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A RAPID ASSAY METHOD OF COLLAGENASE ACTIVITY USING ^{14}C-LABELED SOLUBLE COLLAGEN AS SUBSTRATE

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Summary

A rapid assay method for vertebrate collagenase (EC 3.4.24.3) activity has been developed using ^{14}C-labeled soluble collagen as substrate. The method is based on the incubation of collagen with enzyme in the presence of glucose to prevent collagen fibril formation followed by selective extraction of the enzyme digestion products into dioxane at a final concentration of 50%.

The rate of reaction was about 10 times higher than that obtained by the conventional method using reconstituted collagen fibrils as substrate and the relationship between enzyme activity and concentration was linear over a wider range. When the method was applied to the assay of human granulocyte collagenase, the results showed good correlation with those obtained by the conventional gel method.

Introduction

The most widely employed current assay method for animal collagenase (EC 3.4.24.3) uses radioactive reconstituted collagen fibrils as substrate and measures the release of soluble radioactivity from the fibrils after enzyme digestion [1]. The method is sensitive, specific for collagenase and convenient for routine assays of a large number of samples. However, it has some disadvantages, for example: (1) prior to enzyme digestion, at least 1—2-h preincubation is required to prepare a collagen substrate gel; (2) background radioactivity is rather

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high due to an equilibrium reaction between soluble and fibril collagen (usually 10—15% of total) and varies depending on pH, ionic strength, ionic species and type of contaminant present in samples tested.

Attempts to use soluble collagen as substrate for collagenase assay have been reported [1,2]. These were based on carrying out the enzyme reaction in solution and measuring the decrease in fibril formation at an elevated temperature after stopping the reaction by adding inhibitor. The method is useful for routine assays but applicable only to samples which contain no contaminants which interfere in collagen gel formation.

Recent studies on fibrillogenesis of collagen in our laboratory [3,4] showed that glucose could prevent collagen gel formation at neutral pH even at 37—40°C. No indication of denaturation of collagen was observed during the treatment. Moreover, glucose did not affect collagenase activity. These observations suggested that collagenase activity could be assayed using soluble collagen as substrate at temperatures at or close to physiological, if selective separation of the enzyme digests from the undigested collagen was feasible.

This paper describes studies on the relative solubilities of native and denatured collagen in dioxane at various concentrations and the application of the results to the assay of collagenase activity.

Materials

^{14}C-labeled collagen fibrils and solutions. ^{14}C-glycine labeled guinea pig skin collagen (1200 cpm/mg) was prepared as described previously [5], and dissolved in 0.005 M acetic acid to make a final concentration of 0.4% (w/v). For the conventional gel method of collagenase assay [5], 0.2 ml of the collagen solution was mixed with an equal volume of 0.1 M Tris · HCl buffer, pH 7.8, containing 0.4 M NaCl and 10 mM $CaCl_2$ and incubated at 37°C overnight to form collagen fibrils.

For enzyme assay using soluble collagen as substrate, 0.1 ml of the collagen solution was mixed with an equal volume of the same buffer to which was added glucose to a final concentration of 1 M to prevent gel formation during incubation at 35°C.

Tadpole collagenase. Cultured medium of tadpole skin explants was concentrated as reported previously [5] and used as crude enzyme.

Purified enzyme was prepared from the concentrated medium by affinity chromatography followed by Sephadex G-75 superfine column chromatography as reported [5].

Granulocyte collagenase. Polymorphonuclear leucocytes were isolated [6] from 5-ml samples of heparinized bloods from patients with various diseases, hospitalized in the University Hospital, Okayama University. The cells were suspended in 1.5 ml of 0.05 M Tris · HCl buffer, pH 7.8, containing 0.2 M NaCl and 5 mM $CaCl_2$ and frozen and thawed, three times. The resultant preparation was centrifuged at 10 000 × g for 20 min at 4°C and 25—200 μl of the supernatant containing 0.5—1.5 mg protein [7] per ml was used for collagenase assay.

Methods

Collagenase assay with soluble collagen substrate (solution method)

Principle. The present assay method is based on the incubation of ^{14}C-labeled soluble collagen with enzyme preparation at a temperature between the denaturation temperatures of native collagen and its enzyme digests.

