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Formations of Extracellular Isoamylase and Intracellular α-Glucosidase and Amylase(s) by *Pseudomonas* SB15 and a Mutant Strain

TOSHIYUKI SUGIMOTO, AKINORI AMEMURA, AND TOKUYA HARADA

The Institute of Scientific and Industrial Research, Osaka University, Suita-shi, Osaka, Japan

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Pseudomonas SB15, which produces extracellular isoamylase, was found to produce intracellular α-glucosidase and amylase(s) when grown on maltose. A mutant strain (MS1) derived from it, which formed isoamylase constitutively, also produced these intracellular enzymes constitutively. The activities of the enzymes produced in the mutant strain were much greater than those induced in the parent strain.

The extracellular isoamylase formed by *Pseudomonas* SB15 (2, 3, 5, 6, 9, 10) is useful for production of amylose from starch and also, when used in combination with β-amylase, for production of maltose. This enzyme has also been useful for elucidating the structures of amylopectin (3) and glycogen (2). No other amylase besides isoamylase could be detected in culture filtrates of the organism (6). However, it is probable that when grown on starch this organism produces other amylases which can hydrolyze the amylose formed by the action of isoamylase. Therefore, the intracellular amylases of the organism were examined. In this work, the formation of intracellular α-glucosidase and amylase by *Pseudomonas* SB15 and its mutant strain were compared with that of isoamylase.

MATERIALS AND METHODS

Culture. *Pseudomonas* SB15 was used as the parent strain. The medium used consisted of the following (in 100 ml): carbohydrate, 2 g; sodium glutamate, 0.4 g; $(NH_4)_2HPO_4$, 0.15 g; KH_2PO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $FeCl_3 \cdot 6H_2O$, 10 mg; $MnCl_2 \cdot 4H_2O$, 10 mg; NaCl, 10 mg. The pH was adjusted to 7.0 with 0.01 N hydrochloric acid. Volumes of 95 ml of the medium in 500-ml conical flasks were inoculated with 5 ml of a suspension of bacteria grown in the same medium. The cultures were incubated at 32 C with shaking for 5 days unless otherwise stated. Cells were harvested by centrifugation, washed with water, dehydrated with acetone, and weighed.

Isolation of the mutant. *Pseudomonas* SB15 cells in the late-log phase of growth, in medium containing 1% polypeptone (Takeda), 0.5% yeast extract, and 0.5% NaCl (pH 7.0), were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine under the optimal conditions for *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine mutagenesis reported by Adelberg et al. (1). The cells were washed and incubated overnight in 5 ml of medium containing 2% glucose, 0.1% polypeptone, 0.1% yeast extract, and 0.1% K_2HPO_4 (pH 7.0) with shaking. The culture medium was then diluted with an appropriate volume of saline. The samples were plated on agar medium with the same constituents as the medium described above and incubated for 2 days at 32 C.

The following modification of the test paper method (5) was used to select colonies which produced isoamylase constitutively. Filter paper was dipped into a 1% solution of waxy maize amylopectin containing 0.1 M acetate buffer, pH 3.5, and the wet paper was placed on the colonies formed on agar culture medium and incubated at 40 C for 30 min. Then the paper was treated with iodine vapor. Spots giving a blue color indicated the locations of colonies which produced isoamylase constitutively. These colonies were preserved by the replica method. One colony which produces large amounts of isoamylase was selected and designated as MS1.

Preparation of a crude extract of intracellular amylases. Cells were harvested after incubation for 5 days in glucose or maltose medium. After the cells had been washed three times with saline they gave no amylase activity, but when subjected to ultrasonic vibration they showed α-glucosidase and amylase activities. The optimal conditions for the latter treatment were 20 kc for 30 min at 5 C. A crude extract was obtained by sonication of about 5 to 10 ml of cell suspension containing 5 mg (dry weight) of cells per ml. The supernatant obtained by centrifugation of the sonicated preparation for 20 min at 10,000 rpm was used as the crude extract.

Determination of isoamylase, α-glucosidase, and amylase activities. For estimation of isoamylase a reaction mixture containing 1 ml of 1.0% waxy maize amylopectin and 0.2 ml of 0.5 ml acetate buffer (pH 3.5) was used. For assay of intracellular amylase the mixture contained 1 ml of 1% corn amylose (Sigma Chemical Co.) and 0.2 ml of 0.5 M acetate

buffer (pH 5.5), and the reaction mixture for assay of α-glucosidase contained 1 ml of 0.01 M *p*-nitrophenyl-α-glucoside and 0.2 ml of 0.5 M acetate buffer (pH 5.5). These mixtures were incubated with 0.2 ml of enzyme solution at 40 C for 1 h. The reducing power or *p*-nitrophenol of 1-ml samples of the digests was measured by the method of Somogyi and Nelson (8) or spectrophotometrically at 400 nm, respectively. Each enzyme activity was expressed as micromoles of aldehyde group released per hour.

