

Raw-starch-digesting and thermostable α -amylase from the yeast *Cryptococcus* sp. S-2: purification, characterization, cloning and sequencing

Haruyuki IEFUJI*, Mariko CHINO, Miyoshi KATO and Yuzuru IIMURA

National Research Institute of Brewing, 3-7-1 Kagamiyama, Higashi-hiroshima, 739 Japan

A starch-degrading enzyme produced by the yeast *Cryptococcus* sp. S-2 was purified in only one step by using an α -cyclodextrin–Sephacrose 6B column, and was characterized as an α -amylase (EC 3.2.1.1). The molecular mass and isoelectric point of purified α -amylase (AMY-CS2) were estimated to be 66 kDa and 4.2 respectively. AMY-CS2 has raw-starch-digesting and raw-starch-absorbing activities. Furthermore it was shown to be thermostable. An open reading frame of the cDNA specified 611 amino acids, including a putative signal peptide of 20 amino acids. The N-terminal region of AMY-CS2 (from the N-terminus to position

496) had 49.7% similarity with the whole region of α -amylase from *Aspergillus oryzae* (Taka-amylase), whereas the C-terminal region had a sequence that was similar to the C-terminal region of glucoamylase G1 from *A. niger*. In addition, putative raw-starch-binding motifs exist in some amylolytic enzymes. A mutant AMY-CS2 that lacks the C-terminal domain lost not only its ability to bind or digest raw starch, but also its thermostability. Consequently it is possible that the putative raw-starch-binding domain of AMY-CS2 plays a role not only in the molecule's raw-starch-digesting ability but also in its thermostability.

INTRODUCTION

The enzymic hydrolysis of starch is catalysed by α -amylases (EC 3.2.1.1), glucoamylases (EC 3.2.1.3), β -amylases (EC 3.2.1.2.) and debranching enzymes such as pullulanases (EC 3.2.1.41). With respect to the digestion of raw starch by amylases, the raw-starch-binding and raw-starch-digesting abilities of glucoamylase G1 from *Aspergillus niger* have been intensively studied [1–5]. Some α -amylases also have an ability to digest raw starch [6–8], but little research has been done on the digestibility of raw starches by α -amylases. In contrast, thermostable α -amylases are of interest because of their potential industrial applications. Several thermostable α -amylases have been purified from thermophilic bacteria [9–12] and possible factors in their thermostability have been investigated. However, these factors remain unclear.

In a previous study we isolated a yeast that formed digesting haloes on a starch plate. This yeast was identified as a *Cryptococcus* species from its morphological and physiological characteristics [13]. When *Cryptococcus* sp. S-2 (strain CS2) was cultivated on plates containing raw corn starch or soluble starch, large digesting haloes formed around its colonies, whereas the α -amylase-producing yeasts, genera *Schwanniomyces* [14,15], formed digesting haloes only on plates containing soluble starch [13]. These results indicated that strain CS2 might secrete some amylases that digest raw starch. This report describes the purification and characterization of the raw-starch-digesting, thermostable α -amylase produced by strain CS2.

MATERIALS AND METHODS

Strains and plasmids

Strain CS2 [13] was used to produce amylase and to act as the DNA and mRNA source of the gene libraries. *Escherichia coli* strains HB101 and JM109 were employed as the hosts of plasmid vector pUC18 and pUC118, and pBluescript II SK(+) was used

for DNA manipulation and gene library construction. To construct the cDNA library we used *E. coli* strain Y1090 as host, and phage λ gt11 as vector. *Saccharomyces cerevisiae* DBY746 (a, *ura3*, *trp1*, *his3*, *leu2*) served as the host strain for the transformation, and vector pYCDE1, containing the yeast 2- μ origin of replication, the phosphoribosyl anthranilate isomerase gene, the alcohol dehydrogenase 1 gene promoter, the iso-1-cytochrome *c* gene terminator and an *EcoRI* cloning site, served as the yeast expression vector.

Media

A medium of 1.0% (w/v) maltose and 0.5% yeast extract (pH 4.5) was used for strain CS2 cultivation and amylase production, and a Luria–Bertani medium containing 100 μ g/ml ampicillin was used to cultivate *E. coli*. *S. cerevisiae* was cultivated in a minimal medium of 0.67% yeast nitrogen base without amino acids (Difco), 0.5% glucose and appropriate supplements, or in a medium containing 1.0% (w/v) yeast extract, 2.0% (w/v) polypeptone and 2.0% (w/v) glucose (YPD medium).

