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Amperometric Assay for Collagenase

Amplification by the Use of Glucose Oxidase Conjugated to Insoluble Collagen

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Abstract

A sensitive and rapid amperometric assay for collagenase has been developed. The substrate for the assay is glucose oxidase covalently linked to insoluble collagen with dimethylsuberimidate. The collagenase cleaves the insoluble collagen–glucose oxidase conjugate into smaller, soluble fragments that have glucose oxidase activity. That activity is proportional to the collagenase activity hydrolyzing the insoluble conjugate. In the absence of collagenase, no glucose oxidase activity is found in the soluble phase. Glucose oxidase activity was assayed by measuring amperometrically the rate at which hydrogen peroxide is produced. The kinetics follow that proposed for a soluble enzyme acting on an insoluble substrate.

Index Entries: Amperometric assay, for collagenase; assay, amperometric for collagenase; collagenase, amperometric assay for; glucose oxidase, in amperometric assay for collagenase; collagen, amperometric assay for collagenase.

Introduction

Methods developed for measuring collagenase are generally based on the use of collagen labeled with radioactive isotopes, ^{14}C (*1*, *2*) or ^{3}H (*3–7*), or with non-isotopic fluorescent or dye molecules (*8–11*).

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In this communication we describe an enzyme-amplified, amperometric method for measuring collagenase activity. The substrate is insoluble collagen linked covalently to glucose oxidase. While linked to the insoluble collagen, the glucose oxidase activity is absent in the soluble phase. The collagenase releases glucose oxidase bound to the soluble degradation products of collagen. The amount of glucose oxidase becoming soluble is proportional to the activity of collagenase. The application of covalently linked enzymes as versatile labels and powerful amplifiers of weak signals is now becoming widely used in enzyme-linked assay systems (*12, 13*).

Materials and Methods

Reagents

Insoluble collagen (Type I from bovine achilles tendon), collagenase (Type III, fraction "A" from *Clostridium histolyticum*), β-D-glucose, and dimethylsuberimidate (Sigma); highly purified glucose oxidase from *Aspergillus niger* (Miles).

Covalent Labeling of Insoluble Collagen With Glucose Oxidase

A suspension of insoluble particulate collagen (400 mg) was heated in 50 mL of 0.5*M* $NaHCO_3$ to 60°C and maintained at that temperature while it was continuously stirred. The suspension was centrifuged at 15,000 rpm in a Sorvall refrigerated centrifuge for 5 min. The supernatant was decanted; the pellet was resuspended in 10 mL 0.5*M* $NaHCO_3$ and was vigorously stirred at room temperature for 5 min. Next, 5 mL of 20 m*M* dimethylsuberimidate in 0.5*M* $NaHCO_3$ was added to the stirred suspension, and the stirring was continued for 5 more min. The treated suspension was centrifuged at 15,000 rpm for 3 min and immediately afterward the supernatant was decanted and 5 mL glucose oxidase solution (1050 U/mL) was added to the pellet. The pellet was mechanically dispersed and stirred at room temperature for 15 min, and placed at 4°C for 12–18 h. This suspension was centrifuged at 15,000 rpm for 5 min, the supernatant was decanted, and the pellet washed by suspending and stirring it in 50 mL 1*M* NaCl, 0.1*M* sodium phosphate, pH 5.5, at room temperature for 5 min.

Dimethylsuberimidate was used as the crosslinker because its imidate functional group reacts specifically with an amino group (*15*) and maintains the charge characteristics of the unmodified amino group (*16*).

The suspension was centrifuged at 10,000 rpm for 5 min, the supernatant was discarded, and the pellet was resuspended in 50 mL 1*M* NaCl, 0.10*M* sodium phosphate, pH 5.5, and the pellet was washed three more times with 50 mL aliquots of the same solution and then three times with 50 mL portions of 0.15*M* NaCl in 0.1*M* sodium phosphate, pH 5.5, according to the procedure just described. After the final wash, the pellet of collagen–glucose oxidase conjugate was suspended in TES buffer (0.05*M* TES (*N*-tris-[hydroxymethyl]methyl-2-aminoethane sulfonic acid), 2 m*M* $CaCl_2$, pH 7.5).

