Chemical Pathways of Peptide Degradation. VI. Effect of the Primary Sequence on the Pathways of Degradation of Aspartyl Residues in Model Hexapeptides

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The influence of the primary sequence on the degradation of Asp[4] residues (e.g., formation of the cyclic imide and Asp-X and/or X-Asp amide bond hydrolysis) was investigated using Val-Tyr-Y-Asp-X-Ala hexapeptides. These reactions were proposed to involve cyclization, which would duly be sensitive to steric hindrance. The effects on the rates of individual degradation routes and product distribution under both acidic and alkaline conditions were assessed upon substitutions made on the C-terminal side (X) and on the N-terminal side (Y) of the Asp residue. As expected, the rate of intramolecular formation of cyclic imide and, thus, the product yield were most affected by the size of the amino acid on the C-terminal side of the Asp residue. However, such structural changes had little or no impact on the rate of Asp-X and Y-Asp amide bond hydrolysis. In the former case, the substituted site was one atom removed from the reaction site, accounting for the diminished steric effect observed. As for the latter, the site of substitution was not a participant in the reaction itself, and hence, the rate was unperturbed by this modification. Placing Ser and Val C terminally to the Asp residue prompted racemization and peptide bond hydrolysis to occur under alkaline conditions. N-Terminal substitution of Pro with Gly had no effect on the rate of isomerization via cyclic imide formation but greatly enhanced the rate of Y-Asp amide bond hydrolysis.

KEY WORDS: cyclic imide; isomerization; Asp-X amide hydrolysis; Y-Asp amide hydrolysis; primary sequence effect.

INTRODUCTION

Various chemical reactions are known to affect the properties of peptides and proteins (1). Understanding the fundamental factors modulating the chemical instability of polypeptides is enormously important and essential for their formulation and development as pharmaceutical products. In contrast to conventional organic molecules, polypeptides possess structural complexity, which tends to complicate their chemical behavior. Perturbations in the sequence and the conformation of the polypeptide structure have been implicated in altering significantly the chemical reactivity of proteins (2–4). Patel and Borchardt (5) were able to demonstrate the sequence specificity for the rate of deamidation of Asn[4] in a hexapeptide via a cyclic imide intermediate. It was observed that under neutral to basic conditions, the rate of deamidation was enhanced by substituting Gly on the C-terminal side of the Asn residue. In contrast, the rate was significantly diminished when a considerably more bulky Val residue was placed at this position. The authors attributed this finding to the extent of steric interference exerted by the substituted residue. The results reported by Borchardt and Patel (5) are consistent with data published earlier by other investigators (6–8). In addition to steric factors, enhancement in the rate of deamidation can also be achieved through intramolecular catalysis by hydroxyl and imidazole moieties in Ser and His, respectively (6,9).

Similarly, sequence specificity should also apply to the rate of degradation of the Asp residue under neutral to basic conditions, since the latter reaction also proceeds via the formation of a cyclic intermediate. Indeed, it has been demonstrated that the rate of formation of cyclic imide occurred rapidly when Gly, which imparts minimal steric hindrance to the nucleophilic attack, flanked the Asp on the carboxyl side (6,9,10). The substitution of Ser and His at the same site also rendered a substantial increase in the rate of succinimide formation, for these residues were able to participate as general base/acid catalysts (9–11). Except for the fact that the Asp-Pro peptide bond is known to be particularly acid-labile (12–14), limited literature exists regarding the effect of primary structure on the degradation of Asp-containing peptides under acidic conditions.

The intent of this study was to examine the influence of primary structure on the kinetics and mechanism of the degradation of Asp residues in model hexapeptides. Systematic modifications were made to amino acids that flank the Asp residue C- and N-terminally. The objective of this investigation was to determine the effects of C-terminal and N-terminal substitutions on the rates of formation of cyclic imide and Asp-X and/or Y-Asp amide bond hydrolysis and to assess the product distribution of the degradation of the hexapeptides. According to an earlier study (15), the Asp-Gly hexapeptide predominantly underwent Asp-X amide bond hydrolysis under highly acidic conditions and exclusively isomerized via cyclic imide intermediate at alkaline pH at 37°C. Thus, the kinetic studies on these hexapeptide analogues were conducted at pH 1 and 10 to demarcate the influence of primary sequence on the formation of cyclic imide and Asp-X and/or Y-Asp amide bond hydrolysis.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade and used as received from the commercial suppliers. Sodium chloride, sodium hydroxide, HPLC-grade acetonitrile, and hydrochloric acid were purchased from Fisher Scientific (Fair Lawn, NJ). HPLC-grade trifluoroacetic acid was obtained from Pierce

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[4] Unless otherwise noted, all amino acids listed are L enantiomers of the 20 common amino acids and referred to by their three-letter abbreviations. Asu is used as an abbreviation of the cyclic imide form of Asp.

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Chemicals (Rockford, IL). Trizma base, porcine liver esterase, and sodium borate were purchased from Sigma Chemical Company (St. Louis, MO).

