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Biochemical characterization of a novel extracellular pullulan 6-glucanohydrolase from *Bacillus circulans* S-1

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Abstract

Bacillus circulans S-1 extracellular pullulan 6-glucanohydrolase (EP) (EC 3.2.1.41) has been characterized with a purified enzyme of 140 kDa. The N-terminal amino acid sequence of the purified enzyme was P-L-N-M-S-Q-P. The enzyme displayed a temperature optimum of around 60℃ and a pH optimum of around pH 9.0. The enzyme was stable to incubation from pH 4.0 to pH 11.0 at 4℃ for 48 h. The presence of substrate allowed the protection of the enzyme from heat inactivation. The activity of the enzyme was stimulated by several metal ions such as Mn^{2+} and Ca^{2+}. The enzyme had an apparent Km of 7.92 mg/ml for pullulan. The purified enzyme completely hydrolysed pullulan to maltotriose.

Key words – pullulanase, extracellular pullulan 6-glucanohydrolase (EP), (*Bacillus circulans* S-1)

Introduction

Pullulan 6-glucanohydrolase (EC 3.2.1.41) has recently been receiving much attention as it is important for starch conversion bioprocesses. The enzyme can be used together with glucoamylase to improve the efficiency of starch saccharification for the production of high glucose syrups or with β-amylase to increase the maltose content in high maltose syrups. The enzyme has an activity to hydrolyze the α-1,6-glucosidic linkages of pullulan and produces maltotriose as the end product[1]. This enzyme was first found in *Klebsiella pneumoniae* by Bender and Wallenfels[2]. Pullulanases can split the α-1,6-glucosidic linkages of branched polysaccharides, such as amylopectin or glycogen. Isoamylase also hydrolyzes the α-1,6-glucosidic inter chain linkages of certain branched α-D-glucans but it cannot hydrolyze pullulan.

In the last decade, a number of pullulanase producers have een reported, especially among thermophilic bacteria [3-8]. Most enzymes from thermophilic bacteria are Type II pullulanases, which hydrolyze the α-1,6-glucosidic linkages of pullulan, as well as the α-1,4-linkages of other polysaccharides. Pullulanases of Type I, which exclusively hydrolyze the α-1,6-linkages of pullulan, are produced by *K. pneumoniae*[2], *Bacteroides thetaiotaomicron* 95-1[9], alkalophilic *Bacillus* sp. KSM-1876[10], *Thermus aquaticus* YT-1[4], and *Bacillus acidopullulyticus*[11]. Pullulanases from *K. pneumoniae* and *B. acidopullulyticus* are active in narrow ranges of pH between 4.0 and 6.0, and have no stability in high pH. To our knowledge, there is

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few reports on microbial alkaline pullulanases of Type I [10,12], with a broad pH stability. We have screened for alkalophilic microorganisms that produce extracellular, alkaline pullulanases having broad pH stability[13]. Compared to most of the microorganisims investigated these isolates secreted extremely high concentrations of pullulanase in batch culture. Up to 80% of the total enzyme was detected in the culture fluid and the remaining about 20% of the enzyme was detected in cell extracts[13].

We report here on the purification and characterization of Type I alkaline pullulanase, having broad pH stability, from the culture of the alkalophilic *Bacillus* sp. S-1.

MATERIALS AND METHODS

Materials

Pullulan (M_r=65,000), panose, and isopanose were from Hayashibara Co. Ltd. (Okayama, Japan). Maltooligosaccharides were purchased from Nihon Shokuhin Kako Co. (Tokyo, Japan), and α-, β-, and γ-cyclodextrins were kindly supplied by Dr. H.Taniguchi, Iwate Fermentation and Brewing Research Institute, Japan. Shimpak Ultron 300-C4 column (pore size of 5 μm, 4.6 mm×150 mm) for reverse-phase high pressure liquid chromatography (HPLC) was purchased from Shinwa Co.Ltd.(Tokyo, Japan).

