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Substrate preferences of glutamic-acid-specific endopeptidases assessed by synthetic Peptide Substrates based on intramolecular fluorescence quenching

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The substrate preferences of the easily available Glu/Asp-specific enzymes from *Staphyllococcus aureus* (V8), *Bacillus licheniformis* and *Streptomyces griseus* have been extensively investigated using a series of synthetic peptide substrates, containing an N-terminal anthraniloyl group and a 3-nitrotyrosine close to the C-terminus, allowing the fluorimetric monitoring of substrate hydrolysis by the decrease in intramolecular quenching. All three enzymes hydrolysed Glu-Xaa peptide bonds approximately 1000-fold faster than Asp-Xaa bonds and they are consequently more appropriately termed Glu-specific enzymes. The difference in k_{cat}/K_m for the hydrolysis of substrates with Glu and Asp is primarily due to a difference in k_{cat}. The enzymes appear to hydrolyse all types of Glu-Xaa bonds, although those with Xaa as Asp and, in particular, Xaa as Pro, are hydrolysed with very low rates. The influence of the nature of the amino acid residues at the substrate positions P_2, P_3, P_4, P'_1 and P'_2 has been determined and it is shown that the enzyme from *S. griseus* exhibits the most narrow substrate preference. The results are useful in connection with fragmentation of proteins for sequencing purposes as well as for cleavage of fusion proteins.

Endopeptidases can be divided into several groups based on their substrate specificities. A number of such enzymes, e.g. the subtilisins, are non-specific, although they exhibit pronounced substrate preferences [2]. The specific endopeptidases include those participating in blood clotting and hormone processing, as well as a group with specificity for particular amino acid residues at the P_1 position, i.e. Arg [3], Lys [4], Arg/Lys [5], Pro [6], and Glu/Asp [7–14]. This latter group is widely employed for fragmentation of proteins prior to amino acid sequence determinations and as catalysts for synthesis of peptide bonds [15–17]. However, the extensive literature on sequence analysis shows numerous examples of inefficient cleavage at the appropriate amino acid residue due to unfavourable interactions between enzyme and other positions of the substrate, signifying major contributions of the other primary binding site, i.e. P'_1, as well as the secondary binding sites, i.e. P_2, P_3 ... P_n and P'_2, P'_3 ... P'_n. Such contributions to catalysis have previously been investigated with several enzymes but not in a systematic manner due to lack of a sufficiently large number of suitable substrates. Recently, it was demonstrated that peptide substrates, based on the principle of intramolecular quenching, easily can be synthesized [18]. Furthermore, the optimal choice of fluorophore and quencher allowed the monitoring of cleavage of substrates sufficiently long to span the active site of a protease comprising up to nine subsites and consequently, it provides the basis for systematic investigations of enzyme-substrate interactions at each subsite.

Until 1987, the Glu/Asp-specific proteases were only represented by the Ser endopeptidase from *Staphylococcus aureus* [7, 8, 14]. Since then, similar enzymes have been isolated and two of these, one from *Streptomyces griseus* [11, 13], the other from *Bacillus licheniformis* [12], are available in large amounts from commercial extracts. Here we describe an extensive comparison of the substrate preferences of these enzymes.

MATERIALS

The Glu/Asp-specific endopeptidase from *S. aureus* was from ICN Biochemicals. Mes, Hepes, Bicine, Ches and Caps were obtained from Sigma. The Glu/Asp-specific endopeptidases from *B. licheniformis* and *S. griseus* were isolated as previously described [12, 13]. The anthraniloyl and 3-nitrotyrosine substrates were synthesized as previously described [18].

