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Characterization of pullulan-hydrolysing enzyme from an alkalopsychrotrophic *Micrococcus* sp.

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Summary. A pullulan-hydrolysing enzyme of *Micrococcus* sp. 207 was purified to an electrophoretically homogeneous state by chromatography on DEAE-Toyopearl, α-cyclodextrin-Sepharose and Asahipak GS-520P. The purified enzyme was free of α-amylase activity. The molecular weight of the enzyme as estimated by SDS-PAGE was 120,000 and the pI value as determined by isoelectric focusing was 4.9. The enzyme was most active at pH 8.0 and 50° C. The enzyme was activated by the addition of $CaCl_2$, but its thermoresistance increased after removing free Ca^{2+} ions. The enzyme could hydrolyse the α-1,6-linkages of amylopectins, glycogens and pullulan and the K_m value for pullulan was about 0.018%. Pullulan at concentrations above 0.012% inhibited the enzyme activity and the activity was competitively inhibited by cyclodextrins.

Introduction

Pullulanase (EC 3.2.1.41, pullulan 6-glucanohydrolase) specifically hydrolyses the α-1,6-glucosidic linkages in pullulan and amylopectins. This enzyme is used to elucidate the chemical structure of polysaccharides and also to improve the production yield of glucose, maltose, etc. obtained from starch with amylases by debranching the substrate. Since Bender and Wallenfels (1961) first discovered this enzyme from *Aerobacter aerogenes* (*Klebsiella pneumoniae*), several microbial pullulanases have been purified and characterized by many investigators (Ueda and Nanri 1967; Walker 1968; Yokobayashi et al. 1970; Gunja-Smith et al. 1970; Yagisawa et al. 1972; Nakamura et al. 1975) and thermostable pullulanases have been studied in recent years (Hyun and Zeikus 1985; Plant et al. 1986).

We have studied alkalopsychrotrophic bacteria capable of growth at both pH 10.0 and 0° C (Kimura and Horikoshi 1988). One strain, *Micrococcus* sp. 207 (Kimura and Horikoshi 1989a), was found to produce amylase and pullulanase extracellularly. Both enzymes were produced maximally after cell growth at 18° C for 4 days in an optimized medium at pH 9.7 (Kimura and Horikoshi 1989b).

This paper describes the characterization of the pullulan-hydrolysing enzyme (PHE) produced by strain 207. In a previous paper (Kimura and Horikoshi 1989c), we reported the purification and some properties of two amylases produced by the strain. One of the amylases (enzyme I) also exhibited pullulan-hydrolysing activity (PHA), but the characteristics of the PHA were not examined. In this paper we compare the properties of the PHA of enzyme I to those of the newly purified PHE. There has been a previous report of a pullulanase from *Micrococcus* (Sakai 1981), but it was not purified and characterized. This is the first report of a PHE from an alkalopsychrotrophic bacterium.

Materials and methods

Bacterial strain and growth conditions. Micrococcus sp. 207 was cultured as described before (Kimura and Horikoshi 1989b).

Enzyme and protein assays. The PHA was determined using a standard reaction mixture (0.5 ml) which contained the enzyme solution and 0.1% pullulan in 40 mM N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid) buffer (pH 7.5) and 5 mM $CaCl_2$. The reaction mixture was incubated at 30° C for 10 min and reducing sugar was determined according to the Somogyi-Nelson method (Robyt and Whelan 1972). One unit (U) was defined as the amount of enzyme that released 1.0 μmol reducing sugar equivalent to glucose per minute. Protein was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif., USA), with bovine serum albumin as the standard.

Gel electrophoresis and electrofocusing. The purity of the enzyme was determined by polyacrylamide gel electrophoresis (PAGE) and sodium dodecylsulphate (SDS)-PAGE, and the isoelectric point (pI) of the purified enzyme was determined by isoelectric focusing as described previously (Kimura and Horikoshi 1989c).

