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HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHIC INHIBITORY ASSAY OF α_1-PROTEINASE INHIBITOR*

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SUMMARY

Two size exclusion columns (Bio-Sil TSK 250, Spherogel TSK 2000 SW) were utilized in a high-performance size exclusion chromatographic assay to determine the proteinase inhibitory capacity of α_1-proteinase inhibitor (α_1-PI). Values from assays using both columns agreed well with the standard spectrophotometric inhibitory assays. Moreover, the Spherogel TSK 2000 SW column enabled determination of the proteinase inhibitory capacity in an analogous manner to that of the spectrophotometric assay, which is the measurement of the residual proteinase after interaction with α_1-PI. Nanogram to milligram amounts of protein, namely, plasma α_1-PI, elastase, trypsin and their corresponding complexes with the inhibitor, were fractionated in less than fifteen min.

INTRODUCTION**

α_1-PI is the major serine proteinase inhibitor in mammalian plasma. Among the proteinases inhibited by α_1-PI are trypsin, chymotrypsin and elastase. Although the mechanism of inhibition has not been elucidated, inhibition entails the formation of a 1:1 molar complex between α_1-PI and proteinase. The standard assays used to determine the proteinase inhibitory capacity of α_1-PI are spectrophotometric procedures which measure residual proteinase activity. This is accomplished by measuring the change of absorbance at a particular wavelength which occurs upon hydrolysis of the substrate. Presented in this paper is a versatile and rapid method of HPSEC which can be used not only to assay for proteinase inhibitory capacity, but also to study inhibitor–proteinase interactions such as complex formation. Conventional column gel filtration requires large (milligram) amounts of proteins and extended fractio-

* Preliminary results of this study were presented at the *Federation of American Societies for Experimental Biology Meeting in New Orleans, LA* (A. Feste and J. C. Gan, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 41 (1982) 1017).

** The abbreviations used are: α_1-PI = α_1-proteinase inhibitor; HPSEC = high-performance size exclusion chromatography; BAEE = N-α-benzoyl-L-arginine ethyl ester; NBA = N-*tert.*-butyloxycarbonyl-L-alanine-*p*-nitrophenyl ester; SDS–PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; EIC = elastase inhibitory capacity; TIC = trypsin inhibitory capacity.

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nation times (18–24 h), whereas, HPSEC used nanogram (analytical) to milligram (preparative) quantities and requires less than 15 min for fractionation. Sodium dodecylsulfate polyacrylamide gel electrophoresis, although it also only requires microgram amounts of proteins, is both laborious and time consuming.

MATERIALS AND METHODS

Apparatus

The size exclusion chromatographic system employed consisted of a Beckman Model 110 A pump and an Altex Model 210 injection valve. Two different columns were tested separately; one was a Bio-Rad TSK Bio-Sil 250 column (300 × 7.5 mm, fractionation range, 5000–300,000), and the other was a Beckman Spherogel TSK 2000 SW column (300 × 7.5 mm, fractionation range, 1500–70,000). The proteins were detected by their absorbance at 230 nm with an Hitachi Model 100-10 variable wavelength spectrophotometer and their areas were determined with a Hewlett-Packard recording integrator. The chart speed was 0.5 cm/min. The attenuation setting is 3 in the experiments using the 2000 column, while that for the 250 column is indicated in the legends of the appropriate figures.

Buffers

The mobile phase was either 0.05 *M* or 0.1 *M* sodium phosphate buffer, pH 6.5, which had been made from deionized-redistilled water. The buffer solution was Millipore filtered (0.20 μm) and degassed under house vacuum. The proteins were eluted at a flow-rate of 1.0 ml/min. All other buffer solutions used were Millipore filtered and degassed under vacuum. Protein stock solutions were filtered with disposable Amicon Sterilets (0.20 μm).

Proteins

α_1-PI was purified from out-dated human plasma as described previously[1]. Porcine pancreatic elastase and bovine pancreatic trypsin were obtained from Sigma. Protein concentrations were determined by using their extinction coefficients at 280 nm ($A^{1\%}_{280}$) which for α_1-PI[2], trypisin[3], and elastase[4] were 5.3, 15.4 and 20.2, respectively.

