyphenoloxidase before activity determination. No differences in activity between the

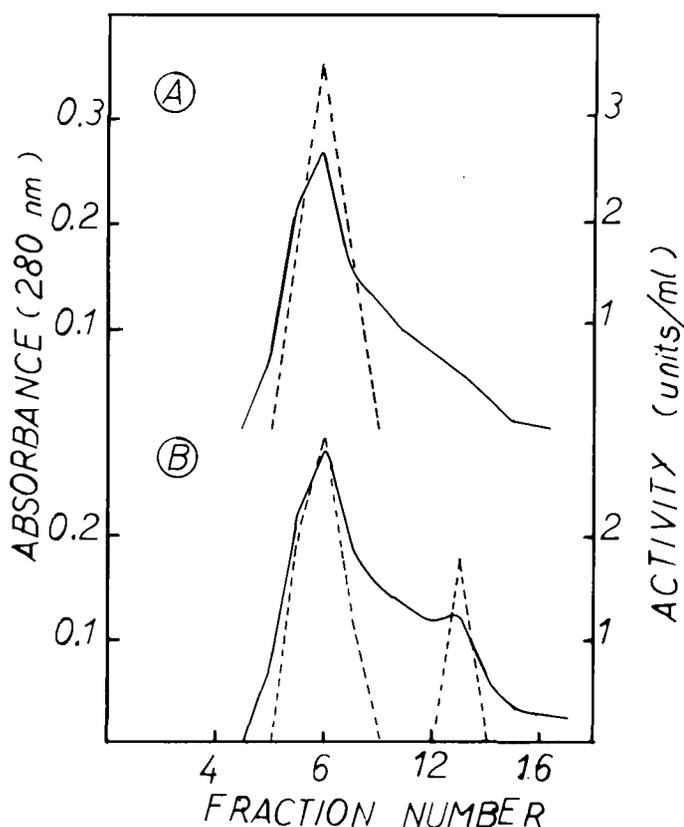


Fig. 7. Sephadex G-200 elution pattern of the H-enzyme from barley. The chromatography was performed under the same conditions as in Fig. 4. A) 10 mg of enzyme dissolved in 1 ml of 100 mM potassium phosphate buffer pH 7.0 and loaded onto the column immediately. B) 10 mg of enzyme dissolved in 1 ml of the same buffer and incubated for 48 hr at 4°C before chromatography.

control and the main assay were observed. Polyphenoloxidase did not contain DNA.

In order to check the possibility of the presence of polyphenoloxidase association-dissociation, the H-enzyme purified from barley by gel filtration (Step 4 purification) was submitted to rechromatography on a Sephadex G-200 column. Two experiments were performed. In one experiment, 10 mg of the purified H-enzyme were dissolved in 1 ml of 0.1M potassium phosphate buffer pH 7.0 and immediately loaded onto the column. In another experiment, the same quantity of enzyme was incubated at 4°C for 48 hr before chromatography. The elution patterns of the H-enzyme with and without previous incubation are shown in Figs. 7A and 7B. It is noted that the H-enzyme was partially converted into the L-enzyme after 48 hr incubation. The experiment was repeated with a higher concentration (46.5 mg) of the steeped-barley H-enzyme. The elution pattern of the H-enzyme submitted to gel filtration without previous incubation is presented in Fig. 8. This experiment showed that the dissociation of the H-enzyme was

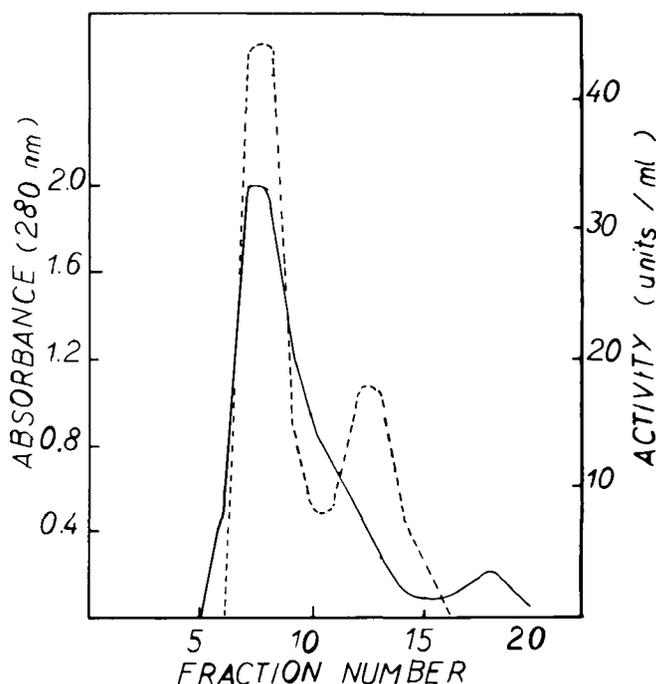


Fig. 8. Sephadex G-200 rechromatography of the steeped-barley H-enzyme (46.5 mg) purified by gel filtration (Step 4 purification). The experiment was performed under the same conditions as in Fig. 4.

