BBA 66037

# APPLICATION OF A RAPID COLORIMETRIC ASSAY TO DETECT ALTERATIONS IN RAT PROLINE IMINO PEPTIDASE

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(Received July 21st, 1969)

SUMMARY

A modification of a colorimetric assay previously used for *Clostridium histolyticum* collagenase (EC 3.4.4.19) based on the cleavage of a chromophore-acyl-pentapeptide was employed to demonstrate enzymatic activity in rat liver, lung, and heart homogenates. Proline imino peptidase (EC 3.4.1.4) displayed maximum activity at 37° and pH 8.5. Liver enzyme activity per gram liver was significantly elevated in male albino rats fed a commercial atherogenic diet and treated concurrently with parathyroid extract (U.S.P.) (25 U.S.P. units per rat every other day). Proline imino peptidase activity demonstrated by this method is operable under physiological conditions and does not require *de novo* synthesis or extrusion of ferment from viable tissue culture to produce measurable enzymatic activity.

## INTRODUCTION

Connective tissue serves in the body to hold the cells of the body in a proper geometric and metabolically functional array. Collagen imparts the necessary tensile strength to this connective tissue. However under various physiological and pathological conditions the connective tissue framework undergoes remodelling. This process requires the destruction of mature collagen and the synthesis of collagen to meet the new geometric and functional demands of the organism.

Although the biosynthetic mechanisms involved in organic matrix synthesis have been studied extensively[1,2]; comparatively little is known about the organic matrix resorptive mechanism necessary to degrade collagen under various physiological and pathological conditions. The biochemical nature of the resorptive process has received attention only in the past decade. Although the exact mechanism of resorption remains a mystery, an enzymatic nature is suggested by the peptide collagen breakdown products. Since most proteolytic enzymes are incapable of breaking down mature collagen, the presence of specific collagen catabolic enzymes in animal tissues is required for connective tissue degradation.

Two types of mammalian collagen degrading enzymes have been reported.

Collagenolytic activity with an alkaline optimum pH has been isolated and demonstrated using the tissue culture technique[3-5]. Another collagenolytic factor with an acid optimum pH has been demonstrated in rat bone and liver homogenates and has been shown to be localized in subcellular particles resembling lysosomes[6-8].

In most previous reports, mammalian collagenolytic activity has been detected through the use of a labeled native collagen. ESPEY AND RONDELL[9] have reported the use of a synthetic substrate to estimate rabbit collagenolytic activity. This system, using a ninhydrin colorimetric analysis to determine the quantity of substrate digested, is reported to be useful in estimating nonspecific mammalian collagenolytic activity. This report describes the application of rapid direct technique previously described for the estimation of bacterial collagenase (EC 3.4.4.19)[10] for the detection of changes in a rat proline imino peptidase (EC 3.4.1.4).

## MATERIALS AND METHODS

Male albino Sprague–Dawley rats were used throughout this study.

*Tissue homogenate preparation.* Liver homogenates (5%, w/v) equivalent to 15 mg of protein per ml and lung homogenates (10%, w/v) equivalent to 8 mg of protein per ml were prepared in cold 0.01 M calcium acetate using a coaxial homogenizer. Heart homogenates (10%, w/v), equivalent to 13 mg of protein per ml were prepared by freezing the tissue in liquid nitrogen, pulverizing, and homogenizing in cold 0.01 M calcium acetate in a coaxial homogenizer.

*Enzyme assay.* A modification of the method of WUNSCH AND HEIDRICH[10] was employed. The substrate solution was prepared by dissolving 7.5 mg of 4-phenyl-*azo*-benzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine (Mann Res. Lab., New York, N.Y.) in 0.1 ml methanol and diluting to 10 ml with acetate–veranol buffer (pH 8.5). Enzymatic activity was quantitized by the amount of chromophore-acyl-dipeptide, 4-phenyl-*azo*-benzyloxycarbonyl-L-prolyl-leucine, present in the reaction mixture after incubation. Liver, lung or heart homogenate (0.5 ml) and substrate solution (2.0 ml) were preincubated separately at 37° for 15 min and then mixed. Following incubation for 15 min at 37° under air, 0.5 ml of the liver reaction mixture or 1.0 ml of the lung or heart reaction mixture were pipetted into 1 ml of 0.5% aqueous citric acid solution. Ethyl acetate (5.0 ml) was added and the sample was shaken for 1 min. The sample was centrifuged at 3000 rev./min for 20 min to remove water droplets from the organic phase. The sample was read against an incubated blank without enzyme at 320 m$\mu$ in a Beckman DB Spectrophotometer.

