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A Micro-assay Method for Parasite Proteinase Activity Using [^{3}H]-Labeled Globin as a Substrate

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Abstract

A micro-assay method for acidic proteinase activity has been developed using [^{3}H]-labeled globin as a substrate to investigate parasite proteinases hydrolyzing host hemoglobin. The method is based on the incubation of globin with enzyme, followed by determination of the radioactivity of enzyme digestion products soluble in 80% dioxane. This method is sensitive enough to detect microgram levels of globin fragments and applicable to a variety of acidic proteinases at a nanogram level.

Key words: *Fasciola* sp., [^{3}H]-labeled globin, micro-assay, parasite, proteinases

Introduction

It has been known that a variety of parasitic helminths possess proteolytic enzymes which show a potent activity against their host hemoglobin (Timms and Bueding, 1959; Sauer and Senft, 1972; Oya and Noguchi, 1977; Dresden and Deelder, 1979; Rupova and Keilová, 1979; Hamajima and Yamagami, 1981; Maki *et al.*, 1982; Swamy and Jaffe, 1983; Sato and Suzuki, 1983). These proteinases are supposed to play a physiological role in the life cycle of the parasites such as development and reproduction, providing nutritions of amino acids from their host proteins including hemoglobin. Furthermore, these proteinases, once purified, can be candidates for marker proteins (antigens) of the parasites, and employed for various immunodiagnosis such as skin test (Senft and Maddison, 1975; Senft *et al.*, 1979) or radioallergosorbent test (Aoki, 1980; Sato *et al.*, 1981).

Currently available assay methods for these proteinase activities are based on the use of hemoglobin as a substrate and the determination of the increase in the trichloroacetic acid-soluble cleavage products by measuring either absorbance at 280 nm or chromophore developed with the phenol reagent. These conventional methods, however, are applicable only when a substantial amount of sample enzyme is available and often affected by the presence of interfering materials such as sulfhydryl compounds. Attempts have been reported to develop a micro-assay method for proteinases using radioactive proteins as substrates (Hill *et al.*, 1970; Williams and Lin, 1971; Maliopoulou *et al.*, 1980). However, all these methods are based on the use of trichloroacetic acid as a precipitant, which causes an unavoidable, severe quenching in the assay of radioactivity. An attempt to employ a synthetic substrate with a carboxy-terminal fluorescent leaving group has also been reported (Dresden *et al.*, 1982).

In this paper, we report a simple and sensitive micro-assay method for proteolytic enzymes using [^{3}H]-labeled human globin as a substrate, which was developed, aiming to purify parasite proteinases in limited quantities.

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

Materials and Methods

Materials.

Porcine pepsin was obtained from Boehringer Mannheim (Germany) and cathepsin D from Sigma (St. Louis, USA). [^{3}H]-labeled acetic anhydride was purchased from Radiochemical Centre (Amersham, UK). Sephadex G-50 and Sephadex G-75 were from Pharmacia Fine Chemicals (Uppsala, Sweden) and urea and dioxane from Nakarai Chemicals Ltd. (Kyoto). The other reagents used were of analytical grade.

Preparation of globin from human red blood cells.

Isolation and crystallization of hemoglobin from human blood cells were carried out according to the method of Rossi-Fanelli and Antonini (1955). Blood after removal of leucocytes (230 ml) was diluted with 1 volume of 1% NaCl solution, and centrifuged at 1500 x *g* for 10 min to precipitate red blood cells, which was repeatedly washed with chilled saline solution. The cells were hemolyzed by adding an equal volume of distilled water containing 10% toluene. After removal of the debris of the cells by centrifugation, the supernatant was dialyzed against saturated ammonium sulfate solution to make a final concentration of 70% saturation. Hemoglobin thus precipitated was extensively dialyzed against distilled water and lyophilized.

The splitting of globin from hemoglobin was carried out by a modification of the method described by Rossi-Fanelli *et al.* (1958). Two percent chilled hemoglobin solution was added dropwise to the chilled acetone-HCl solution with vigorous stirring. The resulting solution was allowed to stand for 30 min, and the supernatant containing heme was removed by aspiration. The pellet was repeatedly washed with chilled acetone-HCl solution, dissolved in and dialyzed against distilled water at 4°C, and then dialyzed against 1 mM sodium bicarbonate containing 0.1 mM DTT. Native globin remaining in the supernatant after centrifugation was dialyzed against distilled water and lyophilized.

