J. Biochem. 120, 974-981 (1996)

Characterization of the S_1 Subsite Specificity of Aspergillopepsin I by Site-Directed Mutagenesis[1]

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Received for publication, June 19, 1996

The structural determinants of S_1 substrate specificity of aspergillopepsin I (API; EC 3.4.23.18), an aspartic proteinase from *Aspergillus saitoi*, were investigated by site-directed mutagenesis. Aspartic proteinases generally favor hydrophobic amino acids at P_1 and P_1'. However, API accommodates a Lys residue at P_1, which leads to activation of trypsinogen. On the basis of amino acid sequence alignments of aspartic proteinases, Asp-76 and Ser-78 of API are conserved only in fungal enzymes with the ability to activate trypsinogen, and are located in the active-site flap. Site-directed mutants (D76N, D76E, D76S, D76T, S78A, and ΔS78) were constructed, overexpressed in *Escherichia coli* cells and purified for comparative studies using natural and synthetic substrates. Substitution of Asp-76 to Ser or Thr and deletion of Ser-78, corresponding to the mammalian aspartic proteinases, caused drastic decreases in the activities towards substrates containing a basic amino acid residue at P_1. In contrast, substrates with a hydrophobic residue at P_1 were effectively hydrolyzed by each mutant enzyme. These results demonstrate that Asp-76 and Ser-78 residues on the active site flap play important roles in the recognition of a basic amino acid residue at the P_1 position.

Key words: aspartic proteinase, aspergillopepsin I, site-directed mutagenesis, substrate specificity, trypsinogen activation.

Aspartic proteinase [EC 3.4.23.-] comprise a group of enzymes whose proteolytic activities are dependent on two aspartyl residues (*1*). They are widely distributed in various organisms, being found in mammalians, plants, microorganisms, and retroviruses, in which they fulfil a variety of roles. There is a good evidence that most of them belong to a family of homologous enzymes similar in structure (*2*). These enzymes are bilobal, each lobe having a similar fold, with a deep and extended cleft which can accommodate at least seven amino acid residues of a substrate in the S_4-S_3' subsites (*3*). Each domain contributes one aspartyl residue, corresponding to Asp-32 and Asp-215 of pepsin, to the catalytic center of the extended binding site (*4*). The "flap," an antiparallel β-hairpin comprising residues 72 to 83 (penicillopepsin numbering), projects across the cleft forming a channel into which a substrate binds. However, these enzymes have distinct preferences for cleaving the bond between hydrophobic residues occupying the S_1-S_1' subsites. For example, renin [EC 3.4.23.15] is characterized by very specific hydrolysis of angiotensinogen at Leu^{10}-Leu^{11} (*5*) and chymosin [EC 3.4.23.4] digests κ-casein at Phe^{105}-Met^{106} for milk-clotting (*6*). Thus, the differences in substrate specificity may be a consequence of alterations in the structure of substrate binding sites.

An aspartic proteinase from *Aspergillus saitoi*, aspergillopepsin I (API; EC 3.4.23.18) (*7-10*) is a monomeric protein with a relative molecular mass of 34,302. API generally favors hydrophobic residues at P_1 and P_1' (*11-13*), but also accepts a Lys residue in the P_1 position, which leads to activation of trypsinogen by cleavage of the Lys^6-Ile^7 bond (*14, 15*). We isolated and characterized its genomic DNA and cDNA and expressed it in yeast *Saccharomyces cerevisiae* cells (*16*). API and other fungal aspartic proteinases such as penicillopepsin [EC 3.4.23.20], rhizopuspepsin [EC 3.4.23.21], and endothiapepsin [EC 3.4.23.22] display significant similarities in their amino acid sequences. Although no three-dimensional structure of API is available, the substrate binding sites may be identified by analogy with those of other aspartic proteinases such as penicillopepsin (*17-19*), endothiapepsin (*20-23*), and rhizopuspepsin (*24-27*). Aspartic proteinases generally have broad primary specificity, but the fungal and mammalian enzymes can differ dramatically from each other in catalytic efficiency against substrates containing a Lys residue at the P_1 position (*28*). Based on the sequence alignment and structure of aspartic proteinases, the major

[1] This work was suppurted in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: P_1, P_2, P_3, *etc.* and P_1', P_2', P_3', *etc.*, designate amino acid residues of substrate on the amino-terminal and carboxy-terminal sides of the scissile peptide bond, respectively, and S_1, S_2, S_3, *etc.* and S_1', S_2', S_3', *etc.*, are the corresponding subsites of the proteinase (*51*). Substitution mutants are indicated by the single-letter amino acid code for the wild-type residue followed by their position and the code for the replacement residue. Deletion is indicated by Δ followed by the wild-type residue and its position.

TABLE I. **Comparison of the amino acid sequence of the active-site flap in the aspartic proteinase family.** The amino acid sequences of aspergillopepsin I (*16*), penicillopepsin (*52*), rhizopuspepsin (*53*), endothiapepsin (*54*), candidapepsin (*55*), mucorpepsin (*56*), porcine pepsin (*39*), and human cathepsin D (*57*) are aligned. These enzymes are classified into two groups (class I and II). Class I enzymes have the ability to activate trypsinogen and class II enzymes have no activity for trypsinogen activation. The mutated positions are indicated in boldface characters.