Enzyme reaction was stopped by adding inhibitor and the products were completely denatured by incubating at the same temperature, then extracted into 50% dioxane (v/v) at room temperature for the determination of radioactivity.

Procedure. 0.2 ml of ^{14}C-labeled collagen solution containing glucose (see Materials) was incubated with 0.2 ml of enzyme preparation at 35°C for various times. The enzyme reaction was stopped by adding 20 μl of 80 mM *o*-phenanthroline dissolved in 50% dioxane, and the resultant solution was kept at the same temperature for 60 min, then placed at room temperature (22—28°C). The reaction products thus denatured were extracted by vigorous shaking after addition of 0.4 ml dioxane and centrifuged at 6000 rev./min for 10 min to precipitate the residual undigested collagen. 0.5 ml of the supernatant was added to 10 ml of Bray's solution [8] and assayed for radioactivity.

The conventional assay method with ^{14}C-labeled reconstituted collagen fibrils (gel method)

Collagenase activity was assayed by measuring the release of radioactive peptides from ^{14}C-labeled reconstituted guinea pig skin collagen fibrils as described previously [1,5]. Enzyme reaction was carried out at 35°C or 37°C.

Disc electrophoresis

Disc electrophoresis of collagen degradation products was carried out according to the method of Nagai et al [9].

Results

Solubility of native and denatured collagen in dioxane with various concentrations.

Since dioxane is used as solvent in Bray's solution the solubility of native and denatured collagen in various concentrations of dioxane was compared at room temperature, using ^{14}C-labeled material.

Native collagen was found to be completely precipitated by 33% (v/v) dioxane at pH 7.8, while the denatured form (heated at 45°C for 10 min) remained soluble, even at 50%, then became precipitable at higher concentrations (Fig. 1a). However, at acidic pH, native collagen did not precipitate, even at 60% dioxane (Fig. 1b).

Since 50% dioxane was apparently effective for the separation of the denatured form from native collagen at neutral pH, the solubility of various amounts of collagen (10—400 μg) in 50% dioxane was examined. Native collagen was virtually completely insoluble, but the denatured form was completely soluble under the same condition, regardless of the amount tested (Fig. 2a). When dioxane was added to mixtures of native and denatured collagen in solu-

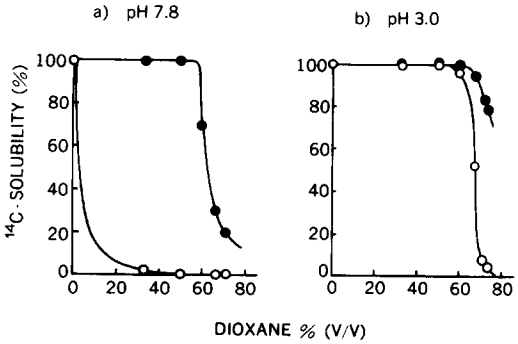

Fig. 1. Comparison of the solubility of native and denatured collagen at various concentrations of dioxane at neutral and acidic pH. To a series of 0.4-ml samples of 0.1% ^{14}C-labeled native (○) and heat-denatured (●) collagen (2400 cpm per sample) in 0.05 M Tris · HCl buffer, pH 7.8, containing 0.2 M NaCl, 5 mM $CaCl_2$ and 0.25 M glucose (Tris/NaCl/$CaCl_2$/glucose) (a) and in 0.05 M acetic acid (b) dioxane was added at room temperature to give the concentrations indicated. After vigorous shaking, the resultant solution was centrifuged at 6000 rev./min for 10 min and the amount of collagen in the supernatant was estimated by measuring radioactivity.

tion to make a final concentration of 50%, the denatured form was quantitatively recovered in the supernatant (Fig. 2b).

In order to test for interference by non-collagenous materials of the type commonly encountered during collagenase assay in the extraction of denatured collagen, 400 μg of heat-denatured ^{14}C-labeled collagen was mixed with 1.7 mg of lyophilized culture media of tadpole skin explants and immediately extracted with 50% dioxane. No indication of interference of the materials in collagen extraction was observed, a recovery of 99%, based on radioactivity, being obtained.

