Cloning and sequencing of a gene encoding acidophilic amylase from *Bacillus acidocaldarius*

TEIJA T. KOIVULA,¹ HARRI HEMILÄ,² RAIMO PAKKANEN,³ MERVİ SİBAKOV⁴ and ILKKA PALVA^{5*}

^{1,2}Departments of Genetics¹ and Public Health,² University of Helsinki, Helsinki, Finland

³Valio Bioproducts Ltd, Turku, Finland

⁴Valio Ltd, Helsinki, Finland

⁵Agricultural Research Center, Jokioinen 31600, Finland

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Two starch-degrading enzymes produced by *Bacillus acidocaldarius* (renamed as *Alicyclobacillus acidocaldarius*) were identified. According to SDS-PAGE, the apparent molecular masses of the enzymes were 90 and 160 kDa. Eight peptide fragments and the N-terminal end of the 90 kDa polypeptide were sequenced. An oligonucleotide, based on the amino acid sequence of a peptide fragment of the 90 kDa protein, was used to screen a λ gt10 bank of *B. acidocaldarius*, and the region encoding the 90 kDa protein was cloned. Unexpectedly, the ORF continued upstream of the N terminus of the 90 kDa protein. The entire ORF was 1301 amino acids (aa) long (calculated molecular mass 140 kDa) and it was preceded by a putative ribosomal binding site and a promoter. Computer analysis showed that the 1301 aa protein was closely related to an α -amylase-pullulanase of *Clostridium thermohydrosulfuricum*. We suggest that the starch-degrading 160 kDa protein of *B. acidocaldarius* is an α -amylase-pullulanase, and the 90 kDa protein is a cleavage product of the 160 kDa protein. Another ORF, apparently in the same transcription unit, was found downstream from the amylase gene. It encoded a protein that was closely related to the maltose-binding protein of *Escherichia coli*.

Introduction

Many *Bacillus* spp. produce starch-hydrolysing enzymes, including α -amylases which are of great importance industrially (Vihinen & Mäntsälä, 1989). α -Amylases hydrolyse the internal α -1,4-glucosidic linkages of starch at random. Usually, the α -1,6-linkages are not hydrolysed by the α -amylases, but the enzymes can bypass the α -1,6-linkages and produce branched dextrans in addition to linear oligosaccharides as end-products. However, certain α -amylases can also hydrolyse α -1,6-glucosidic linkages (Sakano *et al.*, 1985).

Bacillus acidocaldarius (renamed as *Alicyclobacillus acidocaldarius*; Wisotzkey *et al.*, 1992) grows in acidic and hot conditions (60–70 °C) and therefore it is a potential source of exoenzymes which are active at low pH and/or high temperature. *B. acidocaldarius* ATCC 27009 produces a starch-degrading enzyme which

has been partially characterized. It is a thermoacidophilic endoamylase with a pH optimum of 4.5 and temperature optimum of 60–63 °C (Boyer *et al.*, 1979). The enzyme is not released to growth medium, but has been extracted from plate cultures (Boyer *et al.*, 1979). Three other thermophilic and acidophilic starch-degrading enzymes have been characterized from *Bacillus* spp. These enzymes have been isolated from *B. acidocaldarius* strains Agnano 101 (Buonocore *et al.*, 1976) and A-2 (Kanno, 1986), and from *Bacillus* sp. 11-1S (Uchino, 1982). The pH optima of the three enzymes ranged from 2.0 to 3.5, temperature optima between 70 and 75 °C and apparent molecular masses from 54 to 68 kDa. The molecular mass of the *B. acidocaldarius* ATCC 27009 amylase has not been determined. Thus far none of the genes encoding these four proteins have been cloned and sequenced. Essentially, all industrially important bacterial α -amylases have their pH optima close to neutral. However, some processes (e.g. silage treatment) require enzymes that are active at low pH and therefore there is also a need for acidotolerant enzymes. In this paper we describe the cloning and characterization of the gene encoding the thermoacidophilic amylase of *B. acidocaldarius*.

* Author for correspondence. Tel. 16 88270; fax 16 84550.

The nucleotide sequence data reported in this paper have been submitted to the EMBL and assigned the accession number X62835.

Methods

Bacterial strains, plasmids and culture conditions. *B. acidocaldarius* ATCC 27009 was obtained from the American Type Culture Collection (Rockville, MD, USA). The culture was grown on solid medium for enzyme preparation, and in liquid medium for DNA isolation (Darland & Brock, 1971). *Escherichia coli* DH5 α F' was used as a cloning host for M13mp18, M13mp19, pUC19 and pBR322-based DNA constructions which were made for sequencing. *E. coli* strains were grown in Luria broth supplemented with ampicillin (100 μ g ml⁻¹).

Enzyme assays. The Phadebas amylase test (for food applications, unbuffered; Pharmacia) was used for quantitative amylase activity measurements. The Phadebas test is based on a blue insoluble polysaccharide polymer, which is broken down by amylase thus releasing the blue colour. A plate assay was used for rapid screening of amylase activity. In this assay samples were applied into 4 mm wells made in agar plates containing 0.2% soluble starch (Merck), 20 mM-CaCl₂, 50 mM-Na acetate (pH 5.0) and 1.5% (w/v) agar (Difco). The plates were incubated at 55 °C for 2–10 h and starch degradation was detected by spreading 10 mM-KI/I₂ solution on the plates. Zymography was used for the detection of amylase activity in SDS-polyacrylamide gels (Laemmli, 1970) as follows. After SDS-PAGE, the gel was washed in 50 mM-Na acetate (pH 5.0) for 15 min and laid onto a starch-agar plate. The plate was incubated at 55 °C for 10 h and the amylase activity was detected by staining the plate with 10 mM-KI/I₂ solution.

