A systematic series of synthetic chromophoric substrates for aspartic proteinases

Ben M. DUNN,*† Melba JIMENEZ,* Benne F. PARTEN,* Martin J. VALLER,† Carole E. ROLPH† and John KAY†

*Department of Biochemistry and Molecular Biology, University of Florida College of Medicine, Box J-245, Gainesville, FL 32610, U.S.A., and †Department of Biochemistry, University College, P.O. Box 78, Cardiff, CF1 1XL, Wales, U.K.

The hydrolysis of the chromogenic peptide Pro-Thr-Glu-Phe-Phe(4-NO₂)-Arg-Leu at the Phe-Phe(4-NO₂) bond by nine aspartic proteinases of animal origin and seven enzymes from micro-organisms is described [Phe(4-NO₂) is *p*-nitro-L-phenylalanine]. A further series of six peptides was synthesized in which the residue in the P₃ position was systematically varied from hydrophobic to hydrophilic. The Phe-Phe(4-NO₂) bond was established as the only peptide bond cleaved, and kinetic constants were obtained for the hydrolysis of these peptide substrates by a representative selection of aspartic proteinases of animal and microbial origin. The value of these water-soluble substrates for structure-function investigations is discussed.

INTRODUCTION

Aspartic proteinases are important not only in the control of a number of biological systems (e.g. renin in hypertension, pepsin and gastricsin in the gastric phase of digestion, cathepsin D in tissue protein turnover), but are also vital ingredients in several processes of commercial value (e.g. chymosin for cheese-making). Extensive sequence homologies have been observed between the enzymes belonging to this family (for a review, see Kay, 1985). Tertiary structures have been determined by X-ray crystallography for three microbial aspartic proteinases, namely penicillopepsin (James & Sielecki, 1983), *Endothia parasitica* proteinase (Pearl & Blundell, 1984), *Rhizopus chinensis* proteinase (Bott *et al.*, 1982), and the mammalian homologue, pig pepsin (Andreeva *et al.*, 1984). All have broadly similar structures and this, together with the sequence homologies, would appear to indicate that all members of this family are likely to have similar three-dimensional conformations overall. Therefore all should operate through a common catalytic mechanism for the hydrolysis of substrates.

However, substantial differences in substrate specificity, pH optimum and stability have been shown to exist among the various members of the family. All of the enzymes appear to have an extended active-site cleft (Tang *et al.*, 1978), which can accommodate at least seven amino acids of a substrate in the S₄-S₃' subsites (nomenclature of Schechter & Berger, 1967), so that cleavage can occur (usually) between two hydrophobic residues occupying the S₁-S₁' sites (Powers *et al.*, 1977). Indeed, secondary binding has a large effect on the catalytic efficiency of a number of the enzymes (Fruton, 1970; Hofmann & Hodges, 1982; Blum *et al.*, 1985). The differences in specificity, and therefore activity, at various pH values may then be dependent on the nature of the amino acid side chains that make up the subsites in these enzymes.

Investigations into such differences have been facilitated for other proteolytic enzymes by the ready availability of chromogenic peptide substrates from commercial sources (e.g. for the blood-coagulation serine proteinases). However, the few such substrates that are available commercially for aspartic proteinases suffer from problems of poor aqueous solubility and lack of sensitivity. For example, the peptide Leu-Ser-Phe(4-NO₂)-Nle-Ala-Leu-OMe has been examined as a substrate for several mammalian enzymes and the fungal proteinases from *Mucor pusillus* and *Mucor miehei* (Martin *et al.*, 1982; Martin, 1984). However, this peptide is very poorly soluble and has limited value, since its use is restricted largely to studies at very low pH values. Similarly, the peptides Phe-Gly-His-Phe(4-NO₂)-Phe-Ala-Phe-OMe for pig pepsin (Rich & Sun, 1980) and Phe-Ala-Ala-Phe(4-NO₂)-Phe-Val-Leu-O4MP for cathepsin D (Agarwal & Rich, 1983) required the presence of organic solvent in the reaction mixture to ensure adequate solubility, even at low pH values.

The logic used in this laboratory to overcome such technical difficulties while, at the same time, retaining all of the features that are considered desirable in a peptide substrate, has been described previously for pig pepsin (Dunn *et al.*, 1984). Thus the readily soluble peptide Pro-Thr-Glu-Phe-Phe(4-NO₂)-Arg-Leu was synthesized to be sufficiently long to occupy each of the subsites of the active-site cleft such that the Phe-Phe(4-NO₂) pair would fit into S₁-S₁' and would thus preclude kinetic complications, such as multiple substrate binding and transpeptidation (Blum *et al.*, 1985), that have been found to occur with shorter (di- or tri-peptide) substrates.

