Cloning and expression of the carboxypeptidase gene from *Aspergillus saitoi* and determination of the catalytic residues by site-directed mutagenesis

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Carboxypeptidase from *Aspergillus saitoi* removes acidic, neutral and basic amino acids as well as proline from the C-terminal position at pH 2–5. *cpdS*, a cDNA encoding *A. saitoi* carboxypeptidase, was cloned and expressed. Analysis of the 1816-nucleotide sequence revealed a single open reading frame coding for 523 amino acids. When *A. saitoi* carboxypeptidase cDNA was expressed in yeast cells, carboxypeptidase activity was detected in the cell extract and was immunostained with a 72 kDa protein with polyclonal anti-(*A. saitoi* carboxypeptidase) serum. The recombinant enzyme treated with glycopeptidase F migrated with an apparent molecular mass of 60 kDa on SDS/PAGE, which was the same as that of the de-N-glycosylated carboxypeptidase from *A. saitoi*. Site-directed mutagenesis of the *cpdS* indicated that Ser-153, Asp-357 and His-436 residues were essential for the enzymic catalysis. It can be concluded that *A. saitoi* carboxypeptidase has a catalytic triad comprising Asp-His-Ser and is a member of serine carboxypeptidase family (EC 3.4.16.1).

INTRODUCTION

Carboxypeptidase of *Aspergillus saitoi* (designated *A. phoenicis* by Ballou [1]) is an exopeptidase that releases most amino acid residues, including proline, from the C-termini of peptides and proteins at acidic pH [2–5]. *A. saitoi* carboxypeptidase has been used for automatic C-terminal amino acid sequence analyses of α-amylase from cultured rice cells [6] and *Serratia marcesens* serine proteinase cloned in *Escherichia coli* [7]. The *A. saitoi* carboxypeptidase is a glycoprotein that contains both N- and O-linked sugar chains. The N-linked oligosaccharides are unique structures of $Man_{10}GlcNAc_2$ [8] and $Man_{11}GlcNAc_2$ [9]. Deglycosylation of the carboxypeptidase with endo-β-*N*-acetylglucosaminidase and α-mannosidase, however, did not affect the enzymic properties of catalytic activity, pH or thermal stability, or resistivity to peptic protease digestion [8,9].

A. saitoi carboxypeptidase has been classified as a serine carboxypeptidase (EC 3.4.16.1). Some amino acid sequences of serine carboxypeptidases have been defined, such as yeast carboxypeptidase Y [10], yeast *KEX1* protein [11], malt carboxypeptidase I [12], malt carboxypeptidase II [13] and wheat carboxypeptidase II [14], but no definition has yet been made of mould carboxypeptidase. Biochemical studies of serine carboxypeptidases indicated that a serine [15] and a histidine [16] are essential for catalysis. Liao and Remington [17] showed that the enzyme from wheat has a catalytic triad comprising Asp-His-Ser that is similar in arrangement to those of the serine proteinases.

We had, however, doubted the finding that *A. saitoi* carboxypeptidase is in the category of a serine carboxypeptidase because: (1) the optimum pH of *A. saitoi* carboxypeptidase is lower than other serine carboxypeptidases; (2) *A. saitoi* carboxypeptidase is not inhibited by serine protease inhibitors such as di-isopropyl fluorophosphate, tosyl-L-phenylalanylchloromethane ('TPCK'), tosyl-L-lysylchloromethane ('TLCK') or *N*-benzyloxycarbonyl-L-phenylalanine chloromethane (ZPCK); (3) two catalytically active groups on the *A. saitoi* carboxypeptidase with $pK_{e1} = 2.3$ and $pK_{e2} = 4.9$ were recognized to be important in the enzyme action [3]. The pK_a of 2.3 could represent participation of an ionized carboxyl group, whereas the pK_a of 4.9 could be in accord with the ionization of a carboxyl group or an imidazole group. These pK_a values are very close to those of pepsin (EC 3.4.23.1): one is around 1 and the other is between 4 and 5 [18]. Lastly, (4) some chemicals modifying the carboxyl groups inhibit the *A. saitoi* carboxypeptidase activity. Pyridine-2-azo-*p*-dimethylaniline (PAD), known as an inhibitor of several aspartic proteinases, pepsin and aspergillopepsin I (EC 3.4.23.18), by forming the complex with zinc(II) [19], also bound to *A. saitoi* carboxypeptidase (Y. Chiba, A. Kawasaki and E. Ichishima, unpublished work). The formation of this complex is released from the enzyme by hydrocinnamic acid. It was speculated that zinc(II)–PAD was bound to two catalytic carboxylate groups in the active site of the aspartic proteinases. These results led us to consider that the *A. saitoi* carboxypeptidase had two catalytic carboxyl groups like an aspartic proteinase.

