RADIOMETRIC ASSAY OF PROTEOLYSIS BY CRUDE PLANT EXTRACTS

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SUMMARY

Hydrolysis of bovine serum albumin and [^{125}I]bovine serum albumin by crude plant extracts was measured by the Lowry method and liquid scintillation spectrometry. Measurement of protein hydrolysis by [^{125}I] is faster, more sensitive, and more specific than by the Lowry and probably other colorimetric methods. In addition, the [^{125}I] method can measure hydrolysis by crude extracts of endogenous plant proteins.

INTRODUCTION

Measurement of proteolytic enzyme activity in crude extracts of plant tissues assumes increasing importance because of current interest in normal and pathological metabolism (1). However, commonly used methods for estimating proteolytic activity do not measure proteolysis, lack sensitivity or reproducibility, or suffer interference from non-protein phenols that abound in woody plant enzyme extracts (2,3).

Nardi (4) demonstrated the potential of applying radioisotope techniques to measurement of proteolytic activity. We modified his method to develop a reliable, sensitive method for estimating protein hydrolysis by crude plant extracts. The method quantifies protein hydrolysis by measuring [^{125}I] contained in soluble residues.

METHODS

Crude extracts were prepared from fresh, ungerminated jack pine (*Pinus banksiana*) and peanut (*Arachis hypogaea*) seeds. 500 mg of milled jack pine and an equal amount of diced peanut seeds were each homogenized in 3.0 ml of 0.1M Tris-HCl buffer, pH 7.5, which contained 0.1M sucrose (5). Extracts were centrifuged (15,000 xg, 30 min) and dialyzed (as indicated) overnight against extraction buffer.

Reaction mixtures contained: (1) 4.0 ml of 0.5M Tris-Citrate buffer, pH 3.0; (2) 1.0 ml of 0.1% (w/v) fatty acid-free BSA[1/] (Miles Laboratories) in 0.1M Tris-HCl buffer, pH 7.5; 2.0 ml extract as noted above; and (4) either 2.0 ml (88, 800 DPM) of [^{125}I]BSA (New England Nuclear, sp. ac. 0.412 mCi per mg protein) in 0.1M Tris-HCl buffer, pH 7.5; or 2.0 ml 0.1M Tris-HCl buffer, pH 7.5. The [^{125}I]BSA was prepared by diluting the purchased stock solution. The reaction was started by adding extract.

Control mixtures were prepared as were reaction mixtures, except buffer was substituted for extract.

Reactions were sampled immediately after extract was added and hourly thereafter by adding 1 ml of reaction mixture to 1 ml of 10% (w/v) TCA in distilled water. Controls were similarly sampled. Precipitated protein was removed by centrifuging (15,000 xg, 30 min) more than an hour after the reaction was terminated. If the reaction mixtures and controls contained [^{125}I]BSA, 1 ml of supernatant was added to 15 ml of scintillation grade toluene and triton X-100 containing PPO (4.7 g/l) and POPOP (0.4 g/l). [^{125}I] was determined with a Beckman LS-150 liquid scintillation spectrometer equipped with a wide [^{14}C] Isoset. Samples were counted to a statistical error of 1% or for 10 minutes, whichever occurred first.

Gross count rates for reaction mixtures were corrected by subtracting count rates for controls. These net CPM's were corrected for decay, but not converted to DPM because of only minor variation in external standard ratios between samples.

If reaction mixtures did not contain [^{125}I]BSA, TCA soluble hydrolysis products were estimated by the Lowry (6) method with a Beckman DU spectrophotometer. We chose the Lowry method as a sensitive, colorimetric means of quantifying TCA soluble protein hydrolysis products (7,8,9).

Similar methods were used in preliminary experiments that tested effects

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1/ Abbreviations: BSA -- Bovine serum albumin; TCA -- trichloroacetic acid; PPO -- 2,5-diphenyloxazole; POPOP -- 1,4-bis-(2-(4-methyl-5-phenyloxazolyl))-benzene.

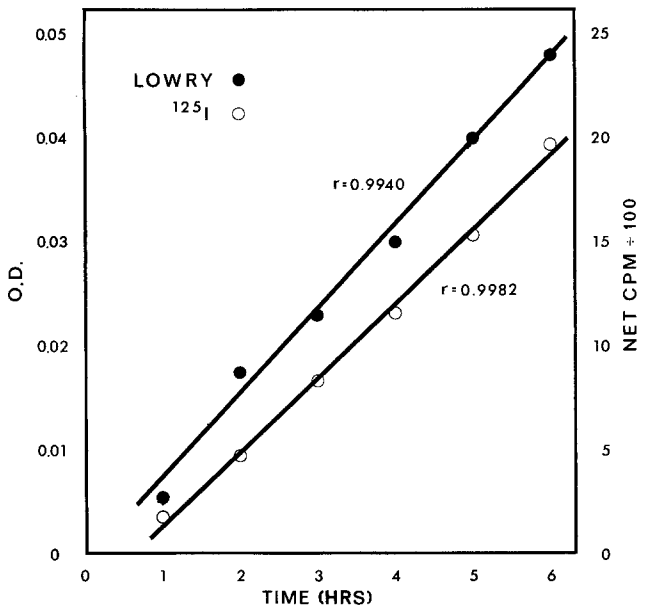

Fig. 1. Hydrolysis of bovine serum albumin by crude peanut extract as estimated with the Lowry and [^{125}I] methods. Extracts for use with the Lowry method were dialyzed. Linear regression analyses were based on means of 5 replications.

of substrate and extract level, pH of extraction and reaction buffers, and dialysis.