The present paper extends these original findings by detailing the suitability of this substrate for use with a wide variety of aspartic proteinases of both animal and microbial origin. Additionally, further substrates based on this parent peptide have been designed and synthesized in order to examine the effect of secondary

Abbreviations used: Phe(4-NO₂), *p*-nitro-L-phenylalanine; Boc, t-butoxycarbonyl; Nle, norleucine; OMe, methyl ester; O4MP, 4-hydroxymethylpyridine ester; Bzl, benzyl; CM-, carboxymethyl.

† To whom correspondence and requests for reprints should be addressed.

Table 1. Amino acid compositions of the synthetic peptide substrates Lys-Pro-Xaa-Glu-Phe-Phe(4-NO₂)-Arg-Leu after purification by cation-exchange chromatography and reversed-phase microBondapak C₁₈ h.p.l.c.

Samples were hydrolysed with 6 M-HCl in sealed, evacuated, tubes at 115 °C for 24 h. Analyses were performed on a Beckman 120C amino acid analyser with manual sample injectors and a Columbia Scientific Industries integrator operating on the 570 nm channel only. Ratios were calculated by summing the amounts of the amino acids present and dividing by the expected total number of amino acids to give an average amount per residue. Each amino acid amount was then divided by this average to give the ratio.

Amino acid	Xaa . . .	Calculated ratio					
		Ile	Val	Ala	Pro	His	Lys
Lys		0.99	1.02	1.06	1.16	0.91	1.98
Phe(4-NO ₂)		0.88	0.85	0.93	0.87	0.87	0.83
His		—	—	—	—	1.17	—
Arg		1.23	1.19	1.19	1.13	1.16	1.26
Glu		0.93	1.14	0.98	1.06	0.96	0.98
Pro		*	*	*	1.97	*	*
Ala		—	—	1.20	—	—	—
Val		—	0.75	—	—	—	—
Ile		0.90	—	—	—	—	—
Leu		1.03	1.02	0.90	1.02	1.02	0.99
Phe		1.04	0.90	0.89	0.99	0.99	0.97

* Proline was present in all peptides, but in general the *A*₅₇₀ was too small to permit accurate quantification.

binding in the active-site cleft on the cleavage of the Phe-Phe(4-NO₂) bond. Previous reports had indicated that interactions at the S₃ subsite might be of especial significance in determining the specificity of enzyme interaction with substrates and inhibitors (Fruton, 1970; Rich & Bernatowicz, 1982; Blum *et al.*, 1985). Consequently, a series of peptides of general structure Lys-Pro-Xaa-Glu-Phe-Phe(4-NO₂)-Arg-Leu was synthesized where Xaa = Ile, Val, Ala, Pro, His or Lys. The interaction of these substrates with a number of aspartic proteinases is described.

EXPERIMENTAL

Peptide synthesis

All peptides were synthesized by the stepwise solid-phase procedure (Barany & Merrifield, 1979) with a Vega model 50 peptide synthesizer to automate the reagent-addition, solvent-washing and mixing procedures. Amino acid derivatives with α -N-t-Boc protection were from Chemical Dynamics (South Plainfield, NJ, U.S.A.) or Vega Biochemicals (Tucson, AZ, U.S.A.). Trifluoroacetic acid was from Eastman (Rochester, NY, U.S.A.) and was redistilled before use. Bio-Beads (1% cross-linked polystyrene with 0.69 mequiv. of chloromethyl substitution/g of dry resin) were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Di-isopropylethylamine and dicyclohexylcarbodi-imide were from Eastman.

The parent peptide Pro-Thr-Glu-Phe-Phe(4-NO₂)-Arg-Leu was synthesized as described previously (Dunn *et al.*, 1984). The peptides of general sequence Lys-Pro-Xaa-Glu-Phe-Phe(4-NO₂)-Arg-Leu were synthesized by the following strategy. α -Boc-Leu-resin was prepared by the caesium salt method (Gisin, 1973) with a substitution level of 0.26 mmol/g of dry resin. A 5 g portion of this resin was placed into the reaction vessel of the synthesizer and elongation was carried out by

the dicyclohexylcarbodi-imide/hydroxybenzotriazole method (Dunn *et al.*, 1983) to yield:

α -Boc-(α -Bzl)Glu-Phe-Phe(4-NO₂)

-(ω -tosyl)Arg-Leu-resin

The sample was dried by washing with methanol and evacuation before being split into six portions by weight. These were each placed into a smaller reaction vessel and each synthesis was continued by addition of Boc-protected isoleucine, valine, alanine, proline, histidine or lysine to give the P₃ substituent of the six different peptides. The synthesis was completed in each case by the addition of proline and lysine.

