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APPLICATION OF TRINITROPHENYLATION FOR THE MEASUREMENT OF α-AMINO RESIDUES RESULTING FROM PEPTIC DIGESTION

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Summary

A sensitive and precise method for the measurement of peptic activity on protein substrate is described. α-Amino residues formed by pepsin digestion are photometrically measured by comparing the absorbances of digested and non-digested material which has been trinitrophenylated. The usual problem of high reagent-blank absorbance is eliminated by using an anion exchange resin, Dowex 1-X8. In contrast to Anson's method, the procedure requires only 1/100 the quantity of protein substrate for analysis.

It was proved to be particularly useful for the estimation of initial rates of proteolysis.

Introduction

Although research into the detection and estimation of protease activity has been augmented greatly by the use of enzyme-specific, low-molecular-weight synthetic substrates, Anson's method [1], introduced nearly 40 years ago, is still commonly used as the ultimate measure of activity on protein substrate. It is based on the spectrophotometric measurement at 280 nm of the trichloroacetic-acid-soluble products. Disadvantages of Anson's method include the impossibility of either detecting initial peptide bond cleavage or measuring initial reaction rate. Lin et al. utilized the trinitrophenylation method for the measurement of proteolytic enzyme activity [2]. Lysyl ε-amino residues in protein substrate were alkylated by reductive methylation prior to assay. Newly formed amino residues of protease digestion was trinitrophenylated using 2,4,6-trinitrobenzene sulfonic acid (Tnb-sulfonic acid) and determined spectrophotometrically. Their method is theoretically very useful for the

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measurement of protein digest; however, there remains the usual problem of high reagent-blank absorbance.

During the course of the present study a more precise method was devised as described in this paper. An anion exchange resin, Dowex 1-X8, was utilized prior to spectrophotometric determination after trinitrophenylation in order to remove high reagent-blank absorbance. This procedure was applicable to the measurement of initial velocity of peptic proteolysis and enabled us to measure a few peptide bonds cleaved by pepsin digestion.

Materials and Methods

Chemicals

Tnb-sulfonic acid, sodium salt, was obtained from Wako Chemical Industries (Osaka, Japan). Porcine pepsin, 3 times crystallised (2600 units/mg protein), was purchased from Calbiochem. (Los Angeles, Calif.) and used without additional purification. The supplier of the hemoglobin substrate powder was Nutritional Biochemical Corp. (Cleaveland, Ohio) while that other porcine serum albumin was Miles Laboratories Inc. (Kankakee, Ill.). All other reagents used were analytical grade.

Assay procedure of peptic digestion and trinitrophenylation

In each of several conical polypropylene tubes 0.2 ml of substrate, protein solution adjusted to pH 1.8 with HCl, was incubated at 37°C for 30 min. Porcine pepsin diluted with 0.016 M HCl was kept for 5 min at room temperature for activation. A 20-μl aliquot of the enzyme solution was added to each tube, and the mixture allowed to react at 37°C for an appropriate period. The mixtures remained at pH 1.8—2.0 throughout the reaction without buffering. The enzyme reaction was stopped by the addition of 1.0 ml of 0.1 M borate buffer, pH 9.0. A 0.1-ml aliquot of 0.03 M Tnb-sulfonic acid solution was then added to each tube for trinitrophenylation, and the reaction was allowed to proceed for 60 min at 40°C. The arylation was terminated by the addition of 0.2 ml of formic acid. About 0.15 g of Dowex 1-X8 resin, 100—200 mesh in acetate form, was put in each tube, and they were mixed gently for 15 s on a vortex mixer. Absorbance readings at 340 nm were made on the supernatants, and the difference between values for digested and nondigested substrate was determined. The numbers of amino residues resulting from digestion are calculated on the assumption that the extinction coefficient of newly formed amino residues at 340 nm is $1.05 \cdot 10^4$ $cm^{-1} \cdot M^{-1}$ [3]. The molecular weight and molar extinction coefficient at 278 nm of porcine pepsin were assumed to be 34 000 and $5.1 \cdot 10^4$ [4], respectively. In those samples not subjected to resin treatment, the arylation was stopped by adding 2 ml of formic acid to the reaction mixture, and absorbance was read at 340 nm.

