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New Type of Pullulanase from *Bacillus stearothermophilus* and Molecular Cloning and Expression of the Gene in *Bacillus subtilis*

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A new type of pullulanase which mainly produced panose from pullulan was found in *Bacillus stearothermophilus* and purified. The enzyme can hydrolyze pullulan efficiently and only hydrolyzes a small amount of starch. When pullulan was used as a substrate, the main product was panose and small amounts of glucose and maltose were simultaneously produced. By using pTB522 as a vector plasmid, the enzyme gene was cloned and expressed in *Bacillus subtilis*. Since the enzyme from the recombinant plasmid carrier could convert pullulan into not only panose but also glucose and maltose, we concluded that these reactions were due to the single enzyme. The new pullulanase, with a molecular weight of 62,000, was fairly thermostable. The optimum temperature was 60 to 65°C, and about 90% of the enzyme activity was retained even after treatment at 60°C for 60 min. The optimum pH for the enzyme was 6.0.

Four types of pullulan-hydrolyzing enzymes have been reported as follows; (i) the type of glucoamylase (EC 3.2.1.3) (22) hydrolyzing pullulan from the nonreducing ends to produce glucose; (ii) the type of pullulanase (EC 3.2.1.41) (2) from *Klebsiella pneumoniae*, hydrolyzing (1→6)-α-D-glucosidic linkages of pullulan to produce maltotriose; (iii) the type of isopullulanase (EC 3.2.1.57) (18) from *Aspergillus niger*, hydrolyzing (1→4)-α-D-glucosidic linkages of pullulan to produce isopanose (*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→6)-D-glucopyranose); and (iv) the type of neopullulanase (tentative designation in this paper) hydrolyzing (1→4)-α-D-glucosidic linkages of pullulan to produce panose (*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose). The enzyme activity has been reported for *Thermoactinomyces vulgaris* α-amylase (19). These action patterns are diagrammatically shown in Fig. 1.

Among these types, *Klebsiella* pullulanase is the most typical pullulan-hydrolyzing enzyme, and the enzyme gene from *Klebsiella aerogenes* has been cloned and sequenced (11). Most of the α-amylases found in nature cannot hydrolyze pullulan. In contrast, *Thermoactinomyces* α-amylase can hydrolyze not only starch but also pullulan. However, the enzyme can mainly function as a starch-saccharyfying α-amylase, and the pullulan-hydrolyzing activity is known to be a side effect (16, 17, 19).

For the purpose of efficient production of panose, we isolated many pullulan-hydrolyzing thermophilic bacteria and finally found a new pullulanase in *Bacillus stearothermophilus* that produced panose from pullulan. The thermostable enzyme was obviously different from the pullulan-hydrolyzing enzymes previously reported (2, 18, 19, 22).

In this paper we describe the purification and characterization of the new type of pullulanase. Molecular cloning of the enzyme gene in *Bacillus subtilis* and the action pattern of the new pullulanase are also described.

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MATERIALS AND METHODS

Media. L broth and L agar have been described previously (8). LS broth was L broth supplemented with 1.0% (wt/vol) soluble starch. PLL agar contained 10 g of pullulan (molecular weight, 50,000; Hayashibara Co., Ltd., Okayama, Japan), 2 g of tryptone, 2 g of yeast extract, 2 g of NaCl, and 15 g of agar in 1 liter of deionized water; it was adjusted to pH 7.3 with NaOH.

Bacterial strains and plasmid. *B. subtilis* TN106 (*arg-15 hsdR hsdM* Amy$^-$) (14) was transformed with chromosomal DNA from *B. subtilis* MT-2 (*trpC2 leuC7 hsdR hsdM* Npr$^-$) (20). The transformant *B. subtilis* NA-1 (*arg-15 hsdR hsdM* Amy$^-$ Npr$^-$) was used as a host strain. pTB522 (encoding resistance to tetracycline) (9) was used as a vector plasmid.

Assay of pullulanase activity. Pullulanase activity was assayed as described by Hyun and Zeikus (6), with slight modification. The reaction mixture (500 μl) was consisted of 1% pullulan in 0.2 M sodium acetate buffer (pH 6.0) and the enzyme. The reaction was stopped after an appropriate incubation period at 60°C by the addition of 3,5-dinitrosalicylic acid reagent (1 ml) (1). One unit of pullulanase activity was defined as the amount of enzyme which released 1 μmol of reducing sugar as glucose per min under the assay conditions described above.

