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Purification and Characterization of Pullulanase from *Klebsiella pneumoniae* NFB-320

Seung Seok Yoo† and Ju-Hyun Yu*

Bioproducts Research Center, Yonsei University, Seoul 120-749, Korea
**Dept. of Biotechnology, Yonsei University, Seoul 120-749, Korea*

Abstract

Pullulanase was produced from the *Klebsiella pneumoniae* NFB-320 with the composition of 1.0% pullulan, 1.5% yeast extract, 0.2% K_2HPO_4 and 0.02% $MgSO_4 \cdot 7H_2O$(pH 5.5). The optimum temperature for activity of the pullulanase was 30°C and the highest yield of the enzyme was obtained after cell growth at 30°C for 18hr, and maintained until 24hr cultivation. The pullulanase was successively purified 52.6 folds with 7.8% yield by acetone precipitation, DEAE-cellulose column chromatography and gel filtrations. The purified enzyme hydrolyzed pullulan into maltotriose exclusively. Chemical and physical properties of purified pullulanase from *Klebsiella pneumoniae* NFB-320 were examined. The optimum pH and temperature for enzyme activity were 5.0 and 60°C, respectively. The enzyme was stable between pH 4 and 7, and up to 50°C. The effect of modification on the rate of enzyme reaction was studied with various chemicals and metal ions. The enzyme has been found to be inactivated by I_2 and N-bromosuccinimide(NBS), which probably indicated the involvement of tryptophan residues in the active center of the enzyme.

Key words: *Klebsiella pneumoniae*, pullulanase, purification, enzymatic properties

INTRODUCTION

Most of the starches that are useful in industry contain amylopectins ranged from 75 to 80%. To apply these into food, chemistry, clothes or detergent market, starches are required to degrade in the form of syrup such as maltotriose, maltose, and glucose(1,2). Amylopectins are partially degraded by α-amylases and generate α-limit dextrin because the enzyme can not degrade α-(1→6) glycosidic linkage as well as same α-(1→4) linkage near the branching point. β-Amylases also stop the degrading activity at α-(1→6) branch point and produce β-limit dextrin(3,4). While glucoamylases mainly cleave both α-(1→4) and α-(1→6) linkages, the reaction rate is very slow. Therefore, debranching enzymes provide lots of benefit in these cases. Among the amylases, α-(1→6) linkages are cleaved by isoamylase and pullulanase(5,6).

Pullulanase(pullulan 6-glucanhydrolase, EC 3.2.1.41) is a debranching enzyme that specifically cleaves such α-(1→6) linkages in starch, amylopectin, pullulan and related oligosaccharides. It is an industrially important enzyme, which is generally used in combination with saccharifying amylases such as glucoamylase, fungal α-amylase and β-amylase for the production of various sugar syrups, because it improves the saccharification and the production yield(7). Moreover, it has gained significant attention as a useful tool for structural studies of carbohydrates(3).

Since pullulanase was found from *Aerobacter aerogenes* in 1962 by Bender and Wallenfels(5), it has been reported from bacteria such as *Escherichia, Bacillus*(8), *Clostridium*(9), and from yeast, and also from fungi like *Aspergillus niger*(10). There are two types of pullulanase, intracellular and extracellular one, each of molecular weight or characteristics is unique although the reaction mechanism of both are common. Specially, the pullulanase produced from *Klebsiella pneumoniae*, that is Gram negative bacteria, is extraordinarily released into the culture medium from the cells. Pugsley et al.(11) proposed the generated enzyme initially bound to the outer membrane of the cells and then subsequently released into the medium.

In the present paper, pullulanase isolated from *Klebsiella pneumoniae* NFB-320 was purified and characterized associated with the temperature, pH, and the

†Corresponding author

active sites.

MATERIALS AND METHODS

Strain and cultivation

Klebsiella pneumoniae NFB-320 was used as the strain to produce pullulanase. The basal culture medium used for obtaining the pullulanase consisted of 1.0% pullulan, 1.5% yeast extract(Difco, Detroit, MI), 0.2% dipotassium phosphate and 0.02% magnesium sulfate(Sigma, St. Louis, MO). The pH of growth medium was adjusted to 5.5. *Klebsiella pneumoniae* NFB-320 was cultivated at 30°C for 18 hours at the rate of 200rpm. The seed culture was incubated at 37°C for overnight with continuous shaking and then was inoculated at a concentration of 0.5%(v/v).