Enzyme assays

α -Amylase activity was assayed by measuring the reducing sugar released with starch as substrate. The reaction mixture consisted of 100 mM phosphate buffer, pH 6.0, containing 1% (w/v) soluble starch (E. Merck, Darmstadt, Germany) and 0.1 ml of enzyme solution at 37 °C. The reaction was initiated by addition of the enzyme solution. After incubation at 37 °C for 30 min, the reaction was terminated by cooling on ice and the amount of reducing sugar released was measured by the Somogyi–Nelson method [16] with maltose as a standard. One unit of α -amylase activity was defined as the amount of enzyme that produced reducing sugar equivalent to 1 μ mol of maltose per min under the conditions described above.

Abbreviations used: AMY-CS2, α -amylase from *Cryptococcus* sp. S-2; G_n , oligosaccharide consisting of n glucose units; strain CS2, *Cryptococcus* sp. S-2; Taka-amylase, α -amylase from *Aspergillus oryzae*.

* To whom correspondence should be addressed.

The nucleotide sequences of DNA (1958 bp) and mRNA (802 bases) for AMY-CS2 will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers D83540 and D83541 respectively.

Purification of enzyme

α -Cyclodextrin was coupled with Sepharose 6B gel (Pharmacia) in accordance with 'Affinity chromatography, principles and methods' (Pharmacia). A 4 ml portion of the resulting wet gel was packed into a glass column and used for the purification of the enzyme as follows. Strain CS2 was cultivated in the amylase-production medium at 25 °C for 3 days under aerobic conditions. After incubation, the supernatant of the culture fluid (1.5 litres) was concentrated to approx. 30 ml by ultrafiltration (Amicon HIP10-20 and YM-10). Then the concentrated culture medium was loaded on to the α -cyclodextrin-coupled Sepharose 6B column. After the column had been washed with 40 ml of 0.2 M NaCl in 0.2 M sodium sulphate buffer, pH 7.0, and 20 ml of water, the bound enzyme was eluted with 5 ml of a solution of 1.2 M maltose in water. These procedures were all done below 10 °C and the eluate was dialysed against water and concentrated by freeze-drying.

Determination of protein concentration

The protein concentration was determined by the dye-binding assay of Bradford [17] with BSA (Sigma) as a standard.

Electrophoresis

SDS/PAGE was performed by the method of Laemmli [18], with the resultant protein bands being stained with Coomassie Brilliant Blue R-250. The molecular mass standards were phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α -lactalbumin (14.4 kDa).

Isoelectric focusing (IEF) was also performed by using the Phastsystem with PhastGel IEF 3-9 (Pharmacia). A broad pI calibration kit (Pharmacia) furnished pI standard proteins. Protein bands were stained as for SDS/PAGE.

Chromatography of hydrolysis products

G17 short-chain amylose was digested by purified strain CS2 amylase at 37 °C for 10 min, 30 min, 1 h, 6 h and 18 h. Sugar products at each time point were identified by paper chromatography with Whatman no. 1 filter paper and developed by the ascending chromatography technique in a solvent system of *n*-butanol/pyridine/water (6:4:3, by vol.) at room temperature. Sugars in the paper were detected by the dipping method with silver nitrate/sodium hydroxide reagent.

Measurement of optical rotation

The optical rotation of the starch was measured by the method of Hyun and Zeikus [19]. A reaction mixture consisting of the enzyme and a 1% (w/v) soluble starch solution in 100 mM sodium acetate buffer, pH 6.0, was incubated at 37 °C. At various times, the optical rotation of the mixture at the sodium line was measured with a Schmidt Haensch polarimeter.

When the optical rotation became approximately constant, 10 mg/ml solid sodium carbonate was added and the mutarotation of the hydrolysate measured. α -Amylase from *A. oryzae* (Taka-amylase; Sankyo Co., Tokyo, Japan) and β -amylase from barley (Type II-B; Sigma) were employed as the controls.

Effect of pH

The effect of pH on amylase activity was determined for pH 1.0–9.0 at 37 °C after a 30 min reaction. The pH of the

reaction solution was adjusted with the following buffers: pH 1.0–3.0, 100 mM sodium acetate/HCl; pH 4.0–5.0, 100 mM sodium acetate; pH 6.0–7.0, 100 mM sodium phosphate; and pH 8.0–9.0, 50 mM Tris/HCl.