Assay for Glucose Oxidase

Hydrogen peroxide produced from the glucose oxidase catalyzed oxidation of glucose was monitored amperometrically using a YSI-Clark 2510 electrode, with a polarizing voltage of 700 mV supplied by a YSI Model 25 oxidase meter (Yellow Springs Instrument Co., Yellow Springs, OH). The amperometric response was displayed on a Fisher Recordall chart recorder (Series 5000). The tip of the electrode was immersed in a well-stirred glucose solution (5 mL, 0.1*M* β-D-glucose in 0.15*M* NaCl, 0.1*M* sodium phosphate buffer, pH 5.5). The reaction was carried out at 25°C and was initiated by adding 100–200 μL of enzyme solution (*14*). Glucose oxidase activity was expressed in changes in nanoamperes per min (ΔnA/min).

Assay for Collagenase

Suspensions of insoluble collagen–glucose oxidase conjugate (0.6 mL, 10 mg/mL) were pipeted into 1.5 mL Eppendorf Micro test tubes. Next, 6.1 mL aliquots of collagenase were added, and the test tubes were capped and vigorously shaken. At various intervals, the tubes were centrifuged in an Eppendorf microcentrifuge, model 5412, for 1 min. The supernatant (100–200 μL) was assayed for glucose oxidase activity amperometrically.

Clostridial collagenase preferentially cleaves collagen in the helical regions at the NA-gly bond, where NA is most frequently a neutral amino acid, in the sequence Pro-NA-Gly-Pro. The specific activity of collagen–glucose oxidase conjugate was determined to be 25.7 nmol glucose oxidized/min/mg conjugate.

Results and Disucssion

Release of Glucose Oxidase from Collagen–Glucose Oxidase Conjugate by Collagenase

Treatment of the insoluble collagen–glucose oxidase conjugate with collagenase, released glucose oxidase activity into the soluble (supernatant) phase (Fig. 1, line A). On the other hand, when collagenase was omitted from the assay mixture, there was virtually no glucose oxidase activity observed in the soluble phase (Fig. 1, line B).

When suspensions of insoluble collagen–glucose oxidase were incubated with constant amounts of collagenase for varying lengths of time, we found, after removing the insoluble materials by centrifugation, that the supernatant contained amounts of glucose oxidase activity that increased proportionally to the time of incubation (Fig. 2).

Effect of Collagenase Concentration [E] on the Release of Glucose Oxidase from the Insoluble Collagen–Glucose Oxidase Conjugate

Fixed amounts of insoluble collagen–glucose oxidase conjugate were incubated with increasing amounts of collagenase for a series of fixed times. At the end of the