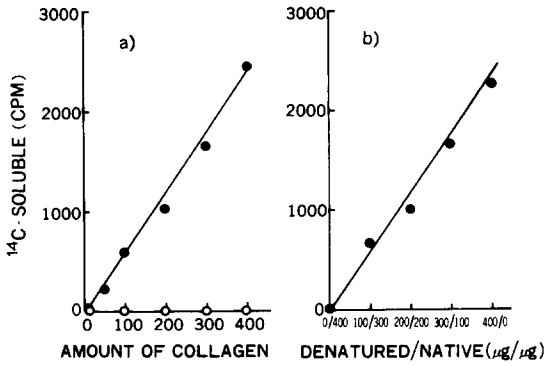

Fig. 2. Solubility of denatured collagen in 50% dioxane at pH 7.8. 0.4 ml of dioxane was added to a series of equal volumes of ^{14}C-labeled native (○) or denatured (●) collagen dissolved at various concentrations in Tris/NaCl/$CaCl_2$/glucose buffer (see Fig. 1 legend) (a) and to a series of mixed solutions (400 μg/0.4 ml in total) of ^{14}C-labeled native and denatured collagen as indicated (b). Solubility of collagen was estimated as described in Fig. 1.

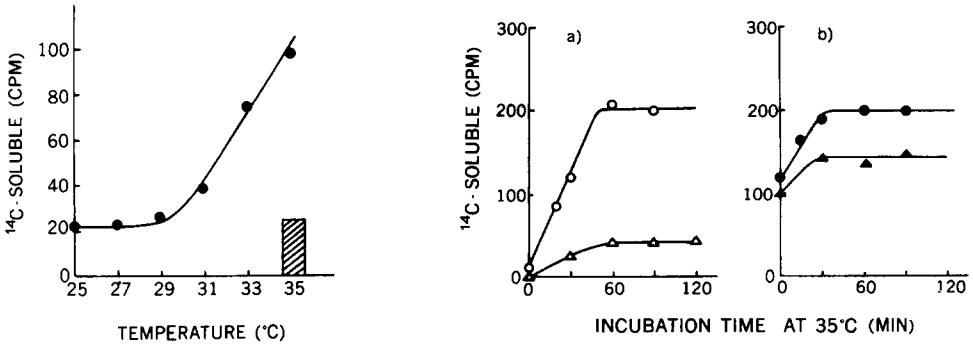

Fig. 3. Effect of temperature on the solubility of collagen in 50% dioxane at pH 7.8. 0.1% ^{14}C-labeled native collagen (480 cpm/400 μg) in Tris/NaCl/$CaCl_2$/glucose buffer (see Fig. 1 legend) was incubated at different temperatures for 2 h, then equal volumes of dioxane preincubated at corresponding temperatures were added and ^{14}C-radioactivity of the supernatant measured. The solubility of collagen in 50% dioxane at 25°C after incubating at 35°C for 2 h is shown as a bar.

Fig. 4. Degree of denaturation of collagenase digest of collagen as a function of incubation time at 35°C. ^{14}C-labeled collagen (480 cpm/400 μg) was incubated with purified (○) and crude (●) tadpole collagenase and human granulocyte extracts (patient Nos. 22: △; and 30: ▲) at 25°C for 22 h (a) and at 35°C for 1 h (b) in a total volume of 0.4 ml. After stopping the enzyne reaction by adding 20 μl of 80 mM *o*-phenanthroline solution, the reaction mixture was placed at 35°C for 0—120 min, then cooled to room temperature (22°—28°C). The degree of denaturation was estimated by measuring ^{14}C-radioactivity soluble in 50% dioxane as indicated in Fig. 1.

