Expression of periplasmic α -amylase of *Xanthomonas campestris* K-11151 in *Escherichia coli* and its action on maltose

Jun-ichi Abe, Yuko Shibata,[†] Mami Fujisue and Susumu Hizukuri

Author for correspondence: Susumu Hizukuri. Tel: +81 992 85 8640. Fax: +81 992 85 8640.
e-mail: hizukuri@chem.agri.kagoshima-u.ac.jp

Department of Biochemical
Science and Technology,
Faculty of Agriculture,
Kagoshima University,
Korimoto-1 21-24,
Kagoshima 890, Japan

A gene encoding the periplasmic α -amylase of *Xanthomonas campestris* K-11151 was cloned into *Escherichia coli* using pUC19 as a vector. An ORF of 1578 bp was deduced to be the amylase structural gene. The primary structure of the enzyme had little identity with other α -amylases, except with the enzyme from *Bacillus megaterium*. The enzyme was expressed in *E. coli* from the *lac* promoter of pUC19 and was found to be transported to the periplasmic space. The expressed enzyme showed the same thermal stability, optimum temperature and substrate specificity as the enzyme from *X. campestris*. The enzyme formed maltotetraose, but not 6'- nor 6''-maltosyl-maltose, from maltose by the reverse reaction, and the tetraose was then hydrolysed to maltotriose and glucose. The addition of maltotriose enhanced the production of glucose from maltose. In addition, maltose was formed by the condensation of glucose by the enzyme. Thus, the periplasmic α -amylase of *X. campestris* was shown to produce glucose from maltose by hydrolysing maltotetraose and possibly higher maltooligosaccharides, which were the products of a condensation reaction, as a major pathway, and by direct hydrolysis of maltose as a minor pathway.

Keywords: heterologous expression, cyclodextrinase, maltose hydrolysis, condensation, dot-matrix analysis

INTRODUCTION

α -Amylase is an important enzyme, not only for the starch industry, but also for structural studies on starch-related saccharides. *Xanthomonas campestris* K-11151, isolated from soil, produces an α -amylase which has unique substrate specificity (Abe *et al.*, 1994). It can hydrolyse α -cyclodextrin (α -CD) into maltose and glucose at the same rate as that for soluble starch, and pullulan into panose with two-thirds of the velocity for soluble starch. Thus, the enzyme appears to have the mixed substrate specificity of α -amylase, cyclodextrinase and neopullulanase. In addition, we found that the enzyme can produce glucose from maltose, which is not a normal substrate of α -

amylases. It would therefore be interesting to investigate the relationship between the structure and function of this enzyme in detail.

Here, we cloned the α -amylase gene into *Escherichia coli* and sequenced it, deduced the primary enzyme sequence, and compared it to those of other amylases. We confirmed that the enzyme expressed in *E. coli* had the same properties as the original enzyme and then tried to find out the mechanism for glucose production from maltose by the enzyme.

METHODS

Materials. *X. campestris* K-11151 has been maintained in our laboratory (Abe *et al.*, 1994). *E. coli* XL-1 Blue [$\Delta(lac)$ *endA1* *gyrA96* *hsdR17* ($r_k^- m_k^+$) *recA1* *supE44* *thi-1* [F' *lacI^q* *lacZ* $\Delta M15$ *proAB* Tn10(*tet*^r)]} and JM109 [$\Delta(lac-proAB)$ *endA1* *gyrA96* *hsdR17* λ^- *relA1* *supE44* *thi* [F' *lacI^q* *lacZ* $\Delta M15$ *proAB* *traD36*]] were used for the construction of a gene library and for subcloning and general DNA manipulation, respectively. Plasmids pUC18 and pUC19 were used as vectors.

[†] **Present address:** Enzymology Research Laboratory, Nippon Gene Co. Ltd, Tonyamachi 1-29, Toyama 930, Japan.

Abbreviation: CD, cyclodextrin.

The DDBJ accession number for the sequence reported in this paper is D38228.

Glucose was from Wako Pure Chemicals, and maltose, maltotriose, maltotetraose, maltopentaose and pullulan of the highest grade were kindly donated by Hayashibara Biochemical Laboratory. Glucose and oligosaccharides were purified on Shandon ODS-Hypersil 5 using water as eluant. Maltitol and maltotriitol were respectively prepared by the reduction of maltose and maltotriose with sodium borohydride and were purified on ODS-Hypersil 5. CDs were donated by Nihon Shokuhinkako. Soluble starch and glycogen (rabbit liver) were purchased from Katayama Chemical and Sigma. Potato amylopectin and amylose were isolated from starch by the method of Lansky *et al.* (1949) with minor modifications (Hizukuri *et al.*, 1981).