Purification of pullulanase

Cell free extracts were obtained at 13,000rpm for 20min by centrifugation and the supernatant was adopted as crude enzyme solution. Cold acetone was added to the crude enzyme solution to 50% saturation with continuous stirring, followed by standing the enzyme at 4°C for overnight. The resulting precipitates were collected by centrifugation at 10,000rpm for 10min, and dried by vacuum evaporator, then dissolved in a small amount of 0.05M sodium acetate buffer adjusted to pH 6.0.

The enzyme solution obtained from the acetone precipitation was applied to a DEAE-cellulose column(3.5 ×25cm) equilibrated with 0.05M sodium acetate buffer (pH 6.0). After the column had been washed with the same buffer, the enzyme was eluted from the column by linearly increasing the sodium chloride concentration in the range of the buffer from 0 to 0.5M.

The enzyme solution was concentrated by ultrafiltration through a PM-10 membrane and was applied to a Sephadex G-100 column(1.6×80cm) previously equilibrated with 0.05M sodium acetate buffer(pH 6.0). Each of 4ml fractions of effluent was collected at a flow rate of 20ml per hour. The enzyme active fractions were pooled and concentrated by ultrafiltration as mentioned above.

The partially purified enzyme solution was repeatedly applied to the second Sephadex G-100 column(1.6×6.5 cm). Elution was also performed with the same buffer. The active fractions were pooled, concentrated and stocked for further analysis.

Enzyme assay

Pullulanase activity was assayed by measuring the amount of reducing sugar degraded from pullulan. The reaction mixture consisting of 200μl enzyme solution and 400μl sodium acetate buffer(50mM, pH 6.0) containing 1% pullulan, was incubated at 40°C for 30min. Reducing sugar generated from pullulan after reaction was measured by Somogyi-Nelson method(12,13). One unit of pullulanase activity was defined as the amount of enzyme causing an 0.01 increase in absorbance at 520 nm for one minute.

Determination of protein concentration

During the course of enzyme purification, the protein eluted from the column was measured in absorbance at 280nm and each concentration was measured by Bradford method(14) with bovine serum albumin(BSA) as a standard.

Thin layer chromatography

To confirm the products degraded from pullulan, the mixture of each 1.0% pullulan and enzyme solution(1 : 1, v/v) was reacted at 40°C for 1hr, 6hrs and 24hrs. Macromolecules not cleaved were removed by centrifugation after adding two volume of methanol. Followed by vacuum drying, reaction mixtures were resuspended to small amount of distilled water, and then developed on DC-Fertigplatten Kieselgel 60(Merck, Rahway, NJ) with solvent system of n-buthanol : pyridine : water(6 : 4 : 3). After drying, each spot was detected by spraying with diphenylamine-alanine-phosphoric acid(15).

RESULTS AND DISCUSSION

Culturing the microorganism

The cell growth pattern was shown in Fig. 1 with the production of the extracellular enzyme. To estimate the products and consumed material by the strain, the change of the pH in medium was also measured along with the growing of the cells. The formation of pullulanase with the cell growth was fairly well corresponded. The pullulanase was subsequently produced during the exponential phase of the cell growth and largely accumulated right after stationary phase.

The pH of the medium was not dramatically changed during culturing the microorganism, but slight increase

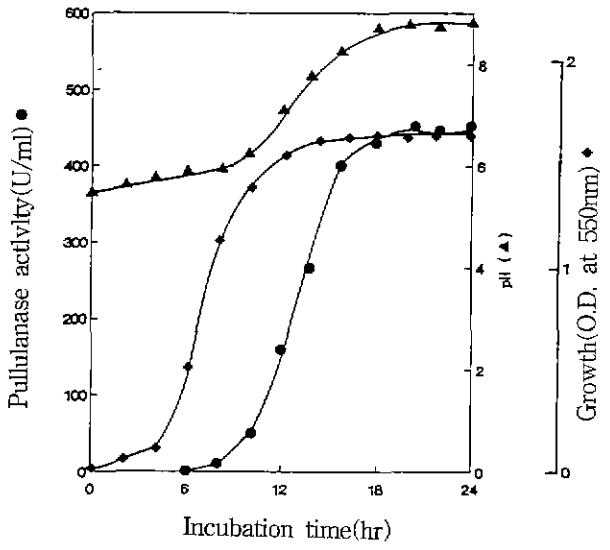

Fig. 1. Time course of the pullulanase production by *Klebsiella pneumoniae* NFB-320.

of the pH was observed when pullulanase was released and accumulated. Therefore, the cells were removed from the medium, and the solution was obtained for purification after incubating for 18 hours due to no apparent increase of the enzyme activity. The study of pullulanase from *Aerobacter aerogenes* by Ohba and Ueda(7) also showed the similar maximal cultivation time. However, Takasaki(16) demonstrated *Bacillus* species required 40 hours to provide the maximal pullulanase activity in that case.