Purification of panose-producing pullulanase. After centrifugation (8,000 × *g*, 10 min) of a culture broth, ammonium sulfate (final 35% saturation) was added to the supernatant, and the solution was kept at 4°C for 2 h. The precipitate was removed by centrifugation (16,000 × *g*, 20 min). Ammonium sulfate was again added to the supernatant to a final concentration of 60% saturation, and it was kept at 4°C for another 2 h. After centrifugation (16,000 × *g*, 20 min), the precipitate was disolved in about 100 ml of 50 mM Tris hydrochloride buffer (pH 8.0) and dialyzed against the same buffer. The enzyme solution was applied to a column (3.0 by 16 cm) of DEAE-cellulose (Whatman DE32) that had been equilibrated with the same buffer. Active enzyme was eluted with a 0 to 0.6 M NaCl gradient. The enzyme fraction was concentrated and applied to a column (2.0 by 125 cm) of

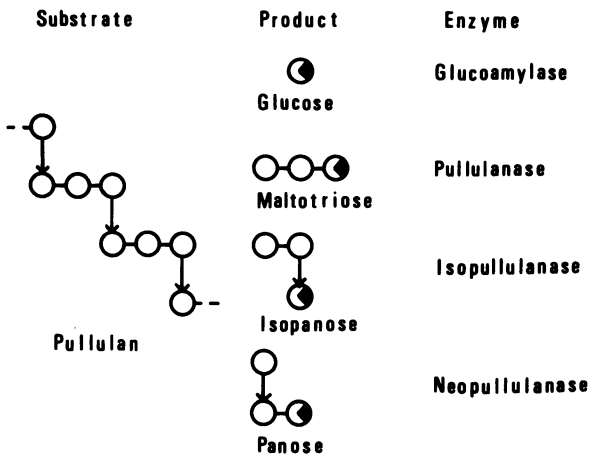

FIG. 1. Enzymatic hydrolysis of pullulan. Symbols: ○, glucose; ◔, glucose with reducing end; ——, α-1,4 linkage; ↓, α-1,6 linkage.

Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden). This gel filtration step was repeated twice. Active fractions were concentrated and applied to an affinity chromatography column (1.5 by 4.0 cm) with Sepharose 6B (Pharmacia) coupled with α-cyclodextrin (Hayashibara Co., Ltd.) as a ligand. A protein peak containing panose-producing pullulanase activity was eluted with a 0 to 0.3 M NaCl gradient.

Analysis of pullulan and starch hydrolysis products. Papar chromatography was carried out in the ascending mode on Whatman no. 1 filter paper with a solvent mixture of *n*-butanol–pyridine–water (6:4:3, by volume), and sugars were detected by spraying with silver nitrate solution (21). High-performance liquid chromatography (LC-6A, Shimazu, Kyoto, Japan; ZORBAX-NH_2 column, Du Pont Co., Wilmington, Del.) were also employed for the analysis of reaction products. Quantitative analysis of glucose and reducing sugar in the hydrolysate was performed as described above for the assay of pullulanase activity. Total carbohydrate was assayed by the phenol-sulfuric acid method (3).

Detection of pullulanase-producing colonies on plates. Pullulanase-producing colonies were selected on PLL agar plates by the method of Morgan et al. (13).

Preparation of plasmid and chromosomal DNA. Either the rapid alkaline extraction method or CsCl-ethidium bromide equilibrium density gradient centrifugation (8) was used to prepare plasmid DNA, whereas chromosomal DNA was prepared as described elsewhere (10).

Transformation. For the transformation of *B. subtilis* with plasmid DNA or chromosomal DNA, competent cells were prepared as described previously (10).

Protein assay. The protein concentration was measured by the method of Lowry et al. with bovine serum albumin as the standard (12).

Other procedures. Procedures for digestion of DNA with restriction endonucleases, ligation of DNA with T4 DNA ligase, agarose gel electrophoresis, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis are described elsewhere (4, 7, 8). Unless otherwise specified, all chemicals used in this work came from sources described in the previous papers (4, 7, 8).