METHODS

The hydrolysis of substrates, based on intramolecular quenching, containing the anthraniloyl and 3-nitrotyrosine groups were assayed by monitoring the fluorescence emission at 420 nm upon excitation at 320 nm, using a Perkin-Elmer LS 50 fluorimeter thermostated at 25°C. The substrate (50 µl 2–20 µM substrate in *N*,*N*-dimethylformamide) was added to 2.45 ml buffer (see below) followed by 10–50 µl enzyme. The initial rate was determined and related to the total increase in fluorescence at complete hydrolysis. With some substrates,

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Abbreviations. ABz, anthraniloyl; BL-GSE, Glu-specific endopeptidase from *Bacillus licheniformis*; SA-GSE, Glu-specific endopeptidase from *Staphylococcus aureus*; SG-GSE, Glu-specific endopeptidase from *Streptomyces griseus*. The binding-site notation is that of Schechter and Berger [1].

Table 1. The kinetic parameters for the BL-GSE- catalyzed hydrolysis of aminobenzoyl/3-nitrotyrosine substrates. Standard deviations, see Table 3.

Substrate	Kinetic parameters		
	k_{cat}	K_m	k_{cat}/K_m
	min^{-1}	μM	$min^{-1} \cdot mM^{-1}$
ABz-Ala-Ala-Glu↓Ala-Phe-TyrNO_2-Asp-OH	1900	5.0	380000
ABz-Ala-Ala-Glu↓Val-TyrNO_2-Asp-OH	500	4.5	110000
ABz-Ala-Phe-Ala-Phe-Glu↓Val-Phe-TyrNO_2-Asp-OH	220	0.18	1200000
ABz-Ala-Phe-Ala-Phe-Asp↓Val-Phe-TyrNO_2-Asp-OH	1.4	1.2	1200

the kinetic parameters k_{cat} and K_m were determined graphically from Lineweaver-Burk plots of initial rates at different concentrations of substrate. With most substrates, only k_{cat}/K_m was determined from initial rates using the relationship $k_{cat}/K_m = V_o/(S_o E_o)$, which is valid at $S_o \ll K_m$. The validity of the equation was ascertained for each substrate by performing duplicate measurements at a minimum of two substrate concentrations, typically 0.1 μM and 0.2 μM. The substrate preferences of the three enzymes investigated were determined at their respective pH optima [11, 12, 14] in the following buffers: 0.05 M Bicine and 2 mM $CaCl_2$, pH 8.0, with the Glu-specific endopeptidase from *B. licheniformis* (BL-GSE); 0.05 M Ches, pH 9.0, with the Glu-specific endopeptidase from *S. griseus* (SG-GSE); 0.05 M Hepes, pH 7.3, with the Glu-specific endopeptidase from *S. aureus* (SA-GSE).

The site of cleavage in the anthraniloyl substrates was determined when 50–80% of the substrate had been consumed, by identification of the products by amino acid analysis after separation by HPLC, using equipment from Waters Associates, a Vydac C_{18} column and 0.1% trifluoroacetic acid as buffer A and 90% CH_3CN + 10% of buffer A (buffer B). The reactions were carried out in the following way: approximately 0.4 μmol substrate was dissolved in 5 μl *N,N*-dimethylformamide and added to the appropriate buffer (see above), followed by an appropriate amount of enzyme. The separated components were analysed by amino acid analysis after hydrolysis with 6 M HCl at 110°C for 24 h.

RESULTS AND DISCUSSION

The substrate preferences of three Glu-specific endopeptidases were determined at their respective pH optima [11, 12, 14], in the case of BL-GSE in the presence of $CaCl_2$. With one of the enzymes, BL-GSE, k_{cat} and K_m values were determined from the initial rates at several substrate concentrations, above and below K_m (see Table 1). The k_{cat} values for the hydrolysis of ABz-Ala-Ala-Glu↓Ala-Phe-TyrNO_2-Asp-OH and ABz-Ala-Ala-Glu↓Val-TyrNO_2-Asp-OH, both with ABz-Ala-Ala-Glu as acyl component, were 1900 min^{-1} and 500 min^{-1}, respectively (ABz, anthraniloyl). The K_m values were around 5 μM with both substrates and this allowed the direct determination of k_{cat}/K_m from initial rates at single substrate concentrations below 0.5 μM, i.e. $S_o \ll K_m$, by employing the equation $k_{cat}/K_m = V_o/(S_o E_o)$. However, the K_m value of 0.18 μM for the hydrolysis of ABz-Ala-Phe-Ala-Phe-Glu↓Val-Phe-TyrNO_2-Asp-OH with BL-GSE suggested that even lower concentrations of substrate are required in some cases. Consequently, with all substrates the validity of the equation was confirmed by determination of k_{cat}/K_m at different concentrations of substrate, typically 0.1 μM and 0.2 μM.

The specificity of all three enzymes for peptide bonds at the carboxyl side of Glu was confirmed by identification of the hydrolysis products from the following substrates: ABz-Ala-Ala-Glu↓Arg-TyrNO_2-Asp-OH; ABz-Ala-Ala-Glu↓Ala-Phe-TyrNO_2-Asp-OH; ABz-Ala-Ala-Glu↓Ala-Ser-TyrNO_2-Asp-OH; ABz-Gly-Ala-Ala-Glu↓TyrNO_2-Asp-OH; ABz-Ala-Phe-Ala-Phe-Glu↓Val-Phe-TyrNO_2-Asp-OH. At a reaction time when 50–80% of the substrate had been consumed, two new peptides, corresponding to cleavage at Glu, accounted for more than 95% of the products. With the substrate ABz-Ala-Ala-Glu↓Glu↓TyrNO_2-Asp-OH, containing neighbouring Glu residues, SA-GSE and BL-GSE cleaved specifically the Glu↓TyrNO_2 bond, whereas SG-GSE cleaved at Glu-Glu as well as at Glu↓TyrNO_2 in a 1:3 ratio. With the substrate ABz-Ala-Phe-Ala-Phe-Asp↓Val-Phe-TyrNO_2-Asp-OH, SA-GSE and BL-GSE cleaved specifically the Asp-Val bond whereas SG-GSE in addition, and with an approximately equal rate, cleaved at the Phe-Asp bond. Thus, it appears that BL-GSE and SA-GSE cleave at the likewise negatively charged Asp in substrates without Glu, whereas SG-GSE does not exhibit a similar preference but equally well cleaves peptide bonds with a hydrophobic amino acid residue in the P_1 position.

Elongation of the substrate in the N-terminal direction of the scissile bond increased the k_{cat}/K_m values with all three enzymes (Table 2). Thus, the k_{cat}/K_m values for the hydrolysis of ABz-Gly-Gly-Ala-Ala-Glu↓TyrNO_2-Asp-OH were 16 times, 280 times and 4 times higher than those of ABz-Ala-Glu↓TyrNO_2-Asp-OH using BL-GSE, SG-GSE and SA-GSE, respectively. Similar effects were observed by increasing the length of the substrate in the C-terminal direction from the scissile bond (Table 2); the k_{cat}/K_m values for the hydrolysis of ABz-Ala-Ala-Glu↓Ala-Ala-TyrNO_2-Asp-OH were 4-times, 54-times and 27-times higher than those of ABz-Ala-Ala-Glu↓TyrNO_2-Asp-OH using BL-GSE, SG-GSE and SA-GSE, respectively.

The results listed in Table 2 suggest that the binding sites of these Glu-specific proteases comprise at least six subsites, i.e. S_1, S_2, S_3, S_4, S'_1 and S'_2, and the properties of these were therefore investigated by determination of their substrate preferences using six series of substrates which were systematically varied, with amino acids representing different properties known to influence the enzymatic activity: Ala, Val and Phe represented a hydrophobic series, Ser an uncharged hydrophilic residue, Arg a positively charged residue, Asp a negatively charged residue and Pro a secondary amino acid.

BL-GSE was essentially specific for substrates with Glu at the P_1 position, since the hydrolysis at Asp, Phe and Ala in corresponding substrates was at rates of only 0.1%, 0.006% and 0.001% of the value obtained with Glu (Table 3). These results are consistent with the specific cleavage at Glu observed

Table 2. The influence of substrate chain length on k_{cat}/K_m using BL-GSE, SG-GSE and SA-GSE. Standard deviations, see Table 3.

Substrate	k_{cat}/K_m with		
	BL-GSE	SG-GSE	SA-GSE
	$min^{-1} \cdot mM^{-1}$		
ABz-Ala-Glu↓TyrNO$_2$-Asp-OH	1500	2	0.041
ABz-Gly-Ala-Ala-Glu↓TyrNO$_2$-Asp-OH	7400	330	1.8
ABz-Gly-Gly-Ala-Ala-Glu↓TyrNO$_2$-Asp-OH	24000	560	0.17
ABz-Ala-Ala-Glu↓TyrNO$_2$-Asp-OH	13000	1700	0.48
ABz-Ala-Ala-Glu↓Ala-TyrNO$_2$-Asp-OH	36000	4600	5.1
ABz-Ala-Ala-Glu↓Ala-Ala-TyrNO$_2$-Asp-OH	46000	91000	13

Table 3. Substrate preferences of BL-GSE, SG-GSE and SA-GSE. The standard deviations with k_{cat}/K_m values in the range 1 – 100, 100 – 20000 and above 20000 $min^{-1} \cdot mM^{-1}$, are less than 9%, 5% and 3%, respectively.

Position	Substrate	k_{cat}/K_m with		
		BL-GSE	SG-GSE	SA-GSE
		$min^{-1} \cdot mM^{-1}$		
P_1	ABz-Ala-Phe-Ala-Phe-Asp↓Val-Phe-TyrNO$_2$-Asp-OH	1200	190	0.49
	ABz-Ala-Phe-Ala-Phe-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	1200000	20000	610
	ABz-Ala-Phe-Ala-Phe-Phe-Val-Phe-TyrNO$_2$-Asp-OH	75	200	2.0
	ABz-Ala-Phe-Ala-Phe-Ala-Val-Phe-TyrNO$_2$-Asp-OH	13	160	0.054
P_2	ABz-Ala-Phe-Ala-Ala-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	350000	53000	44
	ABz-Ala-Phe-Ala-Val-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	380000	180000	75
	ABz-Ala-Phe-Ala-Phe-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	1200000	20000	610
	ABz-Ala-Phe-Ala-Ser-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	130000	8500	33
	ABz-Ala-Phe-Ala-Asp-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	130000	160	12
	ABz-Ala-Phe-Ala-Arg-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	230000	29000	150
	ABz-Ala-Phe-Ala-Pro-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	560000	190000	29
P_3	ABz-Ala-Phe-Ala-Ala-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	390000	44000	140
	ABz-Ala-Phe-Val-Ala-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	120000	180000	510
	ABz-Ala-Phe-Phe-Ala-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	120000	120000	64
	ABz-Ala-Phe-Ser-Ala-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	260000	43000	140
	ABz-Ala-Phe-Asp-Ala-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	42000	7500	25
	ABz-Ala-Phe-Arg-Ala-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	130000	160000	300
	ABz-Ala-Phe-Pro-Ala-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	25000	43	0.79
P_4	ABz-Ala-Ala-Ala-Ala-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	250000	23000	100
	ABz-Ala-Val-Ala-Ala-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	300000	24000	110
	ABz-Ala-Phe-Ala-Ala-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	390000	44000	140
	ABz-Ala-Ser-Ala-Ala-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	230000	11000	48
	ABz-Ala-Asp-Ala-Ala-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	430000	120000	170
	ABz-Ala-Arg-Ala-Ala-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	63000	330	32
	ABz-Ala-Pro-Ala-Ala-Glu↓Val-Phe-TyrNO$_2$-Asp-OH	170000	34000	60
P'_1	ABz-Ala-Ala-Glu↓Ala-TyrNO$_2$-Asp-OH	36000	4600	5.1
	ABz-Ala-Ala-Glu↓Val-TyrNO$_2$-Asp-OH	89000	4800	11
	ABz-Ala-Ala-Glu↓Phe-TyrNO$_2$-Asp-OH	57000	3800	17
	ABz-Ala-Ala-Glu↓Ser-TyrNO$_2$-Asp-OH	84000	11000	6.0
	ABz-Ala-Ala-Glu↓Asp-TyrNO$_2$-Asp-OH	1200	110	0.11
	ABz-Ala-Ala-Glu↓Arg-TyrNO$_2$-Asp-OH	87000	25000	3.8
	ABz-Ala-Ala-Glu↓Pro-TyrNO$_2$-Asp-OH	3.8	41	< 0.1
P'_2	ABz-Ala-Ala-Glu↓Ala-Ala-TyrNO$_2$-Asp-OH	46000	91000	13
	ABz-Ala-Ala-Glu↓Ala-Val-TyrNO$_2$-Asp-OH	12000	37000	1.9
	ABz-Ala-Ala-Glu↓Ala-Phe-TyrNO$_2$-Asp-OH	380000	38000	24
	ABz-Ala-Ala-Glu↓Ala-Ser-TyrNO$_2$-Asp-OH	58000	58000	9.4
	ABz-Ala-Ala-Glu↓Ala-Asp-TyrNO$_2$-Asp-OH	38000	7300	12
	ABz-Ala-Ala-Glu↓Ala-Arg-TyrNO$_2$-Asp-OH	4900	5700	14
	ABz-Ala-Ala-Glu↓Ala-Pro-TyrNO$_2$-Asp-OH	7.4	2.0	< 0.1

by separation of the reaction products by HPLC (see above), as well as the previous observation that BL-GSE preferentially cleaves at Asp only in peptides without Glu [12]. The 1000-fold higher k_{cat}/K_m value for cleavage at Glu compared with Asp, was primarily due to an effect on k_{cat}, the K_m value being only sevenfold higher for cleavage at Asp. Thus, peptide segments containing Asp appear to bind fairly well to the active site of the enzyme, but the rate of cleavage is slow.

BL-GSE exhibited little selectivity with respect to the P_2, P_3 and P_4 positions, suggesting that the corresponding side-chain-binding sites are rather poorly defined. The least-tolerated amino acid residues were Ser, Asp and Arg in the P_2 position, Asp and Pro in the P_3 position and Arg in the P_4 position. On the C-terminal side of the scissile bond, the enzyme essentially did not accept Pro in the P'_1 and P'_2 positions and it only poorly accepted Asp in $P'_1$and Arg in P'_2.

The k_{cat}/K_m values obtained with SG-GSE were generally lower than those obtained with BL-GSE. This enzyme was also essentially specific for substrates with Glu at the P_1 position, but the rates with Asp, Phe and Ala in the corresponding position were 1%, 1% and 0.8% of the values obtained with Glu and these relative values were significantly higher than those obtained with BL-GSE. Thus, the enzyme is less specific for Glu than BL-GSE and, importantly, it exhibits no preference for Asp over Phe and Ala which is also consistent with the results of the HPLC study (see above). The substrate preference of SG-GSE is similar to BL-GSE but appears to be somewhat more restricted. The enzyme only slowly hydrolysed substrates with Ser and in particular Asp in P_2, Asp and in particular Pro in P_3, Arg in P_4, Asp and Pro in P'_1 and Pro in P'_2.

The k_{cat}/K_m values obtained with BA-GSE were extremely low relative to those obtained with the two other Glu-specific enzymes. The enzyme was essentially as unspecific as BL-GSE, since the relative rates were low only with Pro in P_3, Asp and Pro in P'_1 and Pro in P'_2. The broad substrate preference with respect to the amino acid residue at the P'_1 position has previously been shown by digestion of synthetic peptides, but without determination of the relative rates [19].