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Materials. Pullulan (Mn 5×10^4) and β-amylase was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Cyclodextrins (CDs) were obtained from Nihon Shokuhin Kako Co. (Tokyo, Japan). All other chemicals used were of reagent grade.

Purification of the enzymes. The basal buffer used throughout all steps of the purification consisted of 20 mM TRIS-acetate buffer (pH 8.5) containing 1 mM $CaCl_2$. Protein in the culture supernatant (2.95 l) was concentrated by addition of solid ammonium sulphate to 75% saturation. The precipitate formed was collected by centrifugation and dissolved in buffer. After dialysis, the sample solution (183 ml) was applied to a column (22 i.d. × 200 mm) of DEAE-Toyopearlpak 650S (Tosoh Corp., Tokyo, Japan) equilibrated with the buffer containing 0.1 M NaCl. After washing with the same buffer, the enzyme was eluted from the column by a linear NaCl gradient (0.1–0.5 M). The fractions, which had only PHA, were pooled (55 ml), and applied to a column (10 i.d. × 75 mm) of α-CD-Sepharose 6B equilibrated with the buffer containing 0.1 M NaCl. After washing the column with the same buffer, the enzyme was eluted with buffer containing 0.5% α-CD. To remove α-CD, the active fractions were pooled, adsorbed to a small amount of DEAE-Toyopearl, and eluted with the buffer containing 0.5 M NaCl. This eluate was concentrated by ultrafiltration (Diaflo PM10 membrane, Amicon Corp., Amicon, Md., USA), and fractionated by gel chromatography on a prepacked column of Asahipak GS-520P (21.5 i.d. × 500 mm, Asahi Chemical Industry Co., Tokyo, Japan) using the buffer containing 0.1 M NaCl at a flow rate of 2 ml/min.

Purified enzyme I was prepared by the method described previously (Kimura and Horikoshi 1989c).

Results

Purity, molecular weight and isoelectric point of the PHE

The results of the purification are summarized in Table 1. The final yield of the PHE was 5.5%. Figure 1 illustrates the purity of the enzyme. A single protein band was obtained by PAGE and also by SDS-PAGE. The molecular weight of the PHE was estimated to be about 120,000 by SDS-PAGE. The molecular weight was also estimated to be about 140,000 by gel chromatography. The pI value of the PHE was estimated as 4.9.

Effects of temperature and pH

Figure 2 compares the effects of temperature and pH on the activity of the purified PHE and the PHA of enzyme I. Both enzymes exhibited optimum activity at 50° C and at pH 8.0. Figure 3 compares the temperature stability of the PHE and enzyme I. Both enzymes in the presence of added $CaCl_2$ were stable at 40° C for at least 30 min. Each enzyme was examined after dialysis against 20 mM TRIS-acetate buffer (pH 8.5). With each enzyme, about 55–59% of the original activity was retained after 30 min at 70° C. More than 90% of the original activity remained within the pH range from 5.0 to 10.0 in the presence of $CaCl_2$. In the absence of $CaCl_2$, about 30% of the activity was lost at pH 6.0–7.0 and about 70% was lost at pH 10.0 after 1 h at 30° C.

Table 1. Purification of the pullulan-hydrolysing enzyme from *Micrococcus* sp. 207

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Supernatant	454	1679	3.7	100
$(NH_4)_2SO_4$ (75% S)	198	1169	5.9	69.6
DEAE-Toyopearl	54.0	151.1	2.8	9.0
α-CD-Sepharose 6B	10.8	119.2	11.0	7.1
Asahipak GS-520P	5.5	92.3	16.7	5.5