RESULTS

Isolation of thermophilic bacteria that secrete panose-producing pullulanase. Eighteen pullulan-hydrolyzing thermophilic bacteria were isolated on PLL agar at 60°C from samples of compost or soil. These strains were incubated overnight one by one in LS broth at 60°C. After centrifugation, the culture supernatants were used for the assay of extracellular pullulan-hydrolyzing enzyme. Pullulan (1%) was hydrolyzed with these enzymes, and the sugar compositions of the products were analyzed by paper chromatography. Among these strains, thermophilic bacterium TRS40, which secreted panose-producing pullulanase (see below), was isolated. Strain TRS40 was a strictly aerobic, gram-positive, endospore-forming bacillus. The maximum growth temperature for the strain at pH 7 was 65°C. Hence, strain TRS40 could be classified as *B. stearothermophilus*.

Production and purification of the pullulanase. As a preliminary experiment, several carbohydrates such as pullulan, starch, maltose, and glucose were tested at various concentrations for the maximum production of panose-producing pullulanase. Finally, we selected LSII broth, which contained 2.5% soluble starch in L broth. Cell growth and enzyme production reached maximum levels at 60°C after 6 and 16 h, respectively. The 16-h culture broth was used for enzyme purification. About 1,700-fold purification was attained with a yield of 4% after five purification steps (Table 1). Figure 2 shows the sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns for various steps of enzyme purification. The molecular weight of the panose-producing pullulanase was estimated as 62,000 (Fig. 2).

Action pattern of the pullulanase. Pullulan and starch were hydrolyzed with the panose-producing pullulanase. This enzyme hydrolyzed pullulan efficiently, but hydrolyzed less starch (Fig. 3). In fact, the final hydrolysis percentages, (reducing sugar/total carbohydrate) × 100, of pullulan and starch with the pullulanase were 45 and 13%, respectively. Since the density of each spot was not proportional to the sugar concentration, the sugar composition of pullulan hydrolysate (8 h) was quantitatively analyzed by high-performance liquid chromatography; the weight percentages of glucose, maltose, and panose were 9.4, 19.1, and 71.5%,

TABLE 1. Purification of pullulanase from *B. stearothermophilus* TRS40

Purification step	Total vol (ml)	Activity (U/ml)	Total activity (U)	Protein concn (mg/ml)	Total protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	3,980	0.0952	379	4.20	16,700	0.0227	1	100
$(NH_4)_2SO_4$ treatment	103	2.20	227	8.35	860	0.263	11.6	59.9
DEAE column chromatography	80	1.62	130	1.98	158	0.818	36.0	34.3
First gel filtration	72	1.44	104	0.543	39.1	2.65	117	27.4
Second gel filtration	24	1.76	42.2	0.531	12.7	3.31	146	11.1
Affinity column chromatography	15	1.01	15.2	0.026	0.39	38.8	1,710	4.0

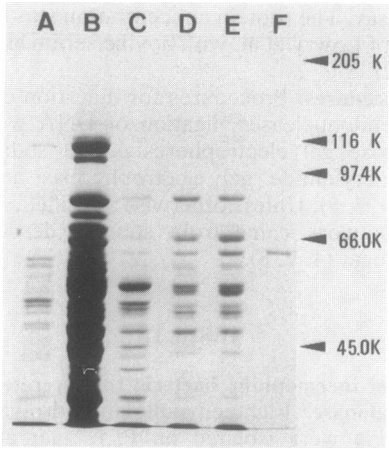

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis pattern of pullulanase of various steps of purification. Lanes: A, culture supernatant; B, salting-out fractions by $(NH_4)_2SO_4$; C, DEAE column pooled fractions; D, first gel filtration pooled fractions; E, second gel filtration pooled fractions; F, affinity column pooled fractions.

