Cloning and nucleotide sequence of an extracellular α -amylase gene from *Aeromonas hydrophila* MCC-1

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(Received 26 April 1993; revised 27 July 1993; accepted 10 August 1993)

A gene encoding the extracellular α -amylase of *Aeromonas hydrophila* MCC-1 was cloned and expressed using its own promoter on the recombinant plasmid pCA101. Subcellular fractionation of *Escherichia coli* JA221 carrying pCA101 revealed that approximately 60% of the amylase activity was localized in the periplasmic space. The extracellular amylase was purified to homogeneity, identified as an α -type and its amino-terminal sequence was determined. Nucleotide sequence analysis predicted a 443 amino acid ORF and 24 amino acids at the amino terminus of the sequence that are not found in the secreted protein. This 24 amino acid sequence has many of the characteristics common to known signal peptides. The predicted amino acid sequence has considerable similarity with mammalian, invertebrate and Streptomycete α -amylases. Most of the amino acid residues that are involved in catalytic activity, substrate binding and calcium binding in several α -amylases were also present in *A. hydrophila* α -amylase at the corresponding positions.

Introduction

Many Gram-negative bacteria possess the capacity to secrete certain proteins into the growth medium. Extracellular proteins produced by these bacteria have to cross an envelope, which is composed of two distinct membranes separated by the periplasm. Although much attention has been focused on the export process, the mechanism by which Gram-negative bacteria secrete proteins across the envelope is not well understood. Several specific and independent export pathways appear to be used for the secretion of different proteins in various Gram-negative bacteria (Pugsley *et al.*, 1990).

Aeromonas hydrophila, a member of the family Vibrionaceae, possesses the capacity to secrete various extracellular proteins, including aerolysin (Howard & Buckley, 1985), enterotoxin (Chakraborty *et al.*, 1984), phospholipase (Nord *et al.*, 1975), amylase (Gobius & Pemberton, 1988), acyltransferase (MacIntyre & Buckley, 1978), chitinase (Yabuki *et al.*, 1986) and proteases (Leung & Stevenson, 1988), which can play important

roles in pathogenesis, degradation of macromolecules and other interactions with the environment. Because of the significance of these toxins and exoenzymes secreted by *A. hydrophila*, this organism has been chosen for the study of secretion mechanisms. Previous studies have reported that when aerolysin, acyltransferase, protease and amylase structural genes are expressed in *Escherichia coli*, these exoproteins are secreted only to the periplasm, indicating that specific functions, absent in *E. coli*, were necessary for the second step of transfer through the outer membrane and release into the medium (Chakraborty *et al.*, 1986; Gobius & Pemberton, 1988; Howard & Buckley, 1986; Rivero *et al.*, 1990; Thornton *et al.*, 1988). The need for other gene products is supported by the observation that pleiotropic export mutants of *A. hydrophila* have been isolated which accumulate aerolysin and protease in the periplasm (Howard & Buckley, 1983). Recently, mutants defective in translocation across the outer membrane have been isolated and genes required for the successful translocation of proteins across the cell envelope have been cloned (Jiang & Howard, 1991, 1992). As an approach to study the secretion of proteins in *A. hydrophila* and *E. coli*, we have cloned the genes encoding exoproteins from *A. hydrophila* into *E. coli* (Chang *et al.*, 1992; Chen *et al.*, 1991). In this paper, molecular cloning of an extracellular α -amylase from *A. hydrophila*, as well as the secretion and locations of the cloned amylase in *E. coli* are described. The derived amino acid sequence for the encoded

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The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number L19299.

polypeptide is also compared with those of prokaryotic and eukaryotic origins.

Methods

Bacterial strains and plasmids. *A. hydrophila* MCC-1 was used. It was isolated initially in this laboratory from soil samples, and identified by the Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan, Republic of China. *E. coli* JA221 (Beggs, 1978) and *E. coli* JM109 (Yanisch-Perron *et al.*, 1985) were used as hosts for recombinant plasmids. Plasmid pBR322 (Bolivar *et al.*, 1977) was used in cloning experiments. Plasmids pUC18 and pUC19 (Vieira & Messing, 1982) were used in subcloning experiments.