	Residue number	
	70	83
Class I		
Aspergillopepsin I	WDISYGDGSSASGD	
Penicillopepsin	WSISYGDGSSASGN	
Rhizopuspepsin	WSISYGDGSSASGI	
Endothiapepsin	WSISYGDGSSSSGD	
Candidapepsin	F YIGYGDGSSSQGT	
Class II		
Mucorpepsin	L NITYGTG-GANGI	
Porcine pepsin	L SITYGTG-SMTGI	
Human cathepsin D	F DIHYGSG-SLSGY	

differences in the S_1 subsite between the fungal and mammalian enzymes can be localized at position 76 [77, where the number in the parentheses denotes penicillopepsin numbering] and 78 [79] on the active site flap (Table I). These residues may be involved in the recognition of basic amino acid residues at the P_1 position. In the preceding paper (*16*), it was reported that Asp-76 of API was the Lys binding site, based on trypsinogen activation by the mutant with Asp-76 converted to Ser, expressed in *Saccharomyces cerevisiae*.

In order to obtain further information about the primary specificity of API, we have constructed an API expression system in *Escherichia coli*, prepared mutants at position 76 and 78 of API by site-directed mutagenesis, and compared their molecular and enzymatic properties. We show that the nature of the active site flap is important for determining the S_1 substrate specificity of aspartic proteinases.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, T4 DNA ligase, alkaline phosphatase, T4 polynucleotide kinase, and *Taq* DNA polymerase were purchased from Takara Shuzo (Kyoto), and T4 DNA ligase was from Gibco BRL (Gaithersberg, Maryland). Pro-Thr-Glu-Phe-Phe(NO_2)-Arg-Leu was obtained from Calbiochem (San Diego, CA, USA). Pro-Thr-Glu-Lys-Phe(NO_2)-Arg-Leu and Ac-Ala-Ala-Lys-Phe(NO_2)-Ala-Ala-amide were from Tana Laboratories (Houston, TX, USA). Bovine trypsinogen was from Sigma Chemical (St. Louis, USA).

Strains, Plasmids, and Media—Aspergillus saitoi ATCC14332 (now designated *Aspergillus phoenicis*) was used as a source of native API. *E. coli* DH5α [*supE*44 Δ*lacU*169(ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1] was used for plasmid isolation and cloning. *E. coli* JM105 (*endA*1 *supE sbcB*15 *thi rpsL* Δ(*lac-proAB*)/F' [*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) was used to propagate bacteriophage M13 vectors. *E. coli* BL21(DE3) [*hsdS gal* (λc*Its*857 *ind*1 *Sam*7 *nin*5 *lac*UV5-T7gene)] was the host strain for protein expression. Plasmids pUC118, M13-mp18, and pET12a were purchased from Takara Shuzo. *E. coli* cells were grown in LB broth (1% bacto trypton, 1% bacto yeast extract, and 1% NaCl).

Construction of Expression Plasmid for API—The expression vector for proAPI, pETAP, was constructed by inserting the API cDNA fragment (*16*) into the *Nde*I/*Bam*HI site of pET12a (*29*). The cDNA was amplified by PCR using the oligonucleotide 5'-GTCCATATGGCACCG-GCTCCCAC-3' to delete the signal sequence and introduce a translation initiation codon. The resulting cDNA fragment was sequenced in order to confirm the absence of undesired mutations, and inserted into pET12a.

Site-Directed Mutagenesis—Site-directed mutagenesis was performed by the method of Kunkel *et al.* (*30*). API cDNA was subcloned into M13mp18 to serve as a template for mutagenesis. The following mutagenic primers were used: D76N, 5'-CGAGCTCCCGTTACCGTAAGA-3'; D76E, 5'-TGGCCGAGCTCCCTTCACCGTAAG-3'; D76S, 5'-TG-GCCGAGCTTCCGGAACCGTAGGAG-3'; D76T, 5'-CGA-GCTGCCGGTACCGTAGGAG-3'; S78A, 5'-CGTCTCCG-CTAGCCGAGGCGCCGTCACCG-3'; ΔS78, 5'-CTCCGC-TGGCGGATCCGTCACCGTA-3'. The mutation was verified by DNA sequencing before subcloning the gene into the expression vector pET12a.

Protein Expression and Purification of Recombinant API—E. coli strain BL21(DE3) transformed with pETAP was cultured at 37°C in a 3-liter flask containing 0.5 liter of LB medium with 50 μg/ml ampicillin until A_{600} reached 0.6, then isopropyl β-D-galactopyranoside (IPTG) was added to a final concentration of 0.1 mM and the incubation was continued for 4 h. The cells were harvested by centrifugation and resuspended in 50 ml of TE buffer (50 mM Tris-HCl, pH 8.0, containing 2 mM EDTA). Lysozyme (50 mg) was then added and the suspension was allowed to stand at 30°C for 15 min. Triton X-100 was added to the suspension to final concentration of 1%, and the cells were ruptured by sonication. The cell homogenate was centrifuged at 6,000 $\times$ *g* for 10 min. The pellet, which contained most of the proAPI as inclusion bodies, was washed by resuspending it in 50 ml of TE buffer, followed by centrifugation. The washed inclusion bodies were dissolved in 50 ml of TE buffer containing 8 M urea and the solution was allowed to stand at room temperature for 1 h. Solid NaCl was added to the solution to a final concentration of 0.5 M and the pH was adjusted to 10.5 with NaOH. The solution was dialyzed stepwise against 500 ml each of 20 mM sodium bicarbonate (pH 10.5) containing 4, 2, 1, 0.5, and 0 M urea successively for 3 h each at room temperature. Then the dialysis buffer was changed to 2 liters of 20 mM sodium phosphate, pH 7.0 and dialysis was continued at 4°C for 8 h. The dialysate was centrifuged at 15,000 $\times$ *g* for 15 min and the supernatant was used as crude proAPI preparation.

