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Contribution from the Institute of Pharmaceutical Sciences,
Hiroshima University School of Medicine, Kasumi, Hiroshima-shi, Japan

Sequential Hydrolysis of Peptides with β-Hydroxoaquotriethylenetetraminecobalt(III) Ion

EIICHI KIMURA

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Hydrolysis of various peptides with β-Co(trien)OH$(H_2O)^{2+}$, I, according to Scheme I has been studied for the purpose of developing an analytical method of sequential degradation of peptides. Initial chelation of peptides with I can be accomplished without much difficulty. Hydrolysis of II occurs fairly smoothly with the majority of the tested peptides. However, several peptide complexes containing proline or amino acids with a free carboxyl group, a potential ligand, strongly resisted the hydrolysis, which might present the most serious problem in this method. A solid-state method which runs the hydrolysis on weak cationic exchange resin, as illustrated in Scheme II, has demonstrated a partial success in the stepwise degradation.

Introduction

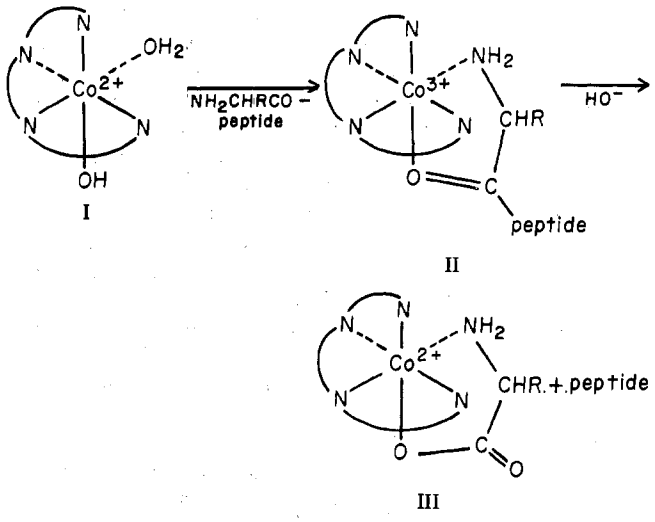
Cobalt(III) complex cations, *cis*-Co(tetramine)OH$(H_2O)^{2+}$, with two cis reactive sites, promote selective N-terminal hydrolysis of peptides.[1-6] The mechanism involves interaction of the two vacant sites with a free N-terminal peptide to form a carbonyl coordinated complex, through which the activated peptide bond is hydrolyzed ~10^6 times more rapidly than uncoordinated ones.[7-10]

Because of the N-terminal specificity and a large rate enhancement effect, the cobalt complex, $CoN_4OH(H_2O)^{2+}$, was often compared with the natural amino peptidases.[9] The main features of the model reaction are that the inorganic complex functioning like a catalyst simultaneously takes up N-terminal amino acid in a stable chelate ion, $CoN_4(AA)^{n+}$, and that no reactive species regenerates after the one cycle.

In view of those unique features and its simplicity compared with current chemical procedures,[11] we had started investigating its potentialities for the N-terminal determination and sequential analyses of polypeptides. The matters to be investigated were (1) to know if there are substrate specificities, since peptides with potentially coordinating side chains, such as $-NH_2$, -SH, or $-CO_2^-$, may seriously interfere with the chelate catalytic action, (2) to seek the optimum reaction conditions and the product separation and identification method on an analytical scale, and (3) to identify problems in applying the method to tri- or longer peptides.

Very recently in similar studies to evaluate its potential, Fenn and Bradbury reported several problems, particularly the slow rate of the complex formation of a sterically hindered peptide, Ala-Leu-Glyol, though the subsequent hydrolysis step occurred as fast as for other simple peptides.[12] In an analogous investigation Girgis and Legg observed that Asp-Gly underwent little hydrolysis with the two-site complex.[13] It has not been clarified whether this is due to the failure of the initial interaction of the peptide with the metal complex or the lack of the subsequent hydrolysis step. Herein are reported our results as well as the scope and limitation of this technique for the stepwise degradation of peptides.

