The Action of Proteolytic Enzymes on N,N-Dimethyl Proteins

BASIS FOR A MICROASSAY FOR PROTEOLYTIC ENZYMES*

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SUMMARY

N,N-Dimethyl proteins were prepared by reductive methylation of their amino groups with formaldehyde and NaBH₄. Proteolysis of these alkylated proteins by trypsin, α-chymotrypsin, subtilisin, pepsin, and fungal protease was determined by direct measurement of the bonds split, with the use of trinitrobenzenesulfonic acid to determine directly the appearance of new terminal amino groups. The low blank values obtained with N,N-dimethyl proteins has resulted in a greatly increased sensitivity and accuracy not possible with unmodified proteins.

On the basis of these studies, an assay of proteolytic activity is described, with N,N-dimethylcasein or N,N-dimethylhemoglobin as substrate, which is from 10 to several hundred times more sensitive than the standard caseinolytic assay of Kunitz.

Proteolytic enzymes and their action on various substrates have been the subject of many studies (1–5). Enzymatic hydrolyses of proteins are complex, difficult to quantitate, and, in general, have not permitted calculation of kinetic constants. Comparisons of different enzymes having different substrate specificities have, for the most part, been based upon assays essentially like the one described by Kunitz (1) over 20 years ago. Such assays are applicable to enzymes having different specificities but are relatively insensitive with low levels of enzyme. In addition, because they rely upon the solubilization of peptides and do not measure each bond split, the results with different enzymes are not necessarily comparable. Quantitative calculations are possible with low molecular weight substrates, amides, or esters, since all cleavages can be measured equally (2, 4, 5). However, these hydrolyses are not necessarily an accurate reflection of an enzyme's activity against protein substrates and are not suitable for use with mixtures of enzymes with different specificities.

During the course of the present study we devised a simple, sensitive, spectrophotometric assay basically like that of Kunitz (1) but designed to measure proteolysis directly from the appearance of new terminal amino groups. Several well known methods are capable of detecting such newly formed amino groups, but with each of these the high blank reading resulting from the use of protein substrates already containing large numbers of amino groups has made such procedures impractical. One procedure, based on the use of salmine, a protein containing few amino groups, has been used to assay trypsin (6). Salmine, however, cannot be used for most other enzymes because of its peculiar amino acid composition. A convenient procedure to decrease or to eliminate amino groups in proteins has recently been reported (7). This procedure is adaptable to the preparation of large quantities of such substrates. It results in the conversion of primary amino groups into dimethylamino groups, a change which does not affect many properties of the protein but does prevent its reaction with TBS*. TBS has been shown to be a sensitive reagent for the determination of protein amino groups (8–10).

MATERIALS

TBS, sodium borohydride, swine skin gelatin (type I), and crystallized hemoglobin (type I) were obtained from Sigma. Hammersten quality casein was purchased from Nutritional Biochemicals, and analyzed reagent grade 37% formaldehyde solution was obtained from J. T. Baker Chemical Company, Phillipsburg, New Jersey.

Trypsin (salt free, crystallized), α-chymotrypsin (twice crystallized), and pepsin (twice crystallized) were obtained from Worthington. Fungal protease from Aspergillus oryzae (370,000 hydrenit units per g) was obtained from the Miles Laboratories, Inc. (Elkhart, Indiana), and subtilisin (Nagarse, lyophilized and crystalline) was from Teikoku Chemical Industry Company, Japan.

* The abbreviation used is: TBS, trinitrobenzenesulfonic acid.
METHODS

Preparation of Substrates—Dimethyl substrates were prepared by a modification of the procedure recently described by Means and Feeney (7). Casein (1.5 g) was warmed to dissolve in 150 ml of 0.1 M borate buffer, pH 9.0, and then cooled to 0°. The slightly cloudy solution was rapidly stirred, and 300 mg of sodium borohydride were then added. A few drops of 2-octanol were also added to prevent any subsequent tendency to foam. Formaldehyde (3 ml) was then added in 100-μl increments over a period of 30 min. After a few additional minutes, the solution was acidified to pH 6 by the addition of 50% acetic acid and dialyzed against deionized water. The desalted protein was then lyophilized and stored at -20° as a fluffy white powder.

Dimethylgelatin was prepared from gelatin by the same procedure. Reductive methylation of hemoglobin was done in a similar manner, except the protein was treated before alkylation with a few drops of hydrogen peroxide and gently warmed in order to decrease its visible absorbance.

Samples for amino acid analysis were prepared by hydrolysis in sealed, evacuated tubes with 6 N HCl at 110° for 22 hours. Analyses were done with a Technicon autoanalyzer, with the 10-hour run for alkaline amino acids recently described (7).