U, units; CD, cyclodextrin

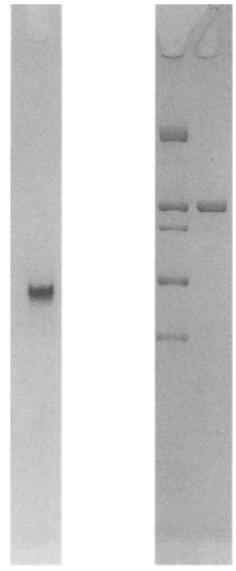

Fig. 1. Polyacrylamide gel electrophoresis (PAGE) of the pullulan-hydrolysing enzyme (PHE): *lane 1*, PAGE; *lane 2*, SDS-PAGE; *M*, standard marker proteins for SDS-PAGE; (from above) myosin, 200,000; β-galactosidase (*Escherichia coli*), 116,000; phosphorylase b (rabbit muscle), 97,000; albumin (bovine serum), 66,000; ovalbumin (egg white), 43,000

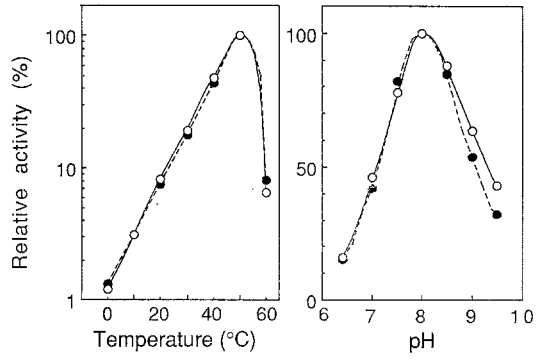

Fig. 2. Effects of temperature and pH on pullulan-hydrolysing activity (PHA). The PHA of PHE (○) and enzyme I (●) were assayed at various tmeperatures in 40 mM N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid) buffer (pH 7.5) (*left*) or at various pH values in 20 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis-Tris Propane) buffer (*right*)

Effects of chemical reagents and metal ions

Table 2 shows the effects of various chemical reagents on the PHE and enzyme I. Both enzyme preparations were inhibited by SDS, *N*-bromosuccimide (NBS) and ethylenediaminetetraacetic acid (EDTA). Enzyme I was

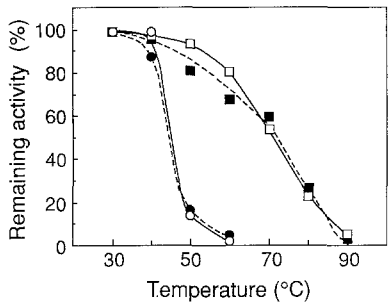

Fig. 3. Effect of temperature on enzyme stability. The purified PHE (*open*) and enzyme I (*closed*), previously dialysed against water, were incubated for 30 min at various temperatures in 20 mM TRIS-acetate buffer (pH 8.5) with 5 mM $CaCl_2$ (○) or without $CaCl_2$ (□). The remaining activity was assayed in the standard reaction mixture

Table 2. Effect of various chemical reagents on the pullulan-hydrolysing enzyme (PHE) from *Micrococcus* sp. 207

Reagent	(*c*)	Remaining PHA (%)			
		PHE		Enzyme I	
		$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$
None		100	100	100	100
Urea	(1 M)	110.3	92.5	109.5	74.0
SDS	(0.1%)	44.6	25.1	73.9	42.7
	(1%)	6.0	0.4	29.7	7.9
pCMB	(5 mM)	100.9	92.6	96.9	78.5
NBS	(1 mM)	120.0	93.7	—	77.5
	(5 mM)	23.0	33.1	8.4	7.1
IAA	(5 mM)	104.7	92.2	87.4	83.7
Cys	(5 mM)	110.4	107.1	141.4	107.3
EDTA	(5 mM)	—	69.7	—	5.1

The enzymes were incubated for 30 min at 30° C in the presence of various chemical reagents as indicated. The remaining pullulan-hydrolysing activity (PHA) was assayed in reaction mixtures containing 5 mM $CaCl_2$: —, not tested; SDS, sodium dodecyl sulphate; pCMB, *p*-chloromercuribenzoate; NBS, *N*-bromosuccimide; IAA, indoleacetic acid; EDTA, ethylenediaminetetraacetic acid

more stable in the presence of SDS but less stable in the presence of NBS or EDTA than the PHE. Both enzymes were inhibited by the bivalent metal ions Co^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Sn^{2+}, Ni^{2+} and Zn^{2+}. Only Ca^{2+} ions stimulated the activity of both enzymes.

