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Engineering the substrate specificity of rhizopuspepsin: The role of Asp 77 of fungal aspartic proteinases in facilitating the cleavage of oligopeptide substrates with lysine in P_1

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Abstract

Rhizopuspepsin and other fungal aspartic proteinases are distinct from the mammalian enzymes in that they are able to cleave substrates with lysine in the P_1 position. Sequence and structural comparisons suggest that two aspartic acid residues, Asp 30 and Asp 77 (pig pepsin numbering), may be responsible for generating this unique specificity. Asp 30 and Asp 77 were changed to the corresponding residues in porcine pepsin, Ile 30 and Thr 77, to create single and double mutants. The zymogen forms of the wild-type and mutant enzymes were overexpressed in *Escherichia coli* as inclusion bodies. Following solubilization, denaturation, refolding, activation, and purification to homogeneity, structural and kinetic comparisons were made. The mutant enzymes exhibited a high degree of structural similarity to the wild-type recombinant protein and a native isozyme. The catalytic activities of the recombinant proteins were analyzed with chromogenic substrates containing lysine in the P_1, P_2, or P_3 positions. Mutation of Asp 77 resulted in a loss of 7 kcal mol^{-1} of transition-state stabilization energy in the hydrolysis of the substrate containing lysine in P_1. An inhibitor containing the positively charged P_1-lysine side chain inhibited only the enzymes containing Asp 77. Inhibition of the Asp 77 mutants of rhizopuspepsin and several mammalian enzymes was restored upon acetylation of the lysine side chain. These results suggest that an exploitation of the specific electrostatic interaction of Asp 77 in the active site of fungal enzymes may lead to the design of compounds that preferentially inhibit a variety of related *Candida* proteinases in immunocompromised patients.

Keywords: aspartic proteinase; chromogenic substrate; electrostatic interactions; hydrogen bonding; inhibitors; rhizopuspepsin; substrate specificity

Rhizopuspepsin, an aspartic proteinase from the fungus *Rhizopus chinensis*, has been the subject of many substrate, inhibitor, and crystallographic studies. The insights gained from the native structure (Suguna et al., 1987a) and many different inhibitor complexes of rhizopuspepsin (Kinemage 1; Suguna et al., 1987b, 1992; Parris et al., 1992) and other fungal enzymes (Cooper et al., 1992; Lunney et al., 1993) have led to proposals for the reaction mechanism (Davies, 1990) and the rationalization of subsite preferences of the aspartic proteinases (Rao et al., 1993; Scarborough et al., 1993). The goal has been to use this information to design targeted therapeutics for renin and the HIV proteinase. Further clinical interest in rhizopuspepsin comes from its similarity to the secreted aspartic proteinases of several opportunistic *Candida* species (Morrison et al., 1993; Fusek et al., 1994). The knowledge gleaned from studying rhizopuspepsin has the potential to foster the design of anti-fungal agents for use in treating vaginal infections and immunocompromised AIDS, organ transplant, and cancer patients (Samaranayake & Holmstrup, 1989; Saral, 1991; Paya, 1993).

The primary specificity of aspartic proteinases is for cleavage between large hydrophobic-hydrophobic junctions, such as Phe-Phe (Fruton, 1976). Rhizopuspepsin and other fungal enzymes are distinct from the mammalian enzymes in that they also have a trypsin-like ability to cleave substrates with lysine (Balbaa et al., 1993) and to bind inhibitors with lysine and ornithine in the P_1 position (Salituro et al., 1987). Based on the

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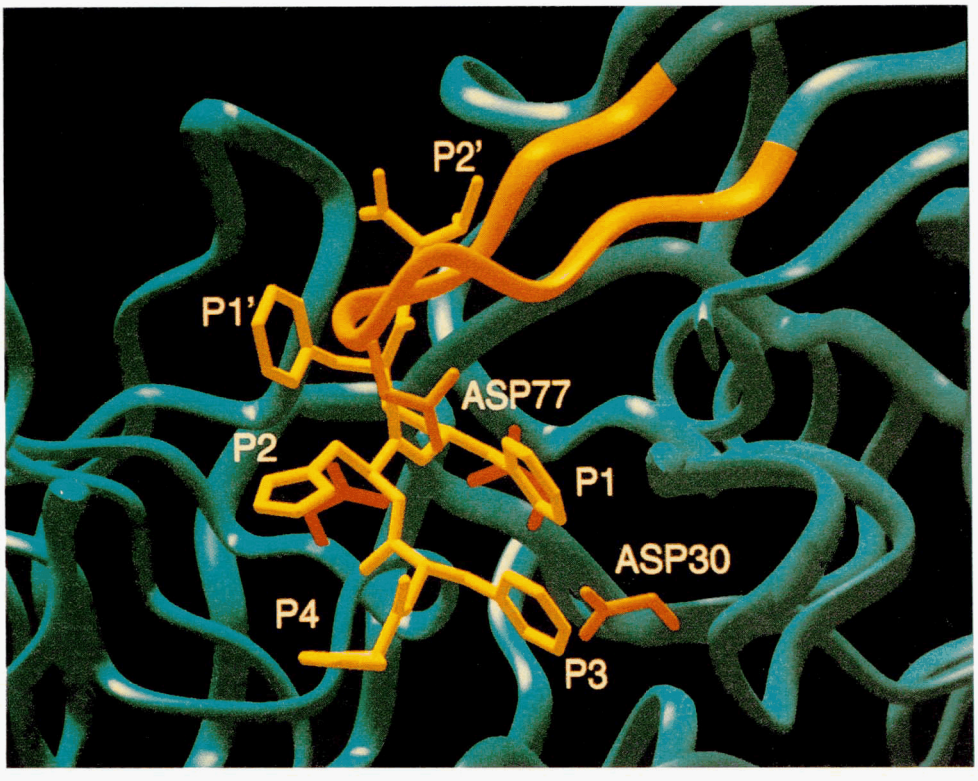

Fig. 1. Ribbon representation of rhizopuspepsin complexed with a reduced peptide bond inhibitor indicating the nomenclature of Schechter and Berger (1967). Catalytic aspartic acid residues, Asp 32 and Asp 215, are shown in red. The flap that extends over the active site and the inhibitor are shown in orange and yellow, respectively. Asp 30 and Asp 77 were mutated to Ile and Thr, respectively.

complex of penicillopepsin with an inhibitor containing lysine in P_1 (James et al., 1985), Asp 77 has been implicated in mediating this activity.

Sequence and structural comparisons, however, suggest that Asp 30 may also play a role (Table 1; Fig. 1). Besides the two catalytic aspartic acids, Asp 32 and Asp 215, rhizopuspepsin has additional aspartic acid residues at positions 30, 37, and 77. Asp 30 is situated at the boundary between the S_3 and S_1 subsites and has the potential to interact with positively charged residues in the P_3 and the P_1 positions of the substrate. Asp 37 is located in the S_2' subsite and probably does not directly affect primary specificity. Asp 77, which is also present in the *Candida* enzymes, is positioned at the end of the flap, points down into the active site cavity, and has the potential for interacting with the P_3, P_2, and P_1 positions of the substrate.

In an analogous manner to the studies on the lysine specificity of trypsin (Graf et al., 1988), mutations were made to the corresponding residues in porcine pepsin, Ile 30 and Thr 77. These experiments were performed in an effort to validate the importance of Asp 77 identified crystallographically and to quantitate the energetic contributions of both residues to substrate specificity. The proteins were overexpressed in *Escherichia coli*, refolded from inclusion bodies, activated, and purified for structural and kinetic comparisons. A series of systematically substituted substrates with lysine in either P_1, P_2, or P_3 was assayed and analyzed by double mutant cycles (Carter et al., 1984; Wells, 1990). Inhibitors containing lysine derivatives in P_1 were also studied to investigate the potential for selective inhibition of fungal aspartic proteinases.

Results

Mutagenesis, expression, and refolding

DNA sequencing of the entire coding region of each mutant protein showed the presence of only the desired base changes. Overexpression of the rhizopuspepsinogens in *E. coli* resulted in the formation of inclusion bodies. The purified inclusion bodies (0.5–1 g for 4 L of culture) contained rhizopuspepsinogen that was >95% pure with a molecular weight of approximately 43 kDa as determined by SDS-PAGE.