Preparation and purification of EP

The pullulanase-hyperproducing *Bacillus circulans* S-1 was isolated from a soil sample collected in the Yusung Hot Spring area, Korea[13]. *B. circulans* S-1 was cultivated aerobically at 50℃ and pH 9.0 for 2 days in a 5-liter stirred-tank fermentor (Korea Fermentor Co. Ltd, Inchon, Korea). The working volume was 3 liters and a speed of the impeller of 350 rpm was maintained. The medium consisted of (w/v): 1% potato starch, 0.5% yeast extract, 0.1% K_2HPO_4, 0.1% $MnCl_2$, 0.2% $MgSO_4 7H_2O$, and 1% Na_2CO_3 (pH 9.0). The culture broth obtained was centrifuged, and the supernatant was used for purification of EP.

Unless otherwise stated, all procedures were done out at 4℃. After a 2-day cultivation, cells were removed from the culture medium by centrifugation (6,000×g, 15 min) at 4℃. The crude enzyme was fractionated with 20 to 70% saturation of ammonium sulfate. The precipitates were dissolved in 120 ml of 50 mM Tris-HCl buffer (pH 8.0) and dialyzed against the same buffer overnight. The dialysate was chromatographed sequentially on a column of DEAE-Toyopearl M650 (2.3 cm×25 cm; Tosoh Co. Ltd., Tokyo, Japan) equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The column was washed with a large volume of the equilibration buffer, and proteins were eluted in 5-ml fractions at a flow rate of 30 ml/hr with a gradient of 0 to 1 M NaCl in the same buffer, as shown in Fig. 1A. The active enzyme fractions (tubes nos 44-58) were combined and concentrated by ultrafiltration (Amicon PM30; Amicon Co. Ltd., Danvers, MA, USA) and dialyzed against the same buffer overnight.

The retentate (5 ml) was applied to a column of Mono-Q (1.2 cm×12 cm; Pharmacia LKB, Upssala, Sweden) equilibrated with 50 mM Tris-HCl buffer (pH 8.0). After washing the column with the same buffer, proteins were eluted with a gradient of 0.3-0.45 M NaCl in the same buffer. Active fractions (tubes Nos 42-53) were pooled and concentrated by ultrafiltration (Amicon PM30), and the concentrate was dialyzed against the same buffer containing 0.15 M NaCl. The dialyzed sample (1 ml) was put on a column of Sepharose 4B (FPLC, Pharmacia LKB, Upssala, Sweden) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. The active enzyme fractions (tubes 36-41) were collected, concentrated, and dialyzed against 50 mM Tris-HCl buffer (pH 8.0). The dialyzed enzyme was used as pure EP. For analysis of the N-terminal amino acid sequence, the purified EP was further ultra-purified by chromatography on a column of Ultron 300-C4, using an HPLC System Gold (Beckman, San Ramon, CA, USA).

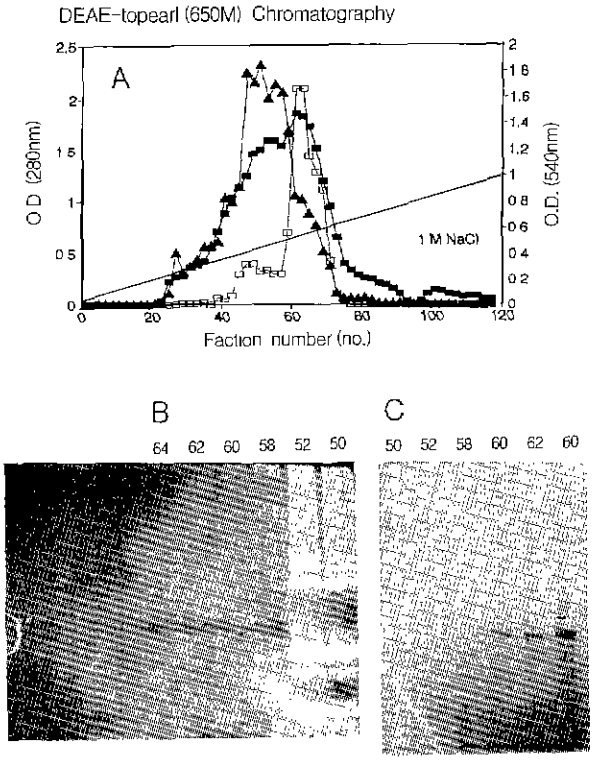

Fig. 1. Separation of the EP using DEAE column.
A) Chromatogram of the crude enzyme purified by ammonium sulfate precipitation (20-70%). —, NaCl gradient; ■, protein concentration; ▲, pullulanase activity; □, amylase activity.
B) Pullulanase activity staining after native-PAGE. Pullulanase activity is a strong blue and amylase activity is white on the weak blue background. C) Protein staining after pullulanase activity staining described in B).

