The Synthesis of *N*-Terminal Acetylpeptides of Ovalbumin and Mammalian Heart Cytochrome c

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In previous publications from our laboratory,[1,2] it has been reported that *N*-acetylglycylserine and *N*-acetylglycylserylglycylisoleucylalanine can be isolated from the enzymatic hydrolysates of ovalbumin; it has also been suggested that these *N*-acetylpeptides are derived from the *N*-terminal position of the protein, because many attempts over the past ten years, to find the terminal group by the usual analysis methods have been unsuccessful. It seems to be important to confirm the structure of such an unusual *N*-terminal peptide by chemical synthesis. *N*-Acetylglycyl-L-serine and *N*-acetyl-L-serylglycine have thus been synthesized. Comparisons of their chromatographic behaviors and of the products of the N→O acyl shift reaction with those of the natural acetylpeptide have been made.

Like ovalbumin, for the *N*-terminal group of mammalian (bovine and equine) heart cytochrome c, conflicting results had also been presented until *N*-acetylglycylaspartylvalylglutamic acid was isolated in a fairly good yield from the enzymatic digest of the cytochrome.[3] The structure of the isolated *N*-acetyltetrapeptide was confirmed by comparison with the synthetic material. The synthesis of the acetyltetrapeptide will be described and the troubles encountered during its synthesis will be discussed in the present communication.

Experimental[4]

The Synthesis of Acetylpeptide.—*N-Chloroacetyl-O-benzyl-L-serine.*—*O*-Benzyl-L-serine[5] (9.8 g.) was dissolved in a mixture of 2 N sodium hydroxide and 2 N sodium carbonate (55 ml.); then into this solution chloroacetyl chloride (8.5 g.) was stirred portion by portion at 0—5°C. After the addition was complete, the reaction mixture was stirred at room temperature for half an hour. The pH of the solution was adjusted to 2.8 with 6 N hydrochloric acid, and white precipitates were collected as a waxy material. Yield, 11 g. (80%). Recrystallization from ethylacetate gave fine crystals. M. p. 114—115°C.

Found: N, 4.77. Calcd. for $C_{12}H_{14}O_4NCl \cdot H_2O$: N, 4.83%.

N-Glycyl-O-benzyl-L-serine. — Absolute methanol (200 ml.) in an ice-salt bath was saturated with

1) K. Narita, *Biochem. Biophys. Res. Comm.*, **5**, 160 (1961).
2) K. Narita and J. Ishii, *J. Biochem.*, **52**, 367 (1962).
3) K. Titani, K. Narita and K. Okunuki, *J. Biochem.*, **51**, 350 (1962).
4) All melting points are uncorrected. The R_f values listed were measured on a paper chromatogram developed with *n*-butanol-acetic acid-water (4:1:1, v/v). The specific optical rotation was measured in an acetic acid solution.
5) K. Okawa, This Bulletin, **29**, 486 (1956).

dry ammonia, and pulverized *N*-chloroacetyl-*O*-benzyl-L-serine (9 g.) was added. *N*-Chloroacetyl-*O*-benzyl-L-serine is soluble in pure methanol, but sparingly soluble in the methanol-ammonia. Thus the suspension in a stoppered bottle was allowed to stand at room temperature for thirteen days with occasional shaking. The insoluble material (2.6 g.), which was the starting material, was removed by filtration. When the filtrate was concentrated in vacuo, a crystalline material was obtained. Yield, 4.3 g. When the above recovered starting material was subjected to further amination, an additional 2.2 g. of the dipeptide derivative was obtained. The total yield was 78%. Recrystallization from water-acetone gave fine crystals. M. p. 192—193°C.

Found: C, 57.87; H, 6.39; N, 10.82. Calcd. for $C_{12}H_{16}O_4N_2$: C, 57.13; H, 6.39; N, 11.11%.

