Effective Purification of 1,4-β-Endoglucanase (EG66) from a Marine Mollusc, *Patinopecten yessoensis*, by Cellulose Column Chromatography

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Abstract: 1,4-β-Endoglucanase was purified from the hepatopancreas of the marine mollusc, *Patinopecten yessoensis*, by cellulose column chromatography. The enzyme (EG66) showed the *M*<sub>e</sub> of 66K on SDS-PAGE, and the hydrolytic activity was maximal at pH 6.0. EG66 readily hydrolyzed glucomannan and 1,3,1,4-glucan as well as CMC and hydroxethyl cellulose. On the other hand, crystalline cellulose, xylan, xyloglucan, laminaran, 1,3,1,6-glucan, galactomannan, alginate, methyl cellulose and hydroxypropylmethyl cellulose were not hydrolyzed at all. N-Terminal amino acid sequence of EG66, which was determined as GATNVQIT NEWPGGFQGTTF, had a motif common to the other molluscan endoglucanases.

Keywords: endoglucanase, cellulose, mollusc, *Patinopecten*

The existence of glycanases including cellulase and amylase have been reported in several marine molluscs.¹⁻³ Onishi and colleagues revealed that the crude extract obtained from 50 species of shellfish had diverse enzymatic activity that degraded laminaran, fucoidan, alginic acid, methyl cellulose, and xylan.⁴ With respect to the molluscan enzymes, 1,4-β-endoglucanase and cellulase were cloned from *Mytilus edulis, Haliotis discus hannai, Caricula japonica* and *Ampullaria crossean*.⁵⁻¹⁰ The endoglucanase from *Mytilus* is a 19K protein that belongs to the glycoside hydrolase family (GHF) 45, subfamily 2,¹¹ while the cellulase from *Haliotis* is a 66K protein that is classified under GHF 9.¹² These data suggested that molluscs might have several types of β-glucanases as their genomic transcripts.

The scallop *Patinopecten yessoensis* (Hotate shell) is one of the major marine products in the northeastern Japan. Though the hepatopancreas (midgut gland) of the shell is not edible and has been discarded, it could possibly be considered a rich source of carbohydrolases. In our previous work two molecular species of β-glucanases were recognized in the extract of the organ, and one species of 1,4-β-endoglucanase with *M*<sub>e</sub> 43K (EG43) was purified.¹³ On purifying EG43 we separated another endoglucanase with a larger molecular size (66K). The larger species of the enzyme (EG66) seemed to have bindability to insoluble cellulose.¹⁴ In this study we describe the purification of EG66 by using cellulose as an adsorbent, and the enzymatic properties of it including hydrolytic activity towards hemicelluloses, water-soluble cellulose derivatives and cellooligosaccharides. Barley β-glucan and 1,3,1,6-glucan (*Aureobasidium* extracellular polysaccharide) were donated by ADEKA Co. (Japan). Glucomannan (konjac) was purchased from Orihiro Co. (Japan). Xyloglucan was donated by Dainippon Sumitomo Pharm. Co. (Japan). Various grades of METOLOSE (nonionic water-soluble cellulose ethers) were donated by Shin-etsu Chemical Co. (Japan). The other reagents were purchased from Wako Pure Chemical Co. (Japan). Internal organ of the scallop (*Patinopecten yessoensis*) was generously provided by Seiho Shoji Co. (Aomori City, Japan). The midgut gland (hepatopancreas) was separated from the surrounding tissues and frozen at −80°C until use. Purification of EG66 was carried out at a low temperature. Frozen hepatopancreas (100 g) was gently thawed in 200 mL of ice-chilled water containing 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min, and was homogenized using a Waring blender. The homogenate was centrifuged (× 7000 g, 20 min) and ammonium sulfate was added to the supernatant up to 75% saturation. The mixture was centrifuged (× 7000 g, 20 min), and the pellet was dissolved in 40 mM phosphate buffer, pH 7.0, containing 0.5 mM NaCl and 1 mM PMSF. The insoluble substances were removed by centrifugation and the supernatants were applied to a column of cellulose (Whatman CF11, 2.2 × 25 cm) that had been equilibrated with 40 mM phosphate buffer, pH 7.0, containing 0.5 mM NaCl. After the column was washed sufficiently with the same buffer containing the salt, adsorbed proteins were eluted by an isocratic flow of 500 mL of 40 mM phosphate buffer, pH 7.0. Fractions containing EG66 were pooled, adjusted to 0.8 M salt with ammonium sulfate, then applied to a short column of Butyl Toyopearl 650M (TOSOH, 1.2 × 3.5 cm) that had been equilibrated in 40 mM phosphate buffer, pH 7.0, containing 0.8 M ammonium sulfate. EG66 was eluted in a small volume of the buffer without the salt. The concentrate of the enzyme...
was loaded onto a gel permeation column of Sephacryl S-100 (Pharmacia, 1.6 × 82 cm) that was run in 40 mM phosphate buffer, pH 7.0. Proteins were determined by the method of Lowry et al. SDS-PAGE was performed according to the method described by Laemmli. On endo-