After the completion of assembly of each of the peptide chains, Lys-Pro-Xaa-Glu-Phe-Phe(4-NO₂)-Arg-Leu, 1–3 mg of each peptidyl-resin was hydrolysed with 0.5 ml of 12 M-HCl and 0.5 ml of propionic acid *in vacuo* at 115 °C for 24 h. The resulting compositions gave amino acid ratios ranging from 0.5 to 1.6 per residue at this stage of preparation.

The completed peptidyl-resins (1.5–2.5 g) were deprotected and the peptides were cleaved from the resin by treatment with anhydrous HF as described previously (Dunn *et al.*, 1983). The peptides were extracted from the dry resin with 50% (v/v) acetic acid/water. A sample from each of the extracts was hydrolysed with 6 M-HCl at 115 °C for 24 h and the resulting analyses indicated that cleavage and extraction had removed at least 90% of the peptide from the resin in all cases.

Purification of peptides

Each of the crude peptides was purified by ion-exchange chromatography on a column (1 cm × 30 cm) of CM-Sephadex. The samples were loaded in 0.01 M-ammonium acetate buffer, pH 5.0, with the conductivity adjusted by dilution to be no higher than that of the starting buffer. After loading, the columns were washed with starting buffer (100 ml) and then a gradient of ammonium acetate buffer from 0.01 M to 0.5 M (200 ml

Table 2. Analysis of products obtained after incubation of synthetic peptides with various aspartic proteinases

Individual substrates, Lys-Pro-Xaa-Glu-Phe-Phe(4-NO₂)-Arg-Leu (in the amounts shown), were incubated with the appropriate aspartic proteinase (1–10 µg) for 1 h at 37 °C in 0.5 ml of 0.1 M-sodium formate buffer, pH 3.1. Reaction products were analysed by microbondapak C₁₈ reversed-phase h.p.l.c. using essentially the same conditions as were used for the purification of the individual substrates. Peaks detected at 254 nm were characterized by amino acid analysis. In all cases the pentapeptide product had the expected composition of Lys-Pro-Xaa-Glu-Phe (emerging at various times of elution according to the nature of the Xaa residue). Similarly the tripeptide product was always found to consist of Phe(4-NO₂)-Arg-Leu (emerging at 12 min of elution in every case). Recoveries of peptides varying from 40 to 80% are typical of reversed-phase (C₁₈) columns.

Enzyme used	Xaa	Substrate (nmol)	Pentapeptide product (nmol)	Tripeptide product (nmol)
Human gastricsin	Val	200	177	175
Yeast proteinase A	Pro	100	47	48
<i>Endothia parasitica</i> proteinase	Ile	125	49	60
Pig pepsin	Ala	315	261	308

each) was applied. Samples from the peaks resulting from chromatography of each of the peptides were hydrolysed for determination of the amino acid compositions (results not shown). Each pool displaying the composition closest to the theoretical was then examined by reverse-phase h.p.l.c. on a Waters liquid chromatograph using a microBondapak C₁₈ cartridge in a Z-Module radial-compression system. Buffers for h.p.l.c. were 0.02 M-ammonium acetate, pH 4.5, with 10% (v/v) methanol (Buffer A) and methanol (h.p.l.c. grade; Buffer B). In general, peptides were examined with a 20 min 20–100% (v/v) Buffer B gradient run at 1.5 ml/min. In cases where more than one peak was obtained, final purification was achieved using isocratic elution conditions. The compositions of the purified peptides are given in Table 1. The yield of each peptide was 20–40% after purification.

Enzymes

Pig pepsin was obtained by activation of pig pepsinogen by the method of Rajagopalan *et al.* (1966). Bovine chymosin and chicken pepsin were prepared by acid activation after isolation of their respective zymogens as described by Foltmann (1966) and Kostka *et al.* (1977) respectively. Cathepsin D was prepared from human spleen as described by Afting & Becker (1981). Human pepsin 3 and pig gastricsin, human gastricsin and human slow-moving proteinase were generously given by Dr. A. P. Ryle, University of Edinburgh Medical School, Edinburgh, Scotland, U.K., Professor J. Tang, Oklahoma Medical Research Foundation, Oklahoma City, OK, U.S.A., and Dr. I. M. Samloff, VA Medical Centre, Sepulveda, CA, U.S.A., respectively. The aspartic proteinases from *Mucor pusillus* and *Endothia parasitica* were isolated by Dr. S. I. Foundling, Birkbeck College, University of London, London, U.K., as described by Sonkuti & Babel (1968) and Williams *et al.* (1972) respectively. Yeast proteinase A was prepared as described previously (Dreyer *et al.*, 1985). The proteinases from *Mucor miehei* and *Scytalidium lignicolum* were kindly given by Dr. M. K. Harboe, Christian Hansen's Laboratory, Copenhagen, Denmark, and Dr. K. Oda, University of Osaka Prefecture, Osaka, Japan, respectively. Penicillopepsin and bovine cathepsin D were generously supplied by Dr. T. Hofmann, University of Toronto, Toronto, Canada, and Dr. V. Turk, J. Stefan

Institute, Ljubljana, Yugoslavia, respectively, whereas the aspartic proteinase from *Rhizopus chinensis* was a gift from Dr. R. Bott and Dr. D. Davies, National Institutes of Health, Bethesda, MD, U.S.A.