The Asp-Gly hexapeptide (L-Val-L-Tyr-L-Pro-L-Asp-L-Gly-L-Ala) was synthesized by Dr. Madhup Dhaon (Abbott Laboratories, North Chicago, IL). L-Val-L-Tyr-L-Pro-L-Asu-L-Gly-L-Ala (Asp-Gly cyclic imide), a major side product in the synthesis of the Asp-Gly hexapeptide, was isolated during reversed-phase preparative purification of the Asp-hexapeptide. L-Val-L-Tyr-L-Pro-L-Asp-L-Ser-L-Ala (Asp-Ser hexapeptide), L-Val-L-Tyr-L-Pro-L-Asp-L-Val-L-Ala (Asp-Val hexapeptide), and L-Val-L-Tyr-L-Gly-L-Asp-L-Gly-Ala (Gly-Asp hexapeptide) were purchased from Peninsula Laboratories (Belmont, CA). L-Val-L-Tyr-L-Pro, L-Val-L-Tyr, L-Ser-L-Ala, L-Val-L-Tyr-L-Pro-D-Asp-L-Val-L-Ala, and L-Val-L-Ala were obtained from Bachem Bioscience Inc. (Philadelphia, PA). L-Val-L-Tyr-L-Pro-L-Asp, L-Gly-L-Ala, L-Asp-L-Gly-L-Ala, L-Val-L-Tyr-L-Gly-L-Asp, L-Val-L-Tyr-L-Gly, L-Asp-L-Ser-L-Ala, and L-Asp-L-Val-L-Ala were synthesized by the Biochemical Service Laboratory at the University of Kansas, Lawrence.

HPLC Equipment

The HPLC system consisted of Shimadzu LC-6A pumps, a SCL-6A controller, a CR4A Chromatopac integrator, a SPD-6A UV detector, and an ISS-100 Perkin Elmer autoinjector. All peptides were purified using a semipreparative Hypersil ODS column (10 × 250 mm, 10-μm resin). Analytical separations of peptides and their degradation products were performed on Hypersil ODS C_{18} (4.6 × 250-mm, 5-μm resin) and Spherisorb C_{18} (4.6 × 50-mm, 3-μm resin) columns.

Buffer Solutions

The buffers used were as follows: 0.08 *M* HCl; pH 1.1; and 0.05 *M* sodium borate, pH 10.0. The pH adjustments of the buffer solutions were made with hydrochloric acid and sodium hydroxide. A constant ionic strength of 0.5 *M* was maintained for each buffer by adding an appropriate amount of sodium chloride. All the buffers were prepared at 70°C. The pH readings were recorded using a POPE Model 1501 pH/ion meter.

Kinetic Measurements

All kinetic experiments were carried out in aqueous buffer solutions at 70°C. A sufficient quantity of purified hexapeptide was added to 5.0 mL of buffer solution to yield an initial concentration of 2×10^{-4} *M*. Aliquots of 250 μL of the resulting solution were then transferred to 1-mL ampoules, flame-sealed, and stored in the oven. The kinetics studies of C- and N-terminally substituted analogues (Val-Tyr-*Y*-Asp-*X*-Ala, where *X* = Gly, Ser, Val and *Y* = Gly) were carried out at pH 1.1 (0.08 *M* HCl) and pH 10.0 (0.05 *M* borate), *m* = 0.5 *M*, at 70°C. At various intervals, ampoules were removed, cooled, and frozen prior to being analyzed by HPLC.

HPLC Analysis

Peptide analyses were performed on the HPLC system described above. The separation methodologies used for all peptides were as follows: Asp-Gly hexapeptide [mobile phase, 12% acetonitrile, 0.1% (v/v) trifluoroacetic acid (TFA) in water; flow, 1 mL/min; column, Hypersil ODS C_{18} (4.6 × 250 mm, 5 μm)], Asp-Ser hexapeptide [mobile phase, 14% methanol, 0.1% (v/v) TFA; flow, 1 mL/min; column, Spherisorb C_{18} (4.6 × 50 mm, 3μm)], Asp-Val hexapeptide (pH 1.1) [mobile phase, 15% methanol, 0.1% (v/v) TFA; flow, 1.4 mL/min; column, Spherisorb C_{18} (4.6 × 50 mm, 3 μm)], Asp-Val hexapeptide (pH 10.0) [mobile phase, 12% acetonitrile, 0.1% (v/v) TFA; flow, 1.3 mL/min; column, Hypersil ODS C_{18} (4.6 × 250 mm, 5 μm)], and Gly-Asp hexapeptide [mobile phase, 8% methanol, 0.1% (v/v) TFA; flow, 1 mL/min; column, Spherisorb C_{18} (4.6 × 50 mm, 3 μm)]. UV detection of eluents was performed at 214 nm. The peptides were quantitated by measuring the peak areas.

Characterization of Degradation Products

All purchased peptides were purified and subjected to FAB-mass spectrometry for identity confirmation. These peptides were then used as HPLC standards to identify degradation peaks. The isoAsp peptides were generated by deamidation of corresponding Asn peptides in ammonium formate solution at pH 10.0 and 70°C. The peaks that were supposedly isoAsp peptides were purified by semipreparative HPLC, lyophilized, and analyzed by FAB-mass spectrometry. These were used as standard samples in identification of kinetically produced isoAsp peptides.

Both FAB-mass spectrometry and esterase hydrolysis studies were used in an attempt to identify a product formed during the degradation of the Asp-Ser hexapeptide at pH 10.0. The FAB-mass spectrum of this product showed a major molecular ion (MH^+) at 651, which corresponds not only to the starting L-Asp-Ser hexapeptide but also to the D-Asp-Ser hexapeptide and Asp-isoSer hexapeptide (O-peptide) resulting from racemization and N,O-acyl migration reactions, respectively. Since FAB-mass spectrometry alone could not distinguish between these two possible candidates, an esterase hydrolysis study was carried out on the degradation product. Approximately 1 μmol of peptide in 1 mL of 50 m*M* Tris buffer, pH 8.0, was incubated with approximately 120 U of a porcine esterase (220 U/mg) for 48 hr at 25°C. Incubation of a negative control, a mixture of the starting L-Asp-Ser hexapeptide and esterase in buffer, was also performed. Analysis was carried out on a C_{18} reversed-phase HPLC column (Spherisorb, 4.6 × 50 mm, 3 μm) using a mobile phase consisting of 14% methanol/0.1% (v/v) TFA in water.