Scheme I



Results and Discussion

Dipeptides. A variety of dipeptides were subjected to hydrolysis for the purpose of investigating substrate specificities and finding a suitable analytical method using minimum amounts of peptides. We first employed the earlier experimental conditions.[1,2]

Reaction of dipeptide (1 μmol) and β-Co(trien)OH$(H_2O)^{2+}$, I,[14] where [reactants] = 10^{-3}-10^{-2} *M*, formed Co(trien)(dipeptide)$^{n+}$, II, in ~10 min at 60° in a pH 7-8 buffer solution (2,4,6-collidine-$HClO_4$). Other buffer systems which contained coordinating agents such as carbonate, phosphate, or tris(hydroxymethyl)aminomethane were sometimes found to interfere seriously with this process. The visible color changed from the initial pink (λ_{max} 508 and 360 nm) characteristic of I to orange (λ_{max} 490 and 345 nm) smoothly, indicating the conversion to the CoN_5O complex.[7] The formation of the peptide complex II was also followed by tlc on silica gel. When the reaction mixture was eluted

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(12) M. D. Fenn and J. H. Bradbury, *Anal. Biochem.*, 49, 498 (1972).
(13) A. Y. Girgis and J. I. Legg, *J. Amer. Chem. Soc.*, 94, 8420 (1972).
(14) The abbreviation "trien" is used throughout this paper for triethylenetetramine.

with 1-butanol-acetic acid-water (4:1:1) or 2-propanol-water (7:3), the cobalt-coordinated species II remained on the starting line but the free peptides and amino acids moved in the normal manner. With the development of the orange color, the free peptide was disappearing but no cleaved amino acid residue was seen yet at this stage. Application of 1 drop of Na_2S aqueous solution to the orange spot on a silica gel plate immediately freed the peptide from chelation, which then could be eluted in a normal way.

After 10–20 min the dipeptide complex II started to decompose to the final chelate product III and the residual amino acid, both of which were easily identified by tlc. The visible spectral change helps little in pursuing this conversion since II and III have indistinguishable electronic absorptions.[7] The completion of the hydrolysis of the peptide complex, as was known by the disappearance of the chelated dipeptide on tlc, took generally more than 4 hr under the employed conditions. We found identification of the N-terminal amino acid was more convenient as free amino acid than as its complex, because of versatility and sensitivity (practically 0.002 μmol of free amino acids was enough for semiquantitative identification on tlc). Thus one could determine the sequence of dipeptides with a quantity less than 1 μmol and even before the hydrolysis completes.

The dipeptides which were hydrolyzed by I included Gly-Gly, Gly-Ala, Gly-Leu, Gly-Phe, Gly-Ser, Gly-His, Gly-Asp, Ala-Gly, Ala-Leu, Leu-Gly, Leu-Tyr, Phe-Gly, Trp-Gly, Trp-Ala, Trp-LeuNH_2, His-Gly, His-Tyr, Glm-Gly, Glu-Phe, Pro-Gly, and Lys-Gly. The yields were measured and are tabulated in Table I. The analytical procedure involved product separation by ppc or tlc and development of the ninhydrin color, whose densities at 560 nm were measured against standards with a transmission densitometer.

The studies using dipeptides showed, in the first place, that the N-terminal amino acids with ligating side chains, such as aspartic acid, glutamic acid, lysine, or histidine do not prohibitively interfere with this process, though the hydrolysis rate generally decreased. Girgis and Legg saw almost no hydrolysis with Asp-Gly at pH 7.5.[13] We found that Asp-Arg coordinated to the cobalt ion but the subsequent hydrolysis hardly occurred in pH 7.4 collidine buffer. Higher concentration of OH^- ion was required to cause the cleavage. The analogous reaction pattern was seen with Glu-Phe. β-Ala-Nle was found to be slowly hydrolyzed only with the presence of a large excess complex.