N-Acetylglycyl-O-benzyl-L-serine.—Glycyl-*O*-benzyl-L-serine was acetylated in a usual manner using acetic anhydride and aqueous alkali. The acetylated dipeptide derivative was recrystallized from water. Yield, 74%. M. p. 105—108°C.

Found: N, 8.73. Calcd. for $C_{14}H_{18}O_5N_2 \cdot H_2O$: N, 8.97%.

N-Acetylglycyl-L-serine.— *N*-Acetylglycyl-*O*-benzyl-L-serine (2g.) was hydrogenated on palladium-charcoal in 75% *t*-butanol (60 ml.) in a 90% yield. Recrystallization from water gave pure acetyldipeptide. M. p. 166—167.5°C (decomp.).

Found: C, 40.80; H, 5.96; N, 13.45. Calcd. for $C_7H_{14}O_5N_2$: C, 41.17; H, 5.92; N, 13.72%.

Carbobenzoxy-L-serylglycine Benzyl Ester.—Carbobenzoxy-L-serine (6.4 g.) was dissolved in a warm chloroform-dioxane mixture (2 : 1, v/v, 45 ml.) and was coupled with glycine benzyl ester by dicyclohexylcarbodiimide (5.4 g.). After the mixture had been kept at room temperature overnight, dicyclohexylurea (4.6 g.) was filtered off. The filtrate was then concentrated and the residue dissolved in ethylacetate (150 ml.). The solution was washed successively with 0.6 N hydrochloric acid, 5% sodium hydrogen carbonate and water, and finally dried. After the removal of additional precipitates of the urea derivative, the solution was partly evaporated and petroleum ether was added. When the crude product (9.1 g.) was recrystallized from ethylacetate, 7.4 g. (72%) of the product was obtained. M. p. 109—110°C (lit. m. p. 102°C[6]).

Found: C, 62.84; H, 6.09; N, 7.14. Calcd. for $C_{20}H_{22}O_6N_2$: C, 62.16; N, 5.74; N, 7.25%.

N-Acetyl-L-serylglycine.—L-Serylglycine (m. p. 204—207°C, decomp.) was obtained from the carbobenzoxydipeptide benzylester by catalytic hydrogenation on palladium-charcoal in 75% *t*-butanol for 5 hr. Into an aqueous triethylamine solution (15% solution, 10 ml.), L-serylglycine (570 mg.) was dissolved, and then acetic anhydride (0.55 g.) was added. The reaction mixture was stirred for half an hour at 0—2°C and then kept for an additional thirty minutes at room temperature. The syrupy product which was obtained by the concentration of the above mixture below 38°C was dissolved in water (2 ml.) and placed on the top of a column of Dowex 50-X2 (H-form, 1×14 cm.). The column was then washed with water until the effluent became neutral. The effluent (30 ml.) was lyophilized. Yield, 480 mg. (68%). M. p. 161—163°C (decomp.).

Found: C, 40.98; H, 5.93. Calcd. for $C_7H_{12}O_5N_2$: C, 41.17; H, 5.92%.

Carbobenzoxy-L-valyl-L-glutamic Acid Dibenzyl Ester.—Crystalline carbobenzoxy-L-valine (6.6 g.) and L-glutamic acid dibenzyl ester *p*-toluenesulfonate (15.8 g.) were dissolved in methylene chloride (40 ml.) containing triethylamine (1.5 g.). Into this solution, dicyclohexylcarbodiimide (5.5 g.) in a small amount of the same solvent was then added on an ice-bath. Dicyclohexylurea precipitated immediately. After it had been allowed to stand in an ice-bath, the reaction mixture was left at room temperature for an additional fifteen hours. The urea derivative was removed by filtration, and the filtrate was successively washed with 0.1 N hydrochloric acid, water, 5% sodium hydrogen carbonate and water. It was then dried over anhydrous sodium sulfate, and the solution was evaporated to dryness in vacuo. The crystalline residue was recrystallized from ethylacetate. Yield, 11.2 g. (76.2%). M. p. 148°C. $[\alpha]_D^{27}$ −23.9° (*c* 2.0).