A buffer containing a high concentration of reducing agent and 8 M urea was used to solubilize the inclusion bodies. Two refolding methods were tried in order to maximize the yields for subsequent activation to the mature enzyme. Refolding by the rapid dilution procedure of Chen et al. (1991) resulted in primarily polymeric material that was difficult to activate completely into an active, monomeric state (data not shown). Even though there was some precipitation, the largest yields of monomeric zymogen capable of activation were obtained when the proteins were refolded by dialysis. Size-exclusion chromatography was used to separate the monomeric form of the zymogen from polymeric material and contaminating *E. coli* proteins.

Table 1. *Partial sequence alignment of several aspartic proteinases*[a]

HPEP	^{28}TVVFDTGSSN^{37}	^{74}TYGTG^{78}
PPEP	TVIFDTGSSN	TYGTG
CATE	TVIFDTGSSN	QYGTG
CATD	TVVFDTGSSN	HYGSG
HREN	KVVFDTGSSN	RYSTG
RCAP	NLDFDTGSSD	SYGDG
CAAP	NVIVDTGSSD	GYGDG
CTAP	TVVIDTGSSD	EYGDL
CPAP	TVIIDTGSSD	RYGDG

[a] This alignment was obtained using the PILEUP program, a module in the GCG Sequence Analysis Software Package (Devereux et al., 1984). The sequences are HPEP, human pepsin (Sogawa et al., 1983); PPEP, porcine pepsin (Chen et al., 1975); CATE, human cathepsin E (Azuma et al., 1992); CATD, human cathepsin D (Faust et al., 1985); HREN, human renin (Hobart et al., 1984); RCAP, *Rhizopus chinensis* aspartic proteinase (Chen et al., 1991); CAAP, *Candida albicans* aspartic proteinase (Hube et al., 1991); CTAP, *Candida tropicalis* asapartic proteinase (Togni et al., 1991); CPAP, *Candida parapsilosis* (de Viragh et al., 1993).

Yields at this stage of purification ranged from 15 to 35 mg for a 4-L preparation.

Activation and purification

Activation of the zymogens, to proteolytically competent forms around 35 kDa, was accomplished by lowering the pH of the solution. Sequence analysis of the wild-type recombinant (WT-REC) and Asp 30 Ile proteins, activated at pH 2.0, confirmed the N-termini of these proteins to be Thr-Ser-Thr-Gly-Gly-Ile-Val-Pro-Asp-. This sequence represents an extension of naturally occurring rhizopuspepsin by nine amino acids. The activation of the D77T and the D30I/D77T proteins at pH 2, however, produced a mixed population of N-termini: the 9-amino acid extension and a 15-amino acid extension, Asn-Lys-His-Lys-Ile-Asn-Thr-Ser-Thr-Gly-Gly-Ile-Val-Pro-Asp-.

Experiments varying temperature, pH, and protein concentration were performed in an attempt to optimize the activation conditions for each of the recombinant proteins and to obtain fully mature N-termini. These optimized conditions, however, still resulted in the nine-amino acid activation intermediate for each of the proteins. Increased yields of the nine-amino acid extended form were obtained for the D77T and the D30I/D77T proteins when the activation was performed at 37 °C and pH 3.0 and 3.5, respectively. The three additional positively charged residues proved to be fortuitous in separating the two activation intermediates by ion exchange. Interestingly, only upon mutation of Asp 77 to Thr was there a notable effect on the overall pI value of the protein observed (Fig. 2). Recombinant rhizopuspepsins were obtained in a highly purified form with yields ranging from 1 to 5 mg for 4 L of culture. All structural and kinetic comparisons were made using the Thr-Ser-Thr-intermediate of the rhizopuspepsins.

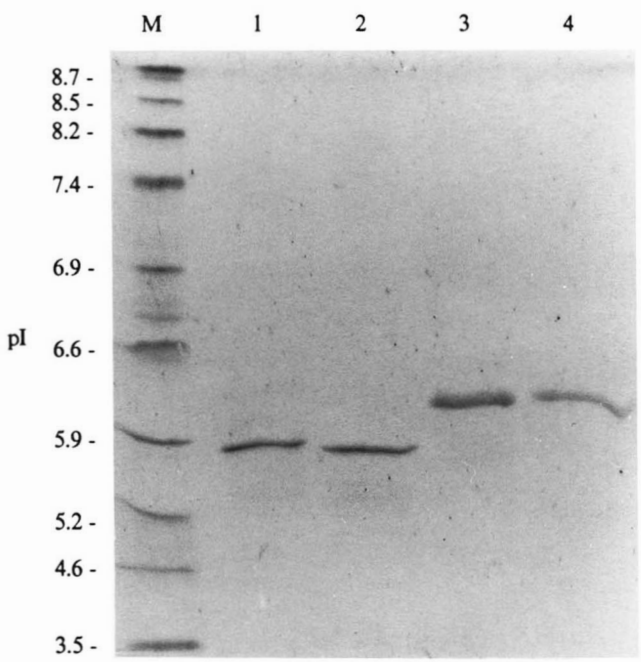

Fig. 2. Isoelectric focusing analysis of the purified recombinant rhizopuspepsins. M, pI markers; 1, wild-type (WT-REC); 2, Asp 30 Ile; 3, Asp 77 Thr; 4, Asp 30I/D77 Thr. The top marker band has a pI of 9.3 and was focused at the electrode/gel interface.

Structural comparisons

Mass spectrometry

The mass for each protein was determined to be as follows: isozyme pI 6, 34,173; WT-REC, 34,627; D30I, 34,638; D77T, 34,914; and D30I/D77T, 34,856. The sizes of the recombinant proteins are consistent with the addition of nine amino acids to the N-terminus of the native isozyme. The values for the WT-REC and D30I proteins are slightly lower than expected. This difference may be the result of C-terminal processing.

Fluorescence spectroscopy

Upon the addition of sufficient guanidinium hydrochloride (Gdn-HCl) to cause unfolding, the intrinsic fluorescence signal at 350 nm shifted to longer wavelengths with a 75% decrease in intensity (Fig. 3A). Analysis of the transition curves (Fig. 3B) by the method of Jackson et al. (1993) is presented in Table 2. Despite the lack of full reversibility of the folding process (data not shown), the pI 6 isozyme and wild-type recombinant proteins exhibit unfolding parameters that are indistinguishable from each other. The decrease in $[\text{Gdn-HCl}]_{50\%}$ of the two single mutants suggests a slight decrease in stability from the wild-type enzymes, WT-REC and isozyme pI 6. Further loss of stability is seen in the double mutant.

CD

Even though there are slight wavelength shifts in the spectra (Fig. 4), the mutants as a whole are, within experimental error, structurally similar to each other and the WT-REC enzyme. These shifts may be due to slight changes in the α-helix/β-sheet ratios. The differences seen between the proteins from 200 to 205 nm cannot be considered to be significant because of the

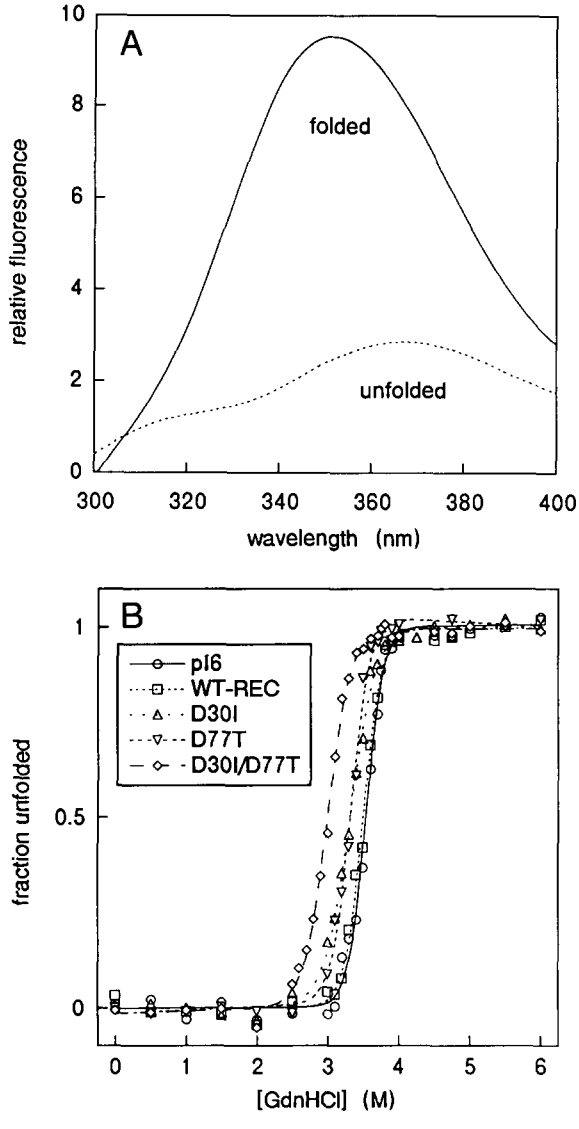

Fig. 3. **A:** Fluorescence emission spectra for folded (0 M Gdn-HCl) and unfolded (6 M Gdn-HCl) wild-type recombinant rhizopuspepsin. **B:** Gdn-HCl induced unfolding of the naturally occurring isozyme pI 6 and the recombinant forms of rhizopuspepsin monitored by the change in intrinsic fluorescence at 350 nm. Each point represents the average of duplicate samples.

high degree of signal fluctuation in this region on the instrument used.

