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Use of Trinitrophenylation for Quantification of Protease and Peptidase Activities

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Abstract—A sensitive and precise method for quantifying protease and peptidase activities is suggested. N-Terminal amino groups of peptides which are formed during hydrolysis of the substrates react with trinitrobenzenesulfonic acid (TNBS), and the trinitrophenyl (TNP) derivatives are determined spectrophotometrically. Spontaneous hydrolysis of TNBS is considerably diminished on trinitrophenylation at pH 7.4 rather than at pH 9-10 as is usually used. The trinitrophenylation method can be used to determine the initial rate of hydrolysis and the kinetics of reactions catalyzed by proteases and peptidases.

Key words: trinitrophenylation, protease, peptidase, amino group, modification

Anson's method, suggested over sixty yeas ago, is still widely used for the determination of protease activity on protein substrates [1]. It is based on the spectrophotometric assay of the proteolysis products, trichloroacetic acid (TCA)-soluble peptides. However, this method is not useful for the determination of peptidase activity and its sensitivity is not sufficient for determining initial rates and reaction kinetics.

Lin et al. [2] used 2,4,6-trinitrobenzenesulfonic acid (TNBS) for the determination of proteolytic activity. The large molar absorption coefficient and its rather constant value for the various trinitrophenyl (TNP)-amino acids provide the high sensitivity and suitability of this method for various protein substrates. Absorption of the initial solution is decreased by alkylation of the substrate amino groups with formaldehyde before the enzymatic reaction. However, the problem of a side reaction remained. Picric acid, having strong absorption at 340 nm, the wavelength at which the reaction products are monitored, is formed during the hydrolysis of TNBS. Mikuni-Takagaki and Hotta added into samples the anion exchanger Dowex 1×8 in acetic form stopping the trinitrophenylation by formic acid [3]. Carboxylic protein groups are not charged near pH 1.6, while picric acid is ionized and bound on the anion exchanger under these conditions. Peptides formed during the hydrolysis by pepsin of alkylated pig albumin were found not to be adsorbed on the ion exchanger, and it does not affect the results of the enzymatic reaction. They demonstrated that it is possible to determine the initial rate of proteolysis and the Michaelis constant for the reaction. Nevertheless, the use of this heterogeneous system is rather complicated.

Proteins are usually reacted with TNBS at pH 9-10 [3, 4] because the rate of formation of TNP products is largest under these conditions, but the rate of spontaneous hydrolysis of TNBS is also rather large at these pH values [5]. In this work, the reaction of N-terminal amino groups of peptides, the products of substrate proteolysis, was done at pH 7.4. The rate of formation of TNP derivatives is sufficient for quantification of forming amino groups, and the hydrolysis of TNBS is considerably diminished compared with the more alkaline conditions. This avoids the need for using the anion exchanger.

MATERIALS AND METHODS

Chymotrypsin was obtained from Samson (St. Petersburg, Russia). The preparation contained 50% active enzyme by weight as determined by titration of the active sites by N-trans-cinnamoylimidazole [6].

Bovine serum albumin (BSA) and melittin (MT) were used as the substrates. BSA has $A^{1\%}_{1\ cm,\ 278} = 6.6$ [7] and molecular mass 66 kD. Melittin was purified from the bee venom by reverse-phase HPLC (RP HPLC) on a Kiloprep (USA) chromatograph using a Waters Nucleosil

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C-18 (USA) preparative column (10 × 60 cm) with 15-20 μm particles and pores of 30-nm average diameter. The protein was eluted by a linear gradient of acetonitrile (0-90%) in water with the addition 0.1% trifluoroacetic acid and was then lyophilized. The purity of MT was not less 95%. Its molecular mass is 2850 daltons [8]. MT has a single aromatic residue (tryptophan) and its ε_{280} was taken as 5,600 $M^{-1}\cdot cm^{-1}$ as for tryptophan [9]. Other reagents from Sigma (USA) were used without additional purification.

