Effect of High Tyrosine Content on the Determination of Tryptophan in Protein by the Acidic Ninhydrin Method

APPLICATION TO CHICKEN OVOINHIBITOR

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Colorimetric determination of tryptophan in intact proteins by the acidic ninhydrin method of Gaitonde & Dovey (1970) gives high apparent tryptophan contents for proteins having high tyrosine/tryptophan ratios. Correction for this interference by tyrosine can be achieved by plotting the ratio of observed to expected tryptophan content as a function of tyrosine/tryptophan ratio for proteins of known composition. The equation of the line is:

$$(\text{Trp}_{\text{observed}}/\text{Trp}_{\text{expected}}) = 1.013 + 0.034 (\text{Tyr}/\text{Trp})$$

Application of this correction to chicken ovomucoid, which contains 17 tyrosine residues per molecule, gave results that agree with tryptophan content determined by other methods.

Various methods used for determination of tryptophan in proteins and other biomolecules, as well as their limitations, have recently been reviewed (Friedman & Finley, 1971). Because tryptophan is either absent from, or present only in small amounts in, most naturally occurring proteinase inhibitors (Vogel *et al.*, 1968; Feeney & Allison, 1969; Kassell, 1970*a,b*; Laskowski & Sealock, 1971), determination of tryptophan in these proteins is often difficult.

Until recently, it was thought that chicken ovomucoid lacked tryptophan, since it resembles most other proteinase inhibitors in amino acid composition (Rhodes *et al.*, 1960; Tomimatsu *et al.*, 1966; Davis *et al.*, 1969). However, none of these analyses clearly demonstrated the presence or absence of tryptophan in this inhibitor. Liu *et al.* (1971) found that chicken ovomucoid exhibits characteristic tryptophan fluorescence, and determined that it contains one tryptophan residue per molecule. We had observed independently that the tryptophan fluorescence is intrinsic to the molecule and not due to a bound contaminant (Zahnley & Davis, 1971), and had attempted to verify our results by the method of Gaitonde & Dovey (1970). This method, which employs reaction with ninhydrin in strong acid, indicated that 1 mol of inhibitor contained 1.5 mol of tryptophan. We have determined that a correction is required when this method is applied to proteins such as chicken ovomucoid, which have high ratios of tyrosine to tryptophan. For such proteins, abnormally high tryptophan contents are obtained. When a correction is applied by means of a 'correction curve' described, the corrected value for tryptophan in the inhibitor

agrees well with the results obtained by other methods (Liu *et al.*, 1971; Barth *et al.*, 1972*b*).

Materials and Methods

The chicken ovomucoid used was purified as described previously (Zahnley & Davis, 1970). Chicken ovomucoid was prepared by Davis *et al.* (1971). Chicken egg-white lysozyme, purified in this laboratory by the method of Alderton *et al.* (1945), was pure by the criterion of starch-gel electrophoresis. Crystallized human serum albumin was Mann lot P 1480 (Schwarz/Mann, Orangeburg, N.Y., U.S.A.).

A 6M solution of guanidine hydrochloric (Mann Ultra Pure: lot T 4626) had an absorbance at pH 4 of 0.002 at 280 nm and 0.015 at 225 nm. Other chemicals were reagent-grade commercial products. Solutions were prepared in distilled water subsequently deionized by passage through a Barnstead demineralizer, boiled, and stored in glass.

Absorption measurements were obtained with a Cary 15 spectrophotometer. A Radiometer pHM 26 pH-meter equipped with a Beckman 39030 thin-probe combination electrode was used for pH measurements.

Ninhydrin reagent *b* of Gaitonde & Dovey (1970) was used for determination of tryptophan. The sample volume was decreased to compensate for the water resulting from the change from 98–100% to 88% formic acid. Lysozyme was used to construct a standard curve. As specified by Gaitonde & Dovey (1970), samples were read against a reagent blank containing the acid and ninhydrin heated under

identical conditions. Controls of protein heated in acid without ninhydrin gave no absorption above 380nm when measured against either distilled water or the acid mixture.

To remove possible bound contaminants, a sample of inhibitor (0.80ml of ~0.5mM) in 6M-guanidine-HCl was dialysed against approx. 5vol. of the same solvent at pH5.5, and then at pH3. The guanidine-HCl was then removed by dialysis against distilled water.

Results

The absorption produced in the reaction by a given amount of lysozyme agreed with the values obtained by Gaitonde & Dovey (1970). The chicken ovo-inhibitor was analysed, chicken ovomucoid (6 Tyr: 0 Trp) and human serum albumin (18 Tyr: 1 Trp) being used as comparison proteins. The presence of tryptophan in our inhibitor preparations, first detected by fluorescence, was confirmed. The inhibitor and human serum albumin gave absorption spectra (maximum at 380–385nm) typical of tryptophan-containing proteins after the reaction with ninhydrin in strong acid (Fig. 1). Gaitonde & Dovey (1970) found that proteins containing tyrosine but not tryptophan (insulin, ribonuclease) gave no typical yellow product in the reaction even though free tyrosine gave a product with some absorption at 385nm ($\epsilon = 400$). Similarly, we found that chicken ovomucoid gave a brown colour but no absorption in the 350–450nm range.

