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Purification and Characterization of the Extracellular α-Amylase from *Streptococcus bovis* JB1

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The extracellular α-amylase (1,4-α-D-glucanglucanohydrolase; EC 3.2.1.1) from maltose-grown *Streptococcus bovis* JB1 was purified to apparent homogeneity by ion-exchange chromatography (Mono Q). The enzyme had an isoelectric point of 4.50 and an apparent molecular mass of 77,000 Da, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme was rich in acidic and hydrophobic amino acids. The 15-amino-acid NH_2-terminal sequence was 40% homologous with the *Bacillus subtilis* saccharifying α-amylase and 27% homologous with the *Clostridium acetobutylicum* α-amylase. α-Amylase activity on soluble starch was optimal at pH 5.0 to 6.0. The enzyme was relatively stable between pH 5.5 and 8.5 and at temperatures below 50°C. When soluble potato starch was used as the substrate, the enzyme had a K_m of 0.88 mg · ml^{-1} and a k_{cat} of 2,510 μmol of reducing sugar · min^{-1} · mg of protein^{-1}. The enzyme exhibited neither pullulanase nor dextranase activity and was 40 to 70% as active on amylopectin as on amylose. The major end products of amylose hydrolysis were maltose, maltotriose, and maltotetraose.

The diverse population of microorganisms that inhabit the rumen of livestock is largely responsible for digestion in these animals. Under normal conditions, ruminal microbes establish a balanced fermentation and supply the animal host with most of the nutrients required for growth. If the diets of animals are radically altered, for instance, by rapidly switching from a forage to a grain diet, the ruminal fermentation can become unbalanced, resulting in digestive disorders, which may include lactate acidosis (1, 18, 19, 25). Lactate acidosis is believed to result from the rapid fermentation of starch, followed by lowered ruminal pH, due to the "overproduction" of lactic acid (10, 25).

Though probably not the only cause of lactate acidosis, *Streptococcus bovis* appears to be an important causative agent of this digestive disorder. This gram-positive bacterium is a normal inhabitant of the rumen that rapidly proliferates when animals are switched from forage to grain diets. It is normally present in the rumen at about 10^7 per ml, but can proliferate to about 10^{10} per ml during unbalanced ruminal fermentation (13, 15).

S. bovis is among the most amylolytic bacteria found in the rumen. When grown in media containing starch, it rapidly produces an α-amylase-like enzyme(s) (6, 30), converts starch to maltooligosaccharides, and ferments these oligosaccharides (6, 7). The production of the amylases appears to be regulated. When grown in medium containing either starch or maltose as the sole carbon source, the organism produced about 12-fold more amylase activity than when cultured in medium containing glucose as the sole carbon source (6). The cellular distribution of the enzyme is also dependent on growth substrate. When grown in starch-containing medium, about 80% of the total amylase activity is cell associated; when grown in medium containing either glucose or maltose, greater than 90% of the total amylase activity is found in the culture broth (6). This report describes the purification and partial characterization of the extracellular amylase produced by *S. bovis* JB1.

MATERIALS AND METHODS

Organism and growth conditions. *S. bovis* JB1 was obtained from R. B. Hespell, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill. The organism was grown anaerobically for 24 h at 39°C in a complex medium containing 0.3% maltose as the carbon and energy source (12). The stationary-growth-phase cultures were harvested by centrifugation (10,000 × *g* for 15 min at 4°C), and the cell-free supernatant was used as the source of enzyme.

Enzyme assay. Amylase activity was determined by measuring the increase in reducing sugar formed by the enzymatic hydrolysis of soluble potato starch. A 0.02-ml enzyme sample was mixed with 0.5 ml of 1% (wt/vol) soluble starch in 0.1 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer (pH 5.5), and the mixture was incubated at 39°C for 10 min. The amount of reducing sugar generated was quantified by the dinitrosalicylic acid method (3). The production of reducing sugar was linear throughout the 10-min assay period. One unit of amylase activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugars per min. Maltose was used as the standard. Several other substrates were tested under the same conditions for relative amylase activity. When maltose was used as the substrate, the amount of glucose liberated was quantified by the glucose oxidase-peroxidase method (2). Protein concentrations were estimated by the method of Lowry et al. (17), with cytochrome *c* as the standard.

