Nucleotide sequence of the α -amylase-pullulanase gene from *Clostridium thermohydrosulfuricum*

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The nucleotide sequence of the gene (*apu*) encoding the thermostable α -amylase-pullulanase of *Clostridium thermohydrosulfuricum* was determined. An open reading frame of 4425 bp was present. The deduced polypeptide (*M_r* 165600), including a 31 amino acid putative signal sequence, comprised 1475 amino acids, with no cysteine residues. The structural gene was preceded by the consensus promoter sequence TTGACA TATAAT, a putative regulatory sequence and a putative ribosome-binding sequence AAAGGGGG. The codon usage resembled that of *Bacillus* genes. The deduced sequence of the mature *apu* product showed similarities to various amylolytic enzymes, especially the neopullulanase of *Bacillus stearothermophilus*, whereas the signal sequence showed similarity to those of the α -amylases of *B. stearothermophilus* and *B. subtilis*. Three regions thought to be highly conserved in the primary structure of α -amylases could also be distinguished in the *apu* product, two being partly 'duplicated' in this α -1,4/ α -1,6-active enzyme.

Introduction

α -Amylases (EC 3.2.1.1) and pullulanases (EC 3.2.1.41) (Fogarty, 1983) are used industrially in the processing of starch. α -Amylases degrade starch by cleaving its internal α -1,4 linkages and usually produce as final products a mixture consisting of maltose, malto-oligosaccharides and α -limit dextrins, the last containing all the α -1,6 branch sites initially present in the starch. Pullulanases debranch starch by cleaving the α -1,6 linkages in its amylopectin component. When used in combination with a pullulanase, α -amylases leave virtually no limit dextrin, and higher yields of the desired end products can be obtained.

Bacillus subtilis (Takasaki, 1987), *Thermoanaerobium brockii* (Coleman *et al.*, 1987), *Thermoanaerobium* sp. (Plant *et al.*, 1987) *Clostridium thermohydrosulfuricum* (Melasniemi, 1988) and *Bacillus circulans* (Sata *et al.*, 1989), produce amylolytic enzymes having both α -1,4 and α -1,6 activities in the same molecule. The gene for

the α -amylase-pullulanase of *C. thermohydrosulfuricum* was cloned in *Escherichia coli* (Melasniemi & Paloheimo, 1989) as an initial step towards producing the enzyme in an industrially better-suited host organism. Both to test different promoter and signal sequence combinations and to understand better the α -amylase/pullulanase character of the enzyme and the relation between the enzyme and its satellite protein (Melasniemi, 1988; Melasniemi & Paloheimo, 1989), the gene (*apu*) coding for the α -amylase-pullulanase was sequenced as reported in this paper.

Methods

Plasmids and phages. The plasmids pALK351 and pALK353 containing the α -amylase-pullulanase (*apu*) gene on a 7 kb *EcoRI* insert (Melasniemi & Paloheimo, 1989) were used as sources of DNA in the sequence determination. For the preparation of the plasmid DNA, the method of Birnboim & Doly (1979) was used. The phage vectors used for sequencing were M13mp18 and M13mp19 (Norrander *et al.*, 1983).

Bacterial strains and culture conditions. *E. coli* JM109 (Yanisch-Perron *et al.*, 1985) harbouring pALK351 or pALK353 (DSM 5355) were grown in Luria-Bertani medium (Maniatis *et al.*, 1982) containing ampicillin (50 μ g ml⁻¹). *E. coli* JM109 used for the transfection of M13 phage was maintained on M9 plates (Maniatis *et al.*, 1982) containing thiamin (15 μ g ml⁻¹) and grown in Luria-Bertani medium.

DNA sequencing. Single-stranded DNA for sequencing was obtained by the M13 procedure of Messing (1983). The *apu* gene was cloned in M13mp18 and M13mp19 as several restriction fragments using restriction endonucleases *EcoRI*, *BamHI*, *PstI*, *HindIII*, *AccI*, *KpnI*

Abbreviations: *apu*, α -amylase-pullulanase gene of *C. thermohydrosulfuricum*; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M28471.

and *SphI* and single-stranded DNA was purified. The DNA was sequenced by the dideoxy chain-termination method of Sanger *et al.* (1977) using a Sequenase kit (United States Biochemical Corporation) and [α - 35 S]thio-dATP (Amersham). The universal 17-mer primer included in the kit and 17-mer oligonucleotides synthesized by a 381A DNA Synthesizer (Applied Biosystems) were used as primers. The whole sequence was determined in both directions with overlapping partial sequences on both strands. For the analysis of the DNA sequence the MicroGenie computer program (Beckman) was used.

Results and Discussion

Nucleotide sequence and deduced amino acid sequence

In previous experiments the coding and regulatory sequences of the *C. thermohydrosulfuricum* *apu* gene were localized to a 6.2 kb *BamHI*–*EcoRI* DNA fragment (Melasniemi & Paloheimo, 1989). The determined nucleotide sequence of the gene, together with the deduced amino acid sequence, is shown in Fig. 1. Only one long open reading frame was present. It codes for a polypeptide with 1475 amino acids and a calculated M_r of 165 600. This is close to the M_r (165 000) determined by SDS-PAGE for the biggest *apu* polypeptide produced in *E. coli* (Melasniemi & Paloheimo, 1989) and compatible with the M_r (180 000–190 000) observed for the extracellular α -amylase-pullulanase produced by *C. thermohydrosulfuricum* (Melasniemi, 1988), considering its carbohydrate content of 11–12%.

The N-terminal end of the deduced *apu* gene product (Fig. 1) comprised a 31 amino acid sequence with the characteristics of a typical signal sequence (McConnell *et al.*, 1986): three positively charged amino acids near the N-terminus, a hydrophobic core, the consensus signal peptidase cleavage sequence Ala-X-Ala and in addition a helix-breaking proline preceding the cleavage site. The signal sequence of the deduced *apu* product (Fig. 1) was followed by the N-terminal amino acid sequence of the extracellular enzyme purified from *C. thermohydrosulfuricum* (Melasniemi, 1988). The N-terminal amino acid sequence earlier reported contained an Ile residue at position +2, but the nucleotide sequence clearly indicates a Thr residue at this position. However, the ATA codon for Ile can be changed to the ACA codon for Thr by a single point mutation. Because of this the DNA sequence at this point must be checked from another clone.