Second, the C terminus has a marked effect on the hydrolysis rate, as in the case of Pro-Leu, Gly-Pro, or Gly-Asp, which was earlier found by Girgis and Legg.[13] The tlc revealed that those peptides coordinated to the cobalt ion, as fast as other dipeptides, but the following reactions especially of the Pro-Leu and Gly-Pro complex were so slow and that even after 4 hr of reaction time at pH 7.4 and 60° no cleavage was detected.

Third, the treatment of dipeptides with equimolar complex I did not always quantitatively yield the intermediate complexes II. While Gly-Gly or Ala-Gly upon mixture with an equimolar amount of complex I turned into the chelate complex nearly completely within ~10 min at 60°, a considerable amount of Leu-Gly or Trp-Ala remained uncoordinated. It is not certain whether this is due to a thermodynamic reason (*e.g.*, peptides with bulky substituents disfavor the complex formation) or a kinetic reason (*e.g.*, the coordination of the sterically hindered peptides is slow). Those dipeptides with a sterically crowded group required more than a 3 molar ratio of the complex I for their complete chelation at pH 7.5 and 60°, which is reflected in the yield of N-terminal cleavage product, as seen in Table I.

Table I. Yields of N-Terminal Cleavage Reactions Using Co(trien)(OH)$(H_2O)^{2+}$

Peptide[a]	10^3 × [complex], *M*	Product (yield, %)
Gly-Gly	8	Gly (48 × 2)
Gly-Leu	8	Gly (38), Leu
Gly-Leu	25	Gly (44), Leu
Gly-Phe	8	Gly (24), Phe (24)
Gly-Phe	25	Gly (49), Phe (51)
Gly-PheNH_2	8	Gly (39), PheNH_2, Phe
Gly-PheNH_2	25	Gly (45), Phe
Gly-Asp	8	Gly (12), Asp
Gly-Asp	25	Gly (20), Asp
Gly-His	8	Gly (23), His
Gly-His	25	Gly (42), His
Gly-Pro	8	None
Ala-Gly	8	Ala, Gly (47)
Ala-Gly	25	Ala, Gly (45)
Ala-Leu	8	Ala (28), Leu (24)
Leu-Gly	8	Leu, Gly (30)
Leu-Gly	25	Leu (52), Gly (49)
His-Tyr	8	His, Tyr (23)
Trp-Ala	8	Trp (17), Ala
Trp-Ala	25	Trp (44), Ala
Glm-Gly[b]	8	Glm, Gly (25)
Glu-Phe	8	Almost none
Glu-Phe[c]	25	Glu, Phe (30)
Asp-Arg	8	Asp (trace), Arg
Asp-Arg[c]	25	Asp (10), Arg
β-Ala-Nle	8	None
β-Ala-Nle	25	β-Ala (10), Nle
Gly-Gly-Gly	8	Gly (43), Gly-Gly
Gly-Gly-Ala	8	Gly (38), Gly-Ala
Gly-Leu-Tyr	8	Gly (11), Leu-Tyr
Gly-Leu-Tyr	25	Gly (12), Leu-Tyr
Ala-Gly-Gly	8	Ala (37), Gly-Gly
Leu-Gly-Gly	8	Leu (17), Gly-Gly, Gly
Leu-Gly-Gly	25	Leu (42), Gly-Gly, Gly
Leu-Gly-Phe	8	Leu (25), Gly-Phe
Leu-Gly-Phe	25	Leu (51), Gly-Phe, Gly, Phe
Glutathione	8	None
Gly-Gly-Gly-Gly	8	Gly (27), Gly-Gly-Gly

[a] Reaction conditions: [peptides] = 8×10^{-3} *M*, 61°, pH 7.4 in 0.1 *M* collidine buffer solution, reaction time 4 hr. [b] Glutaminylglycine. [c] pH 9.5 in triethylamine buffer.

