THE MECHANISM OF ACTION OF MALT BETA-GLUCANASES

IV. The Preparation and Properties of Laminarinase from Germinated Barley[1]

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ABSTRACT

Purified laminarinase was prepared from a 0.6% sodium chloride extract of germinated barley. The fraction which precipitated between 10 and 40% saturation in ammonium sulfate was dialyzed and the albumin fraction was heated for 2 hr. at 50°C. The heated enzyme was further purified by chromatography on DEAE and CM-cellulose ion exchange columns.

The purified laminarinase was soluble in both water and dilute salt solutions and was quite stable at 50°C. Its activity was stimulated by sodium chloride and by citrate-phosphate buffer. The enzyme exhibited a broad pH curve with an atypical sharp optimum at pH 4.59. It hydrolyzed laminarin in a random fashion, producing sugars which when chromatographed moved with glucose, laminaribiose, and gentiobiose, as well as higher oligosaccharides.

Germinated barley contains enzymes able to hydrolyze several beta-linked glucose polysaccharides. Preece and co-workers (13), and Meredith and co-workers (3) reported the presence of endo-beta-glucanase activity in germinated barley. In addition, endo-carboxymethyl cellulase (5,15), exo-carboxymethyl cellulase (11), exo-beta-glucanase (2, 14), laminarinase (4), transglycosidase (5), cellobiase (4), laminaribiase (4), and gentiobiase (11) activities have been reported. Bass and co-workers (1,2) concluded from chromatographic studies of the hydrolysis products that the degradation of barley beta-glucan by extracts of germinated barley involved the actions of endo-beta-glucanase, exo-beta-glucanase, and cellobiase. Luchsinger and co-workers (8,9,11) concluded from studies of solubility, pH optima, and heat stability that germinated barley contained two distinct endo-beta-glucanases in addition to other beta-glucanase activities.

Separation of green malt beta-glucanases by salt fractionation and dialysis (9) or by column separations on activated alumina (4) and cellulose ion exchange resins[3] have generally resulted in preparations containing two or more beta-glucanase activities. The purpose of this paper is to describe a method to prepare laminarinase from germinated

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barley free of other beta-glucanase activities and to present certain aspects of its action.

Materials and Methods

Enzyme Source. Germinated barley was a gift from the Kurth Malting Co., Milwaukee, Wisconsin.

Substrates. 1. Laminarin I.L. No. 24 was purchased from The Institute of Seaweed Research, Inveresk, Midlothian, Scotland, United Kingdom. A 0.714% stock solution (w/v) adjusted to pH 4.60 was used.

2. Carboxymethyl cellulose (CMC) was a gift from the Hercules Powder Company, Wilmington 99, Delaware. (a) A stock solution containing 0.6% CMC-7HP (w/v) adjusted to pH 4.70 was used for measuring viscosity-decreasing activity. (b) A stock solution containing 1.9% CMC-4MP (w/v) adjusted to pH 4.70 was used for measuring reducing-power activity.

3. Barley beta-glucan was prepared as described previously (12).

Ion Exchange Celluloses (Selectacel). 1. DEAE-cellulose was washed as described by Peterson and Sober (12) and equilibrated with 0.001*M* sodium phosphate buffer, pH 7.00.

2. CM-cellulose was washed as described by Peterson and Sober (12), except that 0.5*N* hydrochloric acid and 0.5 *N* sodium hydroxide (rather than the 1*N* reagents) were used, and the product was equilibrated with 0.001*M* sodium phosphate buffer, pH 4.00.

3. P-cellulose was prepared in the same manner except that 0.1*N* hydrochloric acid and 0.1*N* sodium hydroxide were used.

Determination of Enzyme Activities. Viscosity-decreasing activity was determined at pH 4.70 and 30°C. by measuring the change in viscosity of the substrate in Ostwald No. 200 viscometers. Reaction mixtures contained 7 ml. of substrate solution, 2 ml. of 0.2*M* sodium acetate buffer, and 1 ml. of enzyme solution. One unit of enzyme activity is the amount required to increase the reciprocal of the specific viscosity of the 10-ml. reaction mixture by 0.01/30 min.