The pullulanase assay was based on the Somogyi–Nelson method (Nelson, 1944; Somogyi, 1952) that detects the quantity of reducing sugars released during the hydrolysis of pullulan. The reaction mixture contained 0.5% pullulan (Sigma) in 0.15 M-Na acetate buffer (pH 5.0) and 5 mM-CaCl₂. The reaction was allowed to proceed at 55 °C except in the case of temperature optimum determination where the reaction temperature varied from 25 to 75 °C. The pullulan plate assay was used for rapid screening of pullulanase activity. The pullulan plates were similar to the starch plates but 0.2% pullulan was added instead of starch. Samples were applied onto the pullulan plates and incubated at 55 °C for 18 h. Pullulan degradation was detected by spreading the plates with 94% (v/v) ethanol, followed by incubation at 4 °C for 2–4 h. Transparent halos around the holes, against the white background, indicated pullulan hydrolysis.

Purification of the enzyme and determination of peptide sequences. Plate cultures of *B. acidocaldarius* were suspended in 0.5 M-Na acetate (pH 5.0), incubated for 30 min at 4 °C, and the cells were removed by centrifugation at 8000 *g* for 20 min. The supernatant was centrifuged again at 40000 *g* for 30 min and proteins of the cleared medium were precipitated at 0 °C for 30 min by slow addition of (NH₄)₂SO₄ to a final concentration of 70% (w/v). The precipitate was collected by centrifugation at 10000 *g* for 20 min. The pellet was dissolved in 20 mM-Bis-Tris (pH 5.8), applied onto a Bio-Gel P-200 (Sigma) column (1.5 × 45 cm), and eluted with the same buffer. Fractions containing amylase activity were pooled and concentrated by ultrafiltration in a Novacell–Omegacell apparatus (Filtron). The concentrate was rechromatographed in a Superose 12 HR 10/30 column (Pharmacia, Sweden) in 20 mM-Bis-Tris (pH 5.8). Fractions containing amylase were concentrated as above. The proteins were further separated by 10% SDS-PAGE. The gel was treated with 1 M-KCl to visualize protein bands and the band corresponding to the amylase activity was excised. The protein was electroeluted from the gel using an ISCO model 1750 electrophoretic concentrator as described elsewhere (Kalkkinen, 1986). The eluate was freeze-dried and the solid material was dissolved in 50 mM-Tris/HCl (pH 9.0). Lysylendopeptidase (Wako) was added to a final concentration of 3 μ g ml⁻¹ and the mixture was incubated at 30 °C for 18 h. The resulting peptides were separated by reverse phase chromatography on a Vydac 218 TPB5 (0.46 × 15 cm) column

connected to a Varian 5000 liquid chromatograph. The peptides were eluted using a linear gradient of acetonitrile (0–60% in 90 min) in 0.1% trifluoroacetic acid.

For N-terminal amino acid sequence analysis, amylase was transferred electrophoretically onto a polyvinylidene difluoride membrane (Mozdzanowski & Speicher, 1990) and sequenced in a gas/pulsed-liquid sequencer (Kalkkinen & Tilgmann, 1988). The purified peptides from the lysylendopeptidase digestion were applied on polybrene pretreated fibreglass filters and sequenced.

Determination of the isoelectric point. Chromatofocusing was performed using Mono P 5/20 column according to the manufacturer's instructions (Pharmacia). α -Amylase activity and pH of each fraction was measured. Isoelectric focusing was done on 1% agarose gels containing pH 3–10 ampholyte (Pharmacia). The enzyme activity was detected by zymography. In isoelectric focusing, standard proteins (pI range 3.5–9.3) by Pharmacia were used. For isoelectric point determinations the enzyme was eluted from the *B. acidocaldarius* cells by Na acetate and precipitated by (NH₄)₂SO₄ (see above).

Determination of pH optimum. Enzyme activity of the Na acetate-eluted proteins of *B. acidocaldarius* cultures was measured using the Phadebas amylase test at 37 and 60 °C in 0.1 M-citric acid, 0.2 M-Na₂HPO₄. The pH range was 2–7 in steps of 1 pH unit.

DNA methods and sequence analysis. The λ gt10 gene bank of *B. acidocaldarius* ATCC 27009 chromosomal DNA was constructed as follows. Chromosomal DNA from *B. acidocaldarius* was isolated according to Marmur (1961). The DNA was digested partially with *Hae*III. DNA fragments of 5.0 to 6.5 kb, collected from an agarose gel, were methylated with *Eco*RI methylase and ligated to *Eco*RI linkers, and inserted into *Eco*RI-digested λ gt10 using Amersham cDNA cloning system according to the instructions of the supplier.

Hybridizations were done as described previously (Koivula *et al.*, 1991). Sequencing was performed by the method of Sanger *et al.* (1977) and both strands were sequenced. The oligonucleotides (Table 1) were synthesized with an Applied Biosystems DNA-synthesizer Model 381A. Plasmid purifications, DNA digestions and other DNA manipulations were performed according to established methods (Sambrook *et al.*, 1989).

The nucleotide sequences derived from sequencing gels were assembled with the ASSEMBLER program of the PCGENE sequence analysis package (Intelligenetics). Protein sequence databases were searched with the FASTA program (Pearson & Lipman, 1988). Amino acid sequences were aligned with the BESTFIT program of the GCG package (Devereux *et al.*, 1984).