The amino groups of the substrate were modified before proteolysis by the method of Meens and Feeney [10]. $NaBH_4$ (38 mg) was dissolved in 2 ml of water and added to 250 mg of BSA dissolved in 50 ml of 0.1 M borate buffer, pH 9.0. Then 1.7 mmol of CH_2O was immediately added as a 40% solution. $NaBH_4$ and CH_2O were again added after 20 min in the same quantities. After 1 h, the solution was dialyzed against water for 16 h.

MT (4 mg) was dissolved in 2 ml of 0.05 M borate buffer, pH 9.0, and 30 μl of the $NaBH_4$ solution in water (7 mg/ml) and 30 μl of 8% CH_2O solution were added. Formic acid (96%) diluted tenfold was added to pH 6-7, and then the sample was subjected to gel filtration on a Sephadex G-25 column (1 × 50 cm) equilibrated with 0.5 M phosphate buffer, pH 7.4.

BSA and its modified derivative were subjected to SDS-PAGE according to Laemmli using 10% gel [11].

The number of TNP groups in peptides (the products of hydrolysis of the substrates) was determined in test tubes with rubber stoppers.

The kinetics of BSA proteolysis was determined with the addition of 80 μl of 0.5 M phosphate buffer, pH 7.4, to 150 μl of aqueous solution of the modified protein (4.8 mg/ml). This solution was incubated for 5 min at 37°C. Then 20 μl of chymotrypsin solution in this buffer (0.11 mg/ml) were added to each test tube. The reaction was stopped by addition of 100 μl of phenylmethylsulfonyl fluoride (PMSF) solution in ethanol (2 mg/ml) after selected times. Sodium TNBS solution (50 μl, 15 mg/ml) in water was added to each test tube and incubated for 1 h at 37°C. Trinitrophenylation was stopped by addition of 2 ml HCOOH and the absorption at 340 nm was measured. The blank solution was prepared in the same manner but the order of reagent addition was changed (enzyme, PMSF, substrate, TNBS, HCOOH).

The number of amino groups reacting with TNBS was determined as $n = A_{340}/\varepsilon_{340}cl$, where A_{340} is the absorption of the solution at 340 nm, ε_{340} is the average molar absorption coefficient of TNP amino acids (10,500 $M^{-1}\cdot cm^{-1}$ [3]), c is the molar substrate concentration, and l is the optical path length (1 cm).

For the determination of K_m, the enzymatic reaction was stopped after times in the range 5-60 min. The enzyme concentration was one fifteenth that used for the overall kinetic curve of hydrolysis.

For the determination of kinetic parameters of the enzymatic hydrolysis of MT, 0.5 ml of the modified peptide solution in 0.5 M phosphate buffer, pH 7.4, was incubated for 5 min at 37°C, and then 15 μl of the chymotrypsin solution (28 μg/ml) in the same buffer was added. The reaction was stopped after 3, 5, 10, 15, and 20 min using 50 μl of PMSF solution in ethanol (2 mg/ml). Then 0.5 ml of TNBS solution (2 mg/ml) was added and the samples incubated for 35 min at 37°C. The order of addition of the reagents in the control was changed as for BSA. The reaction with TNBS was stopped with 2 ml HCOOH, and the absorption at 340 nm was measured. The value of k_{cat} was determined as the number of cleaved peptide bonds per minute per enzyme molecule.

The products of the enzymatic hydrolysis of MT were determined using a Dionex chromatograph (USA) and a Vydac 218 TP54 analytical column (4 × 250 mm). The gradient elution conditions were the same as for the preparative isolation of melittin. The optical absorbance was monitored at 214 and 280 nm. The kinetic data were statistically treated according to the method of Cornish–Bowden [12].

RESULTS AND DISCUSSION

The proposed method has the advantage over the determination of TNBS at more alkaline pH (9-10) that the ratio of the absorption of the product at 340 nm that of the spontaneous hydrolysis of TNBS is much greater. Preliminary experiments showed that the absorption from the spontaneous hydrolysis of TNBS at pH 7.4 (hydrolysis conditions: 1 h, 37°C) was about 3 times less than at pH 9.0 and 4 times less than at pH 10.0.

