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

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A starch-degrading enzyme produced by the yeast *Cryptococcus* sp. S-2 was purified in only one step by using an α -cyclodextrin– Sepharose 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-starchabsorbing 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

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 rawstarch-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

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 rawstarch-binding motifs exist insome 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.

for DNA manipulation and gene library construction. To construct the cDNA library we used *E*. *coli* strain Y1090 as host, and phage λgt11 as vector. *Saccharomyces cereisiae* DBY746 (**a**, *ura*3, *trp*1, *his*3, *leu*2) served as the host strain for the transformation, and vector p YCDE1, containing the yeast $2-\mu$ origin of replication, the phosphoribosyl anthranilate isomerase gene, the alcohol dehydrogenase 1 gene promoter, the iso-1-cytochrome *c* gene terminator and an *Eco*RI 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 $100 \mu g/ml$ ampicillinwas used tocultivate *E*. *coli*. *S*. *cereisiae* 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 1μ mol of maltose per min under the conditions described above.

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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*.

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 amylaseproduction 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 nbutanol/pyridine/water $(6:4:3, \text{ by vol.})$ 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_d) was defined by the following equation:

 $r_{\rm d}({\%}) = (A_1/A_0) \times 100$

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 $(0.45 \mu m)$ 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:

$$
r_{\rm a}({\%}) = [(A - B)/A] \times 100
$$

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 *Eco*RI} *NotI/BamHI* linkers (Takara) to its 5' and 3' ends and ligation with *Eco*RI-digested λgt11 DNA (Stratagene). The DNA was packed by using an *in itro* 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 *Sau*3AI and size-fractionated in a sucrose gradient. Fragments with sizes between 6 and 8 kb were then ligated to the *Bam*HI-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

*Eco*RI-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 *Bam*HI digestion, were opposite to one another. *S*. *cereisiae* 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*. *cereisiae* 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-starchbinding domain of the C-terminus was constructed by using a PCR amplification method. The forward primer was 5'-CAGG-AATTCGGCGGCCGCGGATCCGTCAAG-3', according to the upstream sequence, and contained an *Eco*RI}*Not*I}*Bam*HI 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 *Eco*RI 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 Takaamylase).

After the *Eco*RI-digested PCR product was ligated to the yeast expression vector pYCDE1, *S*. *cereisiae* 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 $(7.5 \text{ mm} \times 7.5 \text{ cm})$; 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 rawstarch-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.

Figure 2 Paper chromatography of hydrolysis products of G₁₇ short-chain amylose produced with the AMY-CS2

The reactions were performed at 37 $^{\circ}$ C for the durations shown. Sugar products were developed on Whatman no. 1 filter paper by ascending chromatography in a solvent system of nbutanol/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–Sepharose 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–Sepharose 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–Sepharose 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 (*D*), Takaamylase* (\Box) and *β*-amylase from barley (\diamond) 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 (\bigcirc) and presence (\bullet) 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 (\square) and presence (\blacksquare) 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 temperatures 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 Hg^{2+} , Ag^+ , Cu^{2+} and Zn^{2+} 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 $(1\pm 0.2\%)$. Results are expressed as means \pm S.D for three determinations.

Cloning of cDNA

We obtained eight positive cDNA clones after immuno-screening of 20000 individual λgt11 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 \pm S.D. ($n=3$).


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\label{thm:main} \begin{small} getcccycttsqcctqgtd\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{arg}+q\textit{271 ATCGATGGGGGCTCGCCTAACGGATACACGCCCGACGGATCCGCTTACCACGGCTCAGGACATCTAACGACATCAACCCCCACHTLE D G G S P N G Y T P D G S A Y H G Y W A Q D I Y E I N P H
541 CACCCGTTCTGCGAGATCGACTACAACAACCGCAGGAGCATCCTCGATTGCTGGGAGGGTGACGAGATCGTGCCGCTCGACCTCCGGCTGAGATCGTGCGGCTGAGATCGTGCGGCTGAGATCGTCGGCTGAGATCGTGCGGCTGAGATCGTGCGGCTGAGATCGTGCGGCTGAGATCGTGCGGCTGAGATCGTGCGGCTGAGATCGGCTGCGGCTGG
631 ACCGAGGACTCGGACGTGCAGTCCATCTTCAACTCGTGGATCAGCAACTTGATCCAGACGTACAGCCTGCGAATCGACTCGCGAGCCTGGAATCGACTCGCGAGCCCTGGAATCGACTCG
                                                    L R I D S<br>Region II<br>TTAACGGCAGCCCG<br>F N G S P<br>pn III
721 CTCCAGCAGTCCGGCTCCTTCTTCTTCCGGGTTTCAACCAGGCCGCAGGTGGCATGTRCATGGTCGGCGAGGTCTCTCTCTCTTCTTCCCGGGTTCACCAGGTCGAGGTCGCAGGTCGGCGAGGTCTC
нии<br>cgatgagactacag
991 GTCTGACTGCTGCACACGCTTCTTGGGAGgtaagttaactttcgatacatcggcttgctatgccagtgctgatagtcgccagCTC<br>316 S D C S D T T L L G S
1981 ACGTGGTCCTCCtaggtctgggtataagatgctgctaggccatgacggtcggacaacctctgccacactgatgtggtagctcgttgagct<br>628 T W S S *
   tgacctgggcaaagaggagagaagattacggcgcttacaagagagacaaccaaaattaccggctggactttgttggggttctagaggatg<br>cgaggagtggctgcttagcggccacaaggggacaaatgcatgaaagatatgaaagggaaa
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Figure 6 Nucleotide and deduced amino acid sequences of the cDNA and genomic DNA of the AMY-CS2 gene