Results and Discussion

Characterization and purification of the thermoacidophilic amylase of B. acidocaldarius

Previously, no amylase activity had been observed in culture supernatant when *B. acidocaldarius* was grown in liquid culture, but amylase activity could be released from cells grown on plates by washing with 0.5 M-Na acetate buffer (pH 5.0) (Boyer *et al.*, 1979). This suggests that the protein is associated with the cytoplasmic membrane or cell wall at least under the growth conditions tested. We also isolated amylase activity by washing cells grown on plates. The proteins eluted from *B. acidocaldarius* cells by 0.5 M-Na acetate were precipitated with (NH₄)₂SO₄, subjected to SDS-PAGE and

Fig. 1. SDS-PAGE of the $(\text{NH}_4)_2\text{SO}_4$ -precipitated proteins. Lanes 1 and 2 represent a Coomassie Brilliant Blue-stained gel and zymogram, respectively. The arrows indicate the positions of the bands corresponding to amylase activity. The sizes of the molecular mass markers (Pharmacia) are indicated on the left.

analysed by zymography. This assay revealed two bands with apparent molecular masses of 90 and 160 kDa (Fig. 1). The same bands of starch degradation were observed when SDS-PAGE was performed under non-reducing conditions (data not shown). Therefore, the 160 kDa form was apparently not a disulphide-linked dimer of the 90 kDa form. The amount of protein in the 90 kDa and 160 kDa bands was approximately the same, yet the starch-degrading activity of the 160 kDa protein was much higher (Fig. 1). Thus, the 160 kDa form appears to have a much higher specific activity, assuming that both proteins are refolded with similar efficiency. Several attempts to separate the proteins by chromatography failed due to low expression levels and poor recoveries of the proteins. In chromatofocusing and isoelectric focusing followed by zymography, the 90 kDa and 160 kDa polypeptides were detected as a broad peak and a band, respectively, at pI 4.8 (data not shown). The measurement of amylase activity of the cell wash medium at different pH gave a symmetric curve with a relatively sharp optimum at approximately pH 4.8 (at 60 °C). The optimal pH reported by Boyer *et al.* (1979) was 4.5. The amylase activity was fourfold higher at 60 °C than at 37 °C. According to Boyer *et al.* (1979) the optimal temperature for the amylase was 60–63 °C.

The relationship between the 90 kDa and 160 kDa amylases was not clear. The results obtained by chromatofocusing and isoelectric focusing are consistent with the possibility that the two amylases may either be closely related or the smaller amylase could be a breakdown product of the larger. The molecular mass of the *B. acidocaldarius* ATCC 27009 amylase had not been determined previously but the protein was retained by a 30 kDa cut-off membrane (Boyer *et al.*, 1979). The

molecular mass of the amylases from two other *B. acidocaldarius* strains has been shown to be approximately 67 kDa (Buonocore *et al.*, 1976; Kanno, 1986).

Peptide sequences from the purified 90 kDa polypeptide

SDS-PAGE was performed to separate the 90 kDa and 160 kDa proteins for N-terminal sequence analysis, but only the 90 kDa protein could be purified in sufficient amounts. The separated protein was electrotransferred onto a polyvinylidene difluoride membrane and sequenced. A single amino acid sequence of Asp-Ile-Asn-Asp-Tyr (DINDY) was obtained. Furthermore, eight peptides cleaved by lysylendopeptidase from the 90 kDa protein were purified and sequenced (Fig. 2). The sequences of the peptides were determined to design oligonucleotide probes for the cloning of the gene.

Cloning of the amylase-encoding region

The amylase-encoding region was cloned as two fragments: C fragment and N fragment (Fig. 3). First, the C fragment was cloned from a λ gt10 gene bank of *B. acidocaldarius* chromosomal DNA by plaque hybridization. The oligonucleotide 371 (Table 1) was synthesized according to the deduced DNA sequence of one of the lysylendopeptidase-cleaved peptides (Table 1, Fig. 2). It hybridized with six plaques out of 7000 screened. Restriction enzyme digestions of the DNA revealed that the inserts of four clones were of the same size (4.3 kb). One of the cloned regions was sequenced from M13-based constructs. According to the sequence, the cloned region (C fragment) was 4.2 kb long and contained an ORF of 1177 amino acids (aa) with no complete N terminus (Figs 2 and 3). In the middle of the fragment the amino acid sequence DINDY, the N terminus of the 90 kDa protein, could be recognized (bp 4692, Fig. 2). The size of the polypeptide, deduced from the DNA sequence, starting with DINDY is 92 kDa; hereafter the smaller amylase is designated the 92 kDa amylase. However, the ORF continued to the 5' end of the C fragment, which indicated that the 92 kDa amylase is most probably a cleavage product of a larger protein.

