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Purification and characterization of thermostable β-amylase and pullulanase from high-yielding *Clostridium thermosulfurogenes* SV2

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Thermostable β-amylase and pullulanase, secreted by the thermophilic anaerobic bacterium *Clostridium thermosulfurogenes* strain SV2, were purified by salting out with ammonium sulphate, DEAE-cellulose column chromatography, and gel filtration using Sephadex G-200. Maltose was identified as a major hydrolysis product of starch by β-amylase, and maltotriose was identified as a major hydrolysis product of pullulan by pullulanase. The molecular masses of native β-amylase and pullulanase were determined to be 180 and 100 kDa by gel filtration, and 210 and 80 kDa by SDS–PAGE, respectively. The temperature optima of purified β-amylase and pullulanase were 70 and 75 °C, respectively, and both enzymes were completely stable at 70 °C for 2 h. The presence of starch further increased the stability of both the enzymes to 80 °C and both displayed a pH activity optimum of 6.0. The starch hydrolysis products formed by β-amylase action had β-anomeric form.

Key words: Anaerobic bacterium, β-Amylase, pullulanase, thermophilic bacterium, thermostable enzymes.

Maltose and malto-oligosaccharides have applications in food, beverage and pharmaceutical industries. They are produced by the hydrolysis of starch using amylases from higher plants such as barley, sweet potato, soybean and wheat, and also from certain mesophilic bacteria. However, amylases are very expensive and thermally unstable (Hyun & Zeikus 1985a, 1985b; Shen *et al*. 1988). Thermoanaerobic organisms show promise for the production of thermostable and thermoactive enzymes (Zeikus 1979), and efforts have been made to isolate thermoanaerobic bacteria producing thermostable amylolytic and pullulolytic enzymes (Hyun & Zeikus 1985a, 1985b; Koch *et al*. 1987; Nipkov *et al*. 1989; Canganella *et al*. 1994; Swamy *et al*. 1994; Swamy & Seenayya 1996a). Most of the thermophilic microorganisms so far reported produce either β-amylase (Shen *et al*. 1988; Nipkov *et al*. 1989) or pullulanase (Hyun & Zeikus 1985b; Melasniemi 1987; Saha *et al*. 1988). Hyun & Zeikus (1985a) reported both β-amylase and pullulanase from *Clostridium thermosulfurogenes*; however, the yield of pullulanase was comparatively very low.

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β-amylase (EC3.2.1.2) hydrolyses the α-1,4 glucan bonds in amylosaccharide chains from the non-recuding ends and generates maltose (Shen *et al*. 1988). Pullulanase (EC3.2.1.41) hydrolyses the α-1,6 glucosidic linkages of pullulan, amylopectin and related polysaccharides and produces maltotriose. It would be advantageous to have microorganisms that produce both β-amylase and pullulanase simultaneously because the use of these enzymes together would improve the yield of maltose (Norman 1979). We have undertaken the study of *C. thermosulfurogenes* SV2 that produces high yields of thermostable β-amylase and pullulanase. In the present study, we report on the purification and characterization of these enzymes.

Materials and Methods

Organism and Growth Conditions

C. thermosulfurogenes SV2 was routinely grown anaerobically at 60 °C in 120-ml serum vials that contained 20 ml of pre-reduced medium composed of (g/l): soluble starch, 5; NH_4Cl, 1; $MgCl_2$, 0.2; $NaH_2PO_4.7H_2O$, 0.3; peptone, 10; yeast extract, 3; resazurin, 0.002; $FeSO_4$, traces; and an N_2 gas head space. The stationary growth-phase cultures were harvested by centrifugation

(10,000 × *g* for 15 min at 4 °C), and the enzymes from cell-free supernatants were used in further purification processes.

Enzyme Assays

The routine enzyme assays for pullulanase and β-amylase activities involved measuring the reducing sugars resulting from the hydrolysis of pullulan and soluble starch, respectively. The reaction mixture (3 ml) consisted of 0.5 ml of pullulan (1% w/v) (for pullulanase assay) or soluble starch (2% w/v) (for β-amylase assay) and 0.5 ml of appropriately diluted enzyme source in 2.0 ml of 0.1 M phosphate buffer (pH 6.0). After incubation for 30 min at 70 °C for β-amylase and at 75 °C for pullulanase, the reaction was stopped and the reducing sugars were measured by the addition of 1 ml of 3,5-dinitrosalicylic acid reagent. A separate blank was setup for each sample to correct the non-enzymatic release of sugars. One unit of pullulanase or β-amylase was defined as the amount of enzyme which released 1 μmol of reducing sugars as glucose min^{-1} under the standard assay conditions described above.