Purification of the pullulanase

The results of purification for pullulanase from *Klebsiella pneumoniae* NFB-320 were summarized in Table 1. For the preparation of enzyme solution, cells were removed by centrifugation before acetone precipitation. The supernatant was used as crude enzyme solution for further purification.

After two times of cold acetone was added to the crude enzyme solution, the recovery yield of total activity was about 61%, and the specific activity reached 2.5 times higher. These were applied to a DEAE-cellulose column. When the enzyme was eluted from the column by linearly increasing the sodium chloride concentration, pullulanase activity was largely obtained at approximately 0.2M of gradient. Eluted protein solution from the column was fractionated in 10ml, and the yield of enzyme fraction showed pullulanase activity was 25%, and 329U/mg of specific activity. Through the DEAE column chromatograph, enzyme was partially purified to 9.2 times than that of the crude ones.

The enzyme solution eluted by DEAE-cellulose column was concentrated, and continually applied to a Sephadex column. Successful purification was performed by another step of gel chromatograph with the same column except shorter length. The specific activity of the pullulanase fraction after second gel filtration increased 53 times and recovery against the crude one was 8%.

Thin layer chromatography

To identify the main hydrolysis product of pullulan by pullulanase and compare the purity of the enzyme, 1.0% of the pullulan solution was hydrolyzed with purified pullulanase at 40°C for 1hr, 6hr and 24hr, individually. Appropriate amount of digests were spotted on a TLC plate. The TLC chromatogram was shown in Fig. 2. From the result it was suggested that pullulanase degrade pullulan into maltotriose as the final product, which is consistent with the result by Kuriki et al.(8). It also showed the substrates could be completely hydrolyzed into final products within one hour of reaction.

Effect of pH and pH stability for pullulanase activity

The influence of pH and its stability on pullulanase was examined in the reaction mixture ranged from pH 3 to pH 11 as shown in Fig. 3. The pH dependence on the enzyme stability was determined from the residual activity after incubating for 30 minute at 40°C. The effect

Table 1. Summarization of purification for pullulanase from *Klebsiella pneumoniae* NFB-320

Step	Total protein(mg)	Total activity(U)	Specific activity(U/mg)	Yield(%)	Purification(fold)
Culture supernatant	872.5	31236.0	35.8	100.0	1
Acetone precipitation	211.8	19189.0	90.6	61.4	2.5
DEAE column chromatography	23.3	7676.0	329.4	24.6	9.2
Gel filtration(I)	7.4	3655.6	494.0	11.7	13.8
Gel filtration(II)	1.3	2450.2	1884.8	7.8	52.6

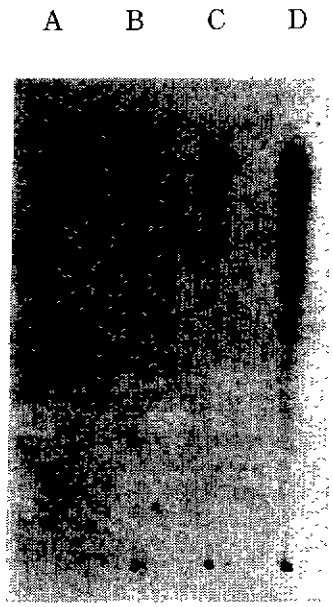

Fig. 2. Thin layer chromatogram of products from pullulan by purified pullulanase.
A: Maltotriose, B: Pullulan digest for 1hr
C: Pullulan digest for 6hr
D: Pullulan digest for 24hr

of pH curve demonstrated the pullulanase activity was highest at pH 5.0. It also showed pullulanase was most stable at a weak acidic pH. In the pH range of 4 to 7, the pullulanase was retained more than 80% of initial activity. However, the activity was dramatically decreased when the pH of the medium dropped below pH 4. *Bacillus stearothermophilus* harboring pullulanase(8) was reported to optimally produce the enzyme at pH 6, while *Clostridium thermohydrosulfuricum*(9) at pH 5.5, *Aerobacter aerogenes*(10) at pH 6.5. These results also support that pullulanase are generally stable at weak acidic conditions.

