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Purification, Characterization, and Nucleotide Sequence of an Intracellular Maltotriose-Producing α-Amylase from *Streptococcus bovis* 148

EIICHI SATOH,[1]* TAI UCHIMURA,[1] TOSHIAKI KUDO,[2] AND KAZUO KOMAGATA[1]

Department of Agricultural Chemistry, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya, Tokyo 156,[1] and Laboratory of Microbiology, The Institute of Physical and Chemical Research (RIKEN), 2-1 Wako, Saitama 351-01,[2] Japan

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An intracellular α-amylase from *Streptococcus bovis* 148 was purified and characterized. The enzyme was induced by maltose and soluble starch and produced about 80% maltotriose from soluble starch. Maltopentaose was hydrolyzed to maltotriose and maltose and maltohexaose was hydrolyzed mainly to maltotriose by the enzyme. Maltotetraose, maltotriose, and maltose were not hydrolyzed. This intracellular enzyme was considered to be a maltotriose-producing enzyme. The enzymatic characteristics and hydrolysis product from soluble starch were different from those of the extracellular raw-starch-hydrolyzing α-amylase of strain 148. The deduced amino acid sequence of the intracellular α-amylase was similar to the sequences of the mature forms of extracellular liquefying α-amylases from *Bacillus* strains, although the intracellular α-amylase did not contain a signal peptide. No homology between the intracellular and extracellular α-amylases of *S. bovis* 148 was observed.

In a previous paper (15), we reported the cloning and expression of two α-amylase genes from *Streptococcus bovis* 148 in *Escherichia coli*. One cloned α-amylase, AmyA (Amy I in the previous paper is renamed AmyA in this paper), was essentially identical to the extracellular raw starch-hydrolyzing α-amylase of *S. bovis* 148. The other α-amylase, AmyB (Amy II in the previous paper is renamed AmyB in this paper), was different from the extracellular enzyme in that AmyB was not adsorbed on raw corn starch and failed to hydrolyze raw starch. This enzyme also showed a unique hydrolysis pattern with soluble starch. The gene encoding AmyB showed no homology to the gene encoding AmyA as determined by Southern blot analysis. AmyB was found to have enzymatic characteristics and DNA homologies different from those of the extracellular α-amylase of this strain. Recently, Whitehead and Cotta (22) reported detecting intracellular amylase activity in *S. bovis* and *Streptococcus salivarius* strains, although the enzymes were not described in detail. This paper is the first paper to report the purification and characterization of an intracellular α-amylase from *S. bovis* 148.

S. bovis 148 was cultured by using glucose, cellobiose, maltose, and soluble starch as carbon sources as described previously (15), and an *S. bovis* 148 cell extract was used for polyacrylamide gel electrophoresis (PAGE). Two bands of amylase activity were detected in a zymogram prepared following PAGE of the maltose-grown *S. bovis* 148 cell extract (Fig. 1A, lane 3). Only one band was evident on the zymogram of the cell-free supernatant from maltose-grown *S. bovis* 148 (Fig. 1A, lane 2), and it displayed the same mobility as one of the two bands produced from the cell extract. Thus, the second band in the cell extract zymogram was presumed to arise from an intracellular amylase. Then raw corn starch adsorption was performed by the previously described procedure (15), and it was effective for separating the two amylases. The extracellular α-amylase was completely adsorbed on the raw corn starch; only the intracellular enzyme was detected in the filtrate (Fig. 1A, lane 4). α-Amylase activity was measured by the 3,5-dinitrosalicylic acid method (8). The extracellular α-amylase of *S. bovis* 148 was produced in large amounts when this strain was grown on soluble starch or maltose as a carbon source, but was not produced when glucose or cellobiose was used (Table 1). Production of the intracellular α-amylase was also induced by either maltose or soluble starch, but not by glucose or cellobiose (Table 1).

The intracellular α-amylase from *S. bovis* 148 was purified by the previously described procedure (15), with some modifications. Maltose-grown *S. bovis* 148 cell extract was treated with raw corn starch, and then the filtrate was used for chromatography on Butyl-TOYOPEARL 650S (TOHSO Co., Tokyo, Japan), Hydroxylapatite Bio-Gel HT (Bio-Rad Laboratories, Richmond, Va.), and DEAE-Sephacel (Pharmacia LKB Biotechnology, Uppsala, Sweden) columns. The active fractions were used for PAGE with a 12.5% polyacrylamide gel, and purified enzyme was obtained by electroextraction from the gel with a Maxyield-NP apparatus (ATTO, Co., Tokyo, Japan). Purification of the enzyme to homogeneity was confirmed by the appearance of a single band on a sodium dodecyl sulfate (SDS)-PAGE gel, and the molecular mass of the purified enzyme was estimated to be 57,000 Da (Fig. 1B). The molecular mass estimated by gel filtration was 55,000 Da, indicating that the intracellular α-amylase exists in a monomeric form. The purified enzyme showed the highest activity at pH 6.5 and 40°C. This enzyme remained stable around neutral pH and lost activity at pH 3.0 to 5.0 and at pH values above 8.0. The intracellular α-amylase was sensitive to temperatures above 45°C, whereas about 90% of the extracellular α-amylase activity remained at 60°C. These effects of pH and temperature on the intracellular α-amylase activity were different from the effects of pH and temperature on the extracellular enzyme of