RESULTS

Both the Lowry and [^{125}I] methods adequately measured proteolytic activity in peanut seed extracts (Fig. 1). Consistent results were obtained with the Lowry method only if extracts were dialyzed. Reactions catalyzed with undialyzed extract demonstrated an induction phase of variable length, and variable reaction rates. Undialyzed extracts proved suitable for the [^{125}I] method.

The Lowry method did not detect jack pine seed proteolytic activity. Only dialyzed extracts were tested because of high background density caused by phenolics. Subsequent tests of reaction media and times (up to 24 hrs) failed to detect activity by the Lowry method.

The [^{125}I] method measured proteolytic activity in either dialyzed or

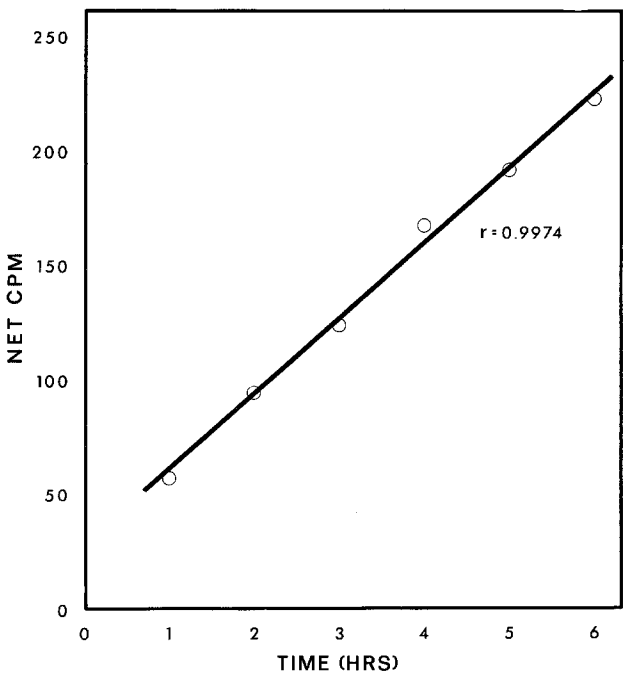

Fig. 2. Hydrolysis of bovine serum albumin by crude, undialyzed jack pine seed extract as estimated with the [^{125}I] method. Negative results were obtained with the Lowry method. Linear regression analysis was based on means of 5 replications.

undialyzed jack pine seed extracts (Fig. 2). The rate of reaction was linear for at least 6 hrs, and essentially linear for as long as 18 hrs.

DISCUSSION

The [^{125}I] method of estimating proteolysis by crude plant extracts is faster and more sensitive than the Lowry and, probably, other colorimetric methods. Sensitivity of the [^{125}I] method can be enhanced by elevating the specific activity of [^{125}I] in the reaction medium. Colorimetric reactions are less easily sensitized. Apparent enzyme activity can most readily be elevated by increasing the specific activity of enzyme in the reaction medium, or measuring a larger volume of hydrolyzate. Volumetric limitations exist in both instances, and elevate the lower limit of resolution of colorimetric methods.

The [^{125}I] method measures only hydrolysis products that originate in

the protein substrate, although endogenous substrates may be simultaneously hydrolyzed. Such specificity eliminates interference, and allows measurement of hydrolysis of any desired protein in crude extracts. Methods exist for iodinating endogenous plant proteins (10). Such iodinated proteins should be preferred in physiological investigations of proteolysis over bovine albumin or other foreign substrates.

Use of [^{125}I]BSA, instead of the more energetic gamma emitter [^{131}I] proposed by Nardi (4) reduces the health hazard. In addition, the longer half-life of [^{125}I] (60 days) as compared to [^{131}I] (8 days) extends substrate life. Finally, the low-energy gamma emission of [^{125}I] lends itself to liquid scintillation spectrometry.

REFERENCES

1. Ryan, C. A. (1973) Ann. Rev. Plant Physiol. 24, 173-196.
2. Greenberg, D. M. (1955) Methods in Enzymology, Vol. 2, pp. 54-64, Academic Press, New York.
3. Haissig, B. E. and Schipper, A. L. (1975) Phytochemistry (In press).
4. Nardi, G. L. (1953) Science 118, 299.
5. Haissig, B. E. and Schipper, A. L. (1972) Anal. Biochem. 48, 129-146.
6. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
7. Duspiva, F. (1939) Protoplasma 32, 211-250.
8. Maggio, R. (1957) J. Cell. Comp. Physiol. 50, 135-143.
9. Jeffery, W. R. (1972) Exp. Cell. Res. 72, 579-582.
10. Bolton, A. E. and Hunter, W. M. (1973) Biochem. J. 133, 529-539.