Reducing-power activity was followed by a modified dinitrosalicylic acid method (10). The reaction mixtures contained 7 ml. of substrate solution, 2 ml. of 0.2*M* sodium acetate buffer, and 1 ml. of enzyme solution. Reducing-power activity on laminarin was determined at pH 4.60 and 30°C. One unit of enzyme activity is the amount required to increase the reducing power of the 10-ml. reaction mixture the equivalent of 1 mg. of glucose per hr. Reducing-power activities on other substrates were determined at pH 4.70 and 30°C. as described previously (11).

Estimation of Protein Concentration. Protein concentration was

estimated from the ultraviolet absorption of the solutions, using the formula given by Kalckar (7).

Preparation of Laminarinase. A 102-g. sample of frozen germinated barley was blended in 240 ml. of 0.6% sodium chloride for 1 min. in a Waring Blendor. The slurry was held for 2 hr. at room temperature, after which it was centrifuged for 20 min. at 1,000 × *g*. A total of 235 ml. of supernatant, pH 5.56, was obtained.

The supernatant was fractionated with ammonium sulfate. The fraction which precipitated between 10 and 40% saturation was dissolved in 0.6% sodium chloride and dialyzed against five successive 3-liter aliquots of distilled water at 0°–4°C., with gentle agitation, for periods of 3, 4, 11, 11, and 11 hr. The precipitate was removed by centrifuging and the albumin fraction, pH 5.31, was heated for 2 hr. at 50°C.

An aliquot of the heated enzyme (105 mg. of protein) was adjusted to pH 7.00 and 0.001*M* sodium phosphate and placed on a DEAE-cellulose column 1.2 cm. diameter by 30 cm. long. Additional 0.001*M* sodium phosphate buffer, pH 7.00, was passed through the column. The elutant was collected in 10-ml. aliquots, using a fraction collector equipped with a 280 mμ scanner and recorder. The elutant in tubes 4 to 10 (the front) contained all the material absorbing at 280 mμ not held on the anion exchange column. The laminarinase activity was present in the DEAE-cellulose front.

The pH of the front was adjusted to 4.00 with hydrochloric acid and placed on a CM-cellulose column (1.2 cm. diameter by 30 cm. long). The column was eluted first with 100 ml. of 0.001*M* sodium phosphate buffer, pH 4.00, and then with a gradient composed of 300 ml. of 0.001*M* sodium phosphate, pH 4.00, and 300 ml. of 0.2*M* sodium phosphate, pH 5.80. After 100 ml. were collected, the gradient was stopped, and elution of the laminarinase was completed with no further gradient (Fig. 1b). The calculated phosphate concentration of the elutant at this stage was 0.003*M*.[4] The laminarinase peak normally appeared after about 150 to 160 ml. of 0.003*M* sodium phosphate had been collected. Usually the solution was made 0.001*M* in GSH.[5]

Results

Representative chromatographic separations of germinated barley extracts on the cation exchangers CM-cellulose and P-cellulose are shown in Fig. 1.

[4] A continuous gradient was used in Fig. 1a; 450 ml. of 0.001*M*, pH 4.00, sodium phosphate buffer and 450 ml. of 0.25*M* sodium phosphate buffer, pH 6.5. Under these conditions laminarinase was eluted from the CM-cellulose column at a concentration of approximately 0.05*M* sodium phosphate.

[5] Occasionally a low recovery of activity after column separation is observed. It was suspected from preliminary experiments that GSH effected a slow reactivation of the laminarinase activity. Later work has shown that the GSH may not have been responsible for the reactivation.