Proteolytic Assays—The enzyme, 0.1 to 2.0 μg, was added to 1 ml of a solution of 0.1% dimethylcasein or other reductively methylated protein substrates at the desired pH and incubated for 10 to 120 min at 38°. Incubations (30 min each) were used for most assays. Reactions were stopped by immersing the samples briefly in a boiling water bath. Then, 1 ml of a solution of 0.1% TBS and 1 ml of a 4% sodium bicarbonate solution, pH 8.5, were added to each sample as in the procedure of Habeeb (9). The samples were then incubated in the dark for 30 min at 50° or for 90 min at 38°. After incubation, 1 ml of a 10% sodium dodecyl sulfate solution and 0.5 ml of a 1 N HCl solution were added to each sample, and the absorbance at 340 μm was determined relative to a blank incubated with all of the components present in the sample except active enzyme.

RESULTS

Preparation of Substrates—Reductive methylation of proteins decreases their reactivity with TBS. The progressive losses of TBS-reactive amino groups with increasing reductive methylation of casein and gelatin are shown in Fig. 1. These extensively methylated substrates contained only a very small fraction of their original primary amino groups. The methylated casein contained only 1.3% of its original lysine content by amino acid analysis. As judged by TBS analysis, large losses of primary amino groups resulted from similar treatment of other proteins (Table I). The procedure is simple and does not involve the use of expensive or unusual reagents. Samples of a few milligrams to 2 Molar extinction coefficients of trinitrophenyl α-amino acids vary from about 1.1 × 10⁴ to 1.5 × 10⁴, depending upon the side chain, with most values near 1.3 × 10⁴. Using this value to calculate the concentration of free amino groups allows the direct expression of proteolytic activity in terms of the number of bonds split.

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several hundred grams can be prepared rapidly, conveniently, and inexpensively.

Reductive methylation does not greatly affect most physicochemical properties of proteins (7). Since none of its charged groups are lost, the solubility of dimethylcasein is not noticeably different from that of casein. Both are insoluble between pH 3.5 and 5.5, and for this reason are poor substrates between these pH values. Dimethylhemoglobin, however, like hemoglobin, is both soluble and a good substrate between these pH values.

Effect of Temperature on TBS Reaction—At 36-38° the reaction of TBS with NH₂-terminal amino groups requires approximately 1½ hour to reach maximum color development (9). Because this was the most time-consuming step, the use of higher temperatures was investigated for the purpose of decreasing this incubation period. Incubation at 60° and above gave higher blank absorbances, and assays done at these temperatures appeared to have proportionately fewer amino groups.

At 50° and 30-min incubation, absorbances of blank and assay samples were nearly identical to those at 38° and 90 min. Results based on the two temperatures were within experimental error of each other. Doing the TBS incubation at 50° allows an assay to be completed in about 1 hour.

Variation of Enzyme and Substrate Concentrations—Different enzymes varied greatly in their proteolytic activity toward dimethyl substrates (Fig. 2). As shown, subtilisin and A. oryzae protease were extremely active. Trypsin and α-chymotrypsin had similar high activities initially but slowed greatly with increasing amounts of enzyme. Under these conditions, increasing the amount of α-chymotrypsin or trypsin above 3 μg did not give proportionate increases in the rate of proteolysis. This was due, perhaps, to their comparatively narrower substrate specificities, and therefore to the relatively smaller number of susceptible peptide bonds in the substrate. From the amino acid composition of casein (13), this deviation from linearity with trypsin appears to coincide very nearly with the point where all susceptible bonds have been cleaved. With chymotrypsin, non-linearity is first observed when only half of the susceptible bonds have been cleaved. A similar lack of linearity with increasing amounts of these enzymes against casein has been described by Kunitz (1).

At low enzyme concentrations, uniform rates of hydrolysis were obtained for relatively long periods with every enzyme but trypsin. Results with chymotrypsin and subtilisin are shown in Fig. 3. It was presumed that the lack of linearity with trypsin was due either to its very narrow substrate specificity and to a lack of susceptible residues in the substrate or to some unknown interaction with the ε-N₂,N-di methyllysine residues.

As indicated by the double reciprocal plot in Fig. 4, increasing the concentration of substrate increased the rate of chymotrypsincatalyzed hydrolysis and readily allowed calculation of its $K_m$ for this substrate. The determined value was 2.82 mg ml⁻¹. With the use of the amino acid composition of whole casein (13), this corresponds to a value of $2.0 \times 10^{-4}$ M, which compares favorably with some of the values reported for low molecular weight substrates. For example, Yoshida, Yamamoto, and Izumiya (14) reported a $K_m$ of $6.7 \times 10^{-3}$ M for Gly₂-Tyr-Gly₄.

