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Methyl-^{14}C-glycinated hemoglobin as a substrate for proteases

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SUMMARY

A simple radioisotopic assay for the proteolytic enzyme activity with methyl-^{14}C-glycinated bovine hemoglobin as the substrate has been developed. The radioactive hemoglobin was prepared by the reaction of hemoglobin with a water-soluble carbodiimide and methyl [^{14}C]glycinate in 5 M guanidine hydrochloride.

Proteolytic enzymes have been generally assayed with hemoglobin or casein as the substrate according to the methods originally developed by Anson[1] and Kunitz[2]. Although these methods which are based on the determination of increase in the trichloroacetic acid-soluble cleavage products either by the phenol reagent or by absorbance at 275 nm are simple, their accuracy and sensitivity are often significantly affected by the presence of interfering materials other than the enzyme and the substrate. Numerous modifications have been introduced to offset this shortcoming, including the use of substrates labeled with dyes[3], radioisotopes[4] and fluorescent compounds[5], but these are not without limitation. Use of a dye protein substrate is limited to narrow pH ranges due to the pH-induced insolubility. Labeling with radioactive iodine gives a substrate with a relatively short half-life, and involves difficulties associated with γ-radiation. Introduction of a radioisotope into proteins by acetylation[6] is inefficient and often expensive. Finally, the fluorimetric assay may be complicated when the effect of a fluorescent compound on the enzyme is determined.

With the increased interest in proteases and their physiological role in biological systems, an assay adaptable to most systems as means of measuring proteolytic activities would be highly desirable. With this view in mind, we have developed a simple and sensitive radioisotopic protease assay which has advantages over the other known methods. The assay involves the use of methyl-^{14}C-glycinated hemoglobin as the substrate. The modification of the carboxyl groups in hemoglobin to the

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$-\overset{O}{\overset{\|}{C}}-N^{14}CH_2-\overset{O}{\overset{\|}{C}}-OCH_3$ form was conveniently attained by treating the protein with a water-soluble carbodiimide and [^{14}C]glycine methyl ester[7,8].

One gram of bovine hemoglobin (Pentex) was treated with 1-ethyl-3-dimethyl-aminopropyl carbodiimide hydrochloride (Ott Chemical; 0.1 M) in the presence of [^{14}C]glycine methyl ester (0.3 M, about 300 μC) and guanidine hydrochloride (5 M) at 25° in a total volume of 100 ml for 3 h, the pH being controlled by a Radiometer pH stat assembly to 4.8. The methyl ester of [^{14}C]glycine (usually 10–20 μC/mmole) was prepared by methylation of [U-^{14}C]glycine (New England Nuclear) after dilution with non-radioactive glycine to a desired specific radioactivity. An equal volume of 1 M sodium acetate, pH 4.8, was added to the reaction mixture, which was then exhaustively dialyzed against distilled water (22 l with three changes in 2 days) and lyophilized. Unreacted [^{14}C]glycine methyl ester can be recovered from the first dialyzate and reused after purification, if desired. Under these conditions, of the 64 carboxyl groups per molecule of bovine hemoglobin theoretically available for reaction, nearly complete conversion (>90%) can be achieved as determined by amino acid analysis of the product with respect to the changes in the ratio of glycine and alanine contents. Tyrosine residues which are partially affected by the modifying reagent can be regenerated by hydroxylamine[10]. However, this treatment is not essential for preparation of the substrate unless the effect of tyrosine residues in the substrate is of specific concern for the proteolytic mechanism. The ^{14}C-labeled modified hemoglobin showed a single major peak upon analysis on polyacrylamide gel electrophoresis (Fig. 1).

A standard assay system for an acid protease such as cathepsin D, contained 1.5 mg of methyl-^{14}C-glycinated hemoglobin (10 000 counts/min), 25 μl of the enzyme and the buffer solution (0.4 M sodium citrate, pH 4.0, or 1.0 M sodium formate, pH 4.0) in a total volume of 0.5 ml. The incubation was allowed to proceed for 30 min at 37°, after which 0.5 ml of 10% trichloroacetic acid was added, followed by 0.3 ml of 2% unmodified hemoglobin solution. These were mixed, centrifuged and a 1-ml aliquot of the supernatant solution was added to 10 ml of Bray's[11] solution, and the radioactivity counted in a liquid scintillation spectrometer. The radioactivity released into the trichloroacetic acid-soluble fraction can be used as a measurement of the enzyme activity. With modification of the reaction pH, the activities of pepsin (at pH 2), papain (at pH 6.5) and trypsin (at pH 8.0) could be similarly assayed.

