Fluorescent oligopeptide substrates for kinetic characterization of the specificity of Astacus protease

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must remain tentative until the three-dimensional structures of these proteins have been determined.

Registry No. HisDCase, 9024-61-7; proHisDCase, 39346-25-3; Glu, 56-86-0; Asp, 56-84-8; Ile, 73-32-5; Phe, 63-91-2; Ser, 56-45-1.

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Fluorescent Oligopeptide Substrates for Kinetic Characterization of the Specificity of *Astacus* Protease†

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ABSTRACT: The design of fluorescent N-dansylated oligopeptides based on the tubulin cleavage pattern by *Astacus* protease yields substrates that are turned over up to 10^5 times faster than those presently available. On the basis of this study, an optimal substrate for *Astacus* protease contains seven or more amino acids and minimally requires at least five amino acids. Direct examination of the formation and breakdown of the ES complex shows its formation occurs within milliseconds at 25 °C. The best heptapeptide substrate, Dns-Pro-Lys-Arg-Ala-Pro-Trp-Val, is cleaved only between the Arg–Ala (P_1–P_1') bond with kinetic parameters k_{cat} = 380 s^{-1} and K_m = 3.7 × 10^{-4} M. The presence of Lys or Arg in the P_1 and P_2 positions yields high-turnover substrates. In the P_3 position, the enzyme prefers Pro > Val > Leu > Ala > Gly, following the same order of preference seen in the tubulin cleavage pattern. Substitution of Leu for Ala in P_1' and of Ser for Pro in P_2' decreases activity by 10^5- and 10^2-fold, respectively. In position P_3', substitution of Trp for Leu leaves the activity unaltered. However, introduction of the Trp fluorophore greatly enhances the sensitivity of the assay due to a 10-fold increase in indole fluorescence for cleavage of any peptide bond between the tryptophan and the dansyl group. Such an energy-transfer-based assay should have widespread use for detection of neutral proteases. The relationship of *Astacus* protease to a recently sequenced bone morphogenetic protein and to metalloproteinases which share the putative zinc binding sequence HExxHxxGxxH is discussed.

Astacus protease, an endopeptidase from the digestive tract of the freshwater crayfish *Astacus fluviatilis*, is a monomeric protein of 200 amino acids (Titani et al., 1987; Vogt et al., 1989). This invertebrate protease has been considered to represent a new family of proteolytic enzymes because of its unusual properties (Zwilling & Neurath, 1981). Specific, naturally occurring inhibitors of *Astacus* protease have not been detected, and catalytically essential amino acid residues have not been identified (Torff et al., 1980).

We recently demonstrated that *Astacus* protease contains 1 mol of catalytically essential zinc per mole of protein (Stöcker et al., 1988). Furthermore, despite the absence of overall

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sequence homology with other zinc enzymes or proteins in general, a short stretch of amino acid residues at positions 90–99 (IIHELMHAIG) is similar to the metal binding site of thermolysin (residues 140–149, VAHELTHAVT). On the basis of its analogy to thermolysin, this sequence could be involved in both ligating the zinc and supplying the general base group for catalysis (Stöcker et al., 1988; Vallee & Auld, 1990a,b).

Thus far, kinetic analyses designed to explore the specificity and the catalytic mechanism of the enzyme have been hampered by the absence of substrates with high catalytic activity. The only synthetic substrates that the enzyme hydrolyzes are some nitroanilide derivatives such as succinyl-$(Ala)_3$-4-nitroanilide (STANA).[1] Originally designed for pancreatic elastase (Bieth et al., 1974), these substrates have been useful for inhibition studies of *Astacus* protease (Stöcker et al., 1988; Wolz & Zwilling, 1989) but are unsuitable for the investigation of the active-center subsite structure because of their low specific activity (k_{cat}/K_m = 20.8 M^{-1} s^{-1} for STANA) (Wolz et al., 1987).

The present study reports the design, synthesis, and use of N-dansylated heptapeptide substrates for the examination of the enzymatic specificity of *Astacus* protease. The structure of these substrates is based on the analysis of the cleavage sites in α- and β-tubulin that have been digested by *Astacus* protease for sequence analysis (Krauhs et al., 1982). The peptides cover the range from positions P_5 to P_4' [nomenclature of Schechter and Berger (1967)] and thus can be used to explore the subsite structure. A fluorescent label allows for the application of radiationless energy transfer (RET) methodology, a very sensitive and versatile approach for studying rapid enzymatic reactions (Auld, 1977, 1987; Lobb & Auld, 1984). Kinetic analyses are based either on RET between a substrate dansyl and tryptophan group to monitor the appearance of the product (Ng & Auld, 1989) or on RET between enzyme tryptophans and the dansyl group of the substrate in order to monitor intermediates in the reaction (Lobb & Auld, 1979). The series of N-dansylated heptapeptides are excellent substrates for *Astacus* protease. The k_{cat}/K_m values of some of them are in the range of those reported for the best substrates of proteases in general.

Materials and Methods

All chemicals were analytical grade and purchased from Fisher Chemical Co. (Medford, MA), Merck (Darmstadt), Eastman Kodak Co. (Rochester, NY), Sigma Chemical Co. (St. Louis, MO), or Serva (Heidelberg).

Astacus protease was purified from the digestive juice of the European freshwater crayfish *Astacus fluviatilis* Fabr. as previously described (Zwilling & Neurath, 1981). Enzyme concentrations were determined by amino acid analysis on a Waters amino acid analyzer using the picotag method (Bidlingmeyer et al., 1984) or calculated from measurements of the optical density at 280 nm using a molar absorptivity constant of ϵ_{280} = 42 800 M^{-1} cm^{-1} (Stöcker et al., 1988).

Precautions were taken to avoid metal contamination (Holmquist, 1988). Buffers and solutions were extracted with 0.01% dithizone, and glassware and plasticware were soaked in 30% nitric acid and rinsed carefully with metal-free water.