Effect of temperature

The effect of temperature on amylase activity was determined in the absence and presence of CaCl₂ (1 mM) from 30 to 90 °C in 100 mM sodium phosphate buffer, pH 6.0, after a 30 min reaction. The thermal stability of amylase was also determined from 30 to 90 °C in the absence and presence of CaCl₂ (1 mM) by incubating the enzyme preparation in 100 mM of sodium phosphate buffer, pH 6.0, for 30 min, then placing it in an ice bath and assaying the residual amylase activity at 37 °C.

Effect of metal ions and chemical reagents

Enzyme assays were performed in the presence of various metal ions (1 mM) or EDTA (10 mM) in 50 mM Mes buffer, pH 6.0. The activity assayed in the absence of metal ions and EDTA was defined as 100%.

Determination of digestion of raw starch

Wheat, corn, rice, potato and sweet-potato raw starches were washed twice with water, centrifuged and lyophilized. Reaction mixtures, each containing 25 mg of one of the raw starches, 1.625 ml of water, 0.25 ml of enzyme solution, 0.625 ml of 200 mM buffers and 0.05 ml of toluene were incubated at 30 °C for 24 and 48 h with shaking in Monod tubes. Phosphate buffers (100 mM, pH 7.0 and 6.0) were used for α -amylase from pig pancreas and amylase from strain CS2 respectively, and 100 mM acetate buffer, pH 5.0, for was used for Taka-amylase. Each enzyme activity was adjusted to 3 units/ml. After the reaction, the mixtures were centrifuged at 16000 *g* for 10 min at 4 °C, and the derived sugar in the supernatant was measured by the phenol/sulphuric acid method [20].

The raw-starch digesting rate (r_a) was defined by the following equation:

$$r_a(\%) = (A_1/A_0) \times 100$$

where A_1 is the molar concentration of sugar in the supernatant after the reaction, and A_0 is the molar concentration of whole raw starch before the reaction.

Adsorbability of amylase on raw corn starch

The desired amounts of amylases were added to the reaction mixture with 50 mM sodium phosphate buffer, pH 6.0, to prepare 1 ml of amylase solution of 2.0 units/ml. Raw corn starch (100 mg) was added to 1 ml of the preparation and shaken gently at 25 °C for 10 min. After filtration through a membrane filter (0.45 μ m pore size), the amylase activity of the filtrate (B) was assayed and compared with that of the original amylase solution (A). The adsorption rate (r_a) was defined by the following equation:

$$r_a(\%) = [(A - B)/A] \times 100$$

Analysis of NH₂-terminal amino acid sequence

The amino acid sequence of the NH₂-terminus was determined by subjecting a sample of the purified enzyme to Edman degradation with an automated protein sequencer (PSQ-2, Shimadzu).

Construction of cDNA and genomic libraries

Strain CS2 was grown at 25 °C in the amylase-producing medium to a phase in which the D_{660} was 6.0, corresponding to the most vigorous amylase production. The cells obtained (200 mg of wet cells) were disrupted in a 2 ml Eppendorf tube by the method of Hoffman [21]. Total RNA was obtained from the aqueous phase by ethanol precipitation. Poly(A)⁺ RNA was then isolated using an oligo(dT)-cellulose column (Pharmacia). Double-stranded cDNA was synthesized from poly(A)⁺ RNA with a cDNA synthesis kit (Pharmacia), followed by attachment of *EcoRI/NotI/BamHI* linkers (Takara) to its 5' and 3' ends and ligation with *EcoRI*-digested λ gt11 DNA (Stratagene). The DNA was packed by using an *in vitro* packaging kit (Stratagene), giving a cDNA library consisting of approx. 1.6×10^6 independent clones.

To construct the genomic library, the chromosomal DNA of strain CS2 was extracted in a similar manner to the total RNA. Purified genomic DNA was then partly digested with *Sau3AI* and size-fractionated in a sucrose gradient. Fragments with sizes between 6 and 8 kb were then ligated to the *BamHI*-digested and dephosphorylated pUC18. The ligation mixtures were used to transform *E. coli* JM109.

Screening of the cDNA library and the genomic DNA library

The cDNA library was screened by using the antibody against α -amylase from CS2 and a SuperScreen immunoscreening system kit (Amersham International). The length of the inserted cDNA of the resultant positive clones was estimated by the PCR method with forward and reverse primers of λ gt11.