DNA manipulation. General DNA manipulation was performed according to Sambrook *et al.* (1989). DNA fragments were recovered from agarose gels using GeneClean II (Bio 101). Plasmids were introduced into *E. coli* by electroporation using a 0.1 cm cell (1250 V, 200 Ω , 25 μ F) on a Gene Pulser (Nippon Bio-Rad). DNA sequencing was carried out on both strands using the Sequenase version 2.0 7-deaza kit (USB) and [35 S]dCTP α S (Amersham), or an ALF DNA sequencer (Pharmacia Biotech) with the AutoRead Sequencing kit after creating nested deletions using ExoIII and S1 nucleases. For Southern blotting, a DNA fragment was labelled with digoxigenin-dUTP (Boehringer Mannheim) according to the manufacturer's instructions. All restriction enzymes were from Nippon Gene. Homologies between two protein sequences were analysed by PALIGN in PC/Gene release 6.6 (IntelliGenetics/Teijin).

Enzyme assays. The amylase and glucose-producing activities were assayed as reported previously by Abe *et al.* (1994). One unit (1 U) is defined as the amount of enzyme that hydrolysed 1 μ mol 1,4- α -glucosidic linkage min^{-1} . Malate dehydrogenase and β -lactamase activities were measured by the methods of Smith (1983) and Bush & Sykes (1984) using L-malic acid and ampicillin as substrates, respectively. Protein was estimated by measuring the A_{280} using a Shimadzu UV-210A spectrophotometer.

Cloning of the α -amylase gene. Chromosomal DNA of *X. campestris* was partially hydrolysed with *Sau*3AI and the fragments of 3–6 kbp were isolated through electrophoresis on 0.8% agarose. The fragments were then ligated into the *Bam*HI site of pUC19. The resulting plasmids were introduced into *E. coli* XL-1 Blue by electroporation, and the recombinants, which grew as white colonies on LB agar supplemented with ampicillin, X-Gal and IPTG, were selected on M9 minimal medium containing ampicillin, IPTG and α -CD as the sole carbon source.

Cell fractionation. The cells of *E. coli* JM109 harbouring pCX213 were grown on LB medium (50 ml) containing ampicillin and IPTG overnight and were fractionated as reported previously by Abe *et al.* (1994).

Gel electrophoresis. Native PAGE and SDS-PAGE were done according to Davis (1964) and Weber & Osborn (1969), respectively, on 7.5% (w/v) gels.

Purification of α -amylase. *X. campestris* K-11151 was cultivated in medium containing 0.5% α -CD, 2% (w/v) polypeptone, 0.2% yeast extract, 0.14% KH_2PO_4 , 0.1% NH_4NO_3 , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% each of NaCl, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 7 p.p.m. $\text{ZnCl}_2 \cdot 7\text{H}_2\text{O}$, 5 p.p.m. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 1 p.p.m. H_3BO_3 at 30 °C for 24 h, and α -amylase was purified as follows.

E. coli JM109(pCX213) was grown on LB agar medium

containing 100 mg ampicillin ml^{-1} and 1 mM IPTG. The cells were then transferred to 5 ml M9 minimal medium supplemented with 1% α -CD instead of glucose as the carbon source, 2 μ g thiamin ml^{-1} , 100 μ g ampicillin ml^{-1} and 0.5 mM IPTG and cultivated at 30 °C overnight. The culture was transferred to 100 ml of the medium and incubated for 18 h. Cells (from 30 $\times$ 100 ml culture) were collected, washed with 30 mM Tris (pH 8.0)/1 mM EDTA/20% (w/v) sucrose by centrifugation, and suspended in the same buffer. Lysozyme was added at a final concentration of 200 μ g ml^{-1} , the mixture was kept at 0 °C for 1 h and then the supernatant was recovered after centrifugation. Pulverized ammonium sulfate was added over 30 min to give 80% saturation. After standing for 1 h, the precipitate was collected by centrifugation, dissolved in 10 mM CHES (pH 8.5)/2 mM CaCl_2 /5 mM mercaptoethanol and dialysed against the same buffer. The solution was passed through a column of CM-Toyopearl 650M (1.7 $\times$ 15 cm), which was equilibrated with the above CHES buffer, to remove lysozyme. The eluant was collected and combined, and its buffer was changed to 50 mM sodium acetate (pH 4.5) containing 5 mM mercaptoethanol and 1.4 M ammonium sulfate. The solution was applied to a column of Phenyl-Toyopearl 650S (1 $\times$ 20 cm). After washing the column with the buffer, enzyme was eluted with a linear gradient of ammonium sulfate (1.4–0 M, total 500 ml). The active fractions were combined and concentrated using an Amicon membrane PM-10, then the buffer was exchanged with 50 mM sodium acetate (pH 4.5)/2 mM CaCl_2 /5 mM mercaptoethanol.

N-terminal sequencing. The N-terminal sequence of the protein was determined using an Applied Biosystems 477A protein sequenator.

Action of enzyme on maltose or glucose. A reaction mixture (5 ml) containing purified maltose or glucose (0.9 M), 2 mM CaCl_2 , 1 mM DTT and enzyme (25 U) in 50 mM sodium acetate (pH 4.5) was incubated at 45 °C, and samples were withdrawn at appropriate time intervals. The products were analysed by HPLC using a Tosoh HLC-803D pump fitted with a column of Wakosil-NH2 (Wako Pure Chemicals), a Tosoh refractometer RI-8000, a Shimadzu Chromatopac C-R6A, and using 75% (v/v) acetonitrile as eluant at 1.0 ml min^{-1} . The tetraose fraction was collected, concentrated, and analysed by high-performance anion-exchange chromatography with a Dionex BioLC pump (Nippon Dionex) fitted with columns of Dionex Carbowac PA1 (4 $\times$ 250 mm, two columns in series) and a pulsed amperometric detector (PAD-1), using 20 mM sodium acetate in 0.15 M sodium hydroxide as eluant. Standard 6 1 - and 6 2 -maltosyl-maltose were prepared by condensation of maltose by *Pseudomonas* isoamylase (Abe *et al.*, 1988).