Tri- and Polypeptides. Reaction of I with an equimolar amount of tri- or tetrapeptide (1 μmol) in pH 7.5 collidine buffer at 60° yielded the corresponding peptide complex II, but an appreciable amount of peptide stayed uncoordinated even after the hydrolysis of II started. A large excess of complex I (~5 μmol) drove the intermediate formation to completion in analogy to the dipeptides.

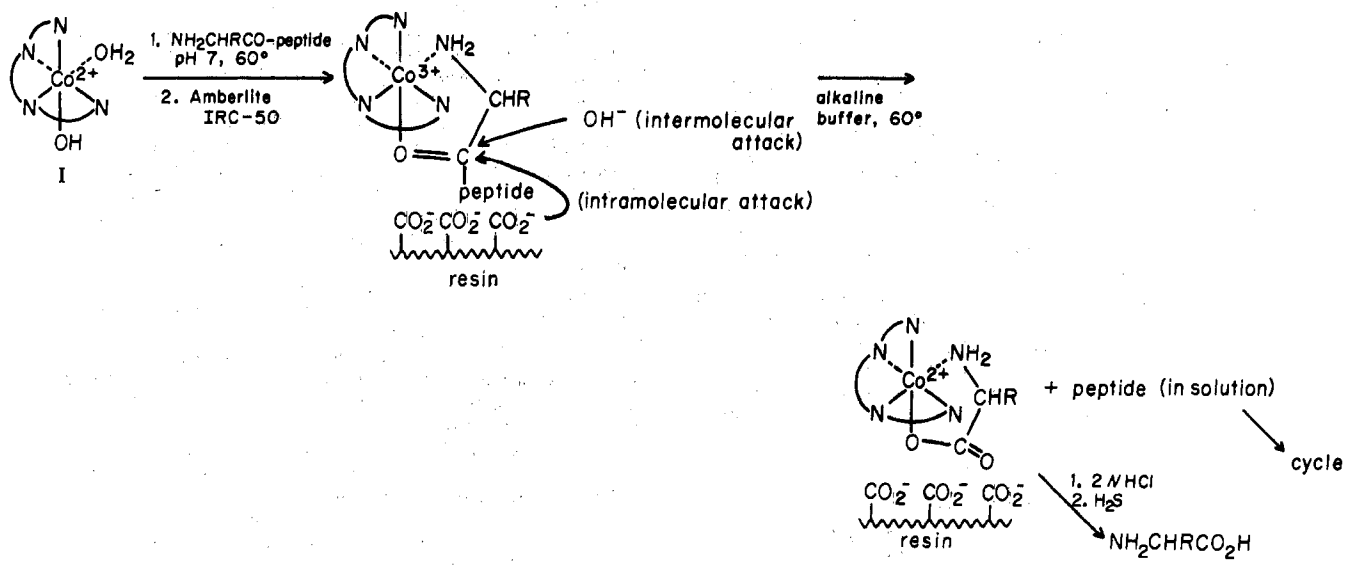
Comparison of the yields of N-terminal cleavage of Gly-Gly, Gly-Gly-Gly, and Gly-Gly-Gly-Gly using the equimolar complex I shows that the longer the peptide, the slower is the hydrolysis rate. The reason could be in the first step, where the longer peptide has more difficulty in forming II, rather than in the second hydrolysis stage.

Meanwhile, there are tetrapeptides like Gly-Pro-Leu-Gly or Pro-Phe-His-Leu, which chelate quantitatively to I but undergo little hydrolysis at pH 7.5 and 60°.

Longer peptides, such as Trp-Met-Asp-PheNH_2, lysyl-bradykinin (Lys-Arg- . . .), or angiotensin II (Asp-Arg- . . .) formed the corresponding complexes II, without much difficulty. The N-terminal amino acids of the first two peptides were cleaved at pH 7.5, but the last one was hardly hydrolyzed.