TABLE II
Purification of Steeped Barley Polyphenoloxidase

Steps	Proteins mg	Total Activity units	Specific Activity units/mg	Purification	Yield %	Catecholase	
						Cresolase	
I. Crude extract from 1000 kernels	600	1800	3.0	1.0	100.0	1.00	
II. Final extract	512	1618	3.2	1.1	89.9	1.00	
III. DEAE cellulose	50	1248	25.0	8.3	69.3	0.93	
IV. G-200 H-enzyme	20	303	15.2	5.1	16.8	1.10	
L-enzyme	22	577	26.2	8.7	32.1	1.20	

avored by a high-enzyme concentration: about 40% of the H-enzyme was converted into the L-enzyme. It is also noted that the steeped-barley H-enzyme could be transformed into the L-enzyme. On the contrary, the steeped-barley L-enzyme could not be converted into the active H-enzyme, even at high L-enzyme concentration, as shown in Fig. 9. So, the H-enzyme dissociation reaction is much easier than the L-enzyme association.

In an attempt to learn about possible inhibitory natural products existing in the crude enzymatic extract, a series of dialysis experiments was carried out in which the enzymatic activity of the retentate was determined with and without the addition of the diffusate. Crude polyphenoloxidase extracted from 60 g of barley with 180 ml of 0.01M phosphate buffer pH 7.0 was centrifuged at $5000 \times g$ for 20 min. The clear supernatant obtained was concentrated by freeze-drying. Afterwards, the lyophilizate was resuspended in the same buffer and dialyzed against the similar buffer. Both the retentate and the diffusate were again freeze-dried. After being redissolved in 0.01M phosphate citrate buffer pH 6.0, the polyphenoloxidase activity of the retentate (40 mg) with and without the addition of the diffusate (30 mg) was determined after various incubation periods. An activity of 0.5 unit/mg of retentate was found and the activity remained unchanged after the addition of the diffusate which was devoid of an inhibitory action.

To obtain further information on the homogeneity of polyphenoloxidase, both L- and H-enzymes isolated by ion-exchange chromatography (Step 3 purification) were collected and analyzed by polyacrylamide-gel electrofocusing. The barley and steeped-barley L-enzyme migrated as a single protein with an isoelectric point (pI) equal to 5.65. On the other hand, the H-enzyme was resolved into two protein staining bands with pI's equal to 5.25 and 5.45. After steeping, the L-protein staining band increased (Figs. 10 and 11).

DISCUSSION

Multiple Forms of Polyphenoloxidase

Polyphenoloxidase isolated from barley, steeped barley, and green malt was resolved into two active forms on Sephadex G-200 column: a higher molecular-weight enzyme (H-enzyme) and a lower molecular-weight one (L-enzyme) as seen in Figs. 4-6. This result is not singular, since the number of polyphenoloxidase

isoenzymes, or forms isolated from different origins, is variable. All these isoenzymes can be differentiated on the basis of their chromatographic behavior, substrate specificities, electrophoretic mobilities, and thermostabilities. A single polyphenoloxidase occurred in *Streptomyces glaucescens* (26), *Nitella mirabilis* (14), the spinach roots (38), and the ocular choroid of four rodent species (48). Two polyphenoloxidases were present in *Neurospora crassa* (12), potato (34), *Aspergillus nidulans* (7), the leaves of sugar cane (8) and spinach (37), and in the membranes of human erythrocytes (46). Generally, more than two polyphenoloxidases were found, for example, in mushrooms (6,21,22), wheat (25,44), and grapes (10). Often a variable number of isoenzymes was due to association-

