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PURIFICATION AND SOME PROPERTIES OF β-1,3-GLUCAN GLUCANOHYDROLASE FROM THE CRYSTALLINE STYLE OF BIVALVIA, *SPISULA SACHALINENSIS*

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SUMMARY

1. β-1,3-Glucan glucanohydrolase, known as laminarinase (EC 3.2.1.6), has been isolated from the crystalline style of Bivalvia, *Spisula sachalinensis*, by means of gel filtration followed by SE-Sephadex column chromatography.

2. The optimum pH for hydrolysis of laminarin was 5.8. Optimum temperature was 45°. Enzyme was relatively thermostable. The enzyme hydrolyzed laminarin; it was not active towards glucobioses and other sugars. On lengthy hydrolysis laminarin gave rise to glucose and laminaribiose. Mol. wt. 22 000.

INTRODUCTION

In a previous paper[1] we reported that the highest laminarinase activity estimated by the increase of reducing sugars in an incubated mixture was found in the crystalline style of Bivalvia, such as *Spisula sachalinensis* and *Mactra sulcataria*. The present paper describes the isolation and purification of laminarinase (β-1,3-glucan glucanohydrolase, EC 3.2.1.6) from *S. sachalinensis*, as well as some of its properties.

MATERIALS AND METHODS

Enzyme source. Animals were collected in summer in Posiet Bay, Japan Sea; only fresh animals were used for the study.

Preparation of enzyme solution. The work was carried out in the cold and within the shortest possible time. The crystalline styles were thoroughly cut with purified sand and extracted with 0.05 M acetate buffer (pH 5.6). The homogeneous mass obtained was centrifuged for 15 min at 9000 rev./min, and the supernatant was lyophilized.

Substrates. Soluble laminarin was purified from *Laminaria cycharioides* by the method of BLACK[2]; fucoidine was removed by means of sedimentation with cetyltrimethylammonium bromide. CM-cellulose, alginic acid, amylopectin, cellobiose,

laminaribiose, gentiobiose, sucrose, trehalose, raffinose, lactose, α-methylglucoside, β-D-phenylglucopyranoside and α-methylmannoside were commercial reagents. CM-chitin was obtained by the method of TRUJILLO[3].

Assay of enzymes. A solution of 0.9 ml of 0.2% laminarin in 0.1 M acetate buffer (pH 5.6) and 0.1 ml of enzyme solution was incubated for 1 h at 37°. The reaction was terminated by boiling the mixture for 5–7 min. The increase of reducing sugar was determined by the method of NELSON[4]. The initial reaction rate was a linear function of enzyme concentration in the range in which assays were obtained. One unit of enzyme was defined as that amount catalyzing the formation of 1 μmole of glucose per min under the conditions described. Specific activity was estimated as units per mg protein. Protein concentration was measured colorimetrically[5]. CM-cellulase, alginase, CM-chitinase and amylase were determined viscosimetrically[6] and, simultaneously, by the increase of reducing sugar. Another activity was estimated by the increase of reducing sugar with subsequent identification of mono- and oligosaccharides in the reaction mixture by paper chromatography.

Paper chromatography. The hydrolysis products were chromatographed on Whatman No. 3 paper in butanol–pyridine–water (6:4:3, by vol.) or ethylacetate–pyridine–acetic acid–water (5:5:1:3, by vol.) systems.

Column chromatography. SE-Sephadex C-50 (Uppsala, Sweden) was treated successively with 1 M NaOH, 0.5 M HCl and 1 M NaOH and equilibrated for 1 day with 0.05 M sodium succinate buffer (pH 5.2).

Gel filtration. Bio-Gel columns P-30 (Bio-Rad, Richmond, Calif., 50–150 mesh) were prepared by the standard procedure as recommended by the manufacturer and equilibrated at pH 5.6 with 0.1 M acetate buffer.

Analytical disc electrophoresis. Analytical disc electrophoresis on 7.5% acrylamide gel was carried out according to the procedure of DAVIS[7] using Tris–glycine buffer (pH 8.9). The protein was stained according to the procedure of CHRAMBACH *et al.*[8]. The location of laminarinase activity in polyacrylamide gels was determined according to procedure B of the method of GABRIEL AND SHU-FONG WANG[9] with laminarin as substrate.

RESULTS

Purification of enzyme

Step I. The lyophilized crude preparation (300 mg) was dissolved in 20 ml of 0.05 M acetate buffer (pH 5.6) and layered on a Bio-Gel P-30 column (4.0 cm × 95 cm packed volume) at room temperature. Elution was carried out with the same buffer; 7-ml fractions were collected every 20 min. The active fractions (Fig. 1, Fractions 14–21, Peak B), including laminarinase, were combined and lyophilized. Peak A containing the main quantity of protein showed also the presence of other carbohydrases found in the crystalline style of *S. sachalinensis:* CM-cellulase, CM-chitinase, alginase and amylase.

Step II. The lyophilized Peak B from P-30 was dissolved in a minimum volume of 0.05 M sodium succinate buffer (pH 5.2) and then dialyzed against the same buffer for 16 h at 0°.