The structural gene was preceded by a sequence identical to the *E. coli* and *B. subtilis* σ^{43} consensus promoter sequences (Rosenberg & Court, 1979; McConnell *et al.*, 1986), the sequence TTGACA being followed by two TATAAT sequences after 10 and 17 nucleotides, respectively (Fig. 1). This putative promoter sequence was preceded by a 13 nucleotide inverted repeat (Fig. 1)

having the characteristics of a transcription terminator (Rosenberg & Court, 1979). This inverted repeat was the longest found in the whole sequence. Some 40 nucleotides downstream from the putative promoter started a seven nucleotide inverted repeat (AAATGTGTTTT-TATCACATTT; Fig. 1) resembling the consensus sequence (AANTGTGANNTNNNNCANATTN) for binding of the cAMP–catabolite activator protein complex of *E. coli* (Crombrugghe *et al.*, 1984). The resemblance of the *apu* inverted repeat to a known bacterial regulatory protein binding site makes it a candidate for the site mediating the observed catabolite repression (Hyun & Zeikus, 1985; Melasniemi, 1987) of the *apu* gene. At a distance of seven nucleotides in front of the gene was the sequence AAAGGGGG (Fig. 1), exhibiting strong complementarity to the 16S rRNA of *Bacillus stearothermophilus* and *B. subtilis* (McConnell *et al.*, 1986). The TAG stop codon terminating the *apu* open reading frame is followed by three other TAG codons in the same reading frame, one, 10 and 15 codons later (Fig. 1), ensuring termination of translation of the gene.

The α -amylase-pullulanase satellite protein is a small protein occurring in a firm, apparently equimolar association with the extracellular enzyme of the native host (Melasniemi, 1988). The sequence coding for the N-terminal end of this protein started some 500 nucleotides after the *apu* open reading frame shown in Fig. 1. Thus, the enzyme and the satellite protein are coded by two adjacent, apparently co-regulated genes.

Purified extracellular α -amylase-pullulanase of *C. thermohydrosulfuricum* (Melasniemi, 1988) contained 11–12% carbohydrate stainable after SDS-PAGE, suggesting a covalent linkage between the polypeptide and the carbohydrate moiety. In *N*-glycosidic linkages of glycoproteins the amino acid sequence next to the glycosylated asparagine is always Asn-X-Ser/Thr (Sharon & Lis, 1981). The deduced amino acid sequence of the *apu* product revealed in total 13 such possible sites for *N*-glycosylation (Fig. 1).

The hydropathy plot (Kyte & Doolittle, 1982) of the *apu* product showed that the signal sequence is the most hydrophobic portion of the protein. No other regions having the length and the hydrophobicity to span a membrane (Kyte & Doolittle, 1982) were found. Thus, it seems that the localization of the enzyme to the surface of *C. thermohydrosulfuricum* cells under some cultural conditions (Melasniemi, 1987), is by other means than via a membrane anchor sequence in the mature polypeptide.

Codon usage

The 37 mol% G + C content of the *apu* gene agrees well with the value of 36–38 mol% reported for several strains

of *C. thermohydrosulfuricum* (Wiegel *et al.*, 1979). However, the G + C content of the third ('wobble') codon position was only 27%, indicating a preference for codons ending in A or T.

The codon usage of the *apu* gene resembled that observed for *Clostridium thermocellum* genes (Schwarz *et al.*, 1988; Grépinet *et al.*, 1988). It was biased and different from the codon usage in the structural genes of *E. coli* (Alff-Steinberger, 1984): for only three out of 17 amino acids (excluding Met, Trp and Cys, the last of which is absent from the protein) was the preferred codon the same as in the *E. coli* genes (data not shown). The preferred *apu* codons were more often those found in rare proteins of *E. coli* than those found in abundant proteins (Grosjean & Fiers, 1982) and in addition the codons preferred in *apu* for Arg (AGA and AGG), Ile (ATA) and Gly (GGA) have been proposed as slowing down modulators of translation in *E. coli* (Grosjean & Fiers, 1982). The exceptional length of the *apu* gene together with the differences in the codon usage presumably makes translation of the gene in *E. coli* slow and prone to premature termination, which, in turn, renders the newly synthesized polypeptides susceptible to proteolytic degradation. These factors may explain the observed degeneracy of the *apu* product in *E. coli* (Melasniemi & Paloheimo, 1989). In contrast to the differences in codon usage between *apu* and *E. coli* genes, a resemblance was observed to the codon usage of *Bacillus* genes (McConnell *et al.*, 1986): 14 amino acids out of the 17 shared a common preference codon in *apu* and in *Bacillus* genes.

Similarities between the *apu* product and other amylolytic enzymes

No general sequence similarity has been found in prokaryotic or eukaryotic signal sequences (McConnell *et al.*, 1986). However, the signal sequence of the *apu* product showed similarity to the signal sequence of the α -amylase of *B. stearothermophilus*, another Gram-positive thermophile, and to that of *B. subtilis* (Fig. 2a).