Biochemical assays

Pullulanase and amylase activities were assayed by measuring the reducing sugar released from pullulan and soluble starch as substrates, respectively[14]. The reaction mixture (1.0 ml) containing pullulan or soluble starch (1%, w/v), 50 mM Tris-HCl (pH 9.0), and enzyme (5-20 μg) was incubated at 50℃ for 30 min. The reducing sugar was measured by the Somogyi-Nelson method[15] or by the dinitrosalicylic acid procedure[16]. One unit (U) of pullulanase or amylase activity was defined as the amount of enzyme that produced 1 μmole of reducing sugar as glucose per min under the conditions described above. Protein were measured by the method of Lowry *et al.*[17] with bovine serum albumin as the standard. The absorbance at 280 nm was used to monitor protein in column eluates.

Native polyacrylamide gel electrophoresis (PAGE) was done using 7.5% (w/v) polyacrylamide gels, as described by Davis[18]. For Activity staining of pullulanase after PAGE, gels were soaked in a solution of 1% (w/v) starch in 50 mM Tris-HCl buffer (pH 9.0) at 50℃ for 30 min, rinsed with water, then stained for amylase or pullulanase activity by flooding with an iodine solution.[19] The bands of amylase activity were seen as white zones and those of pullulanase activity as blue zones, on the weak blue background.

Discontinuous sodium dodecyl sulfate (SDS)-PAGE was done essentially by the method of Laemmli.[20] The bands of protein were stained with Coomassie Blue R250 dye and destained with a solution of 5% methanol-7.5% acetic acid (v/v). Molecular mass markers (Bio-Rad) used were myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (BSA, 67 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). The molecular mass of pullulanase was also measured by calibration of a Sepharose 4B column (1.6 cm×76 cm) with standard proteins which included ovalbumin, BSA, cytochrome C (12.4 kDa), catalase (232 kDa), and thyroglobulin (669 kDa).

Preparation of mouse antiserum against the purified EP (M_r = 140000) is described[13]. Western immunoblotting of protein was done by the method of Gooderham *et al.*[21] using a biotinyl-anti IgG (Vector Laboratories Inc., CA, U.S.A) of goat and streptavidin-horseradish peroxidase system (Promega, Madison, WI, USA).

After ultra-purification of the final sample by reverse phase chromatography using an Ultron 300-C4 column, the N-terminal part of the protein was sequenced using a 470A gas phase protein sequencer equipped with a 120A on-line phenylthiohydantoin analyzer (Applied Biosystems, Warrington, Cheshire, U.K.)

Schiff staining for glycosylation of protein was done. [22] Oligosaccharides produced by enzymatic action were examined by thin layer chromatography (TLC), as described by Kim *et al.*[19].

Effects of pH on the activity and stability

The enzyme activities of the crude enzyme and purified EP were measured as in the pullulanase assay, except that 0.023 U of enzyme was added and the pH was changed using 50 $\mu\ell$ of one of the buffers at various pHs. Buffers used were 30 mM sodium acetate buffer (pH 3.0 - 6.0), 30 mM sodium phosphate buffer (pH 6.0 - 8.0), 30 mM Tris-HCl buffer (pH 8.0 - 10.0), and 30 mM glycine buffer (pH 9.0 - 12.0). The activity found at pH 9.0 was taken as 100%. For pH stability of the enzymes, enzyme solutions kept in 30 mM various buffers of glycine-HCl (pH 2.0 - 3.0) and the buffer system described above for 24 hr at 4℃, and assayed for remaining activity after twofold dilution with 1.0 M Tris-HCl (pH 9.0). The activity of untreated enzyme was expressed as 100%.

