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REACTION OF NINHYDRIN WITH PEPTIDES

Hiroo YONEZAWA*, Masako HASUIKE* and Masashi TATUMOTO*

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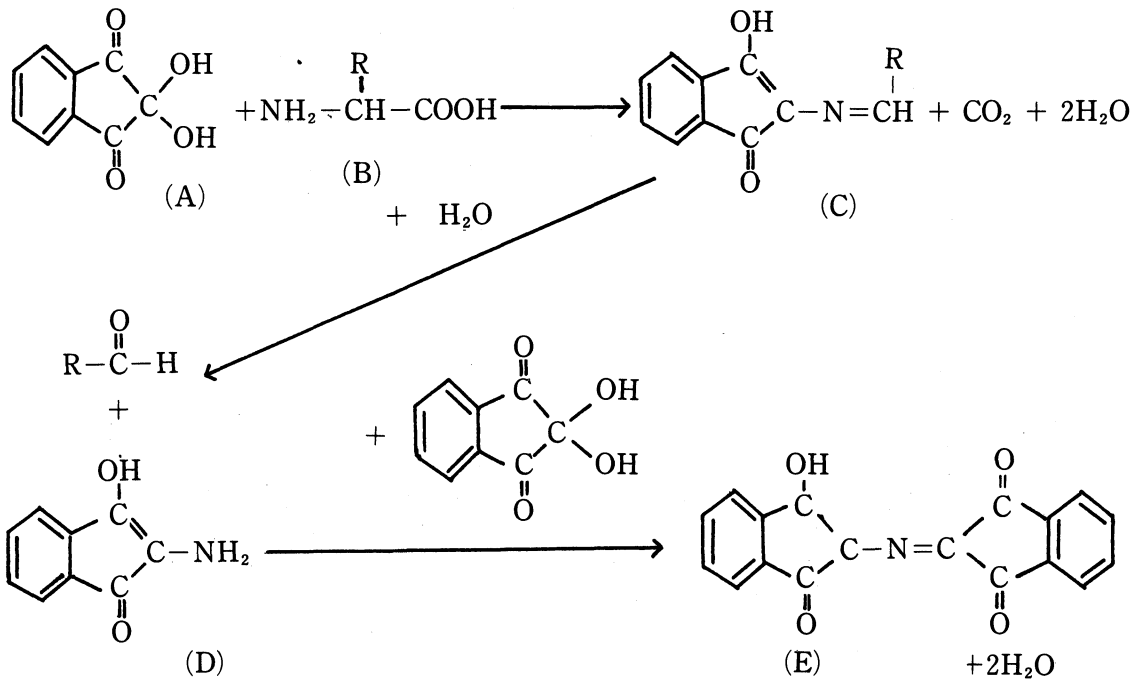
Abstract

The reaction of ninhydrin with various dipeptides and tripeptides has been studied. The color yields of many peptides were larger than that of leucine. The color yield of Ala-Ala-Leu was three times as that of leucine. Ala-Leu was isolated in the reaction mixture of ninhydrin and Ala-Ala-Leu. Then, it is concluded that N-terminal amino acid is sequentially removed from the peptide by the reaction of ninhydrin and peptide. On the other hand, the color yields of glycine containing peptides, Gly-Ala, Ala-Gly, Ala-Gly-Leu were same as that of leucine. In the reaction mixture of Gly-Ala and ninhydrin, N-protected alanine derivative which converted to alanine by hydrolysis with 6 M HCl was contained. The reacion mechanism of ninhydrin and peptides was discussed.

Introduction

The detection and the quantitative estimation of α-amino acids has been accomplished by their reaction with ninhydrin. The reaction products include an aldehyde with one carbon atom less than the α-amino acid and carbon dioxide in stoichiometric amounts and varying amounts of ammonia, hydrindantin and a chromophoric compound, Ruhemann's Purple (RP) (*1*). Many suggestions have been made as to the mechanism of its reaction. McCaldin in his review of the chemistry of ninhydrin suggested the involvement of an intermediate amine (D) (Scheme 1). The initial step of this reaction is a Schiff's base-type condensation of ninhydrin with α-amino acid, followed by decarboxylation and dehydration. From compound C, the amine (D) and the aldehyde are formed by hydrolysis. Finally, a further molecule of ninhydrin condenses with the amine to produce RP (*2*). It is considered the this pathway is current acceptable general mechanism for the ninhydrin reaction

* Department of Chemistry, Faculty of Science, Kagoshima University, Kagoshima, 890 Japan.
Abbreviations : RP, Ruhemann's Purple ; Z, benzyloxycarbonyl ; Boc, t-butyloxycarbonyl.