The deduced amino acid sequence of the mature *apu* product showed several regions similar to sequences in other amylolytic enzymes (Fig. 2; references in the legend). Nakajima *et al.* (1986) have demonstrated four common regions in α -amylases of different origins. The first, second and fourth regions can also be detected in other types of amylolytic enzymes (see e.g. Kuriki & Imanaka, 1989), and these could likewise be distinguished in the mature *apu* product (Fig. 2e, g, j). Most interestingly, considering the dual α -1,4/ α -1,6 activity of the *apu* product, the second and fourth regions were partly 'duplicated'. Thus, within these regions were sequence motifs common to several α -amylases (shaded

in Fig. 2g and j) which were not found in the *apu* product at this position, but were found later in the *apu* sequence (Fig. 2i, l). The 78 amino acid sequence ending immediately after the first region (Fig. 2e) had 63% identity with the amino acid sequence of the neopullulanase of *B. stearothermophilus*. The beginning of this sequence was also similar to the sequences of the isoamylase (EC 3.2.1.68) of *Pseudomonas amyloclavata*, the pullulanase of '*Klebsiella aerogenes*' and the cyclomaltodextrin glucanotransferases (EC 2.4.1.19) of *K. pneumoniae* and *B. macerans*. Somewhat later the sequence was similar to the α -amylase of *B. subtilis*. The 57 amino acid sequence starting one amino acid before the second region had 53% identity with *B. stearothermophilus* neopullulanase (Fig. 2g). Only one amino acid at and two amino acids near the third region (most obvious in mammalian α -amylases) were present in the *apu* product (Fig. 2h). The sequence starting at the fourth region again resembled that of *B. stearothermophilus* neopullulanase (Fig. 2j) and was followed at some distance by a sequence with much similarity also to the cyclomaltodextrin glucanotransferases of *K. pneumoniae* and *B. macerans* (Fig. 2k).

As a whole, the *apu* product most closely resembled the neopullulanase of *B. stearothermophilus*, an enzyme cleaving α -1,4 linkages in pullulan and also α -1,6 linkages in several oligosaccharides (Imanaka & Kuriki, 1989). Several short sequences were also very similar to sequences in *B. macerans* cyclomaltodextrin glucanotransferase, of which enzyme two end products (β - and γ -cyclodextrins) are inhibitors of the α -amylase-pullulanase (H. Melasniemi, unpublished). There were no sequences common to all three debranching enzymes, *P. amyloclavata* isoamylase, '*K. aerogenes*' pullulanase and the *apu* product, that distinguished them from other amylases, except that they all had a short hydrophobic stretch of amino acids near their ends (Fig. 2n) and terminated shortly after this with a basic residue.

The sequence of the C-terminal end of the *apu* product (Fig. 1) is of interest. The prominent sequence of three hydroxyl-bearing Thr residues alternating with hydrophobic amino acids (starting 35 residues before the end) followed shortly after by four Leu residues and four Gln residues, the whole sequence being punctuated and surrounded with several Lys Arg and His residues, is likely to serve a specific function. Because of its hydrophilicity this region is likely to be located on the surface of the molecule, where its positive charge and the hydroxyl groups might participate in the attachment of the enzyme to a negatively charged domain on the surface of *C. thermohydrosulfuricum* cells, on another α -amylase-pullulanase polypeptide (dimerization or aggregation) or on the satellite protein. However, the presence of several His residues (otherwise the rarest of the amino

