An *Aspergillus awamori* acetylesterase: purification of the enzyme, and cloning and sequencing of the gene

Takuya KOSEKI*[1,2], Shinji FURUSE†, Kimio IWANO*, Hiroshi SAKAI‡ and Hiroshi MATSUZAWA‡

*National Research Institute of Brewing, 3-7-1 Kagamiyama, Higashihiroshima 739, Japan, †General Research Laboratory, Ozeki Corporation, 4-9 Imazu Dezaike-cho, Nishinomiya, Hyogo 663, Japan, and ‡Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan

An inducible acetylesterase was purified from the culture medium of *Aspergillus awamori* strain IFO4033 growing on wheat-bran culture by ion-exchange, gel-filtration and hydrophobic-interaction chromatographies. The purified enzyme had an M_r of 31000 and contained Asn-linked oligosaccharides. The enzyme liberated acetic acid from wheat bran, hydrolysed only α-naphthyl acetate and propionate when aromatic esters were used for the substrate, and was tentatively classified as a carboxylic esterase (EC 3.1.1.1). The gene encoding acetylesterase was cloned and sequenced. The deduced amino acid sequence showed that acetylesterase was produced as a 304-amino-acid-residue precursor, which was converted post-translationally into a 275-amino-acid-residue mature protein. Part of the sequence of acetylesterase was similar to the region near the active-site serine of lipases of *Geotrichum candidum* and *Candida cylindracea*. A unique site of putative Asn-linked oligosaccharides was presented.

INTRODUCTION

Xylan-degrading enzymes such as endoxylanases and β-xylosidases greatly participate in biodegradation of hemicelluloses, and are very useful to agricultural and industrial processes. Degradation of hemicelluloses also requires enzyme activities that remove non-xylose substituents, such as *O*-acetyl, arabinose and ferulic, *p*-coumaric and uronic acids, from the xylan backbone, in addition to endoxylanases and β-xylosidases. Acetylxylan esterase activity, capable of cleaving off the *O*-acetyl groups from xylose residues in xylan, has been detected in many cellulolytic and hemicellulolytic micro-organisms, such as *Trichoderma reesei* [1], *Schizophyllum commune* [2,3], *Fibrobacter succinogenes* [4] and *Thermoanaerobacterium* sp. [5]. Efficient and complete degradation of arabinoxylan requires the co-operation of the esterases, as well as other xylanolytic enzymes. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is one of the phenolic acids present in plant cell walls such as those of wheat, barley and rice, and has been shown to be esterified with arabinose in the arabinoxylans of hemicellulose. In addition to hemicellulose, some other polysaccharides are also ferulated, e.g. pectins in sugar beet [6,7] and spinach [8]. A compound released by treatment of wheat bran with *Oxysporus* cellulase was identified as 2-*O*-[5-*O*-(*trans*-feruloyl)-β-L-arabinofuranosyl]-D-xylopyranose [9]. In barley straw, *trans*-*p*-coumaric acid (4-hydroxycinnamic acid) is also linked to arabinoxylans in the same way as *trans*-ferulic acid. Ferulic acid esterases have been purified and characterized from several micro-organisms, including *Streptomyces olivochromogenes* [10], *Aspergillus orysae* [11] and *Aspergillus niger* [12,13], and the specificities of these ferulic acid esterases for a range of methyl ester derivatives of cinnamoyl and benzoyl acids have been examined [13]. The ferulic acid esterase from *A. orysae* releases ferulic, *p*-coumaric and acetic acids from purified wheat-straw arabinoxylan [11].

Therefore, in order to elucidate the role, in the hydrolysis of arabinoxylan, of the acetyl and ferulic acid esterases from *Aspergillus awamori*, which is used widely in *koji*-making (solid-state culture of the mould using rice as the substrate) for *awamori*, a kind of traditional Japanese spirits (*shochu*), we purified an *A. awamori* acetylesterase (ACEA) from culture supernatants. In this report, we describe the purification and characterization of the enzyme, and then the molecular cloning and sequencing of the *aceA* gene encoding it.