Scheme 1. Reaction of ninhydrin and α-amino acid

Approximately same amount of color yield is obtained with given α-amino acid, except proline (*3*). However, the same amount of color yield is not given by each of different peptides. For example, Gly-Gly has same color yield for leucine, but Leu-Leu has twice as many of leucine (*4*). However, The cause of the difference of color yield and the reaction mechanism of ninhydrin and peptide are still unclear. Then, we examined the reaction of ninhydrin with some dipeptides, tripeptides and considered with reaction mechanism.

EXPERIMENTAL

10^{-1} M or 2×10^{-2} M ninhydrin solution was prepared as follows ; $SnCl_2 \cdot 2H_2O$ (0.16 g) was dissolved in 100 ml of citrate buffer (0.2 M pH 5.0) and ninhydrin (3.56 g or 0.71 g) was dissolved in 100 ml of methyl cellosolve, and then, two solutions were mixed before use. Ion exchange chromatography was carried out on Hitachi liquid chromatography, model KLA-5, under these conditions : length of column with spherical resin, 0.9 x 50 cm ; solvent, standard 0.2 M citrate buffer at pH 3.28 (buffer I), pH 4.25 (buffer II), pH 5.28 (buffer III) ; flow rate, 60 ml/hr ; jacket temperature, 55℃.

Synthesis of Peptide

Z-Ala-Ala-OBzl (I) —— To a solution of Z-Ala (1.12 g, 5 mmol) Ala-OBzl·TosOH (1.76 g, 5 mmol) and Et_3N (0.7 ml, 5 mmol) in CH_2Cl_2 (20 ml) was added dicyclohexylcarbodiimide (1.04 g, 5 mmol) at 0 ℃. The reaction mixture was stirred for 1 hr at 0 ℃, and overnight at room temperature. It was evaporated and ethyl acetate was added to the residue. After dicyclohexylurea was filtered off, the filtrate was washed with 2 % HCl, 4 % $NaHCO_3$ and water, dried overe Na_2SO_4, and evaporated to leave an oil, which was crystallized by the addition of petroleum ether. It was recrystallized from ethyl acetate-petroleum ether ; yield, 1.49 g (78%) , mp 136-137℃.

Other Z-dipeptide benyl esters were prepared from the corresponding Z-amino acids and amino acid benzyl ester p-toluenesulfonates as described above.

Ala-Ala (II) —— The I (0.77 g, 2 mmol) was suspended in a mixture of H_2O-acetic acid-methanol (20 ml) and the mixture was hydrogenated in the presence of palladium black. After 24 hr, the catalyst was filtered off, and the filtrate was evaporated *in vacuo* and the resulting crystals were collected with the aid of acetone ; yield, 0.27 g (93%).

Other dipeptides were prepared from the corresponding Z-dipeptide benzyl esters as descrived above.

Boc-Gly-Leu-OBzl (III) —— This was prepared from Boc-Ala (2.65 g, 14 mmol) and Leu-OBzl · TosOH (5.51 g, 14 mmol) as described above; yield of oil, 5.02 g (95%)

Gly-Leu-OBzl·HCl (IV) —— Compound III (5.02 g, 13.3 mmol) was dissolved in 1 M HCl in acetic acid (40 ml). After being left to stand at room temperature for 1 hr, the solution was evaporated and resulting residue was washed by ether ; yield, 4.5 g (95%)

Z-Ala-Gly-Leu-OBzl (V) —— This was prepared from Z-Ala (1.56 g, 7 mmol) and Gly-Leu-OBzl · HCl (2.21 g, 7 mmol) as described above ; yield, 2.50 g (80%) , mp 138-141℃.

Ala-Gly-Leu (VI) —— This was prepared from the V as descrived above ; yield, 1.07 g, (86%)

Ala-Ala-Leu was prepared as described above.