	TTTAGCAGAAAAAGCAGATAAAATGGAGATAACACAAAGGTTAAAGCAGATATAATTTATAGGCGCTTTATAGTAGCC	80
	CTATAAAATTACATATAGTGTATGTATTACACATATTGACAACGAAAAACATATAATTTATAATAGACATGAGGGAACGATTGCGCAAAAATTTGCACAAATAAATGTGTTTT	199
	ATCACATTTATCTTTTGCAATTTGTGAAAGCGATTGCCTAAAAGAAATGGGGTGGGATTTGGGAATTAATTTTTTAAAAACATTTAAGTAAATATATGGATAAAAGGGGATTATAG	318
-31	GTG TTT AAA AGG AGA GCT TTA GGA TTT TTG CTG GCT TTT CTT TTA GTT TTT ACA GCA GTA TTT GGC TCA ATG CCT ATG GAA TTT GCA AAG Met Phe Lys Arg Arg Ala Leu Gly Phe Leu Leu Ala Phe Leu Leu Val Phe Thr Ala Val Phe Gly Ser Met Pro Met Glu Phe Ala Lys	408
-1	GCT GAG ACA GAT ACA GCG CCA GCG ATA GCC AAT GTT GTT GGC AAT TTT CAA TCC AAA CTT GGA GAT TCT GAC TGG AAT ATA AAC AGT GAC Ala Glu Thr Asp Thr Ala Phe Ala Ile Ala Asn Val Val Gly Asn Phe Gln Ser Lys Leu Gly Asp Ser Asp Trp Asn Ile Asn Ser Asp	498
	AAA ACG ATA ATG ACA TAT AAA GGT AAT GGC TTT TAT GAA TTT ACT ACC CCA GTT GCG TTA CCT GCA GGT GAT TAT GAG TAT AAA GTT GCT Lys Thr Ile Met Thr Tyr Lys Gly Asn Gly Phe Tyr Glu Phe Thr Pro Val Ala Leu Pro Ala Gly Asp Tyr Glu Tyr Lys Val Ala	588
30	CTT AAT CAT TCA TGG GAA GGT GGA GGA GTT CCT TCA CAA GGT AAT ATA AGC TTT CAT CTT GAT TCA GAT TCT GTA GTG ACT TTT TAT TAC Leu Asn His Ser Trp Glu Gly Gly Gly Val Pro Ser Gln Gly Asn Leu Ser Phe His Leu Asp Ser Asp Ser Val Val Thr Phe Tyr Tyr	678
60	AAT TAC AAC ACT TCA AGT ATT ACC GAT TCC ACA AAA TAT ACA CCA ATT CCC GAA GAC AAG CTT CCA AGA CTT GTA GGG ACT ATA CAG CCT Asn Tyr Asn Thr Ser Ser Ile Thr Asp Ser Thr Lys Tyr Thr Pro Ile Pro Glu Asp Lys Leu Pro Arg Leu Val Gly Thr Ile Gln Pro	768
90	GCA ATA GGA GCA GGT GAT GAT TGG AAA CCT GAA ACA TCG ACT GCT ATA ATG AGA GAC TAT AAG TTT AAT AAT GTT TAC GAA TAC ACT GCA Ala Ile Gly Ala Gly Asp Asp Trp Lys Pro Glu Thr Ser Thr Ala Ile Met Arg Asp Tyr Lys Phe Asn Asn Val Tyr Glu Tyr Thr Ala	858
120	AAT GTT CCA AAA GGG AAT TAT GAG TTT AAA GTG ACT TTA GGG CCC TCA TGG GAT ATA AAT TAT GGC TTA AAT GGT GAA CAA AAT GGG CCA Asn Val Pro Lys Gly Asn Tyr Glu Phe Lys Val Thr Leu Gly Pro Ser Trp Asp Ile Asn Tyr Gly Leu Asn Gly Glu Gln Asn Gly Pro	948
150	AAT ATT CCT TTG AAT GTA GCC TAT GAT ACT AAG ATT ACA TTT TAC TAT GAT TCG GTT TCA EAT AAT ATA TGG ACA GAT TAT AAT CCA CCT Asn Ile Pro Leu Asn Val Ala Tyr Asp Thr Lys Ile Thr Phe Tyr Tyr Asp Ser Val Ser His Asn Ile Trp Thr Asp Tyr Asn Pro Pro	1038
180	CTT ACA GGA CCT GAT AAT AAC ATA TAT TAT GAC GAT TTA AGA CAT GAC ACC CAT GAC CCA TTC TTC CGC TCG CCT TTC GGT GCA ATA AAA Leu Thr Gly Pro Asp Asn Asn Ile Tyr Tyr Asp Asp Leu Arg His Asp Thr His Asp Pro Phe Phe Arg Ser Pro Phe Gly Ala Ile Lys	1128
210	ACG GGA GAT ACT GTG ACA TTA AGA ATA CAA GCA AAA AAT CAT GAC ATT GAG TCA GCT AAA ATT TCT TAT TGG GAT GAT ATT AAA AAA ACA Thr Gly Asp Thr Val Thr Leu Arg Ile Gln Ala Lys Asn His Asp Ile Glu Ser Ala Lys Ile Ser Tyr Trp Asp Asp Ile Lys Lys Thr	1218
240	AGA ATA GAA GTC CCT ATG TAC AGA ATT GGT CAA AGT CCT GAC GGG AAA TAT GAA TAC TGG GAA GTT AAG TTA AGC TTT GAC CAT CCC ACA Arg Ile Glu Val Pro Met Tyr Arg Ile Gly Gln Ser Pro Asp Gly Lys Tyr Glu Tyr Trp Glu Val Lys Leu Ser Phe Asp His Pro Thr	1308
270	AGA ATT TGG TAT TAC TTT ATA CTT AAA GAC GGG ACC AAA ACT GCT TAT TAC GGA GAT AAC GAT GAA CAA TTA GGT GGC GTA GGT AAA GCC Arg Ile Trp Tyr Tyr Phe Ile Leu Lys Asp Gly Thr Lys Thr Ala Tyr Tyr Gly Asp Asn Asp Glu Gln Leu Gly Gly Val Gly Lys Ala	1398
300	ACA GAT ACA GAA AAT AAG GAC TTC GAA CTT ACT GTC TAC GAT AAA AAC TTA GAC ACC CCT GAT TGG ATG AAA GGG TCA GTA ATG TAT CAA Thr Asp Thr Glu Asn Lys Asp Phe Glu Leu Thr Val Tyr Asp Lys Asn Leu Asp Thr Pro Asp Trp Met Lys Gly Ser Val Met Tyr Gln	1488
330	ATA TTC CCT GAT AGG TTC TTT AAT GGG GAT TCT TCA AAT GAC CAT CTA AAG AAA TAC AGC AGA GGT TTT GAT CCT GTT GAA TAT CAT AGC Ile Phe Pro Asp Arg Phe Phe Asn Gly Asp Ser Ser Asn Asp His Leu Lys Lys Tyr Ser Arg Gly Phe Asp Pro Val Glu Tyr His Ser	1578
360	AAC TGG TAC GAG CTT CCA GAT AAT CCG AAT GAT AAA AAT AAA CTA GGA TAT ACA GGG GAT GGC ATA TGG TCA AAT GAC TTC TTT GGC GGT Asn Trp Tyr Glu Leu Pro Asp Asn Pro Asn Asp Lys Asn Lys Leu Gly Tyr Thr Gly Asp Gly Ile Trp Ser Asn Asp Phe Phe Gly Gly	1668
390	GAT TTA AAA GGT ATA GAT GAT AAA TTG GAT TAT TTA AAA AGC CTT GGA ATA TCA GTC ATT TAT CTC AAT CCA ATT TTT CAA TCA CCT TCT Asp Leu Lys Gly Ile Asp Asp Lys Leu Asp Tyr Leu Lys Ser Leu Gly Ile Ser Val Ile Tyr Leu Asn Pro Ile Phe Gln Ser Pro Ser	1758
420	AAT CAC AGG TAC GAT ACA ACC GAT TAC ACA AAA ATA GAC GAA CTA TTA GGA GAT TTA TCT ACC TTT AAG AAG CTC ATG GAG GAT GCC CAT Asn His Arg Tyr Asp Thr Thr Asp Tyr Thr Lys Ile Asp Glu Leu Leu Gly Asp Leu Ser Thr Phe Lys Lys Leu Met Glu Asp Ala His	1848
450	GCA AAA GGG ATT AAG GTA ATA CTT GAT GGC GTC TTC AAT CAT ACA AGT GAT GAC AGC ATT TAT TTT GAT AGA TAT GGT AAA TAC TTA AAC Ala Lys Gly Ile Lys Val Ile Leu Asp Gly Val Phe Asn His Thr Ser Asp Ser Ile Tyr Phe Asp Arg Tyr Gly Lys Tyr Leu Asn	1938
480	ACG GGA GTT TTA GGT GCT TAT CAA GCA TGG AAA CAG GGA GAT TCA AAG TCT CCA TAT GGT GAC TGG TAT GAG ATA AAG CCT GAC GGT Thr Gly Val Leu Gly Ala Tyr Gln Ala Trp Lys Gln Gly Asp Gln Ser Lys Ser Pro Tyr Gly Asp Trp Tyr Glu Ile Lys Pro Asp Gly	2028
510	ACC TAT GAA GGA TGG TGG GGA TTT GAC AGT TTA CCT GTG ATA AGG CAG ATA AAC GGC AGT GAA TAT AAT GTC AAA ACT TGG GCG GAT TTT Thr Tyr Glu Gly Trp Trp Gly Phe Asp Ser Leu Pro Val Ile Arg Gln Ile Asn Gly Ser Glu Tyr Asn Val Lys Ser Trp Ala Asp Phe	2118
540	ATT ATA AAT AAT CCT AAT GCA ATA TCT AAG TAT TGG TTA AAT CCT GAT GGG GAC AAA AAT GTA GGT GCA GAT GGT TGG AGA TTA GAT GTT Ile Ile Asn Asn Pro Asn Ala Ile Ser Lys Tyr Trp Leu Asn Pro Asp Gly Asp Lys Asn Val Gly Ala Asp Gly Trp Arg Leu Asp Val	2208
570	GCG AAT GAA GTT GCT CAC GAT TTT TGG GTT CAT TTT AGA GGT GCA ATT AAT ACT GTG AAA CCG AAT GCT CCG ATG GTT GCA GAA AAC TGG Ala Asn Glu Val Ala His Asp Phe Trp Val His Phe Arg Gly Ala Ile Asn Thr Val Lys Pro Asn Ala Pro Met Val Ala Glu Asn Trp	2298
600	AAT GAC GCT TCA CTG GAT CTG CTT GGA GAT TCT TTT AAC TCT GTT ATG AAT TAT CTC TTT AGA AAT GCA GTA ATT GAC TTT ATA TTG GAT Asn Asp Ala Ser Leu Asp Leu Leu Gly Asp Ser Phe Asn Ser Val Met Asn Tyr Leu Phe Arg Asn Ala Val Ile Asp Phe Ile Leu Asp	2388
630	AAA TCA TTT GAT GAC GGA AAT GTG GTT CAC AAT CCT ATA GAT GCA GCA AAA CTC GAC CAA AGG CTT ATG AGC ATA TAT GAG AGA TAT CCT Lys Ser Phe Asp Asp Gly Asn Val Val His Asn Pro Ile Asp Ala Ala Lys Leu Asp Gln Arg Leu Met Ser Ile Tyr Glu Arg Tyr Pro	2478