Reductive alkylation of pepsin substrates

Lysyl ϵ-amino residues in protein substrates were dimethylated by the method of Means and Feeney [5] prior to assay. 1.75 mmol of sodium borohydride dissolved in 2.5 ml of water was added to 500 mg protein in 100 ml of 0.2 M borate buffer of pH 9.2, and 2.5 ml solution containing 3.5 mmol of

formaldehyde was added immediately afterwards. Borohydride and formaldehyde were repeatedly put into the solution after a 15 min interval. The reaction mixture was allowed to stand for 1 h in an ice bath, then acidified and dialyzed against distilled water. Dialyzate was lyophilized and stored at −20°C until used.

Modification of Anson's method for measuring peptic activity

1 ml of 0.5% N-dimethylated hemoglobin solution, dialyzed against distilled water and adjusted to pH 1.8, and 1 ml of control buffer were mixed and preincubated at 37°C for 30 min. 0.2 ml of the enzyme solution in 0.016 M HCl activated for 5 min before assay was added to the incubation mixture. After a fixed time, 5 ml of trichloroacetic acid solution was added to each tube. After a 30-min incubation at 37°C, each mixture was filtered through Toyo No. 31 filter paper. Absorbance of the filtrates was read at 280 nm. The enzymatic reaction was effected without buffer salts, as described above in the trinitrophenylation procedure. Moreover, pH changes throughout the reaction are within the range of 0.2 units, a variation which has but negligible effect on peptic activity.

Amino acid analyses and pH measurements

Amino acid analyses were performed with Hitachi Liquid Chromatograph Model 034 using the method of Speckman et al. [6]. pH measurements were made with a Hitachi-Horiba pH meter, Model F-7 ss, on the expanded scale from 1.5 to 2.5 corresponding to potential difference of 100 mV.

Results and Discussion

Reductive alkylation of protein substrates

Protein substrates were reductively alkylated to reduce the number of ϵ-amino groups in lysyl residues and consequently the absorbance of the blank. Degree of alkylation in porcine serum albumin was investigated using an analytical procedure which is dependent upon the interaction of amino acids with 5-dimethylaminonaphthalene-1-sulfonic acid [7]. It was calculated that 5 out of 6 lysyl residues in one mole of porcine serum albumin were alkylated. The modified albumin showed a single, slightly acidic band on disc gel electrophoresis. Fig. 1, (a) and (b), shows the changes in absorbance of various concentrations of the porcine serum albumin (a) and N-dimethylated albumin (b) after trinitrophenylation. The absorbance of trinitrophenylated amino residues in N-dimethylated albumin was 28% of that of the albumin. The digestibilities of both proteins were tested using 200 μg protein and 0.136 μg pepsin, as described under Materials and Methods. Fig. 1, (c) and (d), shows the degree of the digestion of the albumin and N-dimethylated albumin for periods of 30 s to 10 min; the parallel curves showed that the digestibilities of the both proteins were almost equal.

Trinitrophenylation and resin treatment

Trinitrophenylation was performed with Tnb-sulfonic acid according to a modification of the Mokrasch procedure [8]. Incubation was continued for

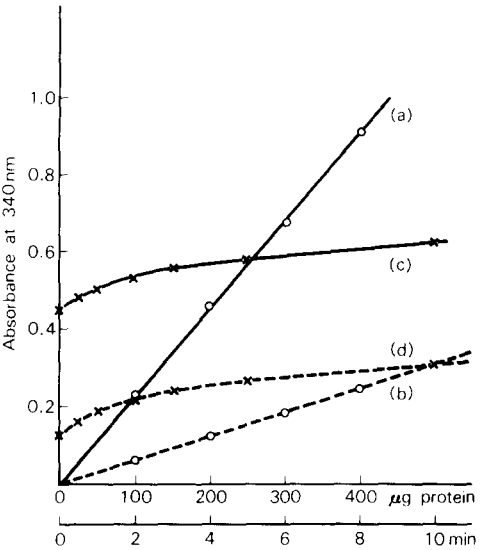
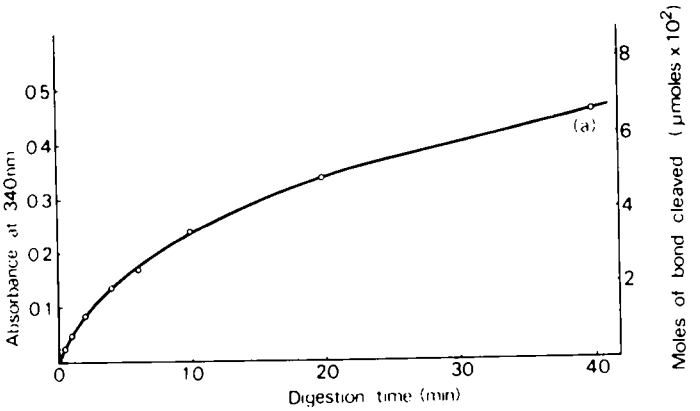
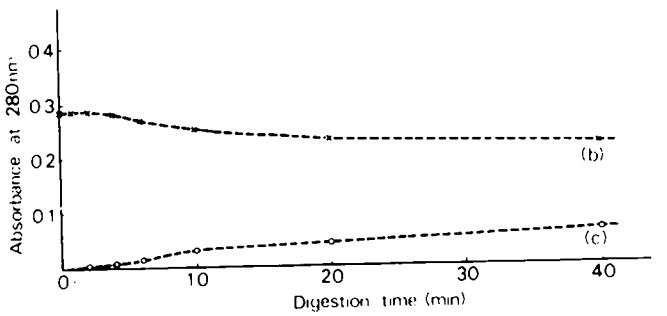


