Extracellular Aspartic Proteinases from *Candida albicans*, *Candida tropicalis*, and *Candida parapsilosis* Yeasts Differ Substantially in Their Specificities†

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ABSTRACT: Extracellular aspartic proteinases have been implicated for some time as virulence factors associated with *Candida* opportunistic fungal infections. Our present knowledge of the enzymatic properties of these proteinases is rather limited. Information on their substrate specificity is important for understanding their roles in invasive *Candida* infections. We have isolated aspartic proteinases from each of the three *Candida* yeasts, *Candida albicans*, *Candida tropicalis*, and *Candida parapsilosis*, and investigated the specificities of these proteinases using a library of synthetic substrates and testing inhibition by pepstatin A. The specificities of these aspartic proteinases are different from those of major human proteinases, including gastric pepsins, renal renin, and cathepsin D. For the peptide substrate, Lys-Pro-Ala-Leu-Phe*Phe(p-NO_2)-Arg-Leu, the values of k_{cat}/K_m were 2.95×10^6 M^{-1} s^{-1} for cleavage by *Candida albicans* proteinase, 1.60×10^6 M^{-1} s^{-1} for cleavage by *Candida tropicalis* proteinase, and 0.59×10^6 M^{-1} s^{-1} for *Candida parapsilosis* proteinase. Substantial differences in specificity among the *Candida* yeast proteinases were identified. For example, *Candida tropicalis* shows large changes in the k_{cat}/K_m value depending on the acidobasic character of the residue occupying the P_2 position (1.6×10^6 M^{-1} s^{-1} for Leu, 0.47×10^6 M^{-1} s^{-1} for Lys, and 0.05×10^6 M^{-1} s^{-1} for Asp at P_2, respectively). *Candida parapsilosis* by comparison is tolerant of these substitutions at P_2 and is highly restrictive at position P_4. The comparison of sequences of these proteinases, taken together with the kinetic data, suggests the participation of as yet unidentified residues of aspartic proteinases in forming the specificity binding pockets.

The role of *Candida* yeast, a true opportunistic pathogen, has long been connected to oral mucosal infections and more specifically to vaginal infections (Sobel,1993). For patients with the HIV virus infection or a lowered immunoresponsiveness, systemic candidamycosis is becoming one of the most serious life-threatening secondary complications (Wade, 1993). Several virulence factors expressed and secreted by the *Candida* yeast have been described in connection with the invasive candidamycoses. The extracellular aspartic proteinase is cited as one of the virulence factors responsible for proteolytic invasion by these yeasts (Krempl-Lamprecht, 1991; Cutler, 1991; Vartivarian & Smith, 1993). The evidence for this role is derived from experiments where a deficiency in the aspartic proteinase has been correlated with a lower level of virulence in some strains of *Candida albicans* (MacDonald & Odds, 1983; Kwon-Chung et al., 1985; Ross et al., 1990). Proteinase deficient strains of *Candida albicans* are found to be less aggressive in *in vivo* experiments (DeBernardis, 1990), and a specific inhibitor of aspartic proteinases, pepstatin A, prevented the cavitation of epidermal corneocytes by *Candida albicans*, *Candida stellatoidea*, and *Candida tropicalis* (Ray & Payne, 1988a). Another example supporting an active role for extracellular proteinases in candidiasis is the correlation of their production with the adherence of the microorganism and cavitation of tissues (Ray & Payne, 1988b).

The study of the kinetic properties of the family of aspartic proteinases frequently has been motivated by their involvement in physiological and pathological processes in humans. The enzymatic properties of the HIV-1 aspartic proteinase (Debouck & Metcalf, 1990), human plasma renin (Thaisrivongs, 1989), and lysosomal cathepsin D (Scarborough, 1993), as well as several other mammalian and fungal aspartic proteinases of commercial interest (Ward & Kodama, 1991), have been studied extensively during the last decade. *Candida* extracellular aspartic proteinases have been isolated and partially characterized from *Candida albicans* (Remold et al., 1968), *Candida tropicalis* (Ruchel et al., 1983), and *Candida parapsilosis* (MacDonald, 1984). The genomic DNA sequences are known for all three proteinases [Huber et al., 1991 (CAAP[1]); Togni et al., 1991 (CTAP); de Viragh et al., 1993 (CPAP)]. All proteinases have the pH optimum close to 3.0.

It has been demonstrated that the *C. albicans* proteinase degrades both macromolecular substrates (Remold et al., 1968) as well as low molecular weight substrates (Capobianco et al., 1992). A fluorogenic method for monitoring the activities of these secreted proteinases has been described recently by Capobianco et al. (1992).

On the basis of three-dimensional structures of other aspartic proteinases, it is clear that the specificity of the proteinase is dictated by a complex system of noncovalent bonds (mainly hydrogen bonds) between the main chain and side chain atoms of the inhibitor and between the side chain and peptidic groups

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[1] Abbreviations: FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate; CAAP, *Candida albicans* aspartic proteinase; CTAP, *Candida tropicalis* aspartic proteinase; CPAP, *Candida parapsilosis* aspartic proteinase; EP, *Endothia parasitica* aspartic proteinase; YPA, *Saccharomyces cerevisiae* vacuolar proteinase A; PP, pig pepsinogen; HR, human renin; HCD, human cathepsin D.

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of the substrate binding cleft in the enzyme [reviewed by Davies (1990)]. Shape complementarity also plays an important role in the tight binding of substrates and inhibitors to the binding pockets of the active site binding cleft. Both the three-dimensional structural data and the specificity data derived from substrate and inhibitor binding studies indicate that the most important residues involved in recognition and specificity are formed by interactions at residue positions P_5–P_3' with specificity pockets S_5–S_3' of the enzyme [nomenclature of Berger and Schecter (1970)]. The most common primary specificity for aspartic proteinases is toward substrates with hydrophobic residues at positions P_1 and P_1' (Fruton, 1976).

Due to the complexity of the interactions between a substrate and the binding sites of these enzymes, only the combination of substrate specificity analysis with the knowledge of the three-dimensional structure for an enzyme has the potential to provide a solid basis for rational inhibitor design. Substrate specificity screening is often the first step in the design of highly specific inhibitors for aspartic proteinases.

The goal of this paper is the characterization of the substrate specificities of extracellular proteinases from the yeasts *Candida albicans*, *Candida tropicalis*, and *Candida parapsilosis* using synthetic substrates and their comparison with other members of the aspartic proteinase family. We focus on the specificity mapping at positions P_5–P_1 and P_2' and P_3'.

EXPERIMENTAL PROCEDURE

Yeast Strains and Their Growth. The following strains of *Candida* yeasts were used for cell growth and enzyme production: strain C-74 of *Candida albicans*, strain ATCC-75 of *Candida tropicalis*, and strain E-18 of *Candida parapsilosis*. Yeast cell cultures were maintained and replated every 6–8 weeks on Sabouraud agar plates. *Candida* yeasts in liquid cultures were grown in sterile medium containing 0.2% bovine serum albumin (BSA) and 1.2% YCB Difco medium, adjusted to pH 4.0. The cultures were grown for 3 days at a temperature of 30 °C, with shaking at 200 rpm. The cells were then pelleted, and the resulting supernatant was sterile filtered. This sterile supernatant was used for all subsequent steps in the enzyme isolation.