RESULTS

Cloning of the α -amylase gene

Using shotgun cloning followed by direct selection for the capability of growth on α -CD medium, two colonies out of 8000 transformants were obtained. The plasmids obtained from them were identical as revealed by restriction enzymic analysis; the plasmid was termed pCX2, and possessed a 4 kbp insert. The restriction map is shown in Fig. 1.

A labelled *Bam*HI fragment (1.7 kbp) from pCX2 hybridized with *Bam*HI- or *Pst*I-digested chromosomal DNA of *X. campestris* K-11151 and in each case gave a single band on Southern blot analysis (data not shown).

Subcloning and expression in *E. coli*

The *Pst*I–*Pst*I fragment of pCX2 was subcloned into pUC19 to give pCX21 and pCX22, which contained the *Pst*I fragment in the same and opposite directions compared to pCX2, respectively (Fig. 1). *E. coli* JM109(pCX21) produced the enzyme when IPTG was included in the medium, while pCX22 did not, even in the presence of IPTG. The amount of enzyme produced by *E. coli* JM109(pCX21) was fourfold higher than that produced by *E. coli* JM109(pCX2). The elimination of the first 800 bp and 1100 bp yielded pCX213 and pCX212, which gave 67-fold and 25% of the productivity of *E. coli* JM109(pCX2), respectively.

A cell-free extract of *E. coli* JM109(pCX213) contained material which corresponded to the purified enzyme from *X. campestris* K-11151 on native PAGE, while that of *E. coli*(pUC19) did not (Fig. 2a).

Seventy-six per cent of α -amylase and β -lactamase activities were recovered in the periplasmic fraction of *E. coli* JM109(pCX213), while 86% of malate dehydrogenase activity was found inside the cell.

Properties of the purified enzyme

Typical purification steps are summarized in Table 1. The purified enzyme from *E. coli* JM109(pCX213) had the same specific activity as that of the original α -amylase from *X. campestris*. The enzyme showed the highest activity for α -CD hydrolysis at 55 °C (pH 4.5, 15 min assay) and the enzyme was stable up to 50 °C (pH 4.5, 30 min preincubation). These properties were the same as those of the enzyme from *X. campestris* K-11151. The purified enzyme hydrolysed soluble starch, amylose and CDs at similar rates and showed two-thirds of the activity on amylopectin and pullulan. Glycogen was a poor substrate. These action specificities were identical to those of the original enzyme (Table 2).

Nucleotide sequence of the α -amylase gene and deduced primary sequence of the enzyme

The whole insert of pCX213 (2200 bp) and a small *Ap*I–*Bam*HI fragment (see Fig. 1) of pCX2 (400 bp) were sequenced (Fig. 3). The GC content of the whole sequenced region was 89%. Two ORFs were found which were at nucleotide positions 505–2097 and 517–2097. We concluded that the latter frame was likely to encode the α -amylase (see Discussion). A putative ribosome-binding site was found 7 bp upstream of the ATG codon but a promoter-like sequence was not found. The ORF consisted of 1581 bp, which coded for 526 amino acid residues. N-terminal sequencing of the enzymes from the original and recombinant strains showed that the sequences were identical from residue 31. The M_r of the mature enzyme calculated from its deduced sequence was 54437, in agreement with the results (M_r 55000) of SDS-PAGE analysis of the enzymes (Fig. 2b).

Fig. 1. Restriction maps and enzyme productivity of clones. **P**►, Direction of *lac* promoter. Relative activities of the recombinants compared to that of pCX2 are shown in parentheses.

Fig. 2. Native PAGE of cell-free extracts (a) and SDS-PAGE of purified enzyme (b). (a) Lanes: 1, extract from *E. coli*(pUC19); 2, extract from *E. coli*(pCX213); 3, purified enzyme from *X. campestris* K-11151. (b) Lanes: 1, M_r markers: BSA (66000), ovalbumin (43000), glyceraldehyde-3-phosphate dehydrogenase (36000), chymotrypsinogen (25700) and myoglobin (17200); 2, purified enzyme of *X. campestris* K-11151; 3, purified enzyme from *E. coli*(pCX213).

The deduced primary sequence of the α -amylase had four consensus regions (DLVINH as region 1, GFRLDAARH as region 2, EFRQ as region 3 and FLSNHD as region 4) for amylases as indicated in Fig. 3.