Molecular weight–elution time (volume) curve

Each of the following standards of known molecular weight was dissolved separately in 0.1 *M* sodium phosphate buffer, pH 6.5 and 20 μl was applied to the Spherogel TSK 2000 SW column. Digitonin, 1229; insulin, 6000; cytochrome *c*, 12,400; trypsin, 24,000; ovalbumin, 45,000; and hexokinase, 96,000, were from Sigma. Glucose oxidase, 154,000, was from Nutritional Biochemicals.

Spectrophotometric assay for trypsin and elastase activity

Trypsin was determined by the rate of hydrolysis of BAEE at 25°C according to the method of Schwert and Takenaka[5]. A 10-μg amount of trypsin in 10 μl of 0.0025 *M* HCl, pH 3.0, was brought to 200 μl with 0.1 *M* Tris-Cl buffer, pH 8.0. A 3-ml volume of the same Tris buffer containing BAEE (1 m*M*) was added and the increase of absorbance at 253 nm was measured.

Elastase activity was determined by the method of Visser and Blout[6] except for substitution of 0.0025 *M* HCl, pH 3.0, for 0.05 *M* sodium acetate buffer, pH 4.0. An amount of 10 μg of elastase (stock solution 1 mg/ml in 0.0025 *M* HCl, pH 3.0) was added to 3.0 ml of 0.01 *M* NBA in 0.05 *M* sodium phosphate buffer, pH 6.5. The increase in absorbance at 347.5 nm was measured.

Trypsin and elastase spectrophotometric inhibitory assays

The standard assay consists of incubating a 1:1 molar ratio of α_1-PI (25 μg) to either trypsin (10 μg) or elastase (10 μg) for 15 min at room temperature in a total of 200 μl of 0.1 *M* Tris–HCl buffer, pH 8.0. The residual enzymatic activity was determined as described above. In other experiments the incubation time was varied from 1 to 30 min.

High-performance size exclusion chromatographic inhibitory assays

Known amounts of α_1-PI, elastase, trypsin and their respective incubation mixtures were applied to the column by syringe suction. Volumes of 400 μl of the protein solutions were passed through a 20-μl sample loop to insure complete loop loading and 20 μl containing 1/20 of the protein was injected. To assess complex formation, 1:1 molar mixtures of α_1-PI and elastase or trypsin were incubated in a total of 400 μl of 0.1 *M* Tris–HCl buffer, pH 8.0, at room temperature (25°C) for 15 min. As in the spectrophotometric assay, the incubation time was also varied from 1 to 30 min.

Semi-preparative separation of elastase from autocatalytic products

Known amounts of elastase in 0.0025 *M* hydrochloric acid, pH 3.0, were frozen, lyopholized and dissolved in 500 μl of 0.1 *M* sodium phosphate buffer, pH 6.5. A 500 μl sample loop was used to inject the sample into either the Bio-Sil TSK 250 column or Spherogel TSK 2000 SW column. Fractions of 500 μl were collected by hand and the absorbance of each fraction was measured at 230 nm.

RESULTS

Purification of elastase

When the commercial preparation of pancreatic elastase (1 μg) was subjected to HPSEC on the Spherogel TSK 2000 column, one major peak first emerged followed by several smaller peaks (Fig. 1a). The major peak accounted for about 76% of the total protein applied; the remaining 24% is represented by the smaller peaks. These smaller molecular weight peptides are apparently autocatalytic enzymatically inactive products. The major protein peak formed a 1:1 molar complex with α_1-PI, whereas the smaller peptides did not interact with the inhibitor. The present results are consistent with our earlier findings, whereby only 75% of elastase prepared commercially was enzymatically active as determined by a specific active site titrant, diethyl-*p*-nitrophenyl phosphate[7].

