The Interaction of Collagenase with Hydroxyapatite and Related Materials and Enzymatic Properties of the Adsorbed Enzyme

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Summary. The commercial collagenase from Clostridium histolyticum was adsorbed on hydroxyapatite, on bovine femur shaft and on enamel and dentin powders. The substrate specificity of the adsorbed enzyme tested, with chromophore substrate, azocoll and native collagen, differed from that obtained with the soluble enzyme. The adsorption and the substrate specificity was also dependent on the adsorbent used. A pretreatment of hydroxyapatite with chondroitin sulphate, hyaluronate and DNA lowered the adsorption of collagenase. Phosphate ion caused desorption of the enzyme from hydroxyapatite. Sodium fluoride caused partial desorption of the enzyme from hydroxyapatite and enamel and dentin powders. Collagenase adsorbed on root surfaces of teeth liberated hydroxyproline containing material.

Key words: Hydroxyapatite — Collagenase — Protein — Apatite interaction.

Introduction

Proteolytic enzymes such as collagenase liberated from microorganisms and/or from the tissue cells take part in tissue degradation of, for example, bone and teeth (Söder and Frostell, 1966; Mäkinen and Paunio, 1966; Schultz-Haudt and Sherp, 1955; Beutner et al., 1966; Riley and Peacock, 1967; Paunio and Mäkinen, 1969; Gross, 1970; Hekkelman, 1973).

It can be suggested that these proteolytic enzymes are able to interact with the inorganic phase of bone or tooth and that these insolubilized enzymes are still biologically active. Water insoluble derivatives of enzymes and biologically active proteins have been dealt with in review articles by Silman and Katehalski (1966) and Goldstein (1970). Glass beds, charcoal, various cellulose and ion exchange resins have been widely used as enzyme carriers. The biologically interesting hydroxyapatite has been used as a carrier of enzymes in only a few studies (Paunio, 1972; Knuutila and Paunio, 1974). This may be due to the fact that the mechanisms of interaction between proteins generally and hydroxyapatite are still not well known. However, the adsorption of different proteins on and their desorption from hydroxyapatite has been widely studied (Ericson and Ericsson, 1967; Norbö and Rölla, 1972; Rölla and Melsen, 1975), especially in connection with hydroxyapatite chromatography (Tiselius et al., 1956; Bernardi and Kawasaki, 1968; Bernardi et al., 1972; Bernardi, 1971).

The possibility that insolubilized enzymes on the tooth surfaces might play an important role in tissue degradation was approached in the present study by using a commercial collagenase enzyme, purified from Clostridium histolyticum, with synthetic hydroxyapatite, the inorganic phases of bone, enamel and dentin as the adsorption bed.

Material and Methods

Adsorption of Collagenase to the Beds. A collagenase (Type 1) from C. histolyticum (Sigma Chemical Company, St. Louis, Mo. USA) was dissolved in 0.01 M Tris-HCl buffer pH 7.0 (1 mg/1 ml). The following bed materials were used: Hydroxyapatite prepared according to the method of Tiselius et al. (1956), bovine femur shaft powder (Ossar*) (Lääke Oy, Turku, Finland), and enamel and dentin powders prepared as described by Paunio et al. (1968). A fraction of 200–230 mesh particle size of these materials was used.

Enzyme adsorption was estimated by mixing 2 ml of the enzyme solution with 50 mg dry bed material. The suspension was centrifuged at (RC-2B Sorwall Superspeed) 10,000 rpm for 10 min at 4° C. The pellet was washed with 2 ml of 0.01 M Tris-HCl buffer pH 7.0 until the supernatant was free of enzyme activity. The...
enzyme activity was determined in each successive supernatant fluid and in the final suspension.

Assays of the Enzyme Activity. The collagenolytic activity was determined in 0.01 M Tris-HCl buffer pH 7.0 in the presence of 0.009 mM CaCl₂. The incubations were carried out at 30 °C.

The assay of the enzyme activity with the chromophore-collagenase substrate (4-phenylazobenzyl-oxy-carbonyl-l-prolyl-l-leucyl-glycyl-l-prolyl-l-arginine dihydrate (Fluka AG, Buchs, Switzerland), was based on the method described by Wünsch and Heidrich (1963). The activities are given as μmol of liberated 4-phenylazobenzyl-oxy-carbonyl-l-prolyl-l-leucine per min, corrected with values obtained from mixtures in which either the substrate or enzyme was omitted.

The enzyme activity against azocoll (Calbiochem AG, Lucerne, Switzerland) was determined by inserting 5 mg of the dry substrate into test tubes to which 1.0 ml of 0.01 M Tris-HCl buffer pH 7.0 and 0.2 ml of the enzyme solution were then added. The absorbance of the liberated dye was measured at 580 nm. The activities are expressed as the increase of the extinction during 1 min reaction time. Controls were as above.