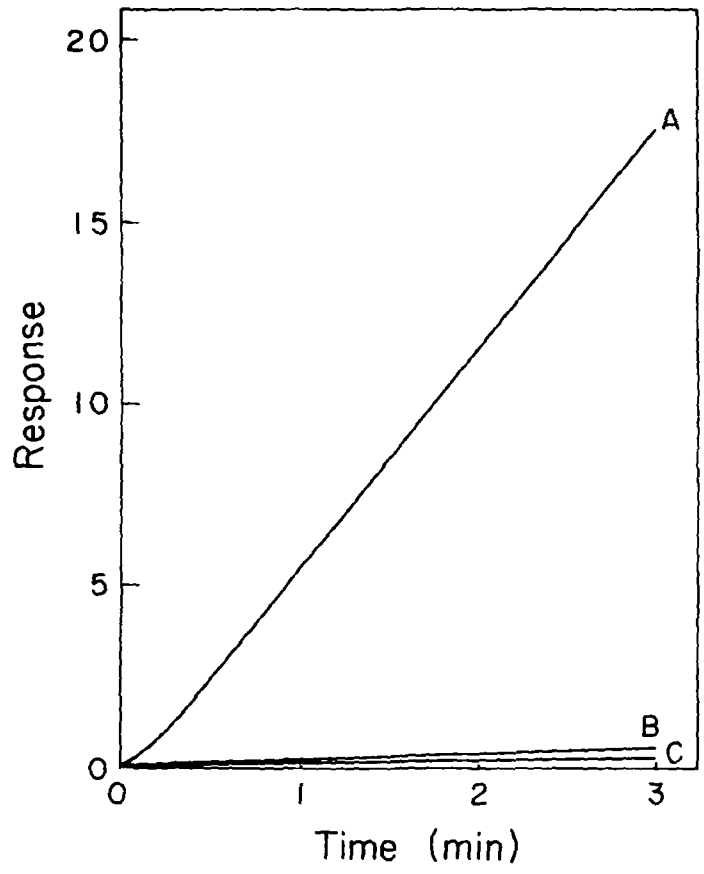

Fig. 1. Measurement of collagenase activity. Amperometric measurement of hydrogen peroxide produced from the oxidation of β-D-glucose by soluble glucose oxidase released from insoluble collagen-glucose oxidase by the action of collagenase. Measurements are in terms of nanoampere unit (nA). Line A: Rate in presence of collagenase. To a 0.6 mL suspension of insoluble collagen–glucose oxidase conjugate (6 mg in TES buffer) was added 0.1 mL enzyme (3.3 mg collagenase in TES). The suspension was incubated at 25°C for 20 min; then it was centrifuged in an Eppendorf centrifuge for 1 min. The supernatant (0.2 mL) was removed and added to the glucose oxidase assay medium. The glucose oxidase activity was monitored amperometrically (details were described in the text). Line B: Rate in absence of collagenase. Conditions were similar to those used in Line A, except that TES buffer was used instead of collagenase solution. Line C: Rate in absence of collagenase and substrate. TES buffer, instead of the supernatant was added to the glucose oxidase assay medium.

incubation periods, the supensions were centrifuged and each supernatant was assayed for glucose oxidase activity. That activity increased hyperbolically with increasing concentration of collagenase (Fig. 3). Such a hyperbolic relationship between rate and enzyme concentration is often observed when either the enzyme or the substrate is insoluble (*17*).

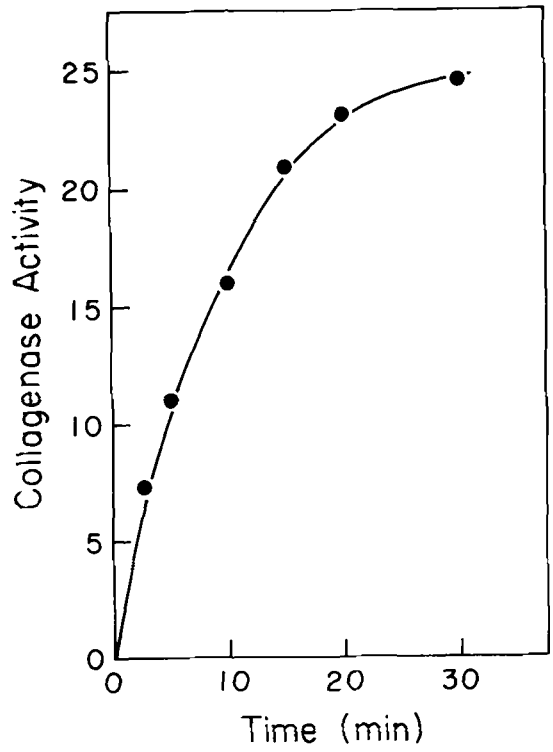

Fig. 2. Time course of collagenase action on insoluble collagen–glucose oxidase conjugate. A suspension of collagen–glucose oxidase conjugate (6 mg) and collagenase (3.3 mg) in a total of 0.7 mL TES were incubated at 25°C for varying times as indicated in the figure. The suspension was then centrifuged for 1 min. The supernatant (0.1 mL) was removed and assayed for glucose oxidase activity amperometrically. Collagenase activity was expressed in terms of the amount of glucose oxidase activity released into the supernatant from insoluble collagen–glucose oxidase conjugate.

Our data appears to fit a kinetic theory developed by McLaren and Packer for enzymes acting on an insoluble substrate (*17*). According to their theory, the reaction rate (v) of a soluble enzyme acting on an insoluble substrate is

$$v = k\cdot(SAS)\cdot[E]/(SAE)\cdot(K_d + [E]) \quad (1)$$

where k = rate constant of the enzyme; K_d = enzyme substrate dissociation constant; $[E]$ = enzyme concentration; SAS = surface area of the substrate; SAE = surface area of the enzyme.

Equation (1) can be arranged as Eq. (2)

$$1/v = (SAE)/k\cdot(SAS) + (SAE)\cdot K_d/k\cdot(SAS)\cdot 1/[E] \quad (2)$$

Since k, K_d, SAS, SAE are constants, a plot of $1/v$ against $1/[E]$ according to Eq. (2) is a straight line. Indeed, when data in Fig. 3 were plotted in a double reciprocal plot, a straight line was obtained (Fig. 4).