The product absorption at 340 nm formed at the subtraction of the spontaneous TNBS hydrolysis absorption from the sum absorption even more at pH 7.4 than at alkaline pH as indicated in the experiments with MT. Apparently it takes place because of the low hydrolysis of TNBS. Therefore the ratio of the product absorption at 340 nm to the absorption of the hydrolysis products at pH 7.4 in several times more than at pH 9-10.

Alkylation of the surface lysine residues before the enzyme reaction can considerably increase the absorption difference between the experimental and control samples [3]. The trinitrophenylation method showed that the BSA molecule has one unmodified lysine residue and MT has 0.2 residue after the reaction with formaldehyde. The modified and native BSA gave single spots on gel electrophoresis.

It should be noted that the picric acid by-product can react with α-amino groups of peptides formed, competing with TNBS, and the rate of this reaction increases with increasing pH. Because the spectrum of the sum of the products might differ from the TNP amino acid spectrum near 340 nm [5], the question arises if it is appropriate to use the molar absorption coefficient of

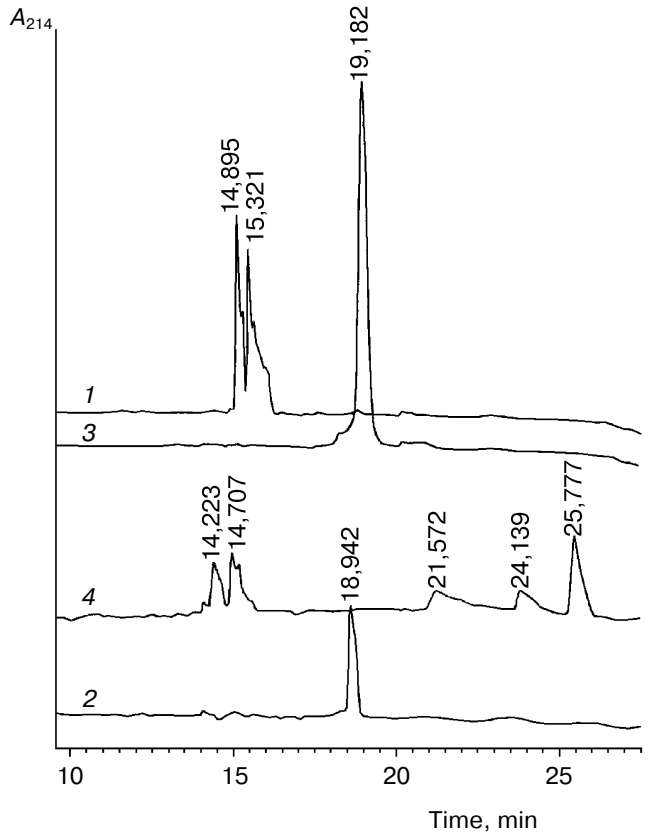

Fig. 1. Hydrolysis of native and modified MT by α-chymotrypsin (RF HPLC): original native (*1*) and modified (*2*) MT; hydrolyzed native (*3*) and hydrolyzed modified (*4*) MT. The numbers by the peaks are the times for elution of the peptides from the column.

TNP amino acids for the determination of the number of modified residues. To solve this problem, model peptides and amino acids or substrates which are cleaved by the proteolytic enzyme giving a known number of fragments can be used. Mikuni-Takagaki and Hotta added the anion exchanger Dowex 1×8 after stopping the trinitrophenylation so that salts of picric acid were not present in the final solution [3]. Because pig albumin was used as the substrate, the true number of modified residues is not known.

In this work, MT was used as one of the substrates. Chymotrypsin is known to cleave a single peptide bond in it [13]. The value of the TNP amino acid molar absorption coefficient permits substrate proteolysis without preliminary alkylation by formaldehyde to be studied because of the small number of superficial lysine residues. Thus, 4.2 residues (3 lysine residues and the N-terminal residue) react with TNBS in unmodified MT. After chymotrypsin hydrolysis, 5.3 residues are trinitrophenylated, i.e., a single peptide bond is cleaved (Fig. 1, curve *3*). This is in agreement with literature data indicating that only the Trp19-Ile20 peptide bond of MT is cleaved by chymotrypsin. This reaction is used as proof of the chymotrypsin purity, i.e., the absence of traces of other proteases [13].