Table 1. Summary of the purification of periplasmic α -amylase expressed by *E. coli* JM109(pCX213)

Step	Activity (U)	Protein (A_{280})	Specific activity (U per A_{280} unit)	Purification (-fold)	Recovery (%)
Crude extract	925	466	1.98	1	
Ammonium sulfate precipitation	2590	51.0	50.7	26	100
CM-Toyopearl 650M column	2450	34.9	70.1	35	95
Phenyl-Toyopearl column	1450	9.65	150	76	56

Table 2. Substrate specificity of enzymes from *X. campestris* K-11151 and *E. coli* JM109(pCX213)

All values are means of at least triplicate determinations, and all standard errors were less than 5%.

Substrate	Relative activity (%)	
	<i>X. campestris</i> K-11151	<i>E. coli</i> JM109(pCX213)
Soluble starch	100	100
Amylose	106	103
Amylopectin	64	61
Pullulan	65	66
α -CD	95	94
β -CD	104	106
γ -CD	111	113
Glycogen	3.2	2.6

Action of the enzyme on maltose or glucose

The activities of the α -amylase on commercial and purified maltose relative to soluble starch were 0.80 and 0.18, respectively (Table 3). One per cent of maltotriose was detected in commercial maltose by HPLC (data not shown). The addition of purified maltotriose at the same ratio to the purified maltose gave a relative activity of 0.74, which was almost the same as that for the commercial maltose (Table 3). The production of glucose was further stimulated by the addition of maltotriose (1:20 ratio of maltotriose to maltose, by mol), which alone was hydrolysed only scarcely (Table 3). Maltotetraose and maltopentaose were shown to be hydrolysed by the enzyme to produce glucose. Although the rate decreased, glucose was produced from a reaction containing a mixture of maltitol and maltotriose (20:1, by mol) as the substrate. The enzyme did not produce any glucose in the reaction mixture which contained maltose and maltotriitol (20:1, by mol) (Table 3).

The enzyme produced glucose from purified maltose gradually for 3 h and then rapidly, in agreement with the decrease of maltose and the appearance of maltotriose and maltotetraose (Fig. 4). The tetraose was neither 6¹- nor 6²-maltosyl-maltose since the retention times of 6¹- and 6²-maltosyl-maltose and maltotetraose were 25.6, 23.6 and 15.6 min, respectively, on the Carbowac PA1 column and the tetraose produced was eluted only at the maltotetraose

position as a single peak. When glucose was used as substrate for the reverse reaction, HPLC analysis of the reaction mixture clearly showed the formation of maltose (Fig. 5).

DISCUSSION

Recombinant plasmid pCX2, which allowed *E. coli* to grow on minimal medium containing α -CD as the sole carbon source, contained a 4 kbp fragment which was shown to originate from *X. campestris* chromosomal DNA by Southern hybridization analysis. The whole structural gene of α -amylase seemed to be subcloned into pCX213 and expressed from the *lac* promoter on a pUC plasmid, since the productivity of the α -amylase of *E. coli* JM109(pCX213) increased 67-fold compared to the *E. coli* JM109(pCX2) enzyme in the presence of IPTG, and native PAGE and SDS-PAGE of the enzyme preparations from *E. coli* JM109(pCX213) showed the same mobilities as those of the purified enzyme from *X. campestris* K-11151.

The enzyme was transported to the periplasmic space of *E. coli* JM109 as in the case of *X. campestris* K-11151, and since the enzyme productivity of *E. coli* JM109(pCX213) was fivefold higher than the original strain and the transformant could grow on a synthetic medium, the enzyme purification became much easier, with higher recovery. The amount of enzyme activity after ammonium precipitation was usually higher than that in the crude enzyme. This might be due to some interfering substance which could be eliminated by fractionation by ammonium sulfate and/or following dialysis. The recoveries of the enzyme were calculated by taking the amount of the enzyme at this step as 100%. The enzyme, which was produced by the transformant in its periplasmic space, had an identical N-terminal sequence to the enzyme from *X. campestris*, indicating that its signal sequence worked properly in *E. coli*, and the enzymes had almost the same properties, i.e. same specific activity, maximum temperature and thermal stability, and substrate specificity, suggesting that the correct folding of the protein occurred.

Although sequencing of the DNA fragments allowed two ORFs, namely nucleotides 505–2097 and 517–2097, to be identified, the second ATG (517–519) was likely to be the initiation codon because: (a) a putative ribosome-binding site was found only in this case (GGAG at –10 to –7), (b) glutamic acid as the second amino acid in a