In this study, we undertook molecular cloning of *A. saitoi* carboxypeptidase cDNA and expression of the recombinant *A. saitoi* carboxypeptidase in *Saccharomyces cerevisiae*. Site-directed mutagenesis was performed to determine the catalytic residues of *A. saitoi* carboxypeptidase.

EXPERIMENTAL

Materials

All chemicals used were of analytical grade and readily available from commercial sources. A pUC118 *A. saitoi* cDNA library was

Abbreviations used: GPD, glyceraldehyde-3-phosphate dehydrogenase; PAD, pyridine-2-azo-*p*-dimethylaniline; ZPCK, *N*-benzyloxycarbonyl-L-phenylalanylchloromethane; Z-Tyr-Leu, *N*-benzyloxycarbonyl-L-tyrosyl-L-leucine.

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The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI Nucleotide Sequence Databases with the following accession number: D25288.

generated in our laboratory as described by Shintani and Ichishima [20]. The synthetic oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. All DNA-modifying enzymes used are commercially available.

cDNA library screening

A cDNA library for 50000 colony-forming units prepared from *A. saitoi* in the pUC118 vector was screened with an end-labelled synthetic oligonucleotide (5′-GTIAATGGIACITCCATICCIG-AIGTIGATTTTGATGTIGGIGAITCCTA-3′) derived from the N-terminal amino acid sequence of *A. saitoi* carboxypeptidase. The filters were hybridized at 42 °C and washed three times with 2 × SSC/0.1 % SDS (1 × SSC: 0.15 M NaCl/0.015 M sodium citrate) for 20 min at 45 °C. Positive colonies were picked and rescreened.

Sequencing of DNA clones

Sequencing was carried out using an Applied Biosystems Inc. 373A model DNA sequencer. The cDNA was subcloned into pUC118 and pUC119, and nested deletions were generated as recommended by a Deletion kit for Kilo-sequence (Takara Shuzo Co., Ltd.). Deleted mutants were sequenced using a *Taq* Dye Primer Cycle Sequencing Kit from Applied Biosystems, Inc. The cDNA sequence was obtained from both strands, and all restriction sites used in subcloning were contained in overlapping sequences.

Expression of recombinant carboxypeptidase

A yeast expression vector pG-3 [21] was cut with *Bam*HI and polymerized with Klenow fragment. Then an *Eco*RI/*Not*I adaptor was added to both ends of the linear pG-3. The cDNA was cloned between the *Not*I sites downstream of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter in the plasmid pG-3. The resulting plasmid pGCP13 was transformed into *S. cerevisiae* YPH250 (*MATa ura3 trp1 his3 leu2*) as described by Ito et al. [22]. Cultured cells were centrifuged, and the precipitate was used for assaying the enzyme activity and Western blotting. Cells transfected with vector alone (pG-3) were used as an internal negative control.