Isolation of Aspartic Extracellular Proteinases. The isolation of the three proteolytic enzymes differed for each strain of yeast. The isolation of *Candida tropicalis* aspartic proteinase (CTAP) started with ammonium sulfate precipitation (60% saturation). The precipitate was pelleted by centrifugation for 1 h at 7000*g*, redissolved in 50 mL of 15 mM sodium citrate buffer (pH 5.6) (buffer A), and dialyzed against the same buffer overnight. The dialysate was then applied to a 20-mL anion-exchange column (DEAE-Trisacryl, IBF, France) and eluted stepwise with 100 mM sodium citrate (pH 5.6). A second chromatography step (FPLC, Mono Q column 5 × 5, Pharmacia, Sweden) was used. Proteinase was loaded in 15 mM sodium citrate buffer (pH 5.6) and eluted by a linear gradient of 15–100 mM sodium citrate (pH 5.6) for 40 min.

In the isolation of *Candida albicans* aspartic proteinase (CAAP) a similar method was used, omitting the salting out step. In place of this procedure, a batch anion-exchange chromatography absorption step on DEAE-Trisacryl resin was used. The proteinase was eluted stepwise with 100 mM sodium citrate buffer, pH 5.6. The final purification of CAAP was achieved in a manner identical to that described in the previous section for CTAP (FPLC). The isolation of *Candida parapsilosis* aspartic proteinases (CPAP1, -2) was done using the previously published procedure (Fusek et al., 1993).

In this study, the major secreted aspartic proteinase expressed by *Candida parapsilosis* (CPAP1) yeast was studied, where the N-terminal sequence corresponded to the sequence pir3:S20705 from the PIR protein database. Yields of proteinases were 4.2, 5.1, and 4.4 mg from 1 L of medium for CAAP, CTAP, and CPAP strains, respectively. The relative concentrations of the proteinases were determined using the BCA protein assay (Bio-Rad, Hercules, CA). The concentration of active enzyme was determined as described elsewhere (Scarborough et al., 1993).

Sequence Analysis and Alignment. The primary sequence comparisons for the three yeast proteinases with other members of the aspartic proteinase family were carried out using the program BESTFIT from the GCG program library (GCG, Madison, WI). Multiple sequence alignments were performed using the program CLUSTALV (Higgins et al., 1992) on a VAX 4600 computer. The following sequence data sets were used for the respective proteinases: CAAP, sw:car3_canal; CTAP, sw:carp_cantr; CPAP, pir3:S20705; *Endothia parasitica* proteinase (EP), sw:carp_crypa; yeast proteinase A (YPA), pir2:A25379; pig pepsinogen (PP), sw:pepa_pig; human renin (HR), sw:reni_human; human cathepsin D (HCD), sw:catd_human. In both analyses the sequences of proenzymes were used. The numbering of sequences used in this paper was based on the sequence of pig pepsinogen and is shown in Figure 2 (Lin et al., 1988). A schematic drawing of the enzyme's substrate binding cleft is shown in Figure 3. This drawing shows the approximate predicted positions of residues that make up the various binding sites of this cleft. This diagram is compiled from the multiple sequence amino acid alignment given in Figure 2 and from the extensive three-dimensional information derived from studies of enzyme–inhibitor complexes, which was reviewed by Davies (1990).

Kinetic Characterization Using Synthetic Substrates. Kinetic measurements were performed on a Hewlett-Packard 8452A diode array spectrophotometer equipped with a multicell transport and thermostated at 37 °C. The chromogenic substrates used in this determination of initial rates of reaction have been described previously by Scarborough et al. (1993) and have a general chemical structure as follows:

$$X_5\text{-}X_4\text{-}X_3\text{-}X_2\text{-}X_1\text{-Phe}(NO_2)\text{-}X_2'\text{-}X_3'$$

The cleavage of the bond X_1-↓-Phe(NO_2) yields a decrease in absorbance in the range 284–324 nm. All measurements were done in 100 mM sodium acetate buffer at pH 3.25. Enzymes were preincubated in the assay buffer equilibrated at 37 °C for 180 s prior to initiating the reaction upon mixing two reaction components. The substrate was aliquoted in 1.5-mL Eppendorf tubes to the desired concentration [S] in 50 μL of H_2O. The enzyme solution was rapidly mixed with substrate, and the resulting reaction volume was immediately transferred to the spectrophotometric cuvette. Kinetic parameters were calculated using the ENZYME KINETICS program package supplied by Hewlet-Packard (Tables 2–6). Values of K_i for pepstatin A were determined by the Linweaver–Burk plot using enzyme concentrations in the subnanomolar range (pepstatin A, Serva, Germany).

RESULTS

Isolation. The three isolated proteins were free of contaminants, as determined by SDS electrophoresis (Figure 1) and by N-terminal sequence analysis (Figure 2).

Comparison of Sequences of Candida Yeast Proteinases with Other Aspartic Proteinases. The values of pairwise

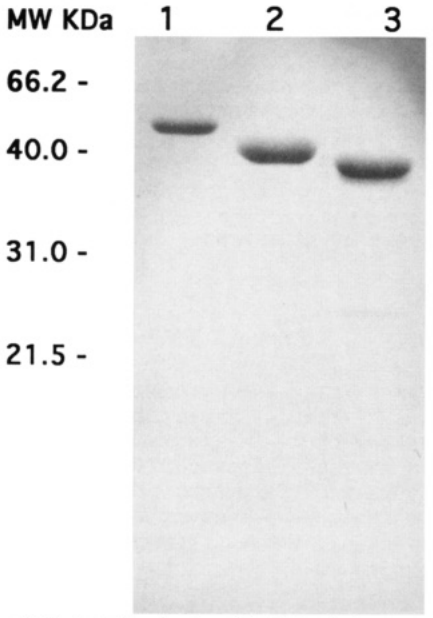

FIGURE 1: SDS–PAGE gel of purified *Candida* yeast proteinases. Coomassie Brilliant Blue R250 stained 15% SDS–PAGE gel of the three purified *Candida* yeast aspartic proteinases: lane 1, CAAP, *Candida albicans*; lane 2 CTAP, *Candida tropicalis*; lane 3, CPAP, *Candida parapsilosis* major secreted proteinase type 1.

Table 1: Values of Pairwise Sequence Similarity/Identity Score[a]

	PP	YPA	EP	HCD	HR	CTAP	CPAP
CAAP	51/31	51/30	51/33	54/31	48/25	65/54	67/54
CTAP	52/33	52/28	48/30	51/28	47/24		67/55
CPAP	55/32	51/30	48/30	51/30	52/27		

[a] Values of pairwise percentage similarity score/percentage identity score for the three *Candida* proteinases as compared to five other aspartic proteinases. CAAP, *Candida albicans*; CTAP, *Candida tropicalis*; CPAP, *Candida parasilosis*; PP, porcine pepsinogen; YPA, yeast proteinase A; EP, *Endothia parasitica*; HCD, human cathepsin D; HR, human renin.

percentage similarity score and pairwise percentage identity score for the three *Candida* proteinases, as compared to five other aspartic proteinases, are summarized in Table 1. The lowest identity score was observed with human renin (25%), and the highest were to pig pepsin (33%) and human cathepsin D (31%).

