Characterization of a new acid stable exo-β-1,3-glucanase of *Rhizoctonia solani* and its action on microbial polysaccharides

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Abstract

A new acid stable exo-β-1,3-glucanase of *Rhizoctonia solani* purified from a commercial source 'Kitarase-M', by a combination of ammonium sulfate precipitation, ion-exchange and gel filtration methods, had specific activity 0.26 U/mg protein, K_m and V_{max} values of 0.78 mg/ml and 0.27 mM/min/mg protein, respectively. It had molecular weight of 62 kDa with optimum activity at 40°C temperature and pH 5.0, with high stability at pH of 3-7. Unique amino acid sequence was found at N-terminal end. The substrate specificity studies confirmed that it is an exo-β-1,3-glucanase. It could hydrolyze curdlan powder to release glucose.

Keywords: characterization; curdlan; exopolysaccharides; exo-β-1,3-glucanase; oligosaccharides; purification, *Rhizoctonia solani*

1. Introduction

Beta-1,3-glucanases are the enzymes, which can cleave the beta glycosidic linkages of glucans. These are having potential application in biotechnology such as cell fusion, transformation and protoplast preparation[1], in brewing industries and during the clarification of slimy must[2]. Microbial exopolysaccharides (EPS) are gaining importance in food as well as other industries, because of their functional properties and are being used to improve the rheology of foods. Curdlan, a water insoluble 1,3-β-glucan produced by *Alcaligenes* sp., has linear molecular structure and exhibits specific gelling properties[3]. The insolubility of curdlan makes it unusable for food and other industrial applications. Polysaccharides of β-glucan group can be hydrolyzed by either exo- or endo-β-glucanases[4,5]. Exo-glucanases degrade the polysaccharides completely and release monosaccharide residues, where as endo-glucanases produce oligosaccharides by partial degradation of the EPS. These oligosaccharides have new functions and they can easily be absorbed by the system than the polysaccharides. Various polysaccharases currently available for the degradation of microbial EPS have been reviewed[6]. However, reports are lacking on the application of commercial enzymes for the degradation of EPS such as curdlan. The commercial enzyme formulations may not be in pure form for specific applications. The present report, therefore, deals with the purification and characterization of exo-β-1,3-glucanase of *Rhizoctonia solani* from a commercial enzyme source and its action pattern on solid curdlan to produce curdlan of low molecular weight with improved solubility. This could increase the use of low molecular weight curdlan for various food and non-food applications.

2. Materials and methods

2.1 Materials

Laminarin of *Laminarium digitatum* (Sigma-Aldrich Co, St. Louis, USA), curdlan (Wako Pure chemicals, Kyoto, Japan), laminarioligosaccharides (Seikagaku Corporation, Tokyo, Japan) and cellooligosaccharides (Yaizu Suisankagaku Co., Ltd, Tokyo Japan) were used in this study. Methyl-β-laminaritetraoside and laminaritetraitol were kindly gifted by Dr. Kitaoka, NFRI, Tsukuba, Japan. Kitarase-M, commercial enzyme Kitarase-M was procured from K-I Corporation, Tokyo, Japan.

2.2 Purification of exo-β-1,3-glucanase by column chromatography

Unless otherwise indicated, the following enzyme purification steps were performed at 4°C. Kitarase-M (50 g) was suspended in 250 ml of Tris-HCl buffer (50 mM, pH 9.0), precipitated with 72.75 g of solid ammonium sulfate (50% saturation) and centrifuged at 10,000 x g for 20 min. The precipitate was dissolved in Tris-HCl buffer (50 mM, pH 9.0) and dialyzed against the same buffer. It was fractionated using DEAE-Toyopearl gel column (ø 1.0 x 10 cm) with Tris-HCl buffer (50 mM, pH 9.0) at a flow rate of 1ml/min, using a linear NaCl gradient (0 to 1M). Absorbance of all the fractions (5 ml/fraction) were measured at 280 nm (UV-210A double beam spectrophotometer, Shimadzu, Japan) and checked for enzyme activity using curdlan as a substrate. The active fractions were pooled, dialyzed against acetate buffer (50 mM, pH 5.0) and fractionated (5 ml/fraction) by CM-Toyopearl gel column using the same buffer. The enzyme was

eluted with a linear NaCl gradient (0-1 M). The enzyme fractions were pooled and concentrated by ultrafiltration (Amicon Ultra 10000 MW cutoff, Millipore). The concentrate was subjected to gel permeation chromatography (GPC) using equilibrated Sephacryl S-200 gel column (ø 1.6 × 60 cm) with phosphate buffer (50 mM, pH 7.0) containing 100 mM NaCl. The flow rate was 0.5 ml/min. GPC was repeated thrice to get a single band in SDS-PAGE. The purified enzyme was stored at -20°C until use.