Enzyme assay

The assay for acid carboxypeptidase activity towards *N*-benzyloxycarbonyl-L-tyrosyl-L-leucine (Z-Tyr-Leu) at pH 3.1 was described previously [2]. One katal of the enzyme was defined as the amount required to liberate 1 mol of leucine from Z-Tyr-Leu/s at 30 °C and pH 3.1, according to the IUPAC and IUB recommendations.

Electrophoresis and immunoblotting

SDS/PAGE was carried out using the buffer system of Laemmli [23] in 12% acrylamide gels. Electroblotting of fractionated proteins on to nitrocellulose membrane was carried out by the method of Towbin et al. [24], and detection was performed essentially according to the method of Hsu et al. [25].

Digestion of N-linked oligosaccharide

Cell extract was denatured in 10 mM sodium phosphate buffer, pH 8.6, containing 0.2% SDS and 0.2% 2-mercaptoethanol while boiling for 5 min. Glycopeptidase F was then added to the cell extract and incubated at 37 °C. After 20 h, the molecular mass of the de-N-glycosylated recombinant carboxypeptidase was calculated by SDS/PAGE and immunostaining.

Purification of recombinant carboxypeptidase

YPH250 cells harbouring pGCP13 were precultured in complete minimum tryptophan dropout medium [26] at 30 °C for 72 h. The preculture was then inoculated in yeast extract/peptone/dextrose medium [26] at 30 °C for 48 h, and the culture was centrifuged at 11000 *g* for 15 min to give a precipitate. The collected cells were lyophilized and homogenized with 0.1% Tween-20 and the inhibitor mixture [27]. The homogenate was centrifuged for 15 min at 11000 *g* and the supernatant was immediately applied to a Sephadex G-100 column that had been equilibrated with 10 mM acetate buffer, pH 5.0, containing 0.2 M NaCl. After dialysis against 10 mM acetate buffer, pH 4.0, the active fraction was applied to an SP-Sephadex C-50 column that had been equilibrated with 10 mM acetate buffer, pH 4.0, and eluted by an increasing NaCl gradient (0–0.3 M). The active fraction was subjected to an HPLC TSKgel G3000SW XL column (Tosoh Corporation) that had been equilibrated with 10 mM acetate buffer, pH 5.0, containing 0.2 M NaCl. Finally the active fraction was collected and dialysed against Milli-Q water at 4 °C and lyophilized.

Native size determination

Purified recombinant carboxypeptidase was passed through an HPLC TSKgel G3000SW XL as described above. Molecular mass was detected in comparison with protein standards of known size (catalase, 240 kDa; aldolase, 158 kDa; BSA, 68 kDa; ovalbumin, 45 kDa).

Site-directed mutagenesis

The mutated enzymes S153A, D357A and H436A were made by using a Sculptor *in vitro* mutagenesis system (Amersham). The oligonucleotide 5′-CATGCCCGCATAAGCTTCGCCGGTG-AT-3′ (oligo S153A), 5′-GAGGAGGTAAGCGAGCCAGC-CG-3′ (oligo D357A) or 5′-CTGCGGGATTTCGGCGCC-AGACAAATACACCG-3′ (oligo H436A) was used to introduced the mutations into the cDNA encoding *A. saitoi* carboxypeptidase. Underlined nucleotides are different from those of the wild type. The mutations were confirmed by sequencing of the mutated sites and surrounding regions. The mutated cDNAs were cloned into the *Not*I site of plasmid pG-3, resulting in plasmid pGCM153 (S153A), pGCM357 (D357A) and pGCM436 (H436A) respectively, and transduced into yeast YPH250 cells.

RESULTS

Sequence analysis of cDNA encoding *A. saitoi* carboxypeptidase

Screening of an *A. saitoi* cDNA library with synthetic oligonucleotide identified five clones by colony hybridization. One of these clones was sequenced completely and was found to contain a 1812 bp cDNA that encoded a complete nucleotide sequence of the carboxypeptidase (Figure 1). The cDNA sequence contains a single open reading frame of 523 amino acids starting at position 46 and ending at 1617. The sequence around the proposed methionine start codon closely matches the consensus sequence for eukaryotic translational initiation sites [28] and the stop codon is found in the frame at position 22. Codon usage of the coding region leads to the choice of C in the third codon position (C: 53%; G: 20%; T: 18%; A: 9%).