* Corresponding author. Present address: Department of Molecular Genetics and Gene Technology, TNO Nutrition and Food Research Institute, Utrechtseweg 48, P.O. Box 360, 3700 AJ Zeist, The Netherlands. Phone: 31 30 694 44 69. Fax: 31 30 694 44 66. E-mail: satoh@voeding.tno.nl.

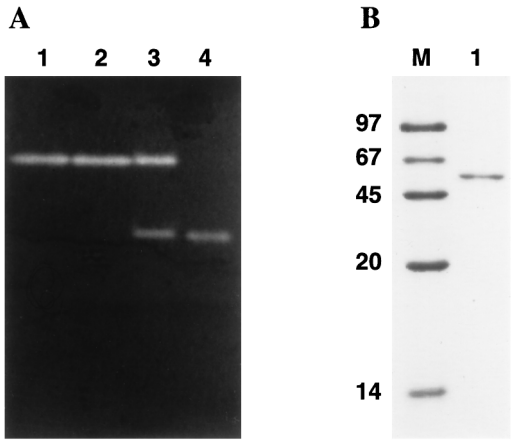

FIG. 1. (A) Separation and detection (by PAGE) of the intracellular and extracellular α-amylases from culture supernatants and cell extracts of *S. bovis* 148 grown on maltose medium. The polyacrylamide gel was incubated in 20 mM sodium phosphate buffer (pH 6.5) containing 1% soluble starch at 40°C for 1 h. The α-amylase activity was detected by staining the gel with a 500 mM I_2 solution. Lane 1, purified extracellular α-amylase; lane 2, supernatant from *S. bovis* cultured on maltose; lane 3, cell extract of maltose-grown cells; lane 4, filtrate of raw-starch-treated cell extract of maltose-grown cells. (B) SDS-PAGE of the purified intracellular α-amylase from *S. bovis* 148. SDS-PAGE was carried out by the method of Laemmli (7). The enzyme was visualized by Coomassie brilliant blue staining. Lane M contained molecular mass markers; sizes (in kilodaltons) are indicated on the left.

S. bovis 148. The α-amylase activity of the purified intracellular enzyme was assayed in the presence of various metal ions and chemical reagents at a concentration of 1 mM. Hg^{2+} completely inhibited the α-amylase activity, and Cu^{2+}, Ag^{2+}, and Pb^{2+} inhibited 70% of the activity. Addition of *p*-chloromercuribenzoate resulted in 90% inhibition of the α-amylase activity, suggesting that cysteine is involved in the catalytic action.

The intracellular α-amylase of *S. bovis* 148 hydrolyzed soluble starch to a large amount of maltotriose and a small amount of maltose, whereas the extracellular α-amylase completely hydrolyzed soluble starch to maltose and glucose (Table 2). The extracellular α-amylase was able to hydrolyze raw starch, but the intracellular enzyme was not. The intracellular α-amylase did not act on maltose, maltotriose, and maltotetraose. This enzyme hydrolyzed maltopentaose to maltotriose and maltose and maltohexaose mainly to maltotriose (Table 3). This hydrolysis pattern of the intracellular α-amylase was consistent with an endo type of amylase.

The gene encoding extracellular α-amylase cloned previously lacked a portion of the C-terminal coding region. A 3.5-kb fragment containing the missing C terminus was obtained by colony hybridization (14). The nucleotide sequences

TABLE 1. Production of intracellular and extracellular α-amylases by *S. bovis* 148 in response to growth substrates

Carbon source	α-Amylase activities (U/mg of cell protein)[a]	
	Intracellular	Extracellular
Glucose	0.02	2.79
Cellobiose	0.01	6.00
Maltose	0.16	36.32
Soluble starch	0.09	52.13

[a] The intracellular and extracellular α-amylases activities were assayed at 30 and 40°C, respectively.