Found: C, 68.66; H, 6.76; N, 5.09. Calcd. for $C_{32}H_{36}O_7N_2$: C, 68.55; H, 6.47; N, 5.00%.

L-Valyl-L-glutamic Acid.—Carbobenzoxy-L-valyl-L-glutamic dibenzyl ester (10 g.) was reduced in 70% acetic acid (150 ml.) in the presence of 10% palladium on charcoal (1.0 g.) for two hours. After the removal of the catalyst by filtration, the filtrate was concentrated under reduced pressure. In order to remove the acetic acid completely, the solid residue was dissolved in a minimal volume of water and the solution evaporated to dryness. The above treatment was repeated, and then the crystalline residue was recrystallized from water-ethanol. Yield, 3.7 g. (84.2%). M. p. 227°C (decomp.). $[\alpha]_D^{27}$ +17.4° (*c* 1.4). R_f. 0.38.

Found: C, 48.84; H, 7.63; N, 11.30. Calcd. for $C_{10}H_{18}O_5N_2$: C, 48.76; H, 7.37; N, 11.38%.

L-Valyl-L-glutamic Acid Dibenzyl Ester p-Toluenesulfonate.—L-Valyl-L-glutamic acid (2.5 g.) *p*-toluenesulfonic acid hydrate (2.2 g.) and benzyl alcohol (20 ml.) were mixed in chloroform (100 ml.), and the mixture was refluxed with Wieland's apparatus (silica gel was used as a desiccant). Within a few hours the partly-insoluble starting material was dissolved entirely. Reflux was continued for ten hours, and then the solvent and the excess benzyl alcohol were removed in vacuo. When the syrupy residue was triturated in ether (150 ml.), a crystalline solid precipitated. It was recrystallized from ethanol-ether. Yield, 5.6 g. (91.8%). M. p. 108～109°C. $[\alpha]_D^{27}$ +0.0° (*c* 2.0). R_f 0.81.

Found: C, 61.66; H, 6.54; N, 4.80. Calcd. for $C_{31}H_{38}O_8N_2S$: C, 62.19; H, 6.40; N, 4.68%.

Carbobenzoxy-L-aspartyl-L-valyl-L-glutamic Acid Tribenzyl Ester. — A mixture of L-valyl-L-glutamic acid dibenzyl ester *p*-toluenesulfonate (3.0 g.), triethylamine (0.5 g.), carbobenzoxy-L-aspartic acid β-benzyl ester[7] (1.6 g.) and dicyclohexylcarbodiimide (1.0 g.) was dissolved in methylene chloride

6) J. S. Fruton, *J. Biol. Chem.*, **146**, 463, (1942).

7) A. Berger and E. Katchalski, *J. Am. Chem. Soc.*, **73**, 4083 (1951).

(30 ml.); this solution was then allowed to stand for fifteen hours at room temperature. The precipitated dicyclohexylurea was filtered off and the filtrate was evaporated to dryness. The residue crystallized immediately. It was recrystallized from methanol. Yield, 2.4 g. (63.2%). M. p. 131°C. $[\alpha]_D^{27}$ −24.0° (*c* 1.9).

Found: C, 66.97; H, 6.34; N, 4.99. Calcd. for $C_{43}H_{47}O_{10}N_3 \cdot CH_3OH$: C, 66.73; H, 6.35; N, 5.19%.

L-Aspartyl-L-valyl-L-glutamic Acid.—Carbobenzoxy-L-aspartyl-L-valyl-L-glutamic acid tribenzyl ester (2.4 g.) was suspended in 70% acetic acid (100 ml.). Hydrogen gas was bubbled into the above solution for three hours in the presence of 10% palladium on charcoal (0.5 g.). The starting tripeptide derivative was dissolved within fifteen minutes. The catalyst was removed by filtration, and the filtrate was evaporated to dryness in vacuo. The residue was dissolved into water and lyophilized. (Attempts to crystallize it were unsuccessful.) Yield, 1.0 g. (89.0%). M. p. 165°C (decomp.). $[\alpha]_D^{27}$ −6.9° (*c* 1.0). R_f 0.18.