2.3 Analysis

Enzyme activity was measured by adding 50 µl of enzyme (in phosphate buffer, pH 7.0) to 200 µl of laminarin solution (0.5 mg/ml). The mixture was incubated at 37°C for 25 min and the reducing sugar content was determined after stopping the reaction by adding copper sulphate reagent and transferring the tubes to ice cold water. Unless otherwise stated, the same method was used throughout the study. One unit (U) of β-1,3-glucanase activity was defined as the amount of enzyme that liberated one µmol of reducing sugar per min under the test conditions. The total carbohydrate and reducing sugar contents of the samples were estimated by the phenol-sulphuric acid method[7] and Somogyi-Nelson method[8], respectively, using glucose as standard. Protein content was determined by the Lowry method[9] using bovine serum albumin as standard. The N-terminal amino acid sequence of the purified enzyme was determined using HP G1005A protein sequencing system after transferring the protein onto PVDF membrane. The sequence similarity was determined at National Center for Biotechnology Information (NCBI) using the BLAST network service[10].

2.4 Characterization of the purified enzyme

The influence of pH on the enzyme activity (pH profile) was determined by varying the pH of the reaction mixture using 100 mM of acetate buffer (pH 3-5), citrate-phosphate buffer (pH 5-7), Tris-HCl buffer (pH 7-9) and phosphate buffer (pH 7.0), individually. To determine pH stability of the enzyme, it was incubated in respective buffers for overnight (18 h) at 25°C and the enzyme assay was carried out at optimum pH by using laminarin solution prepared in acetate buffer (100 mM, pH 5.0). Effect of temperature on enzyme activity was studied by incubating the enzyme-substrate (0.5 mg/ml laminarin) mixture in phosphate buffer pH 7.0, over a range of 30-80°C. To determine the temperature stability, the residual activity of the enzymes was estimated 37°C under the standard conditions after treatment for 60 min in the temperature range of 30 to 80°C. Effect of metallic ions, enzyme inhibitors and chelators on glucanase activity was determined by carrying out the assay after incubating the enzyme with these compounds individually (1 mM concentration) for 60 min at 25°C. The absorption spectrum of purified enzyme was determined using spectrophotometer (UV mini 1240 spectrophotometer, Shimadzu, Japan). The apparent molecular weight of the purified enzyme was determined by SDS-PAGE (10% gel) using Bio-rad mini gel kit. Isoelectric focusing was performed with precasted 'Phastgel-IEF 3-9' using 'Phastsystem' (Pharmacia, Switzerland). Gel containing approx. 2 µg of protein was stained with Coomassie Brilliant Blue R250 and apparent isoelectric point (p*I*) value of the purified enzyme was determined.

The Michaelis-Menten constants (K_m and V_{max}) were determined from the Lineweaver-Burk

representation of data obtained by measuring the initial rate of substrate hydrolysis. Concentrations of laminarin from 0.4 to 8 mg/ml (in phosphate buffer pH-7.0) were used as substrate and 18.5 mU of the enzyme was used in each reaction and the assay was carried at 37°C. Substrate specificity of purified β-1,3-glucanase enzyme was studied using various substrates like curdlan, laminarin, carboxy methyl cellulose (CMC), soluble starch and oligosaccharides of laminarin (1 mg/ml in phosphate buffer pH 7.0).