The putative mature *A. saitoi* carboxypeptidase consists of 471 amino acids (molecular mass of 52453 Da). The underlined

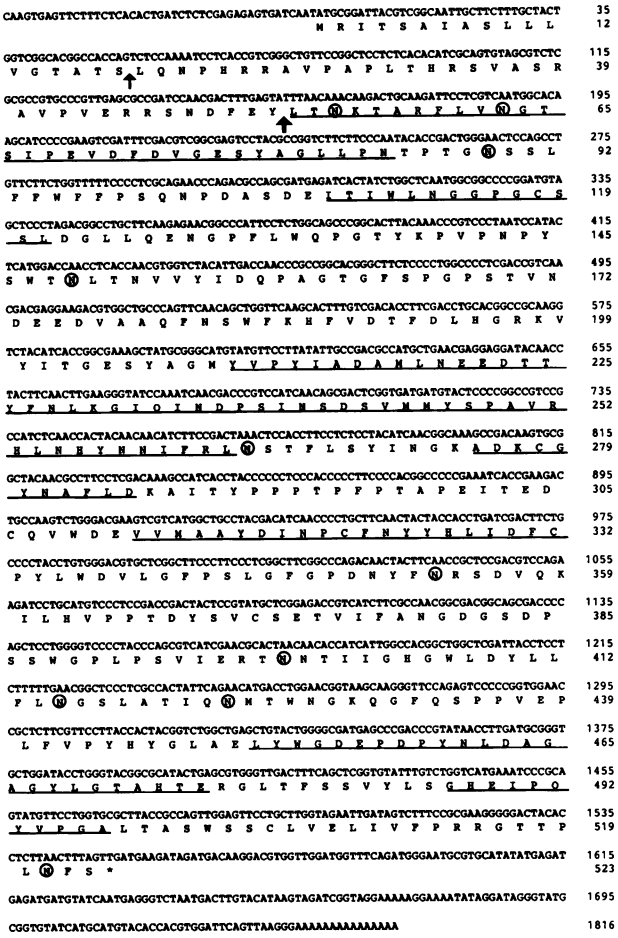

Figure 1 cDNA sequence of *A. saitoi* carboxypeptidase and deduced amino acid sequence

The predicted amino acid sequence of *A. saitoi* carboxypeptidase is shown below the DNA sequence. The standard single-letter symbols are used. Amino acid sequences found by direct sequencing are underlined. A light arrow marks the putative cleavage site of signal peptide according to the weight-matrix approach of von Heijne [30]. A bold arrow indicates the start of the mature enzyme. The amino acid sequences of the potential N-glycosylation site are circled.

sequences of amino acid residues in Figure 1 correspond to the sequences found by amino acid sequencing analyses of native carboxypeptidase from *A. saitoi*. Hydropathy analysis by the algorithm of Kyte and Doolittle [29] indicates that the N-terminal portion of the polypeptide is hydrophobic, and analysis based on the signal sequence cleavage prediction method of von Heijne [30] indicates possible cleavage sites after the 18th amino acid. Since the *A. saitoi* carboxypeptidase is secreted into the medium, the N-terminal sequence is thought to function as a signal sequence.