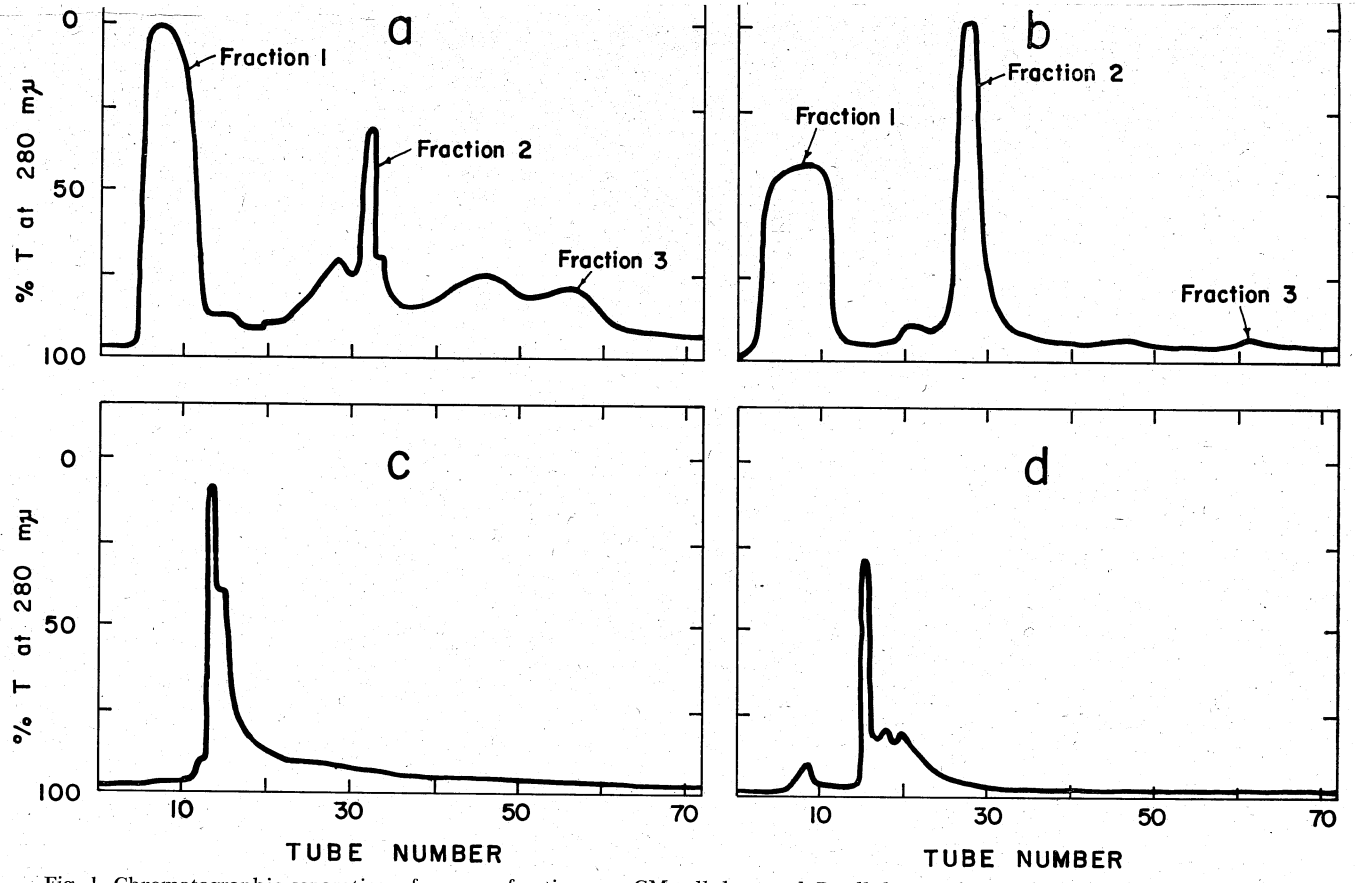

Fig. 1. Chromatographic separation of enzyme fractions on CM-cellulose and P-cellulose cation exchange resins. (a) Crude extract was separated on a CM-cellulose column using a gradient composed of 500 ml. of 0.001*M* sodium phosphate, pH 4.0, and 500 ml. of 0.25*M* sodium phosphate, pH 6.3. (b) The albumin fraction of exhaustively dialyzed extract was separated on a CM-cellulose column using a gradient composed of 450 ml. of 0.005*M* sodium phosphate, pH 4.0, and 450 ml. of 0.25*M* sodium phosphate, pH 6.3. (c) Fraction 2 from separation b (tubes 23–31) was separated on a P-cellulose column using a gradient composed of 500 ml. of 0.1*M* sodium phosphate, pH 4.0, and 500 ml. of 0.1*M* sodium phosphate, pH 6.35. (d) The fraction from the separation in c represented by tubes 12–17 was separated on a P-cellulose column using a gradient composed of 500 ml. of 0.1*M* sodium phosphate, pH 4.90, and 500 ml. of 0.1*M* sodium phosphate, pH 6.20.