2.5 Hydrolysis of exopolysaccharides

The purified exo-β-1,3-glucanase was used to hydrolyze both solid and liquid forms of curdlan, laminarin, carboxymethyl cellulose (CMC), besides oligosaccharides of laminarin and cellulose (all at 1 mg/ml concentration). Equal volumes of the substrate and enzyme solutions having a [E]/[S] ratio of 0.055 U/ml were mixed, incubated at 37°C for various time intervals and subjected to boiling for 5 min to stop the reaction. After cooling, the samples were treated with mixed bed resin AG 501 X 8 (Bio-Rad, Richmond, U.S.A.) for 5-10 min and centrifuged at 7000x g for 5 min to remove the interfering ions. The action pattern of the enzyme was determined by analyzing the clear supernatant both by TLC and HPLC.

To determine the effect of purified β-1,3-glucanase on curdlan powder, samples containing a mixture of 25 mg of curdlan powder and 500 µl of purified enzyme were incubated at 37°C for different time intervals and chilled for 10 min. All the samples were centrifuged at 15,000x g for 10 min to remove unhydrolyzed curdlan and the supernatant was used to detect the derivatives by TLC and HPLC. The remaining solid was washed thrice with deionized water and solubulized using 1N NaOH. The total reducing sugar and carbohydrate content was estimated to determine the degree of polymerization (DP). In some experiments, the samples after incubating for different time periods were boiled by keeping in boiling water bath for 5 min to observe gelling character of curdlan. For TLC, plates coated with silica gel 60 F_{254}, (Merck, Darmstadt, Germany) were used. Ten to twenty µl of the samples along with glucose standard (1% w/v) were spotted on TLC plates (5 x 8 cm) and dried. The chromatography was carried out in a glass chamber saturated with aqueous acetonitrile (75% V/V) for 10-15 min. The plates were sprayed with sulphuric acid-methanol solution (80:20) and dried on a hot plate to detect the derivatives. For quantitative analysis of the derivatives, the reaction mixtures after treating with resin were filtered through 0.45-µm filter (Millex-LH, Millipore, Japan) and analyzed by HPLC system (Jasco Corporation, Japan) using Asahipak NH2P-50 4E column (Shodex, Japan). Degassed aqueous acetonitrile (68%, v/v) was used as a mobile phase with a flow rate of 0.5 ml/min. The oven temperature was maintained at 35ºC and the products were identified using RI-810 detector (Jasco, Japan). A mixture of laminarioligosaccharides was used as standard to detect various oligosaccharides in the reaction mixture.

3. Results and Discussion

3.1 Purification of exo-β-1,3-glucanase from Kitarase-M

Kitarase-M, a commercial enzyme mixture consists mainly β-1,3-glucanase derived from *R. solani*, which can degrade β-D-glucans. In the preliminary studies (data not shown), ammonium sulfate at 50% saturation was found to be satisfactory to precipitate the protein from the sample. Purification of the precipitate by DEAE -Toyopearl gel column showed the presence of three major peaks (Fig. 1). The pooled

glucanase fractions (27-34) obtained at around 0.5 M NaCl concentration were further purified by CM-Toyopearl gel column. As the glucanase could not bind to this gel, it got eluted even before the gradient has started (fractions 5 to 10) (Fig. 2). However, this step could eliminate some impurities from the sample. The enzyme was further purified by Sephacryl S-200 column after equilibrating with phosphate buffer (pH 7.0). The glucanase was eluted in fractions 28 to 35 (Fig. 3). This step was repeated thrice to achieve complete purity. The purification scheme (Table -1) resulted in a 152 fold purification of the enzyme with 30% yield. A substantial increase in specific activity (from 0.002 to 0.265 U/mg protein) was noticed.

3.2 Physico-chemical Properties of the purified enzyme

As shown in Fig.4, activity of the purified enzyme increased with the increase in temperature from 30 to 40°C. The highest activity was observed at 40°C and the activity decreased considerably with further increase in the temperature. Enzyme completely became inactive beyond 60°C. However, variations in the optimum temperature, like 53°C[11] and 55°C[12] for the exo-glucanases and 30°C[13] for the endo-1,3-β-glucanase of *R. solani*, have been reported. This may be due to the difference in structural features of the substrate used[13]. The enzyme purified in our study was found to be highly stable between 30 and 40°C for 1 h in citrate-phosphate buffer (pH 7.0).