For effects of temperature on the activity and stability. The enzyme activities were measured as in the pullulanase assay, except that 0.092 U of enzyme was incubated with 2% pullulan at various temperatures (20 to 95℃). The maximum in each temperature-activity profile was taken as 100%. For thermal stability, the enzyme solutions were kept at various temperatures for 60 min in Tris-HCl (pH 9.0), and then the residual activity was measured.

RESULTS AND DISCUSSION

Enzyme purification

EP was purified 101.6-fold to homogeneity with a specific activity of 744.6 U/mg protein (Table 1). From fractions 40 to 60 eluted from DEAE ion exchange chromatography, two or more peaks were eluted (Fig. 1A). The active fractions were indistinguishable on native-PAGE followed by activity staining and protein staining (Fig. 1B). Pullulanases of the active fractions were further purified by a Mono Q column of FPLC, and a Sepharose 4B column. The single peak of activity eluted was used as purified EP. The purified enzyme migrated a single protein band on SDS-PAGE and native-PAGE (Fig. 2). To obtain the ultra-pure EP for N-terminal amino acid sequencing, further purification was done by reverse phase HPLC using an Ultron 300-C4 column.

Comparison of the EP with other enzymes

The molecular mass of the EP was 140 kDa. A calibrated column of Sepharose 4B gave a molecular mass of 143 kDa. The value was higher than those of any pullulanases reported to date. The molecular masses of pullulanases reported thus far are below 140 kDa: alkalophilic *Bacillus* sp. KSM-1876 (120 kDa)[10], *K. pneumoniae* (80 kDa)[2], *T. aquaticus* YT1 (83 kDa)[4], *B. acidopullulyticus* (115 and 116 kDa)[11], *C. thermosulfurogenes* EM1 (102 kDa)[3], alkalophilic *Bacillus* No. 202-1 (92 kDa)[12], *B. flavocaldarius* KP1228 (55 kDa)[23], alk

Table 1. Purification of *Bacillus circulans* S-1 EP

Fraction	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
Supernatant	551,220	75,250	7.3	100	1
Ammonium sulfate	465,660	52,426	8.9	89	1.2
DEAE-chromatography	217,428	842	228.2	39.4	31.1
FPLC-Mono Q chromatography	181,491	427	425.1	32.9	57.9
Gel permeation	162,320	218	744.6	11.3	101.6

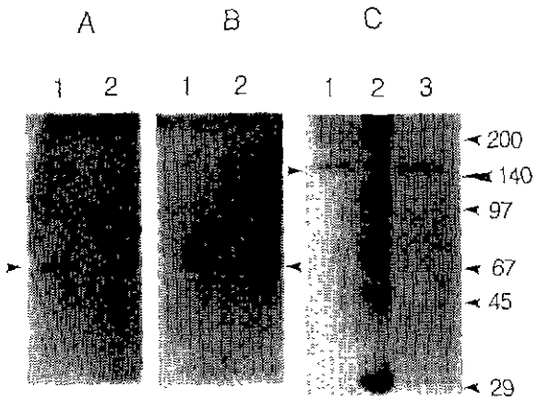

Fig. 2. Electrophoresis of the EP.
A) Native PAGE of the purified EP after activity staining. Purified enzyme was applied in parallel (lanes 1 and 2). B) Activity staining of the purified EP. Purified enzyme was applied in parallel (lane 1 and 2). C) SDS-PAGE of the purified EP. Approximately 10 μg of protein was put on each lane. Arrowheads denote the position of EP. Positions of standard proteins are indicated by (small) arrowheads