The crude proenzyme solution was concentrated by ultrafiltration with DIAFLO YM10 (Amicon), then applied to a Toyopearl HW-55F column (TOSOH; 2.2 cm $\times$ 90 cm) and eluted with 20 mM sodium phosphate, pH 7.0. The active fractions were combined, and further purified by FPLC. The partially purified proAPI was loaded on an anion-exchange RESOURCE Q column (Pharmacia Biotech; 0.64 cm $\times$ 3 cm), and eluted with 20 mM sodium phosphate, pH 7.0, with a linear gradient of 0.1 to 0.6 M NaCl. The peak fractions were dialyzed against 20 mM sodium phosphate, and used as purified proAPI preparation.

API was purified from the crude proAPI as follow. Five

milliliters of 2 M glycine-HCl, pH 2.7, was added to 50 ml of the crude proAPI preparation and incubated at 25°C for 1 h (for 12 h in the cases of D76S, D76T, D76E, S78A, and ⊿S78 mutant). The solution was dialyzed against 1 liter of 20 mM sodium acetate, pH 5.0. The dialysate was applied to a RESOURCE Q column and eluted with 20 mM sodium acetate, pH 5.0, containing a linear gradient of 0 to 1.0 M NaCl. The peak fractions were dialyzed against 20 mM sodium acetate, pH 5.0, and used as purified API. The purity of proteins were examined by SDS-PAGE (*31*).

The mutant enzymes were purified by the same methods, except for the autoactivation time.

Enzyme Assay—Proteolytic activities with milk casein and hemoglobin were assayed at pH 2.7 by a previously described method (*10*). One katal is defined as the amount of enzyme yielding the color equivalent of 1 mol of tyrosine per second with Folin-Ciocalteu's reagent using protein substrates at pH 2.7 and 30°C. Trypsinogen activation was performed as described previously (*16*). One katal is defined as the amount of enzyme forming 1 mol of trypsin per second at pH 3.0 and 30°C. Three chromogenic peptide substrates, Pro-Thr-Glu-Phe-Phe(NO_2)-Arg-Leu (peptide A), Pro-Thr-Glu-Lys-Phe(NO_2)-Arg-Leu (peptide B), and Ac-Ala-Ala-Phe-Phe(NO_2)-Ala-Ala-amide (peptide C), were used as substrates for kinetic analyses. Assays with peptides A and B were performed in 0.1 M sodium acetate, pH 4.5, at 30°C as described by Dunn *et al.* (*32*) and with peptide C, in 0.1 M sodium acetate, pH 4.5, at 30°C by the method of Hofmann and Hodges (*33*). At the end of the reaction, the products were isolated by reverse-phase HPLC on a column of TSK ODS 120-T and identified by amino acid sequence analysis. The rates of reactions were measured for various concentrations of substrate to determined the kinetic constants K_m and V_{max}. Values for k_{cat} were derived from $V_{max} = k_{cat}$ [E], where [E] is the enzyme concentration.

Hydrolysis of Peptides and Isolation of Hydrolysis Products—Angiotensin I (0.40 μmol, 0.52 mg) was dissolved in 1 ml of H_2O, and the pH was adjusted to 3.0 with diluted HCl. These solutions were used as the substrates for analysis of substrate specificity. Purified enzyme (1.4 μg) was added to 100 μl of substrate solution (enzyme/substrate; 1/1,000, mol/mol), and the mixture was incubated at 30°C. The reaction was stopped by addition of 1 μl of concentrated NH_4OH. Samples of hydrolysate were evaporated to dryness in a centrifugal evaporator, and then dissolved in 100 μl of H_2O. Separation and identification of peptides were performed by HPLC on a column (4.0 mm × 250 mm) of TSKgel ODS 120-T (TOSOH) equipped with a Hitachi model L-6200 delivery system. Chromatographic recording was performed at 216 nm with a Hitachi L-4000 UV detector. Each peak was collected, evaporated to dryness and used for the amino acid sequence analysis.

N-Terminal Amino Acid Sequence Analyses—N-terminal amino acid sequences of enzymes were determined by the method of Matsudaira (*34*). Protein samples were subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane using a semi-dry electroblotter (Trans-Blot SD, Bio-Rad, CA, USA). Coomassie Brilliant Blue R-250 stained bands were cut from the membrane and subjected to N-terminal sequence determination on an Applied Biosystems 473A protein sequencer with a 610A data analysis system.

The amino acid sequences of the digests of angiotensin I were determined according to the procedure of Hewick *et al.* (*35*).

CD Measurements—The circular dichroism (CD) measurements were performed as described previously by Yamaguchi *et al.* (*36*). The contents of α-helix and β-structure of the enzymes were calculated according to the SSE-338 program given in Ref. *37*.

