and were dialyzed against the same buffer. Factor XIII was stored at 4°C (5–12 mg protein/ml); the purified *b* subunits at −10°C (3.5 mg/ml).

Though not shown in Fig. 2, it may be mentioned that the DEAE-cellulose chromatographic procedure described can be used simultaneously for the isolation of plasma fibronectin or cold-insoluble globulin.[14] This protein emerges with the NaCl gradient as a peak starting at about 5 $\times 10^{-3}$ mho of conductance.

Microheterogeneity of the *b* Subunit

Isoelectric focusing showed that the *b* subunits, as isolated from pooled human plasma, in spite of apparent homogeneity in regard to size (i.e., in spite of showing a single band in SDS–gel electrophoresis), comprised several species with different pI's (Fig. 6). Treatment with neuraminidase (8 hr, 37°C, 64 units of enzyme/nmol of b_2) removed sialic acid entirely (about 10 mol/mol of b_2) from the protein, shifting the pI's of the remaining species in a more alkaline direction. The observed residual microheterogeneity, showing 2 major and 2 minor bands, with the desialilated material may reflect group-specific differences in the population.

[14] J. Molnar, F. B. Gelder, M. Z. Lai, G. E. Siefring, R. B. Credo, and L. Lorand, *Biochemistry* **18**, 3909 (1979).

[28] Assay of Coagulation Proteases Using Peptide Chromogenic and Fluorogenic Substrates

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Introduction

Amino acid chromogenic and fluorogenic substrates have been used for many years for assaying proteases. The sensitivity of the assay procedures that employ these substrates and the convenience of spectrophotometric or fluorometric measurements has led to their widespread use. Most of the early amino acid chromogenic and fluorogenic substrates are highly selective for the primary specificity-determining (P1) amino acid; thus substrates such as benzoylarginine-*p*-nitroanilide, for assaying trypsin-like proteases, as well as aromatic amino acid *p*-nitrophenyl esters for chymotrypsin-like proteases, have been extensively investigated and

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employed for routine proteolytic enzyme assay. The recognition that both the selectivity of many proteases and their catalytic efficiency depend on interactions with subsite amino acids in the peptide substrate coupled with the availability of amino acid sequences around the cleavage sites in several zymogens of the coagulation and fibrinolytic systems has led to the synthesis and commercial availability of a variety of peptide chromogenic and fluorogenic substrates with much greater selectivity than the single amino acid chromogenic and fluorogenic substrates. Such increased selectivity is required because all of the proteases of these systems are trypsin-like in their primary specificity, and thus discrimination among them without exploitation of the selectivity that results from secondary binding-site interactions is virtually impossible.

A number of monographs have been published reporting procedures for specific protease assays using peptide substrates[1-3] and a comprehensive review of the literature up to 1980 on chromogenic and fluorogenic substrates for coagulation and fibrinolytic system proteases has been published.[4] A bibliography of published reports employing fluorogenic and chromogenic substrates is available from one of the manufacturers of peptide *p*-nitroanilide substrates.[5]

Kinetic constants that describe the hydrolysis of even the commercially available peptide chromogenic and fluorogenic substrates are relatively limited, owing in part to the fact that few of the proteolytic enzymes of the coagulation, fibrinolytic, and kinin systems are commercially available. The data presented in this report are derived from four sources: the limited literature and yet to be published work from three laboratories of the authors of this report. Several reports have listed relative rates of hydrolysis of peptide *p*-nitroanilide substrates by different coagulation proteases at a single substrate concentration, however such data are of very limited use in the design of assay procedures and thus have not been included in this review.

Methods

The peptide *p*-nitroanilide substrates used for these investigations are commercially available and were generously provided by AB Kabi Pep-

[1] M. F. Scully and V. V. Kakkar, eds., "Chromogenic Peptide Substrates: Chemistry and Clinical Usage." Churchill-Livingstone, Edinburgh and London, 1979.
[2] I. Witt, ed., "New Methods for the Analysis of Coagulation Using Chromogenic Substrates." de Gruyter, Berlin, 1977.
[3] H. R. Lijnen, D. Collen, and M. Verstraete, eds., "Synthetic Substrates in Clinical Blood Coagulation Assays." Nijhoff, The Hague, 1980.
[4] R. M. Huseby and R. E. Smith, *Semin. Thromb. Hemostasis* **6**, 175 (1980).
[5] Reference List for Enzyme Substrates, Kabi Diagnostica, Stockholm, Sweden.

tide Research (S-431 22 Molndal, Sweden), Kabi Group Inc. (One Lafayette Place, Greenwich, Connecticut), Boehringer-Mannheim, Gmbh (Postfach 120, D-8132 Tutzing, Federal Republic of Germany), Boehringer Mannheim Biochemicals (7941 Castleway Drive, Indianapolis, Indiana), and Pentapharm Ltd. (CH-4002, Basel, Switzerland). Sources of the substrates used in studies from the literature are found in the original publications.

All peptide *p*-nitroanilides were dissolved in deionized water that had been adjusted to pH 4 with hydrochloric acid. Substrates were stored frozen in this solution and were stable for periods longer than 1 year under these conditions. Substrate concentrations were determined from absorbance at the isosbestic wavelength for the peptide *p*-nitroanilide–*p*-nitroaniline mixtures. Extinction coefficients of 8270 liters/mol/cm in water and 8270 liters/mol/cm in solutions containing 0.01 *M* HEPES–0.01 *M* Tris–0.1 *M* NaCl were employed. Such a procedure was necessary as some peptide *p*-nitroanilide substrates are hygroscopic and thus difficult to weigh accurately.

Protease concentrations were determined by active-site titration using the procedure of Chase and Shaw.[6] Preparations of thrombin were at least 95% active by active-site titration using an extinction coefficient of 1.95 ml/mg/cm at 280 nm to determine protein concentration. Preparations of factor X_a were greater than 80% active by active-site titration using an extinction coefficient of 0.95 ml/mg/cm at 280 nm to estimate the protein concentration. All protease solutions were diluted into a buffer that contained 0.1% polyethylene glycol 6000 to prevent enzyme loss due to adsorption.