Purification of Enzymes

Solid ammonium sulphate was added to 1 l of the clarified culture supernatant to 60% saturation. After standing overnight at 4 °C, and centrifugation (15,000 × *g*, 30 min at 4 °C) the re-suspended precipitate (in 25 ml of 0.1 M phosphate buffer, pH 6.0) was dialysed overnight against the same buffer, and re-centrifuged. The supernatant enzyme solution was concentrated by lyophilization and applied to a DEAE–cellulose column (1.5 × 32.0 cm), previously equilibrated with phosphate buffer (0.1 M, pH 6.0), washed with the same buffer and then eluted step-wise by increasing the concentration of NaCl (0 to 0.5 M) in the same buffer. The fractions (5 ml) were collected and protein concentration was monitored by the absorbance at 280 nm.

The active fractions were pooled, concentrated and subjected separately to gel filtration on a Sephadex G-200 column (1.5 × 72 cm), that had been equilibrated with 0.1 M phosphate buffer (pH 6.0). The proteins were eluted with the same buffer. This gel filtration step was repeated twice.

Determination of Protein

Protein was determined according to the method of Lowry, using BSA (fraction V) as a standard protein, and also by measuring the absorbance at 280 nm.

Identification of Hydrolysis Products

The reaction end products of *C. thermosulfurogenes* SV2 amylase and pullulanase were analysed by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC).

The purified β-amylase and pullulanase, respectively, were incubated with 2% (w/v) soluble starch and 2% (w/v) pullulan at 70 and 75 °C in 0.1 M phosphate buffer (pH 6.0). Samples were withdrawn at various time intervals and the reaction was stopped by heating in boiling water for 5 min. The sugars in these hydrolysates were analysed by TLC and separation on an HPLC column.

TLC was carried out with a solvent system of butanol/acetic acid/water (3:1:1 by volume) (Kim *et al*. 1995), using precoated silica gel plates (Merck, art No. 5553). After developing the products, the sugar spots were visualized by spraying with a reagent consisting of aniline (4 ml), diphenylamine (4 g), acetone (200 ml), and 85% H_3PO_4 (30 ml) (Hansen 1975), and then drying the plates in an oven at 105 °C for 1 h. The HPLC separation system consisted of a Shimadzu RID-6A detector, a Shimadzu CR-4A Chromatopac monitor and an ODS.C18 column (250 × 4.6 mm).

Effects of pH

The relative activities of β-amylase and pullulanase were determined at several pH values (2.0–10.0) under standard assay conditions as described above. To test the pH stabilities, the purified enzymes were separately incubated for 1 h in the above buffers at 70 °C. The remaining activities were then assayed under standard conditions.

Effects of Temperature

The relative activities of β-amylase and pullulanase were determined at various reaction temperatures. To determine the thermostability, enzyme samples in 0.1 M phosphate buffer (pH 6.0) were incubated at various temperatures, and samples were withdrawn for assaying the remaining activities at appropriate time intervals. Effects of different concentrations of starch on thermal stabilities of the enzymes were determined by incubating the reaction mixtures at 80 °C and the remaining activities assayed as described above.

Determination of Molecular Weights

The molecular weights of the purified native β-amylase and pullulanase were estimated by gel filtration chromatography (Andrews 1964). A column (1.5 × 72 cm) of Sephadex G-200 which was equilibrated with 0.1 M phosphate buffer (pH 6.0) was used. SDS–PAGE was performed by the method of Laemmli (1970). The molecular weights of the purified enzymes were estimated from their positions relative to those of standard proteins.

Identification of the Anomeric Form of the Product

The mutarotations of the products formed from soluble starch with the purified *C. thermosulfurogenes* SV2 β-amylase and B. *subtilis* α-amylase (Sigma) were separately examined as described by Hyun & Zeikus (1985a) in a Perkin Elmer Model 214 polarimeter by using the sodium line.

Results

Purification of Enzymes

C. thermosulfurogenes SV2 produced 770 and 910 U of β-amylase and pullulanase (both extracellular and cell bound), respectively per litre. After centrifugation, the crude culture supernatant contained approximately 330 U of β-amylase and 410 U of pullulanase per litre. A summary of the purification is presented in Table 1. After purification, the specific activities of β-amylase and pullulanase, in two separate preparations, were 12.28 and 17.17 U per mg of protein, respectively. The elution profile of the enzymes on the DEAE–cellulose column is shown in Figure 1. Overall, the specific activities of β-amylase and pullulanase increased approximately 86.5 and 98.1-fold, with a yield of 9.5% and 8.8%, respectively.