The genomic DNA library was screened with ³²P-labelled whole cDNA of α -amylase from strain CS2.

Expression of amylase cDNA in *S. cerevisiae*

EcoRI-digested cDNA was ligated to the yeast expression vector pYCDE1. Two such plasmids were obtained; and the directions of their inserted cDNAs, as determined by *BamHI* digestion, were opposite to one another. *S. cerevisiae* DBY746 was transformed with these plasmids by electroporation as described by Becker and Guarente [22] with the use of a gene pulser (Bio-Rad).

Transformed *S. cerevisiae* were grown on a minimal medium agar plate containing 0.5% wheat starch for 5 days at 30 °C, after which amylase expression by a colony was detected by the formation of a halo.

Nucleotide sequencing and computer analysis

Nucleotide sequencing of cDNA inserted in pBluescript II SK(+) plasmids was performed by constructing a nested set of deletions with exonuclease III and mung bean nuclease, then sequencing the deletions with a Dye Primer Cycle Sequencing System (Applied Biosystems) employing single-stranded templates.

A *Taq* Dye Terminator Cycle Sequencing Kit (Applied Biosystems) was used for sequencing analysis of the genomic DNA, with adequate primers designed from the cDNA sequence. A double-stranded pUC18 plasmid containing the genomic DNA was used as a template. Sequences were deduced from overlapping clones, which determined 100% of the sequence in each strand. Sequence data were analysed with DNASIS software (version 3.50, Hitachi Software Engineering Co., Yokohama, Japan).

Construction of the mutant amylase lacking the C-terminal region

The deletion mutant cDNA lacking the putative raw-starch-binding domain of the C-terminus was constructed by using a

PCR amplification method. The forward primer was 5'-CAGG-AATTCGGCGGCCGCGGATCCGTC AAG-3', according to the upstream sequence, and contained an *EcoRI/NotI/BamHI* adapter sequence in the cloned cDNA of amylase from strain CS2; the reverse primer was 5'-ACGAATTCTACACGACAG-TACCCGCGGAGCAGCC-3', corresponding to the sequence between positions 1672 and 1695 of the genomic DNA plus a stop codon and an *EcoRI* site. This PCR product was designed to end at amino acid position 16 from the C-terminus of the putative α -amylase domain (which has high similarity to Taka-amylase).

After the *EcoRI*-digested PCR product was ligated to the yeast expression vector pYCDE1, *S. cerevisiae* DBY746 was transformed with the plasmid in which the inserted PCR product was in the correct direction.

The transformant yeast was cultivated for 3 days at 28 °C and the supernatant of the culture medium was concentrated by ultrafiltration. The concentrate was applied to a DEAE-5PW anion-exchange column (7.5 mm \times 7.5 cm; Tosho Co.), which was eluted with a gradient of 50 mM phosphate buffer, pH 6.0, containing 0.5 M NaCl. The enzyme fraction was further purified with a G 3000-SW gel filtration column (7.5 mm \times 60 cm; Tosho Co.).

RESULTS

Enzyme purification

Strain CS2 formed a large digesting halo around its colonies on the raw starch plate, so this yeast seemed to secrete some raw-starch-digesting amylase.

Amylases with the ability to digest raw starch were shown to adsorb on raw-starch granules and also on α - and β -cyclodextrin [6,23]. Therefore we tried to isolate raw-starch-digesting amylase from strain CS2 by using α -cyclodextrin-Sepharose 6B. When the culture medium of strain CS2 was applied to the α -cyclodextrin-Sepharose 6B column, almost all of the amylase activity in the medium adsorbed on the column. The adsorbed amylase activity on the α -cyclodextrin-Sepharose 6B could not