Dansylated peptide substrates were prepared by Merrifield solid-phase peptide synthesis (Merrifield, 1963) as described (Ng & Auld, 1989). It should be noted that the deformylation conditions of peptides containing tryptophan and lysine can lead to partial formylation of the lysine to varying degrees depending on the amino acids adjacent to the lysine. Characterization of the final products was achieved by amino acid analysis and HPLC. Substrate concentrations were determined spectrophotometrically based on the molar absorptivity of the dansyl group at 340 nm, ϵ_{340} = 4300 M^{-1} cm^{-1}. Spectra of dansyl peptides and of their cleavage products were recorded on a Perkin-Elmer MPF-3 spectrofluorometer.

Initial rates corresponding to less than 10% of the reaction were obtained under steady-state conditions either by stopped-flow fluorescence methods and/or by high-performance liquid chromatography (HPLC). The kinetic parameters were determined by using five to eight substrate concentrations and linear least-squares regression analysis of Lineweaver–Burk plots.

Stopped-flow experiments used a Durrum–Gibson instrument interfaced to a Digital Equipment Corp. computer as described elsewhere (Lobb & Auld, 1980). Assays were performed in 20 mM Hepes buffer, pH 7.8, at 25 °C. Substrate concentrations were in the range from 2.0×10^{-5} to 4×10^{-4} M, and those for the enzyme were from 1.0×10^{-7} to 5.0×10^{-7} M. N-Dansylated oligopeptides that contained a tryptophan on the opposite side of the scissile bond underwent a marked increase in fluorescence at 340 nm upon hydrolysis and could be assayed as described (Ng & Auld, 1989). The initial rate for a given substrate concentration was the average of three to five assays.

The formation and breakdown of ES complexes were observed by using substrates lacking a tryptophan. Excitation of the enzyme tryptophan leads to energy transfer to the substrate dansyl group upon formation of the ES complex. The conditions needed for this assay have been described (Lobb & Auld, 1979).

For HPLC analysis, assays were conducted in 20 mM Hepes buffer, pH 7.8 at room temperature. A 95-μL aliquot of substrate (5×10^{-6}–3×10^{-3} M) was transferred to a 1.5-mL polypropylene tube, and the reaction was initiated by addition of 5 μL of enzyme to give a final concentration ranging from 5×10^{-9} to 1.5×10^{-7} M. For each substrate concentration, five samples were prepared, and the reaction was stopped after 1, 10, 30, 60, or 90 s, respectively, by addition of 5 μL of glacial acetic acid. The samples were analyzed on a Waters Associates liquid chromatography system or on a Dupont HPLC instrument. Peak areas were determined at 225 or 260 nm with a Hewlett Packard 3380 A integrator. The columns used were either a Zorbax ODS PN 880952-702 (4.6 × 250 mm) or a Waters Nova Pak C_{18} (3.9 × 150 mm) reversed-phase column or an Orpegen HD-Sil-18-10-100 (4.6 × 250 mm; Orpegen Heidelberg). Samples (10–50 μL) were eluted with a linear acetonitrile gradient (20–60%) in 0.1% TFA at a flow rate of 1.5 mL min^{-1}. The concentration of products at a given time, P_t, was determined according to the equation:

$$P_t = \frac{P_a + P_b}{P_a + P_b + S_a} \times S_0$$

where P_a, P_b, and S_a are the integrated areas of product A and B and substrate peaks, respectively, and S_0 is the initial substrate concentration (Bond et al., 1986).

The observation of the reactions by thin-layer chromatography (TLC) was performed as previously described (Ng & Auld, 1989). For product analysis by TLC, the reaction

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; STANA, succinyl-$(Ala)_3$-4-nitroanilide; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; RET, radiationless energy transfer; Dns or dansyl, 5-(dimethylamino)-naphthalene-1-sulfonyl; TFA, trifluoroacetic acid; BMP I, bone morphogenetic protein; Cbz, carbobenzoxy; dR, D-arginine; pNA, 4-nitroanilide.

Table I: Peptides Tested as Substrates for *Astacus* Protease

Dns-GGFAG	Cbz-GPGGA-NH_2
Dns-FLA	Dns-PLGIAGdR[a]
Dns-PAG	*b*
Dns-AFFA	Dns-PQGIAGdR
Dns-AAFA	SUC-AAApNA
Dns-GGFA	Dns-KETYSK
Dns-AFLA	Dns-KETYSF

[a] dR = D-arginine. [b] Peptides below this are measurably cleaved.

mixture (0.2-μL aliquots) was spotted on micropolyamide sheets (Schleicher & Schuell) or on silica gel plates (Eastman Kodak). Methanol/water/formic acid (30:20:1), chloroform/methanol/glacial acetic acid (17:2:1), and water/formic acid (200:3) proved to be good solvents for the separation of reaction products on micropolyamide plates. 1-Butanol/water/glacial acetic acid (8:1:1) effectively resolved the spots on silica gel. The dansylated substrate and product were detected with an ultraviolet lamp.

Peptide sequence searches were performed by using the National Biomedical Research Foundation and GenBank/Los Alamos data base files of the Molecular Biology Computer Resource facility at Harvard Medical School.

Results

The search for convenient substrates as a prerequisite for studying the kinetic mechanism was one of the critical objectives during previous investigations of *Astacus* protease. It was known that this enzyme prefers short, aliphatic amino acid residues in the P_1' position (Zwilling et al., 1981), and, thus, the initial substrates chosen in this study contained alanine and glycine residues. Table I lists a number of tri- to heptapeptides that were examined as potential substrates for *Astacus* protease. A number of these peptides are excellent substrates for the metalloproteinases thermolysin, angiotensin converting enzyme (ACE), and carboxypeptidase A as well as for the pancreatic serine proteinases elastase, trypsin, and chymotrypsin. However, *Astacus* protease does not catalyze the hydrolysis of the majority of them. Weak activity is observed with succinyl-$(Ala)_3$-4-nitroanilide (k_{cat}/K_m = 20.8 M^{-1} s^{-1}) and with Dns-KETYSK, Dns-KETYSF, and Dns-PQGIAGdR. For the latter three, the estimated values for k_{cat}/K_m are below 1 M^{-1} s^{-1}.