Found: C, 45.46; H, 6.84; N, 11.20. Calcd. for $C_{14}H_{23}O_8N_3 \cdot 1/2\ H_2O$: C, 45.39; H, 6.35; N, 11.34%.

L-Aspartyl-L-valyl-L-glutamic Acid Tribenzyl Ester p-Toluenesulfonate.—L-Aspartyl-L-valyl-L-glutamic acid (0.8g.), *p*-toluenesulfonic acid hydrate (0.45 g.) and benzyl alcohol (1.0 ml.) were mixed in chloroform (20 ml.). The mixture was then refluxed with Wieland's apparatus. The solvent and the excess benzyl alcohol were distilled off in a high vacuum. The syrupy residue was dissolved in ether (2 ml.), and *n*-hexane was slowly added until crystalline precipitates appeared. These precipitates were collected and recrystallized from ether-*n*-hexane. Yield, 1.35 g. (78.3%). M. p. 181°C. $[\alpha]_D^{27}$ −21.0° (*c* 2.2). R_f 0.85.

Found: C, 62.97; H, 6.10; N, 5.33. Calcd. for $C_{42}H_{49}O_{11}N_3S$: C, 62.95; H, 6.14; N, 5.23%.

Acetylglycyl-L-aspartyl-L-valyl-L-glutamic Acid Tribenzyl Ester.—Into an ice-cooled solution of acetylglycine (0.21 g.) in freshly-distilled dimethylformamide (10 ml.), carbonyldiimidazole (0.3 g.) was stirred. After ten minutes, a solution of L-aspartyl-L-valyl-L-glutamic acid tribenzyl ester *p*-toluenesulfonate (1.2 g.) and an equivalent amount of triethylamine in dimethylformamide (10 ml.) were added. The reaction mixture was then left for four hours at room temperature and diluted with water (20 ml.). The gelatinous product separated was collected by centrifugation, followed by filtration with suction. This was recrystallized from hot ethanol as fine needles. Yield, 0.7 g. (64.2%). $[\alpha]_D^{27}$ −13.0° (*c* 2.1).

Found: C, 63.85; H, 6.20; N, 8.00. Calcd. for $C_{39}H_{46}O_{10}N_4$: C, 64.09; H, 6.36; N, 7.67%.

Acetylglycyl-L-aspartyl-L-valyl-L-glutamic Acid.—Acetylglycyl-L-aspartyl-L-valyl-L-glutamic acid tribenzyl ester (0.5 g.) was reduced over 10% palladium on charcoal (0.2 g.) in 50% acetic acid (20 ml.) for two hours. The catalyst was then removed by filtration, and the filtrate was concentrated in vacuo. The residue was dissolved in water, (20 ml.) and Amberlite IR-120 (H-form, 3 ml.) was poured in while it was being stirred and cooled. The resin was removed by filtration and washed with three 5 ml. portions of water. The combined filtrate and washings were then lyophilized. The colorless, fluffy material which remained was negative to the ninhydrin reaction. It contained three equivalents of carboxyl groups, as determined by titration with sodium methoxide.[8] M. p. 192°C (decomp.). $[\alpha]_D^{27}$ −29° (*c* 1.3). R_f 0.55 (detected with bromocresol green; the paper strip must be free from acetic acid). Amino acid analysis with automatic equipment gave a molar ratio of 0.9 : 1.0 : 1.1 : 1.1 for glycine, aspartic acid, valine and glutamic acid.