CCACCTCGTACATCGGCTCGGCGGCTGTCCAGCGCGTCCGAGCTCGGCTGTCT 60
 ACAGCAACGATCGGTTCTCGGCGGCTGTCCGCACTACGCTCGAAGTTCGCTCGG 120
 ACACGAGATGTGGTGAACACGAGTGTGACCTTCGCGCGGAGAGGTTGACGACT 180
 TCCAGGCTTCGTACGAGCTGAACACGAGTGTGACCTTCGCGCGGAGAGTTCGACATCA 240
 GCGACGAGCGGACCAAGAGTACTTCGCGCGGAGTCCGTCGAGAGCGGACCATCGAT 300
 ACTTCGCGCGGACGAGTACTTCGCGCGTCAAGTTCAGCTGAATCGAGCGGCGCGG 360
 CGCTTCGCGAGGCGCGCGCGCGGCTTTCAAGGCTGTCTCGTCGCGAGCGCGGCA 420
 CCGCGAGGCGCGCGTACGAGTCCGCGCGGCGGACCTTCGCGCGAGGCGTCCGCTTC 480
 —>pCX213
 CATCGAAGCGCAAGCGACCATCGAATGGAGCAAGCATGTCTTCGCTCGGCTTCGCG 540
 M S S S C R F R
 CGGTTGCTGTTCTGCTCGCGCGCGGCTCGGCGCATCGCGCGGTTCCGCGCGTCCG 600
 P L S F L L A A A L G A S A A F A P S V
 CATGCGCGGATCGCGCGGCTCGGCTTCGCGCGTCCGAGGCTCGGCGGTTGATACGAG 660
 H A D A A P A T P R S E G S G V W Y E
 ATCTTCGCGCGGCTGTGATGACCGAGCGAGCGGATCGGCGATCTCAACGCGGTC 720
 I F V R A W Y D T D G D G I G D L N G V
 ACCGCGAAGCTCGACTACCTGCAATCGCTCGGCGTCAAGCGGATCTGGCTGATCGCGATC 780
 T A K L D Y L Q S L G V S G I W L M P I
 AATCGCTCGCGGAGTACCGCGGCTACGACATCAGGCTACGAGGAGATCAACCGCGAG 840
 N P S P S Y H P D T D Y E G I N F Q
 TACGCGAGGATCGCGGACTTCGAGAGCTGTGAGCGAGCGACAGCGCGGATCGAG 900
 Y G T M A D F E K L V S E A H K R G I E
 GTGATCTCGAGCTGGTGTCAACACACAGCGAGCGGATCGGCTTCAGAGCGCGG 960
 V I L D L V I N H T S D Q H P W F K A A
 Region 1
 CTGGATCGGAGGAGCGGCTGCTGCTGCTACCTGGCGAGGCGCGGCTACGAGCTG 1020
 L D P K D A H R S Y T W A G E G T N L
 AAGCGGTCAGCGGCTGGCGCGCGGCGGCTGGCATCGCAAGCGAGCGACTACTG 1080
 K A V S A V G G P A W H A N G K Q H Y L
 GCGGATTCACCGCGGATCGCGGAGCTCAACTACGAGCGGCGGATCGCGCGGAG 1140
 G D F T G A M P D L N Y D E P A V R R E
 ATGATCGCGGTCGCGAAGTTCGCTGCGAGAGGCGCGGATCGGCTTCGCGCTGATGCG 1200
 M I A V G K F W L D K G A D G F R L D A
 Region 2
 GCGCGGACATCTATGAGCTCTGGAAGCGGACAGCGCGGCGGCTGATCGCGAGG 1260
 A R H I Y D D L E S D N G Q P A V I A R
 AATGCGGAGTGTGGAAGAGTTCGCGCGGCTTCGCGCGGCTCGGCTCGGAGCTCTAT 1320
 N A Q W W N E F R Q G L R Q V R P D V Y
 Region 3
 CTGGTCGCGGAGTCTCGCGCGAGCGGCGGCGGAGTGGCGCGGATCTCGCGGACTG 1380
 L V G E V S A K Q P G E L A P Y L P A L
 GGCTCGGTTGCTGCTTCGCTGCGCGAGCGGCTGATCGCGCGGCGGCGAGGAAG 1440
 G S V F D F P L A E Q L I A S A G Q E K
 GCGGCGAGCTCGCGCGGCTGCTACCGGAGAGTACGCGGCTTCGCTGCGCGCGGCGG 1500
 A G K L P A L L T E T Y A A F R A A A G
 GAGCATACGCGGAGCGGCTTCCTTCACACGAGTACGAGCGTGTCTCGAGCGG 1560
 D D Y A D A P L F L S H D Q E R V L S Q
 Region 4
 CTCGCGGCGGATCTCGCGGAGTGTGCGCGGCGGCGGATGCTGCTGAGCTTCGCGGCG 1620
 L G G D L R H M R T A A A M L L T L P G
 CGGCGTACTCTACTACGCGGAGAGATCGGCGGCTGCTCGCGCGAGCGGAGAGAAC 1680
 R P Y L Y Y G E E I G M L G R K P D E N
 CTCGCGGAGCCATGCTGCTGCGAGCGCGCGGCGCGCGGCGGAGCGGCTGGAAG 1740
 L R E P M R W Q A G A P Q R G D S R W K
 CGGTACAGCGTGAAGCAGGCGCGGAGTGTGCTGCGAGCGGAGCGGATCGCGCGAT 1800
 P Y S V K Q G G E V S V Q A E Q D R P D
 TCGCTCTGAGCTTACCGGAGCTGATCATTCGCGCGTGGAGGTGCGCGGCTCGCG 1860
 S L L S F Y R T L I H W R V E V P A L R
 GACGCGCGCTGCGGCTGCTGATACCGCGCGCGCGGCGGCTGCTGCGCTACGAGCGGTC 1920
 D G L R V V D T G A P A L V A Y E R V
 ACAGCGGAGCGCGGCTGCTGCTGCTGCAACCTCTCGCGGAGCGCGGAGCATGAG 1980
 T A D S R A L V V H N L S G K P A S M K
 CTCGCGCGGAGCGCGGAGGCTTCGCGCGGATCGGCTGCGAGTGGCGCTGGCGG 2040
 L G A D R A K A F A A I R L H S A P G A
 ACGCTGACGAGGCGCGGCTCACCGTGCAGCGTACCGACCGTGTGCTGAGTGTGATG 2100
 T L T E G R L T V P A Y A T V V L Q *
 CGTTGAGGACGCGCGGCTTCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 2160
 CGCGCGGCGAGTGGAGCGCGCGGCGGAGGCTGGAGCATGAGAGATCCAGGTGCG 2200
 CGCGCGGAGCTCACGCTGCAATACCTCGCGCGGAGCTCGCGCGCTTCCTTCGCTTC 2280
 CCAAGGATGGGAAATCGCGCGGCGGAGCTCGGATGATGAGCGCGCGGCGGAGTGTG 2340
 CGCGGAGCATGCTTCGCTGATGCGGAGCTGCTGCTGCGAGTGGCTGCTGCGGAG 2400
 CGCGCGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 2460
 GCGCGCGGCGGAGTGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 2520
 TTACGCGCGCTTCGAGCGCGGATGGCGGCTCGAG 2557