The N fragment was thereafter cloned from the λ bank. One clone out of 6000 was found to hybridize with oligonucleotide 393, but not with oligonucleotide 395 (Table 1). This clone contained two *Eco*RI fragments, 3.5 and 1.1 kb in size. The 1.1 kb fragment was part of the C fragment but the 3.5 kb fragment was not (cf. Fig. 3). The nucleotide sequence of the 3.5 kb fragment (N fragment) was determined. According to the sequence, the N fragment was 3.7 kb long and contained a short ORF of 123 codons at the 3' end. The reading frame of

CAAGGGTGGGGAAGGGAACAGGGTCTTGAGAGAAAAGAAAGGATTCGGCAATGAACGATGCAAGAGCACCATTTGACGTCGGGAAGGCTGAGCGTATAATCCAGCAGCACAAGCTTAGTGCAATCGTTTGCAACAGGAGGTTGATGC 3300
M 1

ACGTGAAGTGACGGGTGCCGGAGCCTTGCGACGACCATGTCATGCTGTCAGCGCTTCGGTTCGCGCGCTTTGAAAACACCGCTTCGGTCGGCAGCGAACCGCTTGCGCGCGCAGCGCGCGGATAGTACTAACCGCTGCCCGCGA 3450
H V K W T G A A S L A T T I V M S A A L P F A A A F E N T R S V G T E R V L A A T A A D S T N A S P P 51

CCGGGCGCTCGCAGCGGTGGCAGCAGGAGAGACTCTCGAATGCCGTGCAGATTGTGACGGCAATGGCTTCGCGCTGGAACAGGGGCCACGGCTCCTCGCTCGTGAATGGACTGAACCTCGAGTTTCAAGGTTG 3600
T G A S A G G G T A G E T Y S N A V Q I Y S G N G F R A G N G A T V L A V N G L N L N P S S F K V 101

TGGTATCGAATAGCCTCAACGGCGTGATCGAGCTCACAAGTGATTCGGTCTAACGGGCGCAATTCGCTGCTTCGACCTTCTCGCGGGGATGACGGGCTTGGCGCGGATACGTCACCGCTCACCGCTCGAAAGTGGAGTGATTCGG 3750
V V S N S L N G V I D V T S D S V L T G P N S I A L H L P A G D D G L G A G T Y T V T V E S G G D S 151

TCTCGACGCTTCAGGACGGGATTCAGGTGATGCTTATACGACGGCGACCATTCAGTGGGATGGAATACACATCCGATGGCGGATGTACGTGTGAGATCCGATCCCTCCCTGGACAAGAGGATGACGATCAGGCTTCGAG 3900
V S T P S G G L Q V M P Y T T A D T I Q W D G I Y T S D G A M Y V S D P M P S P G Q E V T I S L R 201

CGTACAGCGGGAACCTGACGAAGTGATCTGAATGCTGGGACACCGCAACAAAGGGTTCGAGTCGAGATGCTCCAGCAAGACGTTTGGACGCTTACGCTTGCTGCGCAGATCCCGCTTCAACCGCGGGAACGATCT 405
A Y S G N L T K V I L N C W D T A Q N K G F Q V E H S P G R T G G P Y Q L W S A T I P A S N G G T I 251

ATTATCGCTTTGACATATACGATGGCACCAGTTTTCGCTGCTCTCAGGTGACGGACTGCACAGCTGTACGACATCAACAACATTCCTGCTGCCGTGGGACGGCTCAGCTTTCGACACTTCAGCGGAATCCCGGTGATACGTTGA 4200
Y Y R F D I Y D G T T G A C T S F A C L S D C G L H T S D D I N M N F P L P V G T V T L S T L Q A N P S D T V 301

CGGTCTCCGACCTGTAGTGACTTCGCGGGAAGGACAGTCAACCGAATCAGCGGTGATCGGTTTCTCAACTGCTGGGCGGAACCGCGCCACAGTCAATGGGAGAACCGGAGCTGGAACGCTGCAAGTTCAGGTCCCAAG 4350
T V S D P V G D F A G S Q Q D Q P N H T V I R F V N S S G E T A A T V N G T M A S W N S V Q F T V P Q 351

GCCTTCCAAAGCGCTGTATCGCGTCGAGTGCACAGCGTCCCAAGBAGCGGATGGGGTGTCAATGTCGAAATGGACAGGATGCGGAGCTTATTGAGGCGCTCTGCCCGCGGATGCAACGCTATGCATGATTCGTTTCAGG 4500
S L P N G G L Y R V E I D T V A K D A D G V V N V E L D R S A E L I V G P L P A M H Q A Y A H D S F Q 401

CGTTCTACCGATCGCTTTCGGAAGCGTGTCCACGGAACCCCATCACGCTTCGCTCGCGGTCGCTCAGCGTGAAGAGTGCAGCGCTTCGCTCTGGGGGACGAGGATCAGTCAGCGGAGATCGACCTGCCGATGCAGAGCTCC 4650
A F Y R S P P F G A V S T G T P T I T L R A P L S V K S A T L R L W G A A D Q S G E I D L P M Q K L 451

AAATGTCGGGAGCAGGTTGGCGCAACAAACCGGCTGCAGGACATCAACGACTACACGTTGGAGCGTGACCATCCCTCGCGCGGATGTGACCAACCGGAGCATGTGGTATCAGTTCGTGACGGAGACGGACACTGGCCAGGTGG 4800
Q M S G D E L A Q Q G T G V Q Q D J N D Y T M W T V T I P A A D V T T P G T M W Y Q A F C V T E T D T G Q V 501

TCTACTACGATGACAAATGGAGTCAAGTGGCGCTGGCCAGGTGGGTGTCTTCGCGGACCGGAGCTACCAGTACGCTGATACGAAACGGGATTCAGACGCGGATTCGAGCGGAGTGGCTGAAGCAGCCCGTGATCGAAATACGCCGG 4950
V Y Y D D N G A Q L E G P G Q V G L S D S G P S Y Q I S V Y E R G F Q T P D W L K H A V I Y E I M P 551