*Protein assay.* Homogenate protein concentration was determined by method of LOWRY *et al.*[11] using bovine plasma albumin (Calbiochem, Los Angeles, Calif.) as standard.

## RESULTS AND DISCUSSION

Many previous demonstrations of animal collagenolytic activity have required *de novo* synthesis or the extrusion of ferment from viable tissue in culture[3,12]. The enzyme measured in the present investigation does not require tissue culture techniques to obtain measurable enzymatic activity.

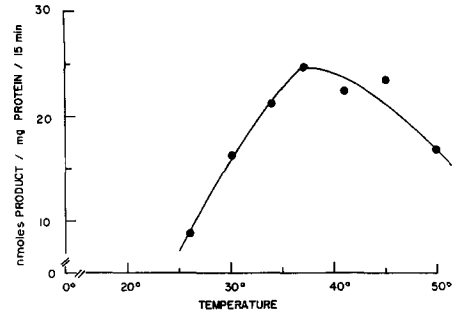


Fig. 1. Variation of liver peptidase activity with temperature of incubation. Aliquots of a pooled homogenate were incubated with substrate at pH 8.5 under air.

Maximum enzyme activity was observed at 37° (Fig. 1). Enzyme activity was temperature dependent below this and activity was retained to 50°. Enzymatic activity dropped markedly at 50° and above.

Enzyme activity was linear with time of incubation up to 30 min (Fig. 2). After this time activity began to level off.

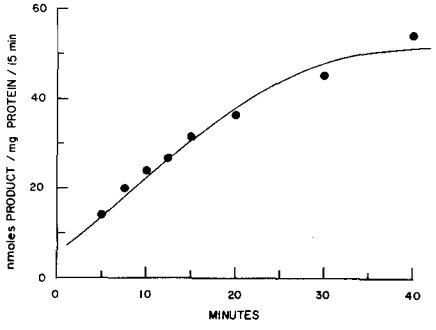


Fig. 2. Variation of liver peptidase activity with time of incubation. Product, 4-phenyl-*azo*-benzyloxycarbonyl-L-prolyl-leucine. Aliquots of a pooled homogenate were incubated with substrate at 37° and pH 8.5 under air.

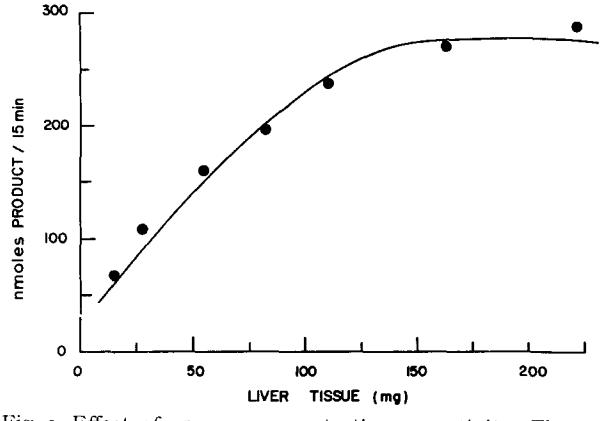


Fig. 3. Effect of enzyme concentration on activity. The enzyme concentration was varied by diluting a homogenate of liver tissue.

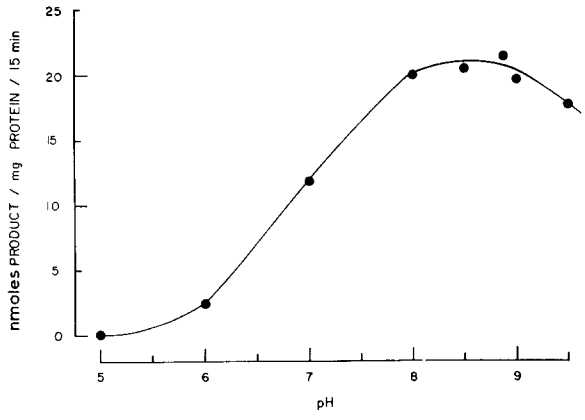


Fig. 4. Effect of pH on peptidase activity. Aliquots of a pooled homogenate were incubated with substrate at 37°.