In the light of the foregoing experimental results, we had

Scheme II



come to a conclusion that a better degradation method was to prepare peptide complexes II with a large excess of I and to treat them, after isolation, with a high-pH buffer solution to accelerate effectively the hydrolysis. This was attempted independently by Fenn and Bradbury.[12]

A Solid-State Method. As one practical approach, we devised a solid-state modification as shown in Scheme II. It consists of (1) quantitative formation of a peptide complex II with a large excess of the complex I at pH 6.5–7 and 50–60°, (2) adsorption of the intermediate II on the CO_2^- resin, which separates II from uncoordinated peptide if any and also inactivates the excess complex I on the resin, (3) hydrolysis on the resin in a suitable alkaline buffer, whereby N-terminal amino acid chelate remains while the residual peptide is released into the solution, for the subsequent analysis, and (4) identification of N-terminal amino acid on tlc after dissociating III from the resin with acid and decomposition with H_2S.

By this method, we determined the sequence of most of the tested dipeptides at pH 8, except for Asp-Arg and Gly-Pro which very slowly underwent hydrolysis at pH 10. It is to be emphasized that, while the reaction is incomplete, on the resin are attached the complexes of the unhydrolyzed dipeptide and of N-terminal amino acid and in the solution is the C-terminal moiety with no contamination of N terminus. Hence, even if the peptides may not be hydrolyzed completely, the sequence can be determined, as in the case of Asp-Arg and Gly-Pro.

At present we are not certain of the mechanism of the peptide hydrolysis on the weak cationic exchange resin. There would seem to be two limiting pathways, *i.e.*, intra- or intermolecular attack of bases at the activated carbonyl as depicted in Scheme II.

Tripeptides were treated similarly. For instance, Leu-Gly-Gly complex II was prepared from 1 μmol of the peptide and 5 μmol of I at pH 6.8 and 60° for 30–60 min. The tlc showed near-quantitative chelation (>95%) without proceeding hydrolysis. The N-terminal hydrolysis on the resin was complete after 4 hr at pH 8 and 60°, and Gly-Gly (90–95% yield) was liberated into the collidine buffer solution with no contaminant of glycine. The advantage is obvious in contrast to the earlier method, where concomitant hydrolysis of Gly-Gly was unavoidable. A trace of Leu-Gly-Gly (~5%) was detected in the solution, which was apparently dissociated from the complex on the resin during the reaction. The similar dissociation was reported with Ala-Leu-Glyol.[12] The degradation cycle was repeated on the freed Gly-Gly, and hence the sequence was determined. This procedure was applied to other peptides such as Gly-Gly-Ala, Leu-Gly-Phe, Gly-Leu-Tyr, and Gly-Gly-Gly-Gly. The first peptide bond of Gly-Leu-Tyr was not completely cleaved under the above reaction conditions, though the total sequence was determined. Also determined by the solid-state method were N-terminal amino acids of Trp-Met-Asp-PheNH_2 and lysyl-bradykinin.

Conclusions

In applying β-Co(trien)$OH(H_2O)^{2+}$, I, to sequential analysis of peptides according to Scheme I, there are several problems.

First, the simplest procedure for degradation which treats a peptide with equimolar I at pH 7.5 and 60° is impractical, since some peptides do not rapidly and quantitatively yield the intermediate complex II. This can be overcome by using a large excess of I at pH ~7 and 50–60°. Under those conditions little hydrolysis occurs.

Second, the hydrolysis of II at pH 7.5 and 60° is not fast enough for the analytical purpose, particularly with sterically crowded peptides like Gly-Pro or peptides with ligating side chains like Asp-Arg. One solution is obviously running the reaction in high pH solution. This generally helped accelerating the cleavage but was not a satisfactory answer for the peptides containing proline or aspartic acid, which seems to present a major difficulty in the use of the method. The problem with aspartic acid might be overcome by protecting the free β-carboxyl group or employing a three-site complex, $Co(dien)X_3$, as discovered by Girgis and Legg.[13]

Third, with some peptides dissociation from the complex II occurs during hydrolysis, particularly at high pH and temperature. We were unable so far to resolve both the second and third problems at the same time.