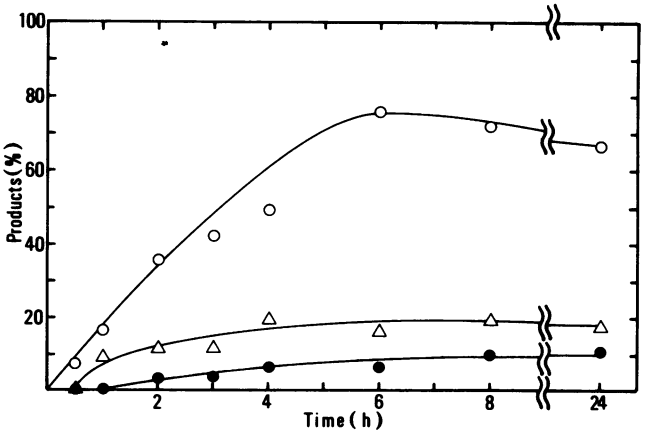

FIG. 4. Time course of the sugar composition of pullulan hydrolysate. The reaction mixture (5 ml), consisting of 2.5 ml of 2% pullulan, 1.0 ml of 1 M sodium acetate buffer (pH 6.0), 1.0 ml of distilled water, and 0.5 ml of purified enzyme, was incubated at 60°C. The sugar composition was analyzed by high-performance liquid chromatography. Symbols: ○, panose; △, maltose; ●, glucose.

respectively (Fig. 4). Although a spot which might correspond to maltotriose was slightly visible with paper chromatography (Fig. 3a), maltotriose could not be clearly detected in the high-performance liquid chromatography system. To confirm that the main product was panose, paper chromatography was carried out. The main product was obviously different from maltotriose and migrated more slowly than did maltotriose (Fig. 5a), and the main spot was completely identical with that of panose (Fig. 5b). Isopanose, which is also a trisaccharide, migrates at nearly the same rate as panose in the paper chromatography system. When glucoamylase attacks panose or isopanose, glucose should be a final product from panose; however, both glucose and isomaltose would be products from isopanose (15). The fact that the final product was only glucose (Fig. 6) indicates that the main product of pullulan hydrolysate with the pullulanase is panose and not isopanose.

Effects of temperature and pH on the activity and stability of the panose-producing pullulanase. The effect of temperature on the stability of the panose-producing pullulanase in the absence of substrate is shown in Fig. 7a. Under these conditions, this enzyme activity was very stable at 60°C for 60 min. The optimum temperature was around 60 to 65°C (Fig. 7b). The thermostability was enhanced and the optimum temperature was slightly increased in the presence of 1 mM EDTA, although the reason is not yet clear. The panose-producing pullulanase was quite stable in the range of pH 6.0 to 9.0, and the optimum pH was 6.0 (Fig. 8a, and b, respectively).

Molecular cloning and expression of the panose-producing pullulanase gene in *B. subtilis*. The chromosomal DNA from *B. stearothermophilus* TRS40 (about 6 μg) was digested with restriction endonuclease *Hin*dIII and ligated with the *Hin*dIII digest (about 2 μg) of pTB522. The ligation mixture was used to transform *B. subtilis* NA-1. Tc^r transformants were transferred by replica plating onto PLL agar for the screening of pullulanase-positive colonies. Among 2,000 colonies tested, one pullulanase-producing colony with a halo was obtained (Fig. 9a). Plasmid DNA was prepared from the

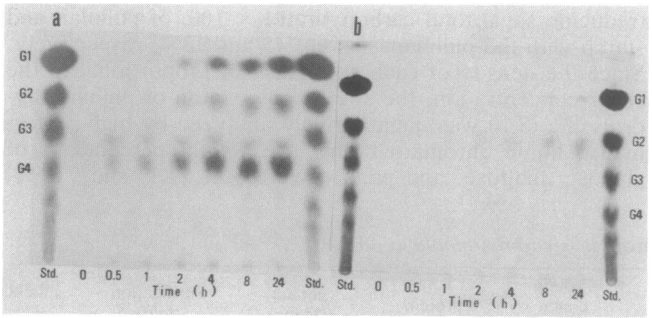

FIG. 3. Time course of pullulan and starch hydrolysis with the pullulanase. (a) Paper chromatography of pullulan hydrolysate, (b) paper chromatography of starch hydrolysate. A 450-μl sample of 1% pullulan or starch in 50 mM sodium acetate buffer (pH 6.0) and 50 μl of purified enzyme were incubated at 60°C; 20 μl of each sample was applied to paper chromatography. Abbreviations: G1, G2, G3, and G4, glucose, maltose, maltotriose, and maltotetraose, respectively; Std., standard mixture of oligosaccharides.