Step III. The dialyzed material (20 mg in 10 ml) was absorbed on a SE-Sephadex column (1.8 cm × 40 cm). Protein was eluted from the column at room

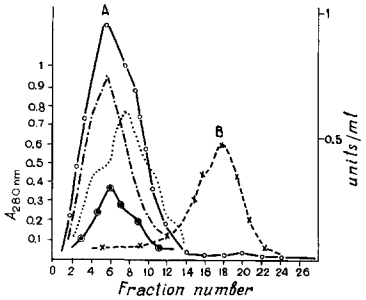


Fig. 1. Gel filtration on Bio-Gel P-30. ○—○, absorption at 280 nm; ×— — —×, laminarinase activity; · · · · · ·, alginase activity; ⊙—⊙, CM-cellulase activity; —·—, amylase activity. For conditions, see text.

temperature by increasing the linear gradient of 1 M NaCl in 0.05 M sodium succinate buffer (pH 5.2); fractions of 3 ml were collected every 20 min. Fig. 2 illustrates a typical elution pattern. Peak IV, containing the minimum quantity of protein, showed the highest laminarinase activity. Laminarinase activity which was also present in Peak III was not the subject of further investigation. Laminarinase (Peak IV) thus obtained showed an approx. 87-fold increase in specific activity in comparison with lyophilized crude powder. The purification results are shown in Table I. The enzyme is homogeneous according to the data of analytical disc electrophoresis[7].

TABLE I

PURIFICATION OF LAMINARINASE FROM CRYSTALLINE STYLE OF *S. sachalinensis*

Step	*Total protein (mg)*	*Specific activity (units/mg protein)*	*Total enzyme units*	*Purification*	*Recovery of enzyme (%)*
1. Lyophilized powder	300	4	1200	(1)	100
2. P-30, Peak B	20	40	800	10	66.7
3. SE-Sephadex, Peak IV	0.6	350	210	87.5	17.5

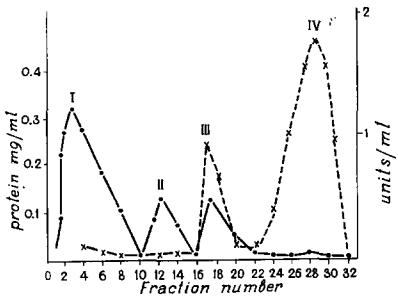
Time-course and optimum pH

Laminarin was digested with enzyme (0.035 unit/ml) under standard conditions. The time curve bent after 4 h of incubation.

The glucanase activity of the enzyme was determined against laminarin at various pH values, using 0.1 M acetate buffer. Maximum activity was observed at pH 5.8, as shown in Fig. 3.

Temperature

The isolated enzyme is relatively stable at usual temperatures. At 20° the enzyme solution loses 8% of its activity over 7 days; at 0° during the same period of time no detectable loss of activity was observed. The loss of activity was 80% after heating the enzyme solution to 60° for 3 min. The optimum temperature for hydrolysis of laminarin by the enzyme is 45°.



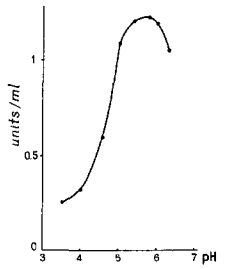


Fig. 2. Chromatography of SE-Sephadex. ●—●, protein, mg/ml; ×— — —×, laminarinase activity. For conditions, see text.

Fig. 3. Effect of pH on laminarinase activity.

Substrate specificity

Laminarinase specifically hydrolyzes laminarin giving rise to glucose, laminaribiose and higher oligosaccharides; after a long incubation, only glucose and laminaribiose remain.

The enzyme had only slight activity in relation to laminaribiose. The hydrolysis rate of laminaribiose is 0.03% of that of laminarin. The following substances were not attacked by the enzyme: CM-cellulose, CM-chitin, alginic acid, amylopectin, cellobiose, gentiobiose, sucrose, trehalose, raffinose, lactose, α-methylglucoside, β-D-phenylglucopyranoside and α-methylmannoside.

Molecular weight

The molecular weight of the enzyme was estimated on a Sephadex G-100 column according to the method of WHITAKER[10]. The molecular weight of the enzyme, determined by graphic interpolation, was equal to 22 000.

DISCUSSION

The crystalline style is commonly considered a unique organ of molluscs and is probably related to a vegetable diet. The crystalline style is perhaps the source of digesting enzymes for Bivalvia. Amylase, cellulase, chitobiase, alginase and lipase are found therein, while proteinase is practically absent[11]. The absence of proteinases in *S. sachalinensis* was shown by KOSLOVSKA AND VASKOVSKY[12]. In the present work, in addition to having established the presence of all the above-mentioned carbohydrases in the crystalline style, we have found the presence of a weak "zosterinase" acting upon the polysaccharide of the Zosteraceae sea plant[13] and CM-chitinase. Ribonuclease and deoxyribonuclease estimated according to the method of RASSKAZOV *et al.*[14] were absent.

The suggested method of laminarinase purification makes it possible to remove all the other carbohydrases at the first step of purification, gel filtration on Bio-Gel P-30, due to the low molecular weight of laminarinase. The further purification of the active fraction on SE-Sephadex makes it possible to obtain laminarinase, with an 87-fold increase in specific activity, free of all the other carbohydrase activities and specific for laminarin. On rechromatography on SE-Sephadex under the described

conditions, the laminarinase obtained was eluted as a single symmetric peak at the same ionic concentration of salt and in the same volume. The laminarinase obtained migrated as a single band on disc electrophoresis in a polyacrylamide gel[7]. At the location of laminarinase activity[9], the single band which coincided with the band stained as protein was obtained[8]. The activity of laminarinase from *S. sachalinensis* is approximately equal to that of exo-β-D-(1 → 3)-glucan glucanohydrolase from Basidiomycete sp. QM806 (ref. 15) and is probably higher than the activity of laminarinase from other sources[16–20]. The enzyme possesses a high activity, low molecular weight and relative heat stability. Moreover, the enzyme source is very peculiar inasmuch as it acts as a laminarinase concentrator in the organism. All these factors serve to make *S. sachalinensis* laminarinase an interesting subject for further study.

Research work on enzyme specificity and investigations for determining its exo- and endo-type activities in comparison with the known laminarinases are under way.

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