Kinetic analysis of the recombinant rhizopuspepsins with substrates

Product analysis confirmed that all peptides were cleaved at the expected positions, that is, between Phe * Nph, Nle * Nph, or Lys * Nph. The enzymes show consistent trends in the kinetic parameters for all the substrates studied upon increase of the assay pH from 3.5 to 5.0: k_{cat} values were independent of pH, whereas two- to fivefold increases in k_{cat}/K_m were observed, mainly as the result of a corresponding decrease in K_m (Table 3). Importantly, all the enzymes have similar k_{cat} values at pH 5.0

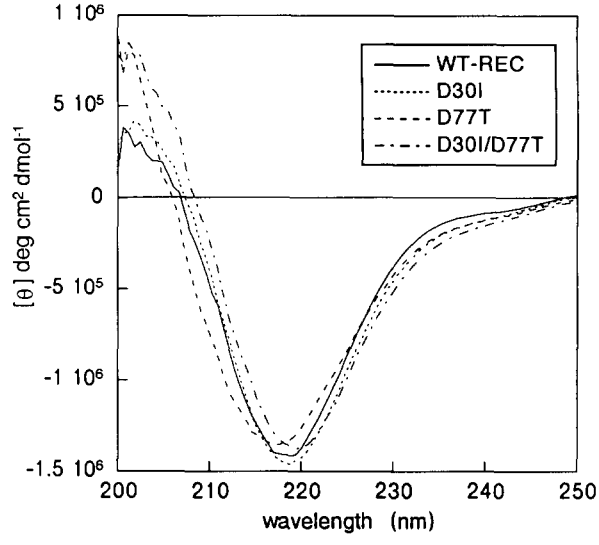

Fig. 4. CD spectra of the recombinant wild-type and the mutant forms of rhizopuspepsin.

for the control peptide 4. This observation confirms the overall similarity seen in the biophysical analysis of the protein folds and establishes the catalytic competence of the enzymes.

Wild-type recombinant

The wild-type enzyme exhibits the characteristic broad specificity seen for similar peptides (Lowther et al., 1991). All substrates, with (1-3) or without (4,5) the lysine residue placed in several alternative positions, were readily cleaved. Even though there is still a preference for the hydrophobic junction (1 and 4), the enzyme is able to cleave the P_1-lysine peptide (3) with equivalent efficiency (k_{cat}).

Asp 30 Ile mutant

This mutant cleaves the substrates, including the P_1-lysine derivative, with nearly identical k_{cat} values to the wild-type enzyme. A two- to threefold increase in K_m is seen with peptides 1-4. This increase results in k_{cat}/K_m values that are in the range seen for porcine pepsin with substrates 1, 2, and 4 (Table 4). The larger changes seen in all the kinetic parameters for peptide 5 are probably due to the loss of hydrophobic interactions when the aromatic phenylalanine is replaced by the aliphatic norleucine.

Asp 77 Thr mutant

The mutation of Asp 77 to Thr resulted in an enzyme that displays reduced k_{cat} values toward this series of substrates. The most significant result of this mutation appears to be the 100-fold increase in the K_m parameter and the parallel decrease in k_{cat}/K_m. This mutant enzyme is also no longer competent to cleave the P_1-lysine peptide as shown by the 10^5-fold decreases in k_{cat}/K_m from 360,000 to 5 M^{-1} s^{-1} at pH 3.5 and 1,660,000 to 13.7 M^{-1} s^{-1} at pH 5.0.

Asp 30 Ile/Asp 77 Thr mutant

The double mutant enzyme is able to cleave all the substrates but with decreased specificity (k_{cat}/K_m). This enzyme is simi-

Table 2. *Gdn-HCl denaturation parameters of native and mutant forms of rhizopuspepsin*[a]

Enzyme	$[\text{Gdn-HCl}]_{50\%}$ (M)	m (kcal mol^{-1} M^{-1})	$\Delta G_{U\text{-}F}^{H_2O}$ (kcal mol^{-1})
pI 6	3.54 ± 0.02	4.8 ± 0.5	17.0 ± 1.8
WT-REC	3.50 ± 0.02	4.6 ± 0.5	16.0 ± 1.7
Asp 30 Ile	3.31 ± 0.02	3.3 ± 0.2	10.9 ± 0.8
Asp 77 Thr	3.33 ± 0.02	4.5 ± 0.4	15.1 ± 1.4
Asp 30 Ile/Asp 77 Thr	3.00 ± 0.01	3.7 ± 0.2	11.0 ± 0.7

[a] Parameters derived from the denaturation curves presented in Fig. 3. Each enzyme was studied from 0 to 6 M Gdn-HCl in 0.1 M sodium formate, pH 3.0. All denaturant concentrations were performed in duplicate. Values were determined by the method of Jackson et al. (1993); curve fit of observed fluorescence, F, versus [denaturant]. The errors represented are the standard errors from the KaleidaGraph program.

Table 3. *Kinetic analysis of wild-type and mutant rhizopuspepsins: systematic substitution of lysine into P_3-P_1*[a]

		WT-REC			Asp 30 Ile		
Peptide[b]	pH	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$) × 10^{-6}	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$) × 10^{-6}
1 KPKAF * XRA	3.5	24 ± 3	19 ± 2	1.24 ± 0.17	29 ± 3	64 ± 2	0.46 ± 0.05
	5.0	28 ± 3	6 ± 1	4.90 ± 0.95	29 ± 3	14 ± 1	2.15 ± 0.30
2 KPAKF * XRA	3.5	36 ± 5	45 ± 7	0.81 ± 0.16	47 ± 7	139 ± 23	0.34 ± 0.07
	5.0	41 ± 5	19 ± 2	2.13 ± 0.34	30 ± 3	25 ± 2	1.22 ± 0.18
3 KPAAK * XRA	3.5	35 ± 4	98 ± 6	0.36 ± 0.05	33 ± 5	395 ± 51	0.08 ± 0.02
	5.0	35 ± 4	21 ± 1	1.66 ± 0.20	40 ± 5	99 ± 9	0.40 ± 0.06
4 KPAAF * XRA	3.5	33 ± 4	15 ± 1	2.17 ± 0.26	15 ± 2	31 ± 1	0.49 ± 0.05
	5.0	34 ± 4	7 ± 1	4.73 ± 0.63	16 ± 2	17 ± 2	0.94 ± 0.17
5 KPAAZ * XRA	3.5	18 ± 2	18 ± 1	1.00 ± 0.12	67 ± 8	173 ± 15	0.39 ± 0.06
	5.0	22 ± 2	10 ± 1	2.26 ± 0.41	56 ± 6	60 ± 5	0.94 ± 0.13

		Asp 77 Thr			Asp 30 Ile/Asp 77 Thr		
Peptide[b]	pH	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$) × 10^{-4}	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$) × 10^{-4}
1 KPKAF * XRA	3.5	6 ± 1	561 ± 115	1.00 ± 0.30	–	–	0.26 ± 0.02[c]
	5.0	6 ± 1	283 ± 25	1.90 ± 0.30	–	–	0.71 ± 0.04[c]
2 KPAKF * XRA	3.5	6 ± 1	487 ± 83	1.12 ± 0.30	–	–	0.67 ± 0.04[c]
	5.0	7 ± 1	296 ± 44	2.50 ± 0.60	9 ± 1	449 ± 36	2.03 ± 0.24
3 KPAAK * XRA	3.5	–	–	5.0 ± 0.7 × 10^{-4}[d]	–	–	0.9 ± 0.1 × 10^{-4}[d]
	5.0	–	–	13.7 ± 2.1 × 10^{-4}[d]	–	–	3.4 ± 0.3 × 10^{-4}[d]
4 KPAAF * XRA	3.5	15 ± 3	408 ± 48	3.80 ± 0.80	–	–	1.90 ± 0.10[c]
	5.0	17 ± 2	228 ± 17	7.30 ± 1.20	13 ± 1	253 ± 25	5.30 ± 0.70
5 KPAAZ * XRA	3.5	2 ± 0.4	297 ± 41	0.80 ± 0.20	–	–	0.07 ± 0.01[c]
	5.0	5 ± 1	289 ± 39	1.60 ± 0.40	1 ± 0.1	462 ± 62	0.21 ± 0.04

[a] Kinetic parameters (±standard deviation) were determined at 37 °C with either 0.1 M sodium formate, pH 3.5, or 0.1 M sodium acetate, pH 5.0, containing NaCl to maintain constant ionic strength.

