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Purification and characterization of a novel thermostable β-amylase from *Clostridium thermosulphurogenes*

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An extracellular β-amylase from *Clostridium thermosulphurogenes* was purified 811-fold to homogeneity, and its general molecular, physico-chemical and catalytic properties were determined. The native enzyme was a tetramer of 210 kDa composed of a single type subunit; its 20 amino acid *N*-terminus displayed 45% homology with *Bacillus polymyxa* β-amylase. The β-amylase was enriched in both acidic and hydrophobic amino acids. The pure enzyme displayed an isoelectric point of 5.1 and a pH activity optimum of 5.5. The optimum temperature for β-amylase activity was 75 °C, and enzyme thermostability at 80 °C was enhanced by substrate and Ca^{2+} addition. The β-amylase hydrolysed amylose to maltose and amylopectin and glycogen to maltose and limit dextrins, and it was inhibited by α- and β-cyclodextrins. The enzyme displayed $k_{cat.}$ and K_m values for boiled soluble starch of 400000 min^{-1} per mol and 1.68 mg/ml, respectively. The enzyme was antigenically distinct from plant β-amylases.

INTRODUCTION

β-Amylase (EC 3.2.1.2) hydrolyses the α-1,4-glucan bonds in amylosaccharide chains from the non-reducing ends and generates maltose. β-Amylases are well characterized in higher plants, and have also been reported in micro-organisms (Takasaki, 1976; Murao *et al.*, 1979; Robyt & French, 1964; Higashihara & Okada, 1974; Shinke *et al.*, 1975; Thomas *et al.*, 1980). However, the well-characterized β-amylases are neither active nor stable at temperatures above 65 °C.

Interest in thermoanaerobic bacteria has increased because of their unexamined potential as sources of thermostable and active industrial enzymes, including amylases (Zeikus, 1979; Brock, 1987). Recently, Hyun & Zeikus (1985*a*) reported that *Clostridium thermosulphurogenes* produced an extremely thermostable and thermoactive extracellular β-amylase. The general mechanism for regulation of β-amylase synthesis, its ability to bind starch and to produce maltose syrups have been reported elsewhere (Hyun & Zeikus, 1985*b*; Saha *et al.*, 1987). The purpose of the present study was to purify the β-amylase to homogeneity and to characterize its molecular and biochemical properties. We report that the enzyme has a high degree of thermostability and glycosylation atypical of other β-amylases.

MATERIALS AND METHODS

Organism and cultivation

The catabolic repression-resistant mutant H-12-1 (Hyun & Zeikus, 1985*b*) derived from *C. thermosulphurogenes* wild strain 4B (ATCC 33743) was used. The culture was routinely grown at 60 °C in 26 ml anaerobic pressure tubes (Bellco Glass, Vineland, NJ, U.S.A.), that contained 10 ml of TYE medium (Hyun & Zeikus, 1985*a*) supplemented with 0.5% soluble starch and a N_2/CO_2 (95:5) gas headspace. The culture was grown in a carboy (18 l capacity) containing 16 l of TYE medium with 0.5% maltose as substrate.

Enzyme assay

The routine enzyme assay for β-amylase activity used a reaction mixture (1 ml) containing boiled soluble starch [2% (w/v)], and sodium acetate buffer (50 mM, pH 6.0). Reducing sugar released by enzymic hydrolysis of soluble starch was determined by the dinitrosalicyclic acid method (Bernfeld, 1955). One unit of β-amylase is defined as the amount of enzyme that produces 1 μmol of reducing sugar as maltose per min under the above conditions. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Purification of β-amylase

All the procedures were performed under aerobic conditions.

Ultrafiltration. The culture broth containing cells was concentrated to about 1.6 l by the standard Millipore Pelican Cassette System used in a recirculating mode with an ultrafiltration membrane (M_r cut-off 100000). The extracellular β-amylase was retained with the cell fraction after filtration and then the cells were separated by centrifugation (15000 *g*, 30 min).