Substrate specificity and reaction kinetics

Table 3 shows the substrate specificity of PHE. Amylose was not hydrolysed. Amylopectins were hydrolysed more extensively than glycogens. After cleavage with PHE, all amylopectins and glycogens were hydrolysed readily by β-amylase. Since enzyme I has both amylase activity and PHA, it readily hydrolysed all substrates listed in Table 3. The hydrolysis rate of pullulan

Table 3. Action of PHE on polysaccharides

Substrate	Hydrolysed glucosidic bonds (%)		
	PHE alone	β-Amylase alone	Subsequent action with β-amylase
Amylose (DP = 18)	—	40.3	41.2
Potato starch	2.1	26.0	44.5
Pullulan	34.7	—	35.7
Potato amylopectin	3.6	21.0	40.7
Waxy corn amylopectin	4.1	28.8	41.1
Oyster glycogen	1.0	17.3	42.0
Bovine liver glycogen	1.7	16.3	43.6
Rabbit liver glycogen	0.8	23.7	43.0

Each substrate (2 mg/ml) was incubated at 30° C with PHE (0.08 U) in 20 mM TRIS-acetate buffer (pH 8.0) for 8 h or with β-amylase (0.4 U) in 0.1 M sodium acetate buffer (pH 5.2) for 15 h. The reducing activity of each sample was measured. A sample digested by PHE was heated at 100° C for 5 min, and was subsequently treated with β-amylase (0.4 U/ml) in 0.1 M sodium acetate buffer (pH 5.2) for 15 h at 30° C. The reducing activity was then measured and the hydrolysis rate was calculated as glucose units; —, not detected

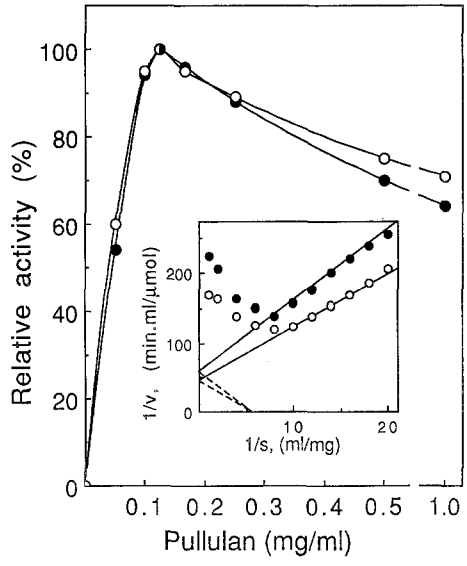

Fig. 4. Effect of pullulan concentration on the activity of the enzymes. The PHA of the purified PHE (5.8 mU) or enzyme I (4.4 mU) was assayed at various concentrations of pullulan in the standard reaction mixture: ○, PHE; ●, enzyme I

with enzyme I was slightly more than that observed with the PHE.

Figure 4 shows the effect of pullulan concentration on the activities of PHE and enzyme I. The enzymes had very similar kinetic profiles and the K_m for pullulan in each case was about 0.018%. Pullulan concentrations above 0.012% inhibited the activity and about 70% of the maximum activity was observed at a substrate concentration of 0.1%.

Reaction products

The final hydrolysis products of both PHE and enzyme I from pullulan were analysed by HPLC according to the method described by Nakamura et al. (1989). About 97% of the product of the PHE reaction was maltotriose (G_3) and the rest was maltotetraose. The maximum yield of G_3 with enzyme I was about 87% and a small amount of glucose and maltose were detected.

To investigate the reaction reversibility, maltose at a concentration of 40% was incubated with 0.2 U of PHE at 30° C and pH 7.5. After 20 h, the sugar composition of the reaction mixture was determined by HPLC. About 1.4% of maltose was converted to tetrasaccharide.