Media and culture conditions. *E. coli* was grown in Luria broth (LB) or on LB agar (1.5%, w/v) (Sambrook *et al.*, 1989). Starch medium contained M9 medium (Sambrook *et al.*, 1989) without glucose, 1% (w/v) soluble starch (extra pure, Ishizu Pharmaceutical Co.), 50 µg ampicillin ml⁻¹, 1 mM-CaCl₂, 1 mM-MgSO₄, and 1.5% agar. Cleared zones after flooding with iodine solution (0.05% I₂ and 0.5% KI) indicated starch hydrolysis. *A. hydrophila* strains were grown at 30 °C in LB or starch medium. Linear malto-oligosaccharides [glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6)], α -amylase from *Bacillus* species and β -amylase from barley were purchased from Sigma.

Construction and screening of the DNA library. All DNA manipulations were carried out in accordance with manufacturers' instructions and as described by Sambrook *et al.* (1989). *A. hydrophila* chromosomal DNA was prepared as described previously (Chen *et al.*, 1991). For the construction of the DNA library, chromosomal DNA from *A. hydrophila* was partially cleaved with *Sau*3A, and fragments of 5–20 kb were isolated by sucrose density gradient centrifugation. The DNA fragments were ligated with T4 DNA ligase to *Bam*HI-digested plasmid pBR322, and the ligation mixture was used to transform *E. coli* JA221 cells made competent by treatment with CaCl₂. After replica-plating on LB agar medium containing ampicillin and tetracycline, about 90% of the clones were tetracycline sensitive (Tc^s), indicating insertion of a foreign DNA fragment. A total of 25000 ampicillin-resistant (Ap^r) Tc^s transformants were kept in a collection. The gene bank was used to transform *E. coli* JA221 and transformants were selected on amylase-selective plates containing starch medium supplemented with 20 µg thiamine ml⁻¹ and 0.01% each of leucine, tryptophan, and threonine. Although *E. coli* has been shown to contain an α -amylase (MalS), which is able to degrade small malto-oligosaccharides (Freundlieb & Boos, 1986), *E. coli* JA221 transformants grew poorly on amylase-selective plates. Colonies growing well on these plates after 16 h of incubation at 37 °C, were isolated and amylase-positive clones were further identified by the production of cleared zones after flooding with iodine solution.

Enzyme activity measurements. Amylase activity was determined at 37 °C, using 0.5% soluble starch as a substrate in 50 mM-sodium phosphate buffer, pH 6.8, containing 5 mM-CaCl₂. The release of reducing sugar from soluble starch was measured with 3,5-dinitrosalicylic acid. One unit of amylase was defined as the amount of enzyme producing 1 µmol of reducing sugar in 1 min as a result of hydrolysis of soluble starch, assayed using glucose as the standard. β -Galactosidase and β -lactamase activities were assayed as described previously (Chang *et al.*, 1992). Protein concentrations were determined using a protein assay kit (Bio-Rad) with bovine serum albumin as standard.

Cellular fractionation. Extracellular, periplasmic and cytoplasmic cell fractions were prepared according to Cornelis *et al.* (1982). An overnight culture of *E. coli* was inoculated into 200 ml of starch medium supplemented with 20 µg of thiamine ml⁻¹ and 0.01% each of

leucine, tryptophan and threonine. The culture was incubated at 37 °C with vigorous shaking. The cells were harvested at the early stationary phase and washed with 10 mM-Tris/HCl (pH 8.0) containing 25% (w/v) sucrose. The washed cells were suspended in the same solution with 1 mM-EDTA, and the suspension was shaken for 10 min at room temperature. After centrifugation at 6000 g for 15 min, the cells were quickly and vigorously suspended in ice-cold water. The suspension was further shaken for 10 min at 4 °C and centrifuged at 8000 g for 10 min. Cells were resuspended in 10 mM-Tris/HCl (pH 8.0) and disrupted by sonication. The extracellular enzyme fraction was the sum of the activities in the culture supernatant, and the wash. The periplasmic fraction was the activity found in the supernatant after treatment with cold water. The cytoplasmic fraction was the activity found in the supernatant after sonication.