[b] Abbreviations used in the substrates are: X, *p*-nitrophenylalanine; Z, norleucine; *, site of cleavage.

[c] k_{cat}/K_m values were determined spectrophotometrically with the assumption that $[S] \ll K_m$; $v = (k_{cat}/K_m)[E]_0[S]_0$.

[d] k_{cat}/K_m values were determined by capillary electrophoresis; $v = (k_{cat}/K_m)[E]_0[S]_0$.

Table 4. *Kinetic analysis of porcine pepsin: Systematic substitution of lysine into P_3-P_1*[a]

		PPEP		
Peptide	pH	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\ s^{-1}$) $\times 10^{-6}$
1 KPKAF * XRA	3.5	–	–	0.0030 ± 0.0005[b]
	5.0	2.4 ± 0.5	122 ± 20	0.019 ± 0.005
2 KPAKF * XRA	3.5	21 ± 5	339 ± 77	0.06 ± 0.02
	5.0	20 ± 3	31 ± 2	0.66 ± 0.11
3 KPAAK * XRA	3.5		[c]	
	5.0		[c]	
4 KPAAF * XRA	3.5	29 ± 6	83 ± 12	0.41 ± 0.09
	5.0	24 ± 5	61 ± 11	0.39 ± 0.10
5 KPAAZ * XRA	3.5	22 ± 4	103 ± 12	0.21 ± 0.04
	5.0	16 ± 3	34 ± 3	0.46 ± 0.09

[a] Assay conditions and abbreviations are the same as those used in Table 3.

[b] k_{cat}/K_m values were determined spectrophotometrically with the assumption that $[S] \ll K_m$; $v = (k_{cat}/K_m)[E]_0[S]_0$.

[c] Cleavage occurred between Nph-Arg (X-R) instead of Phe * Nph (F * X).

lar to the Asp 77 Thr mutant in that it is characterized by the inability to readily cleave the P_1-lysine substrate.

Kinetic analysis of porcine pepsin

Peptides 2, 4, and 5 are readily cleaved with k_{cat} values similar to that of wild-type rhizopuspepsin (Table 4). The k_{cat}/K_m values, however, are two- to fivefold lower. Peptide 1 is poorly cleaved at pH 3.5, consistent with previous observations (Pohl & Dunn, 1988; Rao-Naik & Dunn, unpubl. results). Improvement is seen upon raising the pH, although not to levels seen for the other peptides. Pepsin behaves similarly to rhizopuspepsin with respect to peptide 2 in that the specificity (k_{cat}/K_m) increases without changes in k_{cat}. Importantly, hydrolysis of the P_1-lysine substrate (3) by porcine pepsin did not occur between Lys * Nph. After an extended incubation period with excess enzyme, cleavage was seen between Nph-Arg.

Kinetic analysis of the recombinant rhizopuspepsins with inhibitors

The WT-REC and Asp 30 Ile enzymes were inhibited by both lystatine-based inhibitors (Table 5). The Asp 77 mutants and the mammalian enzymes, however, were not inhibited by the nonacetylated inhibitor. Upon acetylation and removal of the positive charge, inhibition was regained.

Discussion

Authenticity of the recombinant rhizopuspepsins

Several lines of evidence exist to support the conclusion that the recombinant rhizopuspepsins are enzymatically and structurally similar to the native isozymes. Maturation of rhizopuspepsinogen requires catalytic activity. Kinetic comparisons between the two naturally occurring isozymes and the wild-type recombinant enzyme have shown that the nine-amino acid N-terminal extension and differences in pI values do not result in significant deviations in catalytic activity (unpubl. results). These observations suggest that the slight differences seen in the pI values of the mutants will not adversely affect their kinetic analysis.

Even though the refolding process of rhizopuspepsin is not fully reversible, denaturation studies with the aspartic proteinase prochymosin have also shown that the recombinant protein is directly comparable to the native enzyme (Sugrue et al., 1990). Because extensive denaturation studies have not been carried out on mutants of the aspartic proteinases, the trends seen in the change in stability of the large library of mutants and their crystal structures may be useful in rationalizing the change in the denaturation parameters seen for the mutants of rhizopuspepsin (Fig. 3; Table 2). Mutations that have been shown to cause significant changes in stability in comparison to the wild-type protein can be grouped into several categories: (1) insertion or deletion of an amino acid; (2) addition or deletion of disulfide crosslinks; (3) changes made near the ends of loops and α-helices; and (4) the alteration of hydrophobic packing in the core of the protein by the deletion of methylene equivalents (Shortle, 1992). Positions 30 and 77 do not fall within any of the categories mentioned above (Kinemage 1). Because these residues are located on the protein surface and are present in most other mammalian enzymes, the structures were not expected to change significantly (Matthews, 1993).

This discussion, however, still does not explain why differences, particularly between the m and $\Delta G_{U\text{-}F}^{H_2O}$ values of the Asp 30 Ile and Asp 30 Ile/Asp 77 Thr mutant proteins, are observed when comparisons are made to the WT-REC and pI 6 proteins. Several studies have shown that the value is related to the solvent-accessible surface area of the denatured state of the

Table 5. *Inhibition of recombinant rhizopuspepsins and selected mammalian aspartic proteinases by inhibitors of the form Lys-Pro-Ala-Ala-[X]-Ala-Leu-Gly-NH_2*[a]

	K_i (μM)[b]	
Enzyme	[LySta]	[Ac-LySta]
WT-REC	0.18 ± 0.01	0.07 ± 0.01
D30I	2.2 ± 0.1	4.9 ± 0.4
D77T	0%[c]	66.5 ± 3.6
D30I/D77T	0%[c]	21%[c]
HPEP	7%[c]	80%[c]
CATD	0%[c]	46%[c]
CATE	0%[c]	100%[c]
HGAS	2%[c]	66%[c]

[a] [X], [LySta], H_2N-$[CH_2]_4$-$CH(NH_2)$-$CH(OH)CH_2$-COOH (lystatine); [Ac-LySta], CH_3CO-HN-$[CH_2]_4$-$CH(NH_2)$-$CH(OH)CH_2$-COOH; abbreviations for the mammalian enzymes in Table 1; HGAS, human gastricsin.

[b] All assays for the recombinant rhizopuspepsins were performed at pH 5.0, 0.1 M sodium formate. Assay conditions for the mammalian enzymes were: HPEP, pH 3.5, 0.1 M sodium formate; CATD, pH 3.7, 0.1 M sodium formate; CATE, pH 4.6, 0.1 M sodium acetate; HGAS, pH 3.2, 0.1 M sodium formate.

[c] Percent inhibition at 200 μM inhibitor.

protein (Schellman, 1978; Shortle & Meeker, 1989). Mutants that exhibit values less than the wild-type protein show more compact structures and residual structural components when compared to the wild-type proteins by size-exclusion chromatography and CD. These observations suggest that rhizopuspepsin mutants with Ile at position 30 may be able to optimize hydrophobic interactions in the denatured state when compared to enzymes with Asp in this position. These new interactions may make the Asp 30 mutants less stable in water with the equilibrium shifting slightly in favor of the denatured state and a decrease in $\Delta G_{U\text{-}F}^{H_2O}$.