The rate of peptide *p*-nitroanilide hydrolysis was determined from the change in absorbance at 405 nm using an extinction coefficient for *p*-nitroaniline of 9920 liters/mol/cm for this reaction buffer. Data obtained by two authors (R.L. and C.M.J.) are all from solutions consisting of 0.01 *M* HEPES–0.01 *M* Tris-HCl (pH 7.8)–0.1 *M* NaCl–0.1% polyethylene glycol 6000. Polyethylene glycol concentrations less than 2% were without effect on the kinetic parameters being determined; at concentrations greater than 2%, K_m increased essentially linearly with polyethylene glycol concentration. Data provided by another author (U.C.) were determined in a buffer consisting of 0.05 *M* Tris-HCl–0.1 *M* NaCl, pH 8.0. All data are for a temperature of 25°C unless otherwise indicated. Measurements were made using a Cary 219 spectrophotometer at a spectral slit width of 2 nm or less. Data were transferred directly to a digital computer (DEC PDP 11/34a) and initial velocities were estimated using either the direct linear plot-based procedure of Cornish-Bowden[7] or by nonlinear least-squares

[6] T. Chase, Jr. and E. Shaw, *Biochemistry* **8**, 2212 (1969).
[7] A. Cornish-Bowden, *Biochem. J.* **149**, 305 (1975).

fitting to a second-order polynomial. In all but the very lowest substrate concentration mixtures for substrates with Michaelis constants less than 5 μM, less than 2% of the substrate was hydrolyzed in the reactions. Data provided by U.C. were obtained under the same conditions; however, a Beckman Model 25 spectrophotometer was employed and initial velocities were estimated from the slope of a tangent drawn to the reaction-progress curve at zero time. The substrate-concentration range investigated in the laboratories of C.M.J. and U.C. varied from 0.1 to 0.3 $\times$ K_m to at least 5, and as high as 100 $\times$ K_m, depending on the actual value for the Michaelis constant and the solubility of the particular substrate. Data provided by P.L.C. were obtained using a Gemsaec centrifugal analyzer. Initial velocities were calculated from the initial linear portion of the progress curve. Substrate concentrations ranged from $0.2K_m$ to $5K_m$. All reactions were at 37°C. Data from the literature are as described in the specific reports.

Kinetic parameters were determined by unweighted nonlinear least-squares fitting of the simple Michaelis–Menten equation to the data (R.L., C.M.J.), as described by Wilkinson,[8] or a weighted linear regression to the Lineweaver–Burke transform of the Michaelis–Menten equation (U.C. and P.L.C.) as described by Cleland.[9] Substrates for which no independent values for k_{cat} and K_m are given in the tables, but which do have an estimate of k_{cat}/K_m, gave linear dependence of the velocity on substrate concentration. The estimate for k_{cat}/K_m was determined by an unweighted linear regression fit to these data. Data for all substrates were obtained for no fewer than 5 different substrate concentrations, and in some cases, as many as 20 substrate concentrations were employed. In all cases, the Michaelis–Menten equation was assumed to fit the data, although evidence for substrate activation with some of the substrates and thrombin has been observed. Values for the relative standard errors in the kinetic parameter estimates from a single experiment were approximately $\pm 10\%$ for K_m and $\pm 5\%$ for k_{cat}. When several substrate-concentration dependence data sets for thrombin and factor X_a were examined, the standard errors of the mean were approximately $\pm 12\%$ for both parameters. On the basis of these estimates for the "true" error, all parameters are quoted to two significant figures in the tables.

Data for Individual Proteases

Thrombin. Data obtained from the investigation of the hydrolysis of 24 peptide *p*-nitroanilides, one tosyl-arginine-nitrobenzyl ester,[10] and one

[8] G. N. Wilkinson, *Biochem. J.* **80**, 324 (1961).
[9] W. W. Cleland, *Adv. Enzymol.* **29**, 1 (1967).
[10] G. W. E. Plaut, *Haemostasis* **7**, 105 (1978).

thiobenzyl ester[11] by thrombin are given in Table I. Agreement between the kinetic parameters for the hydrolysis of benzoyl-L-arginine-*p*-nitroanilide determined by Takasaki *et al.*[12] and those reported here is very good. Limited data for thrombin for which the active enzyme concentration has been determined precludes more extensive comparisons. In general the values for both K_m and k_{cat} for bovine and human thrombins are very similar, but not identical. Until more extensive intralaboratory comparisons are available, it is impossible to determine whether these differences reflect significant differences between the enzymes from the two species or represent interlaboratory differences.

Effects of pH and solution composition on the hydrolysis of two peptide *p*-nitroanilides, Tos-Gly-L-Pro-L-Arg-*p*NA and H-D-Phe-L-Pip-L-Arg-*p*NA and Cbz-L-Lys-SBzl are given in Table II. The magnitude of the effects from altering buffer composition at constant pH indicate clearly the necessity for control of solution composition and the necessity for knowing solution composition when fundamental information about the properties of the particular enzyme are desired from the kinetic data. Dependence on pH is in general relatively small, between pH 7.8 and 8.5 (R. Lottenberg and C. M. Jackson, unpublished observations). Specific monovalent cation effects on the activity of thrombin have been reported and must be considered in interpreting differences in ionic strength and solution composition.[13]

Factor X_a. Kinetic parameters for the hydrolysis of 25 peptide *p*-nitroanilide substrates by bovine and human factor X_a are given in Table III. Interestingly, the Michaelis constants for all of the substrates investigated for factor X_a lie in the range of 0.1 m*M*, whereas for thrombin, values as low as 0.4 μM have been observed. Solution composition is as described above for the data provided by R. L. and C.M.J. Specific monovalent cation effects on the activity of factor X_a have been reported.[14]

Plasmin and Urokinase. Kinetic data for the hydrolysis of 16 peptide *p*-nitroanilide substrates, 1 thiobenzyl ester,[11] and 1 nitrobenzyl ester[10] by plasmin and urokinase are given in Table IV. Data obtained by U.C. were as described above for thrombin.

Plasminogen–Streptokinase Complex. Data obtained for the hydrolysis of H-D-Phe-L-Leu-L-Lys-*p*NA and Tos-Gly-L-Pro-L-Lys-*p*NA by the plasminogen–streptokinase complex are given in Table V. Data for six different forms of plasminogen that differ in the extent to which portions of

[11] G. D. J. Green and E. Shaw, *Anal. Biochem.* **93**, 223 (1979).
[12] S. Takasaki, K. Kasai, and S. Ishii, *J. Biochem. (Tokyo)* **78**, 1275 (1975).
[13] E. F. Workman and R. L. Lundblad, *Arch. Biochem. Biophys.* **185**, 544 (1978).
[14] C. L. Orthner and D. P. Kosow, *Arch. Biochem. Biophys.* **185**, 400 (1978).