Chilled ethanol was added dropwise to the supernatant fraction while stirring to give a concentration of 20% (v/v) and this solution was left overnight at 4 °C. The precipitate that formed was collected by centrifugation (17000 *g*) for 30 min, dissolved in imidazole buffer (50 mM, pH 6.0) with 5 mM-$CaCl_2$, dialysed against the same buffer and then used as the crude enzyme preparation.

Abbreviation used: PAGE, polyacrylamide-gel electrophoresis.
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Column chromatography. The crude enzyme preparation was loaded onto a DEAE-Sepharose CL-6B column (4.0 cm × 32 cm), previously equilibrated with imidazole buffer (50 mM, pH 6.0) with 5 mM-$CaCl_2$. The column was washed with the same buffer and then eluted stepwise by increasing the concentration of sodium acetate in the same buffer (0.2 M, 0.5 M and 1.0 M). The single activity peak fractions were pooled and concentrated by ultrafiltration (PM 10 membrane, Amicon Co.) and dialysed overnight in 50 mM-imidazole buffer (pH 6.0) with 5 mM-$CaCl_2$.

This solution was then subjected to gel filtration on a Sephacryl S-200 column (1.5 cm × 150 cm) equilibrated with 50 mM-imidazole buffer, pH 6.0 with 5 mM-$CaCl_2$. The enzyme was eluted with the same buffer at a flow rate of 9 ml/h. The single activity protein peak fractions were pooled (except the first few fractions which were slightly contaminated by other proteins), concentrated by ultrafiltration (PM 10), and dialysed against the same buffer. This dialysed enzyme solution was then used as the purified enzyme preparation.

Electrophoresis and molecular mass determination

SDS/polyacrylamide-slab gel electrophoresis (PAGE) was performed as described by Laemmli (1970). The protein standards used were: bovine serum albumin (M_r 66000); egg albumin (45000); glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000); α-lactalbumin (14200). Gels were stained with Coomassie Brilliant Blue G-250. The molecular mass of the native enzyme was determined by gel filtration (Sephacryl S-200) as described by Andrews (1965). Standard proteins used were: apoferritin (M_r 443000); sweet potato β-amylase (200000); yeast alcohol dehydrogenase (150000); bovine serum albumin (66000); and carbonic anhydrase (29000).

Isoelectric point determination

A Servalyt Precote isoelectric-focusing gel (pH 3–10) was used. Samples were run at a constant power of 1 W in an LKB ultraphore isoelectric-focusing apparatus and the gels were stained with Serva Blue W.

Kinetic determinations

The K_m and $V_{max.}$ for β-amylase were determined by the method described by Lineweaver & Burk (1934). The turnover number ($k_{cat.}$) of β-amylase on soluble starch was determined according to the method described by England & Singer (1950).

Antibody production and imunodiffusion

For raising antibodies, rabbits were immunized with 125 μg of purified β-amylase using a novel adjuvant, monophosphoryl lipid A + trehalose dimycolate emulsion (Ribi, 1986). Booster injections with 100 μg of β-amylase protein in this emulsion were given after 6 weeks of primary immunization. Antisera were collected from the animals 1 week later. Immunological characterization of β-amylase was done by the double-immunodiffusion Ouchterlony technique (Ouchterlony, 1953). The gels contained 0.8% agarose in imidazole buffer (50 mM-imidazole, 5 mM-Ca^{2+}, 0.1 M-NaCl, pH 7.0). Enzyme preparations containing 1–20 units of β-amylase activity or 5–50 μg of protein were used against varying amounts of anti-(β-amylase) antibody. The β-amylases from sweet potato and barley (Sigma) were used for comparison.

Chemicals and reagents

All the chemicals were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.). The adjuvant was obtained from RIBI ImmunoChem Research (Hamilton, MT, U.S.A.).