Substrate and inhibitor design

The initial experimental evidence that the fungal aspartic proteinases were distinct from the mammalian enzymes came from the observation that they were able to activate trypsinogen at a Lys-Ile bond (Graham et al., 1973; Morihara & Oka, 1973). Hofmann and his coworkers have confirmed this specificity by using substrates of the form Ac-$(Ala)_m$-Lys * Nph-$(Ala)_n$-amide to study the effects of secondary substrate binding interactions (Balbaa et al., 1993). The substrates used in this study were derived from the highly soluble peptide Lys-Pro-Ala-Lys-Phe * Nph-Arg-Leu (Pohl & Dunn, 1988). This substrate and its P_5-P_1 and P'_2-P'_3 systematic substitution derivatives have been extensively used in the exploration of subsite specificities of rhizopuspepsin (Lowther et al., 1991), pepsin (Pohl & Dunn, 1988; Rao-Naik & Dunn, unpubl. results), cathepsin E (Rao-Naik, unpubl. results), and cathepsin D (Scarborough et al., 1993). Rhizopuspepsin is able to cleave all the substrates in this series with nearly equivalent specificities (k_{cat}/K_m). Changing the P'_3 Leu to Asp, Ala, Arg, or Ser, however, caused a twofold increase in k_{cat}. This information suggested that the substitution of Ala in P'_3 would be advantageous for the study of mutants that may have decreased catalytic activity.

As illustrated in Figure 1 and Kinemage 2, Asp 77 has the potential to interact with the P_3-P_1 residues of a ligand bound in the active site. Asp 30 is located between the S_3 and the S_1 subsites and has the capacity to interact with the P_3 and P_1 backbones and side chains (Suguna et al., 1987b). In addition to the P_1 interactions seen in the P_1-lysine inhibitor complex, crystallographic analyses of penicillopepsin with renin inhibitors have shown that Asp 77 can also interact with a histidine residue in the P_2 position (Blundell et al., 1987). Lysine was substituted into the P_3-P_1 positions of the substrate in order to show definitively the positional requirement for interaction with Asp 77 (Kinemage 2). These peptides were used to determine the influence of a potential interaction with Asp 30. Two peptides (4 and 5) were used as controls representing substrates that did not contain charges in positions P_3-P_1. The Nle peptide was used to mimic the lysine side chain.

The inhibitors were based upon the potent natural product pepstatin (Davies, 1990). The N-terminal half is analogous to peptide 3 containing no positive charges in the P_3 or P_2 positions. The leucine side chain of the statine moiety was changed to the lysine side chain to probe for interactions with Asp 30 and Asp 77. The C-terminal side of pepstatin, which also contains another statine group, was truncated in order to eliminate a secondary binding mode. The removal of the second statine group did not affect the potency of the inhibitors (unpubl. results).

Kinetic analysis

Substrates

The values and pH dependence (pH 3.5 vs. pH 5.0) of the kinetic parameters seen in Table 3 for peptide 3 are directly comparable to those seen previously for rhizopuspepsin (Hofmann et al., 1984; Balbaa et al., 1993). Interestingly, the same pH dependence of k_{cat}/K_m is seen for all the peptides and forms of rhizopuspepsin. Table 6 lists the changes in transition state stabilization energies for the recombinant rhizopuspepsins and porcine pepsin. A negative value indicates that interactions in the transition state are improved at pH 5.0. The similarity in the values, between −0.5 and −1.0 kcal mol^{-1}, over the range of substrates tested implies that the interactions seen between rhizopuspepsin and a substrate may have a general electrostatic component. Asp 30 and Asp 77 do not play a role in this phenomenon.

Interactions between porcine pepsin and this series of substrates also show a dependence on pH (Table 6). The observations with lysine in P_3 and P_2 are probably related to interactions with Glu 13 and Glu 287, respectively (Pohl & Dunn, 1988; Rao-Naik & Dunn, unpubl. data). The dependence seen for peptide

Table 6. *Transition state stabilization energy changes seen with variation in pH from 3.5 to 5.0 for the recombinant rhizopuspepsins and porcine pepsin*[a]

	$\Delta\Delta G_T^*$ (kcal mol^{-1})[b]				
Peptide	WT-REC	Asp 30 Ile	Asp 77 Thr	Asp 30 Ile/Asp 77 Thr	PPEP
1 KPKAF * XRA−0.9	−1.0	−0.4	−0.6	−1.1	
2 KPAKF * XRA	−0.6	−0.8	−0.5	−0.7	−1.5
3 KPAAK * XRA	−0.9	−1.0	−0.6	−0.8	[c]
4 KPAAF * XRA	−0.5	−0.4	−0.4	−0.6	0.0
5 KPAAZ * XRA	−0.5	−0.5	−0.4	−0.7	−0.5

[a] Abbreviations and assay conditions are described in Table 3.

[b] $\Delta\Delta G_T^* = -RT\ln[k_{cat}/K_m(\text{pH } 5.0)/k_{cat}/K_m(\text{pH } 3.5)]$. The standard deviations range from 0.1 to 0.2 kcal mol^{-1}.

[c] Cleavage occurred between Nph-Arg (X-R) instead of Phe * Nph (F * X).

5 may be a result of slight alterations in active site in order to optimize interactions with the Nle residue. The lack of pH dependence for peptide 4 with pepsin suggests that there may be a specific difference between pepsin and rhizopuspepsin. All the substrates in this series contain an arginine in the P_2' position. There is a highly conserved difference between the mammalian and fungal enzymes at residue 37 in the S_2' pocket (Table 1). Rhizopuspepsin contains an Asp at this position, whereas porcine pepsin has an Asn. The analysis of substrates that do not contain an Arg in P_2', however, showed the same pH dependence and does not support the idea that Asp 37 may form a specific electrostatic interaction (Balbaa et al., 1992). The contrasting pH dependence in the kinetic parameters for rhizopuspepsin and porcine pepsin for peptide 4 may also reflect the differences in the pI values of the proteins. Because of the greater change in the surface charges upon raising the pH, rhizopuspepsin may in a general manner attract and facilitate the catalysis of positively charged substrates.

The degree to which Asp 30 and Asp 77 contribute to the specificity of rhizopuspepsin can be analyzed by using double mutant cycles (Carter et al., 1984; Fersht, 1985; Wells, 1990). The changes seen in the free energy of transition state stabilization upon multiple mutations are generally additive providing (1) the residues do not directly contact each other, (2) large structural changes do not occur, or (3) no change in the reaction mechanism has occurred. Kinetic analysis has shown that the rate of exchange of ^{18}O from $H_2{}^{18}O$ into the starting substrate exceeded the rate of incorporation for the reverse peptidolytic reaction (Hyland et al., 1991). This evidence suggests that the free energies calculated by comparing the k_{cat}/K_m parameters for the different rhizopuspepsin species is related to the rate-limiting breakdown of the tetrahedral transition state and not to substrate association or product dissociation events.

The free energies at pH 3.5 and 5.0 for peptides 1–3 are strictly additive (Table 7) and support the structural results suggesting that severe changes in structure have not occurred. Slight deviations from additivity are seen for peptides 4 and 5, but the values do exhibit the same trends. Mutation of Asp 30 to Ile results in an average loss in transition state stabilization free energy of 0.7 kcal mol^{-1} for all peptides. This loss may be attributable to a change in the hydration shell of the protein or the loss of a hydrogen bond to the inhibitor mediated through a water molecule (Fersht et al., 1985). For peptides not containing a lysine in the P_1 position (1, 2, 4, and 5) an average loss in transition state stabilization free energy of 2.3 kcal mol^{-1} is seen when Asp 77 is changed to Thr. The largest effect on mutating position 77 is

Table 7. *Double mutant cycle analysis of the recombinant rhizopuspepsins: Substitution of lysine into P_3-P_1 at pH 3.5 and 5.0*[a]

Peptide	pH 3.5			pH 5.0		
	1,240,000	**0.6**	460,000	4,900,000	**0.5**	2,150,000
	WT		D30I	WT		D30I
1 KPKAF * XRA	**3.0**		**3.2**	**3.4**		**3.5**
	D77T		DBL	D77T		DBL
	10,000	**0.8**	2,600	19,000	**0.6**	7,100
	810,000	**0.5**	340,000	2,130,000	**0.3**	1,220,000
	WT		D30I	WT		D30I
2 KPAKF * XRA	**2.6**		**2.4**	**2.7**		**2.5**
	D77T		DBL	D77T		DBL
	11,200	**0.3**	6,700	25,000	**0.1**	20,300
	360,000	**0.9**	80,000	1,660,000	**0.9**	400,000
	WT		D30I	WT		D30I
3 KPAAK * XRA	**6.9**		**7.0**	**7.2**		**7.2**
	D77T		DBL	D77T		DBL
	5.0	**1.0**	0.9	13.7	**0.9**	3.4
	2,170,000	**0.9**	490,000	4,730,000	**1.0**	940,000
	WT		D30I	WT		D30I
4 KPAAF * XRA	**2.5**		**2.0**	**2.6**		**1.8**
	D77T		DBL	D77T		DBL
	38,000	**0.4**	19,200	73,000	**0.2**	53,000
	1,000,000	**0.6**	390,000	2,260,000	**0.5**	940,000
	WT		D30I	WT		D30I
5 KPAAZ * XRA	**3.0**		**3.9**	**3.0**		**3.8**
	D77T		DBL	D77T		DBL
	8,000	**1.5**	730	16,000	**1.3**	2,100