RESULTS

Overexpression and Purification of Recombinant proAPI—The ATG codon was added in front of the putative pro sequence of API cDNA by the PCR technique. The cDNA fragment was cloned into pET12a, and the resulting plasmid, pETAP was introduced into *E. coli* BL21(DE3) cells. The cells harboring pETAP produced proAPI under the control of T7 promoter, and the enzyme was not active and was mostly insoluble. We therefore developed a procedure for the renaturation of recombinant proAPI into the correctly refolded zymogen, as described in "EXPERIMENTAL PROCEDURES." The inclusion bodies were solubilized with 8 M urea, and dialyzed under alkaline pH. The active proenzyme was purified to homogeneity by two chromatography steps on a Toyopearl HW-55F column and a RESOURCE Q column. The purity of protein was judged by SDS-PAGE (Fig. 1, lane 1). The yields of purified proAPI from inclusion bodies were routinely 10–20%. The amino acid sequence of the N-terminal region was determined as Met-Ala-Pro-Ala-Pro-Thr-Arg-Lys-Gly-, identical to that expected from the construction. ProAPI was converted to the mature form within 5 min by incubating at pH 2.7, 0°C (Fig. 1, lane 3). The conversion was inhibited by pepstatin, an aspartic proteinase inhibitor (Fig. 1, lane 2). These results indicate that API was generated from proAPI by autocatalytic activation.

Purified API was prepared from the crude proAPI preparation by acidification and subsequent chromatography on a RESOURCE Q column, and gave a single band on SDS-PAGE (Fig. 1, lane 4). The N-terminal sequence of recombinant API was found to be Glu-Ala-Ala-Ser-Lys-Gly-Ser-Ala-, which was three amino acid residues longer than that of native API from *Aspergillus saitoi* (Ser-Lys-Gly-Ser-Ala-). These results suggested that autocatalytic cleavage of the propeptide may be occurring in front of glutamine and the product is trimmed by aminopeptidase(s) in the fungal culture or that the activation of the zymogen *in vitro* may be basically different from that *in vivo*. In spite of the difference of N-terminal sequences of the authentic and recombinant APIs, these two enzymes

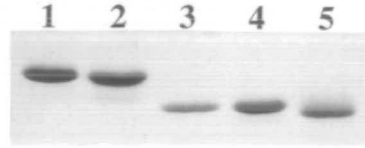

Fig. 1. **Conversion of proAPI to mature API.** Purified proAPI was incubated in 0.2 M glycine-HCl, pH 2.7, in the presence (lane 2) or absence (lane 3) of 0.1 mM pepstatin A at 25°C for 30 min. After the incubation, samples were subjected to SDS/PAGE on a 10% gel. The gel was stained with Coomassie Brilliant Blue R-250. Lane 1, purified proAPI; lane 4, purified mature API; lane 5, native API.

were almost equal in specific activity for hydrolysis of casein (0.138 and 0.139 katal/kg, respectively) and kinetic parameters for hydrolysis of a peptide substrate, Pro-Thr-Glu-Phe-Phe(NO_2)-Arg-Leu, at pH 3.0 (K_m = 0.01 mM and k_{cat} = 15.5 s^{-1} for both enzymes).

Molecular Properties of Mutant APIs—The mutant APIs were purified by the same method as the wild-type API. The purities of the enzymes were judged by SDS-PAGE (Fig. 2). The mobilities of mutant APIs were the same as that of the wild-type enzyme from *E. coli*. Furthermore, the effects of the mutation on the secondary structures of the enzymes were investigated by CD. As Fig. 3 shows, the CD spectra (from 190 to 260 nm) of the enzymes were essentially identical, and the contents of α-helix and β-structure of the wild-type and mutant enzymes were very similar. These results indicated that there was no significant conformational difference between the wild-type and mutant APIs.

Enzymatic Activities—Table II shows a comparison of the specific activities of wild-type and mutant APIs for the hydrolyses of casein and hemoglobin and the activation of bovine trypsinogen by cleaving the Lys^6-Ile^7 bond. The activity of trypsinogen activation fell below the detectable level in the D76S mutant without any change of the proteinase activities for casein and hemoglobin. However, in the D76N mutant both proteolytic and trypsinogen activating activities were approximately half those of wild-type. In the D76E and S78A mutants there are significant decreases in the activity of trypsinogen activation, with very little effect on the proteinase activities for

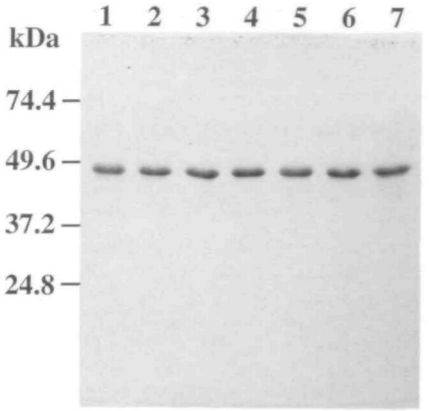

Fig. 2. **SDS/PAGE of purified wild-type and mutant APIs.** Lane 1, wild-type API; lane 2, D76S mutant; lane 3, D76T mutant; lane 4, D76N mutant; lane 5, D76E mutant; lane 6, S78A mutant; lane 7, ΔS78 mutant.