Amylase purification. Ammonium sulfate was added to 200 ml of the clarified culture supernatant to 70% saturation. After 1 to 2 h at 4°C, the precipitate was collected by centrifugation (10,000 × *g* for 15 min at 4°C) and resuspended in 4.0 ml of 0.02 M Tris–0.002 M $CaCl_2$ (pH 7.5). The sample was loaded onto a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column (1.5 by 14 cm) and eluted with the same buffer. The desalted sample was then applied to a Mono Q anion-exchange column (1 by 10 cm; Pharmacia). It

was eluted at a flow rate of 0.5 ml/min, using an NaCl gradient. The amylase activity eluted from the column in a single peak at 0.13 M NaCl.

Electrophoresis and molecular mass determination. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the Pharmacia Phast System in a 10 to 15% gradient Phast Gel. Prior to electrophoresis, the samples were suspended in 0.01 M Tris-HCl–0.001 M EDTA–2.5% SDS–0.01% bromphenol blue (pH 8.0) and heated at 100°C for 5 min. Proteins were stained with silver nitrate. The molecular mass of the purified amylase was estimated from its position relative to those of standard proteins.

Isoelectric point determination. Analytical isoelectric focusing was performed with the Pharmacia Phast System in a Phast Gel IEF 3-9. Proteins were stained with silver nitrate, and the isoelectric point of the purified amylase was estimated from its position relative to those of standard proteins.

Amino acid analysis and NH_2-terminal sequence. The amino acid composition and the NH_2-terminal sequence (15 amino acids) of the purified amylase were determined by the Protein Sequencing Lab, Genetic Engineering Facility, University of Illinois, Urbana.

Measurement of optical rotation. The optical rotation of a hydrolysate of soluble potato starch by the purified *S. bovis* amylase was determined by the method of Hyun and Zeikus (14). A reaction mixture (6 ml), consisting of a 1% starch solution in 0.1 M sodium acetate (pH 6.0) and 47 U of purified enzyme, was added to a 10-cm cell and incubated at room temperature. The optical rotation of the solution was measured at various times in a Perkin-Elmer model 241 polarimeter by use of the sodium line. The mutarotation of the mixture was measured after 30 min by adding 30 mg of solid sodium carbonate to the reaction.

Effects of pH. The relative amylase activity, using 1.0% (wt/vol) soluble potato starch, was determined at various pHs in either 0.1 M Na succinate (pH 4.0 to 6.0), 0.1 M PIPES-HCl (pH 5.5 to 7.5), or 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-HCl (pH 7.0 to 8.5). To test pH stability, the purified enzyme was diluted 10-fold into the above buffers and incubated for 1 h at 39°C. The enzyme was then diluted 10-fold and assayed at pH 5.5 as described above.

Effect of temperature on stability. Enzyme samples were incubated for 1 h at various temperatures between 20 and 60°C in 0.1 M PIPES buffer (pH 5.5). The samples were placed at 39°C for 5 min and then assayed for residual activity.

Effect of metal ions and other chemicals. Enzyme assays were performed in the presence of various metal ions (1 mM) or other reagents (10 mM). The relative activity of the enzyme was compared with the activity obtained in 0.1 M PIPES buffer. The chloride salts of the metals were used, except for Cu^{2+} (sulfate) and Pb^{2+} and Ag^{1+} (nitrate). The response of the dinitrosalicylic acid reagent to maltose was determined in the presence of each metal ion or reagent.

Kinetic determinations. Initial rates of starch hydrolysis were determined at various substrate concentrations (10 to 0.5 mg/ml). The reactions contained 0.09 μg of purified enzyme per ml. The kinetic constants K_m and V_{max} were estimated by the method of Lineweaver and Burk (16), while the kinetic constant k_{cat} was determined by the method of Englard and Singer (11).

Chemicals and reagents. All buffers, enzyme substrates, glucose detection kits, and organic reagents were purchased from Sigma Chemical Co., St. Louis, Mo. All inorganic salts were purchased from Fisher Scientific Co., Fair Lawn, N.J.