ATCGGTTCTCAATAGGCAATATCGCCACGGAGGAGAATCGAATACGCAAAAGGGGATTTATGTAGGGGCGGATGGAACGGAGTCATTAGGCCCATCCAGTTCCACGAGAACTGGGACTCGCGGCCCTATGATCGAATATCTCCGT 5100
D R F Y N G M I A T E E N P N T Q K G I Y V G A D G T E S L G P I Q F H E N W S P P Y D P N I P 601

TATCTGATCCCAAATTCGCAATTCGCGAAGCAATGGCAATGAACTTTGACTTTTCGGAAGTGATTCGAGGCACTCGAGGATAAGCTGGACTACCTGAAGAGCTTTGGAGTCAATACGCTGTATCTGATGCCGCTCTTGAGGCGG 5250
L S D P K I A S L R G N G Q W M I D F F G G D L T G C I E D K L D Y L K S L G V N T L Y L M P V F E A 651

AATCCAATCACAATATGACACGCGCATTTTCAAGATTGACCTGGATTGGAACGACGAGGACTGGCTGAATCTGTACAGGCTGCGCACCGGAAAGGGGTTCCATATCATCTCGACGGGGTGTTCGAAGATACCGGAATGACA 5400
E S N H K Y D T A D T Y F K I D P G F G T G Q D W L N L V Q A A H A K G F H I I L D G V F E D T G S D 701

CGGTATATTCAACAAGTTCGGAACCTTCGCAACGCTGCGTGGCAGCGGTACCTGAAGAACCGCGCTGCTGCGCCCTACTACTCGTGGTACGTTGGAGCGGGAACCACTCAAAACCCATCGGATTCGTTTCAGATCGACA 5550
S V Y F N K F G N F H S N G A W Q A Y L K N Q P S L S P Y S W Y W T G N T S N P Y D S W F Q I D 751

CGCTGCCACTACGGACAGTGAACCCCGCTATCAGCGATTGCTGATGGGAGGACAACTCAGTCGCGCGTGTGGATCCGGGAAGTTCGGGAGGATGGCGCTTGGACTCGGCGGACCAACGGGAATTTCAACACGGCATGGTGG 5700
T L P L T D T S N P A Y Q R F V Y G S D N S V A R V W I R E G A D G W R L D S A D N G N F N T A W W 801

GTGGCTTTCGCGAGCGGTGAATCGATCGATCCCAAGCAGCGATCATCGCGGAGATTCGGGACATCGGACGAATGACAACTGGAACGGATGGTTGACGGGATCGAECTTCGACAGTGAATGAGTACCGATCCGATCCGGAAGCGCGTGA 5850
G G F R Q A V K S I D P N A A I I G E I W D N A T N D N G T D W L T G S T F D S V M N Y Q R F N A V 851

TCGACTTCTTCGCGGCACTGACACGAGGAAAGCTGACGACCCAGCGCTCGAGCTGCGGATTCACACGGAACATGATGCGCTGTACAGTGAATATCCTTCGAGTGTCTACTCGATGATGAACCTTGTGATTCGGAAGACA 6000
I D F F G G T Y N D G N V Q H H A D A A G F N Q C G E L M R L Y S E Y T P L Q S F Y S M M N L V D S Q Q D 901

CCATCGGAGTCTGACCATCTTGGAGAACCGCGGACCGCAGCGGATCTATCCGCGCTCAGCAGGATGAGTACAGCGCGCTCCTCGGCTGAACATGTGGGATCGAGAGGCTGAAGCTTGTATCGGAGTTTCAATTCAGCTTCCCGG 6150
T M R I L T I L E N A P P Q P G D L S A L Q Q D E Y R P S P A A E Q L G I E R L K L V S D F Q F S F P 951

CGCATCCGACCATCTTCAAGCGGACGAGGCGGCTCACTGGTTATTTCGATCCCTCAATCGTCGGAATCTCGTGGGACAACCAAGATTCGATCTCTGAACCACTACCGCAAGCTCGGGGCAATTCGAAACGCCAATCTGTGTC 6300
G D P T I F Y G D E A G L T G T Y S D P L N R R T Y P W D N Q N L D L L N H Y R K L G A I R N A N P V 1001

TTCAGACGGGGATTCACGCCGTTGACGCAAGCGGATGGTGACGCTTTCGAAAGGACCATTCGGAATGGGCGAGATGTCTCGGTTGCGGACGAGGATGCCACGCGCATTCGCGGATCAACAATCAGAACCAAGCTATCACCG 6450
L T Q G D F T P L Y A Q G M V A F A R I R N G R A D V F G V P A E D A T A I V A I N N Q N Q A I T 1051

TGACCATTCGACGGATGGAGCGGTTCGGGACGGTCCACGATGCTGATGAATGAACCAACAGTGGTACAAGTGACAGATGGTGGCATTACACTCAGCTGCAATCGTATCAAGGTGCCATTTGGTGACGCCGAGCAGCGCGGA 6600
V T I P T D G T Y A D G S T M L D E L N N Q W Y K V Q N G G C I T L T L L Q S Y Q G A I L V T P S D A P 1101

TGGCTTATCTGCAAGAGGAGGATTCAGAGCAGGATTCGTCAGGCGCTGCAAGGTGCTGCAAGTATCGCTTCGGGACGAGATTCGGAATGGACAATGGTGCCCTTTGAGCTGTGCTTCTGCGCAGGACTTGAGTGTCACGG 6750
M A Y L Q E E D S Q N E I A W T P V Q G A T I G Y R V R W R Q N P N G Q W V P F G G P V L P A T D L S V T 1151