Determination of Kinetic Rate Constants

The kinetic rate constants were generated by obtaining the best fit of the experimental data using a nonlinear least-squares regression (MINSQ, Salt Lake City, UT). The data set for the kinetics of degradation of Asp-Ser and Asp-Val hexapeptides at pH 10.0 was fitted using LaPlace MicroMath (Salt Lake City, UT). The errors associated with the data presented in Figs. 1 and 2 were estimated to be ±5.

RESULTS AND DISCUSSION

Effect of C-Terminal Substitution

Degradation at an Acidic pH Value

For the Asp-Gly hexapeptide and the C-terminally modified analogues (Asp-Ser and Asp-Val hexapeptides) studied

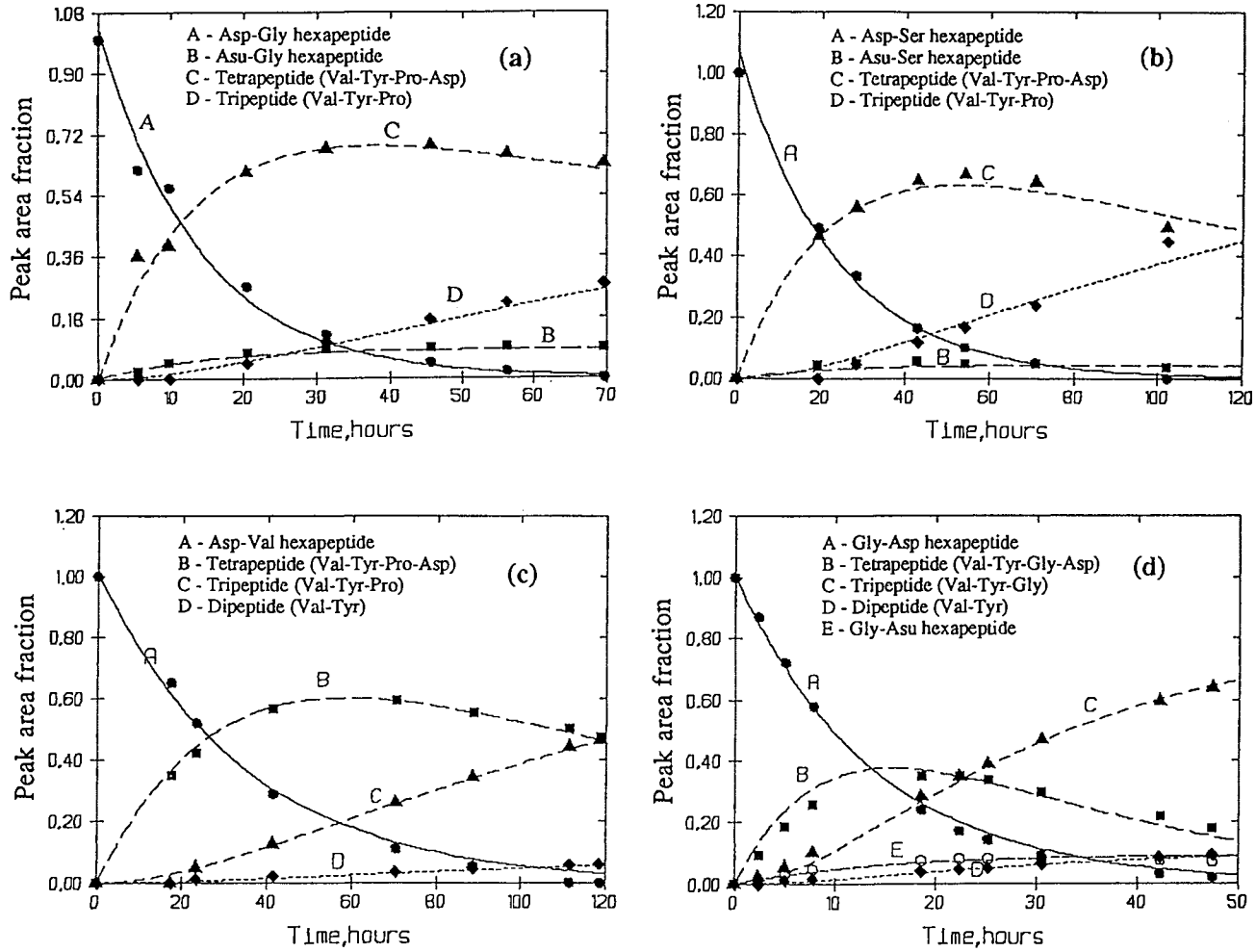


Fig. 1. Kinetic profiles for the degradations of (a) Asp-Gly hexapeptide, (b) Asp-Ser hexapeptide, (c) Asp-Val hexapeptide, and (d) Gly-Asp hexapeptide at pH 1.1, μ = 0.5, and 70°C. The points represent experimental data and the lines are calculated from nonlinear least-squares curve-fitting.