The pH profile of the purified enzyme (Fig. 5) indicated the activity between pH 3.0 and 7.0, with an optimum value at pH 5.0, both in acetate and citrate-phosphate buffers, with laminarin as substrate. The activity of the purified enzyme increased with increase in pH from 3 to 5 and decreased with further increase in pH. Enzyme activity was high at pH 5.0 in acetate buffer and at pH 7.0 in phosphate buffer. However, it was low in citrate buffer at pH 7.0, which may be due to the interference of citrate ions in the estimation of reducing sugars[14]. This is in contrast to the optimum values reported for either exo-glucanases (5.6 - 5.8)[11, 13] or endo-glucanase (5.5)[12] of *R. Solani*. The purified enzyme of the present study was found to be quite stable between pH 3 and 7 in acetate buffer and citrate-phosphate buffer. More than 90% activity was retained at all pH units studied, after 20 h of incubation at 25°C. Contrary to this, stability of the exo-glucanase of *R. solani* was reported to be at pH 10[12]. However, our result is in agreement with the observation that the stability of fungal β-glucanases is commonly between pH 3 and 8.0[11, 15] and stability in acidic pH is a unique feature of the enzyme of our study and probably this is the first report on acid stable glucanase of *R. solani*.

The purity of the enzyme was confirmed with the presence of a single protein band in SDS-PAGE analysis and it had an apparent Mw of 62 kDa (Fig. 6). Variations in the Mw (31.5 - 83.1 kDa) of exo-glucanases from different sources were noticed[11, 16], which might be due to the genetic variations in strain or species or interaction of the enzymes with the gel filtration medium and its concentration. The *pI* of the purified enzyme of our study was found to be 4.5 (Fig. 4 B). However, higher *pI* values of 8.1[11] and 9.8[17] for exo and endo-glucanase of *R. solani*, respectively, were reported. The N-terminal amino acid sequence analysis of the purified enzyme resulted in a unique amino acid sequence of 'Trp-Val-Asn-Gln-Asn-Asn-Phe' (W-V-N-Q-N-N-F). When searched for short and nearly exact match with BLAST software, we could not find any sequence similarity in the data bank at NCBI[10].

The purified β-1,3-glucanase was found to be more specific for compounds having β-1,3-glucosidic linkages, as it did not hydrolyze starch and had shown very weak reaction with CMC. Interestingly, it had five folds higher activity towards laminarin than curdlan. However, a higher reaction rate (4.2%) with curdlan than with laminarin was observed for exo-glucanase of *T. harzianum*[1]. With regard to the effect of metal ions and chelating agents on the activity of purified enzyme, highest activity loss (75%) was noticed with mercury ions. This indicates that the thiol groups of the cysteine residues are involved in the active catalytic site or these groups are essential in maintaining the enzyme structure[18]. Cobalt and ferric ions did not inhibit enzyme activity. Ions of magnesium, zinc, copper, calcium, nickel and manganese showed a remaining activity between 65 and 90 % in the order mentioned. The activity loss with EDTA and 2-mercaptoethanol were found to be 28 and 49%, respectively. Although the purified enzyme had absorption between 240 and 300 nm, absorption maximum was found to be at 280 nm. With laminarin as substrate, the apparent K_m and V_{max} values for the purified β-1,3-glucanase were found to be 0.78 mg/ml and 0.27 mM/ min/mg protein, respectively. The K_m value determined in this study was much lower than the value reported earlier (2.08 mg/ml) for the exo-β-1,3-glucanase of *T. harzianum*[1]. However, with laminarin as a substrate, K_m and V_{max} values for exo-β-1,3-glucanase of *P. anomala* were found to be 0.3 mg/ml and 350 μmol/min/mg, respectively[19].