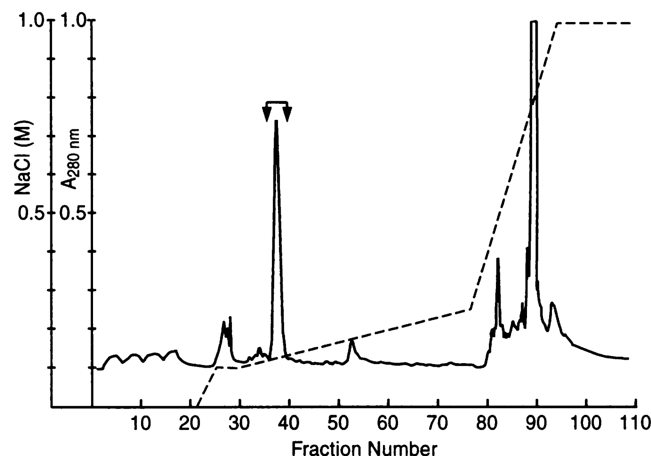

FIG. 1. Purification of the *S. bovis* amylase by Mono Q ion-exchange chromatography. Experimental conditions are given in Materials and Methods. The baseline was initially offset to 0.1 absorbancy unit. The fraction size was 0.5 ml. The α-amylase eluted from the column at about 0.13 M NaCl. Symbols: ——, A_{280}; – –, NaCl gradient.

RESULTS

Purification of amylase. When grown on maltose, *S. bovis* secreted into the culture medium approximately 90% of the amylase activity that it produced. The crude culture supernatant contained about 9.7 U of amylase activity per ml. After ammonium sulfate precipitation and desalting by Sephadex G-25 chromatography, the enzyme preparation contained 160 U of amylase activity per ml. The enzyme was routinely purified to apparent homogeneity by a single anion-exchange chromatography on Mono Q. The elution profile (Fig. 1) showed a single peak of amylase activity. The protein was purified to apparent homogeneity as judged by SDS-PAGE (Fig. 2). If, however, more than 200 ml of starting supernatant was used, the amylase was often slightly contaminated with other proteins after the Mono Q chromatography. In these instances, it was found that the enzyme could be purified to apparent homogeneity by subsequent chromatography on a Sephadex G-75 column (1 by 80 cm; data not shown). A summary of the purification is presented in Table 1. These procedures yielded a pure amylase with a specific activity of 2,650 U/mg of protein. Overall, the specific activity increased about sevenfold, with a 50% yield of activity and a 7% yield of protein.

Optical rotation. The optical rotation of a soluble potato starch hydrolysate was measured with time. Upon addition of solid sodium carbonate, the optical rotation shifted downward, indicating that the hydrolysis products had an α-anomeric configuration (data not shown).

Physical properties. The apparent molecular mass of the purified *S. bovis* α-amylase was 46,000 Da as estimated by gel permeation chromatography on Superose 12. However, when the apparent molecular mass was estimated in the presence of 6 M urea, a value of 75,000 Da was obtained (data not shown). SDS-PAGE of the purified enzyme revealed a single protein band with an approximate molecular mass of 78,000 Da (Fig. 2). Thus, the *S. bovis* α-amylase

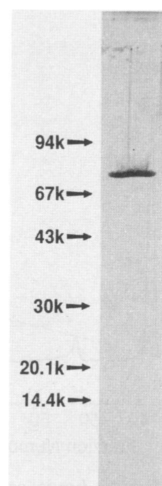

FIG. 2. SDS-PAGE of the purified *S. bovis* amylase. Experimental conditions are given in Materials and Methods. Molecular weight markers are indicated.

appears to be a monomeric protein. The isoelectric point of the enzyme was found to be 4.5 by PAGE-isoelectric focusing (data not shown).

An amino acid analysis of the purified protein showed that the enzyme is rich in both acidic and hydrophobic amino acids and lean in the basic amino acids arginine and histidine (Table 2). Like the *Bacillus subtilis* and *Clostridium acetobutylicum* amylases, the *S. bovis* α-amylase is rich in the hydroxyamino acids. The amino acid analysis did not detect any methionine; however, the NH_2-terminal amino acid sequence detected a methionine residue in the sixth position (Fig. 3).

A comparison of the NH_2-terminal amino acid sequence of the *S. bovis* α-amylase with those of the *B. subtilis* saccharifying amylase and the *C. acetobutylicum* α-amylase revealed 40 and 27% homology, respectively (Fig. 3). The homology appeared after the fourth and seventh amino acids, respectively.