Cathepsin D was prepared from bovine liver by a modification of the method of Barrett[12]. Pepsin, papain and trypsin were obtained from Worthington Biochemicals.

Under the standard assay condition, the radioactivity released by the protease, *e.g.* cathepsin D on methyl-^{14}C-glycinated hemoglobin is proportional to the reaction time up to 30 min, and throughout the range of protein concentration tested as illustrated in Fig. 2. These results are comparable to those obtained by the Anson's hemoglobin-$A_{275\ nm}$ method with 400 counts/min approximately equivalent to 0.08 absorbance response. The maximal cathepsin D activity was observed in the pH range of 3.0–3.2 with the unmodified acid-denatured hemoglobin substrate and pH 4.0 with the modified hemoglobin. A double reciprocal plot showing dependence of the activity on the substrate concentration gave an apparent K_m of $4.1 \cdot 10^{-5}$ M for the unmodified

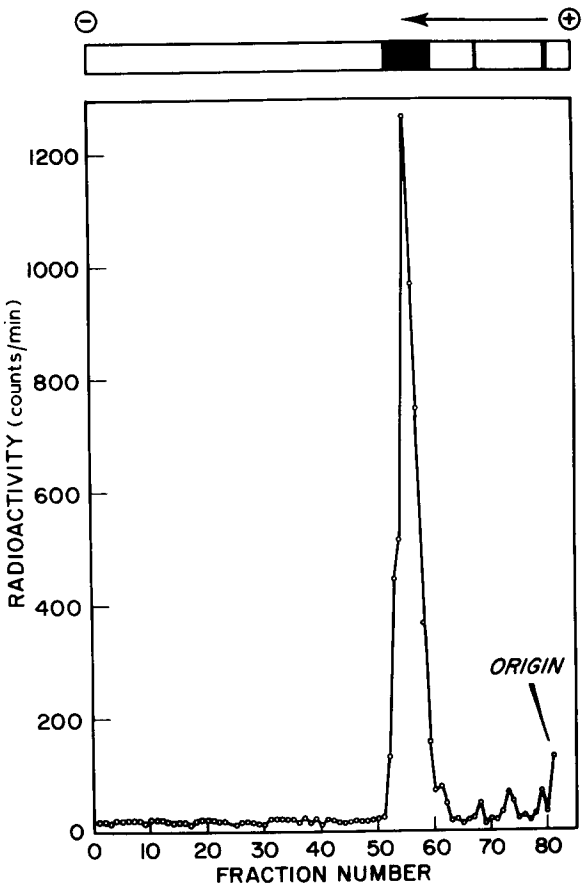

Fig. 1. Polyacrylamide gel electrophoresis of methyl-^{14}C-glycinated hemoglobin preparation. 120 μg of the protein were analyzed in an anionic gel system, pH 9.3. The gel was fractionated with a Savant Autogel divider and each fraction counted in Bray's solution by a liquid scintillation spectrometer. The small peaks which amount to less than 10% of the total radioactivity of the preparation represent the sample trapped at the boundary of the stacking and the separating gel. A duplicate gel was stained with Amido black 10B.

hemoglobin and $1.3 \cdot 10^{-5}$ M for methyl-^{14}C-glycinated hemoglobin. The corresponding values with pepsin were found to be $6.3 \cdot 10^{-5}$ M and $3.3 \cdot 10^{-5}$ M, respectively. It is conceivable that any change in conformation and charge distribution of hemoglobin resulting from the carboxyl group modification does not appreciably affect the affinity and the catalytic mechanism of these proteases toward the protein substrate. However, the carboxyl modification of hemoglobin significantly affects the property of the substrate for trypsin as the apparent K_m shifts from $1.7 \cdot 10^{-3}$ for unmodified hemoglobin to $1.5 \cdot 10^{-5}$ M for the methyl-^{14}C-glycinated hemoglobin.