at pH 1.1, the hydrolysis of Asp-X amide bond constituted the major degradation route (Figs. 1a–c). With the Asp-Gly and Asp-Ser hexapeptides, the cyclic imides (Asu-Gly and Asu-Ser hexapeptides) were also formed, but in lower amounts compared to the Asp-X amide bond hydrolysis products. Insignificant amounts of the Asu-Val hexapeptide were formed from the Asp-Val peptide (Fig. 1d). It should be noted that the tetrapeptides derived from the cleavage of the Asp-X peptide bonds underwent further fragmentation (Y-Asp amide hydrolysis) to yield tripeptides under the experimental conditions (Scheme I). An independent hydrolysis study of the isolated tetrapeptide (Val-Tyr-Pro-Asp) was conducted to confirm that in fact the tripeptides and, in the case of Asp-Val hexapeptide, the dipeptide fragment (Val-Tyr) were generated mainly from the tetrapeptides themselves rather than the starting hexapeptides (Scheme I). These results were not inconsistent with the view that C-terminal peptide bond fission (Asp-X hydrolysis) would be relatively more facile than N-terminal bond fission (Y-Asp hydrolysis), since the former would proceed via an intermediate containing a five-membered ring whose formation is more entropically favorable than that of a six-membered ring (16) that is characteristic of the latter reaction (17). Moreover, the rigid proline residue on the N-terminal side to Asp would, to a certain extent, hinder the cyclization process, further limiting the fission of the Pro-Asp peptide bond. This hypothesis was supported by the results obtained from the N-terminal substitution of Pro with a more flexible residue such as Gly. Considering these two factors together, it was not surprising to note that the starting hexapeptides were cleaved predominantly at the Asp-X bond for all peptides studied. At the level of tetrapeptides, however, C-terminal peptide bond fission was not possible, rendering Y-Asp amide bond hydrolysis the major alternative. For Asp-Ser and Asp-Val hexapeptides, a less significant alternate cleavage reaction at the Tyr-Y peptide bond of tetrapeptide Val-Tyr-Pro-Asp was detected (Figs. 1b and 1c).

Substituting the Gly residue with increasingly large, bulky amino acids made a diminutive, albeit still notable, impact on the hydrolysis of Asp-X amide bond. The Asp-Gly hexapeptide hydrolyzed only 1.6 times faster than the Asp-Ser peptide and 2.3 times faster than the Asp-Val hexapeptide (Table I). The fact that the C-terminal replacement of Gly residue caused any perturbance at all in the rate of