TABLE I
THROMBIN KINETIC PARAMETER SUMMARY

Substrate	Bovine thrombin				Human thrombin			
	K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]	Ref.	K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]	Ref.
1. H-D-Phe-Aze-L-Arg-*p*NA (S-2388)	0.43	48	110	*b*				
2. H-D-Ile-L-Pro-L-Arg-*p*Na (S-2288)	1.1	74	62	*b*				
					3[c]	120[c]	40[c]	*d*
3. H-D-Phe-L-Pip-L-Arg-*p*NA (S-2238)	1.6	95	59	*b*	1.5	90	60	*b*
					4.4	100	23	*e*
	2.7[f]	200	71	*b*	13[f]	180[f]	14[f]	*e*
	9.0[g]	—	—	*d*	7[g]	150[g]	21[g]	*d*
4. H-D-Val-L-Pro-L-Arg-*p*NA (S-2234)	2.0	89	44	*b*				
5. Tos-Gly-L-Pro-L-Arg-*p*NA (Chromozym-TH)	4.0	100	25	*b*	4.2	130	31	*b*
					8.5	130	15	*e*
	5.7[f]	200[f]	36[f]	*b*	13[f]	220[f]	17[f]	*e*
					12[i]	—	—	*h*
6. PyrGlu-L-Pro-L-Arg-*p*NA (S-2366)	39	150	4.1	*b*				
7. Bz-L-Phe-L-Val-L-Arg-*p*NA (S-2160)	18	38	2.1	*b*	83	30	0.36	*e*
					68[f]	45[f]	6.6[f]	*e*
	80[g]	0.068[j]		*d*	110[g]	50[g]	0.45[g]	*d*
8. Tos-Gly-L-Pro-L-Lys-*p*NA (Chromozym-PL)	21	23	1.1	*b*	51	34	0.67	*e*
					60[f]	52[f]	0.87[f]	*e*
25. Cbz-Lys-S-Bzl	40[l]	35[l]	0.88[l]	*k*				
9. Bz-L-Phe-L-Val-L-Arg-*p*NA (Peptide Research Foundation)	72	38	0.53	*b*				
10. Bz-PyroGlu-Gly-L-Arg-*p*NA (S-2405)	66	28	0.44	*b*				
11. H-D-Val-L-Phe-L-Arg-*p*NA (S-2325)	130	22	0.17	*b*				

12. H-D-Pro-L-Phe-L-Arg-*p*NA (S-2302)	130	13	0.10	*b*				
13. Bz-L-Ile-L-Glu-Gly-L-Arg-*p*NA (S-2337) (Pip)	41	2.6	0.065	*b*				
14. H-D-Val-L-Leu-L-Arg-*p*NA (S-2266)	350	19	0.054	*b*	180	9.0	0.052	*e*
15. Cbz-L-Val-Gly-L-Arg-*p*NA	73	3.2	0.045	*b*	150	4.6	0.032	*e*
(Chromozym-TRY)	180[f]	9.0[f]	0.049	*b*	350[f]	8.7[f]	0.025[f]	*e*
16. Bz-L-Ile-L-Glu-Gly-L-Arg-*p*NA (S-2222)	57	0.94	0.017	*b*				
17. L-PyroGlu-Gly-L-Arg-*p*NA			0.0044	*b*	1100	3.1	0.0027	*e*
(S-2444)					1800[f]	6.6[f]	0.0036[f]	*e*
18. H-D-Val-L-Phe-L-Lys-*p*NA (S-2390)			0.0018	*b*				
19. H-D-Val-L-Leu-L-Lys-*p*NA			0.00080	*b*	1400	1.2	0.00086	*e*
(S-2251)					2500[n]	0.67[n]	0.00027[n]	*m*
20. PyrGlu-L-Phe-L-Lys-*p*NA (S-2403)			0.000033	*b*				
21. Bz-L-Arg-*p*NA	120[o]	0.071[o]	0.00063[o]	*b*				
	200[q]	0.12[q]	0.00058[q]	*p*				
22. Bz-L-Pro-L-Phe-L-Arg-*p*NA					490	0.11	0.00022	*e*
(Chromozym PK)					370[f]	0.12[f]	0.00033[f]	*e*
26. PyrGlu-L-Phe-L-Lys-*p*NA					1100[n]	0.07[n]	0.000064[n]	*m*
27. H-L-Ala-L-Phe-L-Lys-*p*NA					1000[n]	0.015[n]	0.000015[n]	*m*
28. Tos-L-Arg-*p*-NitroBzl	14[s]	0.037[j]		*r*				

[a] K_m (μM); k_{cat} (sec^{-1}); k_{cat}/K_m (liters/μmol/sec).
[b] R. Lottenberg and C. M. Jackson (unpublished data).
[c] 37°C, Tris, I = 0.15, pH 8.4.
[d] AB Kabi, manufacturer's literature.
[e] U. Christensen (unpublished data).
[f] 37°C.
[g] 37°C, Tris, I = 0.15, pH 8.3.
[h] Boehringer Mannheim, Gmbh, manufacturer's literature.
[i] 37°C, I = 0.3, pH 8.4.
[j] nM/min/NIH unit.
[k] G. D. J. Green and E. Shaw, *Anal. Biochem.* **93**, 223 (1979).
[l] 0.1 M Tris–0.1 M NaCl, pH 8.0.
[m] D. Collen, H. R. Lijnen, F. De Cock, J. P. Durieux, and A. Loffet, *Biochim. Biophy. Acta* **165**, 158 (1980).
[n] 0.1 M phosphate, pH 7.3.
[o] pH 8.0.
[p] S. Takasaki, K. Kasai, and S. Ishii, *J. Biochem. (Tokyo)* **78**, 1275 (1975).
[q] 0.05 M Tris-HCl, pH 8.2.
[r] G. W. E. Plaut, *Haemostasis* **7**, 105 (1978).
[s] 30°C, 0.1 M Tris-HCl, pH 8.4.

TABLE II
EFFECTS OF SOLUTION COMPOSITION AND pH ON THROMBIN ACTIVITY

Reaction conditions		K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]
Human THROMBIN–Tos-Gly-L-Pro-L-Arg-*p*NA (Chromozym-TH)				
	pH			
0.10 *M* Tris-HCl, 0.0 *M* NaCl	7.95	50	46	0.92
	8.36	43	55	1.3
	8.58	44	50	1.1
	8.76	47	34	0.72
	9.00	54	63	1.2
0.05 *M* Tris-HCl	pH			
0.0 *M* NaCl	8.58	23	32	1.4
0.375 *M* NaCl		11	43	3.9
0.75 *M* NaCl		16	52	3.3
	pH			
0.10 *M* Na Phosphate, 0.0 *M* NaCl	6.50	27	38	1.4
	7.00	14	47	3.4
	7.40	8	42	5.3
	7.74	8	49	6.1
	8.00	8	50	6.3
		8.5	72	8.5
	8.50	9	50	5.6
	pH			
0.05 *M* Na Phosphate, 0.0 *M* NaCl	8.00	6.0	52	8.7
0.15 *M* Na Phosphate		7.5	70	9.3
0.50 *M* Na Phosphate		5.0	69	13.8
	pH			
0.10 *M* PIPES	8.00	9	62	6.9
0.10 *M* Pyrophosphate	8.00	6	42	7.0
0.10 *M* Glycine	8.70	14	54	3.9
0.10 *M* Tricine	8.00	8	94	12.
0.10 *M* Triethanolamine	8.15	16	105	6.6
0.10 *M* MOPS	8.00	12	108	9.0
0.10 *M* HEPES	8.00	11	93	8.5
0.10 *M* TES	8.00	9	102	11.3
Human THROMBIN–D-Phe-L-Pip-L-Arg-*p*NA (S-2238)				
	pH			
0.10 *M* Tris-HCl, 0.0 *M* NaCl	7.95	18.5	23.3	1.3
	8.36	26.5	38.5	1.5
		26.	38.0	1.5

TABLE II (*continued*)

Reaction conditions		K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]
0.10 *M* Tris-HCl, 0.0 *M* NaCl	8.76	30.5	28.3	0.93
	9.00	41.5	33.6	0.81
	pH			
0.05 *M* Tris-HCl, 0.0 *M* NaCl	8.36	15	38.3	2.6
0.25 *M* Tris-HCl		27	43.5	1.6
0.50 *M* Tris-HCl		36	38.1	1.1
	pH			
0.10 *M* Na Phosphate, 0.0 *M* NaCl	6.50	4.5	35.0	7.8
	7.00	4.0	45.0	11
	7.40	4.0	50.8	13
	7.70	4.0	65.0	16
	8.00	4.0	63.3	16
	8.50	6.5	70.0	11
	pH			
0.01 *M* PIPES, 0.0 *M* NaCl	8.00	3.7	34.2	9.2
0.025 *M* PIPES		4.5	38.0	8.4
0.05 *M* PIPES		4.0	36.7	9.2
0.10 *M* PIPES		3.7	37.9	10
0.25 *M* PIPES		3.5	34.1	9.7
0.50 *M* PIPES		4.0	30.8	7.7
Human THROMBIN–Cbz-L-Lys-SBz				
	pH			
0.10 *M* Tris-HCl, 0.0 *M* NaCl	7.95	84	81	0.96
	8.15	75	90	1.2
	8.36	79	82	1.0
	8.76	78	100	1.3
	9.00	84	96	1.1
	pH			
0.10 *M* Na Phosphate, 0.0 *M* NaCl	6.50	54	38	0.70
	7.00	45	66	1.5
	7.40	34	85	2.5
	7.70	30	98	3.3
	8.00	28	107	3.8
	8.50	38	125	3.3
	pH			
0.05 *M* Na Phosphate, 0.0 *M* NaCl	8.50	42	108	2.6
0.375 *M* NaCl		24	109	4.5
0.75 *M* NaCl		29	110	3.8

[a] K_m (μM); k_{cat} (sec^{-1}); k_{cat}/K_m (liters/μmol/sec); reaction temperature 37°.

TABLE III
FACTOR X_a KINETIC PARAMETER SUMMARY

Substrate	Bovine factor X_a				Human factor X_a			
	K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]	Ref.	K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]	Ref.
10. Bz-PyroGlu-Gly-L-Arg-*p*NA (S-2405)	150	260	1.7	*b*				
13. Bz-L-Ile-L-Glu-Gly-L-Arg-*p*NA (S-2337) (Pip)	83	140	1.7	*b*	83	140	1.7	*b*
	120[c]	210[c]	1.8[c]	*b*				
16. Bz-L-Ile-L-Glu-Gly-L-Arg-*p*NA (S-2222)	140	130	0.89	*b*				
	290	130	0.45	*b*				
	300[e]	100[e]	0.33[e]	*d*				
	440[g]	110[g]	0.25[g]	*f*				
5. Tos-Gly-L-Pro-L-Arg-*p*NA (Chromozym-TH)	110	74	0.68	*b*	99	100	1.0	*b*
	220[c]	150[c]	0.67[c]	*b*				
15. Cbz-L-Val-Gly-L-Arg-*p*NA (Chromozym-TRY)	360	60	0.17	*b*				
2. H-D-Ile-L-Pro-L-Arg-*p*NA (S-2288)	1300	170	0.13	*b*				
	2000[h]	110[h]	0.055[h]	*d*				
12. H-D-Pro-L-Phe-L-Arg-*p*NA (S-2302)	700	68	0.097	*b*				
4. H-D-Val-L-Pro-L-Arg-*p*NA (S-2234)			0.070	*b*				
23. Bz-L-Val-Gly-L-Arg-*p*NA (Sigma B-4758)	620	38	0.061	*b*				
11. H-D-Val-L-Phe-L-Arg-*p*NA (S-2325)	690	27	0.039	*b*				
17. L-PyroGlu-Gly-L-Arg-*p*NA (S-2444)			0.032	*b*				
3. H-D-Phe-L-Pip-L-Arg-*p*NA (S-2238)	35	0.78	0.022	*b*				

14. H-D-Val-L-Leu-L-Arg-*p*NA (S-2266)	1000	22	0.022	*b*
6. PyrGlu-L-Pro-L-Arg-*p*NA (S-2366)	1700	24	0.014	*b*
8. Tos-Gly-L-Pro-L-Lys-*p*NA (Chromozym-PL)			0.011	*b*
1. H-D-Phe-Aze-L-Arg-*p*NA (S-2388)	210	2.3	0.011	*b*
18. H-D-Val-L-Phe-L-Lys-*p*NA (S-2390)			0.0060	*b*
22. Bz-L-Pro-L-Phe-L-Arg-*p*NA (Chromozym-PK)	190	1.1	0.0057	*b*
7. Bz-L-Phe-L-Val-L-Arg-*p*NA	33	0.11	0.0033	*b*
(S-2160)	9[g]	0.27[g]	0.03[g]	*f*
9. Bz-L-Phe-L-Val-L-Arg-*p*NA (Protein Res. Found.)	30	0.071	0.0024	*b*
20. PyrGlu-L-Phe-L-Lys-*p*NA (S-2403)			0.0010	*b*
26. PyrGlu-L-Phe-L-Lys-*p*NA	10000[j]	0.72[j]	0.000072[j]	*i*
21. Bz-L-Arg-*p*NA			0.000015	*b*
27. L-Ala-L-Phe-L-Lys-*p*NA	13000[j]	0.14[j]	0.000011[j]	*i*
19. H-D-Val-L-Leu-L-Lys-*p*NA			0.00007	*b*
(S-2251)	1800[j]	0.0064[j]	0.0000036[j]	*i*

[a] K_m (μM); k_{cat} (sec $^{-1}$); k_{cat}/K_m (liters/μmol/sec).
[b] R. Lottenberg and C. M. Jackson (unpublished data).
[c] 37°C.
[d] AB Kabi, manufacturer's literature.
[e] Tris, $I = 0.25$, pH 8.3.
[f] M. J. Lindhout, B. H. M. Kop-Klaassen, and H. C. Hemker, *Biochim. Biophys. Acta* **533**, 342 (1978).
[g] Tris-imidazole, $I = 0.15$, pH 8.2.
[h] 37°C Tris, $I = 0.15$, pH 8.4.
[i] D. Collen, H. R. Lijnen, F. De Cock, J. P. Durieux, and A. Loffet, *Biochim. Biophy. Acta* **165**, 158 (1980).
[j] 0.1 *M* phosphate, pH 7.3.

TABLE IV

PLASMIN AND UROKINASE KINETIC PARAMETER SUMMARY

Substrate	Human plasmin				Urokinase			
	K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]	Ref.	K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]	Ref.
25. Cbz-Lys-SBzl					30[c]	60[c]	2.0[c]	*b*
	24[ee]	50[ee]	2.1[ee]	*d*	50[e]	49[e]	0.98[e]	*d*
26. PyrGlu-L-Phe-L-Lys-*p*NA	29	24	0.83	*f*	1000	0.51	0.00051	*f*
8. Tos-Gly-L-Pro-L-Lys-*p*NA	150	34	0.23	*f*	1800	36	0.020	*f*
(Chromozym-PL)	230[g]	45[g]	0.19[g]	*f*	1600[g]	44[g]	0.027[g]	*f*
	280[i]	76[i]	0.27[i]	*h*				
	290[k]	—	—	*j*				
	280[m]	—	—	*l*				
12. H-D-Pro-L-Phe-L-Arg-*p*NA	140	15	0.10	*f*	2100	0.6	0.00028	*f*
(S-2302)	240[g]	22[g]	0.093[g]	*f*				
5. Tos-Gly-L-Pro-L-Arg-*p*NA	270	27	0.098	*f*	50	5.3	0.11	*f*
(Chromozym-TH)	190	25	0.13	*n*				
	530[g]	45[g]	0.084[g]	*f*	80[g]	9.1[g]	0.12[g]	*f*
27. L-Ala-L-Phe-L-Lys-*p*NA	170[p]	15[p]	0.088[p]	*o*	450	0.028	0.000062	*o*
19. H-D-Val-L-Leu-L-Lys-*p*NA	120	14	0.11	*f*				
(S-2251)	240[p]	12[p]	0.050[p]	*o*	8300[p]	0.63[p]	0.000076[p]	*o*
Miniplasmin (Val442-plasmin)	130[r]	11[r]	0.085[r]	*q*				
	630[g]	23[g]	0.036[g]	*f*				
	320[t]	30[t]	0.094[t]	*s*				
	210[i]	26[i]	0.12[i]	*h*				
Val442-plasmin	280[i]	25[i]	0.093[i]	*h*				
Plasmin B chain	1300[i]	15[i]	0.012[i]	*h*				
22. Bz-L-Pro-L-Phe-L-Arg-*p*NA	200	6.6	0.033	*f*				
(Chromozym-PK)	690[g]	13[g]	0.019[g]	*f*	360[g]	7.2[g]	0.020[g]	*f*
14. H-D-Val-L-Leu-L-Arg-*p*NA	480[r]	12[r]	0.025[r]	*f*			0.00065	*f*
(S-2266)								
2. H-D-Ile-L-Pro-L-Arg-*p*NA	9000[u]	181[u]	0.020[u]	*s*	200[u]	16[u]	0.080[u]	*s*
(S-2288)								
3. H-D-Phe-L-Pip-L-Arg-*p*NA	1200	19	0.016	*f*	1000	4.6	0.0046	*f*
(S-2238)	3500[g]	46[g]	0.013[g]	*f*	1100[g]	10.0[g]	0.0095[g]	*f*

16. Bz-L-Ile-L-Glu-Gly-L-Arg-*p*NA					1000[r]	3.6[r]	0.0036[r]	*f*
(S-2222)					1300[v]	5.8[v]	0.0044[v]	*f*
15. Bz-L-Phe-L-Val-L-Arg-*p*NA	700	8.5	0.012	*f*	670	0.84	0.0013	*f*
(S-2160)					260[g]	0.5[g]	0.0019[g]	*f*
	1100[w]	9[w]	0.082[w]	*r*				
Val442-plasmin	1200[r]	13[r]	0.011[r]	*q*				
17. L-PyroGlu-Gly-L-Arg-*p*NA	1040	8.8	0.0086	*f*	35	10.3	0.29	*f*
(S-2444)					54[g]	17[g]	0.32[g]	*f*
					90[x]	0.00031[g]		*s*
					60[x]	0.00013[z]		*s*
15. Cbz-L-Val-Gly-L-Arg-*p*NA	1400	9.1	0.0067	*f*	570	5.3	0.0094	*f*
(Chromozym-TRY)	1400[g]	8.6[g]	0.0064[g]	*f*				
21. Bz-L-Arg-*p*NA	60	0.09	0.0015	*f*				
24. H-L-Glu-Gly-L-Arg-*p*NA								
					(HMW)200[aa]	21[aa]	0.103[aa]	*h*
					(LMW)270[aa]	16[aa]	0.061[aa]	*h*
28. Tos-L-Arg-*p*-NitroBzl	78[cc]	0.31[dd]		*bb*				

[a] K_m (μM); k_{cat} (sec^{-1}); k_{cat}/K_m (liters/μmol/sec).
[b] G. D. J. Green and E. Shaw, *Anal. Biochem.* **93**, 223 (1979).
[c] 0.1 *M* Tris–0.1 *M* NaCl, pH 8.0.
[d] P. Coleman (unpublished data).
[e] 37°, 0.2 *M* Glycine/Glycinate, pH 8.5.
[f] U. Christensen (unpublished data).
[g] 37°C.
[h] R. C. Wohl, L. Summaria, and K. C. Robbins, *J. Biol. Chem.* **255**, 2005 (1980).
[i] 37°C, 0.05 *M* Tris–0.1 *M* NaCl, pH 7.4.
[j] Boehringer Mannheim Gmbh, manufacturer's literature.
[k] Tris-imidazole, $I = 0.2$, pH 7.5.
[l] M. J. Gallimore, E. Amundsen, A. O. Aasen, M. Larsbraaten, K. Lyngaas, and L. Svendsen, *Thromb. Res.* **14**, 51 (1979).
[m] 0.05 *M* Tris-HCl–0.1 *M* NaCl, pH 7.4.
[n] R. Lottenberg and C. M. Jackson (unpublished data).
[o] D. Collen, H. R. Lijnen, F. De Cock, J. P. Durieux, and A. Loffet, *Biochim. Biophys. Acta* **165**, 158 (1980).
[p] 0.1 *M* Phosphate, pH 7.3.
[q] U. Christensen, L. Sottrup-Jensen, S. Magnusson, T. E. Petersen, and I. Clemmensen, *Biochim. Biophys. Acta* **567**, 472 (1979).
[r] pH 7.8.
[s] AB Kabi, manufacturer's literature.
[t] 37°C, Tris, $I = 0.15$, pH 7.4.
[u] 37°C, Tris, $I = 0.15$, pH = 8.4.
[v] 37°C, pH 7.8.
[w] 37°C, Tris, $I = 0.15$, pH 8.3.
[x] 37°C, 0.05 *M* Tris, $I = 0.05$, pH 8.8.
[y] μM/min/Ploug unit.
[z] μM/min/CTA unit.
[aa] 37°C, 0.05 *M* Tris–0.1 *M* NaCl, pH 9.0.
[bb] G. W. E. Plaut, *Haemostasis* **7**, 105 (1978).
[cc] 30°C, 0.1 *M* Tris-HCl, pH 8.4.
[dd] μM/min/CTA unit/ml.
[ee] 37°C, 0.2 *M* phosphate, 0.2 *M* NaCl, pH 7.5.

TABLE V
PLASMINOGEN/PLASMIN-STREPTOKINASE KINETIC PARAMETER SUMMARY

Substrate	K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]	Ref.
19. H-D-Val-L-Leu-L-Lys-*p*NA (S-2251)				
Plasminogen SK	200[c]	1[d]		*b*
Glu-Plasminogen SK	320[f]	39[f]	0.12[f]	*e*
Val 442-Plasminogen SK	210[f]	29[f]	0.14[f]	*e*
Lys-Plasmin SK	780[f]	52[f]	0.067[f]	*e*
Val 442-Plasmin SK	370[f]	43[f]	0.12[f]	*e*
Plasmin B chain SK	520[f]	36[f]	0.070[f]	*e*
8. Tos-Gly-L-Pro-L-Lys-*p*NA (Chromozym-PL)				
Glu-Plasminogen SK	510[f]	28[f]	0.055[f]	*e*

[a] K_m (μM); k_{cat} (sec^{-1}); k_{cat}/K_m (liters/μmol/sec).
[b] AB Kabi, manufacturer's literature.
[c] Tris buffer, $I = 0.05$, pH 7.4.
[d] $V_{max} = \mu M$/min/CU.
[e] R. C. Wohl, L. Summaria, and K. C. Robbins, *J. Biol. Chem.* **255**, 2005 (1980).
[f] 37°C, 0.05 *M* Tris–0.1 *M* NaCl, pH 7.4.

the plasminogen molecule have been removed from the amino-terminal end are given.

Trypsin and Factor XII$_a$ (*Activated Hageman Factor*). Data for the hydrolysis of 16 peptide *p*-nitroanilides and 2 esters by trypsin, and 8 peptide *p*-nitroanilides and 1 thiobenzyl ester by activated Hageman factor or coagulation factor XII are given in Table VI. Unless otherwise noted in the footnotes to this table, all data for trypsin were provided by Ulla Christensen.

Kallikrein. Data from the literature for the hydrolysis of 4-peptide *p*-nitroanilides by various kallikreins are given in Table VII. No values for k_{cat} are available for these enzymes.

Peptide Fluorogenic Substrates. Data obtained with a variety of peptide fluorogenic substrates are summarized in Table VIII. All data are taken from the literature and no attempt has been made to convert maximum velocities reported by the authors of these papers into values for k_{cat} because insufficient data were available for determining the concentration of active enzyme in the preparations. All references to the sources for these data are given in the footnotes to the table.

Data for Other Coagulation Proteases. McRae *et al.*[15] have investigated the hydrolysis of a large number of peptide thioesters by bovine coagula-

[15] B. J. McRae, K. Kurachi, E. Davie, and J. C. Powers, *Biochemistry* (submitted for publication).

TABLE VI
TRYPSIN AND HAGEMAN FACTOR KINETIC PARAMETER SUMMARY

Substrate	Trypsin				Human Hageman Fragment F			
	K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]	Ref.	K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]	Ref.
5. Tos-Gly-L-Pro-L-Arg-*p*NA (Chromozym-TH)	17	69	4.1	*b*	210[d]	11[d]	0.051[d]	*c*
	20[e]	84[e]	4.2[e]	*b*				
3. H-D-Phe-L-Pip-L-Arg-*p*NA (S-2238)					170[d]	7.3[d]	0.043[d]	*c*
25. Cbz-Lys-SBzl	50[g]	75[g]	1.5[g]	*f*	1700[d]	18[d]	0.011[d]	*c*
	68[i]	57[i]	0.84[i]	*h*				
8. Tos-Gly-L-Pro-L-Lys-*p*NA (Chromozym-PL)	13	17	1.3	*b*				
9. Bz-L-Phe-L-Val-L-Arg-*p*NA (Peptide Research Foundation)	21	22	1.0	*j*				
17. L-PyroGlu-Gly-L-Arg-*p*NA (S-2444)	170	170	1.0	*b*	1100[d]	17[d]	0.015[d]	*c*
15. Cbz-L-Val-Gly-L-Arg-*p*NA (Chromozym-TRY)	77	72	0.93	*b*				
	78	75	0.96	*j*				
	75[t]	—	—	*k*				
	88[e]	84[e]	0.95[e]	*b*				
16. Bz-L-Ile-L-Glu-Gly-L-Arg-*p*NA (S-2222)	190	120	0.61	*b*	180[d]	23[d]	0.13[d]	*c*
	19[e]	270[e]	14[e]	*m*				
7. Bz-L-Phe-L-Val-L-Arg-*p*NA (S-2160)	52	30	0.58	*b*				
	38	28	0.74	*j*				
	21[o]	60[o]	2.9[o]	*m*				
19. H-D-Val-L-Leu-L-Lys-*p*NA (S-2251)	470	22	0.047	*m*				
	400[q]	7.0[q]	0.018[q]	*p*				
2. H-D-Ile-L-Pro-L-Arg-*p*NA (S-2288)					400[r]	23[r]	0.058[r]	*m*
14. H-D-Val-L-Leu-L-Arg-*p*NA (S-2266)	170	37	0.22	*b*	310[d]	9.9[d]	0.032[d]	*c*
12. H-D-Pro-L-Phe-L-Arg-*p*NA (S-2302)	70	15	0.21	*b*	21[d]	14[d]	0.67[d]	*c*
					190[t]	15[t]	0.079[t]	*s*
				(HFa)	190[t]	15[t]	0.079[t]	*s*

(continued)

TABLE VI (*continued*)

Substrate	Trypsin				Human Hageman fragment F			
	K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]	Ref.	K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]	Ref.
22. Bz-L-Pro-L-Phe-L-Arg-*p*NA	120	13	0.11	*b*	710[d]	0.77[d]	0.0011[d]	*c*
(Chromozym-PK)	160[e]	20[e]	0.13[e]	*b*				
26. PyrGlu-L-Phe-L-Lys-*p*NA	200[q]	5.4[q]	0.027[q]	*p*				
27. L-Ala-L-Phe-L-Lys-*p*NA	2000[q]	4.7[q]	0.0024[q]	*p*				
21. Bz-L-Arg-*p*NA	1200[u]	2.7[u]	0.0023[u]	*j*				
	770[uc]	2.4[uc]	0.0031[uc]	*v*				
28. Tos-L-Arg-*p*-NitroBzl	12[y]	140[z]		*x*				

[a] K_m (μM); k_{cat} (sec^{-1}); k_{cat}/K_m (liters/μmol/sec).
[b] U. Christensen (unpublished data).
[c] Y. Hojima, D. L. Tankersley, M. Miller-Andersson, J. V. Pierce, and J. J. Pisano, *Thromb. Res.* **18**, 417 (1980).
[d] 0.05 *M* Tris-HCl, pH 8.0.
[e] 37°C.
[f] G. D. J. Green and E. Shaw, *Anal. Biochem.* **93**, 223 (1979).
[g] 0.1 *M* Tris–0.1 *M* NaCl, pH 8.0.
[h] P. L. Coleman (unpublished data).
[i] 37°C, 0.2 *M* Phosphate–0.2 *M* NaCl, pH 7.5.
[j] R. Lottenberg and C. M. Jackson (unpublished data).
[k] Boehringer Mannheim, manufacturer's literature.
[l] Tris-HCl, pH 7.8.
[m] AB Kabi, manufacturer's literature.
[n] 37°C, Tris, I = 0.15, pH 9.0.
[o] 37°C, Tris, I = 0.15, pH 8.3.
[p] D. Collen, H. R. Lijnen, F. De Cock, J. P. Durieux, and A. Loffet, *Biochim. Biophys. Acta* **165**, 158 (1980).
[q] 0.1 *M* Phosphate, pH 7.3.
[r] 37°C, Tris, I = 0.15, pH 8.4.
[s] M. Silverberg, J. T. Dunn, L. Garen, and A. P. Kaplan, *J. Biol. Chem.* **255**, 7281, (1980).
[t] 0.05 *M* Tris-HCl–0.117 *M* NaCl, pH 7.8.
[u] pH 8.0.
[v] S. Takasaki, K. Kasai, and S. Ishii, *J. Biochem.* (*Tokyo*) **78**, 1275 (1975).
[uc] 0.05 *M* Tris-HCl, pH 8.2.
[x] G. W. E. Plaut, *Haemostasis* **7**, 105 (1978).
[y] 30°C, 0.1 *M* Tris-HCl.
[z] V_{max} = μM/min/mg.

TABLE VII
KALLIKREIN KINETIC PARAMETER SUMMARY

Substrate	K_m[a]	V_{max}	Ref.
22. Bz-L-Pro-L-Phe-L-Arg-*p*NA (Chromozym-PK)			
Human plasma kallikrein	140[c]	—	*b*
12. H-D-Pro-L-Phe-L-Arg-*p*NA (S-2302)			
Human plasma kallikrein	200[e]	6.8 μM/min/PEU	*d*
2. H-D-Ile-L-Pro-L-Arg-*p*NA (S-2288)			
Human plasma kallikrein	1000[f]	1.3 μM/min/U	*d*
14. H-D-Val-L-Leu-L-Arg-*p*NA (S-2266)			
Porcine pancreatic kallikrein	22[g]	0.008 μM/min/KU	*d*
Human urine kallikrein	30[g]		*d*
Human salivary kallikrein	500[g]		*d*

[a] K_m (μM).
[b] Boehringer Mannheim, manufacturer's literature.
[c] $I = 0.15$, pH 7.9.
[d] AB Kabi, manufacturer's literature.
[e] 37°C.
[f] Tris, $I = 0.15$, pH 8.4.
[g] 0.05 M Tris, $I = 0.05$, pH 9.0.

tion factors IX_a, X_a, XI_a, thrombin, and trypsin. To date, these authors have reported the only di- and tripeptide chromogenic substrates to be hydrolyzed by factor IX_a. These dipeptide substrates, carbobenzoxy-L-Phe-L-Arg-isobutyl thioester and carbobenzoxy-L-Trp-L-Arg-isobutyl thioester, are hydrolyzed by bovine factor IX_a with k_{cat}/K_m values of 10,000–20,000 liters/mol/sec. Several tripeptide thiobenzyl esters are also hydrolyzed by factor IX_a with k_{cat}/K_m values of the order 70,000 liters/mol/sec. The original publication should be consulted for detailed information.

Discussion

Although the data base on which conclusions about peptide structure and selectivity might be made is still relatively limited, the fact that k_{cat}/K_m values are approaching values of 10^7 liters/mol/sec indicates clearly that a very high degree of selectivity is achievable. Comparison of k_{cat}/K_m values for the same substrate with different proteases further indicates that discrimination between proteases by using such substrates

TABLE VIII
FLUOROGENIC SUBSTRATES KINETIC PARAMETER SUMMARY

Substrate/enzyme	K_m[a]	V_{max}	Temp.	Ref.
Cbz-L-Ala-L-Ala-L-Lys-4-methoxy-2-naphthylamide				
Plasmin	870[c]	—	37	*b*
D-Ala-L-Leu-L-Lys-7-amino-4-trifluoromethylcoumarin (AFC)				
Plasmin	620[e]	0.067 μM/min/CTAU[e]	25	*d*
L-Ala-L-Phe-L-Lys-7-amino-4-methylcoumarin (MCA)				
Plasmin	45[g]	210 μM/min/CTAU[g]	24	*f*
Urokinase	900[g]	100 μM/min/mg[g]	24	*f*
Thrombin	800[g]	9 μM/min/mg[g]	24	*f*
Suc-Ala-L-Phe-L-Lys-MCA				
Plasmin	400[g]	370 μM/min/CTAU[g]	24	*f*
Urokinase	800[g]	62 μM/min/mg[g]	24	*f*
MeOSuc-Ala-L-Phe-L-Lys-MCA				
Plasmin	440[g]	470 μM/min/CTAU[g]	24	*f*
L-Glu-Gly-L-Arg-MCA				
Urokinase	320[i]	18 μM/min/mg[i]	37	*h*
Boc-L-Glu-L-Lys-L-Lys-MCA				
Bovine plasmin	670[e]	1.9 μM/min/mg[e]	37	*d*
Plasmin	770[e]	3.7 μM/min/mg[e]	37	*d*
Glutaryl-Gly-L-Arg-MCA				
Urokinase	440[i]	18 μM/min/mg[i]	37	*h*
Gly-L-Arg-2-naphthylamide				
Plasmin	1700	0.0006 μM/min/CU	37	*j*
Urokinase	7500	0.000022 μM/min/CTAU	37	*j*
Cbz-Gly-Gly-L-Arg-MCA				
Trypsin	330[l]	3700 μM/min/μM trypsin[l]	25	*k*
Thrombin	110[g]	750 μM/min/mg[g]	24	*f*
Plasmin	450[g]	10 μM/min/CTAU[g]	24	*f*
Urokinase	130[g]	1600 μM/min/mg[g]	24	*f*
Urokinase	170[m]	0.000030 μM/min/IU[m]	25	*k*