[a] WT, wild-type recombinant; D30I, Asp 30 Ile; D77T, Asp 77 Thr; and DBL, Asp 30 Ile/Asp 77 Thr. Numbers above and below the enzyme type are k_{cat}/K_m values in M^{-1} s^{-1} from Table 3. Free energy changes of transition state stabilization, $\Delta\Delta G_T^{\ddagger}$, are shown in bold with standard deviations that range from 0.1 to 0.2 kcal mol^{-1}.

$$\Delta\Delta G_T^{\ddagger} = -RT \ln \frac{k_{cat}/K_m(\text{mutant, mutant 2})}{k_{cat}/K_m(\text{wild-type, mutant 1})}.$$

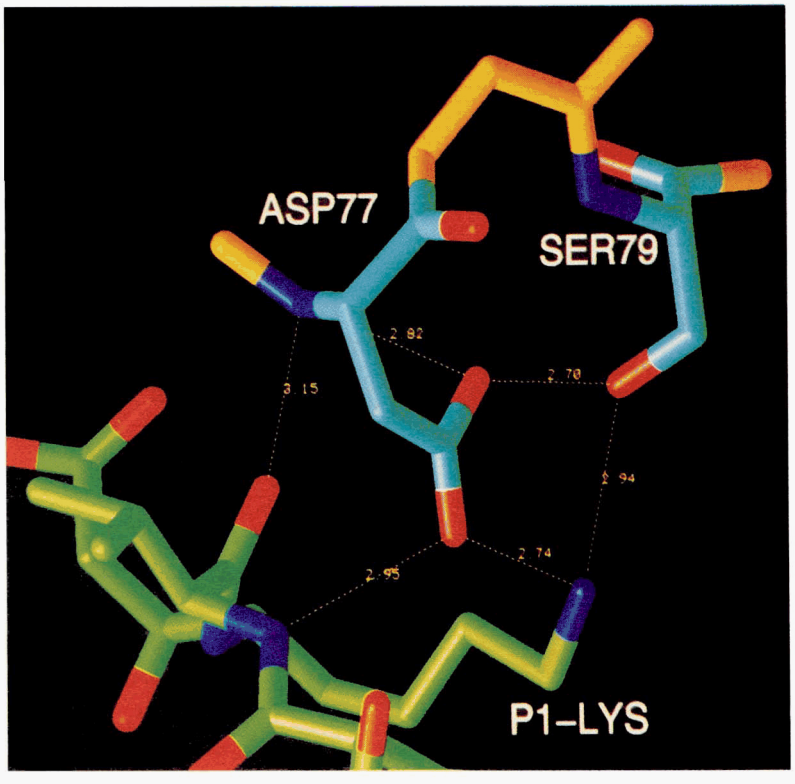

Fig. 5. Hydrogen bonding interactions in penicillopepsin between Asp 77, Ser 79, and the lystatine-based inhibitor containing the lysine side chain in the P_1 position. Yellow dotted lines indicate hydrogen bond distances in angstroms.

clearly observed with the 7 kcal mol^{-1} decrease in transition state stabilization for peptide 3. This large change in free energy indicates that Asp 77 is the crucial residue for enabling cleavage of peptides with lysine in the P_1 position.

In the pepstatin-based inhibitor complex of penicillopepsin (Fig. 5), Asp 77 forms two hydrogen bonds to the inhibitor backbone, one to the lysine side chain and an intraresidue hydrogen bond. The enzyme also forms a hydrogen bond to Asp 77 and to the lysine side chain through Ser 79. The hydrogen bonds to the P_2 NH and carbonyl of the inhibitor are strictly maintained in inhibitor complexes of the aspartic proteinases (Davies, 1990). Extending from this conserved binding mode and the high degree of structural homology in the active site region between rhizopuspepsin and penicillopepsin, a possible explanation for the inability of the Asp 77 mutants and the mammalian enzymes to cleave P_1-lysine substrates can be proposed.

When Asp 77 is mutated, several potential hydrogen bonding interactions are lost, depending on the particular type of substrate bound. When substrates that do not contain a lysine in P_1 (1, 2, 4, and 5) are bound, the Asp 77 intraresidue hydrogen bond and the hydrogen bond between Asp 77 and Ser 79 are lost. The double mutant cycles suggest that loss of these interactions yields a free energy change of 2.3 kcal mol^{-1}. When the P_1-lysine substrate (3) is bound to Asp 77 Thr or the double mutant, two additional hydrogen bonds are lost to the lysine side chain. These combined losses appear to be worth up to 7 kcal mol^{-1}. If the energies related to these hydrogen bonding interactions are additive, the two hydrogen bonds to the lysine side chain are worth approximately 4.7 kcal mol^{-1}. The free energy changes determined by these experiments are in the same range, between 3 and 6 kcal mol^{-1}, as those seen for other mutants of charged hydrogen bond donors and acceptors (Fersht et al., 1985).

Inhibitors

Analysis of the inhibitors with the recombinant rhizopuspepsins and four mammalian enzymes showed that the enzymes containing a Thr at position 77 were not inhibited when the lysine side chain was not acetylated. Upon acetylation and removal of the positive charge, inhibition was regained. This information supports the results from the study of substrates, indicating that rhizopuspepsin is able to bind ligands containing a lysine in P_1 through a specific interaction with Asp 77. The mammalian enzymes, however, cannot accommodate a positive charge in the S_1 pocket.

These results have implications for the design of anti-fungal agents. The *Candida* aspartic proteinases also contain an aspartic acid at position 77. Preliminary evidence that the *Candida* enzymes possess the P_1-lysine specificity comes from their ability to cleave collagen and keratin (Lin et al., 1993). Future mutational and kinetic analysis of the *Candida* enzymes with P_1-lysine-containing substrates and the solution of crystal structures already in progress (Cutfield et al., 1993) are needed to confirm the importance of Asp 77 and other potential interactions in pathogenicity. Observations from this study may also prove to be useful in the study of the paired basic residue-specific aspartic proteinases from yeast and the pituitary (Loh et al., 1985; Azaryan et al., 1993).

Conclusions

A combination of site-directed mutagenesis and kinetic analysis has clearly established the importance of Asp 77 in the unique lysine primary specificity of fungal aspartic proteinases and confirmed the interactions seen in crystallographic analysis. Asp 30,

which is not conserved in the fungal enzymes, was shown not to influence this specificity but may play a role in general electrostatic interactions. The presence of an aspartic acid residue at position 77 has the potential to establish an extensive hydrogen bonding network between the enzyme and the substrate that enables the fungal enzymes, but not the mammalian enzymes because they contain Thr at position 77, to cleave substrates containing lysine in the P_1 position.

Materials and methods

The pet3a expression vector (Studier et al., 1990), containing the rhizopuspepsinogen gene (Chen et al., 1991), was kindly provided by Jordan Tang at the Oklahoma Medical Research Foundation. The hydroxyethylene, $\psi[CH(OH)CH_2]$, containing inhibitor, U85548E, was a gift from Tomi Sawyer. Porcine pepsin was purchased from Sigma and used without further purification. The synthetic oligonucleotides were synthesized by the University of Florida, Interdisciplinary Center for Biotechnology Research (ICBR) DNA Synthesis Core Facility using an Applied Biosystems 394 DNA Synthesizer. Peptide substrates were synthesized by the ICBR Protein Chemistry Core Facility using either an Applied Biosystems 430A Peptide Synthesizer and t-Boc chemistries or an Applied Biosystems 432A SYNERGY Peptide Synthesizer and Fmoc chemistries. All peptides were shown to be >95% pure by reverse-phase HPLC and capillary electrophoresis. Stock solutions of the peptides and U85548E were quantified by amino acid analysis on a Beckman System 6300 high-performance amino acid analyzer following acid hydrolysis. N-terminal sequence analyses of the rhizopuspepsins were performed on Applied Biosystems 470A and 473A protein sequencers. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectroscopic analysis was performed on a Vestec LaserTec Bench Top System (Houston, Texas). Ultrapure 8 M Gdn-HCl in water was purchased from Pierce. All other materials were of the highest commercial grade.