Product analysis

Samples of each of the peptides Lys-Pro-Xaa-Glu-Phe-Phe(4-NO₂)-Arg-Leu (100–1000 nmol) were incubated with enzyme (1–10 µg) in a total volume of 0.5 ml for 1 h at 37 °C before immersion in ice. The products thus generated were examined by h.p.l.c. and amino acid analysis as described previously (Dunn *et al.*, 1984). A portion (10 µl) was first injected into the h.p.l.c. instrument to examine the pattern and to establish conditions for characterization of the products. Peaks observed by detection at 254 nm were collected and hydrolysed for amino acid analysis. The expected peptides arising from cleavage between the phenylalanine and the *p*-nitro-L-phenylalanine residues, i.e. the pentapeptide Lys-Pro-Xaa-Glu-Phe and the tripeptide Phe(4-NO₂)-Arg-Leu, were obtained in every case. Representative examples are presented in Table 2.

Determination of kinetic parameters

Stock substrate solutions were prepared in distilled water or in 0.1 M-sodium formate buffer, pH 3.1, at concentrations ranging from 1.0 to 5.0 mg/ml and portions (5–100 µl) were then diluted into buffer for the rate determinations. In all cases, the rate of hydrolysis was monitored by observing the decrease in *A*₃₀₀ of the solution. The initial linear slopes were measured to yield Δ*A*/min. In all cases, Michaelis–Menten kinetics were observed, and plots of [S]/*v* against [S] permitted the fitting (by the method of unweighted sum of least squares) of unambiguous straight lines. For each determination, initial rates (*v*) were measured with at least six values of the initial substrate concentration ([S]) within the appropriate range. *V*_{max} and *K*_m were derived from these plots, and *k*_{cat} values were obtained from the equation:

$$V_{\max} = k_{\text{cat}}[E]$$

where [E] is the enzyme concentration. The estimated precision of the values is in the range ±5–10%. The concentration of active enzyme was determined in all cases by titration of a measured volume of enzyme against a

Fig. 1. Composite plot of h.p.l.c. analyses obtained by chromatographic samples removed from an incubation mixture of Lys-Pro-Ala-Glu-Phe-Phe(4-NO₂)-Arg-Leu and pig pepsin

Substrate (500 nmol) was incubated at 14 °C in 0.1 M-sodium formate buffer, pH 3.1, for 20 min. Pig pepsin (0.2 µg) was then added to initiate the reaction and again (1 µg) after 100 min. Samples (25 µl) were removed at the times indicated by the numbers to the right of each trace and analysed on a C₁₈ microBondapak column using an isocratic buffer system of 45% buffer B (methanol) and 55% buffer A (20 mM-ammonium acetate buffer, pH 4.5, containing 10% methanol). The injection point is to the left of each trace, and in the $t = 0$ trace a peak of buffer salts was detected. The pentapeptide product, Lys-Pro-Ala-Glu-Phe (labelled P₂) is eluted just after this peak. The tripeptide product, Phe(4-NO₂)-Arg-Leu (P₁), and the uncleaved substrate (S) are labelled accordingly.

solution of isovaleryl-pepstatin, the concentration of which had been precisely quantified by amino acid analysis. The amount of isovaleryl-pepstatin required to yield 100% inhibition was taken to be equal to the amount of active enzyme present in the measured sample.

The change in A_{300} on hydrolysis of these substrates was in the range 1000–2000 M⁻¹ (at pH 3.1).

RESULTS AND DISCUSSION

In the original design of the parent heptapeptide substrate for pig pepsin, Pro-Thr-Glu-Phe-Phe(4-NO₂)-Arg-Leu, residues were chosen for the P₄, P₃, P₂, P₂' and P₃' positions so as to maximize favourable interactions within each of the corresponding subsites in the enzyme. However, since this enzyme has a relatively broad specificity, it is conceivable that oligopeptide substrates of the type described herein might bind in multiple ways within the active site, thus presenting different peptide bonds to the catalytic apparatus. It was considered essential to establish that the same reaction [cleavage of the Phe-Phe(4-NO₂) bond] was being monitored for all of the enzymes and substrates under study. Digestion of all of the substrates by pig pepsin and of representative substrates by the other enzymes was continued for extensive periods and the products of cleavage were separated by h.p.l.c. and identified by amino acid

analysis. No *p*-nitro-L-phenylalanine-containing products were ever obtained other than the tripeptide, Phe(4-NO₂)-Arg-Leu, for any digestion examined (Table 2). Thus the design of this substrate series would appear to ensure binding in the correct orientation.