Found: C, 44.62; H, 6.35; N, 11.82. Calcd. for $C_{18}H_{28}O_{10}N_4$: C, 45.19; H, 6.32; N, 11.71%.

The N→O Acyl Shift Reaction of *N*-Acetylglycylserine and *N*-Acetylserylglycine.—About 2 mg. of the peptide was suspended in 5 ml. of dry dioxane, and hydrogen bromide gas was passed through until the mixture became clear.[2] The solution was refluxed for a few minutes to complete the N→O shift reaction and then evaporated to dryness in vacuo. The residue was dissolved in a minimum volume of water, and an aliquot was used for paper chromatography (*n*-butanol - acetic acid - water 4 : 1 : 1, v/v). The remaining aliquot was made alkaline by the addition of 5% ammonium hydroxide, and then the solution was kept for 2 hr. at room temperature in order to reverse the shifted products (O→N acyl shift). The solution was evaporated at room temperature in an evacuated desiccator. The characterization of the products of the O→N acyl shift reaction was performed by paper chromatography.

The Carboxypeptidase A Digestion of the Acetylglycylaspartylvalylglutamic Acid.—To compare the natural acetyltetrapeptide with the synthetic one, the rate and the extent of the release of amino acids from the peptides by the action of carboxypeptidase A were measured. The crystalline enzyme was purchased from the Worthington Biochemical Corp. (Freehold, New Jersey) and used after three recrystallizations. As has been reported previously,[3] the enzyme hydrolyzed the acetyltetrapeptide most at pH 6.0, in contrast to the usual optimum of pH 8.0, and the digestion was carried out at pH 6.0 (M/7 sodium acetate - M/7 sodium barbiturate - N/10 hydrochloric acid - 8.5% sodium chloride buffer) and at room temperature in the substrate to the enzyme ratio of 1 : 1 by weight. The amino acids released were characterized and estimated as their dinitrophenyl derivatives by paper chromatography.

Results and Discussion

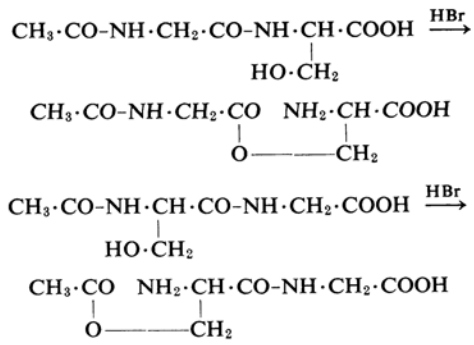
Although it was quite clear from the structural analysis that the acetyldipeptide isolated from the pronase (the trade name of a protease of *Streptomyces griseus*) digest of ovalbumin was acetylglycylserine,[1,2] two acetyldipeptides, which have opposite amino acid sequences were prepared in order to compare them with the natural acetyldipeptide.

8) J. S. Fritz and N. M. Lisicki, *Anal. Chem.*, **23**, 589 (1951).

Unfortunately, the two synthetic acetyldipeptides behaved identically on a paper chromatogram with the natural peptide (R_f 0.46, *n*-butanol - acetic acid - water, 4 : 1 : 1, v/v) ; moreover, the chromatograms of the hydrazinolysates of the three acetyldipeptides, synthetic and natural, were all the same, showing acetylhydrazide (R_f 0.63, pyridine-aniline-water, 9 : 1 : 4, v/v ; 0.45, collidine-picoline-water, 5 : 3 : 2, v/v), glycine hydrazide (R_f 0.37 ; 0.11) and serine hydrazide (R_f 0.46 ; 0.16).[1,2]

In order to distinguish *N*-acetylglycylserine from *N*-acetylserylglycine, the N→O acyl shift reaction was explored in anhydrous dioxane by passing hydrogen bromide gas through. It may be expected that *O*-[acetylglycyl]-serine will be produced from *N*-acetylglycylserine by the shift reaction[2] while *O*-acetylserylglycine is obtained from *N*-acetylserylglycine, and that the R_f values of the above two products are probably different from one another on a paper chromatogram.