Product Formation

TLC analysis of reaction products of *C. thermosulfurogenes* SV2 amylase on starch showed that maltose was the main product. The appearance of maltose as the major hydrolysis product and the relatively small amount of

Table 1. Summary of purification procedure of *Clostridium thermosulfurogenes* SV2 β-amylase* (A) & pullulanase* (P).

Procedure	Total protein (mg)	Total activity (U)		Specific activity (U/mg protein)		Purification factor		Yield (%)	
		A	P	A	P	A	P	A	P
Culture supernatant	2343	334.0	410.0	0.142	0.175	1	1	100	100
Salting out with $(NH_4)_2SO_4$ (60% saturation) and dialysis	261	113.8	181.0	0.436	0.693	3	4	34	44
DEAE–cellulose column chromatography	27.6 18.2	67.3	71.5	2.438	3.928	17.2	22.4	20	17.4
I Sephadex G-200 gel filtration	7.3 5.1	51.6	48.2	7.068	9.451	49.7	54	15.4	11.7
II Sephadex G-200 gel filtration	2.58 2.12	31.7	36.4	12.28	17.17	86.5	98.1	9.5	8.8

*Assayed with 2% soluble starch and 1% pullulan, respectively.
β-Amylase fraction.
Pullulanase fraction.

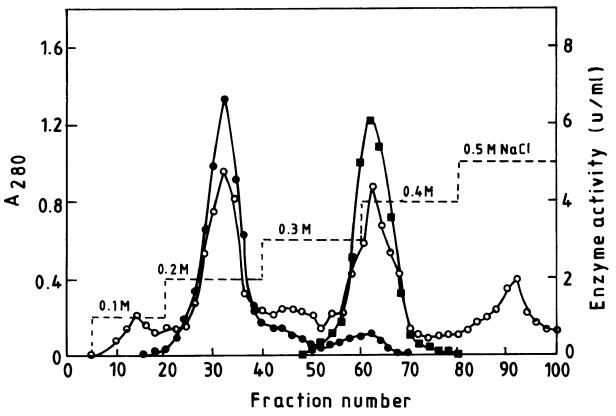


Figure 1. Elution profiles of β-amylase and pullulanase on DEAE–cellulose column chromatography. •, β-amylase activity (U); ■, pullulanase activity (U); ○, protein (A_{280}).

glucose implies that the amylase produced by this microorganism is of the β type. TLC of the hydrolysis products of pullulan by the action of *C. thermosulfurogenes* SV2 pullulanase showed that maltotriose was the main product.

HPLC analysis of starch and pullulan hydrolysates formed by purified β-amylase and pullulanase, respectively, also revealed that maltose and maltotriose were, respectively, the major hydrolysis products.

Influence of pH on Activity and Stability

Both the enzymes showed optimal activity at pH 6.0. The β-amylase was most stable over a broad pH range of 5.5–7.0 and retained about 85 and 61% activities at pH 4.5 and 8.0, respectively. Pullulanase was most stable between pH 6.0 and 7.0, and was reasonably stable between pH 4.00 and 7.5.

Influence of Temperature on Activity and Stability

The optimum temperatures for β-amylase and pullulanase activities were 70 and 75 °C, respectively. Both the enzymes were completely stable at 70 °C for 2 h. About 48 and 68% amylase and pullulanase activities, respectively, remained after 2 h at 75 °C. About 50 and 40% amylase and pullulanase activities, respectively, were lost within 5 min at 80 °C (Figure 2a). However, the presence of 4% starch increased the thermal stability of the enzymes at 80 °C to 2 h (Figure 2b).

Anomeric Form of Product

Figure 3 shows the mutarotation of the hydrolysis products derived from starch with *C. thermosulfurogenes* SV2 β-amylase and *B. subtilis* α-amylase. Upon addition of solid sodium carbonate to the reaction mixture containing the *C. thermosulfurogenes* SV2 β-amylase and soluble starch, the optical rotation shifted upward, indicating that the hydrolysis product had a β-anomeric configuration.

Molecular Weights

The molecular weights of the purified native β-amylase and pullulanase, as determined by the gel filtration method using Sephadex G-200 (Figure 4), were found to be approximately 180 and 100 kDa, respectively, whereas, by SDS–PAGE they were found to be 210 and 80 kDa, respectively.