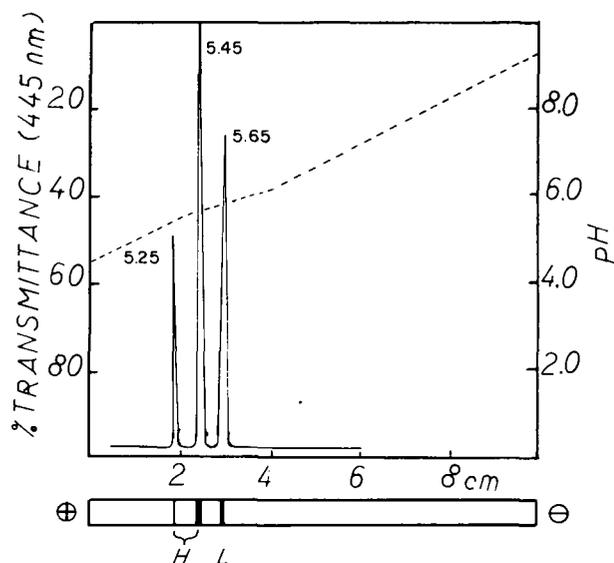


Fig. 10. Polyacrylamide-gel electrofocusing of barley polyphenoloxidase obtained by ion-exchange chromatography (Step 3 purification). Five hundred μg run on gel ($100 \times 5 \text{ mm}$) in the pH range 3.5-10. Abscissa: migration distance. Ordinate: (—) % transmittance and (---) pH gradient.

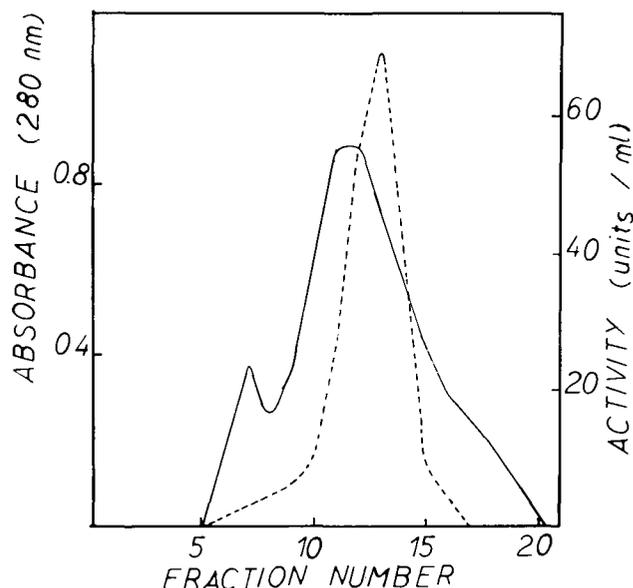


Fig. 9. Sephadex G-200 rechromatography of the steeped-barley L-enzyme (31.1 mg) purified by gel filtration (Step 4 purification). The experiment was performed under the same conditions as in Fig. 4.

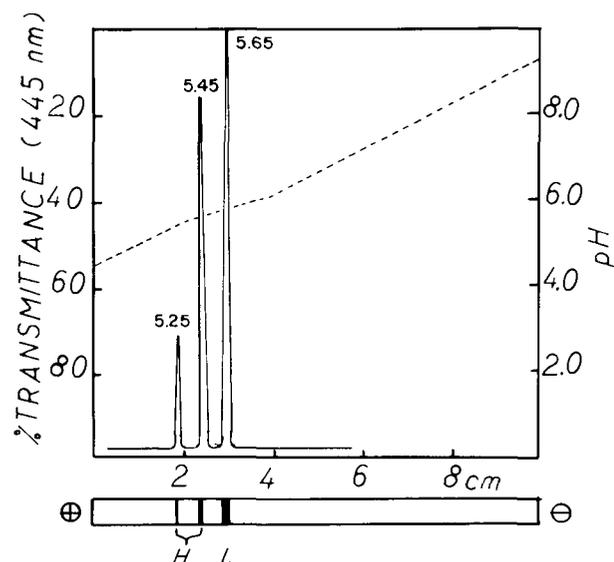


Fig. 11. Polyacrylamide gel electrofocusing of steeped-barley polyphenoloxidase obtained by ion-exchange chromatography (Step 3 purification). Same conditions as in Fig. 10.

dissociation phenomena depending upon the enzyme concentration in solution and other factors (21,22).