Effect of temperature on the solubility of collagen in 50% dioxane at pH 7.8

In order to find an optimal temperature for collagenase assay with soluble collagen, changes in solubility of native collagen in 50% dioxane with increasing temperature were examined. As shown in Fig. 3, native collagen was found to be insoluble up to 29°C (only 20 cpm soluble out of 480 cpm/tube), then the solubility gradually increased up to 20% at 35°C, indicating that denatured collagen could be preferentially extracted into 50% dioxane with low background, below 29°C. Since it is preferable to assay enzyme activity at a higher temperature, native collagen solution was first incubated at 35°C for 2 h, then cooled to 25°C and extracted by adding an equal volume of dioxane at the same temperature. The solubility of the collagen was found to be only a 5%, i.e. as low as that by direct extraction at 25°C.

Changes in solubility of collagenase-digestion products in 50% dioxane with denaturation at 35°C

Since 50% dioxane seemed to be a good solvent for selective extraction of denatured collagen, attempts to employ the solvent for collagenase assay were undertaken. Collagen was first incubated with purified tadpole collagenase at 25°C, under which conditions enzyme digestion products remain in native form. After stopping the reaction by adding *o*-phenanthroline, the mixture was incubated at 35°C for 0—120 min to determine the time required to complete denaturation of the products (Fig. 4). The degree of denaturation was estimated by measuring the amount of material extracted into 50% dioxane after