glucanase assay a reaction mixture containing 0.8% of CMC (low viscosity, ICN Biomedicals Inc., Ohio) and the enzyme in 0.1M sodium acetate buffer, pH 6.0, in a total volume of 1.0 mL was incubated at 30°C. The reaction was stopped by heating for 5 min and reducing sugars were determined by the Somogyi-Nelson method. One katal of the enzyme was defined as the amount of enzyme required to liberate 1 mol of reducing sugar per second under the given conditions. Hemicellulosic carbohydrates were used at 0.1% concentration to avoid stickiness and the hydrolytic ratios (amount of reducing termini released in a period of time) of them were compared to that of CMC determined under the same conditions.

The first cellulose column chromatography was highly effective in the purification of EG66 (Fig. 1 and Table 1). Addition of ethyleneglycol to the buffer caused the rapid elution of proteins from cellulose, but we concluded that the isocratic elution in the phosphate buffer was best to avoid other protein contaminants. Purified enzyme was free from pNP-β-glucosidase activity and showed a protein of Mr 66K on SDS-PAGE (Fig. 1C). EG66 showed the highest hydrolytic activity at 40°C in 100 mM sodium acetate buffer, pH 6.0. The activity was not observed below pH 4.0 or above pH 8.0. The enzyme showed approximately 45% and 5% of activity at 20°C and at 50°C, respectively. After the enzyme was pre-treated for 1 h at 30°C under various pH conditions, over 50% of the activity was kept in the range of pH from 5.0 to 8.0 with the maximum at pH 7.0. Residual activity after pre-incubation for 10 min at 20, 30, 40 and 50°C were 100, 77, 25 and 0% of the maximum, respectively. N-Terminal amino acid sequence of EG66 was determined as GATNVQIT

Table 1. Purification of *Patinopeten* 1,4-β-endoglucanase (EG66).

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total protein (mg)</th>
<th>Total activity (10⁻¹ kat)</th>
<th>Specific activity (kat/kg)</th>
<th>Yield (%)</th>
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<td>1.12</td>
<td>70.0</td>
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<td>206</td>
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</table>
The hydrolysis of cellooligosaccharides by EG66.

(A) Reaction mixtures containing 1% cellooligosaccharides and EG66 in 40 mM phosphate buffer, pH 6.8, were incubated at 30°C for 60 min. Left lane, C5 as the substrate. Middle lane, C6 as the substrate. (B) Reaction mixtures containing 1% C6 and EG66 in 40 mM phosphate buffer, pH 6.8, were incubated at 30°C for the period indicated. In (A) and (B), sugars were developed in isopropanol : 1-butanol : water (12 : 3 : 4) on TLC and visualized with 2 M sulfuric acid containing 0.2% orcinol. M, marker sugars.