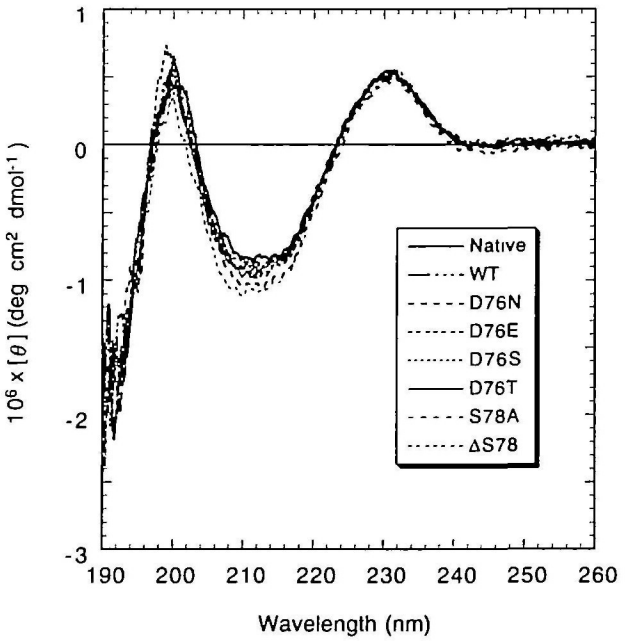

Fig. 3. **CD spectra of wild-type and mutant APIs.** The spectra were measured with a Jasco J-700 spectropolarimeter at room temperature and at the enzyme concentration of 0.2 mg/ml.

TABLE II. **Specific activities of wild-type and mutant APIs for the hydrolyses of casein and hemoglobin and the activation of bovine trypsinogen.** Proteolytic activities were assayed in 50 mM sodium acetate, pH 2.7, 1% casein or hemoglobin at 30°C. The activation of bovine trypsinogen was done with 5 mM trypsinogen in 50 mM sodium citrate, pH 3.0 at 30°C. Parentheses indicate the percentages of the activities of wild-type API.

Enzyme	Specific activity		
	Casein (katal/kg)	Hemoglobin (katal/kg)	Trypsinogen activation (mkatal/kg)
Wild type	0.139 (100)	0.75 (100)	5.1 (100)
D76N	0.060 (43)	0.38 (51)	2.3 (45)
D76E	0.041 (29)	0.45 (60)	0.02 (0.4)
D76S	0.103 (74)	0.40 (53)	N.D.
D76T	0.023 (17)	0.12 (16)	N.D.
S78A	0.142 (102)	0.53 (71)	0.37 (7)
ΔS78	0.010 (7)	0.077 (10)	N.D.

TABLE III. **Kinetic parameters for wild-type and mutant APIs towards the peptide substrates containing phenylalanine or lysine residue at the P_1 position.** Reactions were carried out in 0.1 M sodium acetate, pH 4.5 at 30°C. The kinetic parameters were determined from plots of initial rates *versus* substrate concentration (0.002-0.300 mM for all substrates).

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
PTEF*F(NO_2)RL			
WT	0.007	15.7	2,240
D76N	0.010	8.3	830
D76E	0.057	10.9	191
D76S	0.061	10.8	177
D76T	0.073	5.9	81
S78A	0.017	20.3	1,190
ΔS78	0.107	10.8	101
PTEK*F(NO_2)RL			
WT	0.009	4.5	500
D76N	0.027	3.2	119
D76E	0.050	0.20	4
D76S	0.034	0.040	1.2
D76T	0.065	0.014	0.2
S78A	0.017	0.61	36
ΔS78	0.046	0.036	0.8
Ac-AAK*F(NO_2)AA-NH_2			
WT	0.092	6.8	74
D76N	0.103	3.5	34
D76E	0.171	0.039	0.2
D76S		N.D.	
D76T		N.D.	
S78A	0.130	0.93	7
ΔS78	0.094	0.001	0.01

N.D., not ditected. *Asterisks indicate the scissile peptide bonds.

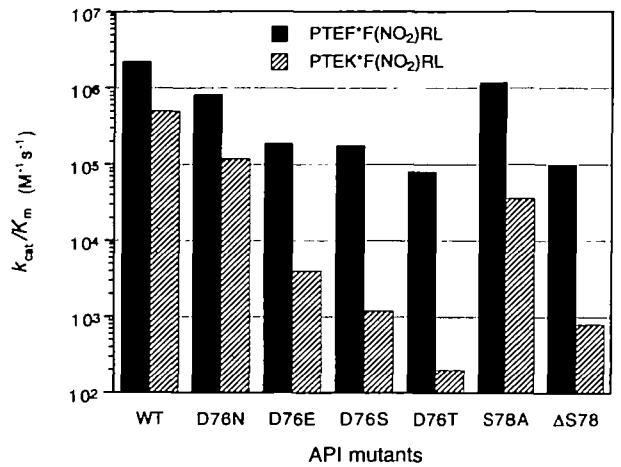

Fig. 4. **Catalytic efficiencies (k_{cat}/K_m) for mutant APIs against peptide substrates having Phe or Lys at the P_1 position.** Data from Table IV.