Purification, amino-terminal amino acid sequence and amino acid analysis of amylase. *A. hydrophila* or *E. coli* JA221 harbouring plasmids pBR322 or pCA101 was grown to stationary phase in 2 l LB medium containing 50 µg ampicillin ml⁻¹, and the extracellular and periplasmic cell fractions were prepared as described above. α -Amylases from the culture supernatant of *A. hydrophila* and from *E. coli* periplasmic or extracellular preparations were purified as follows. The starting material was fractionated with finely powdered ammonium sulphate, and the 25–50% of ammonium sulphate-precipitated material was resuspended in 30 mM-Tris/HCl buffer (pH 8.0) before being applied to a PD-10 column (Pharmacia) to remove salts. The desalting materials were applied to a DEAE-Sepharose CL-6B column (2.6 × 15 cm) equilibrated with 30 mM-Tris/HCl buffer (pH 8.0). The enzyme was eluted with 500 ml of a linear gradient of 0–500 mM-NaCl in the same buffer. The eluted enzyme fraction was concentrated on a membrane (YM3; Amicon) and then subjected to gel filtration on a Sephadex G-100 column (2.2 × 100 cm) that had been equilibrated with 30 mM-Tris/HCl buffer (pH 8.0). The α -amylase was eluted from the column with the same buffer at a flow rate of 20 ml h⁻¹, and the active fractions were collected. Purification was monitored by SDS-PAGE (Laemmli, 1970). The amino-terminal amino acid sequence of α -amylase was determined with an ABI 477A-120A Protein Sequencer after extensive dialysis of the sample against distilled water. The α -amylase was hydrolysed *in vacuo* with 6 M-HCl at 150 °C for 1.5 h, and amino acid analysis was carried out with a Beckman System 6300 High Performance Amino Acid Analyser.

DNA sequencing. All sequences were determined on denatured double-stranded DNA templates by the dideoxy-chain termination method (Sanger *et al.*, 1977), using a Sequenase 2.0 DNA sequencing kit (USB) and [α -³⁵S]dATP [sequencing grade, 12.5 mCi ml⁻¹ (463 MBq ml⁻¹); specific activity, 1000–1500 Ci mmol⁻¹ (37–55.5 TBq mmol⁻¹); New England Nuclear]. Universal and reverse primers were used to obtain the initial sequences within the inserts, and then specific primers for the sequences within the inserts were generated. Conditions for DNA sequencing were as described in the Sequenase brochure (USB). Denaturing PAGE (6%, w/v, polyacrylamide) at 60 W was used to separate the reaction products. The gels were exposed to X-ray film (Kodak Omat-AR) overnight. DNA was sequenced in both directions. In some cases, dITP was used in place of dGTP for a more reliable reading through regions of high G + C content.

Results

Cloning of *A. hydrophila* DNA encoding an extracellular amylase

To clone the gene encoding the extracellular amylase of *A. hydrophila* MCC-1, a genomic library was constructed in *E. coli* JA221. Two amylase-positive clones, designated

Fig. 1. Restriction endonuclease maps of plasmid clones harbouring the amylose gene. A, *Apa*I; B, *Bst*XI; K, *Kpn*I; N, *Nhe*I; P, *Pvu*II; S, *Sac*I; Sa, *Sal*I; Sm, *Sma*I; Sp, *Sph*I. Boxes indicate *A. hydrophila* DNA; thin lines indicate DNA from the plasmid vectors pBR322, pUC18 or pUC19; solid arrows indicate the direction of transcription of the tetracycline promoter (pCA101) or the *lacZp* promoter (pCA1019 and pCA1018); dashed arrows indicate the direction of the putative promoter of the cloned gene. Varying levels of activity are represented by + and ++; - indicates no activity.