In an attempt to design peptide substrates with higher reactivity, we utilized information about the specificity of *Astacus* protease which became available as a result of the sequence analysis of α- and β-tubulin (Krauhs et al., 1982). Digestion of denatured, S-carboxymethylated tubulin yielded a total of 71 cleavage sites. In order to design possible peptide substrates for *Astacus* protease, we analyzed the cleavage pattern of tubulin taking into account the subsites P_4 to P_4'. The frequency, % P_n, for a certain amino acid residue to occupy one of these subsites was calculated from the ratio

$$\%\ P_n = \frac{\text{no. of amino acid X in position } P_n}{\text{no. of amino acid X in tubulin}} \times 100$$

where P_n is a position and X indicates one of 20 amino acids (Keil, 1987). From the values of % P_n, a characteristic pattern can be deduced for the frequency at which certain amino acid residues appear in one of these subsites (Table II). The P_1' subsite is preferentially occupied by small aliphatic residues, with the highest value for alanine (% P_1' = 32). There is a strong preference for a proline in P_2' (% P_2' = 28), and in subsites P_3' and P_4', hydrophobic residues are most frequent, such as phenylalanine (% P_3' = 26) or leucine (% P_3' = 22) and valine (% P_4' = 20). On the N-terminal side of the scissile bond, the preferred amino acids in P_1 and P_2 are arginine (% P_1 = 26), lysine (% P_2 = 21), tyrosine (% P_1 = 24; % P_2 = 21), and asparagine (% P_1 = 26). The distribution of residues in P_3 and P_4 is more variable, although positively charged residues and neutral aliphatic residues are more abundant than negatively charged amino acids (Table II).

On the basis of this information, a series of heptapeptides were synthesized covering subsites P_3 to P_4' with the intent of achieving high catalytic activity toward *Astacus* protease (Table III). After this had been accomplished, a second set of peptides were synthesized, increasing and decreasing the size of a highly turned over peptide substrate (Table IV). In addition, heptapeptides were designed with amino acid inserts which would likely make them poorer substrates based on the tubulin cleavage pattern. In all of the peptides, a dansyl group is attached at the N-terminal amino acid as a prerequisite for the use of these substrates in fluorescent-based assays. A number of peptides were synthesized with a tryptophan residue in the P_3' position. In these substrates, the dansyl and indole fluorophores are separated by five amino acid residues, and

Table II: Cleavage Pattern of Tubulin α and β Chains by *Astacus* Protease[a]

P_4		P_3		P_2		P_1		P_1'		P_2'		P_3'		P_4'	
K	18	P	18	K	21	R	26	A	32	P	28	F	26	V	20
H	17	V	17	Y	21	N	26	T	24	T	15	L	22	P	18
L	14	I	16	M	18	Y	24	S	22	D	15	C	15	T	12
P	13	K	15	P	18	S	16	G	15	I	13	W	13	I	11
G	11	Q	13	L	13	F	14	V	11	H	13	A	11	A	11
M	11	Y	12	W	13	H	13	H	4	V	11	Q	11	C	10
N	10	R	12	Q	11	M	11	M	4	A	9	V	9	H	9
R	9	L	11	N	10	A	8	L	3	N	8	T	8	G	8
T	8	N	10	T	10	K	6	N	3	F	7	M	7	S	8
E	8	H	9	F	9	D	6	I	2	Y	6	R	7	Q	8
F	7	T	7	S	8	Q	5	E	1	E	5	Y	6	N	8
S	6	A	5	R	7	P	5	C	0	Q	5	P	5	Y	6
Y	6	S	4	V	6	L	5	D	0	C	5	G	4	F	5
Q	5	E	3	G	6	G	3	F	0	M	4	E	4	E	4
C	5	G	3	H	4	T	2	K	0	G	3	S	4	M	4
A	5	D	2	D	2	V	2	P	0	S	2	K	3	R	2
I	2	C	0	E	1	E	1	Q	0	L	2	I	2	D	2
D	2	F	0	A	0	C	0	R	0	K	0	D	0	L	2
V	2	M	0	C	0	I	0	W	0	R	0	H	0	K	0
W	0	W	0	I	0	W	0	Y	0	W	0	N	0	W	0

[a] Amino acids are given in one-letter code. C is (carboxymethyl)cysteine. The numbers are values of % P_n, i.e., the frequency at which a certain residue occurs in one of the subsite positions P_4 to P_4'.

Table III: Kinetic Constants for the Hydrolysis of Dansyl Heptapeptide Substrates by *Astacus* Protease

substrate							k_{cat} (s^{-1})	K_m ($\times 10^{-4}$ M)	k_{cat}/K_m (M^{-1} s^{-1})
P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'			
Dns P	K	R	A	P	W	V[a]	380	3.7	1.0×10^6
Dns V	K	R	A	P	W	V[a]	190	2.5	7.6×10^5
Dns L	K	K	A	P	W	V[a]	210	2.9	7.2×10^5
Dns L	K	R	A	P	W	V[a]	210	3.3	6.4×10^5
Dns L	K	R	A	P	L	V[b]	120	2.5	4.8×10^5
Dns A	A	R	A	P	L	V[c]	200	4.8	4.2×10^5
Dns L	K	Y	A	P	W	V[a]	67	2.8	2.4×10^5
Dns L	R	R	A	P	L	G[b]	130	5.8	2.2×10^5
Dns L	K	N	A	P	L	V[b]	180	10.0	1.8×10^5
Dns G	K	Y	A	P	W	V[a]	40	2.7	1.5×10^5
Dns G	K	R	A	P	W	V[b]	2.2	0.15	1.5×10^5
Dns G	K	R	A	P	L	V[b]	2.3	0.17	1.3×10^5
Dns G	K	N	A	P	L	V[c]	56	5.2	1.1×10^5
Dns G	K	N	A	P	L	V[b]	39	4.0	9.6×10^4
Dns G	R	R	A	P	L	G[c]	34	3.9	8.7×10^4
Dns G	P	R	A	P	L	V[b]	20	5.3	3.8×10^4