MATERIALS AND METHODS

Organism and growth conditions

The *A. awamori* strain IFO4033, which is very important in the fermentation of such Japanese spirits as *awamori*, was used in this study. It was grown for 5 days at 30 °C in 0.1 % (w/v) bactotryptone/0.5 % (w/v) yeast extract/0.3 % (w/v) sucrose/0.1 % (w/v) $NaNO_3$/0.001 % (w/v) $FeSO_4$,$7H_2O$, and one of four carbon sources [glucose, xylose, oat spelt xylan and wheat bran, all at a concentration of 2 % (w/v)]. Cultures used for carboxyesterase production, containing approx. 6×10^5 spores in 150 ml of medium, pH 5.5, were shaken at 120 rev./min in 500 ml Erlenmeyer flasks.

Enzyme assays

Acetylesterase activity was assayed as follows: 0.15 ml of 5 mM α-naphthyl acetate dissolved in methanol was added to 1.34 ml of 100 mM sodium phosphate buffer (pH 7.0), and then 0.01 ml of enzyme source was added. The reaction mixture was incubated for 15 min at 37 °C, and terminated by addition of 1.5 ml of 10 % (w/v) SDS containing 0.01 % (w/v) Fast Garnet GBC (Sigma). After incubation at room temperature for 15 min, the optical density at 560 nm was measured [14]. The standard curve was prepared by using α-naphthol. One unit of the enzyme activity

Abbreviation used: ACEA, *Aspergillus awamori* acetylesterase.

[1] Present address: Office of Brewing Technology, Kanto-Shinetsu Regional Taxation Bureau, 1-1-15 Kudanminami, Chiyoda-ku, Tokyo 102, Japan.

[2] To whom correspondence should be addressed.

The nucleotide sequence of the *aceA* gene has been submitted to the DDBJ/EMBL/GenBank Nucleotide Sequence Database under the accession number D87681.

was defined as the amount of enzyme that releases 1 μmol of α-naphthol/min under the above assay conditions.

Ferulic acid esterase activity was assayed with ferulic acid/arabinose/xylose polymer as the substrate, as described by McCallum et al. [15]. One unit of the enzyme activity was defined as the amount of enzyme that releases 1 μmol of ferulic acid/min at pH 6.0 and 50 °C.

Xylanase activity was measured as described by Ito et al. [16]. Culture supernatant (0.1 ml) was added to 0.4 ml of xylan (oat spelt) solution [2 % (w/v) in 50 mM acetate buffer solution (pH 5.0)]. The reaction mixture was incubated for 30 min at 37 °C, and the released reducing sugar was measured by the Somogyi–Nelson method [17] with xylose as the standard. One unit of activity was defined as the amount of xylanase needed to liberate 1 μmol of xylose/min under these assay conditions.

The protein concentration was estimated with the Bio-Rad protein assay kit using BSA (Sigma) as the standard.

Enzyme purification

Step 1. Ammonium sulphate precipitation. The culture was centrifuged at 5000 ***g*** for 15 min to exclude mycelia, and the supernatant was subjected to 40 %-satd.- and 80 %-satd.-$(NH_4)_2SO_4$ fractionation. Step 2. Anion-exchange chromatography. The pellet, after 80 % $(NH_4)_2SO_4$ saturation, was resuspended in 50 mM sodium acetate buffer (pH 6.0), and applied to a Sephadex G-25 NAP-10 column (Pharmacia), preconditioned with the above buffer. After desalting, the crude enzyme solution was added to an HPLC anion-exchange column of DEAE-5PW (21.5 mm × 15 cm; Tosoh Co., Tokyo, Japan), equilibrated with 50 mM sodium acetate buffer (pH 6.0). The sample was eluted with stepped increases in NaCl from 0 to 1.2 M in 50 mM sodium acetate buffer (pH 6.0) at a flow rate of 3 ml/min. Fractions (3 ml each) were collected. Step 3. Gel filtration. The pooled active fractions were concentrated by ultrafiltration and applied on to a gel-filtration column of G2000-SW_{XL} (7.8 mm × 30 cm; Tosoh Co., Tokyo, Japan), and the column was eluted with 20 mM sodium acetate buffer (pH 6.0) containing 0.2 M NaCl at a flow rate of 0.5 ml/min. Fractions (0.5 ml each) were collected and enzyme activities were measured as described below. Step 4. Hydrophobic interaction chromatography. The pooled and concentrated active fractions from the gel-filtration column were fractionated on an HPLC hydrophobic interaction column of Phenyl-5PW (21.5 mm × 15 cm; Tosoh Co., Tokyo, Japan) using a 100–0 %-satd.-$(NH_4)_2SO_4$ gradient in 50 mM sodium phosphate buffer (pH 7.0), and 1.7 M $(NH_4)_2SO_4$ supplemented with the above additives at a flow rate of 3 ml/min. Fractions (3 ml each) were collected.