In order to prepare large amounts of highly active elastase, 5 mg of commercial sample was subjected to HPSEC on the TSK 2000 column. The elution pattern is shown in Fig. 1b, whereby at least 3 peaks emerged. The failure of the peaks to separate completely is apparently due to overloading. To eliminate or minimize con-

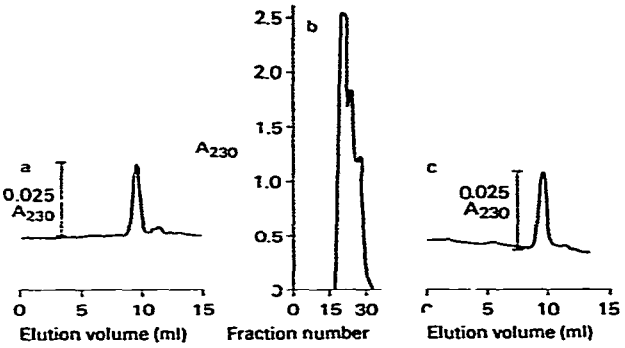

Fig. 1. Separation of elastase from autocatalytic products on the TSK 2000 column. (a) 1.0 μg of elastase from a commercial preparation; (b) 5.0 mg of elastase (commercial preparation), 500-μl fractions were collected, fractions 19 and 20 pooled, and the pH adjusted to 3.0 with 5 *M* hydrochloric acid; (c) 1.0 μg of elastase from pooled fractions 19 and 20. The retention time of the elastase peak was 8.91 min.

tamination with smaller and inactive peptides, only the first two fractions (Tube Nos. 19 and 20) were pooled for subsequent studies. Fig. 1c depicts the elution pattern of 1 μg of the pooled material. As can be seen, the first major peak now represents greater than 96% of the total protein applied. As shown below (Fig. 3), the purified elastase interacts with α_1-PI on a mole to mole basis.

A Bio-Sil TSK 250 column was also utilized to purify the enzyme as shown in Fig. 2. The elastase showed a broader peak whether using analytical (1 μg, Fig. 2a) or preparative (2 mg, Fig. 2b) amounts. When the enzyme was re-chromatographed on the same column, it still showed some degree of heterogeneity (Fig. 2c). These results clearly showed that the TSK 2000 column is superior to the TSK 250 column in separating elastase from its inactive autocatalytic products.

Complex formation between α_1-PI and elastase

Fig. 3 shows a typical elution profile on a TSK 2000 column of (a) α_1-PI (2.5 μg); (b) elastase (1 μg) and varying molar mixtures of inhibitor to enzyme, namely, (c) 0.25:1 (0.625 μg:1 μg); (d) 0.5:1 (1.25 μg:1 μg); (e) 0.75:1 (1.9 μg:1 μg); (f) 1:1 (2.5 μg:1 μg) and (g) 1.1:1 (2.8 μg:1 μg). Although the chromatographic procedure failed* to resolve or separate completely unreacted α_1-PI from its complex (Fig. 3g), definite increases in the areas of the inhibitor peak are seen after interacting with the enzyme. Moreover, there was a concomitant reduction of the corresponding elastase peaks (c, d, e, and f), which can be quantitatively correlated with inhibitory activity of α_1-PI (see Table I). The recorded retention times for α_1-PI–elastase complex, α_1-PI and elastase were 6.46, 6.64 and 8.91 min, respectively.

* The failure to effect a complete separation of unreacted α_1-PI from the complex despite a significant difference in molecular weight may be due to the fact that the complex behaves as a molecule which has a molecular weight which is less than would be predicted from the sum of the 2 molecular weights of the two proteins. Saklatava *et al.*[8] have shown that marked conformational changes do take place in one of both of the proteins upon complexation.

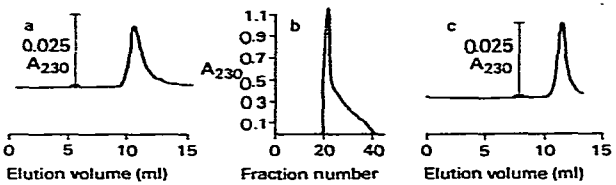

Fig. 2. Separation of elastase from autocatalytic products on the TSK 250 column. (a) 1.0 μg of elastase from a commercial preparation; (b) 2.0 mg of elastase (commercial preparation), 500-μl fractions were collected, fraction 21–33 pooled, and the pH adjusted to 3.0 with 5 *M* hydrochloric acid; (c) 1.0 μg of elastase from pooled fractions 21–23. The retention time of the elastase peak was 10.88 min.