The amount of enzyme activity observed was directly proportional to the amount of enzyme present in the incubation flask (Fig. 3). Under the conditions used in this assay the maximum amount of enzyme which could be determined was that present in 125 mg of liver homogenate.

Proline imino peptidase (EC 3.4.1.4) activity over a pH range from 5 to 9.5 was determined using acetate–veranol buffer (Fig. 4). Optimum activity was observed between pH 8 and 9. There is a broad pH optimum above pH 8 with complete loss of activity at pH 5.0. Bacterial and animals 'collagenases' have pH optima between 6.0 and 9.0 (refs. 3 and 13). The collagenase from rheumatoid synovium demonstrated by EVANSON *et al.*[4] in tissue culture had a similar pH optimum. FULLMER AND LAZARUS[14] reported a collagenase from human, goat, and rat bone which showed major activity between pH 7.0 and 9.0 and only slight activity below pH 6.0. The lack of activity of this enzyme below pH 5.0 clearly distinguishes it from the acid optimum lysosomal enzymes demonstrated by FRANKLAND AND WYNN[6] and WOODS AND NICHOLS[7], while the nonspecific proteinases associated with lysosomal granules have been shown to be active at neutral pH (ref. 15).

Enzymatic activity was demonstrated in liver, lung, and heart tissue (Table I). The greatest specific enzyme activity was observed in the liver. Treatment of the homogenates with ultrasound or Triton X-100 did not alter the total activity of the homogenates.

TABLE I

LIVER, LUNG, AND HEART PEPTIDASE ACTIVITY OBSERVED IN WHOLE RAT HOMOGENATES

Product: 4-phenyl-*azo*-benzyloxycarbonyl-L-prolyl-leucine. Figures are mean ± S. E. (number of animals).

| *Tissue* | *Peptidase activity (nmoles product per mg protein per 15 min)* |
|---|---|
| Liver | 44.54–0.71 (6) |
| Lung | 29.02–1.99 (6) |
| Heart | 13.39–0.91 (6) |

TABLE II

THE EFFECT OF CHRONIC PARATHYROID EXTRACT TREATMENT ON TOTAL LIVER PEPTIDASE ACTIVITY IN THE ATHEROSCLEROTIC RAT

Product: 4-phenyl-*azo*-benzyloxycarbonyl-L-prolyl-leucine. Figures are mean ± S. E. (number of animals).

| *Treatment* | *Liver peptidase activity (μmoles product per g per 15 min)* |
|---|---|
| Parathyroid extract* | 7.39 ± 0.29 (10) |
| Control | 6.11 ± 0.32 (8) |
| Level of significance | $P \leqslant 0.005$** |

* Dose: 25 U.S.P. units parathyroid extract every other day for 3 months.
** Determined *via* Student's *t* test.

Liver imino peptide (EC 3.4.1.4) activity was measured in a related study in which rats were fed an atherogenic diet and treated concurrently with parathyroid extract (U.S.P.) for 3 months. An 18% increase in liver enzyme activity per gram liver tissue was observed following chronic parathyroid extract treatment at a dose of 25 U.S.P. units every other day (Table II).

The enzyme demonstrated in this present report is active under physiological conditions and is capable of degrading the synthetic substrate which is preferentially acted upon by the isolated bacterial collagenase from *Clostridium histolyticum*[10]. The assay procedure employed to demonstrate this mammalian enzyme is direct and quick as compared to those using labeled native collagen substrates, and is sensitive to drug induced changes in total activity.

WUNSCH AND HEIDRICH[10] indicated that the metabolism of this substrate is specific for collagenase activity since they observed no alteration of activity in the presence of other proteolytic enzymes. Thus the use of this assay system offers a rapid and convenient means of measuring and screening for drug-induced changes in mammalian peptidase which cleaves proline rich peptides.

ACKNOWLEDGEMENTS

Supported by U.S. Public Health Service, National Institutes of Health, Grant HE 11578 and the Rhode Island Heart Association. Parathyroid Extract was generously supplied by Eli Lilly and Co.

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