690	CTT CCA GTG TTT TAT TCT ACT ATG AAT CTT TTA GGT TCT CAT GAC ACC ATG AGG ATA TTG ACA GTA TTT GGA TAC AAC TCT GCG GAT GAA	2568
	Leu Pro Val Phe Tyr Ser Thr Met Asn Leu Leu Gly Ser His Asp Thr Met Arg Ile Leu Thr Val Phe Gly Tyr Asn Ser Ala Asp Glu	
	<u>SphI</u>	
720	AAT CAA AAT TCT CAA GCA GCA AAA GAC CTT GCG GTT AAA AGA CTT AAA CTT GCT GCA ATA CTG CAA ATG GGT TAT CCA GGC ATG CCT TCT	2658
	Asn Gln Asn Ser Gln Ala Ala Lys Asp Leu Ala Val Lys Arg Leu Lys Leu Ala Ala Ile Leu Gln Met Gly Tyr Pro Gly Met Pro Ser	
750	ATT TAT TAC GGA GAT GAA GCA GGA CAA TCT GGC GGA AAA GAC CCG GAC AAC AGA AGG ACA TTC CCT TGG GGA AGA GAA GAT ACT GAT TTG	2748
	Ile Tyr Tyr Gly Asp Glu Ala Gly Gln Ser Gly Gly Lys Asp Pro Asp Asn Arg Arg Thr Phe Pro Trp Gly Arg Glu Asp Thr Asp Leu	
780	CAA ACT TTT TTC AAG AAA GTT GTA AAT ATA AGA AAT GAG AAT CAA GTT TTG AAA ACA GGA GAC CTT GAG ACA CTT TAT GCA AAT GGC GAT	2838
	Gln Thr Phe Phe Lys Lys Val Val Asn Ile Arg Asn Glu Asn Gln Val Leu Lys Thr Gly Asp Leu Glu Thr Leu Tyr Ala Asn Gly Asp	
810	GTT TAT GCC TTT GGA AGA AGA ATC ATA AAT GGA AAA GAT ACT TTT GGG AAA TCG TAT CCT GAC AGT GTA GCT ATT GTC GTA ATA AAT AAA	2928
	Val Tyr Ala Phe Gly Arg Arg Ile Ile Asn Gly Lys Asp Thr Phe Gly Lys Ser Tyr Pro Asp Ser Val Ala Ile Val Val Ile Asn Lys	
840	GGT GAC GCA AAA CAG GTT TCT ATA GAT ACC ACT AAA TTT ATA AGA GAT GGA GTT GCT TTT ACA GAT GCC TTA ACT GGC AAG ACA TAC ACG	3018
	Gly Asp Ala Lys Gln Val Ser Ile Asp Thr Thr Lys Phe Ile Arg Asp Gly Val Ala Phe Thr Asp Ala Leu Ser Gly Lys Thr Tyr Thr	
870	GTT CAG GAT GGT AAA ATT GTT GTA GAA GTT GGA TCA ATG GAT GGA GCT ATA CTT ATA TCA GAT ACA GGA CAA AAT TTG ACA GCA CCT CAG	3108
	Val Gln Asp Gly Lys Ile Val Val Glu Val Gly Ser Met Asp Gly Ala Ile Leu Ile Ser Asp Thr Gly Gln Asn Leu Thr Ala Pro Gln	
	<u>AccI</u>	
900	CCA ATA ACA GAC CTT AAA GCA GTC TCA GGG AAT GGC AAA GTA GAC CTT TCC TGG AGT GTA GTA GAT AAA GCA GTA AGC TAT AAT ATT TAC	3198
	Pro Ile Thr Asp Leu Lys Ala Val Ser Gly Asn Gly Lys Val Asp Leu Ser Trp Ser Val Val Asp Lys Ala Val Ser Tyr Asn Ile Tyr	
930	CGT TCT ACA GTT AAA GGT GGG TTA TAT GAA AAA ATA GCT TCA AAT GTT ACG CAA ATT ACC TAT ACT GAT ACA GAG GTT ACC AAT GGT CTA	3288
	Arg Ser Thr Val Lys Gly Gly Leu Tyr Glu Lys Ile Ala Ser Asn Val Thr Gln Ile Thr Tyr Thr Asp Thr Glu Val Thr Asn Gly Leu	
	<u>★</u>	
960	AAG TAT GTG TAT GCT GTA ACC GCT GTA GAT AAT GAT GGA AAT GAG AGT GCT TTA AGC AAT GAA GTT GAG GCA TAT CCA GCA TTT CCT ATT	3378
	Lys Tyr Val Tyr Ala Val Thr Ala Val Asp Asn Asp Gly Asn Glu Ser Ala Leu Ser Asn Glu Val Glu Ala Tyr Pro Ala Phe Pro Ile	
	<u>★</u>	
990	GGC TGG GCG GGA AAT ATG AAT CAA GTA AAT ACA CAT GTA ATA GGT GTA AAT AAT CCA GTT GAA GTG TAT GCT GAA GTG TGG GCT CAA GGG	3468
	Gly Trp Ala Gly Asn Met Asn Gln Val Asn Thr His Val Ile Gly Val Asn Asn Pro Val Glu Val Tyr Ala Glu Val Trp Ala Gln Gly	
1020	CTT ACA GAT AAA CCT GGC CAA GGA GAA AAT ATG ATT GCC CAG TTA GGA TAT AGG TAT ATT GGA GAT ACT GTA GGA GAT GCA GTT TAC AAT	3558
	Leu Thr Asp Lys Pro Gly Gln Gly Glu Asn Met Ile Ala Gln Leu Gly Tyr Arg Tyr Ile Gly Asp Thr Val Gly Asp Ala Val Tyr Asn	
1050	GCC GTG TAC AAT AAG GTA GAA GGT GTT GAA ATA AGT AAG GAC TGG ACA TGG GTT GAT GCA CAA TAT GTA GGT GAT TCT GGA AAT AAT GAT	3648
	Ala Val Tyr Asn Lys Val Glu Gly Val Glu Ile Ser Lys Asp Trp Thr Trp Val Asp Ala Gln Tyr Val Gly Asp Ser Gly Asn Asn Asp	