[a] Analyzed by tryptophan fluorescence changes between substrate and product. [b] HPLC analysis. [c] Analyzed by tryptophan fluorescence changes between enzyme–substrate complex and enzyme plus products.

occur on opposite sides of the cleavage point. In the intact peptide, the indole fluorescence is quenched by the dansyl group, and, thus, cleavage can be monitored continuously by observing the increase of fluorescence during conversion of substrate to product (Ng & Auld, 1989).

The substrates were designed so that *Astacus* protease should cleave at one site only. Only one dansylated product is released in all cases as verified by both TLC and HPLC, indicating that only one peptide bond is hydrolyzed. Amino acid analyses of the reaction products, separated by HPLC, demonstrate that cleavage occurs only on the N-terminal side of the central alanyl residue.

Figure 1 shows the fluorescence change that occurs in the hydrolysis of Dns-VKRAPWV (3.25×10^{-4} M) catalyzed by *Astacus* protease (1.0×10^{-7} M) under stopped-flow fluorescence conditions. The initial rate is calculated from the linear part of the trace which occurs in the first 5 s of the reaction. The reaction is complete in the next 265 s. The double-reciprocal plot for triplicate assays at several substrate concentrations yields kinetic parameters of k_{cat}, 194 s^{-1}, and K_m, 2.5×10^{-4} M (Figure 2).

The heptapeptides designed on the basis of the tubulin cleavage patterns are excellent substrates with k_{cat}/K_m values of 3.8×10^4 to 1×10^6 M^{-1} s^{-1} (Table III). They all follow Michaelis–Menten kinetics with no sign of substrate inhibition up to substrate concentrations of about 10-fold higher than their K_m values.

The influence of the length of the peptide on the rate of hydrolysis catalyzed by *Astacus* protease was studied with the

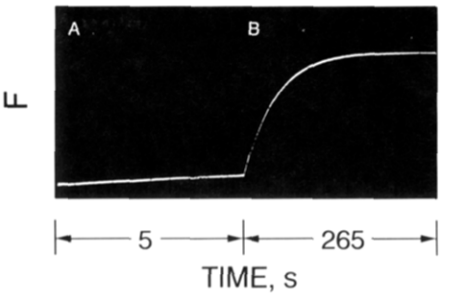

FIGURE 1: Kinetic analysis of a tryptophan-containing dansyl substrate by stopped-flow fluorescence. Assay conditions: 20 mM Hepes, pH 7.8, 25 °C. The substrate to product conversion of the peptide Dns-VKRAPWV (3.25×10^{-4} M) by *Astacus* protease (1.0×10^{-7} M) is monitored by the increase of the tryptophan fluorescence, through a 360-nm band-pass filter after excitation at 285 nm. The first 5 s of the reaction (A) represents the linear part of the trace from which the initial rate is calculated on the basis of the overall fluorescence yield after the completion of the reaction (B).

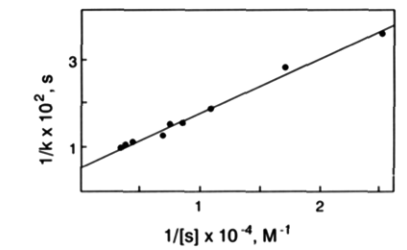

FIGURE 2: Lineweaver–Burk plot for the hydrolysis of Dns-VKRAPWV. Rate constants were calculated from the initial rates, as shown in Figure 1.

nonapeptide Dns-HHLKRAPWV and with the series Dns-LKRAPLV, Dns-LKRAPL, Dns-KRAPL, and Dns-RAPL. The highest k_{cat}/K_m value is obtained for the nonapeptide, 1.4×10^6 M^{-1} s^{-1}. Decreasing the length causes a continuous drop in activity to a minimum k_{cat}/K_m value of 0.6 M^{-1} s^{-1} for the dansyl tetrapeptide Dns-RAPL (Table IV).

Of the single amino acid replacements designed to test the specificity of *Astacus* protease, the substitution of leucine for alanine at the scissile bond has the most dramatic effect. While bovine trypsin readily catalyzes the hydrolysis of both Dns-LKRAPWV and Dns-LKRLPWV, *Astacus* protease does not (Figure 3). Bovine trypsin cleaves the former substrate at both the Lys–Arg and the Arg–Ala bond, favoring the Lys–Arg bond by a ratio of 3:1 (Figure 3a). *Astacus* protease cleaves the Arg–Ala bond at a rate of about 10 times faster than that for bovine trypsin (Figure 3b). Bovine trypsin also cleaves both the Lys–Arg and Arg–Leu bonds in the leucine-substituted heptapeptide, favoring the Lys–Arg bond by a ratio of 8:1 (Figure 3c). On the other hand, *Astacus* protease shows only a slight degree of hydrolysis even at 1000 times more enzyme and a 144 times greater period of incubation