Acetylation of globin with [^{3}H]-labeled acetic anhydride.

The globin was purified by dissolution in 0.2 M acetate buffer, pH 4.5, containing 1 mM EDTA and 1 mM DTT and precipitation at 80% dioxane and 4°C, and then denatured by dissolving in 6 M urea solution. After removal of urea by extensive dialysis against distilled water, the resulting globin was lyophilized (1 g), dissolved in 50 ml of 0.2 M borate buffer, pH 8.0, containing 0.15 M NaCl and allowed to react with 5 mCi of [^{3}H]-labeled acetic anhydride (500 mCi/mmol) at 25°C for 1 hr (Montelaro and Rueckert, 1975). The [^{3}H]-labeled globin was precipitated by adding dioxane to make a final concentration of 80%. The resulting globin was washed to remove unbound radioactivity by repeated precipitation and dissolution with 80% dioxane and the acetate buffer, respectively. The labeled globin gave a specific activity of 5.4 x 10^6 dpm/mg protein. Unlabeled globin, purified as described above, was employed as a carrier protein to adjust specific radioactivity for proteinase assays.

Purification of proteinase from Fasciola sp.

Adult worms (127 g) of *Fasciola* species were minced with scissors and homogenized in 0.05 M sodium acetate buffer, pH 3.9, containing 0.15 M NaCl, 1 mM EDTA, and 10 mM DTT with a Teflon tissue homogenizer. The homogenate was further sonicated at 20 kHz and 150 watts for 15 sec x 4 on ice with a Tomy model UR-200P sonifier (Tokyo) and centrifuged at 12,000 x *g* for 45 min. The pellet was reextracted with the same buffer. The supernatant, filtered through a glass-wool column to remove floating lipids, was subjected to ammonium sulfate fractionation. The precipitate recovered at 50% saturation was dissolved in 0.05 M sodium acetate buffer, pH 3.9, containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT and centrifuged; the supernatant obtained was applied to a Sephadex G-75 column equilibrated with the same buffer.

The effluent fractions showing proteinase activity were pooled, concentrated by ultrafiltration using an Amicon UM-10 membrane filter and rechromatographed on a Sephadex G-50 superfine column. The enzyme fractions were pooled, concentrated, and used as enzyme sourse. The *Fasciola* enzyme was found to be activated by the presence of 10 mM DTT and inhibited by *N*-ethylmaleimide, indicating the enzyme to be of the thiol type. Therefore, assays of globinolytic activity with *Fasciola* enzyme were performed in the presence of 10 mM DTT.

Protein assay.

Protein content was determined according to the method of Hartree (1972) using bovine serum albumin as a standard protein.

Enzyme assays.

Conventional method: To 100 μl of 1% human hemoglobin in 0.2 M acetate buffer, pH 4.5, containing 1 mM EDTA, 20–150 μl of enzyme sample was added (final volume; 500 μl). After incubation at 37°C for 1 hr, the enzyme reaction was terminated by adding an equal volume of 10%trichloroacetic acid and the absorbance at 280 nm of the supernatant was determined.

Micro-assay method: The incubation mixture consisted of 50 μl of [^{3}H]-labeled globin substrate solution (1800 dpm, 300 μg), 5–100 μl of enzyme sample and 0.2 M acetate buffer, pH 4.5, containing 1 mM EDTA and 10 mM DTT in a final volume of 200 μl. Enzyme reaction was allowed to proceed for 20 min at 37°C, and then stopped by adding 800 μl of dioxane. The resulting solution was placed on ice for 5–10 min to complete precipitation of undigested globin and then centrifuged. An aliquot (700 μl) of the supernatant was added to 10 ml of Bray's solution, and assayed for radioactivity using a Beckman LS-9000 liquid scintillation system (Sunada and Nagai, 1980). Globinolytic activities of cathepsin D and pepsin were also assayed in the absence of DTT.

Results and Discussion

Characterization of [^{3}H]-labeled globin as a substrate.