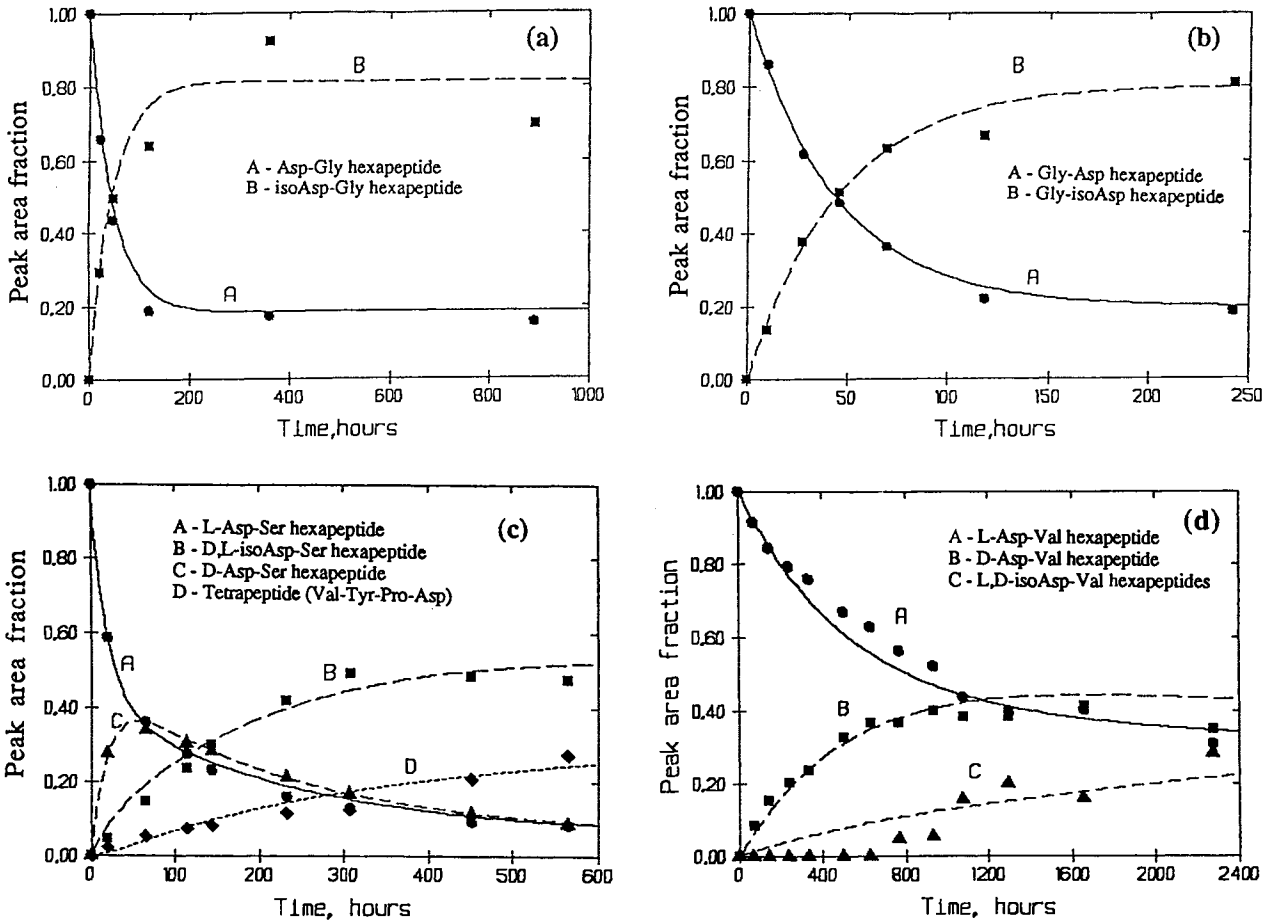


Fig. 2. Kinetic profiles for the degradations of (a) Asp-Gly hexapeptide, (b) Gly-Asp hexapeptide, (c) Asp-Ser hexapeptide (identity of C is tentative), and (d) Asp-Val hexapeptide at pH 10.0 (0.05 *M* borate), μ = 0.5, and 70°C. The lines drawn through the experimental data points are calculated from nonlinear least-squares curve-fitting.

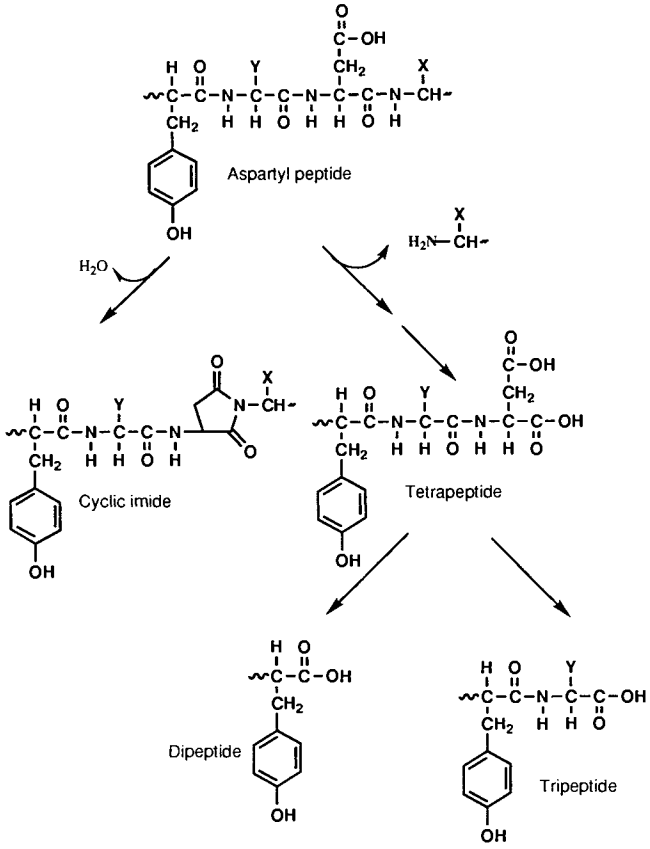
Asp-X amide bond hydrolysis supports the view that this reaction may involve an intramolecular rearrangement leading to the formulation of a cyclic anhydride intermediate, as proposed earlier (15). The effect of the primary sequence on the rate was not pronounced because, unlike the case of cyclic imide formation, the reaction site of Asp-X amide hydrolysis is one atom removed from the potentially interfering side chain of the succeeding amino acid. Nevertheless, if the substituted amino acid was sufficiently large, it could still interfere with the attack by hindering the approach of the nucleophilic species toward the carbonyl center.

Notably, the formation of cyclic imide was more sensitive to the modification made on the C-terminal side because of the closer proximity between the reaction site and the substituted site. When the substituted residue became increasingly more bulky, the rate constants of cyclic imide formation decreased (Table I), indicating that the intramolecular cyclization was effectively impeded by more sterically hindered neighboring residue. Thus, when Gly was replaced with Ser, the rate constant for the formation of cyclic imide decreased by nearly fourfold (Table I), concurring with the results obtained by Stephenson and Clarke (6). Placing Val at the same position diminished the rate of cyclic imide formation even further, such that no cyclic imide was detected (Figs. 1c). The steric factor evidently prevailed as a governing force, based on the observed order of reactivity (Asp-Gly > Asp-Ser ≫ Asp-Val). This ranking is consistent with what Patel and Borchardt found when they examined the influence of primary sequence on the deamidation of Asn-Gly hexapeptide at neutral and basic pH values, where the formation of cyclic imide was involved (5).

The trend of product distribution at pH 1.1 correspondingly reflected that of the rate constants. As the Ser residue C-terminally replaced Gly, the quantity of cyclic imide produced decreased by 5%, while that of tetrapeptide remained relatively constant (Table II). There was an even further reduction in the amount of cyclic imide generated when the more bulky Val was placed in the position of Gly residue (Table II). In contrast, the amount of the tetrapeptide formed did not change significantly (Table II).

Degradation at an Alkaline pH

The degradation of Asp residues at pH 10.0 and 70°C involved predominantly interconversion of Asp to isoAsp via the cyclic imide intermediate (isomerization) for all C-terminally substituted peptides, except the Val-containing analogue (Scheme II and Figs. 2a, c, and d). As expected, the rate of isoAsp formation decreased significantly when Gly



Scheme I. Degradation pathways at pH 1.1 and 70°C.

was substituted with Ser and Val (Table III) due to steric hindrance reducing the ease of formation of cyclic imide, the slow step in the kinetic scheme. The most rapid interconversion occurred with the Asp-Gly sequence, which isomerized three times faster than the Asp-Ser hexapeptide and almost two orders of magnitude faster than the Asp-Val hexapeptide (Table III). Consequently, the ratios of isoAsp/Asp for the Asp-Gly and Asp-Ser hexapeptides were relatively similar (4 to 5), whereas the ratio was less than 1 for the Val hexapeptide analogue (Table II).