TABLE 2. Products of hydrolysis of soluble starch by the intracellular and extracellular α-amylases of *S. bovis* 148[a]

α-Amylase	Product distribution (%)					
	Glucose	Maltose	Maltotriose	Maltotetraose	Maltopentaose	Maltohexaose
Intracellular	0	22.0	78.0	0	0	0
Extracellular	37.1	62.9	0	0	0	0

[a] The purified intracelular and extracellular α-amylases were incubated with 1% soluble starch for 24 h at 30 and 40°C, respectively. The hydrolysis products were analyzed by high-performance liquid chromatography (CCP and 8010 series instrument; TOHSO Co.) by using a TSK gel G-Oligo-PW column (diameter, 7.8 mm; length, 300 mm; TOHSO Co.) and a flow rate of 0.5 ml per min. The data are based on the masses of the products.

of the genes encoding the intracellular and extracellular α-amylases were determined with a *Taq* DNA polymerase and fluorescent dye primers (Applied Biosystems Japan, Co., Ltd., Tokyo, Japan) and were designated *amyB* and *amyA*, respectively. The SwissProt database was used to search for proteins homologous to the deduced amino acid sequences. The deduced amino acid sequence of the intracellular α-amylase corresponded to a 484-residue protein with a calculated molecular mass of 56,650 Da. The molecular mass corresponded to that estimated by SDS-PAGE. On the other hand, a highly hydrophobic 39-amino-acid sequence (amino acids 1 to 39) was found in the deduced amino acid sequence of the extracellular α-amylase. This amino acid sequence is believed to be a signal peptide, whose cleavage between Ala-39 and Asp-40 results in the mature form of the extracellular α-amylase, which is 703 residues long and has a calculated molecular mass of 77,340 Da. This value is similar to the molecular mass of 79,000 Da previously estimated by SDS-PAGE (9). A putative promoter was found in the noncoding regions upstream from both *amyB* and *amyA*. The intracellular α-amylase was 83.2% homologous to the intracellular amylase from *S. bovis* JB1 (22) and, in addition, displayed some homology with the extracellular liquefying α-amylases from *Bacillus* strains. Overall, the levels of homology were 47.5% with the *Bacillus licheniformis* enzyme (3), 46.6% with the *Bacillus stearothermophilus* enzyme (3), and 45.5% with the *Bacillus amyloliquefaciens* enzyme (18). Sequence analysis of the extracellular α-amylase of *S. bovis* 148

TABLE 3. Products of hydrolysis of maltooligosaccharides by the intracellular α-amylase of *S. bovis* 148[a]

Substrate	Product distribution (%)					
	Glucose	Maltose	Maltotriose	Maltotetraose	Maltopentaose	Maltohexaose
Maltohexaose	1.2	16.8	65.9	5.6	10.5	0
Maltopentaose	0	26.0	45.2	0	28.8	0
Maltotetraose	0	0	0	100	0	0
Maltotriose	0	0	100	0	0	0
Maltose	0	100	0	0	0	0
Glucose	100	0	0	0	0	0

[a] The purified intracellular α-amylase was incubated with each substrate at 30°C for 24 h. The hydrolysis products were analyzed by high-performance liquid chromatography as described in Table 2, footnote *a*.

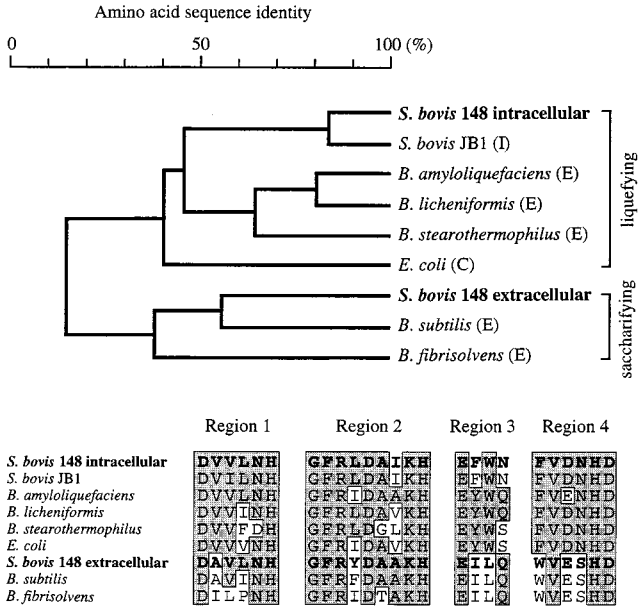

FIG. 2. Dendrogram showing the relationships among the amino acid sequences of α-amylases from different bacterial species. (I), intracellular; (E), extracellular; (C), cytoplasmic. The dendrogram was generated with the CLUSTAL V program (4). Regions conserved among α-amylases from various organisms are shown at the bottom. Similar amino acids are enclosed in boxes.

confirmed its similarity to the extracellular saccharifying α-amylases of *Bacillus subtilis* (23) and *Butyrivibrio fibrisolvens* (13) (levels of similarity, 51.4 and 37.0%, respectively). No homology between the intracellular and extracellular α-amylases of *S. bovis* 148 was observed (Fig. 2).