Comparison of the deduced sequence of *A. saitoi* carboxypeptidase with other known serine carboxypeptidase sequences shows that they share a low degree of similarity (Figure 2): 32.4% with wheat carboxypeptidase II [14], 32.3% with malt carboxypeptidase II [13] and 26.2% with yeast carboxypeptidase Y [10]. However, all of the sequences conserve the catalytic domains (indicated by boxes II to IV in Figure 2) and the domain (box I in the Figure 2) which contains the amino acid residues recognizing the C-terminal carboxylate group of peptide sub-

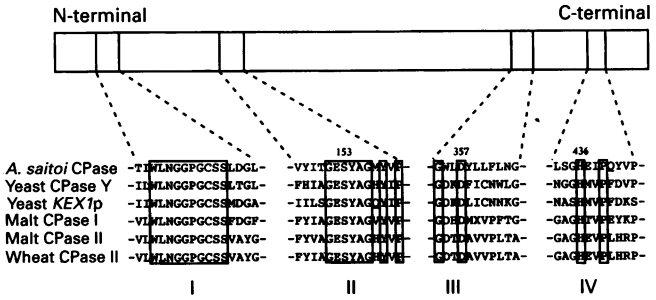

Figure 2 Comparison of the peptide domains reportedly required for enzyme catalysis in eukaryotic serine carboxypeptidases

A. saitoi carboxypeptidase (CPase) is aligned with carboxypeptidase Y [10], *KEX1* protein [11], malt carboxypeptidase I [12], malt carboxypeptidase II [13] and wheat carboxypeptidase II [14] sequences with the differences identified. Amino acids conserved with the serine carboxypeptidases sequence are boxed.

strates [31]. There are also present in the sequence ten potential sites for N-linked glycosylation.

Expression of recombinant carboxypeptidase in yeast cells

The *A. saitoi* carboxypeptidase cDNA was cloned downstream of a GDP promoter, and the resulting plasmid, pGCP13, was used to generate recombinant *A. saitoi* carboxypeptidase protein. No enzymic activity was detected in the culture supernatant. We detected the *A. saitoi* carboxypeptidase activity of the extract obtained from yeast cells transfected with *A. saitoi* carboxypeptidase cDNA in forward orientation (pGCP13), although no activity was observed with the vector alone at pH 3.1. The recombinant *A. saitoi* carboxypeptidase activity is not affected by whether the ZPCK, which is an inhibitor of yeast carboxypeptidase Y, is present or not.

Western-blot analysis of yeast cell extracts shows a 72 kDa protein with rabbit anti-(*A. saitoi* carboxypeptidase) serum, which is consistent with the apparent molecular mass of the native *A. saitoi* carboxypeptidase. Conversely, the extracts obtained from yeast cells transfected with the vector (pG-3) alone or with cDNA in reverse orientation (pGCP31), as negative controls, yielded no stainable protein.

The recombinant carboxypeptidase was treated with glycopeptidase F and was subjected to SDS/PAGE. De-N-glycosylated recombinant enzymes migrated with apparent molecular masses of 62 kDa and 60 kDa, a little larger or equivalent to that of de-N-glycosylated carboxypeptidase from *A. saitoi* (lane 2 in Figure 3).

Characterization of recombinant carboxypeptidase

The recombinant carboxypeptidase was purified using gel-filtration and cation-exchange chromatography (Table 1). The purified recombinant enzyme was homogeneous after SDS/PAGE and had a subunit electrophoretic mobility slightly greater than that of the carboxypeptidase from *A. saitoi* (Figure 4). The recombinant carboxypeptidase was subjected to gel filtration on a TSKgel G3000SW XL column in comparison with standard proteins of known size. The estimated molecular mass of the recombinant carboxypeptidase was 135 kDa (results not shown), which is expected for a dimer.