Analysis of the [^{3}H]-labeled globin showed that about 14% of total radioactivity applied was coupled to the globin, yielding a specific activity of 5.4 x 10^6 dpm/mg protein (0.096 acetyl residue/10^4 daltons). The radioactivity was uniformly labeled on the protein, as observed with elution patterns on a Sephadex G-50 superfine gel column before (Fig. 1) and after (Fig. 2) treatment with pepsin.

Solubility of globin substrate at various concentrations of dioxane.

Fig. 3 shows that the solubility of the globin substrate at pH 4.5 decreased sharply between 40 and 60% dioxane, and only 10% and 6% of the globin remained in the supernatant at 80% and 90% dioxane, respectively. When the solubilities at 80% and 90% dioxane of the

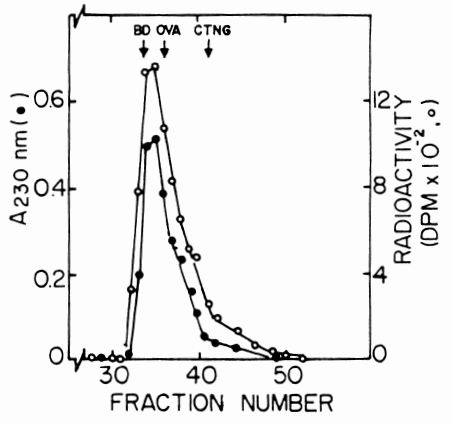

Fig. 1 Sephadex G-50 superfine chromatography of [^{3}H]-labeled globin. One milligram of [^{3}H]-labeled globin was applied to a Sephadex G-50 superfine column (1.6 x 41 cm) equilibrated with 0.02 M sodium acetate buffer, pH 4.5, containing 0.15 M NaCl. Fractions of 0.95 ml were collected at a flow rate of 5.0 ml/hr. An aliquot (500 μl) of each fraction was assayed for radioactivity (○). Absorbance at 230 nm (•). The arrows indicate the elution positions of blue dextran (BD), ovalbumin (OVA) and horse pancreatic chymotrypsinogen (CTNG).

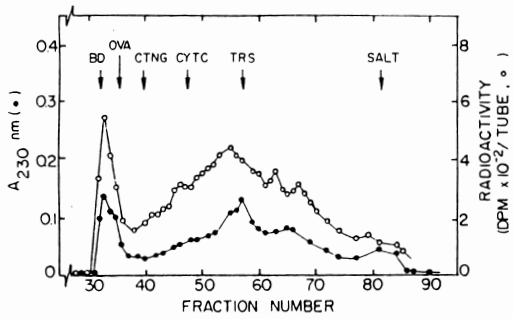

Fig. 2 Distribution of [^{3}H]-labeled acetyl residues in the globin molecule. One milligram of [^{3}H]-labeled globin was partially digested with porcine pepsin at a ratio of enzyme to substrate of 1:50 at pH 4.5 and 37°C for 60 min. The digestion products were chromatographed on a Sephadex G-50 superfine column (1.6 x 41 cm) equilibrated with 0.02 M sodium acetate buffer, pH 4.5, containing 0.15 M NaCl. Fractions of 0.91 ml were collected at a flow rate of 5.0 ml/hr. An aliquot (500 μl) of each fraction was assayed for radioactivity (○). Absorbance at 230 nm (•). The arrows indicate the elution positions of molecular weight markers as shown in Fig. 1. CYTC, horse heart cytochrome c; TRS, trasylol and SALT, barium acetate.

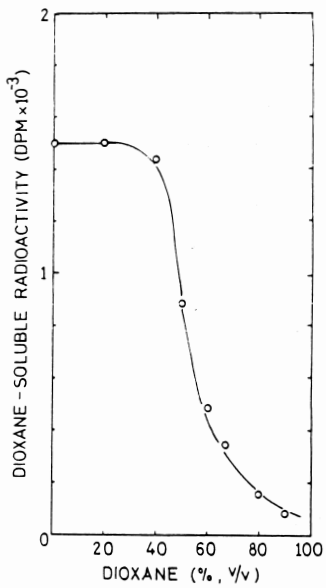

Fig. 3 Solubility of [^{3}H]-labeled globin at various concentrations of dioxane. At room temperature dioxane was added to a series of 50 μl aliquots of [^{3}H]-labeled globin (1500 dpm, 250 μg) dissolved in 0.2 M sodium acetate buffer, pH 4.5, containing 1 mM EDTA to give the concentrations indicated (final 1 ml). After vigorous shaking, the resulting solution was cooled on ice for 5–10 min, centrifuged at 2700 x *g* for 10 min, and the radioactivity in the supernatant (700 μl) was determined.