Influence of pH on activity. The influence of pH on enzymatic activity and stability is shown in Fig. 4. The α-amylase showed optimal activity from pH 5.0 to 6.0 (Fig. 4A). The enzyme was most stable at pH 6.5; however, it was reasonably stable over a broad pH range of 5.5 to 8.5 (Fig. 4B).

Thermal stability. The thermal stability of the enzyme was monitored by measuring the residual enzymatic activity after the enzyme was incubated at the prescribed temperatures for 1 h (Fig. 5). The results showed that the α-amylase was relatively stable to temperatures up to 50°C.

TABLE 1. Summary of purification of α-amylase from *S. bovis*[a]

Fraction	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Purification (fold)	Recovery of activity (%)
Culture supernatant	1,940	5.09	380	1	100
$(NH_4)_2SO_4$ precipitate	1,140	1.58	720	1.9	59
G-25	1,150	1.83	630	1.6	59
Mono-Q	980	0.37	2,650	6.9	50

[a] The substrate was soluble potato starch (1%, wt/vol). The initial culture volume was 200 ml.

TABLE 2. Amino acid composition of the *S. bovis* amylase

Amino acid	No. of residues per molecule of:			
	S. bovis	*B. subtilis* saccharifying α-amylase[a]	*B. licheniformis* liquefying α-amylase[b]	*C. acetobutylicum* amylase[c]
Asx	90	99	62	120
Glx	42	51	45	63
Ser	41	54	26	74
Gly	114	51	45	47
His	8	16	24	16
Arg	8	24	22	14
Thr	44	45	27	47
Ala	65	50	35	62
Pro	22	23	15	22
Tyr	127	27	30	30
Val	46	32	32	45
Met	1[d]	9	7	14
Cys	0	1	0	1
Ile	24	34	20	26
Leu	32	36	28	47
Phe	14	20	20	23
Lys	28	30	28	30
Trp	ND[e]	14	17	ND

[a] From Yang et al. (31).
[b] From Yuuki et al. (32).
[c] From Paquet et al. (24).
[d] None was detected in the original amino acid composition; however, a Met is present in the sixth position of the NH_2-terminal amino acid sequence (Fig. 3).
[e] ND, not determined.

Catalytic properties. The substrate specificity of the purified α-amylase was studied with polysaccharides, maltooligosaccharides, and cyclodextrins as substrates. The relative activities are listed in Table 3. Under the standard assay conditions, the enzyme had no detectable activity against maltose, *p*-nitrophenyl-β-D-glucopyranoside, the maltooligosaccharides as large as maltoheptaose, α-, β-, and γ-cyclodextrins, pullulan, or dextran. Amylose was the best substrate; however, the enzyme showed significant activity towards amylopectin. When amylose was treated with *S. bovis* crude culture filtrates, the only detectable products were maltose, maltotriose, and maltotetraose (6). The purified enzyme showed Michaelis-Menton-type kinetics when soluble potato starch was used as the substrate. As calculated from Lineweaver-Burk plots, the apparent K_m and k_{cat} values at 39°C were 0.88 mg · ml^{-1} and 2,510 μmol of maltose · min^{-1} · mg of $protein^{-1}$ (equivalent to 3,221 mol of reducing sugar [maltose] · s^{-1} · mol of $enzyme^{-1}$).

The following metal ions, at a final concentration of 1.0 mM each, had no effect on the enzyme: Ca^{2+}, Mg^{2+}, Ba^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+}, and Ag^{+}. Mn^{2+} (1 mM) and EDTA, at a final concentration of 10 mM, slightly inhibited the α-amylase activity (19 and 11%, respectively), while EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid] and *o*-phenanthroline had no

I. Asp-Glu-Gln-Val-**Ser**-Met-**Lys**-Glu-**Gly**-**Thr**-Val-**Leu**- Ala-**Ala**-Ala

II. Leu-Thr-Ala-Pro-**Ser**-Ile-**Lys**-Ser-**Gly**-**Thr**-Ile-**Leu**- His-**Ala**-Typ

III. Glu-Leu-Arg-Glu-Asn-Thr-**Lys**-Asp-**Gly**-Val-Met-**Leu**- His-**Ala**-Phe

FIG. 3. Comparison of the NH_2-terminal amino acid sequences of amylases from *S. bovis* (I), *B. subtilis* (II), and *C. acetobutylicum* (III). The data for II were from Yang et al. (31); those for III were from Paquet et al. (24).