For comparative purposes similar experiments were performed using the TSK 250 column. As can be seen in Fig. 4, the elastase peaks, as well as those of the autocatalytic products were not sharply separated (b and d) as those obtained with TSK 2000 column.

Elastase and trypsin inhibitory assays

Table I shows the use of HPSEC on the TSK 2000 column as a means of assaying the capacity of α_1-PI in inhibiting elastase as measured by either the reduction of the elastase peak or increase in the α_1-PI–elastase peak. In contrast to the standard spectrophotometric inhibitory assay which requires about 10 μg of elastase and 25 μg of α_1-PI, the present method needs only 1/10 as much as the above ma-

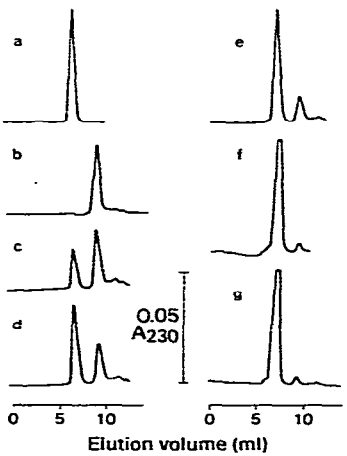

Fig. 3. Demonstration of complex formation between α_1-PI and elastase on the TSK 2000 column. Varying molar ratios of α_1-PI to elastase were incubated under standard conditions (15 min, pH 8.0 at 25°C) and then assayed by both the HPSEC and spectrophotometric methods. (a) 2.5 μg of α_1-PI; retention time, 6.64 min; (b) 1.0 μg of elastase; retention time, 8.91 min; (c) 0.25:1 molar mixture; 0.625 μg α_1-PI μg elastase; retention time of complex, 6.46 min; (d) 0.5:1 molar mixture; 1.25 μg α_1-PI, 1.0 μg elastase; (e) 0.75:1 molar mixture; 1.9 μg α_1-PI, 1.0 μg elastase; (f) 1:1, molar mixture; 2.5 μg α_1-PI, 1.0 μg elastase; (g) 1.1:1 molar mixture, 2.8 μg α_1-PI, 1.0 μg elastase. The percent decrease in the areas of the elastase peaks for c, d, e, f and g were 25.4, 40.3, 63.0, 89.8 and 90.1, respectively. The corresponding values for the spectrophotometric assay (% EIC) were 24, 44.4, 66.6, 93 and 100.

TABLE I

THE USE OF HPSEC (SPHEROGEL TSK 2000 SW) AS A MEANS OF ASSAYING THE INHIBITORY CAPACITY OF α_1-PI AND COMPARISON WITH THE STANDARD SPECTROPHOTOMETRIC ASSAY

Fifty μg of α_1-PI and 20 μg of elastase were incubated in 0.4 ml of 0.1 *M* Tris–HCl buffer, pH 8.0, for as long as 30 min. At various time intervals (1, 5, 10 and 30 min) 20 μl aliquots containing 3.5 μg of complex (2.5 μg of α_1-PI and 1.0 μg elastase) were injected into the column. For the spectrophotometric inhibitory assay, five different samples of 25 μg α_1-PI and 10 μg elastase were incubated in 0.2 ml of 0.1 *M* Tris–HCl buffer, pH 8.0, 25°C. At the end of each incubation period (1, 5, 10, 15 and 30 min), the elastase inhibitory capacity was determined as detailed in the Methods section.

Incubation time (min)	*Area of α_1-PI or α_1-PI elastase complex (arbitrary units)*	*Increase in the area of the complex (%)*		*Area of elastase peak*	*Decrease in the area of elastase (%)*		*Spectrophotometric assay: EIC (%)*
		(A) Observed	*(B) Normalized*		*(A) Observed*	*(B) Normalized*	
0	1,994,000*	–	–	1,523,900*			
1	3,246,424	62.8	91.5	278,012	81.7	89.1	93
5	3,258,503	63.4	92.4	271,911	82.1	90.3	93
10	3,285,811	64.7	94.3	245,724	83.8	92.1	95
15	3,363,492	68.6	100.0	150,949	90.1	100.0	100.0
30	3,351,914	68.1	99.2	160,010	89.5	99.3	100.0

* The areas of 2.5 μg of α_1-PI and 1.0 μg of elastase before formation of the complex.