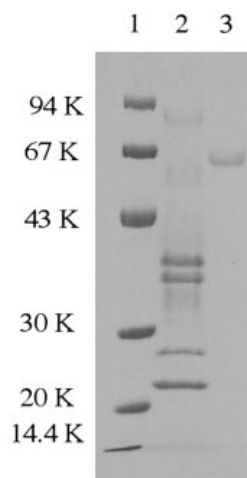


Figure 1 SDS/PAGE of purified AMY-CS2

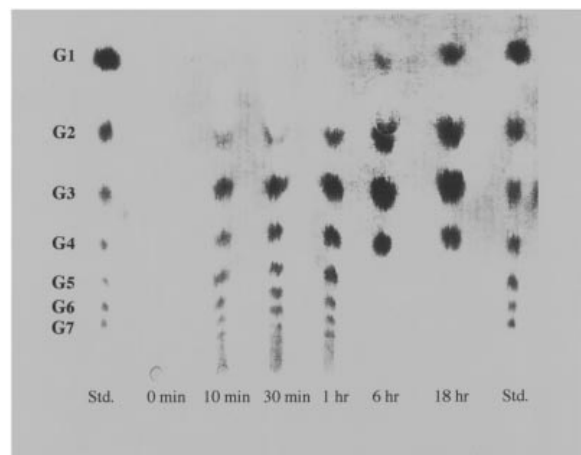
Lane 1, molecular mass markers, phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa) (K, kDa); lane 2, the supernatant of the culture medium; lane 3, the amylase purified with an α -cyclodextrin-Sepharose 6B column.

Table 1 Summary of purification of the AMY-CS2

The initial culture volume was 1,500 ml.

Purification step	Total protein (mg)	Total activity* (units)	Specific activity (units/mg of protein)	Recovery (%)
Supernatant of culture medium	98	1760	18	100
Ultrafilter concentration	75	1460	19.5	83
α -Cyclodextrin–Sephacrose 6B column effluent	0.54	1370	2539	78

* The substrate was soluble corn starch.

**Figure 2** Paper chromatography of hydrolysis products of G_{17} short-chain amylose produced with the AMY-CS2

The reactions were performed at 37 °C for the durations shown. Sugar products were developed on Whatman no. 1 filter paper by ascending chromatography in a solvent system of *n*-butanol/pyridine/water (6:4:3, by vol.) at room temperature. Sugars in the paper were detected by dipping with silver nitrate/sodium hydroxide reagent. Abbreviation: Std., standards.

be eluted by 2 M glucose, acidic buffer (pH 3) or alkaline buffer (pH 9) in 1 M NaCl, but could be specifically eluted by 1.15 M maltose (results not shown). In view of these results, the amylase from strain CS2 was purified by the method described in the Materials and methods section.

The results of SDS/PAGE analysis of the eluate from the α -cyclodextrin–Sephacrose 6B column indicated a single protein with a molecular mass of 66 kDa (Figure 1). This showed that AMY-CS2 could be highly purified in only one step with an α -cyclodextrin–Sephacrose 6B column.

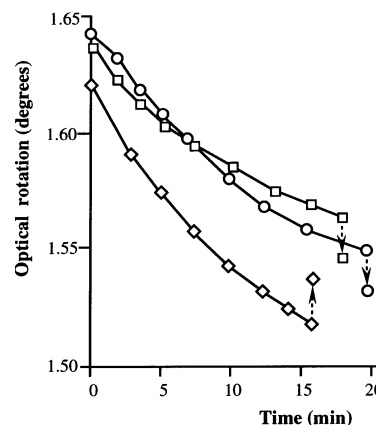
The isoelectric point of this amylase was calculated to be 4.2 by isoelectric focusing (results not shown).

A summary of the α -cyclodextrin–Sephacrose 6B purification is presented in Table 1. This purification increased the specific activity approx. 140-fold, with a 78% yield of activity and a 0.5% yield of protein.

As no additional amylase activity was detected in the culture broth of strain CS2 by HPLC on a DEAE-5PW column, we concluded that the strain produced only the amylase described.

Amylolytic pattern of AMY-CS2

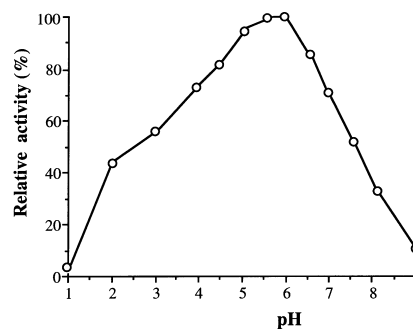
Short-chain amylose (G_{17} , where G represents a glucose unit) was hydrolysed by AMY-CS2 for various durations, and the resultant products were analysed by paper chromatography

**Figure 3** Effect on optical rotation of the action of AMY-CS2 (O), Taka-amylase (□) and β -amylase from barley (◇) on starch

The vertical arrows indicate the change in optical rotation after addition of sodium carbonate to the hydrolysates.

(Figure 2). The sugars produced by the 1 h reaction were G_2 , G_3 , G_4 , G_5 and larger malto-oligosaccharides, whereas little G_1 was observed. The products of the 6 and 18 h reactions were mainly G_1 , G_2 , G_3 and G_4 . These results indicated that the amylase from strain CS2 is an endo-type amylase.