The time course of a representative hydrolysis was examined by using reverse-phase h.p.l.c. to separate and quantify the amounts of substrate and products formed at regular intervals (Fig. 1). The heights of the three peaks derived from these h.p.l.c. traces are plotted in a pseudo-first-order fashion in Fig. 2(b) (for intact substrate), Fig. 2(c) (for the pentapeptide product) and Fig. 2(d) [for the tripeptide, Phe(4-NO₂)-Arg-Leu] respectively. The slopes of all lines were virtually identical, indicating that cleavage of the octapeptide substrate leads directly to the tripeptide and pentapeptide products without the formation of any stable intermediates such as transpeptidation products. The rate of change of A_{300} (Fig. 2a) was approximately equal to the slopes plotted in Figs. 2(b), 2(c) and 2(d), thus demonstrating that the spectrophotometric measurement is an accurate representation of the rate of cleavage of octapeptide into pentapeptide and tripeptide products. Similar analyses were performed for the other substrates (results not shown).

The parent peptide, Pro-Thr-Glu-Phe-Phe(4-NO₂)-Arg-Leu, would appear to be a good substrate not only

Fig. 2. Pseudo-first-order plots [$\log(A_t - A_\infty)$ (a and b) or $\log(A_\infty - A_t)$ (c and d)] for the pig-pepsin-catalysed hydrolysis of Lys-Pro-Ala-Glu-Phe-Phe(4-NO₂)-Arg-Leu

In (a), the A_{300} of the incubation mixture was monitored continuously. The final A_{300} value obtained (A_∞) was subtracted from the absorbance (A_t) measured at any time (t). In (b)–(d), the A_{254} of the peaks labelled S, P₂ and P₁ respectively in Fig. 1 were used as A_t . The absorbance at infinite time, A_∞ , was measured after prolonged incubation. The arrows indicate points at which additions of pig pepsin were made (20 min, 0.2 μ g; 100 min, 1 μ g).

Table 3. Kinetic constants for the hydrolysis of Pro-Thr-Glu-Phe-Phe(4-NO₂)-Arg-Leu by aspartic proteinases of animal origin

All reactions were carried out in 0.1 M-sodium formate buffer, pH 3.1, at 37 °C. The progress of each reaction was monitored by observing the decrease in A_{300} .

Enzyme	K_m (mM)	$k_{cat.}$ (s ⁻¹)	$k_{cat.}/K_m$ (mM ⁻¹ ·s ⁻¹)
Pig pepsin	0.08	89	1110
Human pepsin	0.17	72	425
Chicken pepsin	0.11	17	155
Pig gastricsin	0.10	55	550
Human gastricsin	2.0	9	4
Human cathepsin D	0.55	173	315
Human slow-moving proteinase	0.16	136	855
Bovine chymosin	0.46	51	110
Bovine cathepsin D	0.24	59	245

for pig pepsin as reported previously (Dunn *et al.*, 1984), but for most of the aspartic proteinases (Tables 3 and 4). Most K_m values are less than 1 mM and are similar in magnitude to those observed for oligopeptide substrates (Fruton, 1970; Martin, 1984; Pohl *et al.*, 1983). Notable exceptions include human gastricsin, with a K_m value greater than 2 mM, and the microbial enzymes, such as that from *E. parasitica*, which display K_m values as low as 3 μ M. The large spread of K_m values illustrates how small differences in amino acid sequence between homologous members of an enzyme family can lead to large changes in substrate binding. Substrates having K_m values in the millimolar range or higher are not generally

useful for quantifying enzymes, since the reactions consume large amounts of synthetic peptide. However, although substrates having a low K_m value are suitable for this purpose, the measurement of K_m values in the micromolar range is difficult and imprecise, since the change in absorbance becomes too small to permit accurate kinetic measurements to be made.

The $k_{cat.}$ values reported in Tables 3 and 4 likewise show a considerable range from 1 s⁻¹ (for *Rhizopus chinensis* proteinase) to 173 s⁻¹ (for human cathepsin D). Consequently, the specificity constants ($k_{cat.}/K_m$) indicate that Pro-Thr-Glu-Phe-Phe(4-NO₂)-Arg-Leu is among the best substrates reported for a number of the aspartic

Table 4. Kinetic constants for the hydrolysis of Pro-Thr-Glu-Phe-Phe(4-NO₂)-Arg-Leu by aspartic proteinases of microbial origin

All reactions were carried out in 0.1 M-sodium formate buffer, pH 3.1, at 37 °C. The progress of each reaction was monitored by observing the decrease in A_{300} .