The degradation of native collagen was determined by incubating a reaction mixture consisting of 0.5 ml of the above mentioned Tris-HCl buffer, 0.5 ml enzyme solution, 0.5 ml of the collagenous substrate and 0.2 ml of a 0.1 M CaCl₂ solution in a dialysis sac for 6 h in 300 ml water (adjusted to pH 7.0 with NaOH), and containing 0.009 mM CaCl₂. The outer solution (300 ml) was evaporated to dryness and the hydroxyproline from the solid residue was analyzed as described by Pikkarainen (1968). Collagen was prepared from rat tail according to Byers et al. (1974).

Release of the Enzyme by Phosphate Ions and the Effect of some Compounds on the Adsorption. The effect of phosphate ions on the adsorbed enzyme was tested with a stepwise batch operation. Phosphoric buffers, pH 7.0, at concentrations from 0.001 M to 0.5 M were used. To obtain information concerning the adsorption mechanism, the hydroxyapatite crystals were pretreated with DNA. Fifty mg hydroxyapatite was first mixed with 2 ml of DNA dissolved in water (2.5 mg/ml). The effects of sodium and potassium chloride at concentrations of 0.1 M to 3.0 M on adsorption were also studied.

The effect of the pretreatment of the hydroxyapatite crystals with two connective tissue components, hyalurionate and chondroitin sulphate (Sigma Chemical Company, St. Louis, Mo. USA), on the interaction between the enzyme and the bed was also studied. The amounts of these two components varied from 20 mg to 40 mg, dissolved in 2.0 ml water, and mixed with 50 mg of hydroxyapatite for 15 min. The suspension was centrifuged and the pellet was washed once with 0.01 M Tris-HCl buffer pH 7.0 before the addition of the enzyme solution.

Effect of Sodium Fluoride on the Interaction between the Enzyme and Hydroxyapatite, Enamel or Dentin and the Root Surfaces of Teeth. The ability of sodium fluoride compared to sodium chloride to release the adsorbed enzyme was studied using a stepwise batch operation. Sodium fluoride and sodium chloride at concentrations from 0.05 M to 1.0 M in 2.0 ml of solution were used to elute 50 mg hydroxyapatite, enamel and dentin powders, to which the enzyme was adsorbed. The hydroxyapatite was also pretreated with 0.2, 0.6 and 1.0 M sodium fluoride solution and then the adsorption of the enzyme was studied. The effect of sodium fluoride on the activity of the soluble enzyme was also determined.

The root surfaces of 6 extracted anterior teeth were cleaned carefully with a scaler and washed several times in 0.01 M Tris-HCl buffer pH 7.0. The roots of 2 teeth were incubated together in 2.0 ml of above buffer at 22 °C for 6 h. Each of three incubation buffers were analyzed to determine the enzyme activity with the chromophore-collagenase substrate and the concentration of hydroxyproline. The same roots were then exposed to 2.0 ml of collagenase enzyme (1 mg/ml) for 15 min. The unadsorbed collagenase enzyme was washed away. The teeth were again immersed in 2.0 ml of the buffer for 6 h. The same determinations as above were made. In the third step, the roots were washed 4 times with 0.5 M sodium fluoride solution and then incubated as above. The incubation media were again analysed.

Results

A. Adsorption of Collagenase on Hydroxyapatite

a) The Hydrolysis of Different Substrates Catalyzed by an Adsorbed Enzyme. Collagenase from C. histolyticum was found to adsorb effectively on hydroxyapatite (Fig. 1.). The activity of the unadsorbed enzyme (the activity of the enzyme which was either not adsorbed or was released during the washing procedures) was very small or undetectable in the case of all 3 substrates. However, an interesting finding was obtained, namely a clearly higher rate of hydrolysis by an adsorbed enzyme (II) of the chromophore-collagenase substrate compared to that of azocoll and collagen. The activities of a soluble enzyme (III) measured with these three substrates were used as reference values. The soluble enzyme catalyzed the hydrolysis of all substrates, as shown in Figure 1.

b) Enzymatic Properties of the Adsorbed Enzyme. The effect of the substrate concentration on the rate of hydrolysis catalyzed by the adsorbed enzyme (Fig. 2.) indicated a behavior which, to a certain degree, followed the simple Michaelis–Menten law. However,

![Fig. 1. The rate of the hydrolysis of A: chromophore-collagenase substrate (μmol x min⁻¹), B: azocoll (dE₅₈₀ x min⁻¹) and C: native collagen (μg hydroxyproline x h⁻¹) catalyzed by collagenase from Clostridium histolyticum. Bar I represents the level of the activity of the enzyme which was neither adsorbed or was released during washings. Bar II shows the ability of the adsorbed enzyme to hydrolyze the substrates used and Bar III the same for the soluble enzyme](image-url)