Sequence alignment is shown in Figure 2. The conserved cores of the *Candida* enzymes are homologous or varied conservatively, thus maintaining the general three-dimensional fold of the aspartic proteinases. There are several interesting and specific changes in the *Candida* enzymes relative to the archetypic enzymes of this family. For instance, a large insertion is observed for the surface loop between Cys 45 and Cys 50 in the CAAP and CTAP enzymes. Differences are also observed in the region between residues 102 and 116, which includes the "walls" of the substrate binding pockets S_3 and S_5 in the active site cleft. A further large alteration in the walls of the substrate binding pockets occurs at positions 286–294, which would influence specificity at binding site positions S_2, S_1', and S_3', respectively. It is evident from the alignment that the *Candida* proteinases are missing a second disulfide bridge that is present in the structure of pepsin Cys 206–Cys 210. This disulfide bridge is conserved in all vertebrate aspartic proteinases, but is absent from the vacuolar aspartic proteinase A from *Saccharomyces cerevisae* yeast. *Candida* proteinases, on the other hand, have the disulfide bridge at positions Cys 45–Cys 50. This disulfide is absent from the fungal aspartic proteinases like *Endothia parasitica* and *Mucor pusillus*.

Table 2: Initial Velocities of Cleavage of Variants of Peptide Substrate **1** All at a Concentration of 50 μM[a]

substitution		P_5	P_4	P_3	P_2	P_2'	P_3'
	CAAP	147	175	**264**	**200**	107	297
Ala	CTAP	169	174	**321**	**271**	42	138
	CPAP	119	67	**124**	**257**	112	190
	CAAP	109	**259**	**160**	**120**	**7**	nd
Asp	CTAP	105	**176**	**216**	**147**	**3**	nd
	CPAP	83	**43**	**128**	**169**	**8**	nd
	CAAP	**259**	**108**	**80**	**136**	**264**	**215**
Arg	CTAP	**442**	**210**	**92**	**483**	**321**	**165**
	CPAP	**111**	**8**	**158**	**141**	**124**	**160**
	CAAP	151	120	165	nd	61	233
Ser	CTAP	192	159	263	nd	16	78
	CPAP	99	27	154	nd	73	186
	CAAP	112	**2**	115	**593**	29	**264**
Leu	CTAP	169	**3**	247	**547**	6	**321**
	CPAP	116	**0**	191	**467**	36	**124**

[a] Values for the initial velocities ($\Delta A_{300nm}/s \times 10^6$) of cleavage for substrate variants of the parent peptide substrate **1** (Lys-Pro-Ala-Lys-Phe-Phe(p-NO_2)-Arg-Leu) with the three *Candida* yeasts, CAAP, CTAP, and CPAP. The variants of this substrate are derived by the sequential substitution of residues at positions P_5, P_4, P_3, P_2, P_2', and P_3' by the amino acid residues Ala, Asp, Arg, Ser, and Leu. The peptide substrates identified in boldface type were analyzed further by spectrophotometric measurements and kinetic analysis.

Synthetic Chromogenic Substrates. To determine the specificities of these three extracellular proteinases from Candida yeasts, we have used a library of peptides based on the general peptide structure shown here:

$$X_5\text{-}X_4\text{-}X_3\text{-}X_2\text{-}X_1\text{-Phe}(p\text{-}NO_2)\text{-}X_2'\text{-}X_3'$$

Here, each of the positions X_5–X_2 and X_2' and X_3' was occupied by one of the following amino acid residues: Ala, Asp, Arg, Ser, or Leu. The parent substrate used in this screening was (Dunn et al., 1986; Pohl & Dunn, 1988; Scarborough et al., 1993)

$$P_5\ P_4\ P_3\ P_2\ P_1\ P_1' \qquad P_2'\ P_3'$$

Lys-Pro-Ala-Lys-Phe-Phe(p-NO_2)-Arg-Leu

1

The preliminary screening for the three enzymes was carried out under the following conditions. Each substrate concentration [S] was fixed at 50 μM, and the concentration of enzyme in a 250-μL reaction volume was 0.5 μg. The initial velocities ($\Delta A_{300nm}/s \times 10^6$) were determined. This allowed a general ranking of initial velocities (v) for the three enzymes against the full library of chromogenic peptide substrates. The results of this first screening are summarized in Table 2.

The second parent substrate chosen was a derivative sequence of **1** with a modification in the primary amino acid structure. Peptide **2** introduces a negative charge at the P_2 position and an isoleucine in the P_3 position. Its chemical structure is shown here (Dunn et al., 1986; Pohl & Dunn, 1988):

Lys-Pro-Ile-Glu-Phe-Phe(p-NO_2)-Arg-Leu

2

The results are summarized in Table 3. The last peptide tested had the chemical structure shown here:

Lys-Pro-Ala-Glu-Phe-Phe(p-NO_2)-Ala-Leu

3

On the basis of this first screening, we have chosen a set of peptides for a more detailed kinetic evaluation. This choice