The R_f values of the reaction products of the two synthetic and one natural acetyldipeptides are listed in Table I. It is clear from the table that the shift reaction under the conditions was accompanied by some side reactions. The products of the N→O acyl shift reaction may be recovered as the starting compound by the reverse O→N acyl shift



reaction in an alkaline medium, whereas the products by the side reaction will not be changed under the conditions. As can be seen in Table I, free amino acid resulted from the *C*-terminal amino acid, in some extent as a result of the hydrolytic side reaction. An additional unknown side reaction product, which is ninhydrin-positive, was observed in both cases, but it is not known whether or not the unknown product from acetylglycylserine is the same as that from acetylserylglycine, although their R_f values are quite similar.

At any rate, the behavior of the products of the N→O acyl shift reaction of the natural acetyldipeptide was identical with that of the synthetic acetylglycyl-L-serine. Thus, the

TABLE I. R_f VALUES OF THE PRODUCTS OF N→O ACYL SHIFT REACTION OF *N*-ACETYLGLYCYLSERINE AND *N*-ACETYLSERYLGLYCINE*

	Standard	Reaction products of N→O acyl shift				
		Synthetic *N*-acetylglycylserine		Natural *N*-acetylglycylserine	Synthetic *N*-acetylserylglycine	
		Before O→N shift	After O→N shift	Before O→N shift	Before O→N shift	After O→N shift
Glycylserine	0.14					
Serine	0.16	0.16	0.16	0.16		
Serylglycine	0.17					
Glycine	0.18				0.18	0.18
O-[Acetylglycyl]-serine	0.20	0.20		0.20		
O-Acetylserine	0.31					
O-Acetylserylglycine	0.34				0.33	
N-Acetylglycylserine	*0.46*		*0.46*			
N-Acetylserylglycine	*0.46*					*0.46*
N-Acetylserine	*0.56*					
Unknown**		0.67	0.67	0.60—0.70	0.66	0.66

* *n*-Butanol-acetic acid-water (4 : 1 : 1, v/v) was used as a solvent for descending paper chromatography. The ninhydrin reagent was used for the detection of the products. When quite enough amount of *N*-acetyl derivatives are present on a paper and when prolonged heating with an iron after spraying the reagent was used, they colored as pinkish purple. In this experiment, the chlorine-iodide-starch reaction was also employed after the ninhydrin reaction to identify *N*-acetyl derivatives sharply (R_f values are shown as italic figures). *O*-[Acetylglycyl]-L-serine was a generous gift from Dr. Shumpei Sakakibara of the Institute for Protein Research, Osaka University and the other standard peptides were synthesized by the present authors.

** Color intensity of an unknown spot was weak, especially in the case of the reaction product of *N*-acetylserylglycine.

structure deduced for the natural acetyldipeptide isolated from ovalbumin[1] was confirmed without any ambiguity.

We have demonstrated that cytochromes c from bovine and equine hearts terminated at acetylglycylaspartylvalylglutamic acid.[3] At almost same time Margoliash et al.[9] determined the complete amino acid sequence of equine heart cytochrome, their results are consistent with the *N*-terminal sequence we have described above. In order to confirm the *N*-terminal structure of the cytochrome, we attempted the synthesis of *N*-acetylglycyl-L-aspartyl-L-valyl-L-glutamic acid. The first route to synthesize the acetyltetrapeptide of the problem was as follows:

Cbz-L-aspartic acid β-methyl ester + L-Valyl-L-glutamaic dimethyl ester —DCCD→

Cbz-L-aspartyl-L-valyl-L-glutamic acid trimethyl ester

M. p. 171°C, $[\alpha]_D^{22}$, −19.40 (*c* 0.62, acetic acid), 71%

—H_2/Pd→ L-Aspartyl-L-valyl-L-glutamic acid trimetyl ester + Cbz-glycine —DCCD→

Cbz-glycyl-L-aspartyl-L-valyl-L-glutamic acid trimethyl ester

M. p. 130—137°C, $[\alpha]_D^{22}$ −36.6° (*c* 0.82), 64%

—H_2O→ Cbz-glycyl-L-aspartyl-L-valyl-L-glutamic acid

M. p. 100—111°C, $[\alpha]_D^{22}$ −4.20 (*c* 1.18), 81%

—H_2/Pd→ Glycyl-L-aspartyl-L-valy-L-glutamic acid

$[\alpha]_D^{22}$ +4.7° (*c* 0.64), 89%

—Acetic anhydride→ Acetylglycyl-L-aspartyl-L-valyl-L-glutamic acid

M. p. 140°C, $[\alpha]_D^{22}$ −5.1° (*c* 0.78), 83%

(Cbz and DCCD are the abbreviations for the carbobenzoxy group and for dicyclohexylcarbodiimide respectively.)

The acetylglycyl-L-aspartyl-L-valyl-L-glutamic acid thus synthesized (this acetyltetrapeptide is designated as I to distinguish it from the acetyltetrapeptide II which is prepared by the method described in the "Experimental" section) was shown by amino acid analysis to contain an equimolar amount of the constituent amino acids. The chromatographic behavior (R_f 0.72, *n*-butanol - acetic acid - water, 4 : 1 : 1, v/v) of the synthetic acetyltetrapeptide I on a paper was identical with that of the natural acetyltetrapeptide obtained from bovine and equine heart cytochromes c. *C*-Terminal analyses of both the peptides by the hydrazinolysis method gave only glutamic acid. However, when the synthetic acetyltetrapeptide I was incubated with carboxypeptidase A at pH 6.0, the expected amount of glutamic acid and an unexpectedly small amount of valine were liberated, whereas from the natural acetyltetrapeptide almost quantitative amounts of glutamic acid and valine were released. Therefore, we have considered that the valine residue might be partly racemized during its synthesis. Thus, a bioassay of the valine in the acid hydrolysate of the acetyltetrapeptide I was performed.[10] The estimate showed that it was the natural form. Then the products of partial acid hydrolysate (concentrated hydrochloric acid at room temperature for 48 hr.) of the synthetic acetyltetrapeptide I were compared with those of the natural peptide by paper chromatography and electrophoresis. As is schematically represented in Fig. 1, one main spot, aspartylvalylglutamic acid, could be revealed with the ninhydrin reagent in the case of the natural acetyltetrapeptide. However, in the case of the synthetic peptide I, the aspartylvalylglutamic acid spot was quite faint, and another bluish-green spot appeared with the ninhydrin reagent at a slightly lower R_f value on the chromatogram. This bluish-green spot was located at a more negatively-charged position on the paper electrophoregram at pH 3.6 than that of the tripeptide derived from the natural acetylpeptide. The amino acid analysis of this abnormal spot demonstrated that it also contained equimolar amounts of aspartic acid, valine and glutamic acid. Therefore, it seems to be β-aspartylpeptide, since the pK' value

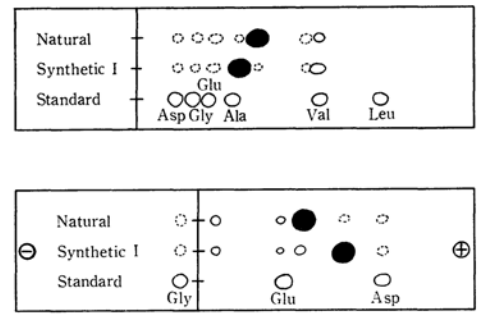


Fig. 1. Paper chromatogram and paper electrophoregram of partial acid hydrolysates of natural and synthetic acetylglycylaspartylvalylglutamic acid I.