RESULTS

Purification

The enzyme purification protocol is summarized in Table 1. β-Amylase was purified 811-fold from the culture-supernatant fraction. The ultrafiltration and ethanol-filtration steps were used for concentration of β-amylase. Although 38% of the enzyme was lost in these steps, they were more practical than acetone (50%) or ammonium sulphate (70% saturated) precipitation. β-Amylase activity was totally stable at 4 °C in the presence of 20% (v/v) ethanol for at least 4 days.

Major contaminating proteins were removed by elution using DEAE-Sepharose CL-6B chromatography with stepwise addition of acetate buffer (Fig. 1). The remaining protein contaminants were removed by gel filtration of Sephacryl S-200. The profiles for β-amylase activity and protein were not exactly coincident (results not shown). The fractions with maximal enzyme activity were collected and further examined for purity by gel filtration and electrophoresis.

The purified enzyme was considered to be homogeneous by the detection of a single band on SDS/PAGE (Fig. 2) and Servalyst polyampholyte isoelectric-focusing gels (results not shown). These procedures purified β-amylase with a high specific activity (4215 units/mg of protein) and a reasonable yield (21%).

Molecular properties

The M_r of purified native β-amylase was determined by gel filtration on Sephacryl S-200. β-Amylase from *C. thermosulphurogenes* displayed an M_r of 210000 (Fig. 3). SDS/PAGE analysis revealed one single band

Table 1. Major steps for purification of β-amylase from *C. thermosulphurogenes*

Steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification (-fold)	Yield (%)
Culture broth	49938	259680	5	1	100
Ultrafiltration	6105	238155	39	8	92
Ethanol precipitation	327	166198	508	98	64
DEAE-Sepharose (CL-6B)	54	108029	2000	385	35
Sephacryl S-200	15	64817	4215	811	21

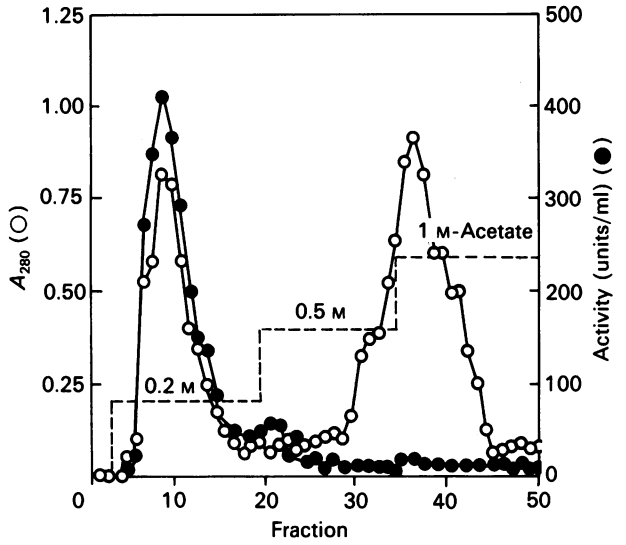

Fig. 1. DEAE-Sepharose CL-6B column chromatography of *C. thermosulphurogenes* β-amylase

The enzyme preparation after ethanol precipitation was applied to a column (2.6 cm × 35 cm) equilibrated with 50 mM-imidazole buffer (pH 6.0 with 5 mM-$CaCl_2$). The column was eluted stepwise with increasing acetate concentrations (----) in equilibrium buffer and 10 ml fractions were collected at a flow rate of 30 ml/h.