From the mutarotation study (Figure 3), which employed

**Figure 4** Effect of pH on the activity of AMY-CS2 towards soluble starch

The relative activity was measured at 37 °C. Sodium acetate/HCl buffer at pH 1–3, sodium acetate buffer at pH 4–5.5, sodium phosphate buffer at pH 6–7 and Tris/HCl buffer at pH 7–9 were used in the enzyme reaction. The maximum activity was defined as 100% of the relative activity.

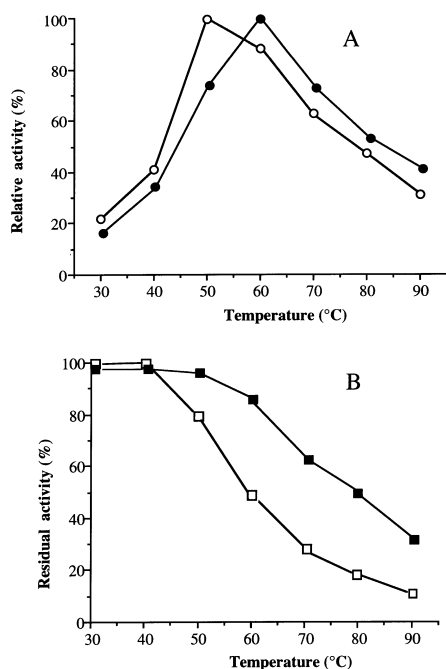


Figure 5 Effect of temperature on the activity (A) and the stability (B) of the AMY-CS2 towards soluble starch

(A) The effect of temperature on the activity was determined in the absence (○) and presence (●) of 2 mM CaCl₂ from 30 to 90 °C in 100 mM sodium phosphate buffer, pH 6.0, after a 30 min reaction. (B) The thermal stability of AMY-CS2 was determined in the absence (□) and presence (■) of 2 mM CaCl₂ by incubating the enzyme preparation in 100 mM sodium phosphate buffer, pH 6.0, for 30 min, then assaying residual amylase activity at 37 °C.

Taka-amylase and barley β -amylase as controls, the downward shift in optical rotation on the addition of base to the starch hydrolysate formed by AMY-CS2 indicates that the hydrolysis products have an α -anomeric configuration. The results presented above provide evidence that AMY-CS2 is an α -amylase.

Effects of pH and temperature on enzyme activity and stability

The optimal activity of AMY-CS2 towards soluble corn starch at 37 °C was obtained at pH 6.0 (Figure 4). The optimum tempera-

tures for enzyme activity towards soluble corn starch in the absence and presence of 1 mM CaCl₂ were 50 and 60 °C respectively (Figure 5A). Thermal stability was monitored by measuring the residual enzymic activity after the enzyme was incubated at 30–90 °C for 30 min (Figure 5B). The loss of activity of AMY-CS2 with increasing temperature was slow, and in the presence of CaCl₂ 34 % activity remained even at 90 °C. From these results, AMY-CS2 seemed to have considerable thermostability.

Effects of metal ions on enzyme activity

The effects of metal ions and EDTA on the enzyme activity were determined at a concentration of 1 mM. Heavy metals such as Hg²⁺, Ag⁺, Cu²⁺ and Zn²⁺ inhibited enzyme activity by 25.0, 51.0, 52.0 and 87.6 % respectively. Na⁺, Mg²⁺, Ca²⁺ and EDTA did not have any influence on the enzyme activity.

Digesting ability towards various raw starches

AMY-CS2 was able to digest various raw starches (Table 2). Compared with the α -amylase from pig pancreas, which has a strong raw-starch-digesting ability, AMY-CS2 had slightly weaker digesting abilities towards wheat starch, corn starch, rice starch and sweet-potato starch but a stronger activity towards potato starch. In contrast, Taka-amylase showed little activity towards these raw starches.

Adsorption on raw starch

AMY-CS2 adsorbed on raw starch but the adsorption rate (57 ± 4.9 %) was weaker than that of α -amylase from porcine pancreas (71 ± 5.7 %). In contrast, adsorption of Taka-amylase on raw starch was negligible (1 ± 0.2 %). Results are expressed as means ± S.D for three determinations.