Inhibition by cyclodextrins

The PHA of both enzymes was inhibited competitively by CDs. The inhibition constants (K_i) for α-CD, β-CD and γ-CD were 1.0×10^{-4}, 3.1×10^{-5} and 1.7×10^{-4} M, respectively, as determined by Dixon plots (Dixon and Webb 1979).

Discussion

The optimum temperature for activity of the PHE was about 50° C (Fig. 2). An Arrhenius plot calculated from the result gave a single line between 0 and 50° C similar to that reported for the amylase described previously (Kimura and Horikoshi 1989c). However, the activation energy was much higher than that of the amylase. The pH optimum for the PHA was 8.0. This value was considerably higher than observed previously with other debranching enzymes, for example the pullulanase of *Micrococcus* sp. (Sakai 1981) which was pH 5.5–6.0. One exception is the pullulanase produced by an alkalophilic *Bacillus* (Nakamura et al. 1975) which had optimum activity at pH 8.5–9.0.

The K_m value of the PHE for pullulan was about 0.018% (Fig. 4). Interestingly, substrate inhibition was observed at quite low concentrations of pullulan, above 0.012%. Such a phenomenon has not been observed with other pullulanases (Marshall 1973; Takasaki 1976). Since substrate inhibition at such a low concentration was observed, we examined the reverse reaction (Abdullah and French 1966) of the PHE. Indeed, a small amount of tetrasaccharide was produced from an excess of maltose. It may have occurred because the true substrate of this enzyme is not pullulan.

Our previous paper described the characteristics of two purified α-amylases produced by strain 207. One of these α-amylases, enzyme I also exhibited PHA which was not characterized. In this paper we have examined the properties of the PHA of enzyme I and compared it with the newly purified PHE (Table 4). These enzymes had different molecular weights and pI values; however, many enzymatic properties were very similar. Especially the temperature stability of both enzymes, with and without $CaCl_2$, were similar and distinct from the amylase activity. The stabilities of the PHE and enzyme I in the presence of some chemical reagents were different. Notably, activity of the PHE was reduced in the presence of SDS more than that of enzyme I, whereas the amylase activity of enzyme I was stable in the presence of SDS and $CaCl_2$. On the other hand, whereas only 30% of the original activity of PHE was lost in the presence of EDTA for 1 h at 30° C, enzyme I lost 95% of its activity under the same conditions. This value was similar to the result obtained with enzyme II but differed from that of the amylase activity of enzyme I. These differences may be due to the interaction of the two enzyme moieties of enzyme I. The stability of PHE in the presence of EDTA seemed to be in conflict with the thermostability observations (Fig. 3). We assume that both PHE and enzyme I change to a more thermostable conformation after removal of Ca^{2+} ions at low temperature. The PHA of both enzymes was inhibited by CDs, while the amylase activity of enzymes I and II was not. This difference may be explained by the hypothesis that enzyme I consists of two discrete enzymes.

Table 4. Properties of two PHEs from *Micrococcus* sp. 207

Property	PHE	Enzyme I
Molecular weight	120,000	185,000
Isoelectric point	4.9	4.8
Optimum pH	8.0	8.0
Optimum temperature	50° C	50° C
K_m for pullulan	0.018%	0.018%
pH stability	5.0–10.0	5.0–10.0
Temperature stability		
40° C ($+Ca^{2+}$)	100%	88%
70° C ($-Ca^{2+}$)	55%	59%
Activator	Ca^{2+}	Ca^{2+}
Inhibition		
Metal ion	Co^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Sn^{2+}, Ni^{2+}, Zn^{2+}	
SDS (0.1%, $+Ca^{2+}$)	55.4%	26.1%
EDTA (5 mM)	30.3%	94.9%
Pullulan (0.1%)	28.7%	35.7%
Cyclodextrins	Competitive	Competitive

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