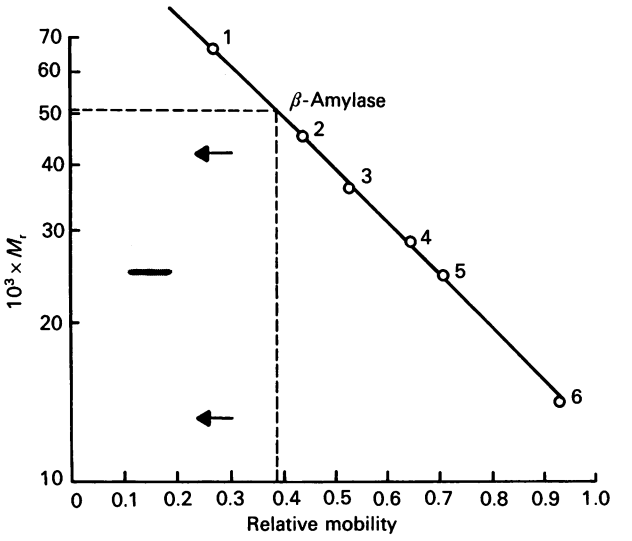

Fig. 2. Subunit molecular-mass determination of β-amylase by SDS/polyacrylamide-gel electrophoresis

Standards: (1) bovine serum albumin (M_r 66000); (2) egg albumin (45000); (3) glyceraldehyde 3-phosphate dehydrogenase (36000); (4) carbonic anhydrase (29000); (5) trypsinogen (24000); and (6) α-lactoalbumin (14200). Insert: photograph of the SDS/PAGE of purified β-amylase showing a single band. A 10% gel was used and stained with Coomassie Brilliant Blue G-250.

with M_r corresponding to 51000 indicating that the enzyme was a tetramer composed of a single type of subunit (Fig. 2). When compared with commercial standards, the isoelectric point of this enzyme was found to be 5.1 (results not shown).

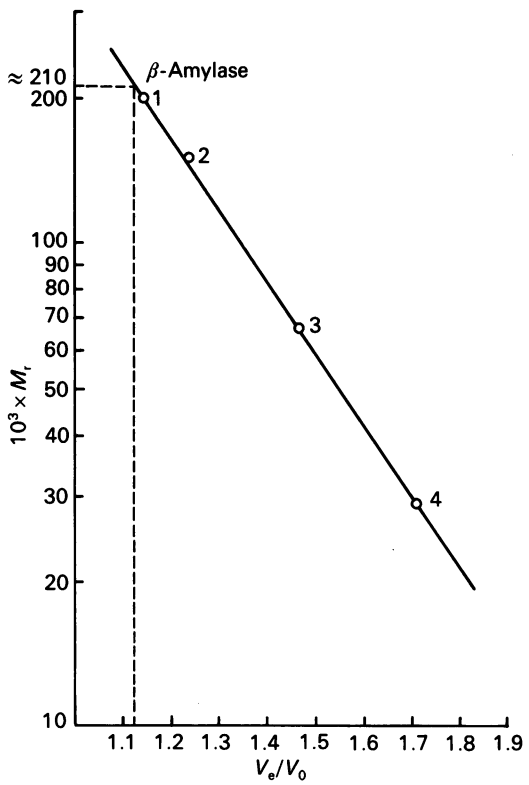

Fig. 3. Molecular-mass determination of native β-amylase by Sephacryl S-200-gel filtration

A column (1.5 cm × 150 cm) was used with a flow rate of 5 ml/h. Standards: (1) sweet potato β-amylase (M_r 200000); (2) yeast alcohol dehydrogenase (150000); (3) bovine serum albumin (66000); and (4) carbonic anhydrase (29000). V_e: elution volume of each protein. V_0: void volume of the column determined with Blue Dextran.

Amino acid composition analysis of purified β-amylase indicated that the enzyme was rich in acidic and hydrophobic amino acids but low in arginine and histidine (Table 2). Comparison of the *N*-terminal amino acid sequences of β-amylase from *C. thermosulphurogenes* with that from *Bacillus polymyxa* showed only 45% homology (Fig. 4).

Immunological characterization of β-amylase of *C. thermosulphurogenes* was done to study its antigenic relationship to eukaryotic enzyme sources. No antigenic cross-reactivity could be detected between β-amylase of sweet potato or barley, and the antiserum prepared from *C. thermosulphurogenes* β-amylase.

