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Note

Rapid benchtop method of alkaline hydrolysis of proteins

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Tryptophan can easily be quantitated by amino acid analyzer or by specific assays[1]. However, tryptophan analysis of proteins requires a hydrolysis step which can be time-consuming or cause variable losses of tryptophan. Despite promising improvements in acid hydrolysis techniques, significant losses of tryptophan still occur, especially in the presence of carbohydrate[2,3]. Alkaline hydrolysis remains the method of choice when one requires precise determination of tryptophan[4,5].

Spies and Chambers[4] originally noted that increased temperature of hydrolysis dramatically accelerated release of tryptophan. Hare[6] described a technique of rapid acid hydrolysis at 155°C, which eliminated the need for sealing samples in thick walled tubes. I adapted this method for alkaline hydrolysis, using a simple benchtop block heater. Following hydrolysis, tryptophan was quantitated with the sensitive, rapid fluorimetric method of Nakamura and Pisano[1]. Thus, the tryptophan content of a protein can be determined in half a day.

EXPERIMENTAL

Lysozyme was obtained from Boehringer (Mannheim, G.F.R.); tryptophan and the albumins were Sigma products (Sigma, St. Louis, MO, U.S.A.). Glutamine synthetase was prepared by Miller's method[7]. Protein concentrations were determined spectrophotometrically using a molar absorptivity of 37,800 for lysozyme (281 nm, 1 m*M* HCl), 44,000 for bovine albumin and 35,200 for human albumin (278 nm, 50 m*M* potassium phosphate, pH 7.0)[8]. Glutamine synthetase concentration was calculated as described[9]. Tryptophan was determined in water with $\varepsilon_{molar} = 5500$ at 278.9 nm.

The hydrolysis solution was 5 *M* NaOH (Baker, Phillipsburg, NJ, U.S.A.) with 3% (v/v) thiodiglycol (Pierce, Rockford, IL, U.S.A.). Solutions were made fresh daily, although stability during longer storage was not tested. Thiodiglycol must be included to prevent variable losses of tryptophan as noted by Oelshlegel *et al.*[10] for hydrolysis at 120°C[5].

Hydrolysis vials were Wheaton 1-dram borosilicate glass vials (No. 224882), with a PTFE on silicone cap liner (Pierce No. 12712). They were used without additional treatment or cleaning. Unlined caps were from PGC Scientific (Rockville, MD, U.S.A.). If the usual rubber-lined caps are supplied, this liner must be removed

before inserting the PTFE/silicone liner. (A narrow metal spatula blade permits easy removal of the rubber liner. The caps should then be boiled in water to remove loosely-adhering particles.)

These vials fit into a heating block (Pierce Reacti-Block C) seated in a standard, small, bench-top heater (Lab-Line Multi-Blok Heater No. 2090, with cover). The temperature was set at 155°C. With the cover in place, the temperature usually varied no more than 1°.

Alkaline hydrolysis

Place 25 μl of protein solution (2–200 nmoles in tryptophan) in the hydrolysis vial. Add 500 μl NaOH–thiodiglycol, and flush the vial of air with a stream of argon or nitrogen for 20 sec. Then screw on the cap with the PTFE liner in place. The cap should be snug, but overtightening may crack the neck of the vial. Place the vial in the heating block for the desired time. As shown in the Results section, taking several time points may give more accurate determinations. Times samples may be obtained by using different vials. However, if the amount of protein available is limited, one may sample from a single vial. If a single time point is chosen, 30–45 min is adequate for most proteins. Silicate extraction from the glass increases considerably when hydrolysis time exceeds 45 min.

After cooling, add 500 μl of 25% acetic acid and then 2.5 ml of 200 m*M* borate, pH 9.0. (Make by titrating boric acid with NaOH.) Recap and centrifuge the vial for 10 min to precipitate insoluble silicates. Use a 100-μl aliquot of the supernatant for tryptophan determination as described by Nakamura and Pisano[1]. If several time points were obtained, extrapolate to infinite hydrolysis time with a double reciprocal plot (Fig. 2).