All routine DNA manipulations and *E. coli* handling procedures were performed as outlined by Sambrook et al. (1989). All plasmids and PCR products were isolated using Magic Miniprep or PCR kits from Promega.

Cloning and mutagenesis

Mutations in the rhizopuspepsinogen gene were made by using a modified version of the overlap extension polymerase chain reaction method of site-directed mutagenesis with the use of TAQ Polymerase or Vent polymerase as previously described (Scarborough & Dunn, 1994). Primers used were: engineering an *Nde* I site, 5′CAT ATG GCA GTT AAC GCT GCC CC3′; Asp 30 → Ile; 5′GA GGA ACC GGT ATC AAA GAT AAG GTT GAA C3′, 5′TTT GAT ACC GGT TCC TCC GAT TTA TG3′; Asp 77 → Thr; 5′GAT ACC GCT AGC AGA AGA GCC AGT ACC ATA AG3′, 5′TCT TCT GCT AGC GGT ATC TTG GC3′; engineering a *Bam*H I site, 5′GGA TCC TTA TTG AGC GAC AGG AGC G3′. The double mutant was generated by repeating this procedure using the Asp 30 Ile mutant gene as the starting template. The entire coding region of rhizopuspepsinogen within the pCR (InVitrogen) or the pGEM-3Z (Promega) vectors was dideoxy-sequenced according to the Sequenase 2.0 kit protocol (United States Biochemical). Mutant genes were transferred to a pET3a expression system vector lacking an *Eco*R V restriction site due to a 375-bp deletion between the *Bam*H I restriction site and ampicillin-resistance gene. The use of this new vector allowed the efficient screening of recombinants because of the unique *Eco*R V restriction site within the rhizopuspepsinogen gene.

Expression

Native and mutant zymogens were expressed and purified from *E. coli* strain BL21 (DE3) as reported with minor changes (Chen et al., 1991). A 1:50 dilution of an overnight culture grown in M9 media (10 μg/mL thiamine, 0.5% casamino acids, 0.2% glucose) containing 50 mg/L ampicillin was made into LB media containing the same amount of ampicillin and grown to an OD_{600} of 0.5. Cultures were grown for an additional 3 h after the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.5 mM. Cells were harvested by centrifugation at 3,500 × *g* for 10 min and resuspended in 4.2 mL of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 1 mM $MgCl_2$ (buffer A) per gram of cells. Following the addition of 80 Kunitz units of DNase (Sigma) per mL of suspension, the cells were lysed by two passes through a French press cell. The resulting slurry was carefully layered over a 27% sucrose cushion and centrifuged at 12,000 × *g* in order to isolate the inclusion bodies that sediment through the sucrose solution (Taylor et al., 1986). Inclusion bodies were washed by resuspension in buffer A containing 1% Triton X-100 and pelleted through sucrose a second time. Purified inclusion bodies were stored at −20 °C until refolding.

Refolding

In order to regain enzymatic activity, the wild-type and mutant recombinant proteins were refolded by a modified procedure for the refolding of prochymosin involving denaturation, reduction, and dialysis (Suzuki et al., 1989). Purified inclusion bodies were dissolved in freshly deionized 8 M urea, 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 10.5, 1 mM EDTA, 1 mM glycine, 500 mM NaCl, and 300 mM β-mercaptoethanol to a final concentration of approximately 1 mg(wet)/mL. After stirring at room temperature for 1 h, the solution was centrifuged at 24,000 × *g* for 30 min to remove undissolved material. The supernatant was dialyzed for two 1-h intervals at room temperature against five times the original volume in 50 mM Tris-HCl, pH 11.0. Dialysis was continued overnight at 4 °C after changing the buffer to 50 mM Tris-HCl pH 7.5. The next morning dialysis was continued for at least 6 h more after the buffer was changed to 50 mM MOPS, pH 7.0. The resulting solution was centrifuged at 24,000 × *g* for 30 min to remove precipitates and concentrated using a Minitan Ultrafiltration system outfitted with low protein binding, PLTK, 10,000 MW cut-off membrane plates (Millipore) and an Amicon pressurized cell with YM10 membranes.

Size-exclusion chromatography

The zymogen was further processed by centrifugation at 45,000 × *g* for 30 min before loading onto a 2.5-cm × 90-cm Sephacryl S300 gel filtration column equilibrated with 50 mM MOPS, pH 7.0, containing 300 mM NaCl. The zymogen was eluted at a flow rate of 25 mL/h, and fractions showing the highest purity

were pooled, concentrated, and buffer exchanged with 10 mM MOPS, pH 7.0.

Activation and ion-exchange chromatography

Native and Asp 30 Ile mutant proteins (0.5 mg/mL) were activated by incubation at room temperature for 15 min after the addition of citric acid, pH 2.0, to a final concentration of 0.1 M. The Asp 77 Thr and the double mutant Asp 30 Ile/Asp 77 Thr zymogens were activated for 24 h in 0.1 M sodium formate, 37 °C at pH 3.0 and 3.5, respectively. After filtering, each enzyme was injected onto a Pharmacia HR5/5 Mono S column equilibrated with 50 mM sodium formate, pH 3.0. The enzyme was eluted with a 25-min gradient to 50 mM sodium formate, pH 3.0, 250 mM NaCl, at a flow rate of 1 mL/min. Enzyme aliquots were quickly frozen and stored at −20 °C.

Structural characterization

N-terminal sequencing

N-terminal sequence analysis of the activated rhizopuspepsins was performed to determine the extent of processing during self-activation. Proteins were electroblotted from 12% Tris-Tricine SDS-PAGE gels (Schägger & von Jagow, 1987) to Immobilon P transfer membranes (Millipore) in 10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.0 containing 20% methanol.

Mass spectrometry

Activated enzymes were analyzed by MALDI-TOF mass spectroscopic analysis. One to ten picomoles of each sample or standard was mixed 1:1 with a fresh solution of 0.05% trifluoroacetic acid, 40% acetonitrile, and saturated sinnapinic acid. The mass spectrum was obtained from the average of at least 50 laser shots (337 nm nitrogen laser, 3 ns pulse width). Time to mass/charge calibration was performed from a calibration curve using bovine carbonic anhydrase II (Sigma, 28,980 Da) generated immediately prior to the rhizopuspepsin samples.

Isoelectric focusing

Isoelectric points of the proteins were determined by electrophoresis on precast gels with a pH gradient from pH 3 to 9 on the Pharmacia PhastSystem.

CD

CD spectra were determined at room temperature in a 0.1-cm-pathlength cell on a Jasco J-500C spectropolarimeter equipped with an IF-500 II computer interface. The polarimeter was standardized with D(+)-camphorsulfonic acid (Chen & Yang, 1977). Samples were diluted into buffer to a final concentration around 0.5 mg/mL in 0.1 M sodium formate, pH 3.0. Just prior to loading into the CD cell, samples were filtered and quantitated by measuring their absorbance at 280 nm. Ellipticity values from an average of 10 scans were converted to the molar ellipticity, $[\theta]$, using the conversion factor $E^{1\%}_{1\,cm} = 12.6$ (Fukumoto et al., 1967) and a molecular weight of 35 kDa. Data points were fit with the smoothing algorithm of the KaleidaGraph program (version 3.0.2, Synergy Software, PCS Inc.).

Fluorescence spectroscopy

Gdn-HCl denaturation curves of the rhizopuspepsins were determined using excitation and emission wavelengths of 280 and 350 nm, respectively, on an SLM Aminco 4800C spectrofluorometer. Proteins were diluted in duplicate to Gdn-HCl concentrations ranging from 0 to 6 M with a final buffer concentration of 0.1 M sodium formate, pH 3.0, and an enzyme concentration around 100 nM. Solutions did not contain reducing agent. Protein/denaturant solutions were equilibrated at 25 °C for at least 1 h prior to spectroscopic measurements.