Enzyme	K_m (mM)	$k_{cat.}$ (s ⁻¹)	$k_{cat.}/K_m$ (mM ⁻¹ ·s ⁻¹)
Yeast proteinase A	0.23	30	130
<i>Mucor miehei</i> proteinase	0.10	5	50
<i>Mucor pusillus</i> proteinase	0.06	16	255
<i>Scytalidium lignicolum</i> proteinase	0.03	3	100
<i>Rhizopus chinensis</i> proteinase	0.008	1	125
Penicillopepsin	0.005	24	4800
<i>Endothia parasitica</i> proteinase	0.003	12	4000

Table 5. Michaelis constants (K_m) for the hydrolysis of Lys-Pro-Xaa-Glu-Phe-Phe(4-NO₂)-Arg-Leu by various aspartic proteinases

All reactions were performed in 0.1 M-sodium formate buffer, pH 3.1, at 37 °C. Abbreviation used: n.d., not determined.

Proteinase	Xaa . . .	K_m (mM)					
		Ile	Val	Ala	Pro	His	Lys
Pig pepsin		0.05	0.06	0.035	0.19	0.415	0.62
Human gastricsin		0.56	0.35	0.68	≥ 2	≥ 2	≥ 2
Human cathepsin D		0.64	0.20	0.84	1.08	≥ 2	≥ 2
Bovine chymosin		0.36	0.28	0.53	≥ 2	≥ 2	≥ 2
Yeast proteinase A		0.29	0.31	0.35	0.16	1.13	n.d.
<i>Mucor pusillus</i> proteinase		0.043	0.055	0.016	0.07	0.035	0.09
<i>Rhizopus chinensis</i> proteinase		< 0.001	0.026	< 0.002	0.039	0.019	0.19
<i>Endothia parasitica</i> proteinase		0.003	0.003	0.002	0.004	< 0.001	0.005

proteinases, reaching a maximum of 4800 mM⁻¹·s⁻¹ for penicillopepsin.

In an attempt to provide an insight into the detailed subsite interactions that may account for the large differences of K_m and $k_{cat.}$ observed for the heptapeptide substrate, a series of new peptides was prepared. These substrates retained the essential features of the parent heptapeptide, but included an eighth residue (lysine) to improve solubility in aqueous buffers still further. In addition, the residue occupying the P₃ position was varied from large hydrophobic amino acids isoleucine and valine, through the less hydrophobic amino acids alanine and proline, and into the hydrophilic amino acids histidine and lysine. This variation in the P₃ residue, occurring within a peptide in which the other six binding residues were maintained constant, was designed to provide information on the preference of the S₃ subsite in the enzymes examined.

These six new substrates were tested with eight representative enzymes, and it can be seen (Table 5) that, for the mammalian enzymes, a relatively hydrophobic residue in the P₃ position yields good substrates, whereas a charged histidine or lysine residue is not accepted particularly well. By contrast, the microbial enzymes (Table 5, lower half) do not display such a large disparity in K_m upon variation of the P₃ residue. This may reflect the fact that the energy derived from interaction of the substrate binding at other subsites is already relatively strong, as indicated by the low K_m value for the

hydrolysis of the parent heptapeptide substrate (Table 4). Alternatively, it is conceivable that the S₃ subsite of each of the microbial enzymes may be much more versatile than their counterparts in the mammalian enzymes. Indeed, examination of the crystallographically determined structure of *Endothia parasitica* proteinase (Hallett *et al.*, 1985) in the S₃ region shows that there are at least two carboxylic acid side chains (Asp-12 and Asp-114), as well as several hydrophobic residues (Ile-117, Ala-13) that are potentially close enough to interact with the P₃ side chain of a substrate or inhibitor. Furthermore, the S₃ site in the *Endothia* enzyme is a large depression that could allow several alternative orientations of the side chain of the amino acid in the P₃ position. If the active sites of the other microbial enzymes were to retain this type of flexibility, it would not be surprising that they can bind all of these substrates so readily. As observed above for the parent heptapeptide, binding (as defined by K_m) is generally much better to the microbial enzymes than to their mammalian counterparts. The versatility of the binding pockets of pig pepsin and the microbial enzymes has been compared (Andreeva *et al.*, 1985), and the somewhat larger size of the pockets in the microbial enzymes has been emphasized. It was suggested that binding interactions in secondary subsites might induce subtle conformation alterations in the orientation at the primary S₁-S₁' subsites, leading to more effective binding.

The $k_{cat.}$ values for this series of substrates (Table 6) also have a wide range. The mammalian enzymes have

Table 6. Catalytic site activities (k_{cat}) for the hydrolysis of Lys-Pro-Xaa-Glu-Phe-Phe(4-NO₂)-Arg-Leu by various aspartic proteinases

All reactions were performed in 0.1 M-sodium formate buffer, pH 3.1, at 37 °C. Abbreviation used: n.d., not determined.