Determination of protein concentration. Protein concentration was estimated by the method of Lowry et al. (7) using bovine serum albumin as standard. Protein concentrations in the effluents from columns were determined from the absorbance at 280 nm, by using a Hitachi 124 UV-VIS spectrophotometer.

Paper chromatography. Sugars were separated by the descending method of Toyo filter paper no. 50 with *n*-butanol-pyridine-water (9:5:7, vol/vol/vol) as solvent. Sugars were detected with silver nitrate reagent.

RESULTS AND DISCUSSION

Formations of isoamylase, α-glucosidase, and amylase(s) by Pseudomonas SB15 and MS1. It has been reported that *Pseudomonas* SB15 produces isoamylase when grown on maltose, isomaltose, starch, or glycogen, but does not when grown on many kinds of carbohydrates including mono- and disaccharides (6). The formation of this enzyme may be inducible. Attempts to isolate a mutant capable of producing isoamylase even when grown on glucose resulted in the isolation of the mutant strain, MS1. It was found that both the parent and mutant strain produced intracellular α-glucosidase and amylase as well as extracellular isoamylase. The growth of strains SB15 and MS1 and their production of extracellular isoamylase and intracellular α-glucosidase and amylase(s) was tested in glucose, galactose, and maltose media (Table 1). Half the cells harvested were used for estimation of growth. The other half was sonicated for estimation of intracellular α-glucosidase and amylase activities.

Growth of MS1 was much less than that of SB15 in all media and in maltose medium at pH 7; the growth of MS1 was so low that there was little change in the pH. Strain SB15 only produced extracellular isoamylase in maltose medium, whereas strain MS1 produced it in all media. No activity could be detected in maltose medium (pH 7), probably because of the lability of the isoamylase under neutral and alkaline conditions (10). The experiments using a pH of 5.5 as initial pH of maltose medium were carried out to clarify the validity of the assumption that the alkalinity of the medium was responsible for the absence of isoamylase. Strain MS1 produced much more isoamylase than strain SB15, although it grew much less. The intracellular activities of α-glucosidase and amylase activities of strain SB15 were much higher in maltose medium than in glucose or galactose medium, whereas those of strain MS1 were high in all media. Amylose derived from amylopectin or glycogen by the action of extracellular isoamylase may be degraded by intracellular α-glucosidase and amylase, and the sugar formed may be utilized as the carbon source for growth. Unlike the parent strain, both extra- and intracellular enzymes are produced constitutively in the mutant.

To see what intracellular enzymes are formed, crude cell extracts of SB15 and MS1

TABLE 1. *Effects of carbon sources on formations of extracellular isoamylase, intracellular α-glucosidase, and an amylase in Pseudomonas SB15 and MS1*[a]

Strain	Carbon source	Cells formed (mg [dry wt]/100 ml)	pH		Extracellular isoamylase activity[b]	Intracellular[b]	
			Initial	Final		α-Glucosidase activity	Amylase activity
SB15	Glucose	106	7.0	4.8	0	1.7	16
SB15	Galactose	104	7.0	4.5	0	2.2	23
SB15	Maltose	113	7.0	4.9	1,270	13.6	116
SB15	Maltose	118	5.5	4.6	1,210	8.1	78
MS1	Glucose	55	7.0	5.4	2,320	40.6	467
MS1	Glactose	76	7.0	4.3	3,230	45.0	422
MS1	Maltose	37	7.0	6.8	0	25.2	260
MS1	Maltose	64	5.5	4.1	4,660	38.3	356

[a] Cultures in medium containing (per 100 ml) 2 g of carbon source, 0.4 g of glutamic acid, and inorganic salts were incubated for 5 days at 32 C. Then the cultures were centrifuged and the supernatant was adjusted to pH 3.5. Isoamylase activity in the supernatant and α-glucosidase and amylase activities in the crude extracts of the cells were measured as described in Materials and Methods.

[b] Expressed as micromoles of aldehyde group per hour per 100 milliliters.