M_r determination

M_r values were estimated by SDS/PAGE using a 10–20 % (w/v) gradient gel (Daiichi Pure Chemicals Co., Tokyo, Japan) by the method of Laemmli [18] and by gel filtration. Proteins were detected with Coomassie staining (Bio-Rad R-250). M_r values were estimated from a plot of log M_r versus mobility using the following standards: phosphorylase *b* (94000), BSA (67000), ovalbumin (43000), carbonic anhydrase (30000), soya bean trypsin inhibitor (20000) and α-lactalbumin (14000).

Endoglycosidase H treatment

To eliminate possible Asn-linked carbohydrate, the enzyme was treated with endoglycosidase H (endo-β-*N*-acetylglucosaminidase H of *Streptomyces plicatus*; Boehringer Mannheim, Germany) as described by Trimble and Maley [19]. An enzyme sample (10 μg of the enzyme in a total volume of 30 μl) in 50 mM sodium phosphate buffer (pH 6.0) was boiled for 10 min in the presence of 0.02 % (w/v) SDS. After cooling, 0.5 m-units of endoglycosidase H was added and the samples were incubated at 37 °C overnight. The products of endoglycosidase H treatment were analysed by SDS/PAGE.

pH optima and stability, and thermal stability

The pH optimum of the purified esterase was determined by performing the assay in pH 3–7 (McIlvaine buffer), pH 7–8 (sodium phosphate buffer), and pH 8–9 (Tris/HCl buffer), all at 100 mM and 37 °C. To analyse the pH stability of acetylesterase activity, the pH of this solution was adjusted using the above-mentioned buffers for pH 3–9 and for pH 9–11 potassium and sodium borate buffer, after which it was incubated for 1 h at 37 °C. The residual acetylesterase activity was assayed at pH 7.0 (100 mM sodium phosphate buffer).

The thermal stability was determined by performing the assay in 100 mM sodium phosphate buffer, pH 7.0, at temperatures of 30, 40, 50, 60 and 70 °C. The residual activity was analysed after 1 h.

Effects of chemical reagents on acetylesterase activity

The effects of chemical reagents on acetylesterase activity was investigated by addition of various compounds to a final concentration of 1 and 10 mM in the reaction mixture usually used for the determination of the enzyme.

Amino acid sequence analysis

Amino acid sequences were analysed by Edman degradation using an automated protein sequencer (PSQ-2, Shimadzu, Kyoto, Japan). Lysylendopeptidase (Wako Pure Chemical Industries, Tokyo, Japan)-digested peptides of acetylesterase were purified by HPLC.

Cloning of the acetylesterase gene

On the basis of the N-terminal amino acid sequence of the intact enzyme and an amino acid sequence of the peptide, two oligonucleotide probes were synthesized to identify by Southern blot analysis the *aceA* gene in DNA restriction fragments from *A. awamori*. A 29-mer degenerate oligonucleotide, designated ACE-1 (5′-TCYGGYTCYCTYCAGCAGGTYACYGAYTT-3′), was designed from a chosen region of the N-terminal amino acid sequence. A 23-mer oligonucleotide mixture based on the internal amino acid sequence, designated ACE-3 (5′-ATGGARTGGT-TYGGYTTYGCYAA-3′), was also designed. A Southern blot of *A. awamori* genomic DNA digested to completion with various restriction enzymes was hybridized at 42 °C as described by Gomi et al. [20] with the ^{32}P-labelled probe. A genomic library was constructed from the *A. awamori* strain IFO4033 by ligating partially *Sau*3AI-digested genomic DNA fragments into the λ replacement vector λEMBL3 cut with *Bam*HI (Stratagene, La Jolla, CA, U.S.A.). The genomic library was screened under low-stringency conditions using ACE-1 or ACE-3 as a probe. Phage DNA mini-preparations were obtained as detailed in Sambrook et al. [21]. Isolated DNA was digested with a restriction enzyme (*Bam*HI, *Eco*RI, and *Sal*I), separated on a 0.8 % (w/v) agarose gel, alkaline-blotted to a Hybond-N^+ membrane (Amersham) and probed at low stringency.