Finally, the separation of the products can be easily achieved with the solid-state method.

With the limited range of our experiments, the reaction conditions described here may not be optimal, and it is clear that more work is required to find the proper pH, buffer, reaction time, temperature, cationic exchange resin, etc., before this technique may become practical.

Experimental Section

Materials. β-[Co(trien)(CO_3)]ClO_4 was prepared in a similar manner to that described by Sargeson and Searle.[15] Peptides were of reagent grade quality, obtained from Protein Research Foundation, Osaka, Japan; Fluka AG, Buchs, Switzerland; or Mann Research Laboratories, New York, N. Y., unless otherwise noted. Amberlite IRC-50 (100 mesh) and CM-Sephadex C-25 were preequilibrated by treating H forms with 0.2 *M* 2,4,6-collidine solution, followed by 0.2 *M* collidine–$HClO_4$ buffer (pH 8). Merck precoated tlc (silica gel F 254) and Toyo filter paper No. 51 were used for chromatographic analyses.

Dipeptide Hydrolysis Reactions. Stock solutions of β-Co(trien)-$OH(H_2O)^{2+}$, I, were prepared before each experiment by treating a weighed amount of β-[Co(trien)(CO_3)]ClO_4 with 6 equiv of 0.5 *N* $HClO_4$ for 30 min and adjusting to pH 7.5 with 0.5 *N* NaOH solution. The final concentration was adjusted by adding CO_2-free water to 0.05 *M*. The titration of Co(trien)$(H_2O)_2^{3+}$ with NaOH solution gave an accurate concentration of the cobalt complex.[2] A dipeptide was dissolved in 0.2 *M* collidine buffer (pH 8) to prepare a stock solution (0.01 *M*). The peptide solution (0.1 ml, 1 μmol) was mixed with 0.02 ml (1 μmol) of the complex solution in a microtube (1-ml content) with a tight cap and the mixture was heated in a constant-temperature bath at 60°. Fixed aliquots were withdrawn at convenient times using a 2- or 4-μl micropipet (Drummond) and applied on a silica gel tlc or a filter paper strip. If necessary, 2 μl of freshly prepared Na_2S aqueous solution (10%) was further applied to the spot on a tlc, whereby the orange-red spot immediately blackened and released the coordinated ligands. These were eluted in the ascending manner using 1-butanol–acetic acid–water (4:1:1) or 2-propanol–water (7:3) as an eluent. The spots were identified by comparison with known marker amino acids after spraying freshly prepared ninhydrin solution (0.25% in water-saturated 1-butanol) and heating at 105° for 5 min.

For measurement of the yields of N-terminal hydrolysis the reaction mixture was treated with 0.03 ml of 10% Na_2S aqueous solution, whereby quantitative decomposition of the complexes occurred. After standing at room temperature for 30 min followed by centrifugation, the known amount of the supernatant solution was spotted on a filter paper or a silica gel plate (2–8 μl), along with various known concentrations of amino acid in collidine buffer. After elution and ninhydrin color developing as described earlier, the color densities of the separated amino acids were measured at 560 nm using a Toyo densitometer. General precautions were taken from those employed by Bull[16] and Rockland.[17] In 0.0025–0.02 μ*M* concentration range, the control amino acids followed Beer's law. Scanning in two opposite directions and taking the average value were needed to get a linear standard curve. In the case of dipeptide hydrolysis, either N or C terminus, whichever separated well from the other species, or both were measured.