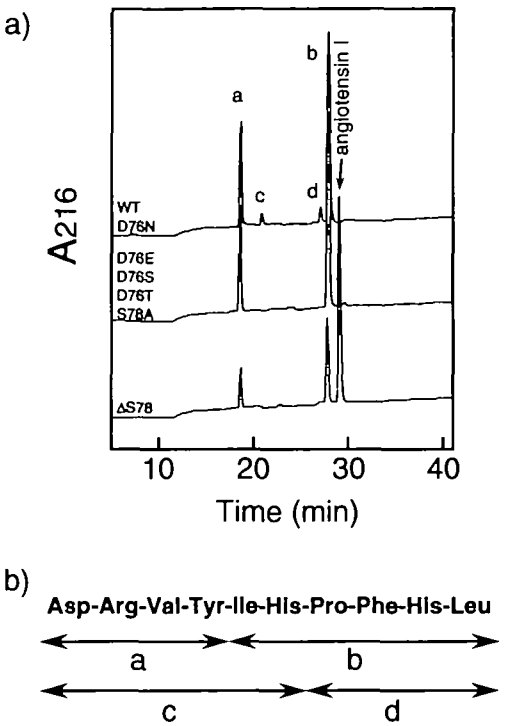

Fig. 5. **HPLC profiles for the digests of angiotensin I with wild-type and mutant APIs (a) and the cleaving sites (b).** Angiotensin I was hydrolyzed with wild-type or mutant API at 30°C, pH 3.0, and the products were subjected to HPLC separation on a reverse-phase column. The peptides were structurally identified.

casein and hemoglobin. The catalytic activities of D76T and ⊿S78 were generally decreased, but there were larger reductions in trypsinogen activating activity, as with D76S and S78A APIs. These results strongly suggest that Aps-76 and Ser-78 of API play an important role in the recognition of the basic amino acid residue at the P_1 position.

Kinetic Studies—To probe the effects of the mutations on specificity at the P_1 position, we used peptide substrates, Pro-Thr-Glu-Phe-Phe(NO_2)-Arg-Leu (peptide A), Pro-Thr-Glu-Lys-Phe(NO_2)-Arg-Leu (peptide B), and Ac-Ala-Ala-Lys-Phe(NO_2)-Ala-Ala-amide (peptide C). Peptide A was cleaved at Phe-Phe(NO_2) bond, which represents pepsin-like activity, and peptides B and C were cleaved at the Lys-Phe(NO_2) bond, which is equivalent to trypsinogen activation. No other bond in these peptides was hydrolyzed by any of the enzymes. From kinetic determination of the k_{cat}/K_m (catalytic efficiency) one can obtain the second-order rate constant for conversion of substrate to product (*38*). Combining the effects due to substrate binding and transition state stabilization, this parameter is useful for assessing altered substrate specificity. Differences in $\log(k_{cat}/K_m)$ provide an accurate measure of the lowering of the transition state activation energy ($\Delta G_T^{\ddagger}$). The k_{cat}, K_m, and k_{cat}/K_m values were determined from the initial rate measurements and the results are listed in Table III and graphically represented in Fig. 4.

The effects of individual mutations on the activity of API towards the substrate that contain Phe at P_1 were considerably less dramatic. The mutations at Asp-76 and Ser-78 led to a 2- to 30-fold lowering of k_{cat}/K_m compared to the wild-type API for this substrate. The decrease in k_{cat}/K_m was largely due to the effect of the mutation on K_m. There was a significant effect of the mutations on activity towards the substrate containing Lys at P_1. For peptide B, the D76E, D76S, D76T, and ⊿S78 mutations caused 130-, 430-, 2,600-, and 650-fold decreases in k_{cat}/K_m value compared to the wild-type API, respectively, whereas the D76N mutant effectively hydrolyzed this substrate. Peptide B has the same sequence except for the substitution of P_1 Phe to Lys. The effect of each mutation on K_m was similar to that in the case of peptide A, indicating that the changes of K_m value caused by the mutation may be independent of the interaction with the P_1 side chain. The decreases in catalytic efficiencies mainly reflect decreases in k_{cat} for the Lys side chain of the substrate. The effects of each mutation on activity towards peptide C were similar to those in the case of peptide B, although there was no detectable cleavage with D76S and D76T mutant APIs.

Cleavage of Angiotensin I—A summary of the actions of wild-type and mutant APIs on angiotensin I is shown in Fig. 5. The wild-type and D76N APIs cleaved the Tyr^4-Ile^5 and His^6-Pro^7 bonds of angiotensin I, while only the Tyr^4-Ile^5 bond was hydrolyzed by D76E, D76S, D76T, S78A, and ⊿S78 APIs. Thus, the mutations of Asp-76 and Ser-78 caused changes of the substrate specificities of API, decreasing the affinity for basic residues at the P_1 position, and making the specificity more restrictive so that only hydrophobic residues are acceptable at the P_1 and P_1' position, as in mammalian aspartic proteinases.