NEWPGGFQGTF, which shared a conserved motif (written in capitals) with Halitosis discus cellulase (\texttt{svad-VtisNhWdGGFGQks}a),

\textit{Corbicularia japonica} cellulase (\texttt{eaepVtitiNsWnGGFGQgsf}32) and Ampullaria crossen \textbeta\textsubscript{1},4-endoglucanase (\texttt{asvstVpvtNhWpGGFGQakv}34).

With regard to the substrate specificity, \textit{Patinopeten} EG66 readily hydrolyzed glucomannan, a linear heteropolysaccharide of \textbeta\textsubscript{1},4-glucose and \textbeta\textsubscript{1},4-mannose, and barley \textbeta\textsubscript{1},4-glucan, a liner polysaccharide of \textbeta\textsubscript{1},3- and \textbeta\textsubscript{1},4-glucose, as well as CMC. The hydrolytic ratios of glucomannan and barley \textbeta\textsubscript{1},4-glucan were 5.0- and 3.4-fold higher respectively than that of CMC. On the other hand galactomannan (guar gum, a linear heteropolysaccharide of \textbeta\textsubscript{1},4-mannose and \textalpha\textsubscript{1},6-galactose) was not hydrolyzed at all. Crystalline cellulose, xylan (beechwood), xyloglucan, laminarin, \textbeta\textsubscript{1},3,1,6-glucan and alginate were not the substrate to EG66 at all. These results suggested that a soluble linear polysaccharide containing \textbeta\textsubscript{1},4-glucose residues could be more preferable to EG66 as the substrate. As to the activity towards cellooligosaccharides, C6 was a better substrate than C5 (Fig. 2A). On the hydrolysis of C6, sugars C2, C3 and C4 emerged at the initial phase of the digestion (Fig. 2B). Glucose was scarcely produced even at the late phase of the reaction, suggesting that C3 was not readily cleavable by the enzyme.

As to the hydrolysis of water-soluble cellulose ethers, hydroxyethyl cellulose (HEC) was hydrolyzed by EG66 with the ratio of 0.6 to CMC. Methyl cellulose (METOLOSE type SM-4000) and hydroxypropylmethyl cellulose (METOLOSE types 60SH-4000, 65SH-4000 and 90SH-4000 with the molar substitution 0.25, 0.15 and 0.20, respectively) were not hydrolyzed. A slight hydrolysis of hydroxyethyl-methyl cellulose was observed, where the hydrolysis of METOLOSE types SEB-04T (molar substitution 0.20) and type SNB-30T (molar substitution 0.30) were 7% and 6% of CMC after 24 h reaction, respectively.

We previously observed that another \textit{Patinopeten} \textbeta\textsubscript{1},4-endoglucanase EG43 did not act on cellooligosaccharides ranging from C3 to C6, nor the nonionic cellulose ethers except HEC of which the hydrolytic ratio was 0.12 to CMC.\textsuperscript{12} In this context EG66 showed more activity to such cellulose substances than EG43. Though their relationship is unknown, the two enzymes may be cooperative in the degradation of \textbeta\textsubscript{1},4-glucan. Existence of several sizes of celluloses (66K, 75K and 100K) were also reported with the digestive gland of abalone.\textsuperscript{9}

EG66 did not act on galactomannan, indicating that the hydrolysis of \textbeta\textsubscript{1},4-mannosidic linkage was negative, while it showed notable activity towards glucomannan. Some of the microbial celluloses are known to act on glucomannan, and the molecular structure of CBM having affinity to oligomannose and glucomannan has been described.\textsuperscript{16,17} \textit{Haliotis} 66K cellulase (GHP9) is speculated to have CBM of family 2 which various carbohydrateases including glucanase, chitinase, xylanase and mannannase have.\textsuperscript{18} The molecular structure of \textit{Patinopeten} EG66 is unclear but it seems possible for the enzyme to have a role in the digestion of marine cellulose and hemicellulosic polysaccharides. The hydrolytic activity of EG66 towards natural marine polysaccharides will be a matter of interest in our future work.

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REFERENCES


13) O.H. Lowry, N.J. Rosenberg, A.L. Farr and R.J. Randall:


