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DETERMINATION OF ELASTASE ACTIVITY BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The action of elastase on elastin was measured by high-performance liquid chromatography with on-line post-column derivatization. After alkaline hydrolysis, a 20-ml sample was injected into a ODS-2 gel column. An elastin-specific dipeptide, Val-Pro, and the internal standard, Gly-Leu, were eluted by a linear gradient of 0 to 10% of 1-propanol in 50 m*M* heptafluorobutyrate (pH 3) at a flow-rate of 1 ml/min. The eluent was reacted with fluorescamine, and the fluorescent products were measured. Retention times for Val-Pro and Gly-Leu were 17.33 and 23.54 min. The peak areas of 0.2–16 μg of Val-Pro gave a straight-line plot. Elastase activity was constant from 6 to 24 h and was 0.95 ± 0.02 (S.D.) μg/h. The method may be useful for the measurement of the elastolytic activity in some diseased tissues.

INTRODUCTION

Elastolytic activity has been identified in the pancreas, polymorphonuclear leukocytes, platelets, alveolar macrophages, fibroblasts, and several microorganisms[1–3]. In some tissues several isoenzymes of elastase also exist. The human and porcine pancreas contain two elastases and two proelastases[1,3]. Polymorphonuclear leukocytes from humans contain at least three isoenzymes[1]. The elastolytic activity measured in pathological specimens may thus be due to the combined effect of many different elastolytic proteases. In addition, the presence of other proteases, such as trypsin and chymotrypsin, will enhance the total elastolytic activity[1,4].

It has been suggested that alterations in elastin breakdown occur in such clinical entities as emphysema, abdominal aortic aneurysms, and pancreatitis[5–9]. Since the disease process may involve several elastases, the determination of the elastolytic activity in these specimens should be performed by solubilizing elastin. However, there have been some technical difficulties with the use of insoluble elastin for the measurement of elastase activity[1]. Gravimetric, nephelometric, and spectrophotometric methods of measuring the amount of soluble elastin have been inaccurate, and are at best semi-quantitative. In order to improve the assay, elastin has been derivatized with various dyes, fluorescent agents and radioactive isotopes[1,2]. However, the

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assays measure a group leaving from elastin and should not be considered elastolytic methods. Although there are several different structural types of elastin, based upon the amino sequence, some dipeptides occur in high frequency in hydrolysates of elastin[10]. One of these dipeptides is L-valyl-L-proline (Val-Pro), and 44 of these doublet sequences are present in 1000 amino acid units of bovine ligamentum nuchae elastin[11]. We report here a method by which the elastolytic activity of a biological specimen can be measured by determining the amount of Val-Pro solubilized from elastin.

EXPERIMENTAL

Materials

The high-performance liquid chromatographic (HPLC) system with an on-line post-column reaction device consisted of an automatic injector (Model 712), an automatic gradient controller (Model 680), two mobile-phase pumps (Models 510 and 501), a 250 × 4.6 mm I.D. Partisil ODS-2 column, two post-column pumps, two mixing T-pieces, two reaction coils, and a fluorescence detector (Model 420), all of which were purchased from the Waters Chromatography Division of Millipore (Milford, MA, U.S.A.).

Bovine ligamentum nuchae elastin, procine pancreatic elastase (Type IV), tris (hydroxymethyl)aminomethane (Tris), L-valyl-L-proline, glycyl-L-leucine, potassium hydroxide, sodium hydroxide, heptafluorobutyric acid, boric acid, sodium borate, and fluorescamine were obtained from Sigma (St. Louis, MO, U.S.A.), perchloric acid and hydrochloric acid from Mallinckrodt (St. Louis, MO, U.S.A.), 1-propanol from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), polypropylene test-tubes from Sarstedt (Princeton, NJ, U.S.A.), and a stainless-steel Swinney filter holder (13 mm) and nylon membranes (0.45 μm) from Rainin Instrument (Woburn, MA, U.S.A.).

Methods

Elastin (9 mg) was weighed out and suspended in 2 ml of sterile 0.2 *M* Tris–HCl buffer (pH 8.0). The hydrolysis of elastin was initiated by adding 28 units of the elastase, and incubating at room temperature. The final reaction volume was 2.2 ml. To obtain the zero time sample, the solution was mixed with a Vortex mixer before 0.5 ml of the reaction mixture was removed. This was immediately mixed with 0.5 ml of 0.4 *M* perchloric acid. At 6, 24, and 48 h, additional 0.5-ml portions of the reaction mixture were similarly withdrawn and added to the centrifuge tubes containing 0.5 ml of 0.4 *M* perchloric acid. After centrifugation at 3000 *g* for 10 min, the supernatant was decanted into 4 ml of 4 *M* potassium hydroxide, and incubated at 110°C for 18 h to obtain complete hydrolysis of the peptides, due to the elastolytic action. After neutralization with perchloric acid, the suspension was centrifuged as before, and the supernatant was decanted into another tube. The potassium perchlorate precipitate was washed twice with 0.5 ml of water and combined with the supernatant. The pooled solution was filtered through a 0.45-μm membrane to remove fine particles.