Two α-amylase activities of *S. bovis* 148 were detected together in the cell extract because of the adherence of the extracellular enzyme to the carbohydrate capsular material surrounding *S. bovis* 148 cells (9). The effectiveness of the raw corn starch adsorption treatment for removing the extracellular α-amylase from the cell extract allowed us to investigate the intracellular α-amylase. The intracellular α-amylase was determined to be inducible by maltose and soluble starch, as is the extracellular α-amylase. The intracellular α-amylase production by *S. bovis* 148 grown on maltose as the carbon source was approximately sevenfold greater than the production by strain 148 grown on glucose. On the substrates tested, however, the production of the extracellular α-amylase always greatly exceeded that of the intracellular enzyme (by 140 to 600 times). The intracellular α-amylase produced about 80% maltotriose from soluble starch. This result suggests that the intracellular enzyme is a maltotriose-producing α-amylase, similar to the enzymes previously reported in *Streptomyces griseus* (19), *B. subtilis* (16), *Microbacterium imperiale* (17), and *Natronococcus amylolyticus* (5, 6).

The deduced amino acid sequence of the intracellular α-amylase was similar to the sequences of the amylase detected in the cell extract of *S. bovis* JB1, the extracellular α-amylases from *Bacillus* strains, and the cytoplasmic α-amylase from *E. coli* (12). The extracellular α-amylases from the *Bacillus* strains contained a putative secretion signal peptide composed of 29 to 34 residues, whereas the *S. bovis* JB1 amylase and the *E. coli* α-amylase did not. Such a signal peptide was not found upstream from the N terminus of the *S. bovis* 148 intracellular α-amylase, and the N terminus showed similarity with the N termini of the mature enzymes from the *Bacillus* strains; thus, it does not seem that the *S. bovis* 148 α-amylase is destined for export. We use the term intracellular in this paper because this enzyme was detected only in the cell extract fraction and not in the extracellular fraction. It is not clear whether this enzyme is located in the cytoplasm or the membrane, because the capsule around the cells precluded preparation of a membrane fraction from *S. bovis* 148. However, a membrane anchor was not found at the end of the intracellular α-amylase, and a hydropathy plot analysis did not indicate a membrane protein or a transmembrane location. These results suggest that the intracellular α-amylase is located in the cytoplasm rather than in the membrane. In contrast, the extracellular α-amylase was found to contain a typical signal peptide. In addition, a putative operator for catabolite repression, similar to that found in *B. subtilis* (21) and *Lactobacillus pentosus* (11), was found upstream from the *amyA* gene. Freer (2) reported the purification and some properties of an extracellular α-amylase from *S. bovis* JB1. The N terminus of the *S. bovis* 148 extracellular α-amylase is almost identical to that of the *S. bovis* JB1 α-amylase. From *S. bovis* JB1, only the gene encoding the intracellular amylase was cloned (1), whereas genes encoding both intracellular and extracellular α-amylases have been cloned from *S. bovis* 148 and analyzed. On the basis of amino acid sequence analysis, all of the bacterial α-amylases described above were classified into the following two groups: liquefying α-amylases and saccharifying α-amylases. The intracellular α-amylase of *S. bovis* 148 is included in the liquefying group, while the extracellular α-amylase is included in the saccharifying group. In agreement with the previous report of highly conserved regions in amylases (10), four regions were found in this study to be common to both the liquefying and saccharifying α-amylases. Although no homology between the *S. bovis* 148 intracellular and extracellular α-amylases was determined, these four regions were well conserved in the two enzymes (Fig. 2).

Walker (20) first reported the presence of cell-bound α-amylase in *S. bovis*, described the inability of this enzyme to digest raw starch, and determined its hydrolysis pattern, which was similar to that of the extracellular α-amylase, but this cell-bound α-amylase was not purified and characterized. Intracellular α-amylases of *Streptococcus* strains have been described so far in a *S. salivalius* strain and in *S. bovis* JB1 (22). However, the role of this intracellular amylase in *S. bovis* in vivo, the relationship between intracellular and extracellular α-amylases, and the significance of a maltotriose-producing α-amylase in *Streptococcus* strains have yet to be determined.

Nucleotide sequence accession numbers. The nucleotide sequences of the extracellular and intracellular α-amylase genes determined in this study have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB000829 and AB000830, respectively.

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