Discussion

The yield of the enzymes in the present study, when compared to any single strain producing both thermostable β-amylase and thermostable pullulanase reported so far, is high. *C. thermosulfurogenes* 4B produced 5.57 U

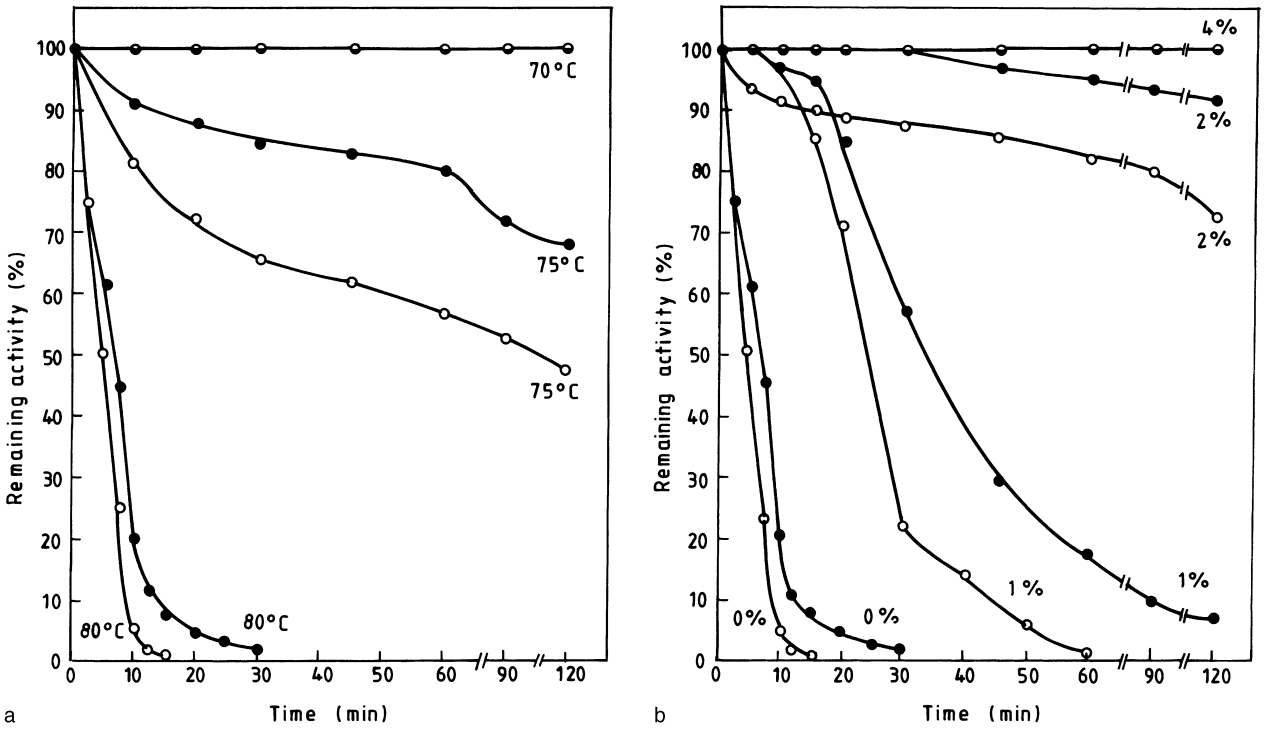
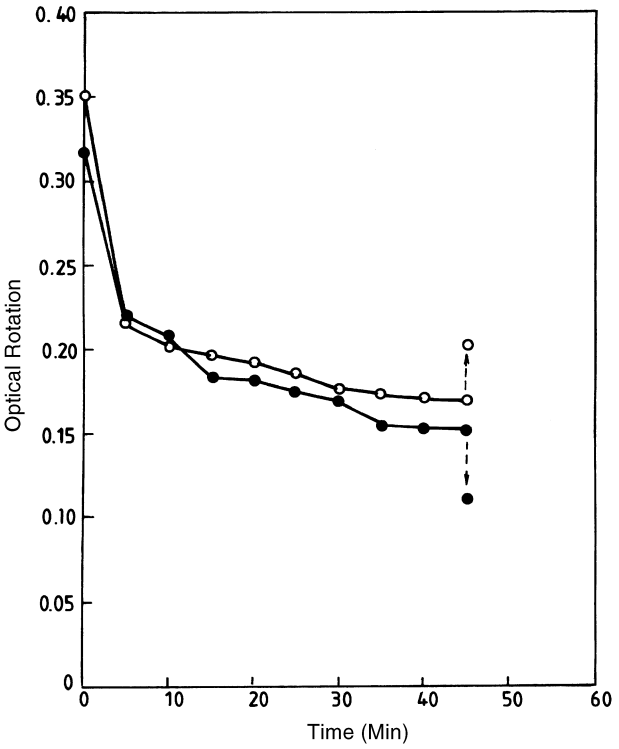