	<u>AccI</u>	
1080	AAA TAC ATG GCT AAA TTT GTA CCT GAT ATG GTA GGC ACA TGG GAA TAC ATT ATG AGA TTT TCT TCT AAC CAA GGC CAT GAT TGG ACG TAT	3738
	Lys Tyr Met Ala Lys Phe Val Pro Asp Met Val Gly Thr Trp Glu Tyr Ile Met Arg Phe Ser Ser Asn Gln Gly His Asp Trp Thr Tyr	
1110	ACA AAA GGA CCA GAT GGG AAA ACA GAT GAA GCA AAA CAG TTT ACT GTC GTG CCA TCA AAT GAT GTA GAA ACA CCT ACA GCT CCA GTC TTA	3828
	Thr Lys Gly Pro Asp Gly Lys Thr Asp Glu Ala Lys Gln Phe Thr Val Val Pro Ser Asn Asp Val Glu Thr Pro Thr Ala Pro Val Leu	
1140	CAA CAA CCA GGA ATT GAA TCC TCA AGA GTT ACA CTT AAC TGG AGT CCG TCA GCT GAT GAT GTT GCT ATT TTC GGC TAC GAA ATC TAT AAG	3918
	Gln Gln Pro Gly Ile Glu Ser Ser Arg Val Thr Leu Asn Trp Ser Pro Ser Ala Asp Asp Val Ala Ile Phe Gly Tyr Glu Ile Tyr Lys	
	<u>★</u>	
1170	TCT TCA AGT GAG ACA GGA CCA TTT ATA AAG ATT GCA ACT GTG TCT GAC AGT GTG TAT AAC TAC GTA GAT ACA GAT GTA GTA AAT GGC AAT	4008
	Ser Ser Ser Glu Thr Gly Pro Phe Ile Lys Ile Ala Thr Val Ser Asp Ser Val Tyr Asn Tyr Val Asp Thr Asp Val Val Asn Gly Asn	
1200	GTG TAT TAT TAT AAA GTT GTG GCA GTT GAT ACC TCC TAT AAT AGG ACA GCA TCA AAT ACA GTA AAA GCG ACA CCT GAT ATA ATA CCT ATA	4098
	Val Tyr Tyr Tyr Lys Val Val Ala Val Asp Thr Ser Tyr Asn Arg Thr Ala Ser Asn Thr Val Lys Ala Thr Pro Asp Ile Ile Pro Ile	
	<u>★</u>	
1230	AAA GTG ACA TTT AAT GTC ACA ATA CCA GAT TAT ACT CCT GAT GAT GGT GTA AAT ATT GCT GGA AAC TTC CCG GAT GCT TTC TGG AAT CCA	4188
	Lys Val Thr Phe Asn Val Thr Ile Pro Asp Tyr Thr Pro Asp Asp Gly Val Asn Ile Ala Gly Asn Phe Pro Asp Ala Phe Trp Asn Pro	
	<u>★</u>	
1260	AAT GCT AAT CAA ATG ACA AAA GCT GGT TCT AAC ACC TAT AGT ATT ACA CTT ACT TTA AAT GAG GGT ACA CAA ATT GAA TAT AAA TAT GCA	4278
	Asn Ala Asn Gln Met Thr Lys Ala Gly Ser Asn Thr Tyr Ser Ile Thr Leu Thr Leu Asn Glu Gly Thr Gln Ile Glu Tyr Lys Tyr Ala	
1290	AGG GGG AGC TGG GAT AAA GTA GAA AAA GGT GAA TAC GGC AAT GAG ATT GAC AAC AGA AAA ATA ACT GTT GTC AAT CAG GGT TCA AAT ACA	4368
	Arg Gly Ser Trp Asp Lys Val Glu Lys Gly Glu Tyr Gly Asn Glu Ile Asp Asn Arg Lys Ile Thr Val Val Asn Gln Gly Ser Asn Thr	
1320	ATG GTT GTA AAT GAT ACA GTG CAA AGA TGG AGA GAT GTG CCA ATA TAT ATC TAT TCT CCA AAA GAC AAG ACT ATT GTT GAT GCA AAT ACG	4458
	Met Val Val Asn Asp Thr Val Gln Arg Trp Arg Asp Val Pro Ile Tyr Ile Tyr Ser Pro Lys Asp Lys Thr Ile Val Asp Ala Asn Thr	
	<u>★</u>	
1350	AGT GAA ATA GAG ATT AAA GGC AAT ACT TAC AAA GGT GCG AAA GTA ACT ATA AAT GAT GAA TCT TTT GTG CAA CAA GAA AAT GGA GTA TTT	4548
	Ser Glu Ile Glu Ile Lys Gly Asn Thr Tyr Lys Gly Ala Lys Val Thr Ile Asn Asp Glu Ser Phe Val Gln Gln Glu Asn Gly Val Phe	
1380	ACA AAA GTA GTT CCC TTG GAA TAT GGT GTA AAT ACT ATC AAA ATA CAT GTA GAG CCA AGT GGT GAC AAG AAT AAT GAA CTT ACA AAA GAT	4638
	Thr Lys Val Val Pro Leu Glu Tyr Gly Val Asn Thr Ile Lys Ile His Val Glu Pro Ser Gly Asp Lys Asn Asn Glu Leu Thr Lys Asp	
1410	ATA ACA ATA ACT GTT ACA AGA GAG AAG CCG GCC AGG AGA CAG AAC CTA CTC CTA CTC CAC CAA CAG AAA CAA CAA AAC CAT CTC AAG AAG	4728
	Ile Thr Ile Thr Val Thr Arg Glu Lys Pro Ala Arg Arg Gln Asn Leu Leu Leu Leu His Gln Gln Lys Gln Gln Asn His Leu Lys Lys	
1440	TAT CAC AAG GCA AAA TAGTTGTAGAAAATAACACAACACACTTACAATAGACAAAACAAAGTAGCAAAAGAACATAAAGACACTTCAAGAAAGAAATACGATTGAC	4839
	Tyr His Lys Ala Lys End End End End	