Cloning of cDNA

We obtained eight positive cDNA clones after immuno-screening of 20000 individual λ gt11 cDNA plaques with anti-AMY-CS2 antibody. Two of the clones were estimated by PCR to have 2.1 kb inserted cDNAs that were of reasonable length to encode

Table 2 Ability of amylases to digest raw starches

Reaction mixtures contained 1 % of various raw starches and 3 units/ml of enzyme. Phosphate buffers (100 mM, pH 6.0 and 7.0) were used for AMY-CS2 and α -amylase from pig pancreas (PPA) respectively, and 100 mM acetate buffer, pH 5, for Taka-amylase (TAA). After incubation for 24 or 48 h the derived sugars in the supernatant were measured by the phenol/sulphuric acid method. The digestion rate was calculated as in the text. The results depicted are means ± S.D. ($n = 3$).

Raw starch	Digestion rate (%)					
	AMY-CS2		PPA		TAA	
	24 h	48 h	24 h	48 h	24 h	48 h
Wheat	42.0 ± 4.5	55.0 ± 6.6	52.5 ± 5.4	55.0 ± 3.6	3.0 ± 1.4	4.0 ± 1.2
Corn	46.0 ± 3.6	48.5 ± 5.2	55.0 ± 4.2	56.0 ± 4.3	1.5 ± 0.4	2.0 ± 0.8
Rice	28.5 ± 4.1	44.0 ± 5.7	36.0 ± 5.0	44.0 ± 6.4	1.0 ± 0.7	1.0 ± 0.2
Potato	7.5 ± 2.5	12.0 ± 3.7	2.5 ± 0.4	4.0 ± 1.4	0.1 ± 0.1	0.1 ± 0.0
Sweet potato	14.0 ± 2.2	24.0 ± 4.6	24.0 ± 2.8	30.0 ± 3.5	0.5 ± 0.3	0.5 ± 0.2

starch because the enzymic product, glucose, would not release that binding; the O-glycosylated flexible linker segment could therefore shift the N-terminal catalytic domain and thus allow it to cleave successive monosaccharides from the ends of the starch. In contrast, the enzymic products of an α -amylase such as AMY-CS2 are maltose and other oligosaccharides. The maltose, and perhaps the other oligosaccharides, could release the enzyme from the starch and allow it to move to a new spot, which is convenient for an endo-type amylase. Consequently the long linker segment might not be necessary for α -amylase.

In addition to being notable for its raw-starch-digesting ability, AMY-CS2 is also notable for its thermostability. The thermostability of α -amylases from *B. stearothermophilus* and *Clostridium thermosulfurogenes* [9] have been studied because of the industrial importance of these enzymes. The distance between the α -amylase consensus region I and region II, the numbers of cysteine residues, and the aliphatic index have been discussed as possible factors that make the α -amylases thermostable. However, the real factors in the thermostability of these α -amylases have not yet been clarified.

We now propose that the C-terminal raw-starch-binding motifs might be related to the thermostability of AMY-CS2 and some other α -amylases. There are some α -amylases that, like AMY-CS2, possess putative raw-starch-binding motifs at their C-terminus. It is interesting that almost all of these α -amylases are from thermophilic bacteria, such as *B. stearothermophilus*, *Cl. thermosulfurogenes* [9], *Streptomyces thermoviolaceus* [10] and *Thermomonospora curvata* [11] and that they are all thermostable. However, these α -amylases have not been shown to have raw-starch-binding and raw-starch-digesting activities because they have not been studied in this respect. The raw-starch-digesting amylase from *Bacillus* sp. B1018 has a sequence that is similar to that of the raw-starch-binding domain of AMY-CS2, and has also been shown to be thermostable [32,33].

As shown above, possession of the putative raw-starch-binding motif by an amylase always seems to be associated with enzymic thermostability. Furthermore the mutant AMY-CS2 that lacked a C-terminal domain was shown to lose not only its abilities to bind and to digest raw starch but also its thermostability. In the light of these observations it is possible that the putative raw-starch-binding domain of these amylases plays a role not only in the raw-starch-digesting ability of these enzymes but also in their thermostability.

Of course, it is reasonable that thermophilic bacteria should produce thermostable α -amylases. However, it is puzzling that yeast strain CS2, which is non-viable over 35 °C, produces such a thermostable α -amylase. The putative raw-starch-binding motif in amylase might accidentally have two functions, namely raw-starch-digesting ability and thermostability. The former function

might be exclusively available to strain CS2 and the latter might be exclusively available for thermophilic bacteria.