Homogeneity of barley and steeped-barley polyphenoloxidase was checked. Polyacrylamide-gel electrofocusing of the H-enzyme produced a pattern of two protein-staining bands with pI's equal to 5.25 and 5.45. On the other hand, the L-enzyme migrated as a single protein with a pI 5.65 (Figs. 10 and 11). The pI's of polyphenoloxidases from grapes (10), membranes of erythrocytes (46) and terrestrial isopods (1) were acidic. But the pI's of frog epidermis polyphenoloxidases were basic (4).

Barley and steeped-barley polyphenoloxidase contained small amounts of RNA which could not be hydrolyzed by RNase. The polyphenoloxidase activity remained unchanged after the addition of an excess of yeast RNA. These experiments suggest that added RNA did not interact with polyphenoloxidase, though previous studies demonstrated that they modified a malt protease activity (15) and they were combined with papain and pepsin (16,17). Attempts to remove bound RNA resulted in loss of polyphenoloxidase activity from apples (3) and soils (30).

Activation of Polyphenoloxidase during Steeping

If the DEAE-cellulose-elution diagrams of barley and steeped-barley polyphenoloxidase (Figs. 1 and 2) are compared, a great difference can be noted: after steeping, the specific activities increased almost three-fold. On the other hand, the enzyme possessed the same net charge before and after steeping and thus was eluted in the same fractions. Likewise, if the Sephadex G-200 elution patterns of polyphenoloxidase (Figs. 4 and 5) are considered, the same conclusion may be reached: the molecular weights of the H- and L-enzymes remained unchanged, but the total activity increased three-fold after steeping. The results confirm our previous finding: polyphenoloxidase activity increased primarily during steeping (20). Before discussing the different mechanisms involved in the increase of polyphenoloxidase activity during steeping, the hypothesis of *de novo* biosynthesis of the enzyme can be discarded, since it was not the enzyme concentration, but rather the specific activity, which was increasing (Tables I and II). Besides, there was no appearance of new forms or different isoenzymes during steeping (Figs. 10 and 11). The conversion of active polyphenoloxidase from a latent proenzyme or from an enzyme with a weaker activity might act via different mechanisms—elimination of an endogenous inhibitor, a limited proteolysis, or a variation in the configuration of the enzyme. All these different processes will be taken into account.

The hypothesis of the elimination of polyphenoloxidase inhibitors leading to the activation of enzyme is possible. Different kinds of inhibitors might intervene, for example, a competitive inhibitor like a phenol substituted with an electron-withdrawing group (11). In a previous study (20), before determining polyphenoloxidase activity, the crude extract was always dialyzed on a column of Sephadex G-25 and submitted to ultrafiltration in order to discard any inhibitory natural products. Moreover, the maximum velocity of the reaction catalyzed by polyphenoloxidase was determined. The continual increase of the maximum velocity during steeping allows these researchers to reject the action of a competitive inhibitor. Another possibility would be the action of a noncompetitive inhibitor—for example, a copper-complexing agent. In this case, the increase of activity during steeping would be caused by an addition of copper to the apoenzyme. This possibility is also ruled out because barley polyphenoloxidase activity was not stimulated by the addition of $10^{-3} M$ $CuSO_4$. In order to confirm that there was no inhibition, a crude extract from barley was dialyzed and the polyphenoloxidase activity of the retentate was determined with and without the addition of the diffusate. Enzyme activity was the same in both cases. So, the action of any competitive, noncompetitive, and dialyzable inhibitors may be eliminated. Another possibility would be a high-molecular-weight protein acting as an inhibitor of polyphenoloxidase, as reported by Bull and Carter (7). This nondialyzable inhibitor was only separated from the *Aspergillus nidulans* enzyme on DEAE-

cellulose chromatography. In the present study, barley polyphenoloxidase was not activated by the method of purification, *i.e.*, ammonium sulfate fractionation, dialysis, DEAE-cellulose, and G-200 chromatographies. Thus the possibility of a high-molecular-weight protein acting as an inhibitor appeared remote.

Many studies have shown that polyphenoloxidases were considered to be activated after their release from membranes of mitochondria or chloroplasts to which they were bound (31). In our case, this possibility is not probable because barley polyphenoloxidase is a nonparticulate enzyme, being 90% soluble in 0.1 M phosphate buffer pH 7.0 and 0.01 M *Tris-HCl* pH 8.6. After centrifuging at $15,000 \times g$ for 60 min, the small quantity of precipitate obtained was devoid of enzymatic activity.