Protein-encoding sequences are indicated in capital letters. The putative signal sequence is underlined and the putative TATA-like sequence is doubly underlined. Conserved sequence regions (I, II, III, IV) of α -amylase are shown in boxes.

AMY-CS2 (molecular mass 66 kDa). Because neither of the inserted cDNAs had *Eco*RI sites, they were cut out by *Eco*RI digestion and subcloned into pUC118. Both were subsequently found to have the same restriction map (results not shown), suggesting that they were identical.

Expression of cDNA in S. cerevisiae

The *Eco*RI-digested 2.1 kb cDNA was ligated to pYcDE-1. The appearance of a digesting halo around an *S*. *cereisiae* colony that was transformed with one of the two produced plasmids (results not shown) confirmed that the cloned cDNA encoded amylase.

Cloning of genomic DNA

As the cloned cDNA in pYcDE-1 was confirmed to encode amylase, we employed it as a probe for screening approx. 11000 individual colonies of the genomic DNA library. Five positive clones were obtained with partly similar restriction maps (results not shown). On the basis of the restriction map of cDNA cloned in pUC118, the 3.7 kb *Hin*dIII fragment was estimated to contain all the coding region for the cDNA and more than the putative promoter region; that fragment was therefore subcloned into pBluescript II $SK(+)$ (2.95 kb) to analyse the genomic DNA sequence.

Analysis of nucleotide and amino acid sequences

The results of the cDNA sequencing, along with the deduced amino acid sequence, are shown in Figure 6. In the cDNA sequence there was a large open reading frame encoding 611 amino acids from the ATG triplet of the initiator codon to the TAG triplet of the stop codon. The amino acid sequences for the two lysyl-endopeptidase-digested peptides were verified at positions 71–86 and 577–589. The N-terminal amino acid sequence was recognized from Leu-21 to Asn-46. Consequently the signal peptide was 20 amino acid residues, from Met-1 to Gly-20. The four conserved sequences (I, II, III, IV), which have been identified in α -amylases and which have been proposed to form the active site of Taka-amylase A [24], were found at positions 144–149, 235–240, 264–268 and 327–332.

The molecular mass of the putative mature protein is 65800 Da, which is almost identical with that estimated by SDS/PAGE (66000 Da).

The genomic DNA contained two introns at positions 867–912 and 1024–1076, and their lengths were 46 and 53 nucleotides respectively. These introns begin with a $GT(G/A)AGT$ sequence and end with an AG sequence, which are general characteristics of an intron.

In the upstream region, the TATAA-like promoter sequence appears at position -59 , which is known to be required for transcription initiation by RNA polymerase II in higher eukaryotes [25]. However, the other CAAT promoter sequence [26] does not appear there.

Codon usage

The percentages of A, T, G and C at the third position in the open reading frame of AMY-CS2 cDNA were 6.0% , 12.5% , 28.8% and 52.6% respectively, indicating that the position is significantly biased to C and G. As strain CS2 is a basidiomycetous yeast and has a G+C content of 67% [13], it seems that the $G + C$ content of AMY-CS2 could be influenced by the $G + C$ content of the total nuclear DNA.

Comparison of the deduced amino acid sequence

The deduced amino acid sequence of AMY-CS2 was compared with the sequences of related enzymes through the SWISS-PROT database. The whole region of Taka-amylase has the highest similarity (49.7 $\%$ match) to the N-terminal region of AMY-CS2 at positions N-terminal to 496. Figure 7 shows these amino acid sequences and their similarity. This N-terminal region of AMY-CS2 also has a high similarity to yeast α -amylases from *Schwanniomyces occidentalis* (48.2%) and *Saccharomycopcis fibrigella* (47.4%).