Standards

The internal standard, glycyl-L-leucine (Gly-Leu), and L-valyl-L-proline (Val-

Pro) were solubilized in water at a concentration of 1 mg/ml and were stored at –70°C. Dilutions with water for the assay were made daily. Val-Pro dilutions varied from 1 to 1000 μg/ml, and 100 μg/ml Gly-Leu were added to each standard. With an injection volume of 20 μl, 0.02–200 μg of Val-Pro, and 2 μg of Gly-Leu were injected into the column.

Chromatographic conditions

The method of Muramoto was modified, and an on-line post-column reaction was used to facilitate the collection of data[12]. The column was equilibrated at room temperature with 50 m*M* heptafluorobutyrate (pH 3.0) before each injection, at a flow-rate of 1 ml/min. After sample or standard injection, the dipeptides were separated by passing the equilibrating solution through the column for 5 min, and then adding 1-propanol to the mobile phase to produce a linear gradient of 0 to 10% of the alcohol in the initial solution over a period of 30 min. The post-column on-line derivatization of the dipeptides was obtained by mixing the mobile phase with 0.2 *M* borate buffer (pH 8.6) at af flow-rate of 50 μl/min. Fluorescamine, in a concentration of 0.2 mg/ml methanol, was then added at 75 μl/min. After mixing, the fluorescamine–Val-Pro reaction proceeded rapidly in the coil. The fluorescent products were determined at an excitation wavelength of 395 nm and with an emission filter with a 455-nm cutoff. Samples and standards were measured in triplicate. The column was again equilibrated with the initial mobile phase, and samples were injected at 50-min intervals.

Calculations

Alkaline hydrolysis of elastin produces a mixture of amino acids and the alkaline-resistant Val-Pro. However, during hydrolysis, the dipeptide has a tendency to cyclize and form a diketopiperazine[11]. Several investigators have demonstrated that

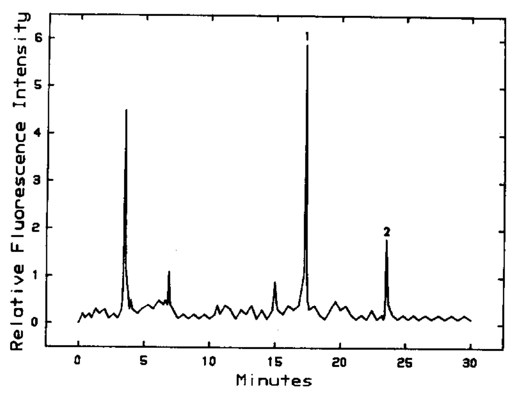

Fig. 1. Elution of 10 μg of L-valyl-L-proline (1) and 2 μg of glycyl-L-leucine (2) at retention times of 17.33 and 23.54 min, respectively. A 20-μl sample was injected into a ODS-2 gel column (250 × 4.6 mm I.D.). The dipeptides were eluted with 50 m*M* heptafluorobutyrate (pH 3) at a flow-rate of 1 ml/min for 5 min, and then by a linear gradient of 0 to 10% of 1-propanol in the heptafluorobutyrate for 30 min. Using two post-column pumps, mixing T-pieces, and reaction coils, the eluent was mixed with borate buffer (pH 8.6) and fluorescamine. The fluorescent products were measured at an excitation wavelength of 395 nm and with a 455-nm cutoff emission filter.

the two forms exist in a 1:1 ratio[10,13,14]. Since the diketopiperazine does not react with fluorescamine, only the Val-Pro peak was measured and used as the index of elastin hydrolysis. The arithmetic mean and standard deviation were calculated for each time period.

RESULTS

The retention times for Val-Pro and Gly-Leu were 17.33 and 23.54 min, respectively (Fig. 1). When the peak areas of the standards of Val-Pro were plotted against the concentrations, a straight line was obtained from 0.2 to 16 μg. The peak areas of the elastolytic products, which corresponded to the retention times of Val-Pro, were determined. The amount of Val-Pro released from elastin was calculated to be 0.95 $\pm$ 0.02 μg/h over the 48-h assay period. At 6 h, the rate was 1.00 μg/h, and at 48 h the rate was 0.94 μg/h.

DISCUSSION

Quantitation of elastolytic activity in biological specimens can be accomplished by measuring the breakdown of elastin, and determining the amount of Val-Pro which has been released. In this study, a "purified" porcine elastase, which contains elastase I, elastase II and trypsin activity, has been used to demonstrate the usefulness of the method. The rate of elastin hydrolysis is essentially constant from 6 to 48 h.

A chromatographic method comprising automatic injection, gradient elution, and on-line post-column derivatization permitted the assay of ten samples or standards in triplicate per 24 h. The elution of Val-Pro and Gly-Leu occurred in less than 25 min. In order to re-equilibrate the column, an additional 25 min was required before the next sample could be injected. This method may be useful in the measurement of the elastolytic activity of tissues and, in particular, specimens containing multiple proteolytic activities, such as the emphysematous lung, aneurysmal aorta, and septic pancreas.

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