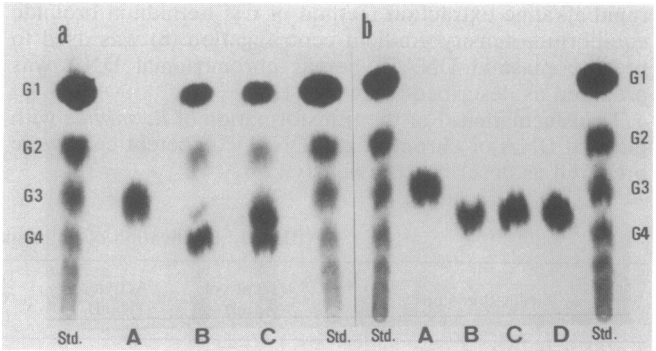

FIG. 5. Paper chromatography of pullulan hydrolysate with the pullulanase. (a) Lanes: A, maltotriose; B, pullulan hydrolysate; C, maltotriose plus pullulan hydrolysate. (b) Lanes: A, maltotriose; B, main product extracted from pullulan hydrolysate; C, panose; D, main product extracted from pullulan hydrolysate plus panose. Abbreviations are as in Fig. 3.

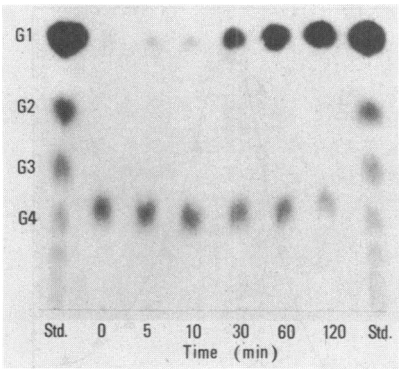

FIG. 6. Proof that the main product of pullulan hydrolysate with the pullulanase is panose. The main product extracted from pullulan hydrolysate was treated with glucoamylase and applied to paper chromatography. Abbreviations are as in Fig. 3.

transformant (Tc^r Pul^+) and designated as pPP1. The transformation of *B. subtilis* NA-1 with pPP1 yielded 100% Tc^r Pul^+ colonies, indicating that the ability to produce pullulanase was associated with the recombinant plasmid. pPP1 DNA was digested with several restriction endonucleases and subjected to agarose gel electrophoresis. *Hin*dIII digestion of pPP1 yielded four fragments; three fragments might have been cloned incidentally (data not shown). The sum of these four *Hin*dIII fragments, estimated as about 17 megadaltons (MDa), was consistent with that obtained for other restriction fragments. To obtain a smaller plasmid that harbors the pullulanase gene, pPP1 was cleaved with *Hin*dIII, ligated with the *Hin*dIII digest of pTB522, and used to transform *B. subtilis* NA-1. A new small recombinant plasmid, pPP10, was obtained from a transformant (Tc^r Pul^+). Plasmid pPP10 was digested with several restriction endonucleases, and the digestion patterns were examined by agarose gel electrophoresis. pPP10 was composed of two *Hin*dIII fragments (7.0 and 2.2 MDa), and the restriction map of pPP10 is shown in Fig. 9b.

Proof that the structural gene of panose-producing pullulanase is coded on the 2.2-MDa *Hin*dIII fragment. To determine whether the structural gene of thermostable panose-producing pullulanase is coded on the 2.2-MDa *Hin*dIII fragment, pullulanase from the recombinant plasmid-carrier strain of *B. subtilis* NA-1 was studied. *B. subtilis* NA-1(pPP10) was cultivated in LSII broth at 37°C for 16 h. The culture supernatant was treated at 60°C for 15 min, and the enzyme was purified. Pullulanase from *B. subtilis* NA-1(pPP10) showed the same electrophoretic mobility as panose-producing pullulanase from *B. stearothermophilus* TRS40 (molecular weight, 62,000) (data not shown). Both pullulan and starch were hydrolyzed with the pullulanase from *B. subtilis* NA-1(pPP10), and the sugar compositions of the hydrolysates were analyzed by papar chromatography and high-performance liquid chromatography. The sugar compositions were completely identical with those of the samples obtained with *B. stearothermophilus* TRS40 (data not shown). Other characteristics of the enzyme were also the same as those of panose-producing pullulanase from the original strain. These facts indicate that the structural gene of the panose-producing pullulanase is cloned in the recombinant plasmid pPP10. Since the purified enzyme from pPP10 carrier produced panose and a small amount of glucose and maltose from pullulan, we concluded that only the one enzyme was responsible for these reactions.