TABLE I

PURIFICATION OF LAMINARINASE

STAGE OF PURIFICATION [a]	ACTIVITY	PROTEIN	SPECIFIC ACTIVITY	YIELD
	units	*mg.*	*units/mg.*	%
1	10,400	587	1.77	
2	5,950	246	2.41	57
3	5,350	37.2	14.4	51
4	4,950	17.7	28.0	48
5	3,770	3.75	101	36

[a] Sodium chloride extract (236 ml., 0.6%) was obtained from 102 g. of germinated barley (stage 1). The fraction soluble in 10% saturated but insoluble in 40% saturated ammonium sulfate solution was collected (stage 2). It was dialyzed and then heated for 2 hr. at 50°C. (stage 3). The preparation was then passed through DEAE-cellulose (stage 4) and then was passed through CM-cellulose (stage 5).

The laminarinase activities at various stages of purification are shown by the results of a representative purification given in Table I.

Effect of Substrate Concentration. The activity increased until a substrate concentration of 0.5% laminarin was reached, and then remained constant at 2.5 units over a concentration range of 0.5–0.8%.

Effect of Ionic Strength and Ionic Composition. The activity of the enzyme in 0.04*M* sodium acetate buffer was increased by 40 to 50% on addition of sodium chloride at concentrations of 0.01 to 0.20*M*. In a buffer containing sodium phosphate (0.04*M*) and sodium citrate (0.02*M*), the activity was 40% higher than in 0.04*M* sodium acetate.

Effect of pH. Laminarinase activity was measured over the pH range 3.90 to 5.85 using phosphate-citrate buffers; the pH values found immediately after the reaction are reported. Each point represents the average of at least three activity determinations using two different enzyme preparations. The results obtained in the presence of 0.01*M* sodium chloride show that the pH optimum is approximately pH 4.59 (Fig. 2).

The substrate concentration required to saturate an enzyme may be affected by the pH at which the reaction is run. Tests showed that the enzyme also was saturated in the presence of 0.5% laminarin at pH 3.92 and 5.83.

A similarly "atypical" pH curve was obtained with crude laminarinase prepared by a limited dialysis of sodium chloride extracts of germinated barley to remove reducing sugars. The crude laminarinase exhibited all the beta-glucanase activities found in the original extract.

Action on Other Beta-Glucans. Purified laminarinase was tested for reducing-power activity on barley beta-glucan, CMC-4MP, cellobiose, and gentiobiose; and for viscosity-decreasing activity on barley beta-glucan and CMC-7HP. The preparation exhibited only traces or no action on these substrates.

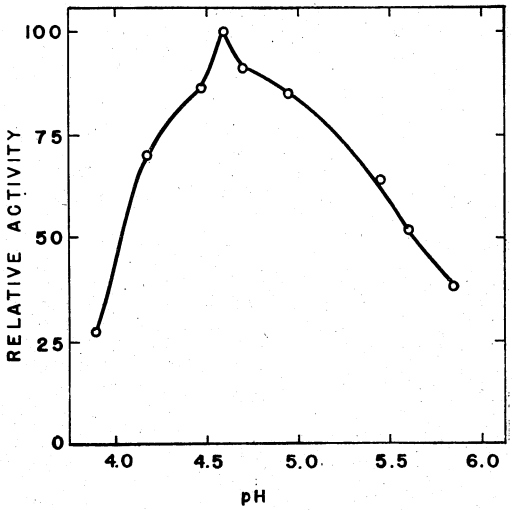

Fig. 2. Effect of pH on the activity of purified laminarinase.