Effect of Reaction Time on Color Yield——To a 0.1 ml of sample solution (10^{-3} M, in citrate buffer pH 5.0) , was added 1 ml of ninhydrin solution (10^{-1} M), and heated at 100℃. The solution was cooled to room temperature, was added ethanol (4 ml) and its optical density determined at 570 nm.

Reaction of Ninhydrin with Ala-Ala-Leu——To a 0.1 ml of Ala-Ala-Leu solution (10^{-2} M, in citrate buffer pH 5.0), was added 1 ml of ninhydrin solution (2×10^{-2} M) and heated for 30 min at 100℃. The reaction mixture was cooled and water (2 ml) was added. The resulting RP was extracted with n-butanol (2 ml x 2) and the aqueous layer was evaporated to dryness. The residue was

applied to ion exchange chromatography. The column was pre-equilibrated with buffer I and developed with buffer III.

Reaction of Ninhydrin with Gly-Ala——To a 0.2 ml of Gly-Ala solution (10^{-2} M, in citrate buffer pH 5.0) was added 2 ml of ninhydrin solution (10^{-1} M) and heated for 1 hr at 100℃. The reaction mixture was cooled and was added water (2 ml) and pH was adjusted to 9-10 with 4 M NaOH. The resulting RP was extracted with n-butanol. To the aqueous layer were added 1 ml of leucine solution (10^{-2}M) and 12 M HCl (4 ml), and heated for 24 hr at 110℃ in a sealded tube. The solution was evaporated to dryness and applied to ion exchange chromatography. The buffer I was applied until 45 minutes and system was changed with the buffer II.

RESULTS AND DISCUSSION

The rates of color development have been determined for leucine and peptides. As shown in Fig. 1 and 2, maximum color yield was obtained with leucine in 5-10 minutes. The peptides were divided into two groups on the basis of their maximal color yield. One group had same color yield and same rate of color development as that of leucine. On the other hand, another group had larger color yield than that of leucine. In the latter group, reaction time of 5 minutes gave same color yield of leucine and color yield was gradually increased untill 60-120

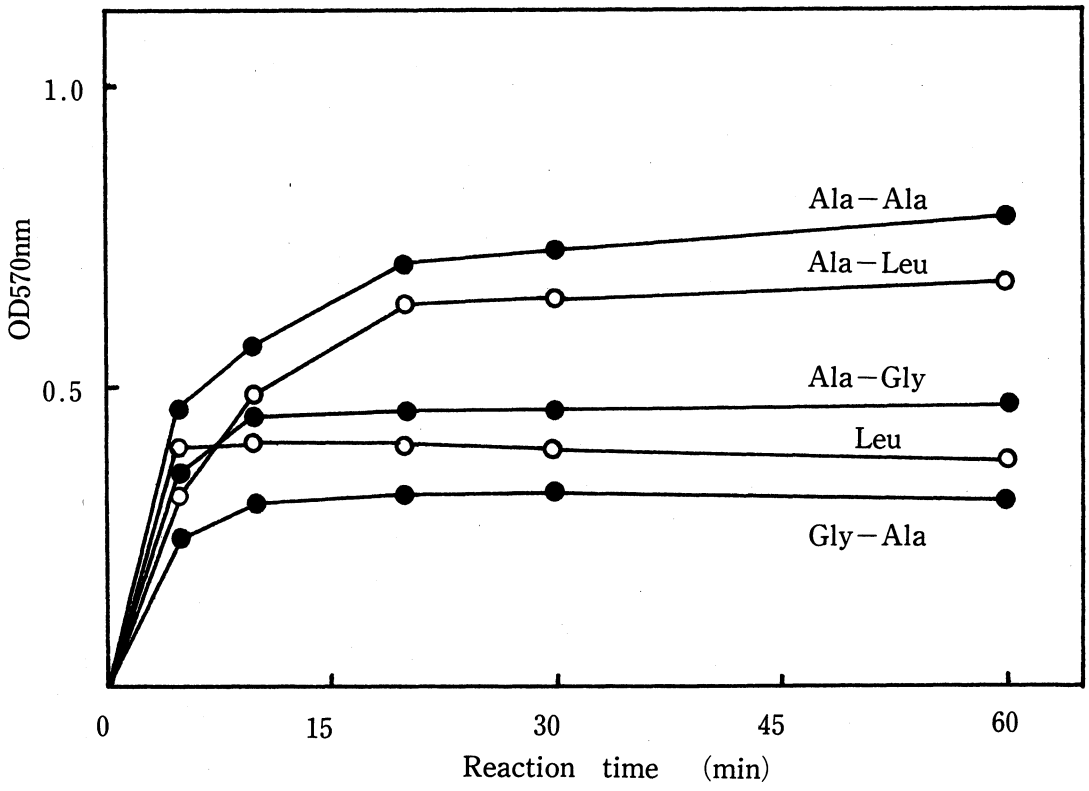


Figure 1. Color development of dipeptides.