DISCUSSION

We have achieved the overproduction and purification of the zymogen of aspergillopepsin I in *E. coli*. The recombinant proAPI, which was accumulated as inclusion bodies, was refolded by solubilization in 8 M urea and subsequent alkaline dialysis. The active proAPI was autocatalytically converted to mature API by acidification, and this process was inhibited by pepstatin, an aspartic proteinase inhibitor. Similar results were observed for pepsinogen (*39*), procathepsin D (*40*), prochymosin (*41*), rhizopuspepsinogen (*42*), and canditropsinogen, the zymogen of aspartic proteinase from *Candida tropicalis* (*43*). The measured K_m

and k_{cat} values against a peptide substrate, Pro-Thr-Glu-Phe-Phe(NO_2)-Arg-Leu, and the CD spectra were indistinguishable between the native and recombinant API, which indicated that recombinant API was enzymatically and structurally identical to native API in spite of the difference in N-terminal sequences of the enzymes. It seems that our expression system is effective for site-directed mutagenesis studies of API.

We have mutated the Asp-76[77] and Ser-78[79] residues of API to test their roles in determining the primary specificity. These residues are conserved only in the enzymes with the ability to activate trypsinogen, such as penicillopepsin and rhizopuspepsin. The three-dimensional enzyme-inhibitor complex structures have been analyzed for endothiapepsin, penicillopepsin and rhizopuspepsin. According to these analyses, the side-chain carboxylate and amide nitrogen of Asp-77 are hydrogen bonded to P_2 amide nitrogen and carbonyl of the inhibitor, respectively, and there is a hydrogen bond between the P_2 carbonyl oxygen and amide nitrogen of Gly-76 (Fig. 6A). When a hydrophobic amino acid occupies the P_1 position of a ligand, the hydrophobic side chain is accommodated in the hydrophobic S_1 pocket, which consists of Asp-31, Tyr-75, Phe-112, and Leu-121 (*19-22, 26, 27*). The three-dimensional structure of the penicillopepsin/Iva-Val-Val-LySta-OEt (Iva: isovaleryl; LySta: 4*S*,3*S*-4,8-diamino-3-hydroxyloctanoic acid) complex revealed that the ε-amino group of the LySta side chain forms a tight ion pair with the carboxylate of Asp-77 and hydrogen bonds with the carboxylate of Asp-77 and hydroxyl group of Ser-79 (Fig. 6A) (*19*). We therefore altered Asp-76 of API to Asn in order to replace the carboxyl group with an uncharged side chain very similar in size to that of Asp, and to Glu so that the carboxyl group would retained but the effective distance to the side chain of the P_1 residue of a substrate would change. The substitution of Ser-78 to Ala was made to examine the effect of hydrogen bonding on P_1 Lys specificity by the removal of the hydroxyl group of the Ser side chain. The D76S and D76T substituted and Ser-78 deleted APIs corresponding to mammalian aspartic proteinases were also constructed.

Each enzyme was overexpressed in *E. coli* and purified by anion-exchange column chromatography. The molecular properties of the mutant APIs, relative molecular mass, N-terminal sequence, and secondary structure, were essentially identical with those of the wild-type API. Each mutant maintained sufficient proteolytic activities for casein and hemoglobin, whereas the specific activity for trypsinogen activation was substantially reduced by the D76S, D76T, and ⊿S78 mutations. Previously, we reported that D76S mutant API expressed in *S. cerevisiae* had no activity for trypsinogen activation and we suggested that the activity was quantatively influenced by the electrostatic interaction between P_1 Lys of the substrate and Asp-76 of API (*16*). However, the D76N mutant still retained trypsinogen activating activity and deletion of Ser-78 (though the charge of Asp-76 is still present) disabled the Lys residue of trypsinogen from accommodation in the S_1 pocket, indicating that the electrostatic interaction was not essential for P_1 Lys specificity.

API digests the His^6-Pro^7 bond in a tetradecapeptide renin substrate (*12, 13*). The wild-type and D76N APIs cleaved the His^6-Pro^7 bond of angiotensin I, whereas no cleaving activity for this bond was found for D76E, D76S, D76T, S78A, and ⊿S78 mutants. The Tyr^4-Ile^5 bond was effectively hydrolyzed by each mutant enzyme. These results are consistent with the kinetic analysis using the peptide substrates: the activities of API toward the sub-