DISCUSSION

We found and purified a panose-producing pullulanase from a newly isolated strain, *B. stearothermophilus* TRS40. The enzyme can hydrolyze pullulan efficiently and hydrolyzes only a small amount of starch (Fig. 3). When pullulan was used as a substrate, the main product was panose; a small amount of glucose and maltose were simultaneously produced.

It has been reported that *Thermoactinomyces* α-amylase hydrolyzed pullulan and produced panose. However, the enzyme was shown to hydrolyze starch up to 50% (19). Hence, the panose-producing pullulanase in this paper should be clearly distinguished from α-amylase and should be considered a new enzyme. In this context, some substrates were subjected to hydrolysis with the panose-producing pullulanase. Although amylose was efficiently hydrolyzed, amylopectin was scarcely hydrolyzed (data not shown). These results are consistent with the above-men-

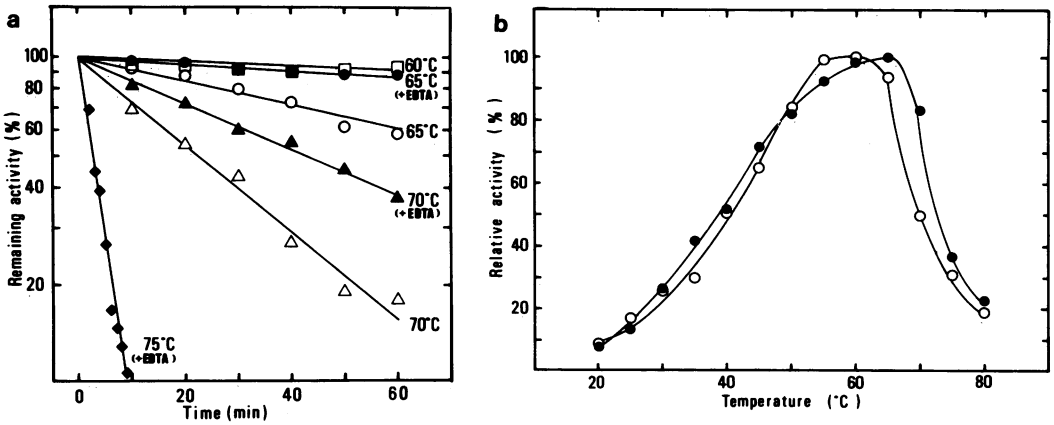

FIG. 7. Effect of temperature on the stability and activity of the panose-producing pullulanase. (a) Thermal stability of the panose-producing pullulanase in the absence of substrate. Partially purified enzyme (specific activity, 1.83 U/mg) in 0.2 M sodium acetate buffer (pH 6.0) was incubated at the indicated temperature, and the remaining activity was assayed. (b) Effect of temperature on the activity of the panose-producing pullulanase. The reaction mixture (500 μl), consisting of 400 μl of 1% pullulan in 0.2 M sodium acetate buffer (pH 6.0), 50 μl of distilled water (○) or 10 mM EDTA (●), and 50 μl of partially purified enzyme (specific activity, 1.28 U/mg), was incubated for 10 min at the indicated temperature before the assay of activity.