Effect of temperature and thermal stability for pullulanase activity

Fig. 4 illustrated the effect of temperature and influence on the pullulanase stability. The pullulanase activity was examined from 20°C to 80°C for 30 minutes. The maximum activity for pullulanase was observed at 60°C, and decreased approximately 35% for each±10°C.

The thermostability of the pullulanase was determined by measuring the residual activity after leaving the enzyme at various temperatures between 20~80°C for 30 minutes. The pullulanase was stable up to 50°C, but inactivated at about 55°C by a half of the total activity, even more completely inactivated above 70°C. The pullulanase could be considered as a relatively thermostable enzyme from the results.

Influence of chemicals and metal ions on the pullulanase activity

The effect of chemicals such as chemical modifier

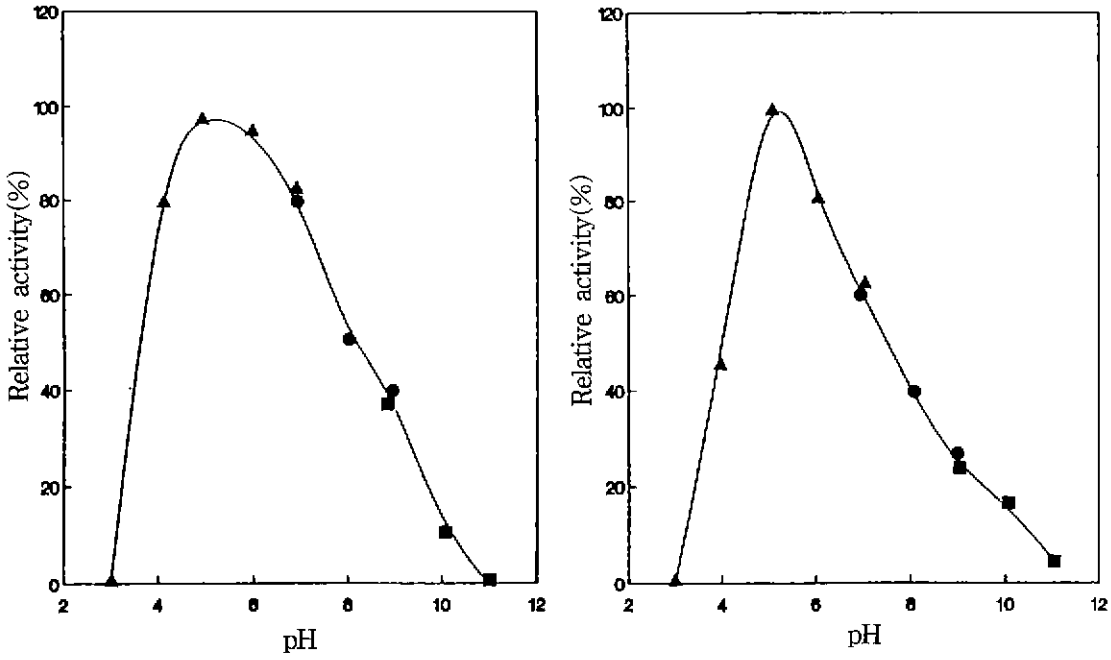

Fig. 3. Effect of pH and pH stability on the pullulanase activity.
pH 3~7: 0.05M sodium acetate buffer, pH 7~9: 0.05M Tris-HCl buffer, pH 9~11: 0.05M glycine-NaOH buffer