It was thought that initial rapid racemization of the starting Asp-containing hexapeptide occurred when Gly was replaced with either Ser or Val C-terminally to the Asp residue (Figs. 2c and d). The identity of D-Asp-Val hexapeptide was confirmed by its reversed-phase retention property in relation to that of a standard and by FAB-mass spectrometry. In the case of the Ser-containing analogue, product C was suspected to be the D epimer of the Asp-Ser hexapeptide (Fig. 2c) based on the results from the esterase cleavage study and FAB-mass spectrometry. There was no evidence of cleavage of the degradation product C by esterase to generate the tetrapeptide (Val-Tyr-Pro-Asp). However, the absence of cleavage could be interpreted as follows: (a) Product C was not the Asp-isoSer hexapeptide (O-peptide) or (b) the supposedly Asp-isoSer hexapeptide was perhaps not the substrate for this particular esterase. Although these results did not by any means unambiguously confirm the identity of product C, they are also not inconsistent with the view that product C could be the D-Asp-Ser hexapeptide. Further support for racemization was derived from the fact that under alkaline conditions, most O-peptides reportedly undergo rapid base-catalyzed ester hydrolysis upon formation to yield fragmentation products, thus they are not usually detectable (18,19). It is plausible to suggest that the Asp-isoSer hexapeptide intermediate might have been involved in the observed production of tetrapeptide as suggested in Scheme III but was too unstable at pH 10.0 to be detected. Interestingly, the suspected D-Asp-Ser hexapeptide was formed rapidly, leveled off after approximately 80 hr, and then degraded at the same rate as the L-Asp-Ser hexapeptide, as evidenced by their overlapping terminal slopes (Fig. 2c). In contrast, the D-Asp-Val epimer did not undergo degradation as rapidly as did the D-Asp-Ser hexapeptide but remained at a relatively constant level (Fig. 2d). However, no epimerized products were detected with the Asp-Gly hexapeptide, probably because the competing hydrolysis of the cyclic imide, affording the L epimers of Asp-Gly and isoAsp-Gly hexapeptides, was much more rapid than racemization.

Table I. Summary of Rate Constants for Degradations of Val-Tyr-*Y*-Asp-*X*-Ala at pH 1.1 and 70°C

	Rate constant (hr^{-1}) × 10^3			
Peptide	CI formation[a]	Tetrapeptide formation[b]	Tripeptide formation[c]	Dipeptide formation[d]
X = Gly *Y* = Pro	6.1 ± 0.9	66.0 ± 2.2	5.9 ± 0.3	—
X = Ser *Y* = Pro	1.8 ± 0.5	41.0 ± 1.3	6.1 ± 0.3	—
X = Val *Y* = Pro (negligible)	1.0×10^{-7}	29.0 ± 0.6	7.8 ± 0.2	1.0 ± 0.2
X = Gly *Y* = Gly	6.6 ± 0.6	65.0 ± 1.4	50.0 ± 1.3	6.6 ± 0.8

[a] Formation of cyclic imide.
[b] Formation of tetrapeptide (Asp-X hydrolysis).
[c] Formation of tripeptide (Y-Asp hydrolysis).
[d] Formation of dipeptide fragment (Val-Tyr).

Table II. Effect of the Primary Sequence of Val-Tyr-*Y*-Asp-*X*-Ala on the Distribution of Degradation Products

Peptide	pH 1.1[a] % cyclic imide	pH 1.1[a] % tetrapeptide	pH 10.0,[a] IsoAsp/Asp ratio
X = Gly *Y* = Pro	9	63	4
X = Ser *Y* = Pro	4	65	5
X = Val *Y* = Pro	ND[b]	60	<1
X = Gly *Y* = Gly	9	20	4

[a] Peptides were incubated at 70°C for 70 hr.
[b] The Asu-Val hexapeptide was not detected by HPLC.

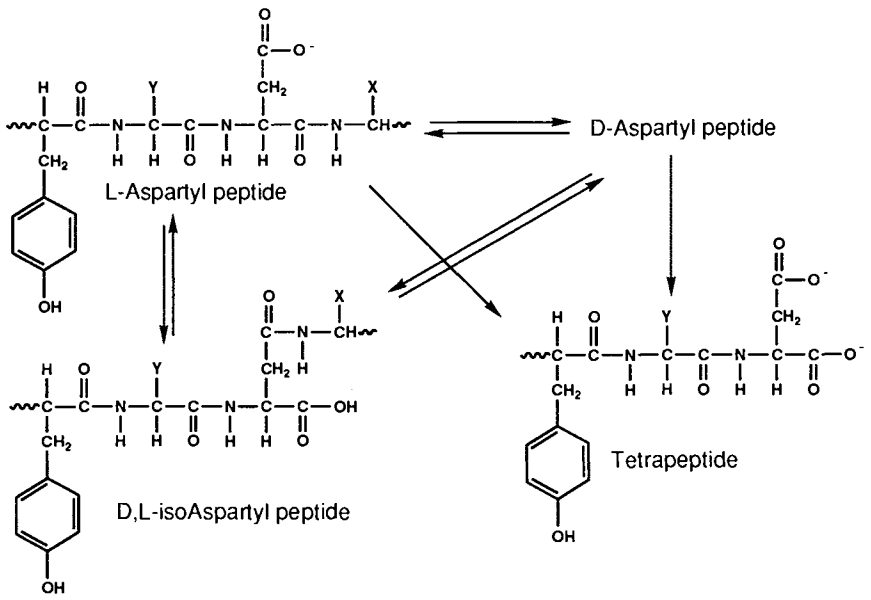
Racemization at the α carbon occurs most favorably in the cyclic imide structure, which imparts resonance stability to the potential formation of a carbanion (8). Other mechanisms include direct proton abstraction from the L-Asp or L-isoAsp peptides. It was noted that the L-Asp-Ser hexapeptide appeared to isomerize and racemize 29 and 25 times, respectively, faster than the L-Asp-Val hexapeptide (Table III). Evidently, increasing the steric hindrance by substituting Ser with Val lowered the rates of both reactions by approximately the same factor. This result tends to suggest that racemization in this case proceeded predominantly through the same cyclic imide intermediate present in the isomerization reaction.