RESULTS AND DISCUSSION

Production of esterases and xylanase

A. awamori strain IFO4033 was grown on various carbon sources as described in the Materials and methods section. The cells were harvested every 24 h for 5 days. Culture supernatants were obtained through removing the cells by centrifugation. Enzyme activity in the culture supernatants was assayed for acetylesterase, ferulic acid esterase and xylanase. Productions of the enzymes were nearly at a maximum at 4 days of culture and highly dependent on the type of carbon sources (Table 1). Acetylesterase activity was produced by all carbon sources except glucose and was 5-fold higher with oat spelt xylan than with wheat bran and xylose. Ferulic acid esterase activity was highest with wheat bran, moderate with oat spelt xylan and absent with xylose and glucose. *A. niger* also produced the highest level of ferulic acid esterase with wheat bran when examined on the same carbon sources [22]. Xylanase activity was highest with oat spelt xylan, moderate with wheat bran and xylose, and absent with glucose. These results suggest that the carbon sources specifically influence the induction of acetylesterase, ferulic acid esterase and xylanase produced by *A. awamori*.

Purification and properties of ACEA

Table 2 summarizes a typical purification of ACEA from *A. awamori*. A 22-fold purification, with a yield of 20% and a specific activity of 13.2 units/mg of protein, was obtained after these steps. The purified ACEA from the hydrophobic-interaction chromatography column migrated on an SDS gel as a single band with an M_r of 31000 (Figure 1, lane 2). After endoglycosidase H treatment, the acetylesterase exhibited an increase in mobility on SDS/PAGE (Figure 1, lane 3).

Table 1 Effects of carbon sources on the production of esterases and xylanase from *A. awamori* IFO4033

Enzyme activity is in units of enzyme activity per ml of culture; n.d., no detectable activity. Values are for 4-day cultures.

Carbon source	Protein (mg/ml)	Enzyme activity (units/ml)		
		Acetylesterase	Ferulic acid esterase	Xylanase
Wheat bran	0.3	0.2	0.8	8.7
Oat spelt xylan	0.4	1.0	0.3	23.1
Xylose	0.3	0.2	n.d.	13.9
Glucose	0.5	n.d.	n.d.	n.d.

Table 2 Purification of *A. awamori* acetylesterase

After *A. awamori* was grown on wheat-bran culture for 3 days at 30 °C, the supernatant was used for the purification. The volume of culture supernatant was 750 ml.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Culture supernatant	235.1	150.0	0.6	100	1.0
$(NH_4)_2SO_4$ fractions (40–80%)	98.8	108.7	1.1	74.5	1.8
DEAE-5PW	26.3	68.4	2.6	49.0	4.3
G-2000SW_{XL}	12.7	54.6	4.3	38.7	7.2
Phenyl-5PW	2.3	30.5	13.2	20.3	22.0

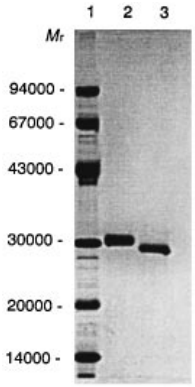

Figure 1 SDS/PAGE of purified ACEA and endoglycosidase-H-treated ACEA

Lane 1, M_r standards, consisting of phosphorylase *b* (94000), BSA (67000), ovalbumin (43000), carbonic anhydrase (30000), soya bean trypsin inhibitor (20000) and α-lactalbumin (14000); lane 2, purified ACEA; lane 3, endoglycosidase-H-treated ACEA.