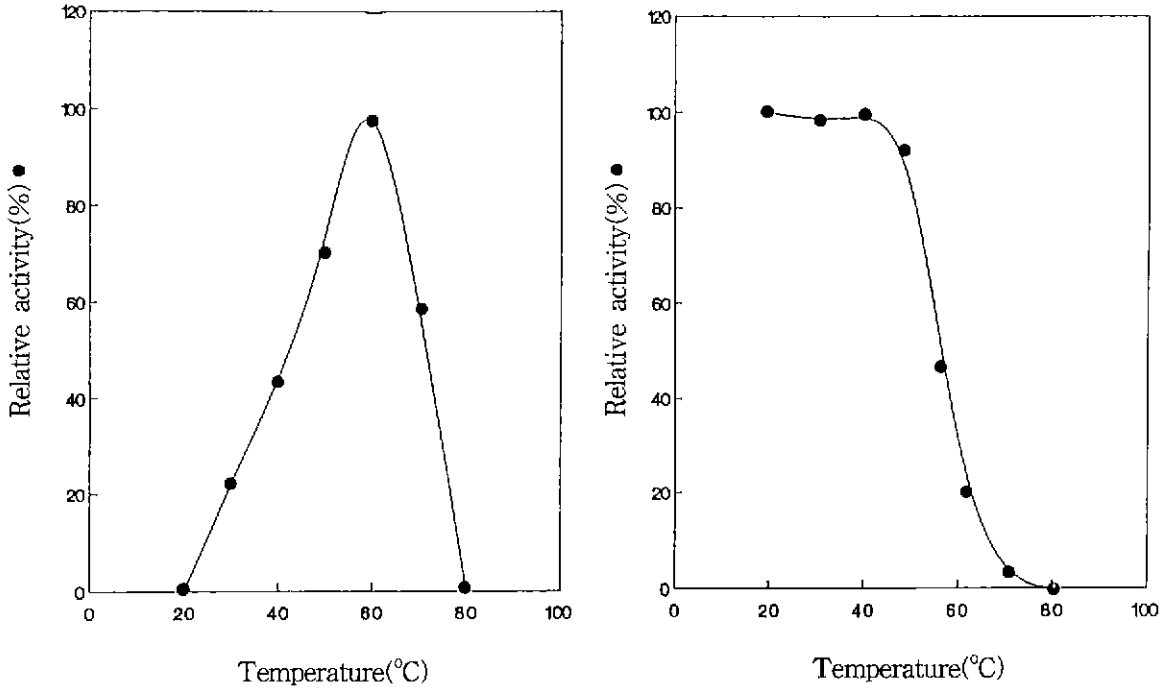

Fig. 4. Effect of temperature and thermal stability on the pullulanase activity.

or reducing agent and metal ions on the stability of pullulanase was demonstrated in Table 2~3. For this purpose, 1.0mM of metal ions and normal dosage of chemicals were added into the pullulanase solution before the enzyme reaction. The relative activity was represented to show the inhibition of the additives. Among the addition of the metal ions, pullulanase activity was strongly inhibited by the Hg^{2+} and Cu^{2-} ions, and also partially suppressed by aluminium and ferrous ions. It was reported Hg^{2+} either reacted with the sulfhydryl of the protein or crosslinked protein itself, and finally changed the conformation of the enzyme structure(17). In the case of the chemicals, the pullulanase activity was strongly inhibited by N-bromosuccinimide(NBS) which related to tryptophan. It was not affected by iodoacetate alkylating methionine, histidine, cysteine and tyrosine. Also, hydroxylamine, which can react carbonyl residue of both aspartic acid and glutamic acid, did not strongly inhibit the enzyme activity. Through the results from the effect of chemicals and metal ions, it was proposed that tryptophan could be strongly related to the active site of the pullulanase, not related with tyrosine, cysteine, histidine and methionine.

Table 2. Effect of metals on the pullulanase activity[1)]

Metal ion(1mM)	Relative activity(%)
None	100
$AgNO_3$	94
$AlCl_3$	67
$MnSO_4$	103
$FeSO_4$	47
$MgCl_2$	105
$ZnSO_4$	95
$CaCl_2$	106
$HgCl_2$	2
$CuSO_4$	20
KCl	108
$NiCl_2$	97
NaCl	101
$BaSO_4$	100

[1)]Incubated at pH 5.0, 30°C for 30min before assaying the remained pullulanase activity

Table 3. Effect of chemicals on the pullulanase activity[1)]

Reagents	Concentration	Relative activity(%)
None		100
Sodium azide	0.1%	94
EDTA	1mM	67
SDS	0.1%	103
Urea	6M	47
β-Mercaptoethanol	25mM	105
PMSF	1mM	95
PCMB	1mM	106
I_2	1mM	2
Iodoacetate	1mM	20
N-bromosuccinimide	1mM	108
H_2O_2	1mM	97
Hydroxylamine	1mM	100

[1)]Incubated at pH 5.0, 30°C for 30min before assaying the remained pullulanase activity

Table 4. Kinetic parameters of the pullulanase against pullulan substrate

Parameters	Values
K_m(mg/ml)	0.63
V_{max}(U/mg)	285.70
V_{max}/K_m(U/ml)	453.49

Kinetic parameters of the pullulanase against pullulan

The kinetic properties of the pullulanase was summarized in Table 4. Substrates hydrolyzing activity for pullulanase was calculated by Lineweaver-Bulk equation(18) using pullulan of the concentration ranged from 0.05M to 0.5M. Pullulanase showed relatively low Michaelis constant and greater maximum velocity as shown in Table 4. The results also supported the pullulanase had relatively high catalytic efficiency.

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