Substrate Inhibition of Trypsin—Unlike chymotrypsin, increasing the concentration of dimethylcasein above 2.5 mg per ml...
decreased the rate of trypsin-catalyzed hydrolysis. This behavior resulted in a nonlinear double reciprocal plot (Fig. 4) typically considered to be a demonstration of inhibition by substrate. In spite of this apparent inhibition, observed catalytic constants were similar to those observed with other substrates. The rate of tryptic hydrolysis of the Arg-Gly bond in oxidized \( \beta \) chain of insulin under similar conditions, for example, is approximately 10- to 20-fold lower than the observed rate of hydrolysis of dimethylcasein (3). The apparent \( K_m \) of trypsin against dimethylcasein is 1.39 mg ml \(^{-1}\) or 3.34 \( \times 10^{-4}\) M, slightly lower than the reported values of 2.5 \( \times 10^{-4}\) M to 4.5 \( \times 10^{-4}\) M for hydrolysis of N-benzoylarginamide (15).

**Determinations of pH Activity Profiles**—To further illustrate the general usefulness of this assay, pH activity profiles of several enzymes were determined. The two enzymes shown in Fig. 5 have acidic pH optima, which necessitated the use of dimethylhemoglobin as substrate, since dimethylcasein was insoluble over part of this range. The optimum activity determined for pepsin was between pH 1.5 and 2.0, in agreement with the results obtained by other workers using other substrates (16). The other enzyme preparation employed was a mixture of at least two acidic fungal proteinases from *A. oryzae*. As shown, it has a relatively broad pH optimum centered at pH 4.5 and, on a weight basis, a lower activity than pepsin.

**DISCUSSION**

Most assays for proteolytic activity are basically like that originally described by Kunitz (1) in 1947. Many modifications of this basic assay have been developed to suit specific needs (6, 17–20). These assays are most useful for enzymes having unknown, undefined, or broad substrate specificities and for enzyme mixtures. Their great suitability for many different enzymes results from the use of protein substrates having many different potentially susceptible bonds. Such assays are often used to compare proteolytic activities of enzymes having different specificities. These assays are not extremely sensitive, however, and are therefore not of great use at low levels of proteolytic activity. Among the reasons for their relative insensitivity, the most important seems to be their failure to measure all bond cleavages. Hydrolysis of small numbers of bonds in such assays would be expected to result in larger peptides, proportionately greater numbers of which would, because of their size, be precipitated by trichloracetic acid and could not be distinguished from uncleaved protein. The characteristics which result in trichloracetic acid precipitability are not well understood but seemingly would not be equally distributed among the cleavage products. Also, since the soluble peptides are usually measured from their absorbance at 280 nm, and such absorbance must vary from one peptide to the next, equal degrees of proteolysis by different enzymes would not result in the same increment of increased absorbance.

By measurement of all newly formed NH\(_2\)-terminals, with the exceptions of proline and hydroxyproline, the method used in the present study affords a greatly increased sensitivity and permits such proteolyses to be expressed in terms of the number of bonds cleaved rather than, as has been common in the past, in terms of "Kunitz units" or other units of rather indefinite meaning.
Such a basis for expressing proteolytic activity appears to be a more meaningful way to compare enzymes with different specificities. The use of ninhydrin or other reagents to detect newly formed amino groups should give similar results and, under certain conditions, may prove even more convenient than TBS. Ammonium and primary amine buffers, such as Tris, it should be pointed out, must be avoided in the present procedure, because they too are detected by TBS.

Reductive methylation of lysyl residues renders them resistant to tryptic hydrolysis (7, 21) and decreases the number of trypsin labile bonds relative to the corresponding unmodified substrate. Failure of trypsin to cleave e-N,N-dimethyllysine bonds has been attributed to failure of these residues to participate in hydrogen bond formation with the enzyme and thus to a failure to bind to the enzyme (21). Supporting this is the lack of trypsin-inhibitory activity of the two dimethyl derivatives of the inhibitory primary amines, benzylamine and butylamine (21). Turkey ovomucoid, a trypsin inhibitor whose amino groups are essential for its activity, is also inactivated by reductive methylation (7). Inhibition of trypsin by higher concentrations of dimethylcasein, however, is observed and may indicate a more complex basis for this inhibition.

Inherent in the use of reductively alkylated proteins is the assumed functional similarity to unalkylated polypeptide chains. The validity of this assumption is not entirely complete for enzymes such as trypsin whose specificity includes lysyl residues. Such an assumption seems to be well founded, however, for most other enzymes. Reductive methylation affects only amino groups, and since their ionic properties are not greatly altered, does not result in a decreased solubility. Other methods for modifying lysyl residues and also retaining their cationic state are not usually suitable for conveniently or economically preparing large quantities of substrate.

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