JA221(pCA101) and JA221(pCA102), were detected in approximately 5000 transformants screened. The DNA inserts of the hybrid plasmids pCA101 and pCA102 were characterized by digestion with restriction enzymes, and physical maps showed that the two plasmid inserts were identical except that the pCA101 insert (9.0 kb) was 2 kb smaller than that of pCA102. Subcloning of various restriction fragments indicated that the amylose gene was located on the 1.7 kb *Nhe*I-*Kpn*I fragment of pCA101. This fragment was subcloned into pUC18 and pUC19 yielding plasmids pCA1018 and pCA1019, respectively (Fig. 1). Although the insert DNA fragment in pCA1018 was oriented in the opposite direction to that in pCA1019 with respect to the *lac* promoter, both plasmids expressed amylose activity in *E. coli*. In the presence of IPTG,

larger colonies were observed on starch medium plates with *E. coli* JM109(pCA1019) than with JM109-(pCA1018).

Cellular distribution of amylose in *E. coli*

Amylose from *A. hydrophila* was secreted into the medium. Only a small amount of amylose activity was measured in the cells (data not shown). To investigate whether amylose expressed from the cloned gene could be secreted by *E. coli*, the localization of amylose activity in *E. coli* JA221(pCA101) in early stationary phase was determined. As a control, *E. coli* JA221 harbouring pBR322 was cultivated under the same conditions. Cell fractions were also assayed for β -lactamase and β -galactosidase activities as markers for periplasmic and cytoplasmic enzymes, respectively. Table 1 shows that essentially no enzymic activities were detected in the culture supernatant of *E. coli* JA221(pBR322); β -lactamase was found predominantly in the periplasmic fraction and β -galactosidase in the cytoplasmic fraction. However, about 40% of total β -lactamase and 30% of total amylose activities were found in the extracellular fraction of *E. coli* JA221(pCA101), and approximately 90% of β -galactosidase in the cytoplasmic fraction.

Purification and properties of amylose

The M_r value of the purified enzyme from *A. hydrophila* was estimated to be 46000 by SDS-PAGE (data not shown). A protein with an identical M_r value was purified from osmotically shocked cells and culture medium of *E. coli* JM109 transformed with pCA1019 (data not shown).

Thin-layer chromatographic analysis revealed that the products of soluble starch hydrolysis by the purified cloned amylose from *E. coli* JM109(pCA1019) were mainly G2 and G3. When G6 was used as a substrate, hydrolysis products of the cloned amylose or *A.*

Table 1. Distribution of amylose and marker enzymes in early stationary phase growth of *E. coli* JA221(pCA101) and *E. coli* JA221(pBR322)

Experiments were performed in triplicate, and the ranges of values are given.

Plasmid	Fraction	Enzyme activity (percentage total enzyme activity)		
		Amylose	β -Lactamase	β -Galactosidase
pBR322	Supernatant	—*	10–16	—
	Periplasmic	—	80–84	—
	Cytoplasmic	—	4–6	100
pCA101	Supernatant	21–33	36–44	4–8
	Periplasmic	54–69	48–54	0–2
	Cytoplasmic	10–13	8–10	90–94

* *E. coli* JA221(pBR322) cells contained no detectable amylose activity.