Finally, placing Ser in the position of the Gly residue introduced an alternate degradation pathway at alkaline pH values. This pathway involved hydrolyzing the Asp-Ser amide bond generating the tetrapeptide Val-Tyr-Pro-Asp (Scheme II and Fig. 2c). The hydrolytic route was not observed with any other hexapeptide analogues under alkaline conditions. Two plausible mechanisms, both of which involved the Ser hydroxyl side chain that has been shown to participate in various types of intramolecular catalyses (9,20), were postulated to account for the formation of tetrapeptide at alkaline pH. The first mechanism involves intramolecular general acid catalysis by the hydroxyl functionality in the attack of the carbonyl carbon of the Asp residue by the carboxylate anion of Asp side chain (Scheme IIIa). This would lead to the formation of the cyclic anhydride intermediate, which rapidly hydrolyzes to form the tetrapeptide and dipeptide fragments. The second mechanism is characterized as the N,O-acyl migration reaction, in which the hydroxyl group participates as an intramolecular nucleophile, attacking the carbonyl carbon of the Asp residue to produce the Asp-isoSer hexapeptide intermediate (Scheme IIIb). This intermediate then undergoes ester hydrolysis to yield the tetrapeptide and dipeptide fragments. Since the mechanisms are kinetically indistinguishable, it is not possible to differentiate the two based on the kinetic data.

Effect of N-Terminal Substitution

Degradation at an Acidic pH

As a result of N-terminal replacement of Pro with a relatively less hindered Gly residue, the rate of N-terminal peptide bond fission (Y-Asp amide bond hydrolysis) was increased by a factor of eight, relative to the Asp-Gly hexapeptide (Table I, Fig. 1d). This is in good agreement with the postulation that the hydrolysis of Y-Asp peptide bond proceeds via a six-membered cyclic anhydride intermediate (17). Such an increase in the rate merely reflected the intramolecular nature of the reaction, which was appropriately sensitive to the steric effect. This ring closure was perhaps facilitated by relief of steric hindrance and added flexibility in the backbone conformation such that the tripeptide surpassed the customarily predominant tetrapeptide to become the major degradation product at pH 1.1 (Fig. 1d). Additionally, hydrolysis at the Tyr-Y amide bond also experienced an



Scheme II. Degradation pathways at pH 10.0 and 70°C.

Table III. Summary of Rate Constants for Degradations of Val-Tyr-*Y*-Asp-*X*-Ala at pH 10.0 and 70°C

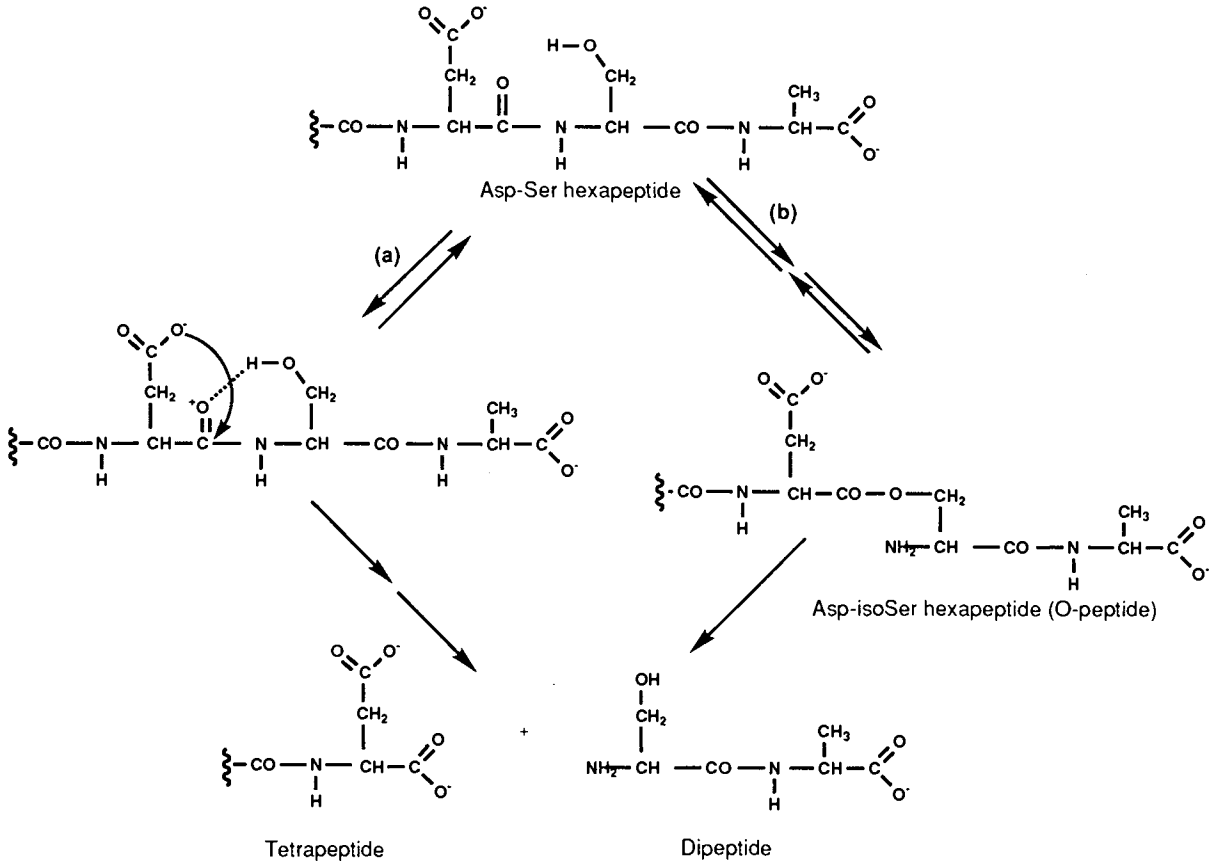
Peptide	Rate constant (hr^{-1}) × 10^3					
	Formation of DL-isoAsp peptides	Regeneration of L-Asp from isoAsp peptides	Formation of D-Asp peptides	Regeneration of L-Asp from D-Asp peptides	Tetrapeptide formation from D-Asp peptides	Tetrapeptide formation from L-Asp peptides
X = Gly *Y* = Pro	18.0 ± 2.1	4.0 ± 1.0	—	—	—	—
X = Gly *Y* = Gly	18.0 ± 0.7	4.4 ± 0.6	—	—	—	—
X = Ser *Y* = Pro	5.8	0.8	27.0	26.0	1.1	1.1
X = Val *Y* = Pro	0.2	4.0×10^{-2}	1.0	0.9	—	—