3.3 Action pattern of purified enzyme

The TLC analysis of the reaction mixture (pH 7.0) containing the enzyme and curdlan indicated the presence of only glucose (data not shown). This might be due to the hydrolysis of the terminal glucosidic bond, which released only glucose from non-reducing end of curdlan. This was also confirmed by the HPLC analysis (Fig. 7). Similarly, only glucose was released from the Methyl-β-D-laminaritetraoside and laminaritetraitol (data not shown). The glucanase of *T. viride* also released glucose from curdlan, indicating that the enzyme isolated was an exo-glucanase[16]. In TLC analysis, curdlan could not be detected because of its insoluble nature at pH 7.0, whereas the same sample could be detected after adjusting the pH to 12, because of its solubility at this pH. Although the purified β-1,3-glucanase could hydrolyze laminarin, it could not hydrolyze laminaribiose (data not shown). Thus, the purified enzyme is considered to be a β -1,3-glucan glucohydrolase (E.C.3.2.1.58) but not glucosidase. The purified enzyme could release glucose from laminaritriose, laminaritetraose, laminaripentaose and laminarin indicating the ability of this enzyme to hydrolyze compounds that are having β-1,3 linkages. This observation is in tune with the report of Tsujisaka et al.[12], who have also noticed liberation of almost glucose during the hydrolysis of laminarin by the exo-glucanase of *R. solani*. Similarly, K5- type yeast killer toxin, of *Pichia anomala* NCYC 434 cells, having exo-β-beta 1,3 glucanase activity was reported[20]. At the same time, our enzyme could not hydrolyze β-1,4 linkages present in cellooligosaccharides such as cellobiose, cellotriose, cellotetraose and cellopentaose indicating the specificity of the enzyme and its affinity for β-1,3 linkages. The enzyme purified in our study had very low affinity towards CMC.

The time course study on the effect of purified enzyme on laminaripentaose was studied. The reaction mixture of laminaripentaose and enzyme was subjected to TLC analysis after 0, 5, 10, 30, 60 min and overnight incubation at 37°C. Release of free glucose and formation of laminaritriose were observed in samples incubated for 10 and 30 min. With further increase in reaction time to 60 min, the laminaritriose was hydrolyzed to produce laminaribiose and glucose. Production of exclusively glucose was observed after overnight incubation at 37°C. This indicated that the enzyme action on laminaripentaose is slow and continuous and could complete the hydrolysis by overnight incubation. Laminarin and laminaripentaose were hydrolyzed with the purified enzyme and the reaction mixtures were analyzed by HPLC and the results are presented in Table 2. Purified enzyme could hydrolyze laminarin and released free glucose within 5 min of interaction between substrate and enzyme. As can be seen from the results, the enzyme could liberate glucose and laminaritriose in 3.9:1 ratio after overnight incubation 37°C. Contrary to this, release of glucose and gentiobiose with the hydrolytic action of exo-1,3-β-glucanase on laminarin was reported and gentiobiose was further degraded to glucose in subsequent stages of hydrolysis[11]. In our study, production of glucose and laminaritriose were observed with the action of the enzyme on laminaripentaose during the first ten minutes. As seen in Table 2, the glucose content increased with the increase in reaction time from 5 to 10 min. Further increase led to the production of laminaribiose, besides glucose and laminaritriose in 2:1:2 ratios. The glucose level increased considerably in the reaction mixture, after 60 min at 37°C, whereas with overnight incubation, laminaritriose was completely hydrolyzed to glucose and laminaribiose (3.2:1). The glucanase nature of the purified enzyme was confirmed with the presence of laminaribiose even after overnight incubation. Release of glucose with both laminarin and laminaripentaose indicated that the enzyme is an exo-enzyme. The purified enzyme showed preference in hydrolyzing the substrate based on the size of the molecule. It could hydrolyze laminaripentaose, laminaritetraose very rapidly over laminaritriose and laminaribiose. With 1:10 dilution, it could hydrolyze laminaripentaose and laminaritetraose completely in 5 min to glucose and laminaribiose or laminaritriose, whereas, overnight incubation was required for complete hydrolysis of laminaritriose. The purified enzyme could hydrolyze curdlan, with glucose as a major end product, indicating that the enzyme is an exo-glucanase. However, the three β-1,3-glucanases isolated from *Acremonium blochii* C59 could hydrolyze the curdlan only at 2-7 %, relative to its action on laminarin[21].