Table IV: Kinetic Constants for the Hydrolysis of Dansyl Peptides of Variable Length by *Astacus* Protease[a]

substrate										k_{cat} (s^{-1})	K_m ($\times 10^{-4}$ M)	k_{cat}/K_m (M^{-1} s^{-1})
P_6	P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'			
Dns	H	H	L	K	R	A	P	W	V[a]	310.0	2.3	1.4×10^6
		Dns	L	K	R	A	P	L	V[b]	120.0	2.5	4.8×10^5
		Dns	L	K	R	A	P	L[b]		43.0	8.7	4.9×10^4
			Dns	K	R	A	P	L[b]		40.0	20.0	2.0×10^4
				Dns	R	A	P	L[b]		0.035	560.0	0.6
		Dns	L	R	R	A	S	L	G[b]	2.7	6.7	4.0×10^3
		Dns	G	R	R	A	S	L	G[b]	0.7	2.3	3.0×10^3
		Dns	G	G	R	A	P	W	V[b]	20.0	50.0	3.9×10^3
		Dns	L	K	R	L	P	W	V[b]			$\sim 1.8 \times 10^1$[c]

[a] Analyzed by the change in fluorescence between substrate and product. [b] HPLC analysis. [c] Calculated from the first-order rate of appearance of product divided by enzyme concentration.

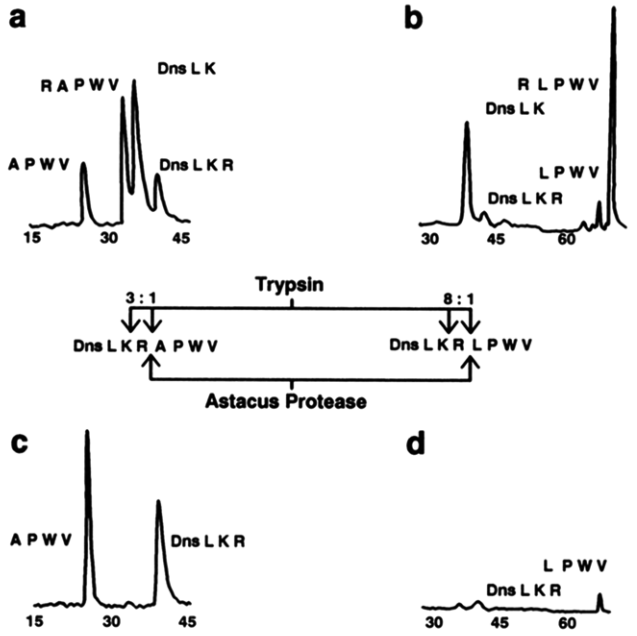

FIGURE 3: HPLC separation of reaction products of the hydrolysis of Dns-LKRAPWV and Dns-LKRLPWV by bovine trypsin and *Astacus* protease. Reactions were carried out in 100-μL assay volume in 20 mM Hepes, pH 7.8, at 25 °C. A linear gradient of 20–60% acetonitrile in 0.1% TFA was used, at a flow rate of 1.5 mL/min on a Waters Nova Pak C_{18} column. The substrate concentration was 1×10^{-4} M. (a) Hydrolysis of Dns-LKRAPWV and (b) Dns-LKRLPWV by bovine trypsin (2×10^{-8} and 3.8×10^{-8} M, respectively) after 30- and 10-min incubation, respectively. In both peptides, trypsin cleaves the Lys–Arg bond and either the Arg–Ala or the Arg–Leu bond. (c) Dns-LKRAPWV is cleaved by *Astacus* protease (5×10^{-9} M, 10-min incubation) only at the Arg–Ala bond. (d) Dns-LKRLPWV after 24-h incubation with *Astacus* protease at 5×10^{-6} M enzyme after prior incubation with benzamidine–Sepharose.

(Figure 3d). The apparent rate of hydrolysis is so low that it requires removal of trace amounts of *Astacus* trypsin from the enzyme sample. Thus, a 200-μL solution of *Astacus* protease, 1×10^{-5} M, treated with 20 μL of benzamidine–Sepharose, yields a turnover number of 9×10^{-5} s^{-1} based on the amount of Dns-LKR present after a 24-h incubation with substrate (Figure 3d).

The non-tryptophan-containing peptides can be used to directly examine the formation and breakdown of ES complexes by measurement of the RET between enzyme tryptophans and the substrate dansyl group. Figure 4 shows the binding of Dns-GKRAPLV to *Astacus* protease and its subsequent hydrolysis. The rapid decrease in protein tryptophan fluorescence to reach a minimum within the mixing time of the instrument indicates rapid equilibration of the ES complex(es) at 25 °C (Figure 4A). This is followed by an increase in fluorescence as the enzyme–substrate complex is converted to products (Figure 4B). Kinetic parameters, $k_{cat} = 6.53$ s^{-1} and $K_m = 6.0 \times 10^{-5}$ M, are calculated from the steady-state region (Figure 4B). These values are only apparent constants because the fluorescent product Dns-GKR binds to the enzyme weakly (see below) and thus interferes with the analysis (Lobb & Auld, 1984).

Four different products, Dns-LKR, Dns-GKR, Dns-AAR, and APLV, were tested for inhibition of *Astacus* protease. Data were obtained from initial rate measurements using the substrate Dns-LKRAPWV at a concentration of 1.1×10^{-5} M ($S/K_m = 0.03$) and an enzyme concentration of 9.7×10^{-8} M. K_i values were calculated from Dixon plots. Dns-AAR and APLV do not inhibit the enzyme up to concentrations of 4×10^{-4} M, whereas Dns-LKR and Dns-GKR inhibit with K_i values of 1.9×10^{-4} and 1.5×10^{-4} M, respectively. The

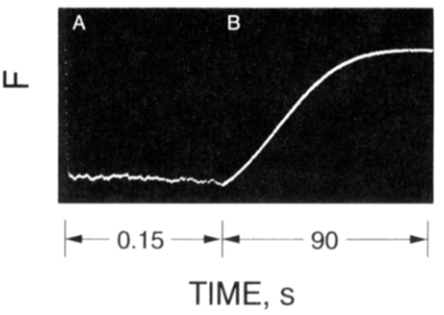

FIGURE 4: Stopped-flow fluorescence observation of the formation and the breakdown of the ES complex of *Astacus* protease, 1.0×10^{-6} M, and Dns-GKRAPLV, 1.1×10^{-4} M, in 20 mM Hepes, pH 7.89 at 25 °C. Excitation was at 285 nm, and the enzyme tryptophan emission was observed with a 360-nm band-pass filter. (A) Rapid equilibration of E and S to form the ES complex; (B) steady-state rate of conversion of the ES complex to enzyme plus products.

combination of Dns-LKR and APLV (both at 1×10^{-4} M) do not alter the inhibitory effect of Dns-LKR.