GATCTCGCACC	GATCTGCCCCCTCTGCCCCCTCTGCTGCCACCGAATGGCATGAAAGC	60
ATTTTCATT	CACATTTCTTAATTAATTCAGCCTGTTCGATTTTCAACCACATCGATTTC	120
TGAATGCTAC	GAGCTGAACGAGGTTGTTTGTGTTCCGTTCTGACTACACGACAGTTATAC	180
	-35 -10	
GTCAATAACA	GATCGAAGGGGAAAATCGTCATGCACAACACACTGTTTCGAACCGCCCTG	240
	RBS METHisAsnThrLeuPheArgThrAlaLeu	10
CTGGCGGCGG	CACTCGGCTCTTTCAGCCACACTGCCAGCGCCGAAGGAGTCATGGTTCAC	300
LeuAlaAlaAla	LeuGlySerPheSerHisThrAlaSerAlaGluGlyValMetValHis	30
	24 ↑	
CTGTTTCAAT	TGGAAGTTCAACGACATCGCCAACGAGTGCAGAGACCGTCTCGGGCCCAAG	360
LeuPheGluTrp	LysPheAsnAspIleAlaAsnGluCysGluThrValLeuGlyProLys	50
GGGTTCGGT	TGGCTCCAGTTCAGCCACCGCCGAGCACAAGCAGGGCAGCCAGGTCTGG	420
GlyPheGlyGly	ValGlnValSerProProAlaGluHisLysGlnGlySerGlnValTrp	70
TGGACCGTCT	ATCAACCGGTACAGTACAAGAATTTCAACAGCTTCGGCGGATGCGAGGCA	480
TrpThrValTyr	GlnProValSerTyrLysAsnPheAsnSerPheGlyGlyCysGluAla	90
GAGCTCAGG	AGCATGATCGCCCGCTGCAACGCGGCGGGGTCAAGGTCTATGCCGACGCC	540
GluLeuArgSer	MetIleAlaArgCysAsnAlaAlaGlyValLysValTyrAlaAspAla	110
GTCTTCAACC	ACATGGCATCGGGTTCGGGCACCGCCACCGCGGCGGCAGCTACAACCTCG	600
ValPheAsnHis	MetAlaSerGlySerGlyThrAlaThrGlyGlyGlySerTyrAsnSer	130
GGGCAATACC	AATATCCCCAGTTCGGCTACAACGACTTCCATCACAGCGGCACATCACC	660
GlyGlnTyrGln	TyrProGlnPheGlyTyrAsnAspPheHisHisSerGlyAspIleThr	150
AACTACGGT	GACAGCAACAACGTCTGGAACGCGCGCCTCTACGGCCTGCCGACCTCAAT	720
AsnTyrGlyAsp	SerAsnAsnValTrpAsnGlyAlaLeuTyrGlyLeuProAspLeuAsn	170
ACCGGCTCAT	CTACGTCCAGGATCAGATCGCCACCTACATGAAGACCCTGCTCGGCTGG	780
ThrGlySerSer	TyrValGlnAspGlnIleAlaThrTyrMetLysThrLeuLeuGlyTrp	190
GGCGTGGCGG	GTTTCGCATCGATCGGCCAAGCACATGGCGCCGGCCGATGTGAAGGCC	840
GlyValAlaGly	PheArgIleAspAlaAlaLysHisMetAlaProAlaAspValLysAla	210
ATCTCGACA	AGGACAGGCCCGGCTACCTGGAGGTGATCGGCGCAGGCGGGGAG	900
IleLeuAspLys	AlaGlySerProArgAlaTyrLeuGluValIleGlyAlaGlyGlyGlu	230
TCCCCGACAT	TTCAGCCGGGCGCTACACCTACATCGACACCGTCACCGAGTTCAAATAC	960
SerProAspIle	SerProGlyArgTyrThrTyrIleAspThrValThrGluPheLysTyr	250
GGCACC	GATCTCGCCGCCAAGTTCAACGGCCAGATCAAGAACCTCAAACCCCTGGGCGAG	1020
GlyThrAspLeu	AlaAlaAsnPheAsnGlyGlnIleLysAsnLeuLysThrLeuGlyGlu	270
AGCTGGGG	CCTGCTCCCTCCAACAAGGCCTTCGTCTCGTGGACAACACGATCCCGAA	1080
SerTrpGlyLeu	LeuProSerAsnLysAlaPheValPheValAspAsnHisAspProGlu	290
CGCGCGCACG	GCGGTGGCGGCATGCTACCTTCATGCAGGGTGCCCGCTACGATCTGGCC	1140
ArgAlaHisGly	GlyGlyGlyMetLeuThrPheMetGlnGlyAlaArgTyrAspLeuAla	310
AACACCTT	CATGCTGGCTGGCCCTATGGCTGGAAGCAGGTGATGTCGGCGTATCGCTTT	1200
AsnThrPheMet	LeuAlaTrpProTyrGlyTrpLysGlnValMetSerAlaTyrArgPhe	330
GAAAACAT	TGGGCACCTATGAAACCGACAAGGGGGCGCCGGGCAGCACGCCCTGCACGGAC	1260
GluAsnMetGly	ThrTyrGluThrAspLysGlyAlaProGlySerThrProCysThrAsp	350
AGCCAGT	TGGAACGACGCGCCGACCATCATGAACATGGTCCTGTTCGCAAC	1320
SerGlnTrpAsn	CysGluGlnArgArgProThrIleMetAsnMetValLeuPheArgAsn	370
CGGACCGA	AGGCCACGCCGTGAGCAACTGGTGGGACAACGGCAACAACAGATCGCCTTC	1380
ArgThrGluGly	GlnProValSerAsnTrpTrpAspAsnGlyAsnAsnGlnIleAlaPhe	390
AGCCGCGT	TACAAGGGCTTCGTCGCCATCAACAACGAGAGCGGCACGCTGGTGGCCCTCGG	1440
SerArgGlyThr	ArgAlaSerSerProSerThrThrArgAlaAlaArgTrpTrpProArg	410
TGCAGACG	CTCGCGCCGCGAGTACTGCAACCTCCTGGGGGCAATGACTACTGCAGCG	1500
CysArgArgSer	ArgProAlaSerThrAlaThrSerTrpGlyAlaMetThrThrAlaAla	430
GCGGTTAT	TGTCACCGTCGACGGCAGCGGCAAGGCCAGCCTGAACGATTCCCCGGCATGAA	1560
AlaValMetSer	ProSerThrAlaAlaAlaArgProAla***	443
CGCGCCGC	CATCATGCCGTTTGACCAAGGCCAGCCCTGCGGCGGTTCCGTCCTGCCGG	1620
→	←	
GCAACAAG	TTTTCAGCAGCATGAACCGTGGCGCGGTAC	1656