Physico-chemical properties

The optimum temperature for the action of purified β-amylase on soluble starch was 75 °C (Fig. 5). Although $CaCl_2$ was not required for enzyme activity, enzyme stability was greatly enhanced by the addition of 5 mM-$CaCl_2$. The enzyme was thermostable for 2 h in the absence of substrate or Ca^{2+} at 70 °C, but not at 80 °C. β-Amylase was thermostable at 80 °C in the presence of either Ca^{2+} (5 mM) or the substrate (1%).

Fig. 6 shows that this β-amylase displayed optimum activity at pH 5.5 with > 80% activity at pH 6.0 or 5.0. The β-amylase showed a broad pH stability over the range of 3.5 to 7.0 at 70 °C in the presence of 5 mM-$CaCl_2$.

Table 2. Amino acid composition of β-amylase (M_r 210000) purified from *C. thermosulphurogenes*

ND: not determined.

Amino acid	Molar ratio
Lys	209
His	78
Arg	62
Asp	753
Thr	492
Ser	452
Glu	358
Pro	270
Gly	453
Ala	442
Cys	ND
Val	341
Met	77
Ile	292
Leu	329
Tyr	202
Phe	236
Try	ND

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      1                   5                  10
I.    Ser-Ile-Ala-Pro-Asn-Phe-Lys-Val-Phe-Val-
II.   Ala-Val-Ala-Asp-Asp-Phe-Gln-Ala-Ser-Val-

      11                  15                 20
I.    Met-Gly-Pro-Leu-Glu-Lys-Val-Thr-Asp-Phe-
II.   Met-Gly-Pro-Leu-Ala-Lys-Ile-Asn-Asp-Trp-
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Fig. 4. Comparison of *N*-terminal amino acid sequence of β-amylases from *C. thermosulphurogenes* (I) and *B. polymyxa* (II)

Values for (II) from Kawazu *et al.* (1987).

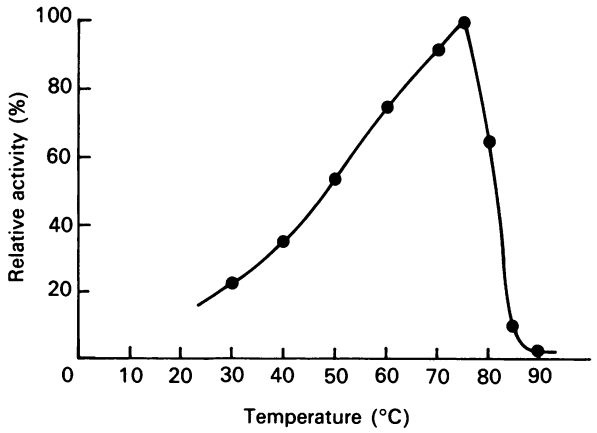

Fig. 5. Effect of temperature on purified β-amylase activity

Enzyme activity was assayed at pH 6.0 and the temperatures indicated by standard methods. Two units of purified β-amylase were used.

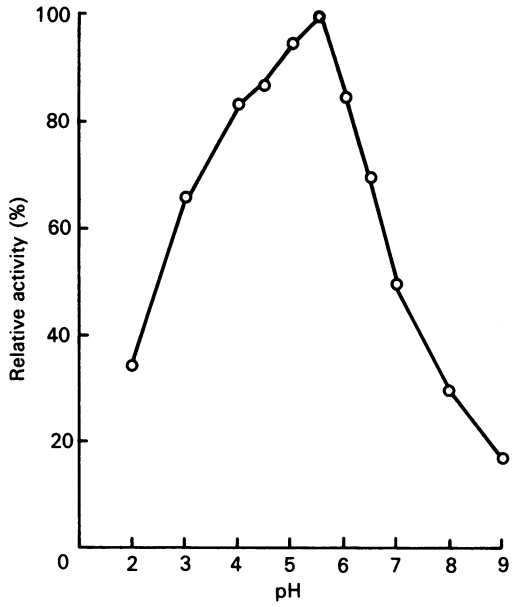

Fig. 6. Effect of pH on purified β-amylase activity

Enzyme activity was assayed at 60 °C and the pH values indicated by standard methods. Two units of purified β-amylase were used.