Hydrolysis of Tripeptides and Polypeptides. The same procedure was generally used as applied to dipeptide reactions. When Leu-Gly-Gly (1 μmol) was treated with 1 μmol of I at pH 7.5 and 60°, hydrolysis of Gly-Gly was found to occur after 1 hr, while the tripeptide complex was being hydrolyzed.

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(16) H. B. Bull, J. W. Hahn, and V. H. Baptist, *J. Amer. Chem. Soc.*, 71, 550 (1949).
(17) L. B. Rackland and M. S. Dunn, *J. Amer. Chem. Soc.*, 71, 4121 (1949).

Dipeptide Hydrolysis by the Solid-State Method. The stock solution of dipeptide (1 μmol or less) was treated with I (5 μmol) for 15 min at pH 6.8–7 and 50°. The resulting solution was stirred with Amberlite IRC-50 cation-exchange resin (150 mg) for 30 min. Then, the solution was syringed out and the resin was washed a few times with pH 8 buffer. Normally one will not see uncoordinated dipeptides in these solutions. The resin was shaken in 0.2 ml of pH 8 buffer solution (0.2 *M* collidine–$HClO_4$) at 60°. At convenient times the solution (4 μl) was spotted on a silica gel plate. Elution and ninhydrin spraying showed gradually increasing concentration of C-terminal amino acid, until no more increase was seen (*ca.* 4 hr required generally, except for peptides having proline and aspartic acid). Use of higher pH (10–11) buffer solution shortened the reaction time to 1–2 hr, but hydrolysis yields of Gly-Pro and Asp-Arg were not much improved. Before the reaction was complete, on the resin were attached complexes of unhydrolyzed dipeptide and N-terminal amino acid. When the reaction was over, only the N-terminal amino acid complex was left. This was determined by desorbing the cationic complexes with 2 *N* HCl, followed by decomposition with H_2S and analysis by tlc.

Tri- and Polypeptide Hydrolysis by the Solid-State Method. The stock solution of tri- or tetrapeptide in pH 6.8–7 collidine buffer (0.1 ml, 1 μmol) was treated with the solution of I (0.1 ml, 5 μmol) for 30–60 min at 60° in a microtube. The following operation, as described above, freed the residual peptides into the solution after the N-terminal cleavage. No amino acid or peptide resulting from further hydrolysis was detected. In the degradation of Leu-Gly-Gly, as the reaction proceeded, a trace of the tripeptide (~5% by comparison with the control on tlc) was liberated into the solution, together with Gly-Gly (~95% after 4 hr). On the resin only the leucine complex remained with no unhydrolyzed tripeptide complex. The solution containing Gly-Gly was adjusted to pH 7 with 0.5 *N* $HClO_4$ and subjected to the second degradative cycle, which yielded glycinato complex on the resin and free glycine in the solution. Complexes of some tetrapeptides such as Pro-Phe-His-Leu and Gly-Pro-Leu-Gly on the resin failed to promote the cleavage of the first peptide bonds at pH 8–10.5 and 60°.

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Registry No. I, 16404-41-4; Gly-Gly, 556-50-3; Gly-Leu, 869-19-2; Gly-Phe, 3321-03-7; Gly-$PheNH_2$, 1510-04-9; Gly-Asp, 4685-12-5; Gly-His, 2489-13-6; Ala-Gly, 687-69-4; Ala-Leu, 3303-34-2; Leu-Gly, 686-50-0; His-Tyr, 35979-00-1; Trp-Ala, 24046-71-7; Glm-Gly, 2650-65-9; Glu-Phe, 20556-22-3; Asp-Arg, 2640-07-5; β-Ala-Nle, 50599-94-5; Gly-Gly-Gly, 556-33-2; Gly-Gly-Ala, 19729-30-7; Gly-Leu-Tyr, 4306-24-5; Ala-Gly-Gly, 3146-40-5; Leu-Gly-Gly, 1187-50-4; Leu-Gly-Phe, 17608-53-6; Gly-Gly-Gly-Gly, 637-84-3.