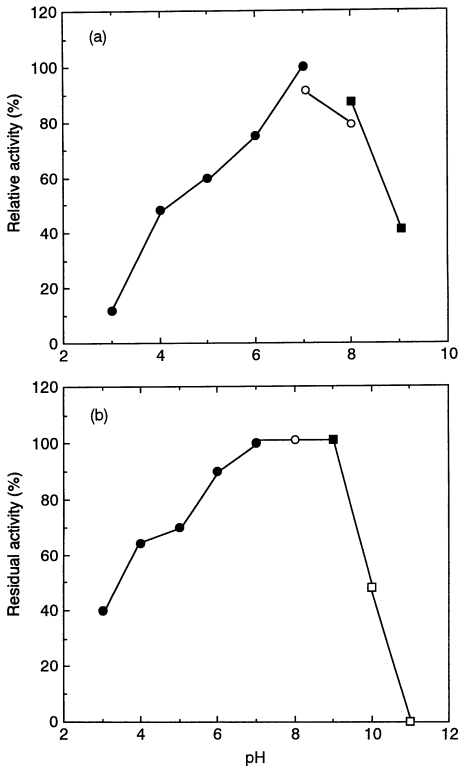

Figure 2 Effects of pH on the (a) activity and (b) stability of ACEA

(**a**) The enzyme activity was measured in McIlvaine buffer (pH 3–7), sodium phosphate buffer (pH 7–8) and Tris/HCl buffer (pH 8–9). (**b**) The residual enzyme activity was measured at pH 7.0 after incubation for 1 h at 37 °C and at the indicated pHs. The concentration of the enzyme in the reaction mixture was 0.05 unit/ml for both (**a**) and (**b**) at 37 °C. (●) McIlvaine buffer; (○) sodium phosphate buffer; (■) Tris/HCl buffer; (□) potassium borate buffer.

Endoglycosidase H hydrolyses Asn-linked high-mannose oligosaccharides, and thus at least part of the carbohydrate was Asn-linked. The presence of Asn-linked oligosaccharides in acetylxylan esterases of *T. reesei* has been reported [1].

The esterase had a pH optimum of 7.0 (Figure 2a) using 5 mM α-naphthyl acetate as the substrate. Regarding the effects of pH

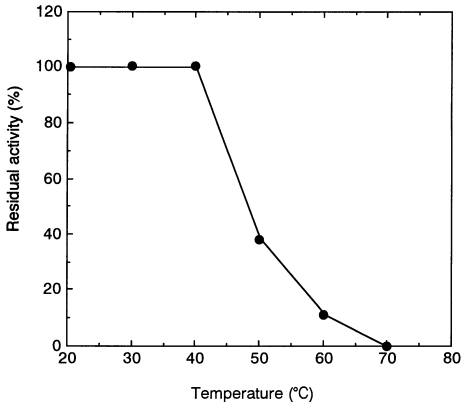

Figure 3 Effect of temperature on the stability of ACEA

The residual enzyme activity was measured after incubation of aliquots of the enzyme in 100 mM sodium phosphate buffer (pH 7.0) at 20–70 °C for 1 h. The concentration of the enzyme in the reaction mixture was 0.05 unit/ml.

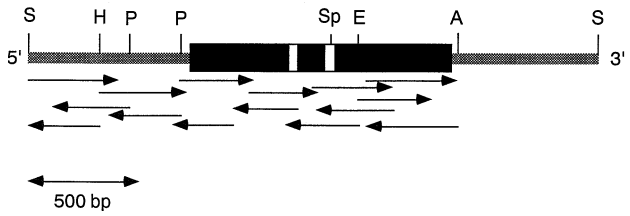

Figure 4 Restriction map of the genomic *aceA* DNA and the sequence strategy

The protein coding region is indicated by the solid (exons) and open (introns) boxes. A, *Acc*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sp, *Sph*I.

and temperature on acetylesterase stability, the enzyme activity was significantly retained from pH 6.0 to 9.0 (Figure 2b) and decreased at temperatures higher than 40 °C (Figure 3).

The acetylesterase activity was fully inhibited by 1 mM di-isopropyl fluorophosphate and 10 mM PMSF, suggesting that the enzyme is a serine esterase. In these experiments, the enzyme (100 ng/ml) was first incubated 30 min at 37 °C in the presence of the inhibitors, and then the remaining acetylesterase activity was measured with the enzyme assay methods described in the Materials and methods section.

The purified enzyme liberated acetic acid from destarched wheat bran. However, the ACEA did not liberate ferulic acid from destarched wheat bran, and also did not liberate fatty acids from α-naphthyl esters longer than butyrate (results not shown).

The N-terminal sequence obtained from the purified ACEA was Ser-Gly-Ser-Leu-Gln-Gln-Val-Thr-Asp-Phe-Gly-Asp-Asn-Pro-Pro-Asn-Val-Gly-Val (APN). The N-terminal sequences of the peptides that were obtained after treatment with lysyl-endopeptidase were Gln-Trp-Ala-Gly-Val-Phe-Gly-Tyr-Asp-Tyr (AP1), Xaa-Met-Glu-Trp-Phe-Gly-Phe-Ala (AP2) and Thr-Glu-Ala-Asn-Thr-Pro-Gln-Thr-Asn-Tyr-Glu-Thr-Thr-Ile-Val-Gly-Asp-Leu-Gln (AP3).