Unfractionated inhibitor (duplicate analyses) and the non-diffusible material remaining after it had been dialysed first against guanidine-HCl and then against water to remove guanidine-HCl were analysed. Apparent tryptophan contents of 1.56, 1.53, and 1.41 mol/mol of inhibitor respectively were obtained, the value of $\epsilon_{\text{Trp}} = 17160$ for protein derived from a lysozyme standard being used. However, inspection of both our data and those of Gaitonde & Dovey (1970) suggested a relationship between tyrosine content and colour yield of tryptophan. When the ratio of the tryptophan content obtained by the Gaitonde & Dovey (1970) method to the expected tryptophan content was plotted against the ratio of tyrosine to tryptophan, a straight line was obtained (Fig. 2). We have designated this line the 'correction curve'. The equation of the line (unweighted least-squares fit) is:

$$\left(\frac{\text{Trp}_{\text{observed}}}{\text{Trp}_{\text{expected}}}\right) = (1.0127 \pm 0.0051) + (0.0335 \pm 0.0005) \left(\frac{\text{Tyr}}{\text{Trp}}\right)$$

Standard errors of the slope and intercept are shown. When the results of the tryptophan analyses for the inhibitor were corrected by using this graph, values

Fig. 1. Absorption spectra of the reaction products of chicken ovo-inhibitor, human serum albumin, ovomucoid and lysozyme with ninhydrin in formic acid-hydrochloric acid

Accepted values for tryptophan contents of the proteins (mol of tryptophan/mol of protein) are: (A) lysozyme, 6; (B) human serum albumin, 1; (C) ovo-inhibitor, 1; (D) ovomucoid, 0. The horizontal bars at the absorption maximum (vertical broken line) show the molar absorption expected for the integral values given. The retentate after dialysis of ovo-inhibitor against guanidine-HCl is shown (.....). Two analyses of ovomucoid are shown (— — —). See the text for other details.

of 0.99, 0.97, and 0.89 mol of tryptophan/mol respectively were obtained. [A tyrosine/tryptophan ratio of 17 was assumed in determining the correction, since the inhibitor contains 17 mol of tyrosine/mol of protein (Tomimatsu *et al.*, 1966; Davis *et al.*, 1969).]

An alternative correction procedure, subtracting the tyrosine contribution calculated from the product of the absorptivity of free tyrosine times the number of mol of tyrosine/mol of protein, gives a range of 1.00–1.16 mol of tryptophan/mol of inhibitor for the same analyses. The correlation of colour yield to

content of other amino acids was poorer than for tyrosine and decreased in the approximate order: histidine, cystine, proline, methionine, phenylalanine.

Fig. 2. 'Excess of tryptophan content' of known proteins as a function of the tyrosine/tryptophan ratio

This calibration curve ('correction curve') was used to correct the inhibitor results (●). R indicates the value for the inhibitor retentate after dialysis against guanidine-HCl (see the text). Proteins: HSA, human serum albumin; BSA, bovine serum albumin; T, bovine trypsin; L, lysozyme. The data for the last three proteins were recalculated from the data presented by Gaitonde & Dovey (1970). Expected values are accepted integral values obtained by other methods.

Discussion

As recently pointed out (Friedman & Finley, 1971; Barth *et al.*, 1972a), determination of tryptophan in proteins remains a problem, especially for proteins such as the chicken ovom inhibitor, where the large tyrosine content makes detection of tryptophan by ultraviolet absorption difficult and unreliable (Light & Smith, 1963; Barth *et al.*, 1972b). Problems similar to those reported by Harrison & Hofmann (1961) were encountered when the colorimetric procedure of Spies & Chambers (1949) was used to determine the tryptophan content of the inhibitor (Davis *et al.*, 1969; Liu *et al.*, 1971).

The results presented here show that the method of Gaitonde & Dovey (1970), which employs reaction of the protein with ninhydrin in formic acid-hydrochloric acid, can give erroneous results with some proteins. This deviation from correct values for tryptophan is significant even at tyrosine/tryptophan ratios well below that of the chicken ovom inhibitor (Fig. 2). To apply the correction described in this paper, the tyrosine content must be known, but it

can be readily determined by other methods. Inclusion of reference proteins having tyrosine/tryptophan ratios similar to the possible ratios expected, as illustrated by the use of human serum albumin in this case, is advisable. The tryptophan content of the inhibitor after correction for tyrosine interference as described here agrees well with the values obtained by fluorescence (Liu *et al.*, 1971; Zahnley & Davis, 1971), oxidation with *N*-bromosuccinimide (Liu *et al.*, 1971) and magnetic circular dichroism (Barth *et al.*, 1972b). Thus the method of Gaitonde & Dovey (1970) can be extended to determination of tryptophan in intact proteins having high tyrosine/tryptophan ratios provided that this interference is taken into account.

Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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