increase in rate due to the replacement of the rigid Pro residue (Table I). It was confirmed, in a manner similar to that used with C-terminally modified analogues, that Val-Tyr and Val-Tyr-Gly were generated mainly from Val-Tyr-Gly-Asp itself (Scheme I).

As anticipated, N-terminal substitution did not perturb the rates of Asp-X hydrolysis and cyclic imide formation (Table I) since the residue which flanks the Asp residue N-terminally was not involved in either reaction. However, while N-terminal modification had no impact on the yield of cyclic imide, the quantity of generated tetrapeptide was much reduced (Table II). This reduction resulted because of a substantial enhancement in the rate of Y-Asp amide bond hydrolysis, probably at both the hexapeptide and the tetrapeptide levels, and that of the Tyr-Y peptide bond hydrolytic route.

Degradation at an Alkaline pH

At pH 10.0, the Gly-Asp hexapeptide predominantly isomerized to form the Gly-isoAsp hexapeptide (Fig. 2b) at a ratio of 4:1 in favor of the isoAsp peptide (Table II). Evi-



Scheme III. Serine-catalyzed formation of tetrapeptide via (a) intramolecular general acid catalysis and (b) intramolecular nucleophilic catalysis.

dently, N-terminal substitution had no impact on the interconversion kinetics since the formation of the cyclic imide was not sensitive to the type of modification in which the substituted amino acid was not involved in the reaction.

SUMMARY AND CONCLUSION

The influence of primary sequence on the chemical reactivity of the model hexapeptides was satisfactorily employed in this study to probe the mechanism of individual degradation pathways. At pH 1.1, all peptides studied underwent two parallel routes of degradation, formation of cyclic imide and Asp-X hydrolysis. C-Terminal substitution of Gly with increasingly more bulky residues (Ser, Val) noticeably perturbed the rate of cyclic imide formation and the amount of cyclic imide being produced. This finding is consistent with the concept that Gly residue imparts minimal steric interference and maximum flexibility, allowing the reaction site to adopt conformations that are more favorable for cyclization. The rate of Asp-X hydrolysis was affected only slightly by C-terminal modification since the reaction site of this degradation pathway is remote from the potentially interfering side chain of the neighboring amino acid.

A substantial increase in the rate of Y-Asp hydrolysis was observed as a result of N-terminal replacement of Pro with Gly residue. The relief of steric hindrance and added flexibility in the backbone conformation must have facilitated the cyclization reaction to generate the postulated six-membered cyclic anhydride intermediate. No structural influence on the rates of formation of cyclic imide and tetrapeptide was noted since the substituted site did not participate in either reaction. However, N-terminal modification rendered the competing Y-Asp amide bond hydrolysis more facile such that the tripeptide emerged as the predominant product of the Gly-Asp hexapeptide at pH 1.1.

At pH 10.0, for all peptides studied, except for the Asp-Val hexapeptide, isomerization of the Asp residue occurred to generate the isoAsp peptides as the major products. Significant racemization was observed only with the Asp-Val and, possibly, Asp-Ser hexapeptides. While the D epimer of Asp-Val hexapeptide remained relatively stable upon formation, the D-Asp-Ser hexapeptide underwent further degradation at a rate that was comparable to that of the L-Asp-Ser hexapeptide. The ratios of isoAsp to Asp were similar for all analogues, except that containing Val, for which the ratio was less than 1. At alkaline pH values, the introduction of the Ser residue C-terminally gave rise to an alternate hydrolytic pathway which might involve the Ser hydroxyl moiety as an intramolecular general acid and/or intramolecular nucleophile.

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