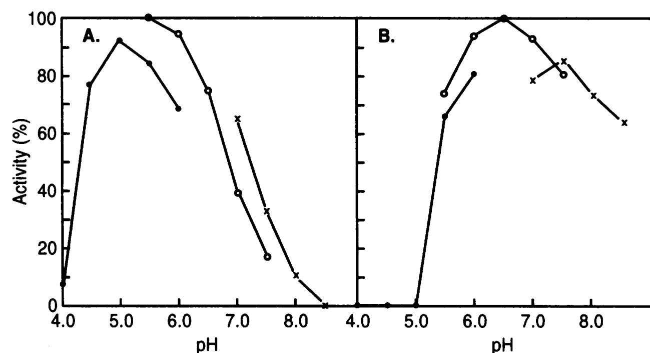

FIG. 4. Effect of pH on *S. bovis* amylase activity. The relative activity (A) and stability (B) were measured in 0.1 M Na succinate (●), 0.1 M PIPES-HCl (○), or 0.1 M HEPES-HCl (×). Experimental conditions are given in Materials and Methods.

effect. Likewise, 2-mercaptoethanol and dithiothreitol had no effect on activity. The α-amylase activity was inhibited by the sulfhydryl reagents Hg^{2+} (19% inhibition) and *p*-chloromercuribenzoic acid (88% inhibition at a final concentration of 1 mM). The inhibition of both Hg^{2+} and *p*-chloromercuribenzoic acid could be reversed by the addition of 10 mM dithiothreitol.

DISCUSSION

The α-amylase was isolated from maltose-grown *S. bovis* JB1 supernatant and purified approximately sevenfold with a yield of 50%. The apparent purity of the enzyme was demonstrated by SDS-PAGE. The purified α-amylase had an acidic isoelectric point of 4.5. This value is similar to those found for α-amylases purified from *C. acetobutylicum* (24), *B. subtilis* (22), and several other bacteria (29). The apparent molecular mass of the α-amylase, when determined by SDS-PAGE, was determined to be 77,000 Da. When gel filtration was employed, the estimated molecular mass was 48,000 Da, unless 6 M urea was included in the buffers, in which case the molecular mass was estimated to be 75,000 Da. Apparently, the enzyme interacts with the agarose gel, resulting in a retardation of its mobility and, thus, an underestimation of its molecular mass. In the presence of 6 M urea, the forces responsible for this interaction appear to be neutralized, resulting in a molecular mass estimation that agreed favorably with that obtained by SDS-PAGE. The discrepancy observed between the apparent molecular mass determined by SDS-PAGE and that determined by gel filtration has been reported previously for several microbial amylases (4, 8, 9, 21, 23, 28).

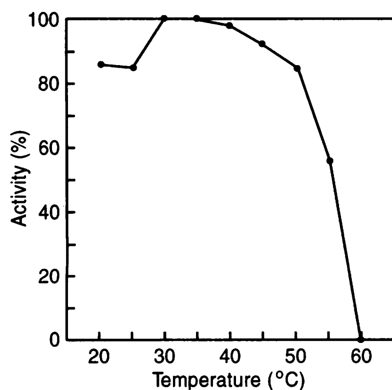

FIG. 5. Effect of temperature on stability of the *S. bovis* amylase. The relative activity was measured at 39°C after preincubation of the enzyme at various temperatures for 1 h in 0.1 M PIPES-HCl buffer (pH 5.5).

TABLE 3. Relative activity of the purified *S. bovis* amylase with different substrates[a]

Substrate	Relative activity (%)
Potato starch (soluble)	100
Potato amylose (type III)	100
Potato amylopectin	70
Corn starch (soluble)	120
Corn amylopectin	40

[a] No activity was observed with pullulan, dextran, cyclodextrins, maltose, *p*-nitrophenyl-β-D-glucopyranoside, or maltodextrins.