Table 3. Glucose-forming activity of α -amylase from *E. coli* JM109(pCX213)

Substrate*	Relative activity†
Commercial maltose	0.80
Maltose	0.18
Maltotriose	0.02
Maltotetraose	0.75
Maltopentaose	0.75
Maltitol	< 0.01
Maltotriitol	< 0.01
Maltose + maltotriose‡	0.74
Maltose + maltotriose§	1.34
Maltose + maltotriitol§	0.03
Maltitol + maltotriose§	0.56

* Substrate concentration was 1 % (w/v) unless indicated.

† Activity for soluble starch was taken as 100. All values are means of at least triplicate determinations, and all standard errors were less than 5%.

‡ The ratio of maltotriose to maltose was 1:100 (by mol).

§ The ratio of maltotriose or maltotriitol to maltose or maltitol was 1:20 (by mol).

signal peptide (encoded by the ORF at 505) does not agree with the general structure of bacterial signal peptides (Pugsley, 1993), and (c) the N-terminal amino acid sequence of the putative signal peptide, particularly the position of the positively charged residue Arg, which was four to five residues away from the first Met, was quite similar to those of avirulence protein (Parker *et al.*, 1992), endoglucanase (Gough *et al.*, 1990) and extracellular α -amylase (Hu *et al.*, 1992) from *X. campestris*; the sequences Met-(X)₃₋₄-Arg- are in accordance with the general signal peptide structure (Pugsley, 1993). We could not find a promoter-like sequence, suggesting that this gene was linked to others; however, the gene was functional under the *lac* promoter on a pUC plasmid and produced 1.3 U (ml culture)⁻¹, fivefold higher than the productivity of the original strain, *X. campestris* K-11151. The deduced primary sequence of the enzyme showed 30 amino acid residues for the signal peptide and 496 amino acid residues for the mature enzyme. The M_r was calculated to be 54437, which was in good agreement with the result of the SDS-PAGE.

The enzyme had four regions (Nakajima *et al.*, 1986) commonly found in many amylases (marked in Fig. 3). We deduced that D197, E225 and D311 corresponded to the essential residues of Taka-amylase (Matsuura *et al.*,

Fig. 3. Nucleotide sequence of the DNA fragment containing the α -amylase gene. A putative ribosome-binding site and a sequence which agreed with the results of N-terminal sequencing of the purified native and recombinant enzymes are singly and doubly underlined, respectively. Regions 1–4 are the consensus sequences found in amylases (see text).

Fig. 4. HPLC chromatogram of the reaction products from maltose after 6 h (a) and determination of each product (b). Products were analysed by HPLC using 75% (v/v) acetonitrile as eluant. A, Reaction products; B, purified maltose used as substrate; G1 and ●, glucose; G2 and ○, maltose; G3 and ▲, maltotriose; G4 and △, maltotetraose. The peaks marked by an asterisk were due to the buffer and/or enzyme.

1984), D206, E230 and D297, respectively, by the comparison of the primary sequences. The low degree of identity (14%) and the result of dot-matrix analysis (Fig. 6a) showed that the periplasmic α -amylase of *X. campestris* was a different enzyme from the reported extracellular enzyme (Hu *et al.*, 1992). The periplasmic enzyme showed 44% identity to the *Bacillus magisterium* α -amylase (Metz *et al.*, 1988; Fig. 6b), which can also act on starch-related materials, pullulan and CDs (Brumm *et al.*, 1991a), but has a slightly different substrate specificity in that the rate for α -CD is less than 20% of those for β - and γ -CDs, and glycogen is a better substrate than pullulan, amylose and amylopectin. The primary sequence of the periplasmic α -amylase from *X. campestris* K-11151 showed a low degree