Fig. 1. Standard curves for porcine serum albumin and N-dimethylated porcine serum albumin. The arylation was stopped by a 2-ml addition of formic acid. The ordinate shows the absorbance at 340 nm corrected for reagent blank; the abscissa for (a) and (b) shows the concentration (μg) of porcine serum albumin (○———○) and N-dimethylated porcine serum albumin (○- - - - -○). The abscissa for (c) and (d) shows the digestion periods of porcine serum albumin (X———X) and N-dimethylated porcine serum albumin (X- - - - -X) using 200 μg albumin and 0.136 μg pepsin (see text).

60 min at 40°C, a condition which elicited full color development. The reaction was stopped by adding 2 ml of formic acid to the trinitrophenylated mixture, and the absorbance at 340 nm was read directly. The reagent blank showed a very high reading, about 0.5 absorbance units. For this reason it is difficult to detect an increase in absorbance caused by the α-amino groups formed during peptic digestion. Several attempts were made to reduce the high absorbance of the reagent blank, and it was shown that resin treatment was useful. The procedure involved the addition of 0.2 ml of formic acid followed by 0.15 g Dowex 1-X8 in the acetate form, and gentle agitation for 15 s. The absorbance of the reagent blank was almost negligible as a result of the resin treatment. The pH of the trinitrophenylated mixture was decreased to about 1.6 by the addition of formic acid. Most of the carboxyl groups in the protein, including terminal carboxyls, released by peptic cleavage are not charged in this pH range. On the other hand, Tnb-sulfonic acid, picric acid and other decomposition products which might be in the mixture are highly acidic and negatively charged in the solution, therefore the disturbing components were adsorbed to the resin. In order to ascertain whether the peptides formed during the digestion are adsorbed by the resin, the following experiments were performed. The progressive changes of the digested and trinitrophenylated N-dimethylated albumin (200 μg) followed by the two treatments with resin and without resin were compared. It was shown that the apparent initial rates and total protein concentrations at least up to 4-min digestion in both cases were the same. Low molecular weight peptide such as angiotensin I, a decapeptide, and the mixture of angiotensin I and N-dimethylated albumin were not adsorbed to the resin under the conditions described above. All the above