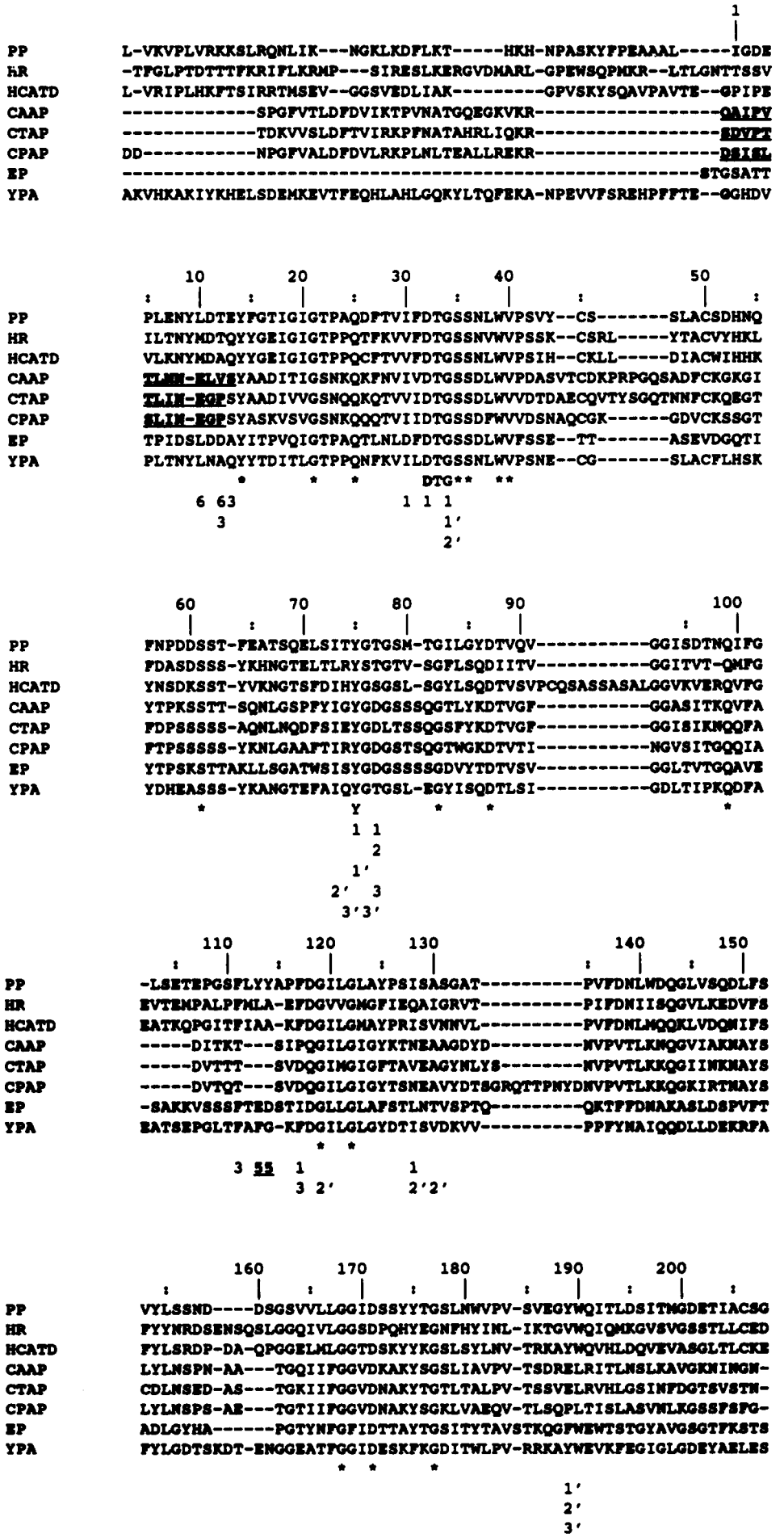
PP L-VKVPLVRKKSLRQNLIK---NGKLKDFLKT-----HKH-NPASKYFPEAAAL-----IGDE
HR -TFGLPTDTTTFKRIFLKRMP---SIRESLKERGVDMARL-GPEWSQPMKR--LTLGNTTSSV
HCATD L-VRIPLHKFTSIRRTMSEV--GGSVEDLIAK---------GPVSKYSQAVPAVTE--GPIPE
CAAP ----------SPGFVTLDFDVIKTPVNATGQEGKVKR----------QAIPV
CTAP ----------TDKVVSLDFTVIRKPFNATAHRLIQKR----------EDVPT
CPAP DD----------NPGFVALDFDVLRKPLNLTEALLREKR----------DSISL
EP ----------STGSATT
YPA AKVHKAKIYKHELSDEMKEVTFEQHLAHLGQKYLTQFEKA-NPEVVFSREHPFFTE--GGHDV
PP PLENYLDTEYFGTIGIGTPAQDFTVIFDTGSSNLWVPSVY--CS-------SLACSDHNQ
HR ILTNYMDTQYYGEIGIGTPPQTFKVVFDTGSSNVWVPSSK--CSRL-----YTACVYHKL
HCATD VLKNYMDAQYYGEIGIGTPPQCFTVVFDTGSSNLWVPSIH--CKLL-----DIACWIHHK
CAAP TLHN-ELVEYAADITIGSNKQKFNVIVDTGSSDLWVPDASVTCDKPRPGQSADFCKGKGI
CTAP TLIN-EGPSYAADIVVGSNQQKQTVVIDTGSSDLWVVDTDAECQVTYSGQTNNFCKQEGT
CPAP SLIN-EGPSYASKVSVGSNKQQQTVIIDTGSSDFWVVDSNAQCGK------GDVCKSSGT
EP TPIDSLDDAYITPVQIGTPAQTLNLDFDTGSSDLWVFSSE--TT-------ASEVDGQTI
YPA PLTNYLNAQYYTDITLGTPPQNFKVILDTGSSNLWVPSNE--CG-------SLACFLHSK
PP FNPDDSST-FEATSQELSITYGTGSM-TGILGYDTVQV----------GGISDTNQIFG
HR FDASDSSS-YKHNGTELTLRYSTGTV-SGFLSQDIITV----------GGITVT-QMFG
HCATD YNSDKSST-YVKNGTSFDIHYGSGSL-SGYLSQDTVSVPCQSASSASALGGVKVERQVFG
CAAP YTPKSSTT-SQNLGSPFYIGYGDGSSSQGTLYKDTVGF----------GGASITKQVFA
CTAP FDPSSSSS-AQNLNQDFSIEYGDLTSSQGSFYKDTVGF----------GGISIKNQQFA
CPAP FTPSSSSS-YKNLGAAFTIRYGDGSTSQGTWGKDTVTI----------NGVSITGQQIA
EP YTPSKSTTAKLLSGATWSISYGDGSSSSGDVYTDTVSV----------GGLTVTGQAVE
YPA YDHEASSS-YKANGTEFAIQYGTGSL-EGYISQDTLSI----------GDLTIPKQDFA
PP -LSETEPGSFLYYAPFDGILGLAYPSISASGAT----------PVFDNLWDQGLVSQDLFS
HR EVTEMPALPFMLA-EFDGVVGMGFIEQAIGRVT----------PIFDNIISQGVLKEDVFS
HCATD EATKQPGITFIAA-KFDGILGMAYPRISVNNVL----------PVFDNLMQQKLVDQNIFS
CAAP -----DITKT---SIPQGILGIGYKTNEAAGDYD---------NVPVTLKNQGVIAKNAYS
CTAP -----DVTTT---SVDQGIMGIGFTAVEAGYNLYS--------NVPVTLKKQGIINKNAYS
CPAP -----DVTQT---SVDQGILGIGYTSNEAVYDTSGRQTTPNYDNVPVTLKKQGKIRTNAYS
EP -SAKKVSSSFTEDSTIDGLLGLAFSTLNTVSPTQ---------QKTFFDNAKASLDSPVFT
YPA EATSEPGLTFAFG-KFDGILGLGYDTISVDKVV----------PPFYNAIQQDLLDEKRFA
PP VYLSSND----DSGSVVLLGGIDSSYYTGSLNWVPV-SVEGYWQITLDSITMGDETIACSG
HR FYYNRDSENSQSLGGQIVLGGSDPQHYEGNFHYINL-IKTGVWQIQMKGVSVGSSTLLCED
HCATD FYLSRDP-DA-QPGGELMLGGTDSKYYKGSLSYLNV-TRKAYWQVHLDQVEVASGLTLCKE
CAAP LYLNSPN-AA---TGQIIFGGVDKAKYSGSLIAVPV-TSDRELRITLNSLKAVGKNINGN-
CTAP CDLNSED-AS---TGKIIFGGVDNAKYTGTLTALPV-TSSVELRVHLGSINFDGTSVSTN-
CPAP LYLNSPS-AE---TGTIIFGGVDNAKYSGKLVAEQV-TLSQPLTISLASVNLKGSSFSPG-
EP ADLGYHA------PGTYNFGFIDTTAYTGSITYTAVSTKQGFWEWTSTGYAVGSGTFKSTS
YPA FYLGDTSKDT-ENGGEATFGGIDESKFKGDITWLPV-RRKAYWEVKFEGIGLGDEYAELES

FIGURE 2: Multiple sequence alignment for the *Candida* yeast aspartic proteinases. A multiple sequence alignment of the three *Candida* yeast aspartic proteinases with sequences of five other aspartic proteinases representative of this family. The numbering shown is for the porcine pepsin sequence. Positions of sequence identity are marked by an asterisk. DTG/D*G defines the following features of these enzymes: **D**, the active site aspartates; TG/*G, the residues of the fireman's grip. Y shows the position of the conserved tyrosine of the flap. The sequences of the three proteinases that were determined by amino terminal sequencing are shown in underlined boldface type. The numbering below the alignment indicates the predicted residues in a given substrate binding pocket of the *Candida* enzymes. Boldface underlined numbering indicates the positions of deletions in the various specificity pockets in the *Candida* enzymes that most likely give rise to different substrate specificities in the yeast enzymes. The deletions are relative to the porcine pepsin structure.