TGGAACCGGATGCATATGCGCAACGTTTGTGTACAAGCGCTGTTTTCGCGCTGTGATCAGCGCCAGTCTCCGGTGTGCGCACCTAAGACGGATTCGCTTCCCGTGAATGGCCGCGGATGAGTCAAGCGGATTCGTAGTGGT 6900
V E R D Y A Q T F A V Q L F S A S D H A Q S P V S A P K T V S T S L A V D V P A V R L S Q P I V S G 1201

GTGTTGGAGATCGATGGTCTCGATCAGCGCGTTTCAGGCGGACGAGTATGTGATACCAAGACGAGGCGCAGGATCGTATGCTCCGTTGCGCAGCGCTCCCAAGTGGCGATTCGCGACGCTATAGGGGAGTTCG 7050
R V V G D R A M V S I T P V S G A T Q Y V I Y Q R Q G D G S Y A P V A T V S T S A G S A A I G E V P 1251

CGCAAGGTCGCGCAACTCGCTTACGCGAGGATTCGCGTGACAGTGGCGTACCTGCAAGTTTCTCGTGGTGAGCTACCGGTGGCTGCGCAAAACGAAGTGGGCAAGCTGTGACCAATCCATTGACCTATCGCTCTGAAAAAGT 7200
A Q G P A N S P H A T I R V T V P V P A G F S S V T Y R V A A Q N E D G Q A V T N P L T L S L S K K 1301

GATCGCTCGGCAAAAGGGCTATCGGAATTTTTCGAGATAACATCTTGATTGCGATCGATGAAAGCGTTATATACTACCGCAACAGGTTTGGCGCAAGCAATGCACAGAGGGGTTGTGTCGTTGAGGAGTGGGAATCGATCGACA 7350
M G N R I D 6

GGTGTGGCGCGCTCGTGTGGCGGCGGACAGTCGCGGCTGTGGAACCTCGAAGCGTGTGCAAAACAGCTCTCCTTCACGAGCTCGTGAGCGCAAAAGGGGAGGCAAGCGCTCTGCCAAAGGCGAGCATTACCGGTTTGGTGGT 7500
R C G R A R V G G R T V A G C G G T S N G G Q N T S P S T S S S A K G E A S A L P K G E T I T V W S 56

GGCAGCAGGCGCGGAT

Fig. 2. Nucleotide sequence and the deduced amino acid sequence of the *B. acidocaldarius* amylase gene. The putative promoter, initiation codon and termination codon are underlined. The RBSs are indicated by double underlining. The deduced amino acid sequence is shown below the nucleotide sequence. Stretches of the amylase that were determined by peptide sequencing are underlined; in the case of an undefined aa in the peptide sequence, a short gap in the line is left under the residue. The putative signal sequence cleavage sites of the amylase are indicated with arrowheads. The *EcoRI* site, at bp 3662, marking the junction of the N and C fragments, and the 16 bp palindrome upstream of ORF2, are marked by a line above the sequence.

Fig. 3. The structure of the cloned region. The *EcoRI* site (E) is located at the junction of the N and C fragments. The 1.1 kb fragment adjacent to the N fragment is indicated by a dotted line. The major ORFs are indicated with arrows. Restriction enzyme sites used in Southern blotting are indicated: B, *Bam*HI; P, *Pst*I; Bg, *Bgl*II; H, *Hind*III.

the ORF matched the reading frame of the 1177 aa ORF of the C fragment (Figs 2 and 3).

Two approaches were used to verify that the separately cloned N and C fragments were colinear in the *B. acidocaldarius* chromosome and that they had been cloned without rearrangements. First, PCR with oligonucleotides 417 and 436 (Table 1) was used to isolate the region joining the N and C fragments from the chromosome. A fragment of 1 kb was obtained and the

DNA sequence was identical with the previously determined joint region. Second, Southern hybridization was used to confirm that there were no rearrangements within the whole sequenced region (data not shown, for enzymes used in digestions, see Fig. 3).

Nucleotide sequence of the amylase gene

The entire sequenced region was 7872 bp long (Fig. 2). The coding region for the amylase was 3903 bp, corresponding to a protein of 1301 aa (Fig. 2). Four basepairs upstream from the putative translation initiation codon (at bp 3297) a sequence similar to the 3' end of the 16S rRNA of *B. subtilis* was recognized and this could serve as a ribosomal binding site (RBS). Further upstream a putative promoter, with -35 (TTGACG) and -10 (TATAAT) regions, was found.

The molecular mass of the protein encoded by the ORF, 140 kDa, is in reasonable agreement with the size of the larger amylase observed in zymography (160 kDa). Hereafter the protein encoded by the entire ORF is designated the 140 kDa amylase. The amino acid

Table 1. Oligonucleotides used for cloning the *B. acidocaldarius* amylase

Number	Sequence*	Corresponds to:
371	5' CAGTCCTGCTGIGTICCGAAICCGGGTCGAT 3'	IDPGFGTQQD
393	5' CCGGCGCCAAGCCCGTCATCCCCGGCAGG 3'	5' end of the C fragment
395	5' GCTTGTGCCGCAGCCACCGCAGACA 3'	3' end of the C fragment
417	5' GGGTTGATCCTGGCTTC 3'	5' end of the C fragment
436	5' CAAGGGTCTTGAGAGAA 3'	3' end of the N fragment

* I, inosine.