Proteinase	Xaa . . .	$k_{\text{cat.}} \text{ (s}^{-1}\text{)}$					
		Ile	Val	Ala	Pro	His	Lys
Pig pepsin		106	19	97	220	10	15
Human gastricsin		8	3	5	< 0.1	< 0.1	< 0.1
Human cathepsin D		124	55	60	25	< 0.1	< 0.1
Bovine chymosin		190	74	20	< 0.1	< 0.1	< 0.1
Yeast proteinase A		54	42	21	14	2	n.d.
<i>Mucor pusillus</i> proteinase		19	15	7	5	3	7
<i>Rhizopus chinensis</i> proteinase		3	4	5	2	4	2
<i>Endothia parasitica</i> proteinase		16	20	16	7	7	21

Table 7. Specificity constants ($k_{\text{cat.}}/K_{\text{m}}$) for the hydrolysis of Lys-Pro-Xaa-Glu-Phe-Phe(4-NO₂)-Arg-Leu by various aspartic proteinases

Proteinase	Xaa...	$k_{\text{cat.}}/K_{\text{m}} \text{ (s}^{-1} \cdot \text{mM}^{-1}\text{)}$					
		Ile	Val	Ala	Pro	His	Lys
Pig pepsin		2120	320	2770	1160	24	24
Human gastricsin		14	9	7	5	0.6	0.5
Human cathepsin D		195	275	70	25	6	7
Bovine chymosin		530	260	40	45	3	3
Yeast proteinase A		185	135	60	90	2	n.d.
<i>Mucor pusillus</i> proteinase		440	275	440	70	85	80
<i>Rhizopus chinensis</i> proteinase		> 3000	155	> 2500	70	210	11
<i>Endothia parasitica</i> proteinase		5330	6660	8000	1750	> 7000	4200

poorer K_{m} values than do their microbial counterparts, but exhibit higher $k_{\text{cat.}}$ values in general. This might be interpreted to indicate that substrate binding energy may be transmitted to catalytic potential (Jencks, 1975). However, this postulate, that the poorer the value of K_{m} the larger the value of $k_{\text{cat.}}$, is not substantiated if a comparison is made within the series of six substrates with a single enzyme. For example, in the case of pig pepsin, the substrates with Xaa = histidine or lysine have the weakest K_{m} values and the lowest values of $k_{\text{cat.}}$. This is also observed for gastricsin, cathepsin D and chymosin. The $k_{\text{cat.}}$ values for the microbial enzymes are again smaller than for the mammalian enzymes, but they display the same constancy as observed for the K_{m} values.

From the specificity constants, $k_{\text{cat.}}/K_{\text{m}}$ (Table 7) it is apparent that extremely effective substrates are now available for almost all of the aspartic proteinases. The introduction of these substrates has greatly facilitated studies of the mechanism of action of these enzymes (Dunn & Fink 1984; Hofmann *et al.*, 1984) and has made it possible to study the binding of competitive inhibitors of these enzymes in a straightforward way (Valler *et al.*, 1985; Powell *et al.*, 1985; Hallett *et al.*, 1985; Dreyer *et al.*, 1985). It should be emphasized that the primary value of these new peptide substrates lies in their use for studies of structure-function relationships in purified enzymes rather than for routine enzyme assays during isolation or extraction.

Finally, it would appear that the S₃ subsite is the location of a great deal of the secondary specificity

exhibited by the mammalian aspartic proteinases, whereas the microbial enzymes are apparently much less rigid in this requirement provided that the S₃ subsite is occupied. Any secondary specificity exhibited in the microbial enzymes must therefore arise from other subsites in the active site. These initial investigations were carried out at a single pH value (of 3.1) in order to maintain continuity with previous investigations (Fruton, 1970; Valler *et al.*, 1985; Dreyer *et al.*, 1985), but it must be recognized that individual enzymes might display different pH-dependencies and this may be a further factor contributing to the observed kinetic variability. Such dependencies are clearly worthy of further investigation.

This work was supported by grants from the Science and Engineering Council (U.K.) (GR/C42521 and 42538), the U.S. National Institutes of Health (no. AM 18865), by a University of Wales-British Council 50th Anniversary Fellowship and by awards from The Wellcome Trust and Burroughs-Wellcome Fund. We are very grateful to our many colleagues throughout the world who generously provided samples of their purified enzymes. It is also a pleasure to acknowledge the patience and secretarial skill of Ms. Kathy Galliers.