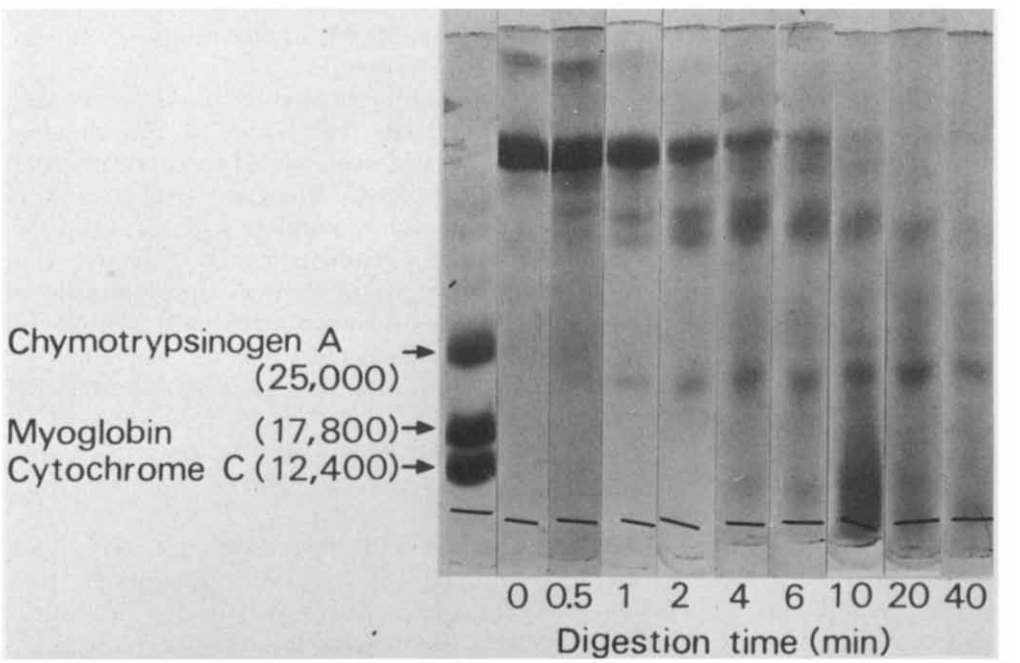


Fig. 2. (A) Kinetic curves for peptic digestion. (a) 200 μg of N-dimethylated albumin were digested with 0.136 μg of pepsin at various times (0—40 min) as described in the text, and the products were detected by the trinitrophenylation method followed by resin treatment (○————○). (b) and (c) 500 μg of N-dimethylated albumin and 0.34 μg of pepsin (2.5 volumes of assay mixture used in (a)) were incubated and 2 ml of 4% trichloroacetic acid were added to the solution after the designated digestion periods. The precipitate was dissolved in 2 ml of 2 M NaOH, and the absorbances of the precipitate (X- - - - -X) and the supernatant (○- - - - -○) were read at 280 nm. Values given are corrected for reagent blank. (B) Sodium dodecyl sulfate disc gel electrophoretic patterns of pepsin digested N-dimethylated albumin.

results indicated that the resin treatment produced no effect on the result of the enzymatic reaction and was useful for reducing the absorbance of the reagent blank.

Kinetic studies

The progressive changes (0—40 min) of the digested N-dimethylated albumin were checked by three methods: (1) trinitrophenylation followed by the resin treatment, (2) trichloroacetic acid precipitation and (3) sodium dodecyl sulfate disc electrophoresis [9] stained by Coomassie Brilliant Blue R-250. The results are shown in Fig. 2(A) and (B). It is clear that the trinitrophenylation method was very sensitive for distinguishing protein cleavage when only a few peptide bonds were cleaved. However, the modification of Anson's method using N-dimethylated albumin as the substrate did not detect such early changes, since the large fragments separated from the protein were trichloroacetic acid-insoluble. Moreover, the absolute amount of peptic proteolysis product cannot be estimated by the method. Sodium dodecyl sulfate electrophoretic patterns revealed that the earlier cleavages were rather restrictive and the products were large fragmented polypeptides. The experimental kinetic curve of (a) in Fig. 2 (A) contained a linear region which permitted accurate measurement of the initial formation rate of the peptic digestion products. K_m value was obtained from the double reciprocal plots using the least squares method. The molar extinction coefficient for trinitrophenyl-amino groups given as $1.05 \cdot 10^4$ at 340 nm by Satake et al. [3] was used for the calculation. The estimated K_m value of N-dimethylated albumin by the trinitrophenylation was 0.29 g/l. This procedure is also applicable to analyze the inhibiting reactions of pepsin inhibitors.

Recently, Schwabe applied an assay, originally reported by Udenfriend et al. [10], which utilized the fluorescing reagent fluorescamine for gauging proteolytic enzyme activity [11]. Though the principle is theoretically the same as that for trinitrophenylation, there remain unsettled problems; the reactivity and the fluorescent yield of the fluorescamine with peptides of differing N-terminal amino acid and structure were significantly different. The trinitrophenylation method is superior to the fluorescent method because of consistent relative color intensities with various amino acids and peptides as reported by Satake et al. [3]. Okuyama and Kasai [12] and recently Blow et al. [13] utilized a modified trinitrophenylation method originally described by Goldfarb [14] for proteolytic activity assay. It is based on the measurement of a stable complex of trinitrophenylated derivatives with sulfite ion at 420 nm. The results obtained with the procedure closely paralleled those from our method.

The advantage of our procedure described is its increased sensitivity. The high reagent-blank absorbance is eliminated by the resin treatment; it is sensitive enough to measure an initial rate of peptic proteolysis with almost 1/50 of the product formation of that in the method described by Lin et al. [2]. Our method is also reliable for the kinetic studies of peptic activity against protein substrates.

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