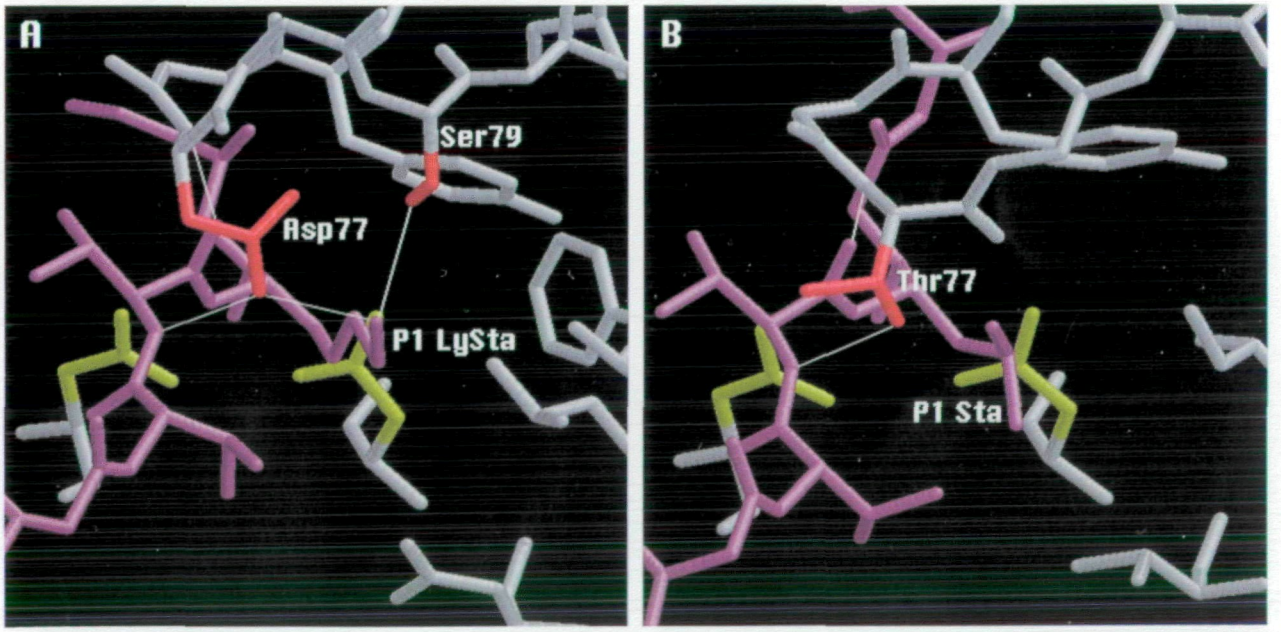

Fig. 6. **Representations of S_1 subsites of penicillopepsin (A) and porcine pepsin (B) with inhibitors.** The crystal structures of these enzymes were retrieved from Brookhaven Protein Data Bank (PDB). (A) Structure of penicillopepsin/Iva-Val-Val-LySta-OEt complex (file 1APT in PDB). (B) Structure of human pepsin/pepstatin (file 1PSO in PDB). The residues corresponding to the mutated residues in API, Asp-76 and Ser-78, are colored red in each depiction. The catalytic residues and inhibitors are shown in yellow and purple, respectively. White lines indicate hydrogen bonds.

strates containing a basic amino acid at the P_1 position are influenced by the mutations at Asp-76 and Ser-78, while this is not the case for a hydrophobic residue at the P_1 position. *Candida albicans* aspartic proteinase (candidapepsin; EC 3.4.23.24) hydrolyzes the His-Thr bond in the fluorogenic substrate 4-(4-dimethylaminophenylazo)benzoyl-γ-aminobutyryl-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-[5-(2-aminoethyl)amino]naphthalene-1-sulfonic acid (*44*) and also activates trypsinogen (*45*). Position 77 and 79 are conserved as Asp and Ser in candidapepsin, respectively. These results indicate that Asp-77 and Ser-79 of fungal enzymes are important for the recognition of a basic amino acid residue at the P_1 position.

A comparison of the Michaelis-Menten kinetic parameters for the hydrolysis of peptides A and B by the site-directed mutants of API suggests that Asp-76 and Ser-78 play important roles in API activity when substrates contain Lys at the P_1 position. The inhibition analysis of D77T mutated rhizopuspepsin using Lys-Pro-Ala-Ala-[X]-Ala-Leu-Gly-NH_2, where [X] is LySta or Ac-LySta, showed that rhizopuspepsin is able to bind substrates containing Lys at the P_1 position through the electrostatic interaction of Asp-77 with P_1 Lys (*46*). However, D76N mutant API retained sufficient activities towards peptides B and C containing Lys at the P_1 position, as well as trypsinogen activation, which indicated that the negative charge of Asp-76 may be dispensable for the recognition of P_1 Lys. On the basis of the data in Table III, although the changes of K_m value caused by the mutations may be independent of the interaction with the P_1 side chain, the D76S, D76T, and ⊿S78 mutations result in large reductions in k_{cat} value *versus* the substrates containing Lys at the P_1 position. It appears that these residues on the active site flap have important roles in the stabilization of the transition state complex. This effect can be explained simply by one or both of two mechanisms: (i) a disturbance of the hydrogen bond network between P_1 Lys and the side chains of Asp-76 and Ser-78, and/or (ii) steric hindrance of substrate binding resulting from the change of flap structure. When D76S or D76T mutant API binds to a substrate containing Lys at the P_1 position, the hydroxyl group of Ser or Thr would be hydrogen bonded to P_2 amide nitrogen of the main chain as in mammalian enzymes (Fig. 6B) (*47-50*), which may cause an alteration of the side-chain torsion angle of Ser or Thr in order to hydrogen bond to the substrate and disable this group from binding to the ε-amino group of P_1 Lys. The substitution of Ser-78 [79] to alanine removes the hydrogen bond between Ser-78 and the ε-amino group of P_1 Lys. The deletion of Ser-78 in API, corresponding to mammalian aspartic proteinases such as pepsin (Fig. 6B), renin and cathepsin D, would give rise to a shorter and warped flap, which may change the direction of the Asp-76 side chain to bind to the main chain of the substrate and/or cause steric hindrance between the resulting flap and the ε-amino group of P_1 Lys. Thus, in the recognition of P_1 Lys of a substrate, Asp-76 and Ser-78 of API appear to contribute to the stabilization of the transition-state complex by forming a hydrogen bond network between P_1 Lys and the side chains of Asp-76 and Ser-78, which may be supported by the unique flap structure of API.

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