The importance of the interaction between enzyme and the side chain at any given position of the substrate is reflected by a dependence of k_{cat}/K_m on the properties of the amino acid occupying this position. All three enzymes exhibited extremely low activities when Pro residues are present in certain, but not all, positions and may be associated with the fact that Pro residues may cause bends in the peptide chain. Therefore, it is probably reasonable to exclude this amino acid residue from the evaluation. The contribution of the side chain to the stability of the transition state is reflected by the $\Delta(\Delta G)$ [20] (see legend of Table 4) between the most and the least preferred amino acid residue (excluding Pro). It would be expected that the $\Delta(\Delta G)$ is highest in the P_1 position for all three enzymes due to the fact that they are specific for Glu at this position. However, this was only the case with BL-GSE and SA-GSE (Table 4); with SG-GSE higher values were observed at other positions although this is not due to specificity for a particular amino acid residue but rather to a poor acceptance of a single amino acid. Thus, the specificities of all three enzymes are determined exclusively by their S_1 sites. The high values of $\Delta(\Delta G)$ at other positions show that some of the side-chain-binding sites are important for the substrate preference, i.e. S'_1 and S'_2 in BL-GSE, S_4, S_2 and S'_1 in SG-GSE and S'_1 in SA-GSE. The highest values of $\Delta(\Delta G)$ are found with SG-GSE, showing that this enzyme exhibits the most pronounced substrate preference. Nevertheless, all side-chain-binding sites, with the exception of the specificity creating S_1, appear to be rather poorly defined since they readily accept hydrophilic or even charged amino acid residues as well as hydrophobic amino acid residues. Similar wide tolerances have previously been observed with other proteolytic enzymes [2].

Table 4. The importance of the substrate side chains at P_1, P_2, P_3, P_4, P'_1 and P'_2 for transition-state stabilisation. A list of preferred and poorly accepted side chains is given. The difference in transition-state stabilisation between the preferred (A) and the least-preferred amino acid residue (B) is given by $\Delta(\Delta G) = RT \ln [(k_{cat}/K_m)_A/(k_{cat}/K_m)_B]$ as previously described in [20]. When the least-preferred amino acid residue is Pro, the second least preferred residue is B. For a particular binding subsite, a poorly accepted amino acid residue is defined as one that results in a k_{cat}/K_m value of less than 2% of the value obtained with the preferred amino acid residue in the same position.

Enzyme	Substrate			
	position	$\Delta(\Delta G)$	preferred	poorly accepted
		kJ mol^{-1}		
BL-GSE	P_4	4.8	Asp	none
	P_3	5.5	Ala	none
	P_2	5.5	Phe	none
	P_1	28	Glu	Ala, Phe, Asp
	P'_1	11	Val	Asp, Pro
	P'_2	11	Phe	Pro
SG-GSE	P_4	15	Asp	Arg
	P_3	7.7	Val	Pro
	P_2	18	Pro	Asp
	P_1	12	Glu	Ala, Phe, Asp
	P'_1	13	Arg	Asp, Pro
	P'_2	6.9	Ala	Pro
SA-GSE	P_4	4.1	Asp	none
	P_3	7.5	Val	Pro
	P_2	9.7	Phe	none
	P_1	23	Glu	Ala, Phe, Asp
	P'_1	12	Phe	Asp, Pro
	P'_2	6.3	Ala	Pro

CONCLUSION

The easy synthesis and use of aminobenzoyl, 3-nitrotyrosine substrates has allowed extensive subsite mappings of three endopeptidases. All three enzymes are essentially specific for cleavage at Glu-Xaa, with other peptide bonds including Asp-Xaa being cleaved at rates more than 1000-fold slower. This is also the case for the enzyme from *S. aureus* (SA-GSE), previously believed to readily cleave at Asp-Xaa bonds in the absence of ammonium bicarbonate [7, 8]. In the case of BL-GSE, it appears that Asp-containing peptides bind rather tightly to the enzyme but with slow cleavage of the Asp-Xaa bond. The use of a substantial number of substrates has delineated the influence of interactions between enzyme and substrate remote from the scissile bond, allowing predictions of approximate cleavage rates of Glu-containing peptide segments. Such information is useful for proper design of fusion proteins as well as for better predictability in the fragmentation of proteins prior to sequence analysis.

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