globin degradation products by *Fasciola* proteinase were compared, more than 85% of the total degradation products remained in the supernatant at 80% dioxane (Fig. 4), while recovery of the products in the supernatant at 90% dioxane was as low as 13–17% of that at 80% dioxane. Therefore, dioxane at a final concentration of 80% was employed to precipitate undigested globin substrate in enzyme assays. Globin degradation products with molecular weights up to 12,000 remained in the supernatant at 80% dioxane (Fig. 4a).

Release of dioxane-soluble fragments from globin substrate by Fasciola proteinase as a function of incubation time or enzyme concentration.

The *Fasciola* proteinase hydrolyzed globin substrate in a linear fashion up to 20–30% digestion of the total substrate under the experimental conditions employed (Fig. 5). Similar results were obtained with pepsin and cathepsin D (data not shown). No marked release of radioactivity was observed with the substrate incubated without enzyme even in the presence of sulfhydryl compound, and the level of the values after 2 hr incubation was not more than 10% of total radioactivity. This is a marked contrast to the trichloroacetic acid precipitation method, the use of which results in a significant increase in the background value by the presence of reducing reagent.

Fig. 6 shows that the sensitivity of the present method for pepsin and the *Fasciola* proteinase is more than 10 fold higher than the conventional method and that a nanogram level of acidic proteinases can be determined if assayed after 1-2 hr incubation at 37°C. A similar result was obtained with cathepsin D (data not shown), indicating that the present

method is widely applicable to the assay of a variety of acidic proteinases.

The micro-assay method reported here enabled us to quantify the activity of *Fasciola* acidic thiol proteinase with much less consumption of the enzyme preparations. This

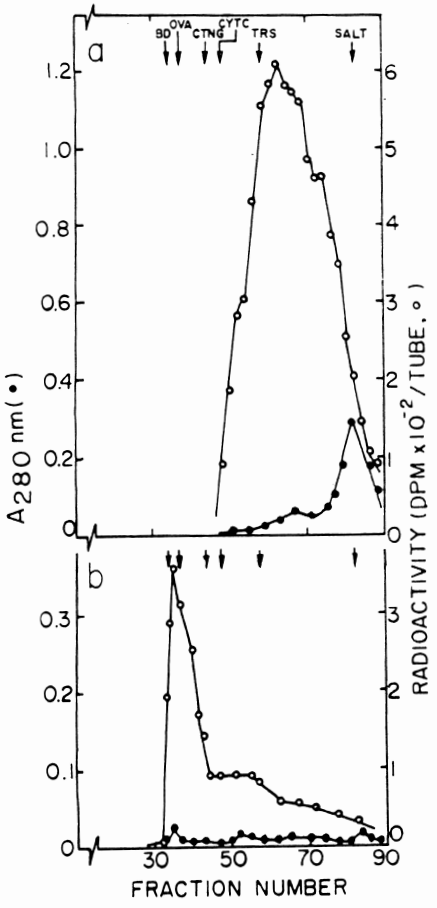

Fig. 4 Sephadex G-50 superfine chromatography of the globin fragments released by *Fasciola* proteinase. [^{3}H]-labeled globin (3.0 mg/ml) was incubated with *Fasciola* proteinase at 37°C and pH 4.5 for 40 min in the presence of 10 mM DTT. After addition of dioxane to make a final concentration of 80%, the dioxane-soluble and insoluble fractions were separated by centrifugation. The supernatant was dried up with an evaporator, dissolved in 0.02 M sodium acetate buffer, pH 4.5, containing 0.15 M NaCl, and applied to a Sephadex G-50 superfine column (1.6 x 41 cm) equilibrated with the same buffer (a). The pellet was dissolved in the same buffer containing 6 M urea. The resulting solution was applied to the same column described above (b). Fractions of 0.93 ml were collected at a flow rate of 3.66 ml/hr. The arrows indicate the elution positions of molecular weight markers as shown in Figs. 1 and 2. The absorbance at 280 nm of the effluent fractions corresponding to salt is ascribed to the reducing reagent (DTT) added to the enzyme mixture.