The three carboxylic acid residues Asp-219, Glu-244 and Asp-312 in AMY-CS2 correspond to the residues Asp-206, Glu-230 and Asp-297 in Taka-amylase [27,28] and the residues (Asp-176, Glu-208 and Asp-269) in the α-amylase of *Bacillus subtilis* [29] that have been shown to be the catalytic residues of these enzymes.

In contrast, the C-terminal region of AMY-CS2 showed a high similarity to the C-terminal region of glucoamylase G1 from *A*. *niger* that is thought to be required for adsorption on raw starch [30]. Similar sequences exist in some amylolytic enzymes [31];

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102030
N-AMY-CS2 LSPAEWRSQSIYQVVTDRFALDNGGNSPSCSGQSELNLYCNGTFAGIIDKLDYIQNMGFT
1020
                                                          30
                                                                            40
                                                                                           50
                                           80
                                                                        100
                                                                                       110
                                                          90120
                             70
                \verb|AIWISPVVKNIDGGSPNGYTPDGSAYHGYWAQDIYEINPHFGGASGLTDLSNALHSRGMY\\External Community
                \verb|AWITPVTAQLPQTTA--Y---GDAYHGYWQQDIYSLNENYGTADDLKALSSALHERGMY\\ADDLKALSS<br>100<br>CET-DYNNE
                                                     80
                                                                    9060
                                                                                                 110
                               70
                                                        150,<br>160
                                         140
                          130
                LMVDVVVNHMAYYCGTDGGCGPGNSVNYGSFTPFNSESYFHPFCEI-DYNNRTSILDCWE
                 . . . . . . . . . x . . .
                                              strikisto strikisto en la tradicio
                                         ---DGAGSSVDYSVFKPFSSQDYFHPFCFIQNYEDQTQVEDCWL
                LMVDVVANHMG-Y---
                                                             XAGSSVDYSVFK<br>130 1<br>10 210
                                                                            -HPFCFIQNYED<br>150 1<br>0 230
                     120
                                                                                             160
                                          200190<sub>1</sub>180
               GDEIVPLVDLRTEDSDVQSIFNSWISNLIQTYNIDGLRIDSLQQSGSFFFPGFNQAAGGM
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                                                                                    ر<br>290
                YMVGEVFNGSPSYVCPYQQAGMPGVLNYPMFFYITNAFQTSSGSMSQLAQGISAMQSDCS
                     :::..:.:.:.:::::.
                                                                      ,,,,,,,,,,,,,
                YCLGEVLDGDPAYTCPYONV=MDGVLNYPIYYPLLNAFKSTSGSMDDLYNMINTVKSDCP
                  230 240 250 260 270 280<br>
310 320 30 330 340 350
             300
                DTTLLGSFLENQDNPRFPSQTSDLTRAQNAIAFTMLQDGIPITYYGQEQHLSGSGVPLNR
                QEQHYAGGNDP<br>340<br>CONTIMBECN
                           300 310 320 330<br>370 380 390 400290
             360
                EALWTSGGYDTSSPLYEMITTVNQLRTLAIKQNGGFVTYKIQVPYTDSNHIVTRKGNSGY
               \begin{minipage}{0.9\textwidth} \begin{minipage}{0.9\textwidth} \begin{itemize} \textbf{G} & \textbf{S} & \textbf{350 360 370 380 390<br>430 440 450 460400
                                                                                       470
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               {\tt QIVGVYTNVGSAGSSSTLSISSSETGFQASEPVMDVLSCTLYHTGTDGSLSFTMTGGLPR}ma a nan man a na matangan sa mang
               \verb|QUYTLSNKGASGDSYTLSLSGA--GYTAGQQLTEVIGCTTVTVGSDGNVPVPAAGGLPR\\430
                                                                    440
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                     \begin{array}{cc} 410 & 420 \\ 490 & \end{array}460
             480
               VFYNATALAESSLCTTY
                 . . . . . . . . . . . . .
               VLYPTEKLAGSKICSSS
                        470
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Figure 7 Amino acid sequence alignment of N-terminal domain of AMY-CS2 with Taka-amylase

Shown are the, N-terminal domain of AMY-CS2 (N-AMY-CS2) and the whole region of Takaamylase (AMY-ASPOR). Identical amino acids and similar amino acids are indicated by two dots and one dot respectively. The catalytic residues of Taka-amylase and the corresponding residues of AMY-CS2 are shown in bold.