Paper chromatography was carried out by descending way using *n*-butanol - acetic acid - water (4 : 1 : 1, v/v). Paper electrophoregram was made after migration for 120 min. at a potential of 3000 V./60 cm. at pH 3.6. Black spot represents aspartylvalylglutamic acid.

9) E. Margoliash, E. L. Smith, G. Kreil and H. Tuppy, *Nature*, **192**, 1125 (1961).

10) The bioassay of valine was kindly performed by the Ajinomoto Research Laboratory.

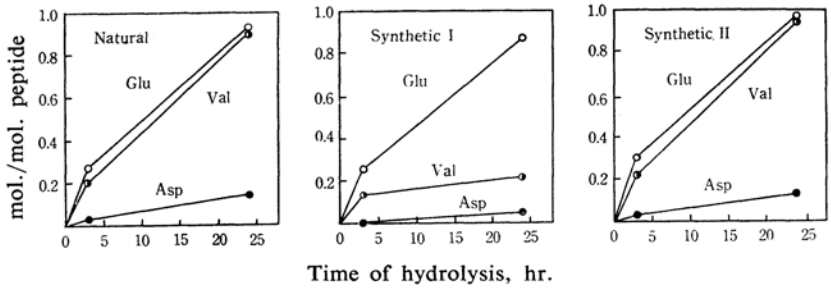


Fig. 2. Releases of amino acids from natural and synthetic acetylglycylaspartylvalylglutamic acids I and II by the action of carboxypeptidase A at pH 6.0. Released amino acids were estimated as their dinitrophenyl derivatives.

of the α-carboxyl group (1.995)[11] is lower than that of the β-carboxyl group (3.910)[11] and the former is more negatively charged than the latter at an acidic medium (pH 3.6). The β-linkage might be produced during the saponification step of the ester groups of carbobenzoxyglycyl-L-aspartyl-L-valyl-L-glutamic acid trimethyl ester via an assumed succinimide-type linkage.[12] It appears probable that the synthetic acetyltetrapeptide I is acetylglycyl-β-L-aspartyl-L-valyl-L-glutamic acid.

Thus we next explored the synthesis of the acetyltetrapeptide II, excluding the saponification step of the ester of the side chain of the aspartyl residue, as is described in the "Experimental" section. Namely, only the benzyl ester was used as the protection of the carboxyl groups, and it was removed by catalytic hydrogenation. Furthermore, to avoid alkaline conditions throughout the synthesis, acetylglycine was coupled with the tripeptide tribenzyl ester, in contrast to the use of acetic anhydride under the alkaline conditions to introduce the acetyl group to the tetrapeptide in the previous synthesis of the acetyltetrapeptide I.

The synthesized acetyltetrapeptide II showed behavior identical to that of the natural one in every respect examined. The releases of amino acids from the three acetyltetrapeptides, natural, synthetic I and II, by the action of carboxypeptidase A at pH 6.0 are shown in Fig. 2.

The infrared absorption spectra of the two synthetic acetylpeptides I and II were the same in the 4000—650 cm^{-1} region, but an appreciable difference could be observed between the two in the 900—400 cm^{-1} region. Such a difference should probably be attributed to the difference in the mode of linkage in aspartic acid residues in the synthetic acetyltetrapeptides.

Summary

In previous studies acetylglycylserine and acetylglycylaspartylalylglutamic acid had been isolated from the enzymatic digests of ovalbumin and cytochromes c of bovine and equine hearts, respectively. Here, in order to confirm the structures of the acetylpeptides isolated, acetylglycyl-L-serine and acetylglycyl-L-aspartyl-L-valyl-L-glutamic acid have been synthesized. It has been proved that the natural acetylpeptides are identical with the synthetic ones in every respect examined. Some troubles encountered during the synthesis of the acetyltetrapeptide have been discussed.

The authors are grateful to the Ajinomoto Co., Ltd., and to Dr. Kunio Fukushima for performing the bioassay of L-valine and the infrared absorption analysis respectively.

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