The specific activity of the recombinant carboxypeptidase towards Z-Tyr-Leu was measured at pH 3.1. The value for

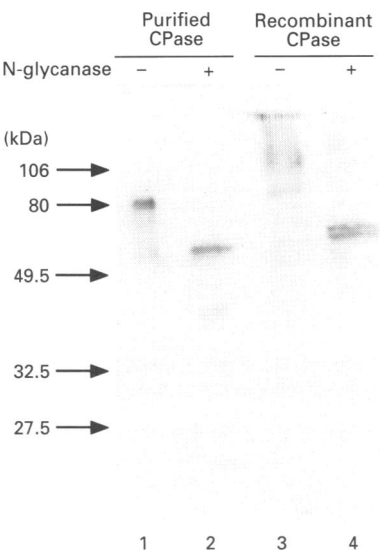

Figure 3 Western-blot analysis of the de-N-glycosylated yeast cell extracts

Samples were separated on an SDS/12%-PAGE gel and immunostained with anti-(*A. saitoi* carboxypeptidase) serum. Lane 1, purified *A. saitoi* carboxypeptidase (CPase); lane 2, as lane 1, treated with glycopeptidase F; lane 3, the extract of the cells carrying pGCP13; lane 4, as lane 3, treated with glycopeptidase F.

Table 1 Purification of the recombinant carboxypeptidase

Purification step	Total protein (mg)	Total activity (nkat)	Specific activity (kat/kg)	Yield (%)
Homogenate	406.0	1380	0.0034	100
Sephadex G-100	6.9	472	0.068	34.2
SP-Sephadex C-50	0.16	467	2.92	33.8
TSKgel G3000SW	0.054	220	4.07	15.9

recombinant carboxypeptidase was 4.1 kat/kg, which was almost the same as that of *A. saitoi* carboxypeptidase, 4.5 kat/kg.

Site-directed mutagenesis

As shown in Figure 2, four conserved regions were observed in the *A. saitoi* carboxypeptidase sequence. To establish whether the Ser-153, Asp-357 and His-436 residues in the conserved regions were essential for activity of the *A. saitoi* carboxypeptidase, we generated point mutations that changed each residue to alanine, by oligonucleotide site-directed mutagenesis. The mutated *cpdS* genes were cloned into the pG-3 plasmid and introduced into yeast YPH250 cells. Mutant carboxypeptidases (S153A, D357A and H436A) were then produced in 5-day cultures. Attempts to detect an inactive protein were made by Western blotting, with the wild-type enzyme as molecular-mass standard. Responses were obtained in all mutants (Figure 5). Next, each cell extract was assayed with Z-Tyr-Leu at pH 3.1; however, no carboxypeptidase activity was detected from any mutant protein. The Asp-138 mutant protein (D138A), as a positive control, completely retained the carboxypeptidase activity (results not shown). These results suggest that the *A. saitoi* carboxypeptidase functionally conserves the catalytic residues (Ser-153, Asp-357 and His-436) of any other carboxypeptidase,

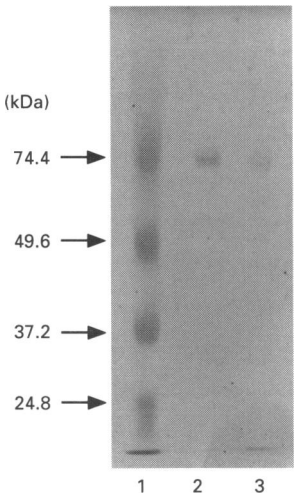

Figure 4 SDS/PAGE of the purified recombinant carboxypeptidase

Electrophoresis was performed in a 12% acrylamide gel. Protein size markers are shown in lane 1. Lane 2: purified *A. saitoi* carboxypeptidase. Lane 3: purified recombinant carboxypeptidase from yeast.

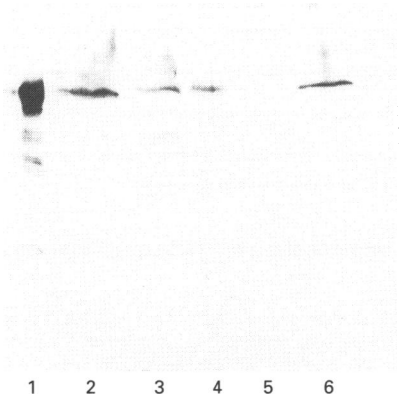

Figure 5 Western-blot analysis of the mutant carboxypeptidases

Samples were separated by SDS/12%-PAGE and immunostained with anti-(*A. saitoi* carboxypeptidase) serum. Lane 1: purified *A. saitoi* carboxypeptidase. Lanes 2, 3, 4, 5 and 6: cell extracts from yeast carrying pGCM153 (S153A), pGCM357 (D357A), pGCM436 (H436A), pG-3 and pGCP13 (wild type) respectively.

and strongly imply that the *A. saitoi* carboxypeptidase is a serine protease with a catalytic triad comprising Asp-His-Ser.