Fig. 1. Nucleotide and deduced amino acid sequence of the *apu* gene. Putative promoter and ribosome-binding sites are denoted by double and single underlinings, respectively. Inverted repeats are denoted by arrows and the N-terminal end of the mature protein is underlined. Restriction sites present also in the polylinker of the M13 vector are shown above the sequence. Possible N-glycosylation sites are shown by an asterisk.

(a) Cth apu -31 M F K R R A L G F L L A F L L V F T A V F G S M P M E F A L A
 Bst amy -34 M L T F H R I I R K G W M F L L A F L L T A S L F C P T G R H A L A
 Bsu amy -33 M F A K R F K T S L L P L F A G F L L L F H L V L A G P A A A S A

(b) Cth apu 49 L P A G D Y 54
 Shy amy 375 L P A G T Y 381
 Rat amy 442 L P A G T Y 447
 Hum amy 449 L P A G T Y 454
 Bma cgt 452 L P A G T Y 457

(c) Cth'apu 240 T G D T V T L R 247
 Shy amy 392 D G D T V T V G 399
 Bma cgt 584 T G D Q V T V R 591

(d) Cth apu 356 V M Y Q I F P D R F F N G D S S N 372
 Bst npl 387 V W Y Q I F P R F A N G N P S I 403
 BarAmy 319 G I P C I F Y D F F N W G F K D 335
 Bma cgt 16 V I Y Q I V T D R F A D G D R T N 32

(e) Cth apu 416 F F G G D L K G I D D K L D Y L K S L G I S V I Y L N P I F Q S P S N H R Y D T T D Y T K I D E L L G D
 Bst npl 170 F F G G D L Q G I I D H L D Y L V D L G I T G I Y L T P I F R S P S N H K Y D T A D Y F E V D P H F G D
 Pam iam 198 Q Y R G T Y Y G A G L K A S Y L A S L G V 218
 Kae pul 345 Q G G S D L K G A F Y R 356
 Bsu amy 40 S P I N Q V K E G N Q G D K S M S N W Y W L Y Q P T S Y Q I G N R Y L G T
 Shy amy 60 K I A G R L G D
 Kpn cgt 47 Y T G G D L R G L I N K L P Y L K S L G V 67
 Bma cgt 49 Y F G G D W Q G I I D K I N D G Y L T G M 69

1
 Cth apu L S T F K K L M E D A H A K G I K V I L D G V F N H T S D 496
 Bst npl K E T L K T L I D R C H E K G I R V M L D A V F N H C G Y E 251
 Pam iam 281 F H N A G I K V Y M D V V Y N H T A E G 300
 Kae pul 590 K Q D L G M N V I M D V V Y N H T A E G 609
 Bsu amy E Q E F K E M C A A A E E Y G I K V I V D A V I N H T T S D 106
 Shy amy R D A F A S M V S A C H A G V K V I A D A V V N H M A A G 97

(f) Cth apu 556 I N G S E Y 561
 Rat amy 235 I K G S E Y 240
 Mou amy 239 I K G S E Y 244
 Hog amy 241 I K G S E Y 246