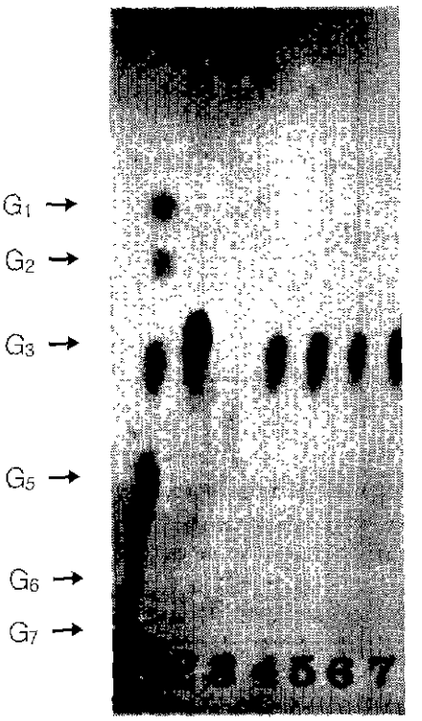

Fig. 3. TLC analysis of the Reaction Products Produced by the EP.
Lane 1, a standard mixture containing a series of maltooligosaccharides; lane 2, panose; lane 3, without samples; lane 4, 5-min; lane 5, 10-min; lane 6, 30-min; lane 7, 60-min incubation.

alopsychrotrophic *Micrococcus* sp. (120 kDa)[24], thermophilic *Bacillus* sp. (100 kDa)[5], *B. thetaiotaomicron* 95-1 (72 kDa)[9], *B. stearothermophilus* (70 kDa)[25] and *T. thalpophilus* (79 kDa[6]. Some pullulanase-like enzymes, such as *B. circulans* F-2 amylase-pullulanse enzyme (220 kDa)[26], *B. subtilis* pullulanase-amylase complex (450 kDa)[27], *Thermoanaerobacter* B6A amylopullulanase (450 kDa)[7], and *C. thermohydrosulfuricum* α-amylase-pullulanase (165 kDa)[8], have been regarded as enzymes of high molecular masses. However, these enzymes are not true pullulanases in that they all have dual activities of amylase and pullulanase. Therefore, EP from *Bacillus circulans* S-1 can be identified as a real pullulanase or pullulanase Type I which has the highest molecular mass.

Physicochemical properties

The isoelectric point of EP was estimated to be 5.5 by isoelectric focussing using a Pharmacia LKB IEF system (data not shown). The pullulanase, when resolved by SDS-PAGE, was negative for staining with the Schiff reagent, indicating that the enzyme is not glycosylated. The ultra-purified sample was treated by Edman degradation. The N-terminal amino acid sequence of the enzyme was Phe-Leu-Asn-Met-Ser-(Trp-Phe).

The optimum temperature for the enzyme reaction was around 60℃ at pH 9.0 (Fig. 4B). The purified EP was stable up to 70℃, after incubation for 60 min at pH 9.0, and the activity was lost completely on heating at 85 ℃ (Fig 4B). However, crude enzyme was stable up to 80 ℃. The enzyme was stabilized by the addition of the substrate, pullulan. As the temperature for optimal activity rose with increasing substrate added, the thermostability of this enzyme seems more enhanced at higher substrate levels. The enzyme was active at 75℃ in 3.0% (w/v) pullulan. Pullulan protected the enzyme from heat inactivation, and this effect was dependent on the