DISCUSSION

In this paper we present the first report of a cDNA sequence of a mould carboxypeptidase. The cDNA of *A. saitoi* carboxypeptidase (*cpdS*) is approximately 1.8 kb long and encodes a preproenzyme consisting of 523 amino acids, where the pre region is a signal peptide. The mature region (471 amino acids) of *A. saitoi* carboxypeptidase is longer than those of yeast carboxypeptidase (421 amino acids) and wheat carboxypeptidase (423 amino acids; A-chain plus B-chain). As shown in Figure 2, similarity of the amino acid sequence is observed in the N-terminal and C-terminal regions, although the central region is

not conserved among the serine carboxypeptidases. The central region of *A. saitoi* carboxypeptidase has a proline-rich domain which is not seen in other serine carboxypeptidases, suggesting that the region may contribute to the stability of the enzyme.

A. saitoi carboxypeptidase is a highly glycosylated enzyme [8,9]. The molecular mass of the recombinant carboxypeptidase was similar to that of the purified carboxypeptidase from *A. saitoi* on SDS/PAGE when *cpdS* was expressed in yeast cells. The result indicates that the composition of N-linked oligosaccharide of recombinant enzyme expressed in yeast cells was nearly the same as that of native carboxypeptidase from *A. saitoi*. De-N-glycosylation treatment with glycopeptidase F decreased the apparent molecular mass to 62 and 60 kDa. The smaller form is the same molecular mass as that of the deglycosylated enzyme from *A. saitoi*. We believe that the larger form of the recombinant carboxypeptidase is the result of the unusual processing in the propeptide; however, we have not purified this larger form yet.

The amino acid sequence alignment shows that the catalytic domain is conserved between the *A. saitoi* carboxypeptidase and serine carboxypeptidases. Alteration of the deduced catalytic residues (Ser-153, Asp-357 or His-436) by site-directed mutagenesis led to loss of all activity of *A. saitoi* carboxypeptidase towards the peptide substrate. This is the first report of the active aspartic acid of a serine carboxypeptidase being identified by site-directed mutagenesis. These results suggest that *A. saitoi* carboxypeptidase has a catalytic residue (Ser, Asp and His) and that the enzyme is correctly placed in the category of serine carboxypeptidases.

This contradiction makes it conceivable that *A. saitoi* carboxypeptidase may have another catalytic mechanism, in spite of the fact that a serine, a histidine and an aspartic acid are required for catalysis; that is to say, a fourth catalytic residue, which has a carboxyl group in the side-chain, may exist. From the analysis of the pH-dependencies of serine carboxypeptidase Y-catalysed hydrolysis and aminolysis reactions, Christensen [32] has proposed a new reaction model in which a possibly ionizing glutamic acid indirectly plays a role in catalysis. The model could explain why catalysis occurred whenever the histidine was protonated at low pH. *A. saitoi* carboxypeptidase may have a similar catalytic mechanism. The pH-dependence for the *A. saitoi* carboxypeptidase-catalysed hydrolysis of Z-Glu-Tyr showed a lower pK_a than that of carboxypeptidase Y, suggesting an aspartic acid residue, despite glutamic acid on the catalysis of carboxypeptidase Y, possibly participating in that of *A. saitoi* carboxypeptidase.