Discussion

The primary specificity of *Astacus* protease was revealed by the pattern of cleavage in the digestion products of α- and β-tubulin (Krauhs et al., 1982). Thus, *Astacus* protease exhibits a strong preference for small uncharged residues (most frequently alanine, threonine, serine, and glycine) in the P_1' position (Table II). However, only 32% of all the X–Ala bonds in the tubulins are cut, indicating the significant influence of secondary binding on the cleavage of a potentially susceptible peptide bond. In addition, small alanyl-containing substrates are turned over very slowly by this enzyme (Table I). For the synthesis of suitable substrates of *Astacus* protease, the analysis of the tubulin cleavage sites was extended beyond the P_1' position to reach from subsites P_4 to P_4'.

The tubulin cleavage pattern proved to be very helpful as a model for substrate design. Eighteen peptides synthesized according to this pattern are excellent substrates (Table III and IV). They are all cleaved at the predicted site and turned over by a factor of 10^3–10^5 times faster than the currently used nitroanilide substrates. The highest observed ratio for k_{cat}/K_m as a measure of specificity, 1.4×10^6 M^{-1} s^{-1}, is clearly in the range of the highest values known for proteases in general [e.g., cf. Morihara (1974), Morgan and Fruton (1978), Harper et al. (1984), and Steinbrink et al. (1985)].

The present study confirms previous evidence that *Astacus* protease has an extended substrate binding site (Zwilling & Neurath, 1981) as well as emphasizing the importance of certain residues to the reactivity. Thus, in the series Dns-LKRAPLV ($k_{cat}/K_m = 4.8 \times 10^5$ M^{-1} s^{-1}) through Dns-RAPL ($k_{cat}/K_m = 0.6$ M^{-1} s^{-1}), the reactivity decreases drastically (Table IV), although each of these substrates contains the same hydrolytic site, an Arg–Ala bond. Although the activity falls continuously with decreasing length of the peptide, a 10^4-fold decrease occurs between the penta- and tetrapeptides. In contrast, if the heptapeptide Dns-LKRAPWV and the nonapeptide Dns-HHLKRAPWV are compared, the elongation by two histidine residues increases k_{cat}/K_m 2-fold, which is due to the improvement of both k_{cat} (314 s^{-1}) and K_m (2.3×10^{-4} M). Hence, we conclude that an optimal substrate for *Astacus* protease should comprise seven or more amino acids and minimally likely requires at least five amino acids.

In general, the predictions of which amino acids will elevate or reduce catalytic activity based on the tubulin cleavage pattern (Table II) are reflected in the corresponding kinetic parameters for the synthetic substrates (Tables III and IV).

The specificity is dominated by a small side-chain-containing residue in position P_1', as reflected in the $\geq 3 \times 10^4$ decrease in activity when leucine replaces alanine (Figure 3, Table IV). Moreover, the present study further stresses the importance of secondary binding interactions to activity. Of particular importance is a proline residue in position P_2'. Upon substitution of this proline by a serine residue, the k_{cat} value drops 50-fold, whereas the value of K_m stays about the same. In terms of k_{cat}/K_m, the proline peptides Dns-LRRAPLG and Dns-GRRAPLG are 55 times and 29 times more active, respectively, than their serine counterparts Dns-LRRASLG and Dns-GRRASLG. It has been suggested that a proline in P_2' might reflect a preference of reverse turns by *Astacus* protease (Krauhs et al., 1982; Sonneborn et al., 1969). Thus, a tilted substrate conformation positively affects k_{cat}, which might be caused by additional strain on the scissile bond in the catalytic step or an enhanced release of product.

The exchange of a tryptophan for leucine in P_3' has only minor consequences on activity as would be expected (Table II). Dns-GKRAPLV and Dns-GKRAPWV have nearly identical k_{cat}/K_m values of 1.3×10^5 and 1.4×10^5 M^{-1} s^{-1}, respectively, and for the corresponding peptides, Dns-LKRAPLV and Dns-LKRAPWV, values of 4.8×10^5 and 6.5×10^5 M^{-1} s^{-1} are obtained (Table III). Thus, introduction of the tryptophan fluorophore does not decrease activity, but it does greatly enhance the assay sensitivity due to the 10-fold increase in the tryptophan fluorescence upon hydrolysis of any peptide bond between the dansyl and tryptophan chromophores (Figure 1). Such RET-based assays should have a widespread use in assaying neutral proteases, particularly those having a need of specificity requirements on both sides of the scissile bond (Ng & Auld, 1989; Stack & Gray, 1989).

Strong influence is contributed by protein subsites S_2 and S_1 on the activity toward peptide substrates. As predicted from the tubulin pattern, the most reactive substrates contained arginine, asparagine, and tyrosine in the P_1 subsites and lysine or arginine in the P_2 subsite. These substrate residues are either positively charged or capable of hydrogen bonding. As a striking example, substitution of lysine by glycine in P_2 causes a 330-fold increase in K_m (Tables III and IV). The importance of positively charged amino acids in P_2 and P_1 supports the proposal that the active-site region of *Astacus* protease may include an accumulation of glutamic acids that can interact with positive charges of the substrate (Stöcker et al., 1988).