Fig. 5. Determination of reaction products from glucose. A reaction mixture containing purified glucose was incubated at 45 °C for 6 h and products were analysed by HPLC. A, Reaction products; B, purified glucose used as substrate. G1, Glucose; G2, maltose; G3, maltotriose. The peaks marked by an asterisk were due to the buffer and/or enzyme.

of identity with cyclodextrinase from *Thermoanaerobacter ethanolicus* (Podkovyrov & Zeikus, 1992) (18%; Fig. 6c) and neopullulanase from *Bacillus stearothermophilus* (Kuriki & Imanaka, 1989) (19%; Fig. 6d), although it has both activities. The latter two enzymes have 48% identity and differ from the general α -amylase in having an additional domain, as indicated by Jaspersen *et al.* (1991). The enzyme showed 13%, 10% and 6% identity, respectively, to Taka-amylase (Toda *et al.*, 1982), *Bacillus subtilis* α -amylase (Yamazaki *et al.*, 1983) and porcine pancreatic α -amylase (Pasero *et al.*, 1986), and similar values were found to most other α -amylases.

In a previous paper, we mentioned that the enzyme has the ability to degrade maltose to glucose (Abe *et al.*, 1994). However, the purified enzyme was found to have a rather low activity on purified maltose (Table 3). In this study, we clearly show that the main pathway of maltose degradation by the enzyme is the hydrolysis of maltotetraose to maltotriose and glucose after condensation of two maltose molecules into a maltotetraose molecule (Fig. 4). It was noted that the condensation product was neither 6¹- nor 6²-maltosyl-maltose, but maltotetraose. Thus, the enzyme could form a 1,4- α -glucosidic linkage via a condensation reaction. This type of condensation was the same as in the case of the *B. megaterium* α -amylase (Brumm *et al.*, 1991b).

The reconstituted commercial maltose, which was prepared by mixing 99 parts purified maltose and 1 part purified maltotriose (by mol), gave almost the same hydrolysis rate as that for commercial maltose (Table 3). Moreover, the addition of maltotriose (1:20 maltotriose to maltose, by mol) greatly stimulated the release of glucose. This suggests that the rather high activity of the

Fig. 6. Dot-matrix analyses of primary sequences of amylases. (a) *X. campestris* K-11151 periplasmic α -amylase (XCPAMY) versus *X. campestris* extracellular α -amylase (Hu et al., 1992); (b) XCPAMY versus *B. megaterium* α -amylase (Metz et al., 1988); (c) XCPAMY versus *T. ethanolicus* cyclodextrinase (Podkovyrov & Zeikus, 1992); (d) XCPAMY versus *B. stearothermophilus* neopullulanase (Kuriki & Imanaka, 1989).

enzyme for the commercial maltose was due to the stimulation of the condensation by the low amount of maltotriose, followed by the release of glucose from maltopentaose. As shown in Table 3, the enzyme has the ability to produce glucose from this sugar. When maltotriitol instead of maltotriose was added to maltose, glucose production decreased drastically, while stimulation was still observed when the reaction mixture contained maltitol and maltotriose (20:1, by mol; Table 3). These results clearly show that maltotriose and maltose are predominantly accommodated at the positions for a donor and an acceptor, respectively, at the active site during condensation of the mixed substrate and the binding affinity of maltotriose to the former site is very strong. Maltotriitol also binds to the donor site, and therefore glucose production from the mixture containing this substrate was reduced. Thus, the substrate-binding site of the enzyme which accommodates the non-reducing terminal glucosyl residue of maltotriose and maltotriitol appears to have very high affinity. Considering both the condensation ability of this enzyme and the cross-contamination of oligosaccharide preparations, we may need to reevaluate thoroughly the action of this enzyme on oligosaccharides in a future study.

During the reaction on maltose, condensation occurs to form maltotetraose and possibly higher maltooligosaccharides; those oligosaccharides and breakdown products from them must then serve as donors or receptors

for further condensation or possible transglucosidation reactions. Thus, the total reaction of glucose formation from maltose could be very difficult to resolve clearly.

In these experiments, we could not identify whether a mechanism for direct hydrolysis of maltose is present; however, we could detect the formation of maltose and maltotriose as products of the reverse action using purified glucose as substrate. This suggests that the enzyme has a possible function in direct maltose breakdown, although the rate would be very low.

ACKNOWLEDGEMENTS

This work was supported in part by Grants-in-Aid for Cooperative Research (A) (no. 05303010) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

- Abe, J., Hizukuri, S., Koizumi, K., Kubota, Y. & Utamura, T. (1988). Enzymic syntheses of doubly-branched cyclomaltoheptaose through the reverse action of *Pseudomonas* isoamylase. *Carbohydr Res* **176**, 87–95.
- Abe, J., Onitsuka, N., Nakano, T., Shibata, Y., Hizukuri, S. & Entani, E. (1994). Purification and characterization of periplasmic α -amylase from *Xanthomonas campestris* K-11151. *J Bacteriol* **176**, 3584–3588.
- Brumm, P. J., Hebeda, R. E. & Teague, W. M. (1991a). Purification and characterization of the commercialized, cloned *Bacillus mega-*