Cloning of the acetylesterase gene

The *aceA* gene was isolated through hybridization using degenerate oligonucleotides designed from the peptide sequences. A 29-mer degenerate oligonucleotide, designated ACE-1, was designed from the N-terminal sequence, Ser-Gly-Ser-Leu-Gln-Gln-Val-Thr-Asp-Phe, of APN. As a secondary probe, ACE-3 was also designed from the sequence, Met-Glu-Trp-Phe-Gly-Phe-Ala, of AP2. The genomic DNA was partially digested with *Sau*3AI in order to produce 10–20 kb fragments. The digested DNA was ligated to *Bam*HI-cut dephosphorylated EMBL3 vector DNA arms. The ligated DNA was packaged *in vitro* using Gigapack II Gold packaging extracts (Stratagene). The packaged phage was used to infect *Escherichia coli* strain P2392, and a total of 10000 plaques were screened with ACE-1 as a probe. Three strongly hybridizing plaques were picked for further analysis. The bacteriophage DNAs were isolated using LambdaSorb Phage Adsorbent (Promega), and the DNAs digested with restriction endonucleases were subjected to a Southern blot analysis using ACE-3 as a probe. A 2.5-kb *Sal*I DNA fragment was isolated from one of the clones and subcloned into pUC119. The restriction map is shown in Figure 4.

Nucleotide sequence of *aceA* and deduced amino acid sequence

Figure 5 shows the determined nucleotide sequence of the 1.8-kb *Sal*I–*Acc*II fragment including the *A. awamori aceA* gene and the deduced amino acid sequence. Two introns predicted on the basis of the fungal consensus sequences, such as donor (GTRNGT), lariat (RTCRAC) and acceptor (YAG) sequences [23], are indicated by broken underlines. A protein of 304 amino acid residues was deduced from the coding region. Four amino acid sequences (APN, AP1, AP2 and AP3) determined chemically on ACEA were found in the deduced amino acid sequence, as shown by boxes in Figure 5. The deduced amino acid sequence showed that mature ACEA was preceded by a putative leader peptide of 29 amino acid residues and that the M_r of the putative mature protein was calculated to be 29416. The leader peptide was a little longer than usual fungal signal sequences [24,25]. Two basic residues were located at the C-terminus of the leader peptide, and thus the cleavage of the bond between Arg^{-1} and Ser^{1} should take place by a KEX2-like processing protease [26]. The putative ACEA contained a unique site of N-glycosylation (Asn^{161}), suggesting that an Asn-linked high-mannose oligosaccharide (Figure 1) is linked with Asn^{161}.

In the 5′-flanking region of the *aceA* gene, there were a typical TATA sequence and two CAAT-like sequences at -99, -123 and -170 upstream of the translation start codon ($+1$) respectively. There was an AATGAA sequence, which is related to the putative polyadenylation signal AATAAA, 12 nucleotides downstream of the stop codon.

Amino acid sequence similarity

Similarity with the deduced amino acid sequence of ACEA was searched for in the SWISS-PROT sequence database. The ACEA showed an identity of 95% with the reported acetylxylan esterase of fungus *Aspergillus niger* [27]. No similarities were found with the published acetylxylan esterases of *Caldocellum saccharolyticum* [28], *Pseudomonas fluorescens* subsp. *cellulosa* [29], *Streptomyces lividans* [30] and *T. reesei* [31].

As described above, the enzyme activity was fully inhibited by di-isopropyl fluorophosphate and PMSF, so the serine residue seemed to be one of the active-site residues. The consensus sequence near the active-site serine of serine enzymes, proteases, lipases and esterases is known to be Gly-Xaa-Ser-Xaa-Gly [32]. On the basis of the Gly-Xaa-Ser-Xaa-Gly sequence, Ser^{119} and Ser^{146} in ACEA were predicted to be the site (Figure 5). By

computer analysis, only the part of the sequence containing Ser^{119} of ACEA was found to be about 28 % identical with the sequences near the active-site serine of two lipases of *Geotrichum candidum* [33,34] and a lipase of *Candida cylindracea* [35] (Figure 6). However, part of the sequence containing Ser^{146} of ACEA did not show appreciable similarities with any serine enzymes. These results suggest Ser^{119} of ACEA is one of the catalytic residues. However, ACEA did not show lipase activity, suggesting that substrate recognition in ACEA differs from those in lipases.

We thank Dr. K. Ito and Dr. H. Iefuji for kindly providing purified xylanase and helpful advice.