Mode of Action of Laminarinase. Laminarin was hydrolyzed with purified laminarinase for periods of 20 min., 1, 3, 7, and 24 hr. The resulting samples were deproteinized by heating and centrifuging; desalted and concentrated; and the products were separated by paper chromatography. The samples were spotted on Whatman No. 1 chromatographic paper and irrigated with n-butanol-acetic acid-water (10:3:3) for 3 and 6 days. Representative chromatograms for 3- and 6-day irrigations are shown in Fig. 3.

Chromatography of Unheated Enzymes on P-Cellulose. The albumin fraction was chromatographed on CM-cellulose without first being heated to remove endo-beta-glucanase activity. Fraction 2 (Fig. 1b), which then contained both endo-beta-glucanase (EBG) and laminarinase activities, was collected and subsequently chromatographed on a P-cellulose column. The P-cellulose column was eluted with a gradient composed of 500 ml. of 0.1*M* sodium phosphate, pH 4.00, and 500 ml. of 0.1*M* sodium phosphate, pH 6.35. The proteins showed signs of separating (Fig. 1c) and the main peak and the shoulders were assayed separately. The fraction in each tube (tubes 12–17) contained both EBG and laminarinase activities. The ratios of the two activities were similar for each portion, which suggested that the two activities were not being separated. Reseparation of the fraction in tubes 12–17 on a P-cellulose column, using a gradient composed of 500 ml. of 0.1*M* sodium phosphate, pH 4.90, and 500 ml. of 0.1*M* sodium phosphate, pH 6.20, resulted in four main peaks (Fig. 1d). There was EBG as well

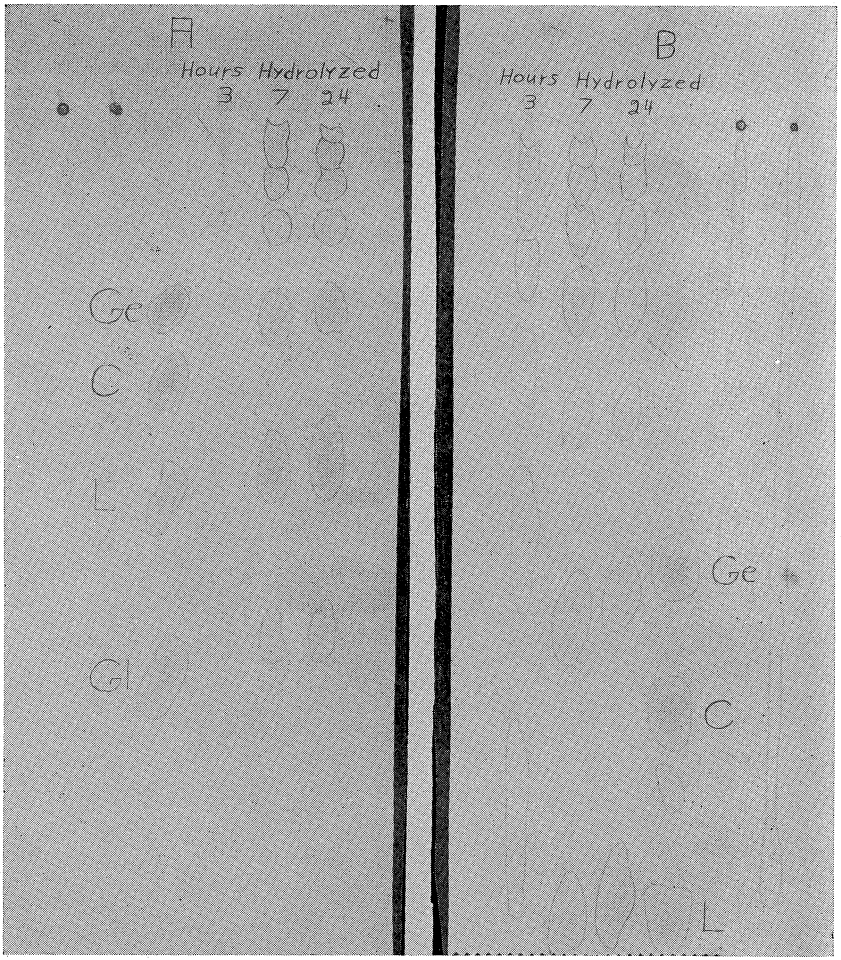

Fig. 3. Separation of enzyme hydrolysis products of laminarin. The chromatogram in part A was irrigated for 3 days and that in part B was irrigated for 6 days (n-butanol-pyridine-water, 10:3:3).