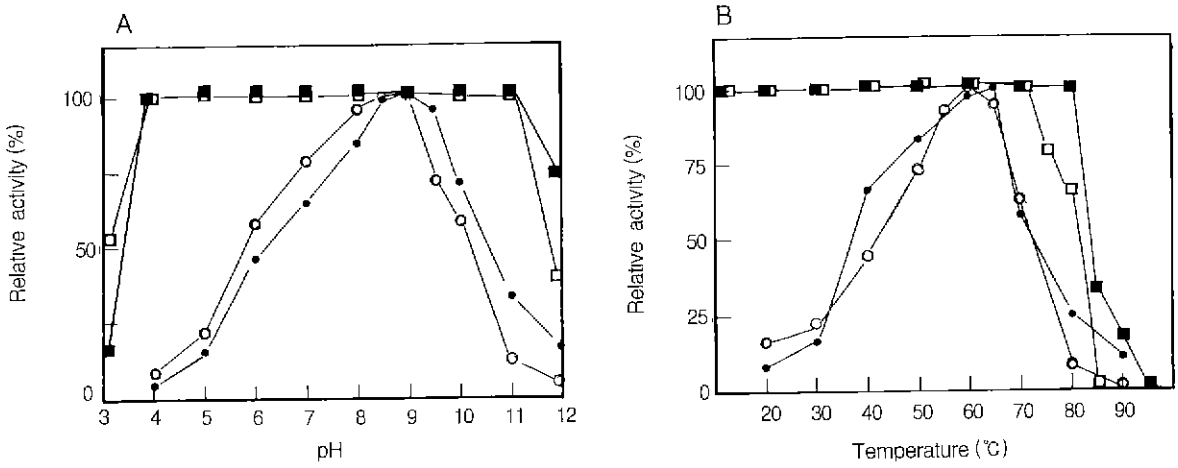

Fig. 4. Effects of pH and Temperature on the Crude Enzyme and Purified EP.
A) Effects of pH on the activity and stability. B) Effects of temperature on the activity and stability. □, stability of purified EP, ■, stability of crude enzyme, ○, activity of purified EP; ●, activity of crude enzyme.

added concentrations. In the absence of pullulan, EP rapidly lost its pullulanase activity at temperatures above 70℃, and the half-life of the enzyme was 72 hr when incubated at pH 9.0 and at 70℃. In the presence of 5% (w/v) starch, pullulanase activity was completely stable at 70℃, and 45% of the original activity remained after heating at 80℃ for 30 min. This property and stability will give it potential as a debranching enzyme in the starch-processing industry[28]. EP was most active at around pH 9.0 (Fig. 4A). This optimum pH is higher than those of other pullulanases from *K. pneumoniae* (pH 6.5)[2], *B. acidpullulyticus* (pH 5.0)[11], alkalophilic *Bacillus* No 202-1 (pH 8.5-9.0)[12], and an alkalopsychrotrophic *Micrococcus* sp. (pH 8.0)[12]. One exception is the pullulanase produced by alkalophilic *Bacillus* sp. KSM-1876 which had an optimum activity at pH 10.0-10.5[10]. EP was stable from pH 4.0 to 11.0 after incubation at 4℃ for 24 hr (Fig. 4A). Thus, the pullulanase from *B. circulans* S-1 is the most alkali-resistant among microbial pullulanases reported thus far. Although many bacteria elaborate starch debranching or pullulanase enzymes, these enzymes are sufficiently narrow in pH range as to preclude their use in enzyme-based starch technology [28]. Allen and Dawson[28] have emphasized that the principle requirements for pullulanases of commercial importance are thermal stability and high activity over a wide range of pH.

Divalent cations, such as Zn^{2+}, Cu^{2+}, and Fe^{2+} ions, inhibited the enzyme activity (Table 2). Ca^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, K^{+}, and Na^{+} ions, each at 1 mM, were neither inhibitory nor stimulatory. Mn^{2+} ions greatly stimulated the EP activity and EDTA was not inhibitory. The purified EP was dialyzed against H_2O or EDTA for 24 hr.

Table 2. Effects of metal ions on enzyme activity

Reagent	Concentration (mM)	Relative activity (%)
No addition	-	100
NaCl	1	110
CaCl2	1	107
CoCl2	1	112
NiCl2	1	106
MgCl2	1	107
ZnCl2	1	71
CuCl2	1	46
CuSO4	1	54
FeSO4	1	94
FeCl3	1	67
MnCl2	1	144
EDTA	1	107

The effects of Mn^{2+} ions and EDTA on the pullulanase activity were then examined (Fig. 5). Dialysis against EDTA did not inhibit the activity, when compared with the control (dialyzed against H_2O). EDTA and EGTA, each at 15 mM, were also not inhibitory under our assay conditions, suggesting that these chemicals did not chelate a possible divalent cation(s) required for the pullulanase activity of PUL-E. Such insensitivity to EDTA and EGTA is also observed with *B. flavocaldarius* pullulanase[32]. However, dialysis against EDTA stimulated of the enzymatic activity of EP in the presence of Mn^{2+}, suggesting that Mn^{2+} ions are required for full activity of the pullulanase. In most cases of the reported pullulanases, Ca^{2+} ions are required for their full activity[2,5,6,24]. It was known that Ca^{2+} ions strongly enhance both the activity and stability of pullulanases from *T. aquaticus*[4] and *T. thalpophilus*[5] at a high temperature. Ca^{2+} ions are without effect on the activities of pullulanases from *Bacillus circulans* S-1 (Table 2) and from *B. flavocaldarius* [29]. The activity of EP was not inhibited by α-cyclodextrin. Furthermore, β- and γ-cyclodextrins, known as possible competitive inhibitors of pullulanase[30], had no effect. This results differentiate EP from other bacterial pullulanases.