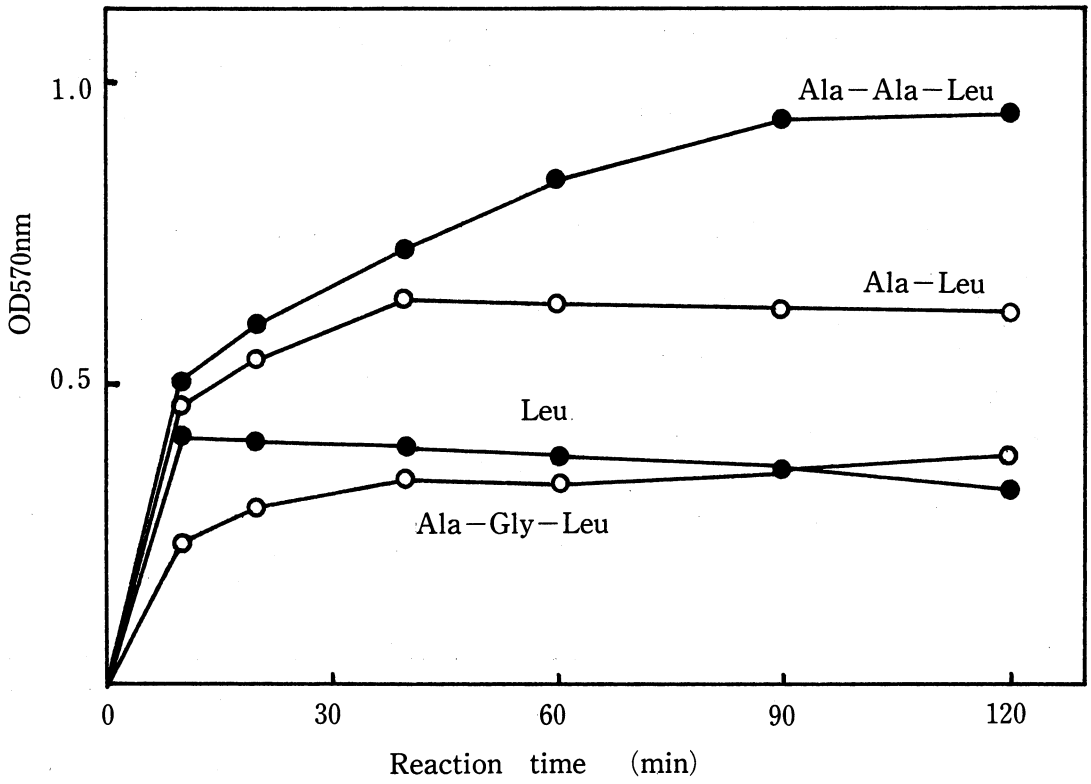


Figure 2. Color development of tripeptides.

Table I. Color yields of peptides

Peptide	Color yield	Peptide	Color yield
Leu	1.0	Ala−Val	1.4
Ala−Ala	1.8	Ala−Tyr	1.3
Ala−Leu	1.7	Ala−Gly	1.2
Ala−Ile	1.7	Ala−Pro	1.0
Ala−Ser	1.7	Gly−Ala	0.8
Ala−Asp	1.6	Ala−Ala−Leu	2.8*
Ala−Lys	1.6	Ala−Gly−Leu	1.1*
Ala−Phe	1.5		

*Reaction time ; 120 minutes

minutes. For example, the color yield obtained with Ala-Leu was 1.7 times as that of leucine (Fig. 1). The color yields of the peptides at the reaction time of 60 minutes are summarized in Table I.