Table 3: Initial Velocities of Cleavage of Parent Substrates 1–3

peptide	CAAP	CTAP	CPAP
1	264	321	124
2	120	120	238
3	80	70	212

[a] Initial velocities ($\Delta A_{300nm}/s \times 10^6$) for the cleavage of parent peptides 1–3 by the three *Candida* yeast aspartic proteinases, CAAP, CTAP, and CPAP. The sequences of the parent peptides are given below. The scissile bond cleaved by the yeast enzymes is indicated by ↓.

P_5 P_4 P_3 P_2 P_1 P_1' P_2' P_3'

{1} Lys-Pro-Ala-Lys-Phe-↓-Phe(p-NO_2)-Arg-Leu

{2} Lys-Pro-**Ile**-**Glu**-Phe-↓-Phe(p-NO_2)-Arg-Leu

{3} Lys-Pro-Ala-**Glu**-Phe-↓-Phe(p-NO_2)-**Ala**-Leu

has focused on substitutions in the parent sequences that gave the most dramatic changes in the initial cleavage rates, as seen in Tables 2 or 3. The results of this determination are summarized in Tables 4–6, respectively.

Inhibition by Pepstatin A. For the determination of the inhibition constants of these three *Candida* proteinases by pepstatin A, peptide **3** was used as a substrate.

The K_i values determined for the *Candida* enzymes were 0.6 ± 0.3 nM for CPAP, 6.0 ± 2.3 nM for CAAP, and 12.0 ± 3.8 nM for CTAP, respectively.

DISCUSSION

We have studied the substrate specificities for three *Candida* yeast aspartic proteinases by measuring k_{cat} and K_m values for a library of synthetic peptides. The initial analysis gave us an indication that specificity differences do exist for each of the individual yeast enzymes. Tables 2 and 3 indicate that these proteinases show a preference for a particular substrate sequence. Peptide **1**, Lys-Pro-Ala-Lys-Phe-Phe(p-NO_2)-**Arg**-Leu, which is rich in basic residues, is the preferred substrate for CAAP and CTAP (Table 2). In contrast to this, peptides **2** Lys-Pro-Ile-**Glu**-Phe-Phe(p-NO_2)-**Arg**-Leu, and **3**, Lys-Pro-Ala-**Glu**-Phe-Phe(p-NO_2)-Ala-Leu, with glutamic acid at the P_2 position are preferred by CPAP (Table 3). The enzyme CPAP shows a preference for an acidic side chain chemical structure in P_2.

Table 4: Kinetic Values for Cleavage of Substrate Peptide **2** and Its Derivative with Ala at P_1[a]

	CAAP			CTAP			CPAP		
peptide	k_{cat} (s^{-1})	K_M (10^{-6} M)	k_{cat}/K_M (s^{-1} M^{-1} × 10^6)	k_{cat} (s^{-1})	K_M (10^{-6} M)	k_{cat}/K_M (s^{-1} M^{-1} × 10^6)	k_{cat} (s^{-1})	K_M (10^{-6} M)	k_{cat}/K_M (s^{-1} M^{-1} × 10^6)
2	5.6 ± 1.0	29.3 ± 7.8	0.52 ± 0.17	3.2 ± 0.6	38.3 ± 8.6	0.04 ± 0.01	3.7 ± 1.4	13.7 ± 2.0	0.27 ± 0.11
2/P_1A		ND		21.9 ± 2.0	119.7 ± 34.0	0.18 ± 0.07	18.2 ± 3.8	43.4 ± 4.8	0.42 ± 0.18

[a] Kinetic values for the cleavage of parent peptide **2** (Lys-Pro-Ile-Glu-Phe-Phe(p-NO_2)-Arg-Leu) and the substrate resulting from the Phe to Ala substitution at the primary specificity position P_1.

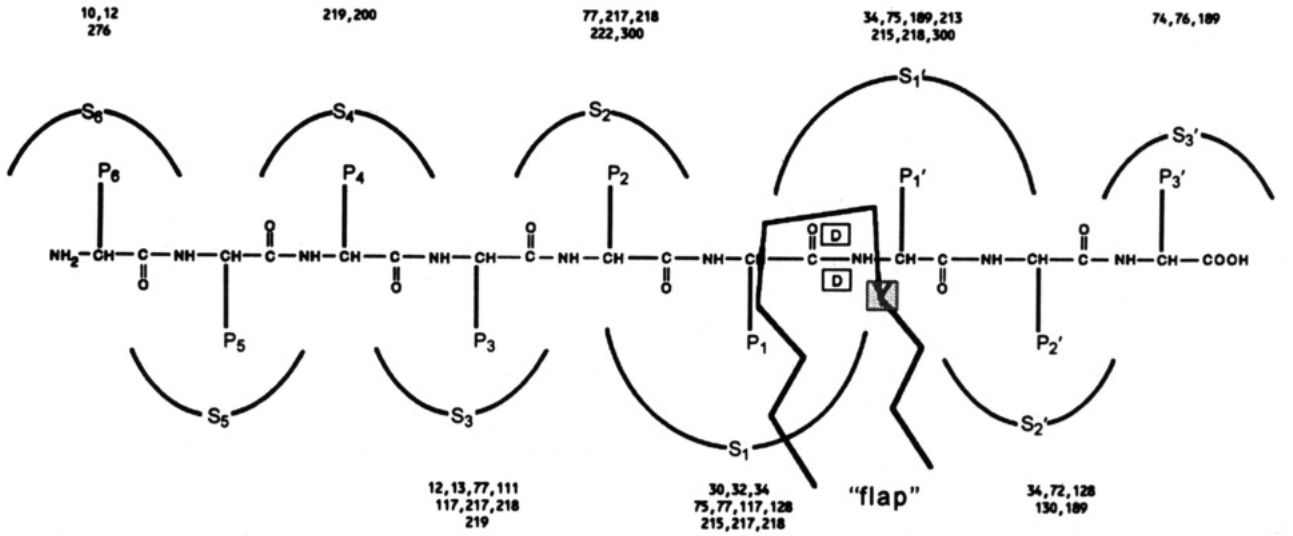

FIGURE 3: Substrate binding sites S_6–S_3'. Diagram of the binding pockets in *Candida* yeast aspartic proteinases. Interactions at residue positions P_6–P_3' with specificity pockets S_6–S_3' of the enzyme are according to the nomenclature of Berger and Schecter (1970). The numbers shown adjacent to each pocket are the predicted residues forming that enzyme specificity pocket. The position of the flap is indicated, and the conserved Tyr is marked by a capital Y (shaded box). The positions of the two active site aspartates are marked by a capital D (open boxes).