Different polyphenoloxidases from animal origin have been reported as activated by a partial proteolysis, leading to active enzymes with lower molecular weights (2,4,39,41,49). This possibility cannot be taken into consideration because the polyphenoloxidase isolated from barley and steeped barley was eluted between the same fractions on Sephadex G-200 chromatography (Figs. 4 and 5).

Another consideration for polyphenoloxidase activation could be a conformational change after varied treatments. Indeed, *in vitro* activation of certain polyphenoloxidase is reported by detergents, urea, acids, bases (23,24,36,37), and oxygen (27). Different physical treatments like heat (13), light (45), and irradiation (35) also activated the enzyme. At least in one case, conformation changes of the enzyme were observed (28). In this case, the activation of steeped-barley polyphenoloxidase could be caused by a conformational change. The activation energy of the enzymatic activation during steeping was determined to be 15.1 kcal/mole (20). This value lay between the values of two known reactions: the irreversible inactivation of polyphenoloxidase during the catechol oxidation with an activation energy of 7.5–15.5 kcal/mole (18) and the thermal denaturation of the enzyme with 80 kcal/mole (33). The first process did not involve any extensive damage to the protein whereas, during the second process, the enzyme suffered marked changes in spatial configuration. The enzyme activation during steeping with an apparent activation energy 15.1 kcal/mole would be due to a small conformational change of the enzyme and this reaction would be simpler than that which happened in *Drosophila* (32, 42).

If the specific activities of the H- and L-enzymes purified by Sephadex G-200 chromatography are compared, it is noted that, before steeping, the specific activity of the H-enzyme was a little higher than that of the L-enzyme (Table I). After steeping, the specific activity of the H-enzyme decreased about 21%, whereas the specific activity of the L-enzyme increased two-fold (Table II). These determinations showed that the L-enzyme was more activated during steeping. So, the conformation of the L-enzyme from barley and steeped barley would be different.

In addition to the activation of the L-enzyme by conformational change, the possibility of the conversion of the H-enzyme to the L-enzyme during steeping (and germination) must be considered. Indeed, the H-enzyme was partially transformed to the L-enzyme after 48 hr incubation (Fig. 7). This reaction was favored by a high concentration of the H-enzyme (Fig. 8). We note that the H-enzyme isolated from barley or steeped barley was able to be converted partially to the L-enzyme (Figs. 7 and 8). This reaction led to an increase in the L-enzyme concentration after steeping and after 5 days germination (Figs. 5 and 6). On the other hand, the L-enzyme could not be converted to the active H-enzyme (Fig. 9).

All these experiments showed that, during steeping, two reactions took place: a) a conversion of the H-enzyme to the L-enzyme and b) an activation of the L-enzyme, probably due to a conformational change.

A similar case could be found in the two spinach polyphenoloxidases. Both were convertible and the lighter enzyme possessed a higher specific activity (37). The polyphenoloxidase

from sugar cane leaves also was resolved into two enzymes on G-200 column and the smaller molecular-weight enzyme was much more active (8).

The present work has revealed a novel mechanism in the control of enzymatic activity. In barley, polyphenoloxidase occurred predominantly under a higher molecular-weight enzyme (H-enzyme) which was not very active. During steeping, the H-enzyme was converted into a lower molecular-weight enzyme (L-enzyme) which possessed a higher enzymatic activity, probably due to a conformational change. Previous studies had shown variations in enzymatic activities during steeping (5,43), but none of the studied enzymes possessed so high an increase in activity as the polyphenoloxidase. According to all these works, steeping is an important physiological process during which the activities of different enzymes in the kernels undergo large variations, as in the case of β -amylase (5), glucose-6-phosphate dehydrogenase (43), and especially polyphenoloxidase.

The molecular weights, spectral, and catalytic properties of polyphenoloxidase will be presented in subsequent publications.

SUMMARY

Polyphenoloxidase was purified from barley, steeped barley, and green malt by using salt fractionation, DEAE-cellulose chromatography, and gel filtration. Polyphenoloxidase was resolved on Sephadex G-200 column into two peaks of activity: a higher molecular-weight enzyme (H) and a lower molecular-weight enzyme (L). The proportion of the H-enzyme decreased during steeping and germination. After steeping, the specific activity of the L-enzyme increased most. The experiments suggest that the increase of the L-enzyme activity during steeping was caused by a conversion of the H-enzyme to the L-enzyme and a conformational change of the latter. When submitted to polyacrylamide-gel-electrofocusing analysis, the H-enzyme was separated into two protein bands and the L-enzyme migrated as a single protein.

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