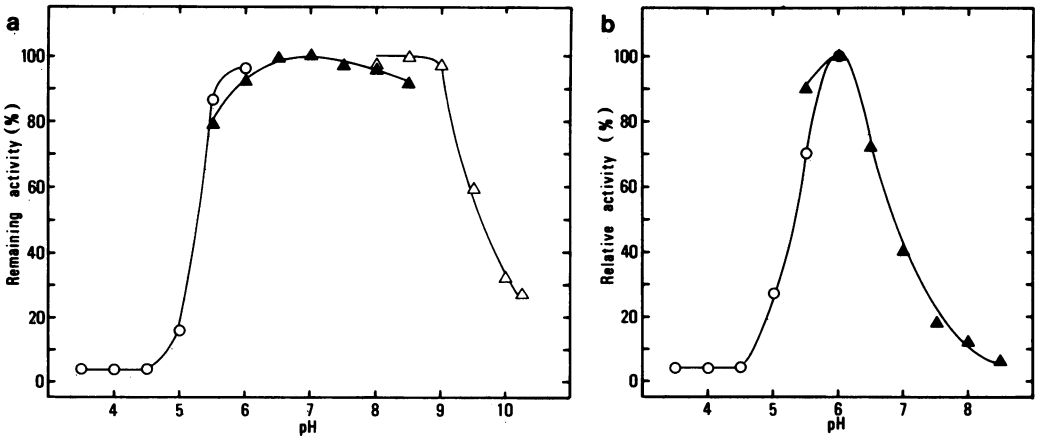

FIG. 8. Effect of pH on the stability and activity of the panose-producing pullulanase. (a) pH stability of the panose-producing pullulanase. Partially purified enzyme (specific activity, 1.28 U/mg) was treated in 0.1 M sodium acetate (○), sodium phosphate (▲), or glycine sodium (△) buffer at 60°C for 60 min before the remaining activity was analyzed. (b) Effect of pH on the activity of panose-producing pullulanase. The reaction mixture (500 μl), consisting of 250 μl of 2% pullulan, 200 μl of 0.2 M sodium acetate buffer (○) or 0.2 M sodium phosphate buffer (▲), and 50 μl of partially purified enzyme (specific activity, 1.28 U/mg), was incubated at 60°C for 30 min before the assay of activity.

tioned argument. The production of glucose and maltose in addition to panose from pullulan was seemingly due to two enzymes: (i) the enzyme that produced panose from pullulan and (ii) the enzyme that produced glucose and maltose from panose. However, highly purified panose-producing pullulanase from either *B. stearothermophilus* TRS40 or *B. subtilis* NA-1 carrying pPP10 (containing the enzyme gene in a 2.2-MDa *Hin*dIII fragment from *B. stearothermophilus* TRS40) catalyzed these two reactions. Since about a 1.2-MDa DNA fragment is needed for the enzyme (molecular weight, 62,000), the possibility that two different enzyme genes were cloned simultaneously in a 2.2-MDa *Hin*dIII fragment would be ruled out. Accordingly, these results indicate that a single enzyme could produce panose, glucose, and maltose from pullulan. For the production of glucose and maltose in addition to panose from pullulan, the enzyme has to hydrolyze (1→6)-α-D-glucosidic linkages besides (1→4)-α-D-glucosidic linkages. The precise action of the panose-producing pullulanase is being studied.

Panose is mildly sweet and inhibits the synthesis of insoluble glucan from sucrose by oral bacteria (5). Therefore, panose might be useful to prevent caries of the teeth. In this context, the thermostable pullulanase is very interesting from the standpoint of industrial application. To further investigate and improve the enzyme characteristics at molecular level, DNA sequencing of the enzyme gene is now in progress.

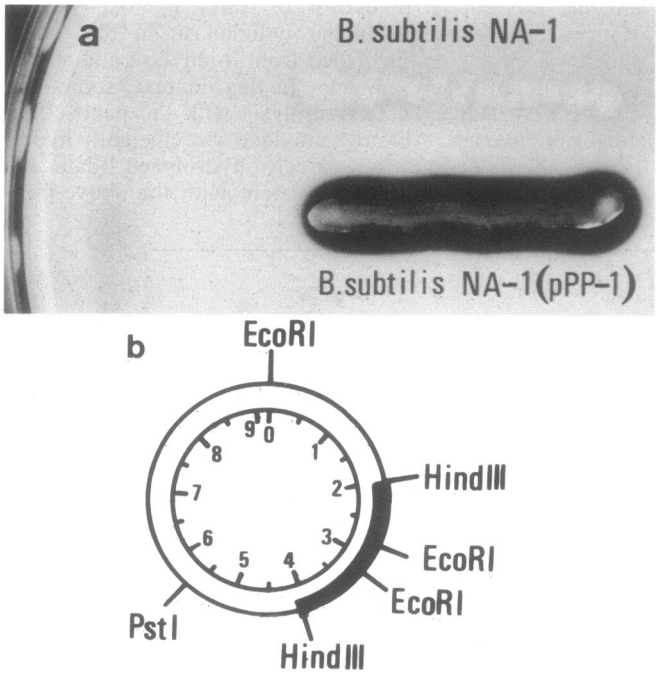

FIG. 9. Molecular cloning and expression of the panose-producing pullulanase in *B. subtilis*. (a) Pullulanase-positive clone with halo on PLL agar. (b) Restriction map of recombinant plasmid pPP10. Arabic numbers inside the circle indicate molecular size in MDa. The heavy line indicates the 2.2-MDa *Hin*dIII fragment from *B. stearothermophilus* TRS40.

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