3.5 Effect of purified enzyme on curdlan powder

Out of curiosity and keeping in view of the insolubility of curdlan in water, it was planned to study the action pattern of the purified enzyme on curdlan powder and the results obtained are presented in Table 2. Although only glucose was detected in TLC analysis, formation of traces of laminaribiose and laminaritriose were also detected by HPLC analysis of the reaction mixture after 6 h treatment, whereas only glucose was detected both by TLC and HPLC in the samples treated for 1 h. The amount of glucose was increased with the increase in reaction time to 18 h. It was surprising to see the action of enzyme on curdlan powder, which was not soluble in the enzyme solution at pH 7.0. The gel formed after heating the

reaction mixture in boiling water was very firm in samples incubated for up to 6 h and in control (untreated), whereas it was very weak in samples that were incubated for 12 and 18 h, due to the formation of low molecular weight curdlan. This clearly indicated that the enzyme could act on curdlan powder and hydrolyze to release the glucose and laminaribiose. The DP of curdlan was found to be 1175, whereas the DP of the insoluble matter obtained after hydrolysis was 505, 404 and 241 for the samples obtained after 1, 6 and 12 h of incubation, respectively. This indicates the formation of curdlan of low molecular weight due to the action of purified glucanase on glycosidic linkages. As a result, the solubility of hydrolyzed curdlan in alkali solution (1 N) increased with increase in reaction time. Samples incubated for 12 and 18 h had better and easy solubility than that of unhydrolyzed curdlan and that of the sample incubated for 1 and 6 h.

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Table 1 Purification of the exo-*β*-1,3-glucanase isolated from "Kitarase-M"

Step	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude enzyme	60	630.0	1.10	0.002	100.0	1.0
DEAE-Toyopearl	30	71.1	1.01	0.014	92.2	8.2
CM-Toyopearl	10	15.0	0.41	0.028	37.6	15.7
Sephacryl S-200	6	1.3	0.33	0.265	30.4	152.0

Table 2 HPLC analysis of derivatives of enzyme hydrolyzed laminarin, laminaripentaose and curdlan powder

Substrate	Incubation period (min)	Compounds identified	Ratio
Laminarin	5	glucose	NA
	1080	glucose: laminaribiose	3.9 :1
Laminaripentaose	5	glucose: laminaritriose	0.7 : 1
	10	glucose: laminaritriose	1.3 : 1
	30	glucose : laminaribiose: laminaritriose	2 :1: 2
	60	glucose: laminaribiose: laminaritriose	7 :1.6:1
	1080	glucose: laminaribiose	3.2 : 1
Curdlan Powder	0	Nil	NA (1175)
	60	glucose	NA (505)
	360	glucose: laminaribiose	6:11 (404)
	720	glucose: laminaritriose	7.8:0.5 (241)
	1080	glucose: laminaribiose: laminaritriose	18 : 0.9: 0.(ND)

NA: not applicable; ND: not determined; Value in the brackets indicate degree of polymerization

Fig.1. Elution profile of the proteins and enzyme activity by DEAE-Toyopearl gel column chromotagraphy (-o-) Enzyme activity; (-) Absorbance at 280 nm; (--) NaCl concentration

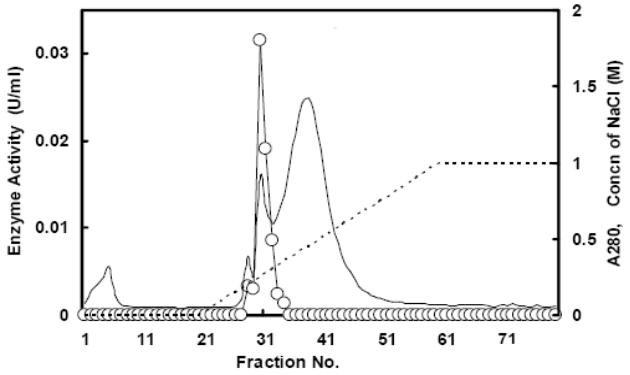

Fig.2. Elution profile of proteins and enzyme activity by CM-Toyopearl column chromatography (□) Enzyme activity; (-) Absorbance at 280 nm

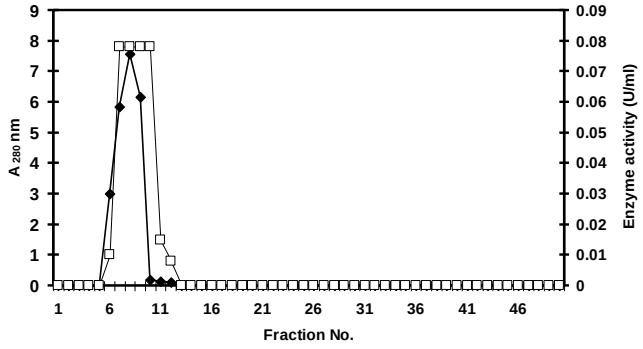

Figure3. Elution profile of proteins and enzyme activity by gel filtration chromatography on Sephacryl S-200 column. (-o-) Enzyme activity; (-) Absorbance at 280 nm