The *S. bovis* α-amylase was rich in both acidic and hydrophobic amino acids and also contained a relatively large amount of hydroxyamino acids and a small amount of arginine and histidine. This general amino acid composition is similar to those of several bacterial and fungal α-amylases (24, 26, 32). A comparison of the NH_2-terminal amino acid sequences of the *S. bovis* α-amylase and those of *C. acetobutylicum* and various *Bacillus* strains revealed homology with both the *C. acetobutylicum* α-amylase (27%) and the saccharifying enzyme of *B. subtilis* (40%). No homology between the *S. bovis* α-amylase and the *Bacillus licheniformis* liquefying α-amylase was detected (24, 31, 32). This suggests that the *S. bovis* α-amylase is related to the saccharifying α-amylases.

The pH for optimal α-amylase activity (pH 5.0 to 6.0) and stability (pH 5.5 to 8.5) was similar to values reported for most bacterial (4, 5, 20, 22, 27) and yeast (8, 9) amylases. The enzyme was stable to temperatures below 50°C.

The activity of the *S. bovis* α-amylase was neither stimulated nor greatly inhibited by various metal ions or chelating agents. *p*-Chloromercuribenzoic acid and, to a much lesser degree, Hg^{2+} inhibited the enzyme activity. The effect of both of these sulfhydryl reagents could be reversed by the addition of the reducing agent dithiothreitol.

It is difficult to accurately compare the kinetic parameters K_m and k_{cat} of various amylases because of variations in the starch origin and method of preparation, as well as in the details of the enzyme assays, e.g., incubation temperature and length of assay. However, the apparent K_m of the *S. bovis* α-amylase for soluble potato starch (0.88 mg · ml^{-1}) was within the range of values (0.35 to 4.3 mg · ml^{-1}) reported in the literature (4, 8, 9, 29) for various amylases. The k_{cat} value of the *S. bovis* α-amylase (2,650 U · mg of protein^{-1}) is one of the highest reported. The k_{cat}s of various amylases are 35 U · mg^{-1} for *Bacillus acidocaldarius* (24), 54.8 U · mg^{-1} for *Bacillus circulans* (28), 87.5 U · mg^{-1} for *C. acetobutylicum* (24), 1,403.2 U · mg^{-1} for *B. subtilis* (22), and 1,560 U · mg^{-1} for red sweet potato (11). It should be noted that the k_{cat} of the *S. bovis* α-amylase was determined at 39°C, the physiological temperature of the rumen. Several of the above-listed k_{cat}s were determined at elevated temperatures. These so-called temperature optima do not reflect a physical property of enzymes, but rather are a function of the assay conditions. Thus, the k_{cat} values might be signifi-

cantly less if this kinetic parameter were determined at the physiological growth temperature of the producing organism rather than at the temperature optima of the enzyme.

When *S. bovis* was grown in medium containing maltose as the carbon source, >90% of the α-amylase activity produced was secreted into the medium. However, when grown in medium containing starch as the carbon source, approximately 78% of the amylase activity was cell associated (6). When soluble starch was treated with cell-free culture fluids from maltose-grown cells, the crude enzyme preparations produced maltose, maltotriose, and maltotetraose. Depending on the time of incubation, either maltose or maltotetraose was the predominant oligomer formed (6, 7). The purified secreted enzyme from maltose-grown cultures produced the same spectrum of oligomers as the cell-free culture fluid (7a). Walker (30) examined the action of the cell-bound amylase produced by starch-grown *S. bovis*. When soluble starch was treated with this enzyme, the predominant oligomer formed was maltotriose. Lesser amounts of maltose and maltotetraose were also produced by this enzyme after prolonged incubations. Also, amylose, maltopentaose, and maltohexaose served equally well as substrates for the cell-associated enzyme from starch-grown cells (30). However, when similar assays (the increase in reducing sugars as measured with the dinitrosalicylic acid reagent) were performed with the purified secreted enzyme from maltose-grown cells, no activity could be detected with either maltose or any of the maltodextrins as substrates. These differences suggest that the amylases produced by *S. bovis* grown on the various carbon sources are not identical. Experiments are under way to purify and characterize the cell-associated amylase produced by starch-grown *S. bovis* cells and critically compare this enzyme with the secreted one produced by maltose-grown cells.

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