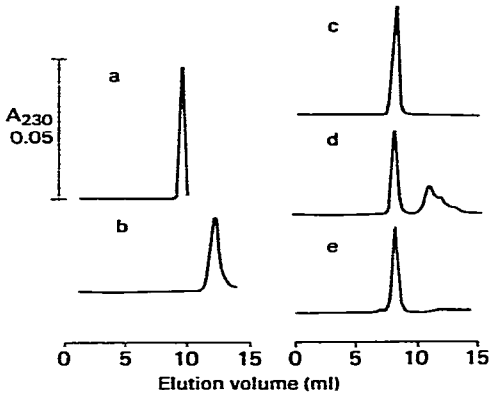

Fig 4. Demonstration of complex formation between α_1-PI and elastase on the TSK 250 column. Varying molar ratios of α_1-PI to elastase were incubated under standard conditions (15 min, pH 8.0, 25°C) and then assayed by the HPSEC method. (a) 2.5 μg α_1-PI; retention time, 8.10 min; (b) 1.0 μg elastase; retention time, 10.88 min; (c) 1:1 molar mixture; 2.5 μg α_1-PI, 1.0 μg elastase, retention time of complex, 8.00 min; (d) 0.5:1 molar mixture; 2.5 μg α_1-PI, 2.0 μg elastase; (e) 2:1 molar mixture; 5.0 μg α_1-PI, 1.0 μg elastase. The attenuation settings for 1, b, c, d, and e were 3, 3, 4, 4 and 5, respectively (sensitivity: 3 > 4 > 5). The percent increase in the areas of the complex peak for c, d, and e were 63.7, 46.7 and 44.3, respectively.

terials, and thus, is more economical. The amount of inactive enzyme* can be directly quantitated since they remain unreacted and elute at the same position even after the addition of relative excess of the inhibitor. Very often, and especially in the case of the elastase assay, the substrate NBA is relatively unstable under the assay conditions and therefore difficulty in obtaining a stable base line reading arises. This problem is obviously obviated when HPSEC is used, since no enzyme substrate is necessary.

Applicability of this procedure for trypsin inhibitory assay is also outlined in Table II and similar results were obtained as for the elastase inhibitory assay.

Molecular weight determination on Spherogel TSK 2000 SW column

The molecular weight estimates of trypsin, elastase, α_1-PI and the α_1-PI–elastase complex are shown in Fig. 5, as obtained from a plot of the retention time (elution volume) and log molecular weight of the reference proteins. The observed molecular weights were as follows: trypsin, 17,000; elastase, 15,000; α_1-PI, 56,000; and α_1-PI–elastase complex, 75,000. The values for α_1-PI and the complex are in agreement with those reported in the literature[9,10] using other methods. On the other hand, elastase and trypsin are both basic proteins and may tend to interact with the column material[11] which account for their longer retention time, reflecting a smaller molecular weight.

DISCUSSION

The results obtained with both the TSK 250 and 2000 column used in the HPSEC assays for determination of the proteinase inhibitory capacity of α_1-PI agreed well with the standard spectrophotometric assays. Moreover, with the TSK

* Refers to inactive enzyme molecules having the same molecular weight as the active species but apparently do not form complex with the inhibitor. Results from several experiments in this laboratory showed that only 5 to 10% of the HPSEC purified elastase is inactive.

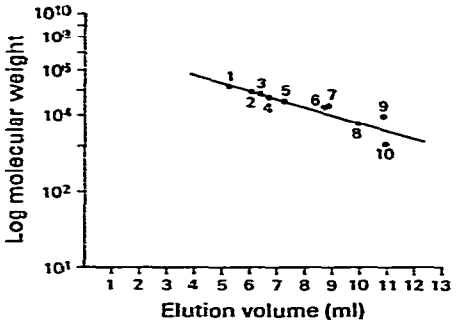

Fig. 5. Molecular weight determination on the TSK 2000 column of α_1-PI and its elastase complex. The following standards of known molecular weight were used: (1) glucose oxidase (154,000 daltons). (2) hexokinase (96,000 daltons). (3) α_1-PI–elastase complex (78,000 daltons), (4) α_1-PI (54,000 daltons). (5) ovalbumin (45,000 daltons), (6) trypsin (24,000 daltons), (7) elastase (24,000 daltons), (8) insulin (6,000 daltons), (9) cytochrome *c* (12,400 daltons), and (10) digitonin (1229 daltons). 3 and 4 were estimated from the plot of log molecular weight and retention time as shown in this figure.

2000 column, the decrease in the area of the elastase peak upon complex formation agreed quantitatively with the residual enzymatic activity determined spectrophotometrically. This allows determination of the proteinase inhibitory capacity of α_1-PI by the HPSEC method according to the same principle as the spectrophotometric assay; *i.e.*, measurement of the residual enzyme (residual activity) after complex formation. Furthermore, the HPSEC (TSK 2000 column) assays allows one to observe the in-

TABLE II

THE USE OF HPSEC (BIO-SIL TSK 250 COLUMN) AS A MEANS OF ASSAYING THE INHIBITORY ACTIVITY OF α_1-PI AND COMPARISON WITH THE STANDARD SPECTROPHOTOMETRIC ASSAY

Eighteen μg of α_1-PI and 8.2 μg of trypsin were incubated in 0.4 ml of 0.1 *M* Tris–acetate buffer, pH 8.0, 25°C, for as long as 30 min. At various time intervals (1, 5, 10, 15 and 30 min) 20 μl aliquots containing 1.3 μg of the complex (0.89 μg of α_1-PI and 0.41 μg of trypsin) were injected into the column. For the spectrophotometric inhibitory assay, five different samples each containing 9 μg α_1-PI and 4.1 μg trypsin were incubated in 0.2 ml of 0.1 *M* Tris–acetate buffer, pH 8.0, 25°C. At the end of each incubation period (1, 5, 10, 15 and 30 min), the trypsin inhibitory capacity was determined as detailed in the Materials and methods section.

Incubation time (min)	*Area of α_1-PI or α_1-PI trypsin complex (arbitrary units)*	*Increase in the areas of the complex (%)*		*Spectrophotometric assay: TIC (%)*	
		*(A) Observed***	*(B) Normalized*	*(A) Observed*	*(B) Normalized*
0	675,546*				
1	1,067,500	58.7	88.5	78.5	80.2
5	1,099,216	62.7	95.7	92.5	94.5
10	1,102,500	63.4	96.7	95.0	97.1
15	1,110,600	64.4	98.3	96.2	98.3
30	1,118,028	65.5	100	97.8	100

* This number represents the area of 0.89 μg of α_1-PI by itself.

** The values were obtained as follows: *e.g.*, at 1 min incubation,

$$\frac{(1{,}067{,}500 - 675{,}546)}{675{,}546} \times 100 = 58.7\%.$$

crease in the area of the complex peak, as well as a decrease in the proteinase peak. In the elastase spectrophotometric inhibitory assay, the %EIC is determined by subtracting the residual elastase activity of the α_1-PI: elastase mixture from the elastase control and then dividing by the same control. If the elastase preparation has some inactive elastase present, the spectrophotometric assay measures only the active elastase. However, in the HPSEC assay, the presence of inactive elastase can be detected using a relative excess of α_1-PI. This explains why in Fig. 3g the observed decrease in the area of the elastase peak is 90.1 % while the % EIC determined spectrophotometrically is 100%.

The HPSEC assay can also be used to study the interaction between α_1-PI and proteinase. The results of both the TSK 250 and 2000 columns clearly show that a 1:1 molar complex between α_1-PI and elastase is formed. Secondly, if one desires to isolate the complex for further study, this can be done in under 8 minutes and quite easily. Conventional column gel filtration can also be used to isolate the complex but larger amounts of protein and longer fractionation times are required. In the case of SDS–PAGE, even when preparative gels are used, extraction of protein from the gels is both laborious and inefficient when compared to HPSEC.

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