In this context, it is important to reflect that there are substantial differences in the acidic–basic character of these proteinases that are utilized during the purification of the three different enzymes. We have determined the isoelectric points of three proteinases, and they were found to be pI = 3.2 for CAAP, pI = 3.6 for CTAP, and pI = 5.6 for CPAP. It is reasonable to propose that the correlation between the isoelectric point of the proteinase and its overall charge recognition in substrate leads to the observable specificities of CAAP and CTAP, which with a lower pI prefer more basic synthetic peptides. CPAP, with a difference in pI of more than 2 pH units, prefers less basic or even neutral substrates.

A second general observation made in this analysis is that the substitution of Ala in any position of the substrates yielded peptides that were readily cleaved by all three proteinases, which may be interpreted as a broad or loose specificity similar to that observed for the gastric pepsins (Fruton, 1970).

Enzyme Substrate Interactions

Substitutions in the Primary Specificity P_1 Position. The preference for hydrophobic residues at positions P_1 and P_1' in aspartic proteinase substrates is well documented (Fruton, 1970). Here we determined the influence of a single substitution at P_1: a Phe to Ala change in the parent peptide sequence (**2**) with the enzymes CTAP and CPAP. Values of kinetic parameters are given in (Table 4). When Ala occupies the primary site P_1, both CTAP and CPAP show an increase in k_{cat}/K_M. This improvement in the specificity constant is largely due to a 5-fold increase in the k_{cat} value; however, it is also accompanied by a smaller increase in the Michaelis constant, K_M. The smaller side chain in the P_1 position results in a substrate with a lower apparent affinity for the enzymes. This is compensated by a lowering of the activation energy.

The only notable change in the primary amino acid structure that could explain this feature is at position 117, which is Phe in most other aspartic proteinases and is known to form part of the S_1 pocket. It is either Asp or Pro in the *Candida* enzymes. The S_1 pocket may be less restricted in volume than is normally the case in aspartic proteinases and thus allows a very loose and broad primary specificity at this position in a substrate.

Substitutions in P_2 and P_3 Positions. The analysis of specificity at position P_2 is shown in Table 5. This subsite was studied using the parent peptide Lys-Pro-Ala-Lys-Phe-Phe-(p-NO_2)-Arg-Leu (**1**) and its substitutions **1**/P_2A, ,**1**/P_2L, **1**/P_2D, and **1**/P_2R, respectively. A common feature of all three *Candida* proteinases is the preference observed for a hydrophobic substitution Leu at P_2. A 5-fold improvement in k_{cat} is observed with CAAP over the parent peptide **1**. Smaller improvements in k_{cat} are also observed for the CPAP and CTAP enzymes. The resultant substrate Lys-Pro-Ala-Leu-Phe-Phe(p-NO_2)-Arg-Leu (**1**/P_2L) so far is the best substrate for all three extracellular *Candida* aspartic proteinases.

When a smaller hydrophobic substitution is made at P_2, there is a corresponding loss in the apparent binding affinity with all of the enzymes, as manifested by the increase in K_M values. With no concomitant improvement in k_{cat}, the resultant specificity constant for this substrate is low. CTAP does not tolerate this change, and k_{cat}/K_M for the **1**/P_2A substrate is 5-fold lower than is observed with the parent substrate **1**. CPAP by comparison is more tolerant of this substitution at P_2. The substitution at P_2 of Asp for Lys in the parent peptide **1**/P_2D (Lys-Pro-Ala-**Lys**-Phe-Phe(p-NO_2)-Arg-Leu) is also acceptable to CPAP, while the specific activities of CAAP and CTAP are decreased as much as 10-fold (CTAP). This

Table 5: Kinetic Values for Cleavage of Substrate 1 and Its Derivatives with Substitutions at P_2 and P_3[a]

	CAAP			CTAP			CPAP		
peptide	k_{cat} (s^{-1})	K_M (10^{-6} M)	k_{cat}/K_M (s^{-1} M^{-1} × 10^6)	k_{cat} (s^{-1})	K_M (10^{-6} M)	k_{cat}/K_M (s^{-1} M^{-1} × 10^6)	k_{cat} (s^{-1})	K_M (10^{-6} M)	k_{cat}/K_M (s^{-1} M^{-1} × 10^6)
1	15.7 ± 3.0	29.3 ± 7.8	0.52 ± 0.17	23.1 ± 4.2	49.0 ± 7.9	0.47 ± 0.12	3.9 ± 0.8	9.1 ± 2.1	0.43 ± 0.13
1/P_2A	37.7 ± 6.8	198.0 ± 18.5	0.19 ± 0.04	27.0 ± 3.0	310.0 ± 24.6	0.09 ± 0.01	7.8 ± 1.8	25.0 ± 6.0	0.31 ± 0.10
1/P_2L	72.5 ± 2.9	24.5 ± 9.2	2.95 ± 0.25	28.0 ± 1.7	17.3 ± 3.7	1.60 ± 0.19	14.5 ± 3.0	24.4 ± 3.9	0.59 ± 0.16
1/P_2D	15.4 ± 3.9	73.0 ± 22.1	0.21 ± 0.08	10.9 ± 2.4	89.1 ± 22.9	0.05 ± 0.02	7.9 ± 1.6	33.9 ± 5.7	0.23 ± 0.06
1/P_2R	4.1 ± 0.7	8.5 ± 1.4	0.49 ± 0.12	28.0 ± 5.2	41.7 ± 6.8	0.69 ± 0.16	4.2 ± 0.9	9.0 ± 1.0	0.47 ± 0.11
1/P_3D	11.2 ± 2.0	42.0 ± 5.0	0.27 ± 0.06	20.6 ± 1.7	126.0 ± 17.0	0.16 ± 0.02	4.5 ± 1.6	31.0 ± 3.0	0.15 ± 0.05
1/P_3R	3.0 ± 0.6	5.7 ± 3.1	0.52 ± 0.30	30.7 ± 2.2	78.1 ± 14.0	0.39 ± 0.05	3.3 ± 0.8	12.0 ± 4.1	0.28 ± 0.12
2	5.6 ± 1.0	29.3 ± 7.8	0.52 ± 0.17	3.2 ± 0.6	38.3 ± 8.6	0.04 ± 0.01	3.7 ± 1.4	13.7 ± 2.0	0.27 ± 0.11
3	78.0 ± 7.4	398.0 ± 35.2	0.20 ± 0.06	19.3 ± 3.4	231.5 ± 19.4	0.08 ± 0.02	36.3 ± 9.1	101.4 ± 16.2	0.36 ± 0.12

[a] Kinetic values for the cleavage of parent peptide **1** (Lys-Pro-Ala-Lys-Phe-Phe(p-NO_2)-Arg-Leu) and its derivatives with amino acid substitutions at positions P_2 and P_3, and kinetic values for the cleavage of parent peptides **2** (Lys-Pro-Ile-Glu-Phe-Phe(p-NO_2)-Arg-Leu) and **3** (Lys-Pro-Ala-Glu-Phe-Phe(p-NO_2)-Ala-Leu).

tolerance of an acidic side chain at P_2 by CPAP is also observed for peptide substrate **2** (Lys-Pro-Ile-**Glu**-Phe-Phe(p-NO_2)-Arg-Leu). The CPAP enzyme demonstrates a broad specificity at the P_2 position, while CAAP and CTAP are more restricted in the amino acids that fit into this binding environment.