However, more study is needed to clarify the relation between the putative raw-starch-binding motif and the enzymic thermostability of amylases.

REFERENCES

- 1 Ueda, S. (1981) Trends Biochem. Sci. **6**, 89–90
- 2 Takahashi, T., Kato, K., Ikegami, Y. and Irie, M. (1985) J. Biochem. **98**, 663–671
- 3 Stoffer, B., Frandsen, T. P., Busk, P. K., Schneider, P., Svendsen, I. and Svensson, B. (1993) Biochem. J. **292**, 197–202
- 4 Belshaw, N. J. and Williamson, G. (1993) Eur. J. Biochem. **211**, 717–724
- 5 Semimaru, T., Goto, M., Furukawa, K. and Hayashida, S. (1995) Appl. Environ. Microbiol. **61**, 2885–2890
- 6 Hayashida, S., Teramoto, Y. and Inoue, T. (1988) Appl. Environ. Microbiol. **54**, 1516–1522
- 7 Hayashida, S., Teramoto, Y., Inoue, T. and Mitsuike, S. (1990) Appl. Environ. Microbiol. **56**, 2584–2586
- 8 Punpeng, B., Nakata, Y., Goto, M., Teamoto, Y. and Hayashida, S. (1992) J. Ferment. Technol. **73**, 108–111
- 9 Bahl, H., Burchhardt, G., Spreinat, A., Haecel, K., Wienecke, A., Schmidt, B. and Antranikian, G. (1991) Appl. Environ. Microbiol. **57**, 1554–1559
- 10 Bahri, S. M. and Ward, J. M. (1990) J. Gen. Microbiol. **136**, 811–818
- 11 Petricek, M., Tichy, P. and Kuncova, M. (1992) Gene **112**, 77–83
- 12 Haecel, K. and Bahl, H. (1989) FEMS Microbiol. Lett. **60**, 333–338
- 13 Iefuji, H., Iimura, Y. and Obata, T. (1994) Biosci. Biotechnol. Biochem. **58**, 2261–2262
- 14 Wang, T. T., Lin, L. L. and Hsu, W. H. (1989) Appl. Environ. Microbiol. **55**, 3167–3172
- 15 Oteng-Gyang, K., Moulin, G. and Galzy, P. (1981) Z. Allg. Microbiol. **21**, 537–544
- 16 Somogyi, M. (1951) J. Biol. Chem. **195**, 19–23
- 17 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
- 18 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
- 19 Hyun, H. H. and Zeikus, J. G. (1985) Appl. Environ. Microbiol. **49**, 1162–1167
- 20 Dubois, M., Giller, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) Anal. Chem. **28**, 350–355
- 21 Hoffman, C. S. and Winston, F. (1987) Gene **57**, 267–272
- 22 Becker, D. M. and Guarente, L. (1991) Methods Enzymol. **194**, 182–187
- 23 Belshaw, N. J. and Williamson, G. (1991) Biochim. Biophys. Acta **1078**, 117–120
- 24 Nakajima, R., Imanaka, T. and Aiba, S. (1986) Appl. Microbiol. Biotechnol. **23**, 355–360
- 25 Benoit, C. and Chambon, P. (1981) Nature (London) **290**, 304–305
- 26 Breathnach, R. and Chambon, P. (1981) Annu. Rev. Biochem. **50**, 349–383
- 27 Matsuura, Y., Kusunoki, M. and Kakudo, M. (1991) Denpun Kagaku **38**, 137–139
- 28 Nagashima, T., Tada, S., Kitamoto, K., Gomi, K., Kumagai, C. and Toda, H. (1992) Biosci. Biotechnol. Biochem. **56**, 207–210
- 29 Takase, K., Matsumoto, H., Mizuno, H. and Yamane, K. (1992) Biochim. Biophys. Acta **1120**, 281–288
- 30 Svensson, B., Larsen, K. and Gunnarsson, A. (1986) Eur. J. Biochem. **154**, 497–502
- 31 Svensson, B., Jespersen, H., Sierks, M. R. and Macgregor, E. A. (1989) Biochem. J. **264**, 309–311
- 32 Itkor, P., Shida, O., Tsukagoshi, N. and Udaka, S. (1989) Agric. Biol. Chem. **53**, 53–60
- 33 Itkor, P., Tsukagoshi, N. and Udaka, S. (1990) Biochem. Biophys. Res. Commun. **166**, 630–636