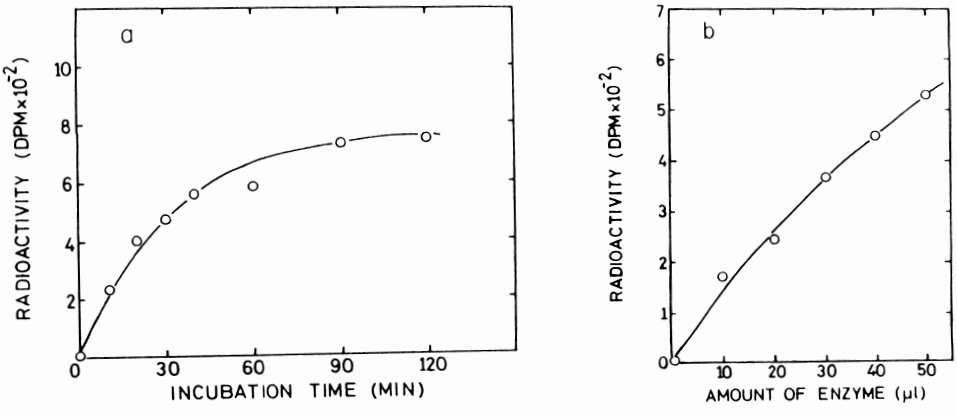

Fig. 5 Kinetic analysis of globinolytic activity of *Fasciola* proteinase as a function of incubation time or enzyme concentration. [^{3}H]-labeled globin (1800 dpm, 300 μg) was incubated with *Fasciola* enzyme (0.8 μg/30 μl) at 37°C for the time indicated (a) or with varying amount of the enzyme for 20 min (b). Other assay conditions were as described in "Methods". The amount of dioxane-soluble globin fragments was estimated by measuring radioactivity after subtracting the background value at each incubation time.

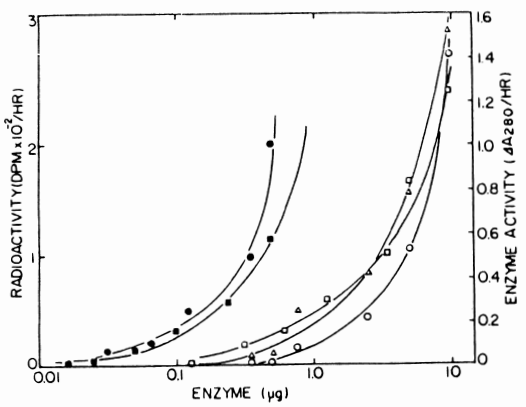

Fig. 6 Comparison of the sensitivity of the micro-assay method with the conventional method. [^{3}H]-labeled globin (closed symbols) was incubated with varying amount of pepsin (square) or *Fasciola* proteinase (circle) at 37°C for 60 min. Hemoglobin (open symbols) was incubated with both enzymes separately at 37°C for 60 min and assayed by the conventional method. Unlabeled globin (triangle) was also assayed in the same way with *Fasciola* proteinase.

method has some advantages as a common assay method of carboxyl proteinases or acidic thiol proteinases:

1) it requires only nanogram levels of enzyme samples,
2) it is sensitive enough to detect microgram levels of globin digests,
3) the presence of thiol compounds, which gives a high background value in the conventional method, does not interfere with this micro-assay method,
4) the use of dioxane, the solvent of Bray's solution, as a precipitant of undigested globin substrate gives scintillation counting efficiencies of tritium as high as 52–54% (Sunada and Nagai, 1980).

The degree of acetylation of the globin substrate is as low as 0.096 residue/10^4 molecules (corresponding to 0.87% of total lysyl residues) and labeling is rather uniform through the globin molecule (see Fig. 2). The pattern of globinolytic activity of parasite proteinase at varying concentration, assayed by the present method is quite similar to that of its hemoglobinolytic activity assayed by the conventional method, except for the sensitivity which is more than 10 fold in the former case (Fig. 6). Furthermore, globin degradation products as large as 12,000 daltons are extractable into the supernatant with 80% dioxane. Therefore, the use of labeled substrate may not affect the results of proteinase assays compared to the use of unlabeled substrate and should be more efficient for preparations of parasite proteinases with a limited amount.

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