Table 3. Substrate specificity of β-amylase purified from *C. thermosulphurogenes*

Substrate [2% (w/v)]	Relative rate of hydrolysis (%)
Soluble starch	100*
Amylose	129
Amylopectin	112
Glycogen (oyster)	150
Pullulan	0

*100% activity corresponds to 1.2 units of β-amylase under standard assay conditions at 75 °C.

Catalytic properties

The purified β-amylase produced maltose and β-limit dextrins as the final end products of soluble-starch hydrolysis. The maximum theoretical value of maltose (62%) was produced by incubating purified β-amylase with soluble starch. The purified β-amylase displayed high catalytic activity with glycogen, amylopectin and soluble starch, but the enzyme did not hydrolyse pullulan (Table 3).

The general enzyme kinetic features of this β-amylase were compared at 60 °C versus 75 °C (Table 4). At 75 °C, the apparent K_m for soluble starch, the $V_{max.}$ and $k_{cat.}$ were 1.68 mg/ml, 122.5 μmol of reducing sugar formed/min·mg of protein^{-1} and 440000 min^{-1} per mol, respectively.

The effects of various metal ions and other compounds on the activity of β-amylase were investigated (Table 5). The enzyme did not require any metal ions for activity. At a concentration of 10 μM-*P*-chloromercuribenzoate, enzyme activity was completely inhibited. This suggests the involvement of essential thiol groups in the active site

Table 4. Kinetic properties of β-amylase purified from *C. thermosulphurogenes*

Soluble starch, pH 6.0 and other reaction conditions as specified in the Materials and methods section.

Temperature (°C)	K_m (mg/ml)	$V_{max.}$ (μmol·min^{-1}·ml^{-1})	$K_{cat.}$ (min^{-1}/mol)
60	2.29	179	400000
75	1.68	197	440000

Table 5. Comparison of different enzyme effectors on purified β-amylase activity

Effector [concn. (mM)]	β-Amylase activity (% remaining)*
None	100
Urea (1.25)	108
Guanidine-HCl (1000)	96
$FeCl_3$ (10)	84
$MgCl_2$ (10)	112
$MnCl_2$ (10)	86
$CaCl_2$ (10)	100
$NiSO_4$ (10)	96
p-Chloromercuribenzote (0.01)	0
Ethanol (10%)	68
$Al_2(SO_{43}$(10)	72

* 100% activity corresponds to 1.2 units of β-amylase assayed at 75 °C.

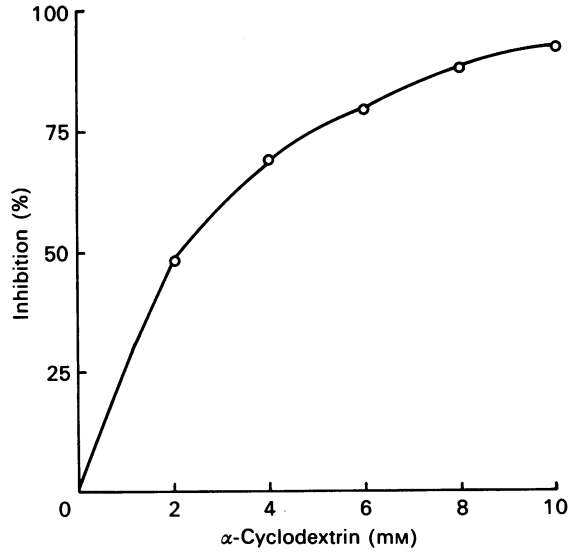

Fig. 7. Inhibition of β-amylase activity by α-cyclodextrin

The enzyme activity was assayed at pH 6.0 and 75 °C and the α-cyclodextrin concentrations indicated by standard methods. Two units of purified β-amylase were used.

for catalytic activity. The inhibition of β-amylase activity by *P*-choloromercuribenzoate was reversed by the addition of dithiothreitol (1 mM) or cysteine (5 mM). Fig. 7 shows the inhibition by α-cyclodextrin of purified β-amylase. The enzyme was competitively inhibited by both α- and β-cyclodextrin.