Denaturation curve analysis. Denaturation curves were analyzed assuming a two-state model where only the native and the denatured states are populated. Fluorescence values for the native and unfolded states, F_N and F_U, were used to determine the equilibrium constant for unfolding, $K_{U\text{-}F}$, and the free energy of unfolding, $\Delta G_{U\text{-}F}$, at different denaturant concentrations using Equation 1.

$$K_{U\text{-}F} = (F_N - F)/(F - F_U) = \exp(-\Delta G_{U\text{-}F}/RT). \quad (1)$$

F is the observed fluorescence, R is the gas constant (1.987 cal mol^{-1} K^{-1}), and T is the absolute temperature. The free energy of unfolding of proteins has been shown to be linearly dependent on denaturant concentration as expressed by Equation 2 (Pace, 1986).

$$\Delta G^{D}_{U\text{-}F} = \Delta G^{H_2O}_{U\text{-}F} - m[\text{denaturant}]. \quad (2)$$

$\Delta G^{D}_{U\text{-}F}$ and $\Delta G^{H_2O}_{U\text{-}F}$ are the free energy of unfolding at denaturant concentration, D, and in water, respectively. The m value is the slope of the transition and is thought to be related to the difference in the degree of accessible surface area between the native and unfolded states (Schellman, 1978). Two methods have been used to calculate the transition point, $[\text{Gdn-HCl}]_{50\%}$, $\Delta G^{H_2O}_{U\text{-}F}$, and m. The method outlined by Pace (1986) employs Equation 2 by plotting $\Delta G^{D}_{U\text{-}F}$ within the transition region (± 1.5 kcal mol^{-1}) versus [denaturant] and linearly extrapolating back, usually quite a long distance, to zero denaturant to obtain $\Delta G^{H_2O}_{U\text{-}F}$. Because small errors in m can lead to large errors in the calculation of $\Delta G^{H_2O}_{U\text{-}F}$ and $[\text{Gdn-HCl}]_{50\%}$, Fersht and coworkers have used a method, represented by Equation 3, which uses all the observed fluorescence data, F, to directly determine typically within ±0.02 M Gdn-HCl (Jackson et al., 1993).

$$\begin{aligned} F = {} & \{(\alpha_N + \beta_N[\text{GdnHCl}]) + (\alpha_U + \beta_U[\text{GdnHCl}]) \\ & \times \exp[m([\text{GdnHCl}] - [\text{GdnHCl}]_{50\%})/RT]\}/ \\ & \{1 + \exp[m([\text{GdnHCl}] - [\text{GdnHCl}]_{50\%})/RT]\}. \end{aligned} \quad (3)$$

This equation combines Equations 1 and 2 and assumes that F_N and F_U are linearly dependent on the denaturant concentration. α_N and α_U are the intercepts and β_N and β_U are the slopes of the baselines at denaturant concentrations before and after the transition region. These parameters, as well as $[\text{Gdn-HCl}]_{50\%}$ and m were allowed to be variables in the KaleidaGraph nonlinear regression analysis program. $\Delta G^{H_2O}_{U\text{-}F}$ values were calculated from Equation 2, where at $[\text{Gdn-HCl}]_{50\%}$, the transition point $\Delta G^{H_2O}_{U\text{-}F} = m[\text{Gdn-HCl}]_{50\%}$.

Kinetic analysis

Inhibitor synthesis

The protected derivative of lystatine (LySta), (3S,4S)-3-hydroxy-4-t-butyloxycarbonylamino-8-benzyloxycarbonylamino octanoic acid, was prepared according to the modified procedure of Jouin et al. (1987). The 8-acetyl derivative of lystatine was obtained after hydrogenolytic debenzylation and acetylation with *N*-acetoxysuccinimide. Inhibitors were synthesized by the fragment condensation of Z-Lys(Z)-Pro-Ala-Ala-OH with H-LySta(Z)-Ala-Leu-Gly-NH_2 and H-LySta(Ac)-Ala-Leu-Gly-NH_2 using diphenylphosphoryl azide (DPPA) as a coupling agent. Coupling products were purified by reverse-phase HPLC, deprotected by hydrogenolysis on Pd/C catalyst, and repurified by HPLC. Synthesis of the fragments was accomplished by solid-phase methodology using standard Fmoc chemistry with *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) activation.

Kinetic assays using chromogenic substrates

The kinetic parameters k_{cat}, K_m, and k_{cat}/K_m were determined by monitoring the decrease in the average absorbance from 284 to 324 nm upon substrate hydrolysis between Phe * Nph, Nle * Nph, or Lys * Nph (Nph, *p*-nitrophenylalanine; Nle = norleucine; *, site of cleavage) (Scarborough et al., 1993; Lowther & Dunn, 1994). The amount of active enzyme was determined by competitive titration with the inhibitor Val-Ser-Gln-Asn-Leuψ[CH(OH)CH_2]Val-Ile-Val (U85548E; Sawyer et al., 1992) under assay conditions of 2% DMSO. In those instances where the K_m values were $\gg$1 mM, the k_{cat}/K_m values were determined by fitting the initial rates of at least six substrate concentrations ranging from 25 to 250 μM to the equation $v = (k_{cat}/K_m)[E]_0[S]_0$ with the assumption that $[S] \ll K_m$. The k_{cat}/K_m values for the cleavage of the P_1-lysine-substituted peptides by the Asp 77 Thr and the Asp 30 Ile/Asp 77 Thr mutants were calculated as above with the initial rates determined by capillary zone electrophoresis (Lowther & Dunn, 1994).

Kinetic assays using competitive inhibitors

Inhibition constants, K_i, were determined by monitoring the competitive inhibition of the hydrolysis of the peptide Lys-Pro-Ala-Lys-Phe * Nph-Arg-Ala for the recombinant rhizopuspepsins and Lys-Pro-Ile-Glu-Phe * Nph-Arg-Leu for the mammalian enzymes (Lunney et al., 1993). All reactions were performed at 37 °C and a final concentration of 0.1 M buffer (Table 5) and 2% DMSO.

Product analysis

All substrates were incubated with enzyme at 37 °C overnight. Cleavage products of the peptides used for analysis of mutants of rhizopuspepsin were determined on a Bio-Rad BioFocus 3000 capillary electrophoresis system (Lowther & Dunn, 1994). All the peptides studied, if cleaved properly, will have the same C-terminal product, Nph-Arg-Ala. This product was purified by reverse-phase HPLC, confirmed by amino acid analysis and used as a retention time standard. The fidelity of the cleavage sites was verified for all forms of rhizopuspepsin and porcine pepsin at pH 3.5 and 5.0.

Capillary zone electrophoresis kinetic assay

The initial rates of cleavage of the peptide containing lysine in P_1 by Asp 77 Thr and Asp 30 Ile/Asp 77 Thr were determined by incubating the enzyme (900 nM) with either 50, 100 or 150 μM substrate for a period of 24 h at 37 °C. A 20-μL aliquot was taken at 0, 1, 3, 5, 7, 12, 15, and 24 h and mixed with 1.5 μL of U85548E to give a final concentration of inhibitor of 5 μM (fivefold molar excess) and stored at −20 °C until the electropherograms were run. The initial linear slopes of the change in intact substrate peak area versus time were converted to M s^{-1} by dividing by the slope (peak area/[*S*]) of a standard curve generated from 30 to 1,000 μM of the substrate (Lowther & Dunn, 1994).

Analysis of transition state effects

In transition state theory, the energy difference between the free enzyme and substrate and the transition state, $\Delta G_T^{\ddagger}$, is related to the energy released upon binding of the substrate, ΔG_S, and the activation energy, $\Delta G^{\ddagger}$, of the chemical steps responsible for bringing the enzyme-substrate complex from the ground state to the transition state (Fersht, 1985):

$$\Delta G_T^{\ddagger} = \Delta G_S + \Delta G^{\ddagger} = RT\ln(k_B T/h) - RT\ln(k_{cat}/K_m). \quad (4)$$

With the assumption that the energies associated with the bond breaking and making steps, $\Delta G^{\ddagger}$, are not significantly affected upon mutation of the enzyme or changes in the assay pH, the discrimination of the wild-type and mutant enzymes for different substrates can be evaluated by their relative binding to the transition state:

$$\Delta\Delta G_T^{\ddagger} = -RT\ln\frac{k_{cat}/K_m(\text{mutant, mutant 2 or pH 5.0})}{k_{cat}/K_m(\text{wild-type, mutant 1 or pH 3.5})}. \quad (5)$$

Molecular graphics

Molecular graphics representations of the inhibitor complexes of rhizopuspepsin (3APR; Suguna et al., 1987b) and penicillopepsin (1APT; James et al., 1985) were generated using the Insight II (version 2.3) from Biosym Technologies, Inc. (San Diego) on a Silicon Graphics Indigo System.

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