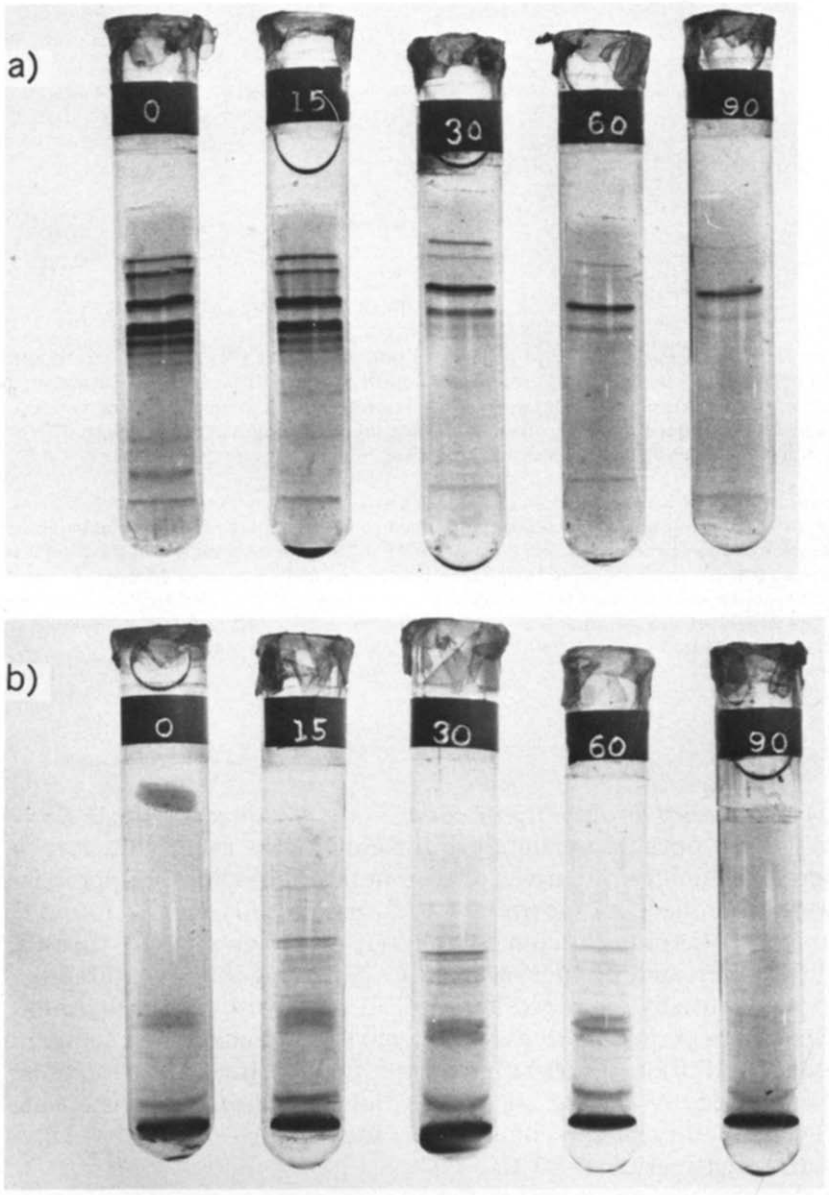

Fig. 5. Disc electrophoresis patterns of collagen degradation products of treatment with crude tadpole collagenase after separating soluble and precipitable fractions in 50% dioxane. The precipitated (a) and soluble (b) fractions in Fig. 4b (●) were subjected to disc electrophoresis at pH 2.3. The former fraction was dissolved in glycine/acetic acid buffer of pH 4.0 and denatured at 45°C for 10 min before electrophoresis. The supernatant was applied to electrophoresis without removing dioxane.

cooling to 25°C. When enzyme reaction mixture (25°C for 22 h) was immediately subjected to extraction into 50% dioxane, no appreciable amount of the products was extracted (see zero time in Fig. 4a). However, the amount of extractable products increased with incubation time up to 60 min, then leveled off. This indicates that 60-min incubation at 35°C is enough to denature col-

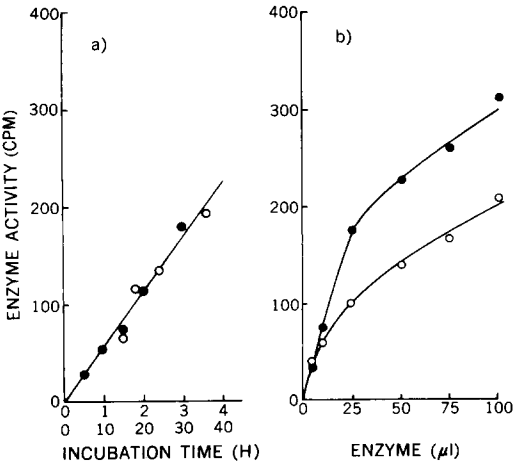

Fig. 6. Comparison of the present solution method with the conventional gel method for the assay of purified tadpole collagenase. a: amount of enzyme used was 10 μl and enzyme reaction was performed at 35°C for 1—4 h and 10—40 h for the solution (•) and gel (○) methods, respectively. b: Incubation times were 2 h and 20 h for the solution (•) and gel (○) methods, respectively.

lagen degradation products (Fig. 4a). Similar results were obtained with human granulocyte collagenase (Fig. 4a).

When enzyme digestion was conducted at 35°C, and the reaction products were extracted at 25°C immediately after stopping the enzyme reaction, the extraction of the products was incomplete (Fig. 4b). However, a further 30—60-min incubation at 35°C resulted in good recovery of the products in the extracts (Fig. 4b).

The 50% dioxane soluble and precipitated fractions (crude tadpole collagenase digest in Fig. 4b) were submitted to disc electrophoresis at pH 2.3. As shown in Fig. 5, collagen degradation products were transferred from the precipitate to the soluble fraction as denaturation proceeded at 35°C, and essentially only undegraded α and β components remained in the precipitate after incubation for 60—90 min at 35°C (Fig. 5a). Similar patterns were obtained when human granulocyte collagenase was employed.

Comparison of collagenase assay methods using soluble and reconstituted fibril collagen as substrates

This newly developed method using soluble collagen as substrate (400 μg collagen/tube) was compared with the conventional gel method using ^{14}C-labeled reconstituted collagen fibrils (800 μg collagen/tube). A linear relationship between enzyme activity and incubation time was observed in both methods, regardless of purity of collagenase preparations (Figs. 6a and 7a). It should be noted, however, that the present method required only 1/10 of the incubation time of the gel method to detect the same level of enzyme activity. Furthermore, the present method was found to be applicable over a wider range of enzyme concentration and used half the amount of collagen substrate, compared with the gel method (Figs. 6b and 7b). Since collagenase activity has gen-