The results obtained for the substitution of lysine for arginine in position P_1 are an apparent contradiction to the results of the tubulin cleavage pattern. Dns-LKKAPWV is cleaved as efficiently by *Astacus* protease as Dns-LKRAPWV, although in the tubulin pattern, arginine is preferred by a four to one ratio over lysine (Table II). The mismatch of the tubulin pattern and the specificity of the enzyme in this case might be due to the unequal distribution of the potentially most reactive cleavage sites, such as Arg–Ala or Lys–Ala bonds. There are, in fact, six Arg–Ala bonds, but only one Lys–Ala bond in α- and β-tubulin which should decrease the overall yield of Lys–X cleavage.

For the P_3 position, the k_{cat}/K_m values decrease in exactly the same fashion as predicted by the tubulin pattern (Pro > Val > Leu > Ala > Gly) (Tables II and III). The best substrate of this series, Dns-PKRAPWV, combines a proline residue in P_3 with another proline in P_2'. A proline-induced bent conformation might promote hydrolysis by exposure and/or strain imposed on the scissile bond.

The substrate binding site for *Astacus* protease seems to be remarkably large and specific for an enzyme that simply serves food digestion. Previous investigations failed to detect proteins homologous to *Astacus* protease. Therefore, the question remained open whether *Astacus* protease has homologues among enzymes from other sources such as intracellular proteases (Bond & Butler, 1987) or whether it is present in organisms other than the decapod Crustacea. We have shown recently that *Astacus* protease contains 1 mol of zinc as well as the amino acid sequence IIHELMHAI that is similar to part of the zinc binding site of the bacterial metalloproteinase thermolysin VAHELTHAV (Figure 5) (Stöcker et al., 1988). In the case of thermolysin, the two histidines are ligands to the active-site zinc, and the glutamic acid provides the general base group for catalysis. The third zinc ligand in thermolysin is another glutamic acid side chain 20 residues removed from the HExxH sequence, and the fourth ligand is a water molecule (Matthews et al., 1972).

	142			146							
Thermolysin	V	V	A	H	E	L	T	H	A	V	T	D	Y	T
Astacus Protease	T	I	I	H	E	L	M	H	A	I	G	F	Y	H
Human BMP 1 *	I	V	V	H	E	L	G	H	V	V	G	F	W	H
Serratia Protease *	T	F	T	H	E	I	G	H	A	L	G	L	S	H
Protease B *	S	F	T	H	E	I	G	H	A	L	G	L	S	H
Ht-d Proteinase	T	M	A	H	E	L	G	H	N	L	G	M	E	H
Human Collagenase *	V	A	A	H	E	L	G	H	S	L	G	L	S	H
Rabbit Collagenase *	V	A	A	H	E	L	G	H	S	L	G	L	S	H
Rat Collagenase *	V	A	A	H	E	L	G	H	S	L	G	L	F	H
Rat Transin *	V	A	A	H	E	L	G	H	S	L	G	L	F	H
Rat Transin 2 *	V	A	A	H	E	L	G	H	S	L	G	L	F	H
Human Stromelysin *	V	A	A	H	E	I	G	H	S	L	G	L	F	H
Human Stromelysin 2 *	V	A	A	H	E	L	G	H	S	L	G	L	F	H
Human Pump 1 *	A	A	T	H	E	L	G	H	S	L	G	M	G	H
Human Collag. IV (72 kDa) *	V	A	A	H	E	F	G	H	A	M	G	L	E	H
Human Collag. IV (92 kDa) *	V	A	A	H	E	F	G	H	A	L	G	L	D	H

FIGURE 5: Zinc proteases having the putative zinc binding site sequence HExxHxxGxxH. Amino acid sequence numbers apply only to the reference protein thermolysin. An asterisk denotes those for which zinc was not measured. References for the reported sequences are thermolysin (Titani et al., 1972), *Astacus* protease (Titani et al., 1987), BMP I (Wozney et al., 1988), *Serratia* protease (Nakahama et al., 1986), protease B (Delepelaire & Wandersman, 1989), Ht-d proteinase (Shannon et al., 1989), transin 2 (Breathnach et al., 1987), stromelysin and transin (Whitham et al., 1986), stromelysin 2 (Muller et al., 1988), pump 1 (Quantin et al., 1989), collagenase IV, 72 kDa (Collier et al., 1988), collagenase IV, 92 kDa (Wilhelm et al., 1989), and collagenases from human (Goldberg et al., 1986), rabbit (Fini et al., 1987), and rat (Matrisian et al., 1985).

Most recently, evidence for the occurrence of a protein with substantial homology to *Astacus* protease, exceeding the above-mentioned active-site region, has been provided through the elucidation of the cDNA sequence of human bone morphogenetic protein I (BMP I), a morphogen that induces bone formation (Wozney et al., 1988; Wang et al., 1988). Domain A of BMP I, comprising 200 amino acids, is 36% identical with *Astacus* protease. However, it is not yet known whether BMP I displays a proteolytic function during the process of bone formation. One indication for possible proteolytic activity is the sequence VVHELGHVV in BMP I, which includes the zinc binding motif HxxxH seen in thermolysin and is strikingly similar to the corresponding sequence found in *Astacus* protease (Figure 5).

It should also be noted that BMP I and *Astacus* protease contain Gly and His residues three and six amino acids removed from the putative second histidyl ligand based on analogy to thermolysin (Figure 5). Neither of these residues is conserved at the corresponding positions of the thermolysin family (Vallee & Auld, 1990a,b). A search of the protein sequences contained in National Biomedical Research Foundation and GenBank/Los Alamos files for the peptide sequence

HExxHxxGxxH yields only a small number of proteins which satisfy these requirements (Figure 5).[2] Most importantly, they are proteolytic enzymes and either have been shown to contain zinc by analytical means, as for the hemorrhagic toxin Ht-d from the venom of western diamondback rattlesnake (Shannon et al., 1989), or are considered to be zinc enzymes, due to their inhibition by chelating agents, as is the case for the collagenases and gelatinases. The majority of these proteases are known to function in the degradation of the extracellular matrix. Besides the potential zinc binding site, these proteases share with *Astacus* protease a preference for proteins containing proline residues one or two amino acids removed from the cleavage site.