DISCUSSION

In general, these results extend fundamental understanding of the biochemical diversity of β-amylase. The enzyme purified from *C. thermosulphurogenes* differs from other described β-amylases because of its high specific activity and extreme thermostability.

The purification scheme employed here was relatively tedious. Selective ultrafiltration of the culture broth allowed 92% recovery of activity during cell and protein concentration. Enzyme precipitation with 20% ethanol was easier than with 50% acetone or saturation with ammonium sulphate. The DEAE-Sepharose chromatography bound contaminating culture medium tryptic-ase and yeast-extract proteins to a greater extent than the β-amylase, but this step resulted in 40% loss of enzyme. The major protein contaminant was separated by Sephacryl S-200. After gel filtration a homogeneous β-amylase was obtained. Since β-amylase binds to raw starch (Saha *et al.* 1987), studies are underway to improve the yield of purified enzyme by the use of raw starch-affinity chromatography and selective desorption techniques.

As expected, the purified β-amylase hydrolysed amylose to maltose and amylopectin, and gelatinized starch to maltose and limit dextrins. The specific activity of β-amylase purified from *C. thermosulphurogenes* was high (4215 units/mg of protein) when compared with the enzyme highly purified from *C. thermoactinomyces* (408 units/mg of protein) by Obi & Odibo (1984) or β-amylase from sweet potato (560 units/mg of protein) (Bernfeld, 1955). Kinetic studies with the purified β-amylase showed a relatively high $k_{cat.}$ and a low K_m value for soluble starch. This may explain the very high specific activity of the enzyme. The $k_{cat.}$ for *C. thermosulphurogenes* β-amylase is higher than that reported (Bernfeld, 1955) for sweet potato (250000 min^{-1} per mol). Inhibition of enzymic activity by P-chloromercuribenzoate was similar to other reported β-amylases that require thiol groups for enzyme active-site function.

The β-amylase purified here displays extreme thermostability when compared with other sources (Takasaki, 1976; Higashihara & Okada, 1974; Shinke *et al.*, 1975). The enzyme did not require metals for activity but Ca^{2+} stabilized the activity on prolonged storage at low temperature (i.e. > 2 days at 4 °C) and enhanced stability at high temperature (> 75 °C). The thermostability of β-amylase purified from *Bacillus cereus* is not enhanced by Ca^{2+} addition (Takasaki, 1976).

The precise molecular mechanisms that account for enzymic activity and stability of amylases at temperatures > 75 °C are not yet clear. In this regard, it is of interest to note that a distinctly different thermostable amylopullulanase recently purified from *C. thermohydrosulphuricum* (Saha *et al.*, 1988) shares some common features with β-amylase, including glycosyl-

ation, a high content of hydrophobic and acidic amino acids, and optimal activity not requiring Ca^{2+}.

The *C. thermosulphurogenes* β-amylase was a tetramer and had an M_r (210000) similar to that of sweet potato (200000). Immunological studies indicated that these two enzymes did not cross-react. Glycosylation is common for glucoamylases, but to our knowledge has not been reported for β-amylases. Bacterial β-amylase such as those purified from *B. cereus* are much smaller in molecular mass (Takasaki, 1976). The *N*-terminal sequence of β-amylase from *C. thermosulphurogenes* also showed limited homology with that reported for *B. polymyxa* (Kawazu *et al.*, 1987).

The structure of β-amylase from *C. thermosulphurogenes* may be unique from other described plant and bacterial β-amylases and it is under detailed investigation. It is of interest now to learn what accounts for high thermostability and activity.

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