Figure 8 Comparision of C-terminal sequences of putative raw-starchbinding motifs

Consensus amino acids are shown in bold. Abbreviations: α CS2, AMY-CS2; α Sli, α -amylase from *Streptomyces limosus*; βCth, β-amylase from *Clostridium thermosulfurogenes*; gaAn, glucoamylase G1 from *A. niger*; mtPs, maltotetraose-forming amylase from *Pseudomonas stuttzeri* ; cAlB, CGTase from alkalophilic *Bacillus* sp. strain 1011.

these sequences have been presumed to be the raw-starch-binding motifs. These regions of similarity are shown in Figure 8.

Characteristics of the mutant strain CS2 amylase lacking the Cterminal putative raw-starch-binding domain

The deletion mutant cDNA lacking the C-terminal putative rawstarch-binding domain of AMY-CS2 was expressed in *S*. *cerei*- *siae* DBY746. The resultant deletion mutant of AMY-CS2, which was purified by HPLC, was examined with respect to its abilities to digest and bind to raw starch, and also with respect to its thermal stability, as described in the Materials and methods section.

This mutant AMY-CS2 was revealed to have little ability to digest or bind to raw starch. It was able to digest only 3% of raw corn starch during a 24 h reaction, and only 2% of the activity was adsorbed on raw corn starch. These values were almost equal to those of Taka-amylase. This mutant amylase also lost its ability to bind to α -cyclodextrin (results not shown).

These results indicate that the C-terminal domain of AMY-CS2 might play a role in binding to raw starch, and consequently AMY-CS2 should have a raw-starch-digesting ability.

The deletion mutant of AMY-CS2 also lost its thermal stability. The residual enzymic activity decreased rapidly at temperatures above 50 °C, falling to 20% at 60 °C and to 5% at 70 °C in the presence of 2 mM CaCl₂.

 It should be noted that we tried to obtain all of the AMY-CS2 expressed by *S*. *cereisiae* DBY746. However, hardly any could be obtained, because the enzyme seemed to be bound in the cell wall and little was excreted. In contrast, the transformed cells themselves possessed high α -amylase activity (results not shown). The *S*. *cereisiae* AMY-CS2 that lacked the C-terminal sequence seemed to be excreted easily through the cell wall, and the cells themselves showed little amylase activity. This suggests that the C-terminal domain of AMY-CS2 might prevent the expressed enzyme from being excreted through the cell wall.

DISCUSSION

We purified a raw-starch-digesting amylase (AMY-CS2) from yeast *Cryptococcus* sp. S-2. The N-terminal region of AMY-CS2 (from the N-terminus to position 496) had 49.7% similarity to the whole region of Taka-amylase, whereas the C-terminal region had a sequence that was similar to the C-terminal region of glucoamylase G1 from *A*. *niger* as well as to putative raw-starchbinding motifs that exist in some amylolytic enzymes [31]. The mutant AMY-CS2 that lacks the C-terminal domain was shown to lose its abilities to both bind to and digest raw starch.

Consequently AMY-CS2 could be represented as a molecule in which the N-terminal region is an α -amylase catalytic domain and the C-terminal region is a raw-starch-binding domain. This structure should give AMY-CS2 the ability to digest raw starch.

The raw-starch-digesting ability of glucoamylase G1 of *A*. *niger* has been well studied. Glucoamylase G1 consists of three functional parts: an N-terminal catalytic site, a highly Oglycosylated linker segment, and a C-terminal domain responsible for binding to raw starch. The linker segment consists of about 70 amino acid residues, within which there are many O-glycosylated Ser and Thr residues. This highly O-glycosylated linker segment was shown to be needed for the efficient digestion of raw starch [5]. In contrast, AMY-CS2 is composed of two functional domains, and lacks a Ser/Thr-rich linker segment. Thus α amylase does not seem to need such a linker segment.

It is noteworthy that AMY-CS2 could easily be purified with α-cyclodextrin–Sepharose gel. When bound to this gel, AMY-CS2 was scarcely liberated by glucose, but was easily liberated by maltose.

Furthermore glucoamylase G1 was shown to bind to β cyclodextrin at the same site at which it binds to raw starch [23]. Therefore it is possible that the binding of the C-terminal domain to raw starch might be easily released by maltose but not by glucose. Hence the following hypothesis can be stated.

Glucoamylase G1 might mainly stay at one point on raw

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 oligosaccarides, 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.

Wenow propose that theC-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 Cterminus. It is interesting that almost all of these α -amylases are from thermophilic bacteria, such as *B*. *stearothermophilus*, *Cl*. *thermosulfurogenes* [9], *Streptomyces thermoiolaceus* [10] and *Thermomonospora curata* [11] and that they are all thermostable. However, these α -amylases have not been shown to have rawstarch-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 rawstarch-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 rawstarch-digesting ability and thermostability. The former function

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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., Haeckel, 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 Haeckel, K. and Bahl, H. (1989) FEMS Micribiol. 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 Benoist, 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