Figure 6 High similarity region of the amino acid sequence of ACEA with those of microbial lipases

Numbering of amino acid residues from the N-terminus of the mature enzyme is shown. Identical amino acid residues are shown in white on a black background. Putative active serine residues are indicated by asterisks. GCL1, *G. candidum* lipase 1; GCL2, *G. candidum* lipase 2; CCL, *C. cylindracea* lipase.

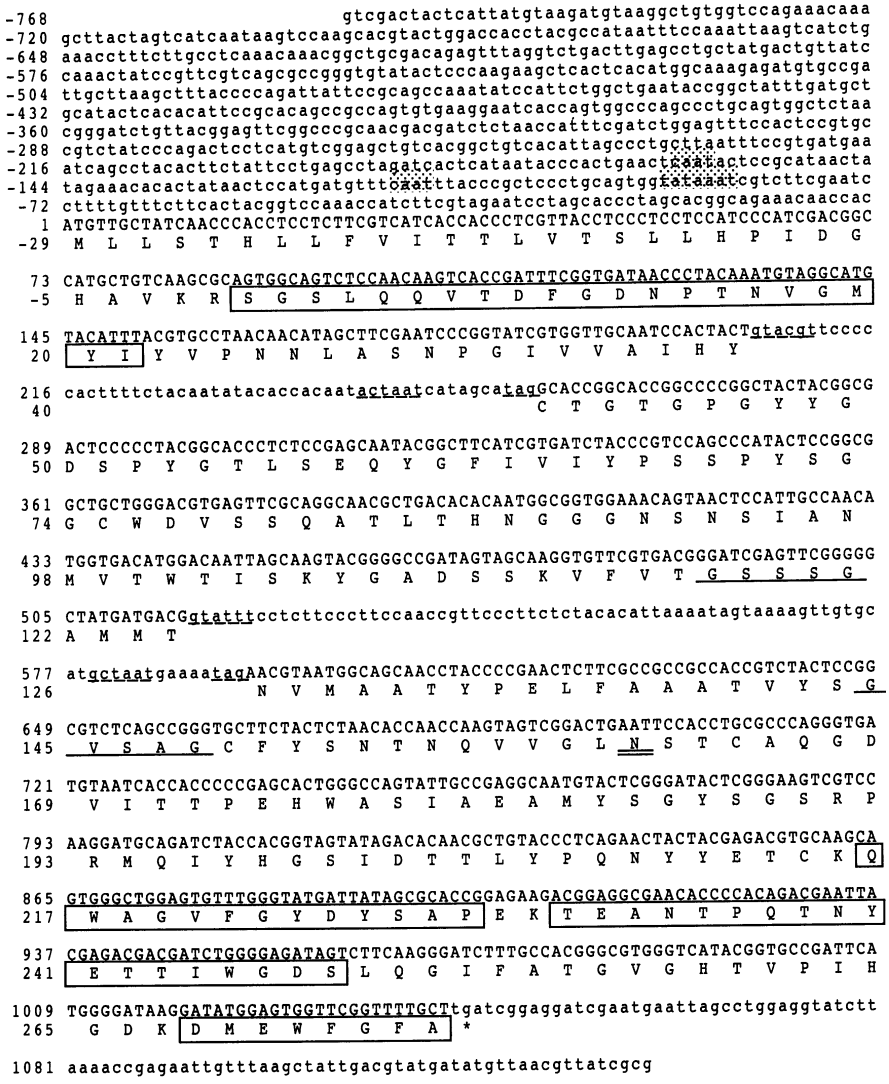

Figure 5 Nucleotide and deduced amino acid sequences of the *aceA* gene from *A. awamori*

The region from *Sal*I to *Acc*II is shown. The putative intron and 5′- and 3′-flanking regions are shown by small letters. The N-terminal amino acid sequence of the mature protein and the amino acid sequences of the three peptides of ACEA, determined by the protein sequencer, are boxed. Two sites having the consensus motif (Gly-Xaa-Ser-Xaa-Gly) of the active-site serine are underlined. A unique N-glycosylation site is doubly underlined. The TATA box and two CAAT-like boxes are hatched.