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REFERENCES

1 Ballou, C. E. (1990) Methods Enzymol. **185**, 440–470
2 Ichishima, E. (1972) Biochim. Biophys. Acta **258**, 274–288
3 Ichishima, E. and Arai, T. (1973) Biochim. Biophys. Acta **293**, 444–450
4 Ichishima, E., Sonoki, S., Hirai, K., Torii, Y. and Yokoyama, S. (1972) J. Biochem. (Tokyo) **72**, 1045–1048
5 Arai, T. and Ichishima, E. (1974) J. Biochem. (Tokyo) **76**, 765–769
6 Chiba, Y., Nieda, Y., Nakajima, T. and Ichishima, E. (1991) Agric. Biol. Chem. **55**, 901–902
7 Yanagida, N., Uozumi, T. and Beppu, T. (1986) J. Bacteriol. **166**, 937–944
8 Chiba, Y., Yamagata, Y., Nakajima, T. and Ichishima, E. (1992) Biosci. Biotech. Biochem. **56**, 1371–1372
9 Chiba, Y., Yamagata, Y., Iijima, S., Nakajima, T. and Ichishima, E. (1993) Curr. Microbiol. **27**, 281–288
10 Valls, L. A., Hunter, C. P., Rothman, J. H. and Stevens, T. H. (1987) Cell **48**, 887–897
11 Dmochowska, A., Dignard, D., Henning, D., Thomas, D. Y. and Bussey, H. (1987) Cell **50**, 573–584
12 Sørensen, S. B., Breddam, K. and Svendsen, I. B. (1986) Carlsberg Res. Commun. **51**, 475–485
13 Sørensen, S. B., Svendsen, I. B. and Breddam, K. (1987) Carlsberg Res. Commun. **52**, 285–295
14 Breddam, K., Sørensen, S. B. and Svendsen, I. B. (1987) Carlsberg Res. Commun. **52**, 297–311
15 Hayashi, R., Moore, S. and Stein, W. H. (1973) J. Biol. Chem. **248**, 8366–8369
16 Bech, L. M. and Breddam, K. (1989) Carlsberg Res. Commun. **54**, 165–171
17 Liao, D.-I. and Remington, S. J. (1990) J. Biol. Chem. **265**, 6528–6531
18 Denburg, J. L., Nelson, R. and Silver, M. S. (1968) J. Am. Chem. Soc. **90**, 479–486
19 Nakatani, H., Hiromi, K., Satoi, S., Oda, K., Murao, S. and Ichishima, E. (1975) Biochim. Biophys. Acta **391**, 415–421
20 Shintani, T. and Ichishima, E. (1994) Biochim. Biophys. Acta **1204**, 257–264
21 Schena, M., Picard, D. and Yamamoto, K. R. (1991) Methods Enzymol. **194**, 389–398
22 Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) J. Bacteriol. **153**, 163–168
23 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
24 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
25 Hsu, S.-M., Raine, L. and Fanger, H. (1981) J. Histochem. Cytochem. **29**, 577–580
26 Treco, D. A. and Lundblad, V. (1990) in Current Protocols in Molecular Biology: Preparation of Yeast Media (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K., eds.), vol. 2, pp. 13.1.1–13.1.7, Greene Publishing Associates and Wiley-Interscience, New York
27 Dunn, B. and Wobbe, C. R. (1990) in Current Protocols in Molecular Biology: Preparation of Protein Extracts from Yeast (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K., eds.), vol. 2, pp. 13.13.1–13.13.9, Greene Publishing Associates and Wiley-Interscience, New York
28 Kozak, M. (1984) Nucleic Acids Res. **12**, 857–872
29 Kyte, J. and Doolittle, R. F. (1982) J. Mol. Biol. **157**, 105–132
30 von Heijne, G. (1986) Nucleic Acids Res. **14**, 4683–4690
31 Mortensen, U. H., Remington, S. J. and Breddam, K. (1994) Biochemistry **33**, 508–517
32 Christensen, U. (1994) Eur. J. Biochem. **220**, 149–153