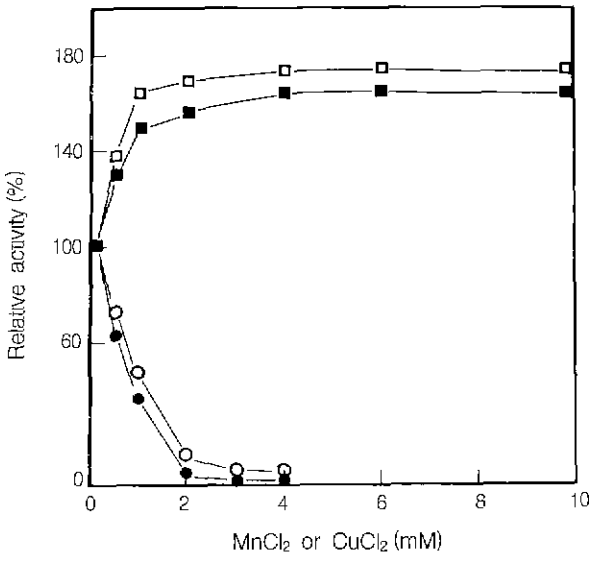

Fig. 5. Effects of $MnCl_2$ and $CuCl_2$ on EP activity.
□, purified EP with $MnCl_2$; ■, crude enzyme with $MnCl_2$; ○, purified EP with $CuCl_2$; ●, purified EP with $CuCl_2$

Substrate specificity

Of the carbohydrate substrates tested, the enzyme hydrolyzed pullulan efficiently. α,β- Limited dextrin, β-limited dextrin, soluble starch, amylopectin, potato starch, and glycogen were also hydrolyzed to some extent. Dextran and α-, β- or γ- cyclodextrins were practically unaffected by the enzyme (Table 3). The enzyme had an apparent Km of 7.92 mg/ml for pullulan, with a Vmax of 396 mg/min/ml; a Km of 1.63 mg/ml for amylopectin, with a Vmax of 37.8 mg/min/ml; and a Km of 3.1 mg/ml for α,β-limited dextran, with a Vmax of 39.6 mg/min/ml.

Maltotriose was detectable as the sole product of hydrolysis of pullulan, indicating that the enzyme had an absolute specificity directed at the $\alpha(1\rightarrow6)$ glucosidic linkages of pullulan (Fig. 3). Low levels of glucose, maltose, and maltotriose were also detectable in the hydrolysis products of amylopectin and soluble starch. This observation is consistent with activity being directed at the $\alpha(1\rightarrow6)$ branch points of amylopectin and starch. Maltose, maltotriose, maltotetraose, and dextran were not hydrolyzed by EP like *B. acidpullulticus*[11], *K. pneumoniae* [2], *T aquaticus* YT-1[4], and *Micrococcus* sp.[12] In addition to pullulanase (pullulanase type I), pullulan-

Table 3. Substrate Specificity of *Bacillus circulans* S-1 EP

Substrate (2%)	Relative activity (%)
Pullulan	100
α,β-Limited dextrin	46.3
Amylose	N.D
Dextran	N.D
β-Limited dextrin	18.3
Amylopectin	23.0
Liver glycogen	17.7

N.D., Not Detected.

hydrolyzing activities have been demonstrated in a glucoamylase that hydrolyze pullulan at the nonreducing end to produce glucose; an isopullulanase that cleaves the α-1,4-glucosidic linkages of pullulan to generate isopanose[31], and a neopullulanase[25] both of which act on the α-1,4-glucosidic linkages of pullulan to panose. Like pullulanases from *K. pneumoniae*[2] and *Bacillus KSM-1876[10]*, the enzyme from *Bacillus circulans* S-1 generated maltotriose, and not panose and isopanose from pullulan. Unlike pullulanases from alkalophilic *Bacillus* KSM-1876[10], and *K. pneumoniae*[2], our EP, as well as the enzyme from alkalophilic *Bacillus* No 202-1 [12], hydrolyzed glycogens.