Table 2. Similarity of the *B. acidocaldarius* 140 kDa amylase with certain polysaccharide-degrading enzymes

Enzyme, species	Region in the 140 kDa amylase (aa)	Identical aa in the region (%)	Reference
α -Amylase-pullulanase, <i>C. thermohydrosulfuricum</i>	12-1301	35	Melasniemi <i>et al.</i> (1990)
	619-1031	49	
	619-695	61	
	834-907	61	
Neopullulanase, <i>B. stearothermophilus</i>	397-1097	36	Kuriki & Imanaka (1989)
Maltodextrin glucosidase, <i>E. coli</i>	383-1106	30	Tapio <i>et al.</i> (1991)
Cyclodextrin glucanotransferase, <i>K. pneumoniae</i>	467-1243	26	Binder <i>et al.</i> (1986)
α -Amylase C, <i>D. thermophilum</i>	535-1078	25	Horinouchi <i>et al.</i> (1988)

Fig. 4. Comparison of the *B. acidocaldarius* 140 kDa amylase with selected polysaccharide-degrading enzymes. The matrix was generated with the COMPARE program of GCG sequence analysis package (Devereux *et al.*, 1984). The length of stretches compared (window) was 30 aa, and 15 identical aa in the window (stringency) led to a dot. Vertical axes: *B. acidocaldarius* amylase. Horizontal axes: Apu, α -amylase-pullulanase; Npl, neopullulanase; MalZ, maltodextrin glucosidase; CGT, cyclodextrin glucanotransferase; AmyC, α -amylase C (for references see Table 2). The entire sequences have been compared. The numbers along the axes indicate the residues of the sequences.

sequence of the N terminus from the 92 kDa protein was found within the coding region (at aa 466). It is noteworthy that the eight internal peptides of the 90 kDa amylase were found in this 140 kDa ORF (Fig. 2). There is no putative RBS or promoter upstream from the N terminus of the 92 kDa amylase, which is consistent with the concept that the smaller amylase may be a cleavage product of the larger. The calculated pI of 4.1 for both the 92 and 140 kDa proteins was slightly lower than that obtained in experimental analysis of the two amylases (pI 4.8, see above). There is also a putative signal sequence at the N terminus of the 140 kDa amylase (Fig. 2).

The 140 kDa amylase appears to be α -amylase-pullulanase

The amino acid sequence encoded by the amylase gene was compared with the sequences of the protein database. The search revealed that the 140 kDa protein was similar to several polysaccharide-degrading enzymes (Table 2, Fig. 4). The closest relative was found to be the α -amylase-pullulanase (Apu) of *Clostridium thermohydrosulfuricum* (Melasniemi, 1988; Melasniemi *et al.*, 1990). The region of similarity between these two proteins covers nearly the entire length of both sequences (Table 2, Fig. 4), and there is up to 60% amino acid identity in two stretches of over 70 aa. It is also noteworthy that the size of the *C. thermohydrosulfuricum* Apu is 1475 aa, i.e. quite close to the size of the 140 kDa amylase (1301 aa). The high degree of similarity between these two proteins suggests that the 140 kDa amylase of *B. acidocaldarius* is α -amylase-pullulanase.

When the *B. acidocaldarius* 140 kDa amylase is aligned with the *C. thermohydrosulfuricum* Apu, the homologous

Fig. 5. Comparison of the *B. acidocaldarius* 140 kDa amylase (Amy) with *C. thermohydrosulfuricum* Apu (Melasniemi *et al.*, 1990). The region of *B. acidocaldarius* amylase that covers the N terminus of the 92 kDa amylase is underlined. Identical (()) and closely related (:) amino acids, with a comparison value ≥ 0.5 in the BESTFIT mutation data matrix (Devereux *et al.*, 1984), are indicated.

region continues well beyond the N terminus of the 92 kDa protein (Figs 4 and 5). Furthermore, the exact N terminus of the smaller amylase (DINDY) is located in a small gap of the Apu sequence (Fig. 5). Usually the insertions and deletions are located on the surface of a protein (Siezen *et al.*, 1991) which suggests that the N terminus of the 92 kDa protein may be located in a loop on the surface of the 140 kDa amylase. Therefore this site could be susceptible to some proteases. There are no data available of the proteases of *B. acidocaldarius*, but subtilisin, a common protease in *Bacillus* spp., cleaves, for example, after Gln (Wells & Estell, 1988), which is the residue preceding the N terminus of the 92 kDa protein. Accordingly, the sequence comparison gives further support to the notion that the 92 kDa amylase may be a cleavage product of the 140 kDa amylase.

Part of the 140 kDa protein is similar to several amylases, of which the *Dictyoglomus thermophilum* α -

amylase is the most closely related (Table 2). It is noteworthy that the region of similarity (aa 535–1078) is included in the 92 kDa protein, which also has amylase activity even though the specific activity of the 92 kDa protein is lower than that of the 140 kDa amylase (Fig. 1). Except for the Apu protein, no other proteins in the protein sequence databases were found to be similar to the N-terminal region (aa 1–500) of the *B. acidocaldarius* amylase.

Tests to show pullulanase activity

The observation that the *B. acidocaldarius* 140 kDa amylase was closely related to *C. thermohydrosulfuricum* α -amylase-pullulanase motivated us to study whether *B. acidocaldarius* amylase has pullulanase activity. First, we tested whether the cell wash medium of *B. acidocaldarius* plate cultures contains pullulanase activity. A sample of cell wash medium was incubated with pullulan and reduced sugars were detected by the Somogyi–Nelson method. A blue colour was detected which indicated that pullulan was hydrolysed by components present in the sample.