RESULTS

Release of tryptophan was followed to assess the rate of hydrolysis of protein. Tryptophan release approaches a maximum at 30–45 min, with release from lysozyme slightly slower than from the other three proteins (Fig. 1). Heating for 90 min or longer leads to a decrease in tryptophan recovery.

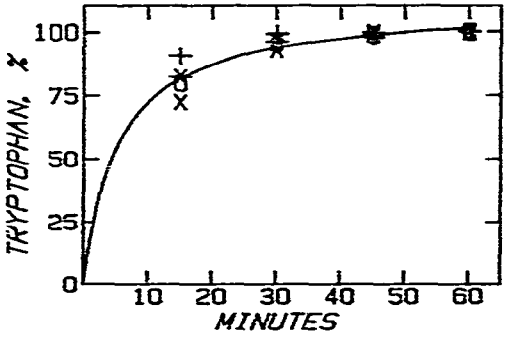

Fig. 1. Release of tryptophan from proteins during alkaline hydrolysis: +, human albumin; ✱, bovine albumin; O, glutamine synthetase; X, lysozyme. To permit plotting the results on one graph, the amount of tryptophan released at 60 min was set at 100%. The curve is the least squares fit to the average of the four proteins. It was fit to a double reciprocal plot, giving

$$\% \text{ Tryptophan} = \frac{110.3\ x\ (\text{min})}{5.238 + \text{min}},\ r = 0.989$$

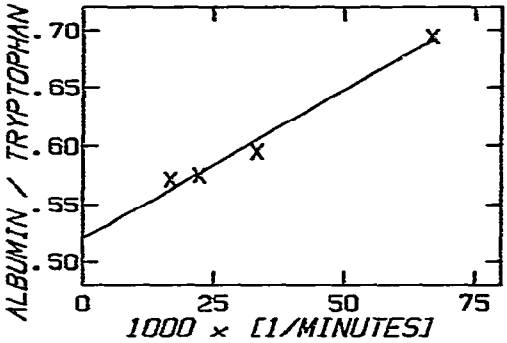

Fig. 2. Release of tryptophan from bovine albumin plotted as a double reciprocal. The fit line is $y = 0.520 + 2.56x$, $r = 0.990$.

The ease of hydrolysis makes it possible to obtain determinations at several times of hydrolysis. Plotting on a double reciprocal plot then permits linear extrapolation to infinite time of hydrolysis (Fig. 2). The results agree with expected values (Table I). If only the 45-min point were used, the average estimated tryptophan content is 10.4% below the extrapolated value. At 30 min, the value is 14.9% below the extrapolated value. These factors can be used to adjust single time point determinations.

TABLE I

TRYPTOPHAN CONTENT OF PROTEINS

Protein	*Measured**	*Expected*
Human albumin	0.94	1
Bovine albumin	1.92	2
Glutamine synthetase	1.95	2**
Lysozyme	6.18	6

* By extrapolation with a double reciprocal plot.
** See Discussion.

DISCUSSION

As reported by Spies and Chambers[4] over 30 years ago, alkaline hydrolysis of proteins proceeds much faster at elevated temperatures. As shown in Fig. 1, recovery of tryptophan from four proteins approaches a maximum by 45 min when hydrolysis occurs at 155°C. The use of the block heater permitted hydrolysis to be conveniently conducted on the lab bench using screw-top vials. Coupled with a fluorimetric assay for tryptophan, the method provides a simple, sensitive procedure for assaying the tryptophan content of proteins. The technique also permits recovery of other acid-labile residues, such as phosphotyrosine[11].

The tryptophan content of glutamine synthetase is two residues per subunit. Previously the content had been assayed by ultraviolet spectroscopy, giving a higher

value of three residues per subunit[12]. Determination with second derivative spectroscopy[13] confirmed that two residues is the correct value[14]. An incorrect, high tryptophan content also resulted from spectral analysis of deoxyribonuclease[5].

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