Fig. 2. The nucleotide and deduced amino acid sequences of the amylase gene of *A. hydrophila*. Numbers refer to the nucleotide or amino acid located at the end of each line. The -10 and -35 regions of the putative promoter are indicated, as is the RBS. Converging arrows indicate inverted repeats. The signal peptidase cleavage site is indicated by a vertical arrow. The stop codon is indicated by asterisks.

deduced amino acid sequence, approximately matched those determined chemically with purified enzymes from the *E. coli* transformant (data not shown).

On the basis of homology to the *E. coli* promoter consensus sequence (McClure, 1985), putative promoter sequences were tentatively assigned to positions 152 through 157 (–35), and 176 through 181 (–10). Between the –10 and –35 regions are 18 nucleotides, the optimal spacing for such regions in this class of promoter sequences. A possible ribosomal binding site (RBS; Shine–Dalgarno sequence) (Shine & Dalgarno, 1974; Stormo *et al.*, 1982), was found to be 9 bp upstream of the presumptive start codon. The translational stop codon TAA is followed by a stable hairpin structure (free energy of $-16.8 \text{ kcal mol}^{-1}$). This structure would be expected to make it an efficient transcriptional terminator.

Codon usage patterns of the α -amylase

The G+C content of the coding region for the *A. hydrophila* MCC-1 α -amylase gene was 63 mol%, whilst that of the third position of the codons was higher (83 mol%). These values are nearly identical to those for glycerophospholipid–cholesterol acyltransferase (GCAT) [62 and 82 mol%, respectively (Thornton *et al.*, 1988)], amylase [62 and 82 mol%, respectively (Gobius & Pemberton, 1988)] and deoxyribonuclease (DNAse) [63 and 87 mol%, respectively (Chang *et al.*, 1992)], and are a little higher than that for aerolysin [58 and 72 mol%, respectively (Howard *et al.*, 1987)] from this species. The codon usage frequency in the α -amylase gene is similar to the pattern for the aforementioned genes of this species (data not shown).