Importantly, if compared to thermolysin, in none of these proteases is there a glutamate residue 20 amino acids removed from the second histidyl ligand to zinc. The function of the conserved Gly and His residues in the formation and stabilization of the proposed catalytic zinc binding site is unknown at present. However, minimally, they may signal a zinc site which is characteristic for these metalloproteinases but different from that of the thermolysin family. Recent studies of a rat protransin fused by its N-terminal propeptide to staphylococcal protein A have shown that transin cannot autoactivate itself if either the first or the third histidines are mutated to leucine or serine, respectively, implying that these residues are critical for proteolytic activity (Sanchez-Lopez et al., 1988).

The design of highly reactive fluorescent substrates for *Astacus* protease can now be used for further mapping of the substrate binding site, for the design of specific inhibitors, and for low-temperature studies of the catalytic mechanism. Moreover, such specific substrates and corresponding inhibitors may be utilized as probes to investigate the question of whether *Astacus* protease has homologues among proteins from other sources as well as for the detection of cryptic enzymatic activity as may be present in BMP I and other proteins of the HExxHxxGxxH type.

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Registry No. DnsPKRAPWV, 129364-28-9; DnsVKRAPWV, 129364-29-0; DnsLKKAPWV, 129364-30-3; DnsLKRAPWV, 129364-31-4; DnsLKRAPLV, 129364-32-5; DnsAARAPLV, 129390-20-1; DnsLKYAPWV, 129364-33-6; DnsLRRAPLG, 129364-34-7; DnsLKNAPLV, 129364-35-8; DnsGKYAPWV, 124479-70-5; DnsGKRAPWV, 129364-36-9; DnsGKRAPLV, 129364-37-0; DnsGKNAPLV, 129364-38-1; DnsGRRAPLG, 129364-39-2; DnsGPRAPLV, 129364-40-5; DnsHHLKRAPWV, 129364-41-6; DnsLKRAPL, 129364-42-7; DnsKRAPL, 129364-43-8; DnsRAPL, 129364-44-9; DnsLRRASLG, 73942-43-5; Protease, 9001-92-7; DnsGRRASLG, 129364-45-0; DnsGGRAPWV, 129364-46-1; DnsLKRLPWV, 129364-47-2.

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[2] The high molecular weight form of human kininogen (Lottspeich et al., 1985) contains the sequence GHGHEQQHGLGHGH. This has not been included in Figure 5 because this peptide is in a particularly histidine-rich region of the protein where there are several GH repeats. The preponderance of histidines in this sequence and the region around it could of course be sites for metal binding.

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Characterization of an Intracellular Hyaluronic Acid Binding Site in Isolated Rat Hepatocytes†

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ABSTRACT: ^{125}I-HA, prepared by chemical modification at the reducing sugar, specifically binds to rat hepatocytes in suspension or culture. Intact hepatocytes have relatively few surface ^{125}I-HA binding sites and show low specific binding. However, permeabilization of hepatocytes with the nonionic detergent digitonin results in increased specific ^{125}I-HA binding (45–65%) and a very large increase in the number of specific ^{125}I-HA binding sites. Scatchard analysis of equilibrium ^{125}I-HA binding to permeabilized hepatocytes in suspension at 4 °C indicates a $K_d = 1.8 \times 10^{-7}$ M and 1.3×10^6 molecules of HA ($M_r \sim 30\,000$) bound per cell at saturation. Hepatocytes in primary culture for 24 h show the same affinity but the total number of HA molecules bound per cell at saturation decreases to $\sim 6.2 \times 10^5$. Increasing the ionic strength above physiologic concentrations decreases ^{125}I-HA binding to permeable cells, whereas decreasing the ionic strength causes a ~4-fold increase. The divalent cation chelator EGTA does not prevent binding nor does it release ^{125}I-HA bound in the presence of 2 mM $CaCl_2$, although higher divalent cation concentrations stimulate ^{125}I-HA binding. Ten millimolar $CaCl_2$ or $MnCl_2$ increases HA binding 3–6-fold compared to EGTA-treated cells. Ten millimolar $MgCl_2$, $SrCl_2$, or $BaCl_2$ increased HA binding by 2-fold. The specific binding of ^{125}I-HA to digitonin-treated hepatocytes at 4 °C increased >10-fold at pH 5.0 as compared to pH 7. The kinetics of ^{125}I-HA binding to intact hepatocytes at 37 °C was rapid and similar to the kinetics of ^{125}I-HA binding at 4 °C ($t_{1/2} \sim 5$ min). Very little ^{125}I-HA was internalized after 4 h at 37 °C (460 molecules cell^{-1} h^{-1}). This rate is extremely slow (~1–3%) compared to the rate of receptor-mediated internalization of other ligands and indicates that HA uptake occurs by a noncoated pit pathway, probably reflecting general membrane pinocytosis. There is no evidence for recycling of the surface HA binding sites or use of the large intracellular reservoir for endocytosis.

Hyaluronic acid (HA)[1] is a ubiquitous component of the mammalian extracellular matrix (Fraser & Laurent, 1989). HA is an important mediator in many biological processes including cell adhesion (Underhill & Dorfman, 1978; Underhill, 1982), morphogenesis (Toole, 1981), wound healing (Toole, 1981; Weigel et al., 1986), tissue remodeling (Toole,

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[1] Abbreviations: HA, hyaluronic acid; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBSS, Hank's balanced salt solution; MES, 4-morpholineethanesulfonic acid.

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