- terium* α -amylase. Part I. Purification and hydrolytic properties. *Starch Staerke* **43**, 315–319.
- Brumm, P. J., Hebeda, R. E. & Teague, W. M. (1991b).** Purification and characterization of the commercialized, cloned *Bacillus megaterium* α -amylase. Part II. Transferase properties. *Starch Staerke* **43**, 319–323.
- Bush, K. & Sykes, R. B. (1984).** β -Lactamase (penicillinase, cephalosporinase). In *Methods of Enzymatic Analysis*, vol. IV, pp. 280–285. Edited by H. U. Bergmeyer, H. J. Bergmeyer & M. Graßl. Weinheim: Verlag Chemie.
- Davis, B. J. (1964).** Disc electrophoresis. II. Method and application to human serum proteins. *Ann NY Acad Sci* **121**, 404–427.
- Gough, C. L., Dow, J. M., Keen, J., Henrissat, B. & Daniels, M. J. (1990).** Nucleotide sequence of the *engXCA* gene encoding the major endoglucanase of *Xanthomonas campestris* pv. *campestris*. *Gene* **89**, 53–59.
- Hizukuri, S., Takeda, Y., Yasuda, M. & Suzuki, A. (1981).** Multibranched nature of amylose and the action of debranching enzyme. *Carbohydr Res* **94**, 205–213.
- Hu, N. T., Hung, M. N., Huang, A. M., Tsai, H. F., Yang, B. Y., Chow, T. Y. & Tseng, Y. H. (1992).** Molecular cloning, characterization and nucleotide sequence of the gene for secreted α -amylase from *Xanthomonas campestris* pv. *campestris*. *J Gen Microbiol* **138**, 1647–1655.
- Jaspersen, H. M., MacGregor, E. A., Sierks, M. R. & Svensson, B. (1991).** Comparison of the domain-level organization of starch hydrolases and related enzymes. *Biochem J* **280**, 51–55.
- Kuriki, T. & Imanaka, T. (1989).** Nucleotide sequence of the neopullulanase gene from *Bacillus stearothermophilus*. *J Gen Microbiol* **135**, 1521–1528.
- Lansky, S., Kooi, M. & Shhoch, T. J. (1949).** Properties of the fractions and linear subfractions from various starches. *J Am Chem Soc* **71**, 4066–4075.
- Matsuura, Y., Kusunoki, M., Harada, W. & Kakudo, M. (1984).** Structure and possible catalytic residues of Taka-amylase A. *J Biochem* **95**, 697–702.
- Metz, R. J., Allen, L. N., Cao, T. M. & Zeman, N. W. (1988).** Nucleotide sequence of an amylase gene from *Bacillus megaterium*. *Nucleic Acids Res* **16**, 5203.
- Nakajima, R., Imanaka, T. & Aiba, S. (1986).** Comparison of amino acid sequences of eleven different α -amylases. *Appl Microbiol Biotechnol* **23**, 355–360.
- Parker, J. E., Barber, C. E., Mi-Jiao, F. & Daniels, M. J. (1992).** Interaction of *Xanthomonas campestris* with *Arabidopsis thaliana*: characterization of a gene from *Xanthomonas campestris* pv. *raphani* that confers avirulence to most *A. thaliana* accessions. *Mol Plant-Microbe Interact* **6**, 216–224.
- Pasero, L., Mazzei-Pierron, Y., Abadie, B., Chicheportiche, Y. & Marchis-Mouren, G. (1986).** Complete amino acid sequence and location of the five disulfide bridges in porcine pancreatic α -amylase. *Biochim Biophys Acta* **869**, 147–157.
- Podkovyrov, S. M. & Zeikus, J. G. (1992).** Structure of the gene encoding cyclomaltodextrinase from *Clostridium thermohydrosulfuricum* 39E and characterization of the enzyme purified from *Escherichia coli*. *J Bacteriol* **174**, 5400–5405.
- Pugsley, A. P. (1993).** The complete general secretory pathway in Gram-negative bacteria. *Microbiol Rev* **57**, 50–108.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Smith, A. F. (1983).** Malate to oxaloacetate reaction. In *Methods of Enzymatic Analysis*, vol. III, pp. 166–171. Edited by H. U. Bergmeyer, H. J. Bergmeyer & M. Graßl. Weinheim: Verlag Chemie.
- Toda, H., Kondo, K. & Narita, K. (1982).** The complete amino acid sequence of Taka-amylase A. *Proc Jpn Acad* **58B**, 208–212.
- Yamazaki, H., Ohmura, K., Nakayama, A., Takeichi, Y., Otozai, K., Yamasaki, M., Tamura, G. & Yamane, K. (1983).** α -Amylase genes (*amyR2* and *amyE*(+)) from an α -amylase-hyperproducing *Bacillus subtilis* strain: molecular cloning and nucleotide sequences. *J Bacteriol* **156**, 327–337.
- Weber, K. & Osborn, M. (1969).** The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J Biol Chem* **244**, 4406–4412.

Received 13 November 1995; revised 3 January 1996; accepted 15 January 1996.