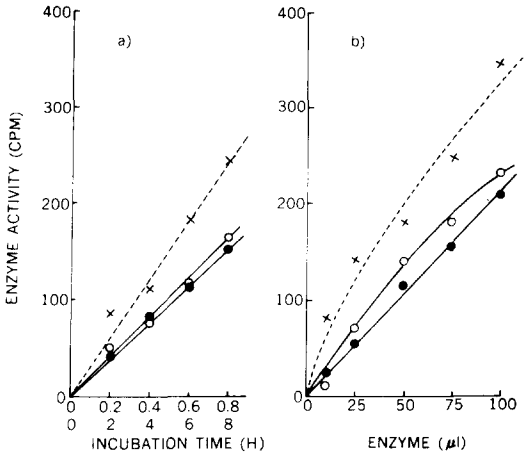

Fig. 7. Comparison of the present and conventional methods for the assay of crude tadpole collagenase. Cultured medium of tadpole skin explants was used as crude enzyme. a: amount of enzyme used was 25 μl and enzyme reaction was performed at 35°C for 0.2—0.8 h and 2—8 h for the solution (•) and gel (○) methods, respectively. b: Incubation time for the solution (•) and gel (○) methods was 0.4 h and 4 h respectively. Enzyme assay at 37°C (X) by the gel method is also indicated.

erally been assayed at 37°C by the gel method, the assay was performed at 37°C for comparison using crude tadpole collagenase. As shown in Fig. 7b, an approximately 40% increase in enzyme activity was obtained, compared with that at 35°C. The relationships between activity and incubation time or enzyme concentration were linear.

Comparison of methods using human granulocyte collagenase

Granulocyte extracts from 37 patients with various diseases were assayed for collagenase activity by the conventional and present methods. A good linear

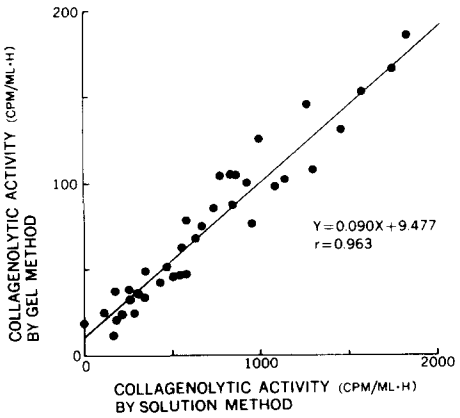

Fig. 8. Relationship between collagenase activities assayed by the gel and solution methods. Collagenase activity in the granulocyte extracts prepared from 5 ml each of bloods from 37 patients with various diseases was assayed by the gel and solution methods. Enzyme reaction was allowed to proceed for 30—40 h in the gel method and for 2—3 h in the solution method using 50—100 μl of the extracts.

relationship between results obtained with both methods was observed, with a correlation coefficient $r = 0.963$ ($P < 0.01$), as shown in Fig. 8. The present method can therefore be employed for the routine assay of granulocyte collagenase with considerable time saving.

Discussion

The collagenase assay method described here was developed to overcome the disadvantages in the conventional method using reconstituted collagen gels, ascribable to the presence of two phases. It provided low backgrounds, rapid reaction rates and linearity as a function of incubation time or enzyme concentration over a wide range. It may also be used for assay at various temperatures below 35°C and at pH values away from neutrality if the reaction products are extracted after adjusting to neutral pH (unpublished data). Since the method is based on the extraction of enzyme digestion products using 50% dioxane, higher counting efficiencies with lower quenching are obtained regardless of the presence of various contaminants in samples. In the conventional method all the reaction mixture, except undigested collagen gels, is added to Bray's solution.

Although little radioactivity (20—25 cpm out of 480 cpm/tube) was extracted even after incubation of native collagen for 16 h at 25°C and 35°C at pH 7.8, a gradual increase in extractable radioactivity up to 70 cpm was observed during incubation at 37°C for 2 h. In these cases extractions were carried out at 25°C. Therefore, the most suitable temperature for enzyme reaction is considered to be 35°C.

It seems unlikely that non-specific proteases affect the present assay method, since a good linear correlation with the conventional gel method was obtained when granulocyte extracts were employed as an enzyme source which are known to contain various proteases [10,11]. Therefore, the method reported here would provide an alternative way to assay collagenase activity or be complementary to the conventional method, with shorter incubation times and more reliable results resulting from low background count rates and less interference by contaminants in samples assayed.

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