All three *Candida* proteinases accept the substitution of Arg at the P_2 position and give values of the specificity constant that are identical to that of the parent peptide **1** that has Lys in P_2. The length of the side chain at P_2 is an important component of specificity recognition at S_2. The longer and more conformationally flexible side chains of Arg and Lys are likely to make hydrophobic interactions with the enzyme at S_2, with the polar nitrogen atoms of Arg or Lys at P_2 extending out into the solvent. Shorter side chains such as Asp are conformationally restricted and so influence binding at S_2 to a much greater extent. It is interesting to note that substrate **3** with a Glu at P_2 is a poor substrate with CTAP; this perhaps indicates that the underlying interaction at Glu P_2 with S_2 in CTAP is one of like charge repulsion between the P_2 acidic Glu and an acidic side chain in the enzyme.

It is interesting to compare the specificity at P_2 in the yeast enzymes with those of the more archetypic aspartic proteinases in the family. Both the *Candida* enzymes and the lysosomal enzyme cathepsin D prefer a hydrophobic residue at P_2 (Scarborough et al., 1993). Cathepsin D also tolerates a negative charge at P_2, but a substitution of Arg in a substrate results in a peptide that is barely cleaved. This apparent specificity is reversed for the *Candida* enzymes CTAP and CTAP, which prefer an Arg at P_2 and reject an acidic side chain at this position. In the long term, it will be interesting to elucidate the three-dimensional structures of these *Candida* enzymes. We may then be able to correlate the substrate specificity differences with the structures of these enzymes (Dunn et al., 1987).

The examination of the sequence alignment indicates that most residues that are necessary to the formation of the S_2 binding pocket are very similar in each enzyme. For example, position 77 is correlated with P_2 specificity and binding in the S_2 pocket (Blundell et al., 1987). This position is conserved to be Asp in all three *Candida* proteinases and in other proteinases such as *Endothiapepsin*, *Rhizopuspepsin*, and *Penicillopepsin*. Position 77 is Ser or Thr in other mammalian aspartic proteinases. If this residue alone dictated the chemical preference of the residue at P_2, then all *Candida* proteinases would have very similar specificities. It quickly becomes apparent that no one residue in the vicinity of the S_2 pocket (hydrophobic 222 and 300) stands out as being unusual or could be assigned to a discrete ionic type interaction or highly oriented polar interaction, which may offer a plausible explanation of why the *Candida* enzymes prefer different acidic–basic chemistry in P_2. It is observed in the crystallographic analysis of several cocrystal complexes of *Endothiapepsin*, with designed inhibitors of human renin, that the side chain orientations show multiple binding modes particularly for the P_5, P_2, and P_3' positions (Blundell et al., 1987). Thus, a residue not typically considered a component of the S_2 binding pocket may contribute to specificity at P_2, particularly when the side chain at P_2 is long and conformationally flexible.

A hypothetical explanation for the acidic–basic specificity of the yeast enzymes comes from a review of aspartic proteinase structure and function. It is known that residue 74 is a component of the S_3' binding pocket (Davies, 1990) and is at the margin of pockets S_2 and S_2'. During the initial fitting steps of substrate binding, residue 74 may come into close proximity with the P_2 residue of substrate. The acidic–basic discriminatory behavior of the enzymes could then be ranked according to the residue identity at position 74. In CTAP we have Glu, and we have Arg in CPAP, Gly in CAAP, and His in cathepsin D. Consistent with this hypothesis, CTAP discriminates against acidic residues in P_2, that is, Asp and Glu substitutions give resultant substrates with very low specificity constants. In contrast, CPAP and CAAP tolerate a variety of side chains at P_2. This may also assist in describing the behavior of cathepsin D. His is found at position 74 and substrates with Glu are cleaved at favorable rates, while the enzyme rejects substrates strongly with His at the P_2 position and less so for Lys or Arg that would extend further into solvent and be less likely to result in charge conflict in the S_2 binding pocket.

The three proteinases show similar behavior with respect to substitutions at the P_3 position (Table 5). The enzymes accept Arg as a substitution at P_3 compared to Ala in the parent substrate **1**. Asp on the other hand is an unfavorable substitution, and K_M increases 3-fold for the CTAP and CPAP enzymes. This behavior mimics the more classic fungal proteinases described by Dunn and coauthors (Dunn et al., 1986). The explanation of this specificity may be found in the amino acid sequence of the enzyme in the region 110–120. This region influences specificity in both the S_1 and S_3 substrate binding pockets of the binding cleft. At position 117, Asp is present in both the CTAP and CPAP enzymes. CTAP and CPAP hydrolyze substrates with Asp in P_3 rather poorly. In CAAP, position 117 is Pro and the conflict of two possible like charges in the S_3 pocket is removed.

Substitutions in the Secondary Specificity P_4, P_5, P_2', and P_3' Positions. The interactions at the P_4 position, which is

Table 6: Kinetic Values for the Cleavage of Substrate Peptide **1** and Its Derivatives at Positions P_4, P_5, and P_2' [a]

	CAAP			CTAP			CPAP		
peptide	k_{cat} (s^{-1})	K_M (10^{-6} M)	k_{cat}/K_M (s^{-1} M^{-1} × 10^6)	k_{cat} (s^{-1})	K_M (10^{-6} M)	k_{cat}/K_M (s^{-1} M^{-1} × 10^6)	k_{cat} (s^{-1})	K_M (10^{-6} M)	k_{cat}/K_M (s^{-1} M^{-1} × 10^6)
1	15.7 ± 3.0	29.3 ± 7.8	0.52 ± 0.17	23.1 ± 4.2	49.0 ± 7.9	0.47 ± 0.12	3.9 ± 0.8	9.1 ± 2.1	0.43 ± 0.13
1/P_4L		NC			NCF (K_M higher than 1 M)			NC	
1/P_4D	12.4 ± 2.1	29.4 ± 3.0	0.42 ± 0.08	8.6 ± 0.6	58.0 ± 12.0	0.15 ± 0.1	20.7 ± 0.2	8.0 ± 3.0	0.09 ± 0.04
1/P_4R	4.9 ± 0.8	10.3 ± 1.9	0.47 ± 0.12	14.6 ± 0.9	69.4 ± 8.5	0.21 ± 0.02	0.2 ± 0.05	5.4 ± 2.0	0.04 ± 0.02
1/P_5R	14.6 ± 2.7	20.5 ± 4.3	0.72 ± 0.2	22.1 ± 1.5	34.4 ± 7.8	0.35 ± 0.09	3.7 ± 1.4	13.7 ± 2.0	0.27 ± 0.11
1/P_2'D		NC			NC			NC	

[a] Kinetic values for the cleavage of parent peptide **1** (Lys-Pro-Ala-Arg-Phe-Phe(p-NO_2)-Arg-Leu) and its derivatives with amino acid substitutions at positions P_4, P_5, and P_2'.