Comparison of amino acid sequence with other α -amylases

Analysis of the predicted polypeptide sequence of the ORF for homology to other sequences in the GenBank database, using the FASTP algorithm of Lipman & Pearson (1985), revealed significant homology to a number of α -amylases from both prokaryotes and eukaryotes. There was a remarkable degree of similarity with α -amylases from mammalian, invertebrate and streptomycete sources (data not shown). Fig. 3 shows that the *A. hydrophila* α -amylase amino acid sequence is similar to those from *Homo sapiens* (human salivary α -amylase; Nishide *et al.*, 1986), *Sus scrofa domestica* (pig pancreas; Kluh, 1981), *Rattus norvegicus* (Norway rat pancreas; MacDonald *et al.*, 1980), *Mus musculus* (mouse pancreas; Hagenbuechle *et al.*, 1980), *Drosophila melanogaster* (Boer & Hickey, 1986) and *Streptomyces* species (Hoshiko *et al.*, 1987; Virolle *et al.*, 1988). In addition to

regions corresponding to the four highly conserved regions (regions I, II, III and IV) reported for other α -amylases (Nakajima *et al.*, 1986; Rogers, 1985), there are a number of other highly conserved regions and residues present in these α -amylases (Fig. 3). These regions include a sequence of Phe–Glu–Trp (positions 32–34 in *A. hydrophila* α -amylase), which was suggested as a possible candidate for interaction with the tendamistat inhibitor (Hofmann *et al.*, 1985).

From the three-dimensional structure of *Aspergillus oryzae* (Taka A) amylase, Matsuura *et al.* (1984) proposed that Glu-230 of region III and Asp-297 of region IV were the important catalytic residues; Tyr-82, Gln-84, Thr-85 (Pro in most α -amylases), Ile-152, Tyr-155, Val-161, Thr-170 (Leu in most other sequences), Leu-173 and Asp-175 were likely to be involved in substrate binding. Similarly, Buisson *et al.* (1987) proposed for porcine pancreatic amylase that Asp-197 and Asp-300 (in regions II and IV, respectively) were the catalytic residues, and that Asn-100 (in region I), Asp-159, Asp-167 and His-201 (in region II) were the calcium-binding residues. All residues corresponding to these catalytic and substrate binding residues, and three of four calcium-binding residues are clearly present in *A. hydrophila* α -amylase (Fig. 3).

Discussion

The gene encoding the extracellular amylase of *A. hydrophila* MCC-1 has been successfully cloned into *E. coli*, and the gene product readily expressed in *E. coli* under the control of its own promoter. The nucleotide sequence analysis revealed a single ORF of 1329 nucleotides. The deduced M_r value of the mature protein, 46000, correlates well with the value determined by SDS-PAGE of the purified α -amylase from *A. hydrophila*. There were no apparent differences in the enzymic characteristics of the purified α -amylase from *A. hydrophila* and that obtained from *E. coli* containing the cloned amylase gene. Moreover, sequences of the 10 amino-terminal amino acids of both proteins were identical, and matched residues 25–34 of the deduced amino acid sequence. A 24 amino acid amino-terminal sequence has many of the characteristics common to the signal sequences of proteins processed by the signal peptidase I type of pathway (Von Heijne, 1988). It has a positively charged amino acid (His) adjacent to a methionine residue at the amino terminus, followed by a hydrophobic amino acid core and Ala at the carboxy terminus. The cleavage sites between Ala and Glu, and the surrounding residues are in accordance with the –3, –1 rule proposed by Von Heijne (1984).

Cloning in *E. coli* of an amylase gene from another *A. hydrophila* strain, JMP636, has been reported (Gobius &

Pemberton, 1988), but whether this amylase belongs to the α -type is unknown. Our cloned α -amylase, which is identified as an α -type, apparently differs from the amylase cloned from strain JMP636. Based on the nucleotide or amino acid sequence, significant divergence exists in JMP636 amylase (data not shown), even though the similarity of codon usage frequencies in the translated reading frame of both amylase genes was observed. Because there is insufficient information on the enzymic properties of these two *A. hydrophila* amylases, it is not clear whether *A. hydrophila* secretes two amylases having the same size but differing considerably in enzymic properties and sequence, or whether the strain used by us or by Gobius & Pemberton is not *hydrophila*. A distinctive property is that the JMP636 amylase gene product was only exported to the periplasm in *E. coli*. Some of the cloned α -amylase was present in the supernatant fluid of cells in early stationary phase (Table 1).