Chymotrypsin, BSA, and MT were taken to show the suitability of trinitrophenylation of both peptides and the proteins and also for enzymes with high and low specificity under conditions near neutrality without the use of an ion-exchanger. However, it was found that modified MT, unlike the native peptide, has not one but the four sites cleaved by chymotrypsin (4.3 residues of TNP amino acids per substrate molecule). Five peptides were also found in the chymotrypsin hydrolyzate of modified MT by HPLC (Fig. 1, curve *4*). This suggests that local conformational changes occur in the environment of these residues after the transformation of the first amine to the second. "Buried" bonds (the primary structure of MT indicates that chymotrypsin could cleave at least 5-6 bonds, but it actually cleaves only one) can be exposed and hydrolyzed by the enzyme. Another possibility is the hydrolysis of the peptide bonds at lysine residues, which

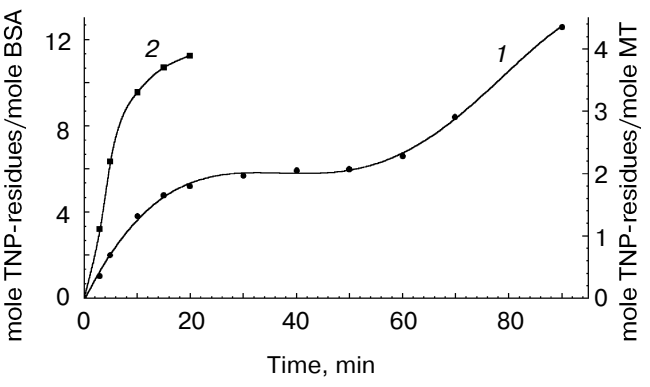

Fig. 2. Kinetic curves of hydrolysis of BSA (*1*) and MT (*2*) by α-chymotrypsin.

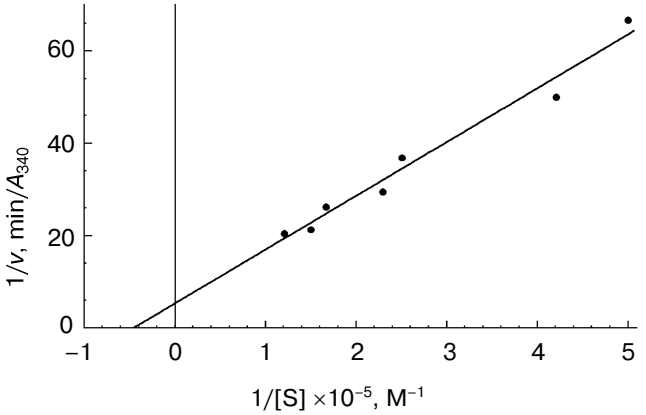

Fig. 3. Kinetics of hydrolysis of MT by α-chymotrypsin in Lineweaver–Burk coordinates.

remain charged after the modification but are made more hydrophobic.

Figure 2 shows kinetic curves of the enzymatic hydrolysis of BSA and MT. The kinetic curve of BSA hydrolysis consists of three regions: an initial straight one (the hydrolysis of superficial peptide bonds), a gently sloping region connected with cleavage of internal bonds, and the terminal bond (the hydrolysis of the bonds of the hydrophobic residues). The initial straight line can be used to determine the initial reaction velocity at different substrate concentrations and the value of K_m, which is 12.5 mg/ml.

Melittin does not have a hydrophobic nucleus. The hydrolysis kinetics of modified MT appears as an ordinary curve with saturation. The value of K_m (23 ± 3 μM) and k_{cat} (3500 ± 500 min^{-1}) were determined from the double reciprocal plot (Fig. 3).

Thus, the enzymatic hydrolysis of a wide group of protein and peptide substrates by both high- and low-specificity proteases can be studied using the trinitrophenylation method under conditions close to neutrality.

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