The bluish purple color formed from ninhydrin and Ala-Leu gave a single spot at the same position as RP by thin layer chromatography. Furthermore, the absorption spectra of this bluish purple color was identical as that of RP. The result showes that two moles of RP is producted from one mole of Ala-Leu. Accordingly, it is concluded that when one mole of Ala-Leu is heated with ninhydrin, one mole of RP is rapidly formed from N-terminal amino acid (alanine), and then deaminated alanine derivative is liberated from the peptide, resulting leucine is reacted to form another one mole of RP (Scheme 2). As shown in Fig. 2, three moles of RP was produced from one mole of Ala-Ala-Leu. The N-terminal amino acid must be sequentially removed from the peptide by the reaction with ninhydrin. When Ala-Ala-Leu was heated with 20 times excess of

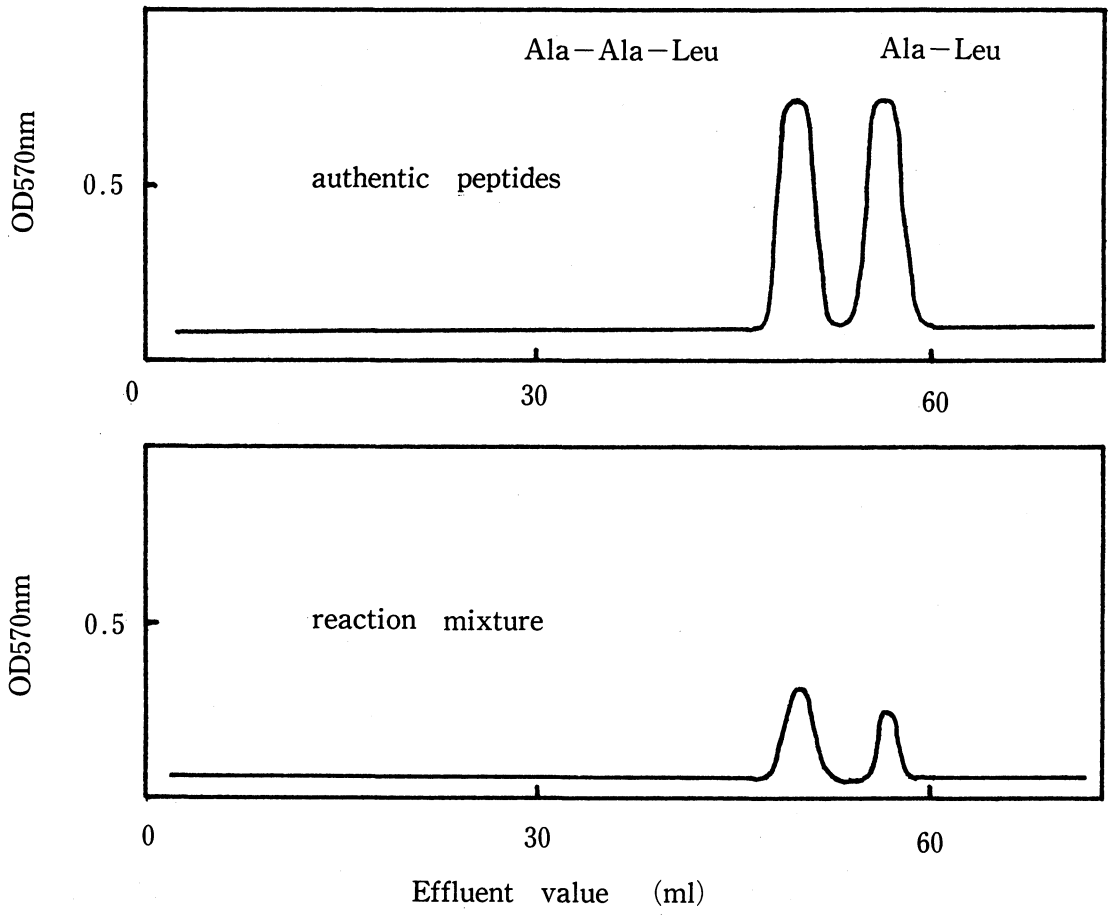
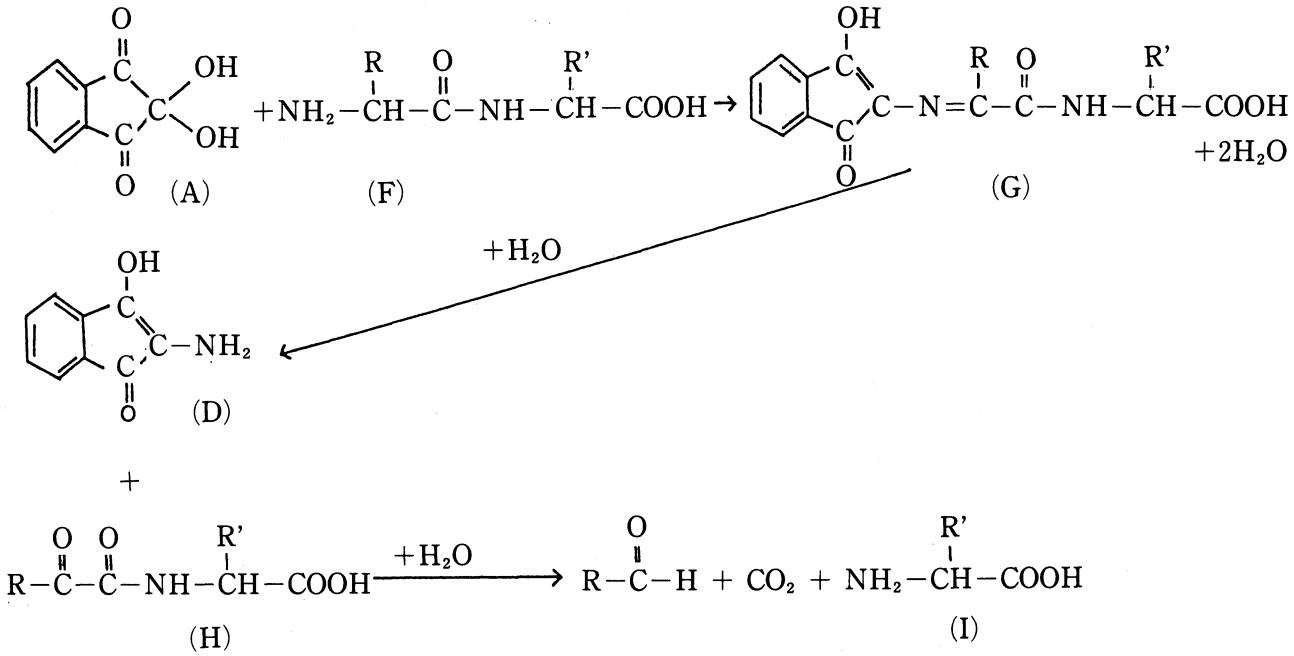