If the *B. acidocaldarius* amylase is also a pullulanase, both enzyme activities would be expected to have similar dependency on temperature: inactivation of the enzyme would cause concomitant loss of both activities. In a crude preparation the effect of temperature on the pullulanase and amylase activities were found to be similar (Fig. 6).

To show that the pullulanase activity observed in the cell wash medium is associated with the amylase, we made further efforts to purify the enzyme. The cell wash medium was fractionated in a Biogel P-100 (Bio-Rad) gel filtration column in 50 mM-Na acetate buffer (pH 5) and 5 mM-CaCl₂. The pullulan- and starch-degrading activities of the fractions were detected semiquantitatively with pullulanase and amylase plate assays. Both activities were detected in the same fractions (data not shown). These biochemical results are consistent with the concept that the *B. acidocaldarius* amylase also has pullulanase activity, even though the proof is not definite.

The purification of the amylase was hampered by small amounts of the protein. The isolation of the amylase by plate cultures was time-consuming and laborious. Therefore we tested whether the enzyme could be isolated from the supernatant of the liquid culture medium. The liquid culture medium described by Darland & Brock (1971) was supplemented with 3–5% soluble starch (Difco) and 0.25% maltose and the cells were grown in a shaker at 50 °C for 1–2 d. Amylase activity was released into the culture supernatant; however, the yields were comparable to those in the plate cultivation. It is noteworthy that the *C. thermohydro-*

Fig. 6. The effect of temperature on amylase (○) and pullulanase (▲) activity released from *B. acidocaldarius* cells. A reaction mixture without enzyme was used as a blank. For pullulanase, the relative activity of 1.0 corresponds to $A_{500} = 0.56$, and for amylase $A_{620} = 2.58$. The samples were incubated for 4 h (amylase) and 8 h (pullulanase).

Fig. 7. Comparison of the ORF2 product with *E. coli* MalE (Duplay *et al.*, 1984). The signal sequence cleavage site of MalE is indicated by an arrowhead. The alignment shows a score of 12 when the statistical significance of the alignment is evaluated by the PCOMPARE program of PCGENE. A score of 3 SD indicates that the odds for obtaining the alignment by chance are less than 1 in 1000 (Feng *et al.*, 1985). Identical (|) and closely related (:) amino acids are indicated.

sulfuricum Apu is released to the culture medium, or associated with the cell surface, depending on the type of carbon source used in the medium (Melasniemi, 1987). The sequence of the *B. acidocaldarius* amylase does not reveal any membrane-anchoring sequence, suggesting an association with the cell wall rather than hydrophobic attachment with the cytoplasmic membrane.

ORF2 is homologous with the maltose-binding protein of *E. coli*

There is an ORF, ORF2, downstream from the amylase gene (Fig. 2). ORF2 lacks the 3'-terminal region of the putative gene. There are two possible RBSs upstream from the putative initiation codon (ATG at 7332 bp). The protein encoded by ORF2 has significant similarity to MalE, the maltose-binding protein of *E. coli* (Fig. 7). In *E. coli* MalE is a periplasmic protein which partici-

pates in maltose transport (Hengge & Boos, 1983; Spurlino *et al.*, 1991). There is no promoter nor transcription terminator in the region between the amylase gene and ORF2 which suggests that the MalE homologue may belong to the same operon with the amylase gene. *E. coli* MalE contains a signal sequence, whereas there is no typical signal sequence in the N terminus of ORF2. It seems possible that the ORF2 protein is bound to the cell membrane of *B. acidocaldarius* and participates in the transport of maltose into the cell. To our knowledge there are no previous reports indicating the location of a maltose transport component in a same operon with amylase.

An attempt to express the amylase gene in B. subtilis

Our preliminary attempt to express the amylase gene in *B. subtilis* was not successful. The entire gene was assembled for expression into the pHP13 vector (Haima *et al.*, 1987). pHP13 has low copy number in *Bacillus* which should minimize possible instability caused by the long insert and the large protein. The plasmid, which contained the gene for the 140 kDa amylase was transformed to *B. subtilis* BRB152 (Sibakov & Palva, 1984) but no starch-degrading activity was detected. Yet, the plasmid had suffered no obvious rearrangements according to restriction enzyme digestions. The inability of *B. subtilis* to produce the enzyme may be caused by several reasons. For example, the promoter may be non-functional in *B. subtilis*, or the enzyme may fold incorrectly at low temperature (37 °C) and neutral pH (pH 7) used in the cultivation.

Conclusions

The gene encoding an amylase from *B. acidocaldarius* was cloned and the encoded protein was found to be closely related to the α -amylase-pullulanase of *C. thermohydrosulfuricum*. It was concluded from the sequence data, with some support from experimental data, that the cloned gene encodes α -amylase-pullulanase. We have been unable to purify the 140 kDa protein, the product of the cloned gene; yet, all the data are consistent with the concept that the 140 kDa protein is identical with the 160 kDa protein first observed in zymography, and the 92 kDa amylase is a cleavage product of this large protein. The 92 kDa protein appears to have lower amylase activity than the 140 kDa protein.

Note added in proof. The 160 kDa amylase of *B. acidocaldarius* ATCC 27009 has recently been purified. The 160 kDa amylase degrades pullulan with a rate that is approximately 100-fold lower than the rate of degradation of soluble starch (B. Brockschmidt &

E. P. Backer, University of Osnabrück, FRG, personal communication).

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