REFERENCES

- Afting, E.-G. & Becker, M.-L. (1981) *Biochem. J.* **197**, 519–522
 Agarwal, N. & Rich, D. H. (1983) *Anal. Biochem.* **130**, 158–165
 Andreeva, N. S., Zdanov, A. S., Gustchina, E. E. & Fedorov, I. A. (1984) *J. Biol. Chem.* **259**, 11353–11365

- Andreeva, N. S., Zdanov, A. S., Gustchina, A. E. & Fedorov, I. A. (1985) in *Aspartic Proteinases and their Inhibitors* (Kostka, V., ed.), pp. 137–150, Walter de Gruyter, Berlin
- Barany, G. & Merrifield, R. B. (1979) in *The Peptides: Analysis, Synthesis & Biology* (Gross, E. & Meienhofer, J. eds.) pp. 1–284, Academic Press, New York
- Blum, M., Cunningham, A., Bendiner, M. & Hofmann, T. (1985) *Biochem. Soc. Trans.* **13**, 1044–1046
- Bott, R., Subramanian, E. & Davies, D. R. (1982) *Biochemistry* **21**, 6956–6962
- Dreyer, T., Valler, M. J., Kay, J., Charlton, P. & Dunn, B. M. (1985) *Biochem. J.* **231**, 777–779
- Dunn, B. M. & Fink, A. L. (1984) *Biochemistry* **23**, 5242–5247
- Dunn, B. M., Lewitt, M. & Pham, C. (1983) *Biochem. J.* **209**, 355–362
- Dunn, B. M., Kammermann, B. & McCurry, K. R. (1984) *Anal. Biochem.* **138**, 68–73
- Foltmann, B. (1966) *C.R. Trav. Lab. Carlsberg* **35**, 143–231
- Fruton, J. S. (1970) *Adv. Enzymol. Relat. Areas. Mol. Biol.* **33**, 401–443
- Gisin, B. F. (1973) *Helv. Chim. Acta* **56**, 1476–1482
- Hallett, A., Jones, D. M., Atrash, B., Szelke, M., Leckie, B., Beattie, S., Dunn, B. M., Valler, M. J., Rolph, C. E., Kay, J., Foundling, S. I., Wood, S. P., Pearl, L. H., Watson, F. E. & Blundell, T. L. (1985) in *Aspartic Proteinases and their Inhibitors* (Kostka, V., ed.), pp. 467–478, Walter de Gruyter, Berlin
- Hofmann, T. & Hodges, R. S. (1982) *Biochem. J.* **203**, 603–610
- Hofmann, T., Dunn, B. M. & Fink, A. L. (1984) *Biochemistry* **23**, 5247–5256
- James, M. N. G. & Sielecki, A. (1983) *J. Mol. Biol.* **163**, 299–361
- Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas. Mol. Biol.* **43**, 219–410
- Kay, J. (1985) in *Aspartic Proteinases and their Inhibitors* (Kostka, V., ed.), pp. 1–17, Walter de Gruyter, Berlin
- Kostka, V., Keilova, H., Gruner, K. & Zbrozek, J. (1977) *Coll. Czech. Chem. Commun.* **42**, 3691–3704
- Martin, P. (1984) *Biochim. Biophys. Acta* **791**, 28–36
- Martin, P., Trieu-Curot, P., Collins, J.-C. & Ribadeau-Dumas (1982) *Eur. J. Biochem.* **122**, 31–39
- Pearl, L. H. & Blundell, T. L. (1984) *FEBS Lett.* **174**, 96–101
- Pohl, J., Baudys, M. & Kostka, V. (1983) *Anal. Biochem.* **133**, 104–109
- Powell, M. J., Holdsworth, R. J., Baker, T. S., Titmas, R. C., Bose, C. C., Phillips, A., Eaton, M., Rolph, C. E., Valler, M. J. & Kay, J. (1985) in *Aspartic Proteinases and their Inhibitors* (Kostka, V., ed.), pp. 479–483, Walter de Gruyter, Berlin
- Powers, J. C., Harley, A. D. & Myers, D. V. (1977) in *Acid Proteinases: Structure, Function and Biology* (Tang, J., ed.), pp. 141–157, Plenum, New York
- Rajagopalan, T. G., Stein, W. H. & Moore, S. J. (1966) *J. Biol. Chem.* **241**, 4295–4297
- Rich, D. H. & Bernatowicz, M. S. (1982) *J. Med. Chem.* **25**, 791–796
- Rich, D. H. & Sun, E. T. O. (1980) *Biochem. Pharmacol.* **29**, 2205–2212
- Schechter, I. & Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157–162
- Sonkuti, G. A. & Babel, F. J. (1968) *J. Bacteriol.* **95**, 1407–1414
- Tang, J., James, M. N. G., Hsu, I.-N., Jenkins, J. A. & Blundell, T. L. (1978) *Nature (London)* **271**, 618–621
- Valler, M. J., Kay, J., Aoyagi, T. & Dunn, B. M. (1985) *J. Enzyme Inhib.* **1**, 77–82
- Williams, D. C., Whitaker, J. R. & Caldwell, P. V. (1972) *Arch. Biochem. Biophys.* **149**, 52–61

Received 22 January 1986/26 March 1986; accepted 15 April 1986