REFERENCES

1 Sundberg, M. and Poutanen, K. (1991) Biotechnol. Appl. Biochem. **13**, 1–11
2 Biely, P., MacKenzie, C. R. and Schneider, H. (1988) Methods Enzymol. **160**, 700–707
3 Halgašová, N., Kutejavá, E. and Timko, J. (1994) Biochem. J. **298**, 751–755
4 McDermid, K. P., Forsberg, C. W. and MacKenzie, C. R. (1990) Appl. Environ. Microbiol. **56**, 3805–3810
5 Shao, W. and Wiegel, J. (1995) Appl. Environ. Microbiol. **61**, 729–733
6 Rombouts, F. M. and Thibault, J.-F. (1986) Carbohydr. Res. **154**, 177–187
7 Phatak, L., Chang, K. C. and Brown, C. (1988) J. Food Sci. **53**, 830–833
8 Fry, S. C. (1982) Biochem. J. **203**, 493–504
9 Smith, M. M. and Hartley, R. D. (1983) Carbohydr. Res. **118**, 65–80
10 Faulds, C. B. and Williamson, G. (1991) J. Gen. Microbiol. **137**, 2339–2345
11 Tenkanen, M., Schuseil, J., Plus, J. and Poutanen, K. (1991) J. Biotechnol. **18**, 69–83
12 Faulds, C. B. and Williamson, G. (1993) Biotechnol. Appl. Biochem. **17**, 349–359
13 Faulds, C. B. and Williamson, G. (1994) Microbiology **140**, 779–787
14 Hespell, R. B. and O'Bryan-Shah, P. J. (1988) Appl. Environ. Microbiol. **54**, 1917–1922
15 McCallum, J. A., Taylor, I. E. P. and Towers, G. H. N. (1991) Anal. Biochem. **196**, 360–366
16 Ito, K., Ogasawara, H., Sugimoto, T. and Ishikawa, T. (1992) Biosci., Biotechnol., Biochem. **56**, 547–550
17 Somogyi, M. (1951) J. Biol. Chem. **195**, 19–23
18 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
19 Trimble, R. B. and Maley, F. (1984) Anal. Biochem. **141**, 515–522
20 Gomi, K., Kitamoto, K. and Kumagai, C. (1991) Gene **108**, 91–98
21 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22 Johnson, K. G., Silva, M. C., MacKenzie, C. R. and Fontana, J. D. (1989) Appl. Biochem. Biotechnol. **20/21**, 245–278
23 Gurr, S. J., Unkles, S. E. and Kinghorn, J. R. (1988) SGM Spec. Publ.: Gene Structure in Eukaryotic Microbes, Vol. 23, (Kinghorn J. R., ed.), pp. 93–139, IRL Press, Oxford, U.K.
24 von Heijne, G. (1983) Eur. J. Biochem. **133**, 17–21
25 von Heijne, G. (1986) Nucleic Acids. Res. **14**, 4683–4690
26 Zhu, Y. S., Zhang, X. Y., Cartwright, C. P. and Tripper, D. J. (1992) Mol. Microbiol. **6**, 511–520
27 de Graaff, L. H., Visser, J., van den Broeck, H. C., Strozyk, F., Kormelink, F. J. M. and Boonman, J. C. (1992) Eur. Patent Appl., Publication Number 0507369A3
28 Luthi, E., Love, D. R., McAnulty, J., Wallace, C., Caughey, A., Saul, D. and Bergquist, P. L. (1990) Appl. Environ. Microbiol. **56**, 1017–1024
29 Ferreira, L. M., Wood, T. M., Williamson, G., Faulds, C., Hazlewood, G. P., Black, G. W. and Gilbert, H. J. (1993) Biochem. J. **294**, 349–355
30 Shareck, F., Biely, P., Morosoli, R. and Kluepfel, D. (1995) Gene **153**, 105–109
31 Margolles-Clark, E., Tenkanen, M., Soderlund, H. and Penttila, M. (1996) Eur. J. Biochem. **237**, 553–560
32 Brenner, S. (1988) Nature (London) **334**, 528–530
33 Shimada, Y., Sugihara, A., Tominaga, Y., Iizumi, T. and Tsunasawa, S. (1989) J. Biochem. (Tokyo) **106**, 383–388
34 Shimada, Y., Sugihara, A., Iizumi, T. and Tominaga, Y. (1990) J. Biochem. (Tokyo) **107**, 703–707
35 Kawaguchi, Y. and Honda, H. (1991) GBR Monographs: Genetic Engineering, No.16, (Alberghina, L. and Schmid, R., eds.), pp. 221–230, VCH, Weinheim, Germany

Received 24 December 1996/24 April 1997; accepted 1 May 1997