Western blotting analysis was done to find whether the pullulanase was in a single enzyme form in the extracellular bacterial system. Culture supernatants were collected from bacterial culture and tested by native PAGE, and then blotted onto a nitrocellulose membrane. As shown in Fig. 6, only one protein corresponding to

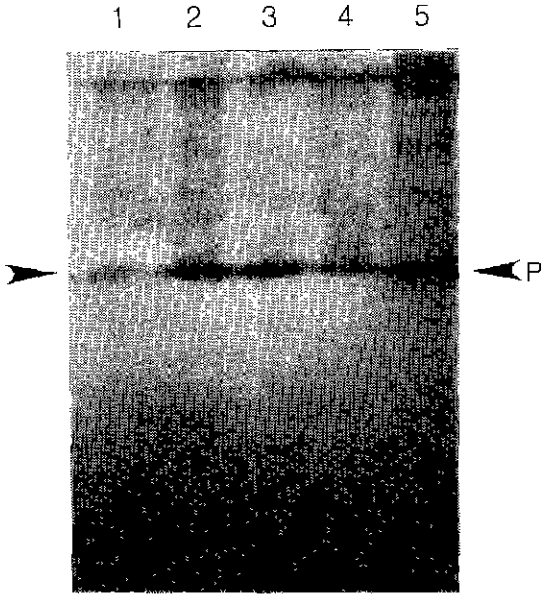

Fig. 6. Western Analysis of Culture Broth with Mouce Antibody.
Lane 1, sample after 6 hr of cultivation; lane 2, 12 hr of cultivation; lane 3, 18 hr of cultivation; lane 4, 24 hr of cultivation; lane 5, 36 hr of cultivation.

pullulanase activity immunoreacted with an anti-pullulanase antibody throughout the cultivation. These results suggested that the pullulanase protein is produced extracellularly as a single enzyme form during cultivation in the case of *B. circulans* S-1. In conclusion, our alkaline pullulanase is characteristically stable in the alkaline pH range with a relatively broad pH stability. As suggested by Ara *et al.*[10], this characteristic of the pullulanase is a prerequisite for use in dishwashing detergents. Current work is focussing on the cloning of the gene encoding EP and aimed at applications of the enzyme in the detergent and starch industries.

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초록 : Bacillus sp. S-1으로부터 생성된 새로운 균체외 pullulanase의 생화학적 특성

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Bacillus circualans S-1으로부터 새로운 균체외 pullulan 6-glucanohydrolase(EP)를 정제하였다. 정제된 EP는 SDS하에서 140kDa의 분자량을 나타내었으며, pI는 5.5이었다. SDS-PAGE에 의해 분석된 이EP는 Schiff staining에 대하여 negative이었으며, 또한 아미노 말단기 순서는 P-L-N-M-S-Q-P이었다. 정제된 EP는 60℃ 부근의 최적온도와 pH 9.0 부근에서 최적 pH를 나타내었으며, pH 4.0에서 pH 11 까지 4℃에서 24시간동안 반응에서도 안정하였다. 또한 기질로 사용된 Pullulan은 열불안정화로부터 효소를 보호하였으며, 그 범위는 기질 농도에 의존하였다. 이 EP의 활성은 Mn^{2+}, Ca^{2+} 이온 에 의하여 활성화되었으며, amylopectin, glycogen, α,β-limited dextrin 및 pullulan의 α-1,6-linkage를 가수분해 하였다. 정제된 EP는 pH 9.0 및 50℃에서 측정하였을 때 pullulan의 경우는 7.92mg/ml의 Km값을 각각 나타내었다. 또한 정제된 EP는 pullulan을 maltotriose까지 완전히 가수분해하였다. 그리고 Mouse anti-serum과 함께 western blotting분석결과 정제된 EP는 배양과정중 단일 형태로 생산되는 것으로 나타났다.