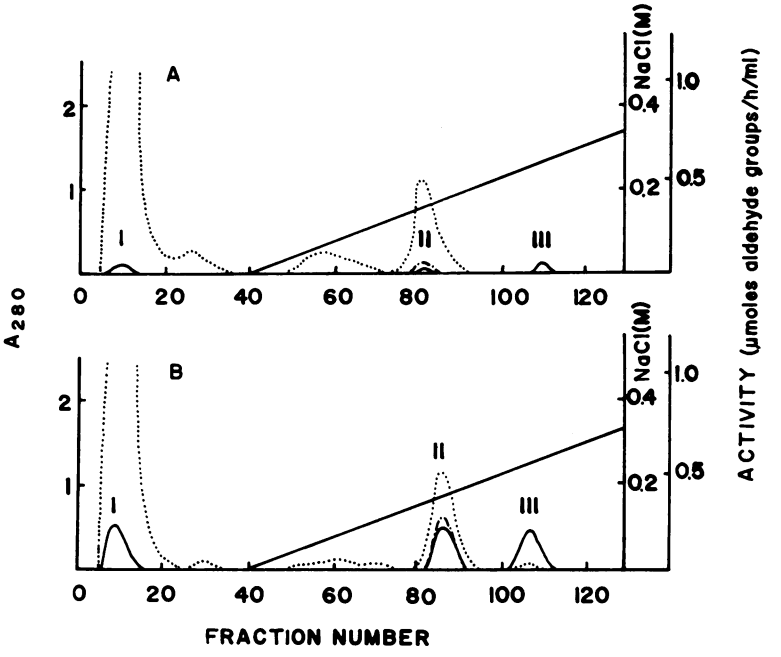

FIG. 1. *Fractionation of α-glucosidase and amylase activities from SB15 cells grown in glucose (A) and maltose (B) media (pH 7.0) by CM-cellulose chromatography. Enzyme extract (20 ml) from cells grown on 100 ml of culture medium was applied to a CM-cellulose column (2.0 by 25 cm) equilibrated with 0.01 M acetate buffer, pH 5.5. The column was washed with the same buffer (150 to 300 ml) and then eluted with a linear gradient of NaCl in the same buffer. The α-glucosidase and amylase activities and amount of protein in each fraction were measured. Symbols: (——), amylase activity; (- - -), α-glucosidase activity; (· · ·), protein.*

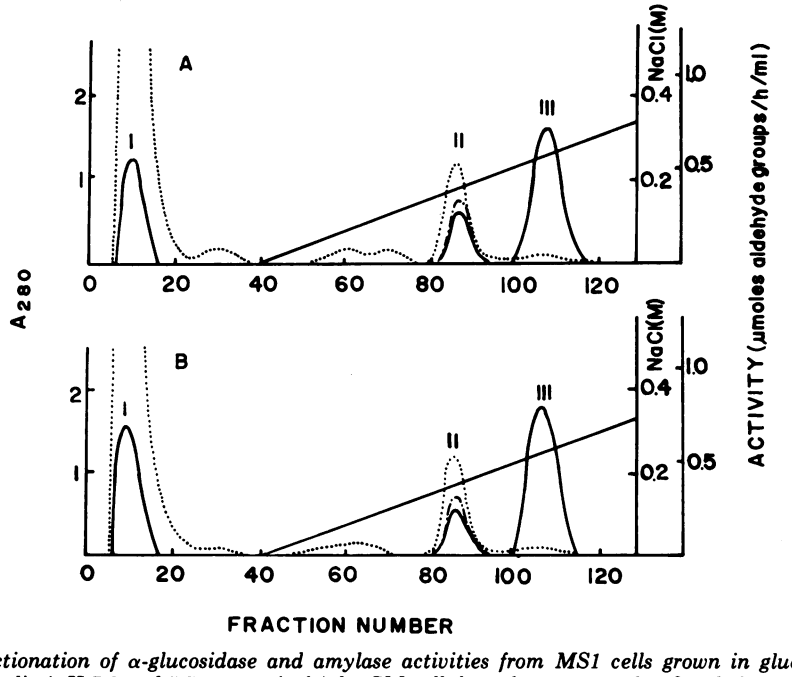

FIG. 2. *Fractionation of α-glucosidase and amylase activities from MS1 cells grown in glucose (A) and maltose (B) media (pH 7.0 and 5.5, respectively) by CM-cellulose chromatography. Symbols: (——), amylase activity; (- - -), α-glucosidase activity; (· · ·), protein. Experimental conditions were as described in the legend to Fig. 1.*

grown on glucose or maltose medium were fractionated on a CM-cellulose column (Fig. 1 and 2). It was found that the intracellular amylase was separated into three fractions (peaks I–III), whereas the α-glucosidase activity was eluted as a single fraction. The enzymatic activities in peaks I–III were much less in extracts of SB15 cells grown on glucose than in those of cells grown on maltose (Fig. 1). However, the activities in peaks I, II, and III were almost the same in extracts of MS1 cells grown on glucose and on maltose (Fig. 2). Thus, all the inducible enzymes in SB15 involved in degradation of starch and glycogen seem to be constitutive enzymes in MS1. It is also interesting that this change from inducible to constitutive enzymes resulted in great increases in the enzyme activities.

No attempt was made to purify the enzyme in peak I, because this fraction seemed to contain a large amount of contaminating protein. The enzyme in peak II was purified and designated an α-glucosidase (A. Amemura, T. Sugimoto, and T. Harada, J. Ferment. Technol., in press). Another enzyme in peak III may be a unique enzyme capable of degrading maltodextrines (DP ≧ 3), cyclodextrines, and amylose well, but scarcely capable of degrading amylopectin and glycogen and not capable of degrading maltose (K. Kato, T. Sugimoto, A. Amemura, and T. Harada, 287th Meeting of Kansai Branch of Agricultural and Chemical Society, Osaka, Japan, 1974, abstr. 4).

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