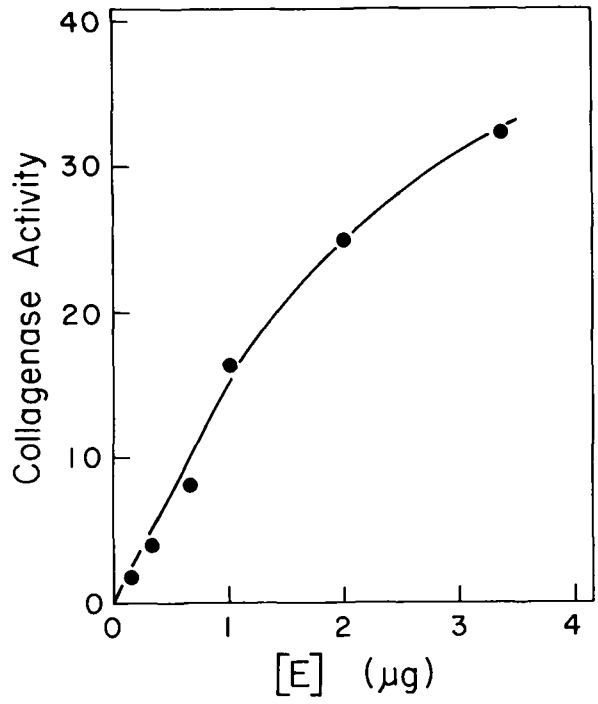

Fig. 3. Relationship between collagenase concentration [*E*] and its activity. To suspensions of insoluble collagen–glucose oxidase conjugate (in 0.6 mL TES) were added 0.1 mL of collagenase (amounts of collagenase added range from 0.1 μg to 3.3 g). These suspensions were incubated at 25°C for 15 min. Then they were centrifuged for 1 min. The supernatant (0.2 mL) was removed and assayed for the released glucose oxidase activity. Collagenase activity was defined as in Fig. 2.

Conclusion

We have developed a sensitive and rapid amperometric assay for collagenase using glucose oxidase as an amplifying label. The assay can be completed in less than 20 min, which is faster than methods previously described that require several hours of incubation. Furthermore, the present method does not use radioactive materials: therefore the possible health hazards associated with the use of radioactive materials are avoided. Finally, the assay system may serve as a convenient method for investigating interaction of a soluble enzyme with an insoluble substrate.

Acknowledgment

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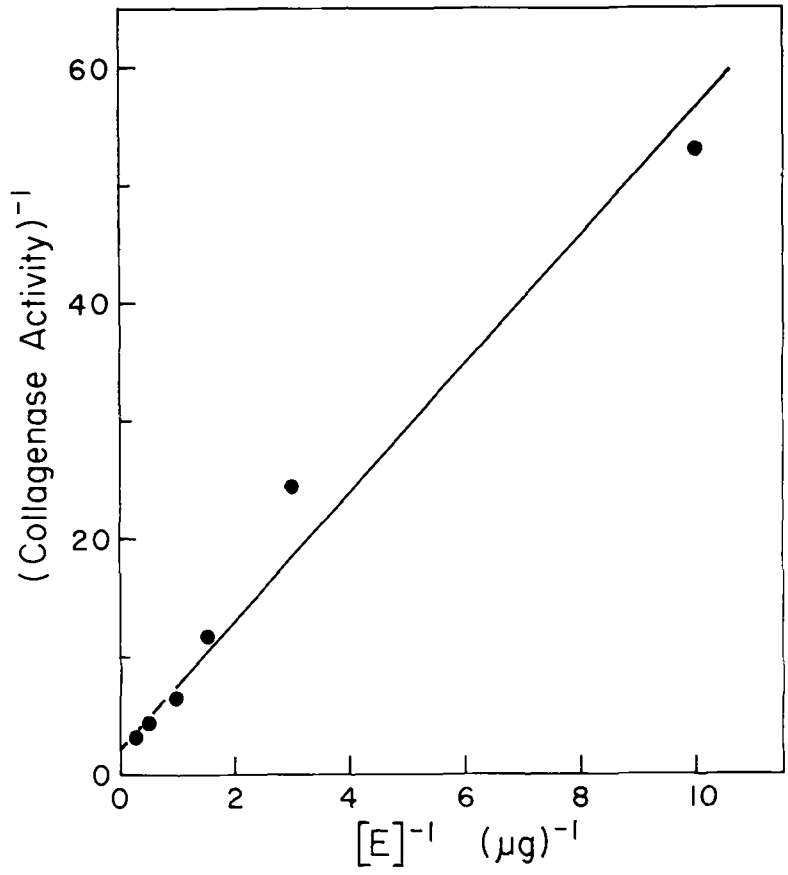

Fig. 4. Reciprocal of collagenase activity plotted against reciprocal of concentration of collagenase. Data were taken from Fig. 3.

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