Pro in the parent peptide **1**, are essential for the tight binding of substrate to these enzymes (Table 6). When Leu was substituted at P_4, we were unable to detect a reasonable cleavage rate with any of the proteinases (CAAP, CTAP, or CPAP). After a more detailed analysis of the CTAP data, it was estimated that a peptide with Leu at P_4 results in a substrate with K_M above 1 M. Other substitutions at the P_4 position showed varied kinetic effects for the three proteinases. Values of k_{cat}/K_M are not dramatically altered when Asp or Arg is substituted for Pro at P_4 with the enzyme CAAP. By comparison, CTAP dislikes an Asp residue at P_4 and is tolerant of Arg. CPAP shows a high degree of restriction at P_4 and rejects both Asp and Arg substitutions at this position; the specificity constant drops 5-fold with the Asp and Arg substitutions. This dramatic change in k_{cat}/K_M is due to the large decrease in the substrate turnover number: the substitution of Arg for Pro in parent substrate **1** produces a decrease in k_{cat} of at least 1 order of magnitude. However, the apparent binding affinity of these substrates is only marginally affected. Several members of the aspartic proteinases show a remarkable rigidity in the specificity at the P_4 position. For example, substrate cleavage analysis of cathepsin D shows that this enzyme is highly selective for Pro at the P_4 position (Scarborough et al., 1991). The *Candida* enzymes also follow this general trend, with CPAP being the most restrictive enzyme in this subclass. It is hypothesized that there is a high degree of shape complementarity of the P_4 side chain to the S_4 binding pocket at this region of the active site cleft in the *Candida* yeast aspartic proteinases.

Data on P_5 specificity show us that the S_5 binding pocket can accommodate a wide variety of amino acid side chain structures without detrimental effects on catalytic rates. Peptide **1**/P_5R produced initial rates of cleavage that were ranked higher than those of the parent peptide **1**. It is evident that Arg is readily accepted in place of Lys, and k_{cat} and K_M are essentially identical to those of the parent peptide. No other substitutions at P_5 were analyzed in detail. It is observed in the cocrystal complexes of *Endothiapepsin* with designed inhibitors that the S_5 binding pocket is extensive and is able to accommodate a wide variety of side chain lengths and charges in part due to its being an open pocket and exposed to solvent (Foundling et al., 1987; Blundell et al., 1987). From these structural studies, it was observed that the P_5 side chain conformations have multiple binding orientations in the enzyme–inhibitor complexes. This in turn is interpreted as a broad enzymatic specificity at the S_5 binding site. It should be noted from the sequence alignment (Figure 2) that certain residues that typically form part of the P_5 pocket in the other aspartic proteinases are absent from the *Candida* enzymes. In particular, residues 113 and 114 are deleted. These prominent changes in the yeast enzymes may dramatically influence and alter substrate binding in this region of the substrate binding cleft.

The final substitution at position P_2' changed Arg to Asp in the parent peptide sequence **1**. The three enzymes had no measurable rates of cleavage for this resultant substrate (Lys-Pro-Ala-Lys-Phe-Phe(p-NO_2)-**Asp**-Leu). The specificity at this position appears to be highly selective. The initial rates of cleavage (Table 2) were also significantly reduced when Ser and Leu were interchanged at this position.

Screening of initial velocities for the cleavage of the synthetic peptides for specificity mapping at the P_3' position indicated to us that modifications here have little or no effect on initial rates of cleavage. The only major change at P_3' was observed with CTAP and the Ser substitution. We chose not to investigate modifications at this position further, but to focus on other regions of the active site binding cleft. In many aspartic proteinases, position P_3' is quite open and exposed to solvent. The *Candida* enzymes appear to be highly tolerant of changes in the chemistry of amino acid side chains at position P_3'.

Inhibition by Pepstatin A. Pepstatin A is the classic competitive tight-binding inhibitor of the aspartic proteinases. It has a predominantly hydrophobic chemical structure and inhibits the archetypic aspartic proteinases like pepsin and cathepsin D with K_i values in the sub-nanomolar range (Umezawa, 1972; Marciniszyn et al., 1976). We found a difference in the inhibition constants of pepstatin A with the three *Candida* proteinases. The K_i value determined for CPAP was approximately 10 times lower than that for CTAP and 20 times lower than that for CAAP. These results indicate that differences in specificity exist in the active site binding clefts of these enzymes. This behavior is also in agreement with the general *in vivo* growth inhibition of the three strains of yeasts when grown in medium containing pepstatin A as an general antifungal agent (M. Fusek and S. I. Foundling, unpublished results).

In this study, we have used a library of chromogenic synthetic substrates to probe the interactions between peptides and enzymes from *Candida* yeasts. In general we have found that differences exist between the three individual enzymes CAAP, CTAP, and CPAP. The enzymes show a broad pattern of specificity, with electrostatic interactions being critical for binding at several positions in the active site cleft. The enzymes show a preference for a long hydrophobic residue at the P_2 position: they accommodate a long positively charged side chain at P_2 well. CAAP and CTAP discriminate against Asp and less so against Glu at P_2, while CPAP tolerates a diversity of charges at this position in the substrate. Specificity is highly selective at P_2', and shape complementarity plays an important part in binding at the P_4 position. The sequence alignment points to several important differences between the yeast enzymes and mammalian aspartic proteinases, particularly in the regions of the binding cleft that forms the S_1 and S_3 pockets (residues 110–120), the S_5' pocket (residues 113 and 114), and the S_2, S_1', and S_3' binding regions (residues 286–

294). This last part of the cleft is important in forming a fairly rigid proline loop in the mammalian enzymes, for example, in cathepsin D and human renin. It is absent from the yeast enzymes. This suggests that the binding cleft is more open in this region, which may imply a loose fit for substrate at positions S_2, S_1', and S_3'. Finally, the changes in the binding pockets of S_1 and S_3 indicate that these regions are more polar than their mammalian counterparts, which are generally hydrophobic environments. The *Candida* enzymes can be considered more versatile than the mammalian counterparts, with the binding cleft being made up of large surface depressions similar to those of the fungal enzyme endothiapepsin. In this way, the enzymes could maintain a greater flexibility for cleaving a variety of extracellular protein substrates.

The importance of having efficient treatments for *Candida* opportunistic yeast infections is increasing, and the search for new therapeutic antifungal agents has become more urgent. The results of this study demonstrate that aspartic proteinases of *Candida albicans*, *Candida tropicalis*, and *Candida parapsilosis* generally differ in their substrate specificities, as well as their sensitivities to pepstatin A. The difference is also observed between *Candida* aspartic proteinases and physiologically occurring human aspartic proteinases. This opens the possibility to the design of a *Candida* proteinase specific inhibitor.

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