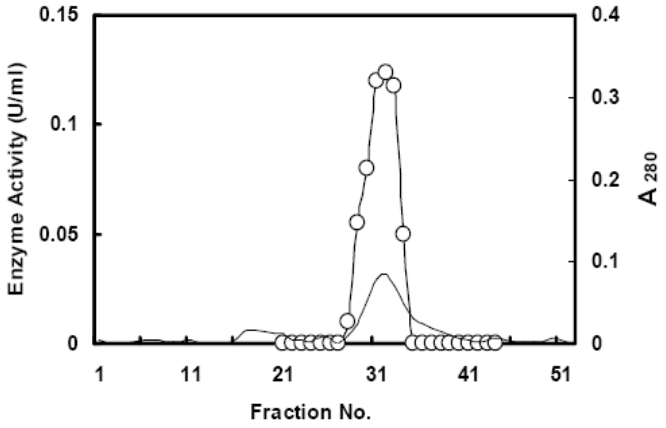

Fig. 4. Temperature profile of purified β-1,3-glucanase with laminarin as a substrate

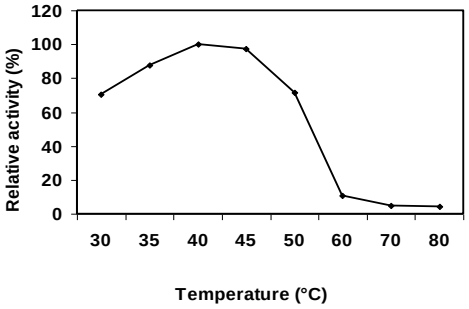

Fig. 5. pH profile of purified β-1,3-glucanase with laminarin as a substrate : (♦) acetate buffer; (■) Tric HCl buffer ; (●) Phosphate buffer ; (▲) citrate-phosphate buffer

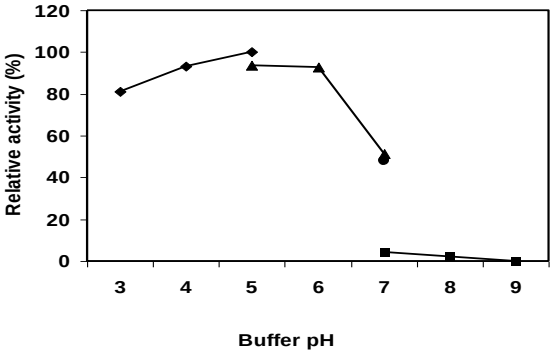

Fig. 6. SDS-PAGE (A) and IEF (B) profiles of purified β-1-3-glucanase

Panel A; E: Purified enzyme, M: Molecular weight standards consisting of myosin (200,000), β- glucosidase (116,000), phosphorylase B (97,400), serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000) and trypsin inhibitor (21,500)

Panel B; E: purified enzyme, M: pI markers - amyloglucosidase (3.5), soybean trypsin inhibitor (4.55), β-lactoglobulin (5.2), bovine carbonic anhydrase (5.58), human carbonic anhydrase (6.55), myoglobin (6.85, 7.35), lentil lectin -acidic (8.15) lentil lectin- middle (8.45) and lentil lectin, base (8.65)

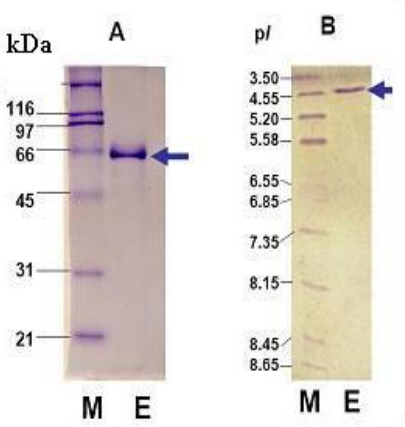

Fig. 7. HPLC analysis of curdlan hydrolysis products