Cbz-Gly-Gly-L-Arg-AFC				
Trypsin	180[l]	8200 μM/min/μM trypsin[l]	25	*k*
Urokinase	190[m]	0.000030 μM/min/IU[m]	25	*k*
Cbz-Gly-L-Pro-L-Arg-4-methoxy-2-naphthylamide				
Thrombin	83[o]	—	37	*n*
Boc-L-Ile-L-Glu-Gly-L-Arg-MCA				
Factor X_a	160[i]	6.1 μM/min/mg[i]	37	*h*
Cbz-L-Phe-L-Arg-MCA				
Plasma kallikrein (bov.)	240[i]	8.0 μM/min/A_{280} = 1.0[i]	37	*h*
Urinary kallikrein (hum.)	280[i]	1.1 μM/min/mg[i]	37	*h*
Pancreatic kallikrein (hog)	530[i]	0.3 μM/min/A_{280} = 1.0[i]	37	*h*
Boc-L-Phe-L-Glu-L-Lys-L-Lys-MCA				
Bovine plasmin	400[i]	0.23 μM/min/mg[i]	37	*h*
H-D-Phe-L-Pro-L-Arg-5-aminoisophthalic acid				
Thrombin	54[o]	—	37	*p*
L-Pro-L-Phe-L-Arg-MCA				
Plasma kallikrein (bov.)	610[i]	13 μM/min/A_{280} = 1.0[i]	37	*h*
Urinary kallikrein (hum.)	220[i]	30 μM/min/mg[i]	37	*h*
Pancreatic kallikrein (hog)	160[i]	12 μM/min/A_{280} = 1.0[i]	37	*h*
Hageman fragment f	54[r]	k_{cat} = 1.9/sec[r]	25	*q*
Cbz-L-Pro-L-Phe-L-Arg-MCA				
Plasma kallikrein (bov.)	470[i]	7.2 μM/min/A_{280} = 1.0[i]	37	*h*
Urinary kallikrein (hum.)	170[i]	7.5 μM/min/mg[i]	37	*h*
Pancreatic kallikrein (hog)	180[i]	4.6 μM/min/A_{280} = 1.0[i]	37	*h*
L-Val-Gly-L-Arg-2-naphthylamide				
Plasmin	900	0.0012 μM/min/CU	37	*j*
Urokinase	600	0.0000048 μM/min/CTAU	37	*j*
Boc-L-Val-Gly-L-Arg-2-naphthylamide				
Plasmin	1600	0.0024 μM/min/CU	37	*j*
Urokinase	1100	0.0000030 μM/min/CTAU	37	*j*
Boc-L-Val-L-Leu-L-Lys-MCA				
Bovine plasmin	250[e]	1.5 μM/min/mg[e]	37	*d*
Plasmin	770[e]	3.7 μM/min/mg[e]	37	*d*

(*continued*)

TABLE VIII *(continued)*

Substrate/enzyme	K_m[a]	V_{max}	Temp.	Ref.
D-Val-L-Leu-L-Lys-AFC				
Plasmin	560[m]	0.088 μM/min/CTAU[m]	25	*k*
H-D-Val-L-Leu-L-Lys-5-aminoisophthalic acid				
Plasmin	250[t]	—	37	*s*
Boc-L-Val-L-Pro-L-Arg-MCA				
Bovine thrombin	21[i]	78 μM/min/mg[i]	37	*h*
Boc-L-Val-Ser-Gly-L-Arg-MCA				
Urokinase	330[i]	9.6 μM/min/mg[i]	37	*h*

[a] $K_m(\mu M)$.
[b] S. A. Clavin, J. L. Bobbit, R. T. Shuman, and E. L. Smithwick, Jr., *Anal. Biochem.* **80,** 355 (1977).
[c] 0.05 *M* Tris-HCl, pH 8.0.
[d] H. Kato, N. Adachi, Y. Ohno, S. Iwanaga, K. Takada, and S. Sakakibara, *J. Biochem. (Tokyo)* **88,** 183 (1980).
[e] 0.05 *M* Tris-HCl–0.15 *M* NaCl, pH 7.4.
[f] P. A. Pierzchala, C. P. Dorn, and M. Zimmerman, *Biochem. J.* **183,** 555 (1979).
[g] 0.05 m*M* TES-NaOH–20% dimethyl sulfoxide, pH 7.5.
[h] S. Iwanaga, T. Morita, H. Kato, T. Harada, N. Adachi, T. Sugo, I. Maruyama, K. Takada, T. Kimura, and S. Sakakibara, *in* "Kinins-II, Biochemistry, Pathophysiology, and Clinical Aspects" (S. Fujii, H. Moriya, and T. Suzuki, eds.), p. 147. Plenum, New York, 1979.
[i] 0.05 *M* Tris-HCl–0.1 *M* NaCl–0.01 *M* calcium chloride, pH 8.0.
[j] W. Nieuwenhuizen, G. Wijngaards, and E. Groeneveld, *Anal. Biochem.* **83,** 143 (1977).
[k] R. E. Smith, E. R. Bissell, A. R. Mitchell, and K. W. Pearson, *Thromb. Res.* **17,** 393 (1980).
[l] 0.45 *M* TES, pH 8.0.
[m] 0.045 *M* TES, pH 8.0.
[n] G. A. Mitchell, P. M. Hudson, R. M. Huseby, S. P. Pochron, and R. J. Gargiulo, *Thromb. Res.* **12,** 219 (1978).
[o] 0.25 *M* Glycine/Glycinate–0.03 *M* NaCl–2 m*M* potassium EDTA.
[p] G. A. Mitchell, R. J. Gargiulo, R. M. Huseby, D. E. Lawson, S. P. Pochron, and J. A. Sehuanes, *Thromb. Res.* **13,** 47 (1978).
[q] Y. Hojima, D. L. Tankersley, M. Miller-Andersson, J. V. Pierce, and J. J. Pisano, *Thromb. Res.* **18,** 417 (1980).
[r] 0.05 *M* Tris-HCl, pH 8.0.
[s] D. E. Lawson, G. A. Mitchell, and R. M. Huseby, *Thromb. Res.* **14,** 323 (1979).
[t] 0.05 *M* Glycine/Glycinate–0.15 *M* NaCl, pH 8.0.

is readily obtainable. In mixtures of proteases, assay using more than one substrate and then solving the set of simultaneous equations that relates the total velocity to that contributed by each enzyme can be done for those mixtures for which the kinetic constants are known for the individual components.[16]

The values for k_{cat} of 100–300 sec^{-1} and the large molar absorptivity for *p*-nitroaniline of 13,300 liters/mol/cm at 380 nm, its absorption maximum, make extremely sensitive assays readily available. It can be anticipated that the increase in sensitivity provided from fluorescence monitoring of the fluorogenic substrates may increase the sensitivity considerably, although the limited amount of information about k_{cat} for the various fluorogenic leaving groups precludes making quantitative statements at this time.

It must be noted that many of the very good substrates for one enzyme, where "good" is judged from a large k_{cat}/K_m and a large k_{cat}, are very poor substrates for one of the other enzymes by these criteria. However, if consideration of the values for K_m are made, these "poor" (low values for k_{cat}) substrates are very frequently good apparent competitive inhibitors of the enzymes by which they are hydrolyzed very slowly. This can greatly facilitate the assay of one protease in the presence of other proteases as described above; however, use of these substrates in coupled assay systems, in which one protease is being generated from its zymogen by another protease, must therefore be made with caution and one must take into account the consequences of this observation.

No consideration of the design of assay procedures using these substrates is given here, since this topic is covered extensively elsewhere.[17–19] It must be noted here, however, that the solubility of some of the chromogenic and fluorogenic substrates is relatively low when one considers the Michaelis constants for the substrates and particular proteases. This is particularly true in solutions of high ionic strength and in the presence of agents such as heparin, which may interact and initiate substrate precipitation.

[16] C. M. Jackson, *Thromb. Haemostasis* **41**, 458 (1979).
[17] A. Fersht, "Enzyme Structure and Mechanism." Freeman, San Francisco, California, 1977.
[18] A. Cornish-Bowden, "Fundamentals of Enzyme Kinetics." Butterworth, London, 1979.
[19] R. D. Allison and D. L. Purich, this series, Vol. 63, p. 3.