Based on analysis of the deduced amino acid sequence of the JMP636 amylase gene and localization of the cloned amylase in *E. coli*, Gobius & Pemberton (1988) predicted that this amylase contained an amino-terminal signal peptide which was removed during its translocation across the cytoplasmic membrane of *E. coli*. They proposed that this enzyme does not contain sufficient information in either the signal peptide or the mature protein to ensure translocation across the outer membrane of *E. coli*. Our data indicate that α -amylase contains a putative signal peptide and that the periplasmic form of the α -amylase assumes a conformation identical to that of α -amylase secreted by *A. hydrophila* MCC-1. The presence of extracellular amylase activity could be due to leakage of the periplasmic proteins through the outer membrane into the supernatant fluid, since approximately 35% of the periplasmic control enzyme, β -lactamase, was also detected in the extracellular fluid. In contrast, the β -galactosidase was predominantly detected in the cytoplasmic fraction, indicating that leakage of cytoplasmic proteins did not occur. These results suggest that α -amylase readily translocates protein across the cytoplasmic membrane and subsequently leaks to the extracellular fluid. Over-expression of the *Bacillus stearothermophilus* α -amylase gene in *E. coli* causes outer membrane damage leading to extracellular secretion of periplasmic proteins (Suominen *et al.*, 1987).

Nakajima *et al.* (1986) identified four conserved regions in the amino acid sequences of eleven α -amylases from a wide range of organisms. The alignment of the amino acid sequence of *A. hydrophila* MCC-1 α -amylase with seven other α -amylases showed not only four regions highly conserved in all enzymes, but also suggested the conservation of several additional regions.

Matsuura *et al.* (1984) proposed a model in which the amylose chain fits into a large cleft that contains the active site of the fungal α -amylase and identified several amino acid residues, which lie on both sides of the cleft which were likely to be involved in substrate binding. These putative active sites and substrate binding residues (Fig. 3) suggest that these residues play important roles in catalytic function and substrate binding. Mammalian α -amylases are inhibited by tendamistat (Hofmann *et al.*, 1985). This inhibition profile is extremely similar to that of the *Streptomyces limosus* α -amylase (Long *et al.*, 1987). The sequence Phe-Glu-Trp, observed in the amino-terminal part of α -amylases from mammals, invertebrates, *S. limosus* and *S. venezuelae*, but absent from the amylases of fungi, plants and most eubacteria, has been suggested as a possible candidate for interaction with the inhibitor. This triplet is also present in the *A. hydrophila* α -amylase sequence at amino acid positions 32–34. It would be of interest to determine the susceptibility of this enzyme to tendamistat. Three putative calcium-binding residues were also observed in the *A. hydrophila* α -amylase, which are consistent with the fact that this enzyme requires calcium ions for activity. In addition, the alignment also revealed several additional stretches of complete identity or a high degree of homology that are present in all eight α -amylases. However, the functional significance of these is unclear.

Comparison of the amino acid sequence of α -amylase from *A. hydrophila*, mammals, invertebrates and streptomycetes revealed that considerable homology extends throughout the entire mature regions, whilst there were no significant similarities between the *A. hydrophila* enzyme and α -amylases derived from *Hordeum vulgare* (barley; Rogers & Milliman, 1983), *A. oryzae* (Toda *et al.*, 1982) and *Bacillus* spp. (Yang *et al.*, 1983), except for four highly conserved regions and a very limited sequence. The amino acid sequence of streptomycete α -amylase has a high degree of similarity with sequences of mammalian and invertebrate amylases which have been reported by Long *et al.* (1987) and Virolle *et al.* (1988). Long *et al.* (1987) suggest that these α -amylases are derived from a common ancestral gene. Our results suggested that these amylases, including that of *A. hydrophila* MCC-1 α -amylase, probably have a common evolutionary origin. However, it remains to be determined whether the mammalian, invertebrate, streptomycete and *Aeromonas* enzymes are derived from an ancestral primitive gene with a recently evolved branch point, or whether the remaining α -amylases have an extremely divergent evolution from an ancient co-ancestral gene.

The authors wish to thank Dr C. G. Kuo for critical reviews of the manuscript. This work was supported by research grants NSC 81-0412-

B-006-524 from the National Science Council, Taiwan, Republic of China.

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