This assay system was found to be especially useful in studying effect of chemical inhibitors on the proteolysis. With the conventional method of Anson, the accuracy of the activity determination is seriously influenced by the properties of the effectors. Extraction of the reaction mixture with an organic solvent before measurement

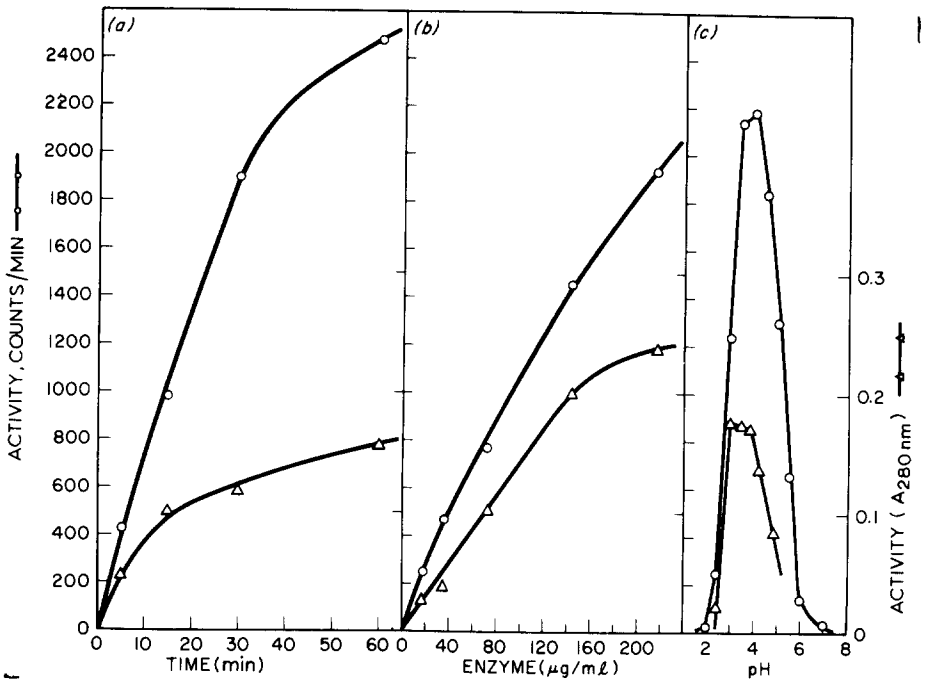

Fig. 2. Reaction of cathepsin D on methyl-^{14}C-glycinated hemoglobin (○—○) and acid-denatured hemoglobin (△—△) as a function of (a) time, (b) protein concentration and (c) pH. Conditions were those as described in the text except that the concentration of unmodified hemoglobin was 10 mg/ml.

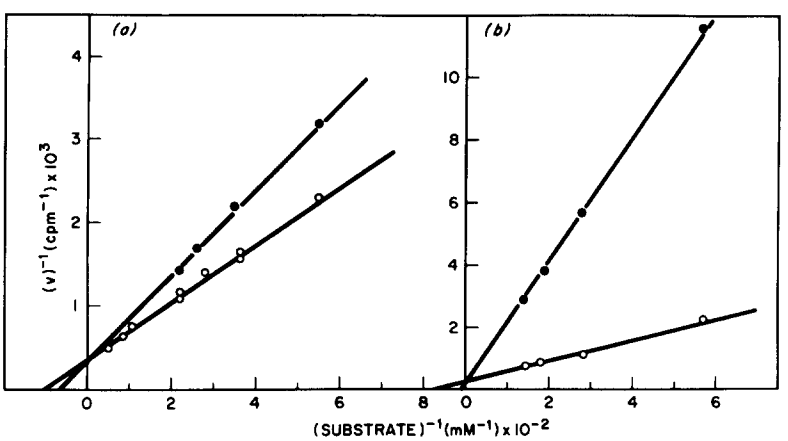

Fig. 3. Double reciprocal plots demonstrating a competitive inhibition of (a) cathepsin D activity on methyl-^{14}C-glycinated hemoglobin by 5 mM phenylpyruvic acid (●—●) (Calbiochem) in the standard assay system. K_i was found to be $0.9 \cdot 10^{-3}$ M; (b) trypsin activity by $3 \cdot 10^{-2}$ mM 2-hydroxystilbamidine isothionate (●—●) (Rhodia, Inc.). K_i, $7 \cdot 10^{-6}$ M. cpm = counts/min.

on hydrolyzates to remove the effector is operable[12], but partial elimination of peptides released from the substrate by the enzyme action may not be excluded. As illustrated in Fig. 3 for the effect of phenyl pyruvate on cathepsin D and 2-hydroxystilbamidine on

trypsin by including suitable controls for correction of quenching of radioactive counting by the effector, the enzyme activity can be reliably determined in the presence of chemical agent(s) without extra treatment.

The sensitivity of the method reported here allows the determination of protease activity even when the quantity of the enzyme source is small as in tissue biopsies or organ cultures. The simplicity of introducing the radioactive label into the protein as described may be applicable to other assay systems, *e.g.* ^{14}C-glycinated collagen for tissue collagenase determination[13].

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