(g) 2
 Cth apu 589 N V G A D G W R L D V A N E V A H D F W V H F R G A I N T V K P N A P M V A E N W N D A S L D L L G D S F
 Bst npl 319 E F D I D G W R L D V A N E I D H E F W R E F R Q E V K A L K P D V Y I L G E I W H D A M P W L R G D Q F
 Pam iam 365 T M G V D G F R F D L A S V 378
 Kae pul 666 D Y K I D G F R F D L M G Y 679
 Bsu amy 167 N D G A D G F R F D A A K H 180
 Bam amy 222 E L S L D G F R I D A A K H 235
 Shy amy 165 S L G V D G F R I D A A K H 178
 Sli amy 168 S L G V D G F R I D A A K H 181

Cth apu N S V M N Y L F 649
 Bst npl D A V M N Y P F 379

(h) 3
 Cth apu 634 L D L L G 638
 Rat amy 232 I D L G G 236
 Hog amy 235 I D L G G 238
 Hum amy 238 I D L G G 241

(i) Cth apu 672 I D A A K 676
 Bam amy 230 I D A A K 234
 Shy amy 173 I D A A K 177
 Sli amy 176 I D A A K 180

(j) 4
 Cth apu 695 S T M N L L G S H D T M R I L T V F G 713
 Bst npl 415 A A F N L L G S H D T S R I L T V C G 433
 Pam iam 498 N S I N F I D V H D G M T 510
 Kae pul 823 E V V N Y V S K H D N Q T 835
 Bsu amy 260 K L V T W V E S H D T Y A 272
 Bli amy 319 K A V T F V D N H D T Q P 331
 Bst amy 322 L A V T F V D N H D T E P 334
 BarBamy 281 K A V T F V D N H D T G S 293
 Bma cgt 277 S G M N L L D N H D M D R 332

+43
 aa

(k) Cth apu 746 G M P S I Y Y G D E A G Q S G G K D P D N R R T F P W G R E D 776 799 G D L E T L 804
 Bst npl 451 G S P C I Y Y G D E I G M T G G N D P E C R 472 159 P W G S E D 164 220 G D K E T L 225
 Kpn cgt 380 G I P A I Y Y G T E 389
 Bma cgt 355 G V P A I Y Y G T E Q Y M T G D G D P N N R 376

(l) Cth apu 964 A V T A V D N 970
 Bli amy 320 A V T F V D N 326
 Bst amy 323 A V T F V D N 329
 BarBamy 282 A V T F V D N 288

(m) Cth apu 1399 S G D K N N 1404
 Rat amy 452 S G D K V N 457
 Hum amy 459 S G D K I N 464

(n) Cth apu 1423 Q N L L L L 1428
 Pam iam 737 Q S L L L L 742
 Kae pul 1059 W S V A V L 1064

acids found in the *apu* product) raises the possibility that the end of the molecule might instead of serving a structural function be involved in substrate binding or even be part of the active site of the enzyme. Regions one, two and four common to α -amylases (Nakajima *et al.*, 1986) all contain a His residue and it is assumed that in the α -amylase of *Aspergillus oryzae* (Matsuura *et al.*, 1984) two of these histidines are involved in substrate binding and one is at the active site. The C-terminal end of the pullulanase of '*K. aerogenes*' bears some resemblance to the end of the *apu* product. It is likewise enriched with basic amino acids, including two His residues. In addition, the four amino acid hydrophobic stretch near the end of the '*K. aerogenes*' pullulanase is preceded by a Ser-Val-Thr-Leu sequence resembling the alternating sequence of hydroxy- and hydrophobic amino acids found near the end of the *apu* product. It will be interesting to see whether the sequences of other α -amylase-pullulanase enzymes show 'duplication' of the second and fourth common regions and whether the C-terminal sequences of other (α -amylase-) pullulanases have characteristics in common with the *C. thermohydrosulfuricum* enzyme.

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Fig. 2. Amino acid sequence similarities between the *apu* product and various amylolytic enzymes. (a) Signal sequence. (b–n) Mature protein. Only those motifs found in addition to the *apu* product in at least two of the enzymes tested are shown. Abbreviations and references: *apu*, α -amylase-pullulanase; *amy*, α -amylase; *npl*, neopullulanase; *iam*, isoamylase; *pul*, pullulanase; *cgt*, cyclomalto-dextrin glucanotransferase; *Cth* (*Clostridium thermohydrosulfuricum*) *apu* (this work); *Bst* (*Bacillus stearothermophilus*) *amy* (referred to by Nakajima *et al.*, 1986, as all others denoted by an asterisk); *Bsu* (*Bacillus subtilis*) *amy* (*); *Bam* (*Bacillus amyloliquefaciens*) *amy* (*); *Bli* (*Bacillus licheniformis*) *amy* (Yuuki *et al.*, 1985); *Shy* (*Streptomyces hygroscopicus*) *amy* (Hoshiko *et al.*, 1987); *Sli* (*Streptomyces limosus*) *amy* (Long *et al.*, 1987); *Bar* (barley) *Aamy* and *Bamy* (amylases A and B; Rogers, 1985); *Rat amy* (*); *Mou* (mouse pancreas) *amy* (*); *Hog* (pancreas) *amy* (*); *Hum* (human salivary) *amy* (*); *Bst npl* (Kuriki & Imanaka, 1989); *Kae* ('*Klebsiella aerogenes*' [*K. pneumoniae*]) *pul* (Katsuragi *et al.*, 1987); *Pam* (*Pseudomonas amyloclavata*) *iam* (Amemura *et al.*, 1988); *Kpn* (*Klebsiella pneumoniae*) *cgt* (Binder *et al.*, 1986); and *Bma* (*Bacillus macerans*) *cgt* (Takano *et al.*, 1986). Numbering starts from the first amino acid of the mature proteins. Numbered bars above the sequence denote common regions in α -amylases (Nakajima *et al.*, 1986) and shading denotes sequence motifs 'duplicated' in the *apu* product.

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