as laminarinase activity present in each of the fractions. However, approximately 90% of the EBG activity was lost during the second separation on P-cellulose.

Separation of laminarinase from EBG was effected by using a gradient consisting of sodium phosphate and sodium chloride. Unheated albumin was placed on P-cellulose and the column was eluted with 50 ml. of 0.01*M* sodium phosphate-0.06*M* sodium chloride solution, pH 4.0, to remove the front. The column was then developed using a gradient consisting of 400 ml. of 0.01*M* sodium phosphate-

0.06*M* sodium chloride, pH 4.8, and 400 ml. of the same reagents at pH 6.0. Laminarinase free of EBG was eluted in a peak collected in tubes 30–35 (pH 4.1 to 4.6). Tubes 36 and 37, representing the forward slope of the peak, contained small amounts of both laminarinase and EBG activities. EBG free of laminarinase was eluted in tubes 38–50 (pH 4.8–5.4). There was no protein peak over this range, but only a low, flat plateau as reflected by the 280-mμ scanner.

Limited experiments showed that the laminarinase prepared by P-cellulose fractionation was stimulated by sodium chloride and exhibited a pH optimum similar to that of the laminarinase described in the previous section. In addition, the preparation exhibited neither viscosity-decreasing activity nor reducing-power activity on barley beta-glucan, carboxymethyl cellulose, cellobiose, or gentiobiose.

Discussion

The results in Table I show that the laminarinase was purified approximately 50- to 60-fold. Laminarinase was not held on DEAE-cellulose under the conditions used (0.001*M* sodium phosphate, pH 7.00), which suggests an isoelectric point above 7. However, certain contaminating proteins were removed, since omission of the DEAE step resulted in a final preparation with approximately one-half the laminarinase activity per mg. of protein. Tests on several substrates showed that the purified laminarinase was essentially free of other beta-glucanase activities initially present.

The enzyme has the characteristics of an albumin, being soluble in both water and dilute salt solutions. The activity of the enzyme was higher in the presence of sodium chloride and sodium acetate, or sodium citrate and sodium phosphate, than in the presence of sodium acetate alone. The enzyme was quite stable to heat; only a small part of the activity was lost during 2 hr. of heating at 50°C. (Table I).

Friedlander and co-workers reported a molecular weight of 3,500 for insoluble laminarin (6). On that basis a K_m of the order of 10^{-4} to $10^{-3}M$ was estimated from the effects of substrate concentration on activity.

The paper-chromatographic experiments supported earlier conclusions by Bass and Meredith that malt laminarinase acts in a random fashion (4). Glucose, laminaribiose, gentiobiose, and slower-running oligosaccharides appeared on the chromatograms (Fig. 3) in the 3-, 7-, and 24-hr. hydrolysates. Other chromatograms showed light but definite oligosaccharide spots after only 20 min. of hydrolysis. The appearance of several oligosaccharides shortly after initiation of the

reaction is consistent with random cleavage by the enzyme.

The pH curve (Fig. 2) appears to be approaching a rather broad peak near pH 4.7 until the sharp distortion with the optimum at pH 4.59 occurs. Both preparations of laminarin exhibited this rather atypical peak. The reason for the distorted pH curve is not immediately apparent. The behavior might suggest that the enzyme is acting on a substrate which does not quite "fit." Under these conditions a sharply higher activity could occur over the very narrow pH range where the ionization of the active site was such that the substrate would fit. A second possibility might be the presence of two enzymes acting on the same substrate, one of which possessed a very narrow pH optimum.

Acknowledgments

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