Figure 2. (a) Thermal stabilities of β-amylase (○) and pullulanase (●) in the absence of starch. In each case, 2.5 U of purified enzyme was incubated in 0.1 M phosphate buffer (pH 6.0) at the temperature indicated and samples were withdrawn at various time intervals as shown and the remaining activities were measured under standard assay conditions. (b) Thermal stabilities of β-amylase (○) and pullulanase (●) in the presence of starch. The experiments were performed as described in (a) except that the enzyme samples were incubated at 80 °C with the indicated amount of starch.



(Hyun & Zeikus 1985a) and its catabolic repression-resistant mutant H-12-1 produced 16.2 U (Shen *et al*. 1988) of β-amylase per ml culture broth. However, the content of pullulanase was less than 0.01 U per ml culture broth. In contrast, *C. thermohydrosulfuricum* produced 0.18 U of thermostable pullulanase per ml of culture broth and 0.01 U of an uncharacterized amylase per ml culture broth (Hyun & Zeikus 1985b), and *C. thermosulfurogenes* SV9 produced 0.64 U of thermostable pullulanase per ml of culture broth (Swamy & Seenayya 1996b).

The molecular weight of β-amylase from strain SV2 was found to be closer to that of sweet potato β-amylase and to that reported for *C. thermosulfurogenes* (Shen *et al*. 1988). The molecular weight of SV2 pullulanase was found to be similar to the pullulanase from *Thermus*

Figure 3. Mutarotation of the products with *C. thermosulfurogenes* SV2 β-amylase (○) and *B. subtilis* α-amylase (●). The reaction mixture containing 2% soluble starch and 2.5 U of purified β-amylase in 0.1 M phosphate buffer (pH 6.0) was added to a 10 cm cell. When the optical rotation of the reaction mixture became approximately constant, ~ 20 mg of solid sodium carbonate was added and the mutarotation of the mixture was measured (dashed lines).

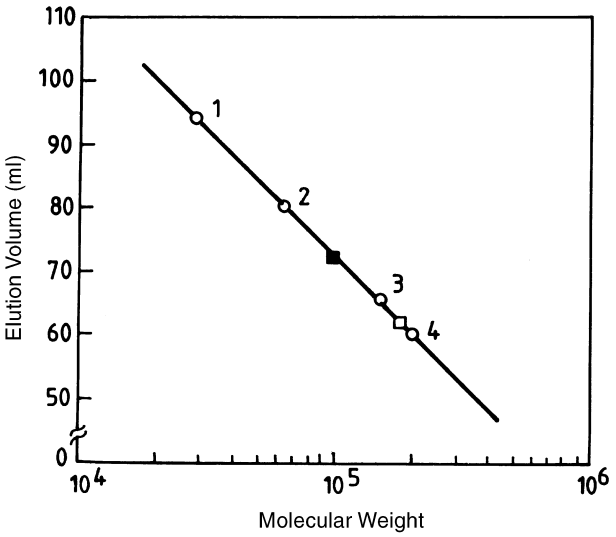


Figure 4. Plot of elution volume against logarithm of molecular weight of proteins on Sephadex G-200 (1.5 × 72 cm) with a flow rate of 6 ml/h and 2 ml fractions. (1) carbonic anhydrase (MW 29,000); (2) BSA (66,000); (3) yeast alcohol dehydrogenase (1,50,000); (4) sweet potato β-amylase (2,00,000); (□) SV2-β-amylase; (■) SV2 pullulanase.

aquaticus YT-1 (Adrian *et al*. 1986) but less than the pullulanase from *C. thermohydrosulfuricum* (Saha *et al*. 1988), *Bacillus* sp. 3183 (Saha *et al*. 1989) and *C. thermosulfurogenes* EM1 (Spreinat & Antranikian 1990).

The temperature optima and heat stability of both the enzymes were comparable to those reported for thermostable β-amylases (Hyun & Zeikus 1985a; Shen *et al*. 1988; Nipkov *et al*. 1989) and pullulanases (Hyun & Zeikus 1985b; Koch *et al*. 1987; *Shen et al*. 1990; Spreinat & Antranikian 1990).

The successful industrial application of these enzymes will depend largely on the improvement of cultivation systems and the availability of higher enzyme yields.

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