Figure 3. Elution patterns of authentic peptides and reaction mixture of Ala−Ala−Leu and ninhydrin.

ninhydrin, Ala-Leu was isolated in the reaction mixture.

On the basis of the reaction of ninhydrin with amino acid, the reaction of ninhydrin with peptides seems to proceed through the following route. Ninhydrin (A) is bonded to N-terminal amino acid of the peptide (F), and G is produced. The G is then hydrolyzed to form the amine (D) and dicarbonyl compound (H). The amine (D) condenses with ninhydrin to produce RP. The H is hydrolyzed and an aldehyde, carbon dioxide, an amino acid are formed. The resulting amino acid reacts with ninhydrin to produce a further mole of RP.



Scheme 2. Reaction of ninhydrin and peptide.

On the other hand, the color yield of glycine containing peptides, Gly-Ala, Ala-Gly, Ala-Gly-Leu were same as that of leucine (Fig. 1 and 2). In these peptides, only N-terminal amino acids react with ninhydrin to produce RP, and resulting deaminated amino acid derivatives may not be liberated from the peptides. The Gly-Ala was heated with a large excess of ninhydrin, then resulting RP and ninhydrin derivatives were extracted with n-butanol and decarded. The hydrolysis (with 6 M HCl) of the ninhydrin negative product remained in aqueous layer gave alanine by ion exchange chromatography. This ninhydrin negative product may be the dicarbonyl compound (H). The color yield of Ala-Pro was same as that of leucine. However, proline gives yellow colors which possess a broad absorption spectrum with approximately maximum at 440 nm. The presence of chromophoric compound which possessed a broad absorption with maximum at 440 nm was

confirmed in the reaction mixture of Ala-Pro and ninhydrin. Then, it is concluded that the N-terminal amino acid is liberated from the peptide same as Ala-Ala.

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