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PROTEINASE INHIBITORY ASSAYS OF SERUM USING HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY

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SUMMARY

A size exclusion column (Spherogel TSK-2000 SW) was utilized in a high-performance size exclusion chromatographic assay to determine the proteinase inhibitory capacity of human sera. Values from assays using this technique agreed well with the standard spectrophotometric inhibitory assays. Nanogram to milligram amounts of protein, namely, α_1-proteinase inhibitor, elastase, trypsin, chymotrypsin and their corresponding complexes with the inhibitor, were fractionated in less than 15 min. The nitrated or oxidized α_1-proteinase inhibitor was shown to retain its ability to form stable complexes with trypsin or chymotrypsin; however, they lost the inhibitory activity against elastase and instead they behaved as common protein substrates for this enzyme. The present chromatographic procedure was unable to detect any peptide released when the native inhibitor and any of the proteinases reacted to form a complex. Moreover, dissociation of the α_1-proteinase inhibitor—elastase complex in an alkaline pH did not result in the formation or release of any peptide.

INTRODUCTION

α_1-Proteinase inhibitor (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 definitely 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 or the inhibitor-containing sample such as the serum itself, are spectrophotometric procedures which measure residual proteinase activity after incubation of the enzyme with the inhibitor or the serum. This is accomplished by measuring the change of absorbance at a particular wavelength which occurs upon enzymatic hydrolysis of an appropriate substrate. Presented in this paper is a versatile and rapid method of high-performance size exclusion chromatography

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(HPSEC) which can be employed without the use of substrates, not only to assay for proteinase inhibitory capacity of pure α_1-PI, but also that of whole or unfractionated serum. Since the assay was able to differentiate the proteinase inhibitory activities of sera from (a) pregnant women, (b) women on oral contraceptives and (c) diluted sera simulating severe α_1-PI deficiency from that of (d) normal male sera, it is anticipated that this method will have a wide range of clinical and diagnostic applications. Moreover, investigations involving inhibitor-proteinase interaction, such as complex formation and dissociation, and digestion of oxidized or nitrated α_1-PI by elastase are readily demonstrable with this technique.

MATERIALS AND METHODS

Apparatus

This size exclusion chromatographic system employed consisted of a Beckman Spherogel TSK-2000 SW column (300 mm × 7.5 mm, fractionation range 1500—70,000), a Beckman Model 110 A pump and an Altex Model 210 injection valve. The proteinases were detected by their absorbance at 230 nm (unless otherwise indicated) with a 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 and the attenuation setting was at 3.

Buffers

The mobile phase was 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 Sterilet (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 and α-chymotrypsin were obtained from Sigma (St. Louis, MO, U.S.A.). Protein concentrations were determined by using their extinction coefficients at 280 nm ($A_{280}^{1\%}$) which for α_1-PI [2], trypsin [3], chymotrypsin [4] and elastase [5] were 5.3, 15.4, 20.7 and 20.2, respectively.

High-performance size exclusion chromatographic inhibitory assays

Known amounts of plasma, serum, α_1-PI, elastase, trypsin, chymotrypsin and their respective incubation mixtures were applied to the column by syringe suction. Using 0.05 *M* Tris—HCl buffer, pH 8.0, 400 μl of the protein solutions were prepared and passed through a 20-μl sample loop to ensure complete loop loading, and 20 μl containing 1/20 of the protein were injected on to the column. The proteins were monitored by their absorbance at 230 nm. To assess complex formation, a 1:1 molar mixture of α_1-PI and elastase or trypsin or chymotrypsin were incubated in a total volume 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 [6], the incubation time was varied in some instances from 1 to 30 min in order to ensure maximum complex formation.

Serum was obtained from a male, a pregnant woman, and a woman receiving oral contraceptives. Each sample was filtered with an Amicon Sterilet (0.2 μm) to remove any particulate matter. Elastase (20 μg) was reacted with various volumes of serum (10—50 μl) in a total of 400 μl of 0.05 *M* Tris—HCl buffer, pH 8.0, for 15 min at 25°C. A 20-μl aliquot was subjected to HPSEC. The proteins were monitored by their absorbance at 280 nm rather than 230 nm. The detector range setting was 0.2 absorbance units and the recorder attenuation setting was 1.0. Corresponding incubation mixtures were assayed by the spectrophotometric procedure described previously [6].

Additional procedures

The spectrophotometric assays for trypsin, chymotrypsin or elastase, the proteinase inhibitory assay for α_1-PI, the nitration with tetranitromethane (TNM) or oxidation with the N-chlorosuccinimide (NCS) of α_1-PI, were described in previous publications [7]. Chymotrypsin in 0.05 *M* Tris—HCl buffer, pH 8.0, was treated with 100-fold molar excess of the inhibitor phenylmethanesulfonyl fluoride for 10 min at 25°C [8], and excess reagent was removed by dialysis against the same buffer in the cold room.

RESULTS

Comparison of the HPSEC and spectrophotometric inhibitory assays of human sera

The HPSEC proteinase inhibitory assay of serum (Fig. 1b) was possible because of the fact that no absorbing (280 nm) material was observed in the area where elastase (Fig. 1c) was eluted. On the other hand, when plasma (Fig. 1a) was used, a major protein peak appeared at the position where the enzyme also elutes. For this reason only sera was utilized for the assay. The inhibitory assay is based simply on the fact that the elastase peak disappears quantitatively when incubated with varying amounts of serum prior to chromatography. As can be seen in Fig. 1d and e, 59 and 100% of elastase (2 μg) disappeared and presumably formed a complex with α_1-PI when incubated for 15 min with 0.5 μl and 1.5 μl of normal male serum, respectively. From Fig. 2, the calculated amount of different serum samples required to inhibit completely 1 mg of elastase were for (a) a normal male, 2.5 ml; (b) a pregnant woman, 0.50 ml; (c) a woman receiving oral contraceptives, 0.55 ml; and (d) normal male serum diluted to 10% with 0.05 *M* Tris—HCl buffer, pH 8.0, 25 ml.

It is evident that the HPSEC assay is superior to the spectrophotometric one since it can achieve 100% inhibition (Fig. 2) while the latter only 70—85%. Moreover, as more serum was incubated with the enzyme, the spectrophotometric assay becomes inefficient as indicated by an apparent decreasing inhibitory activity. This is not the case in the HPSEC assay.

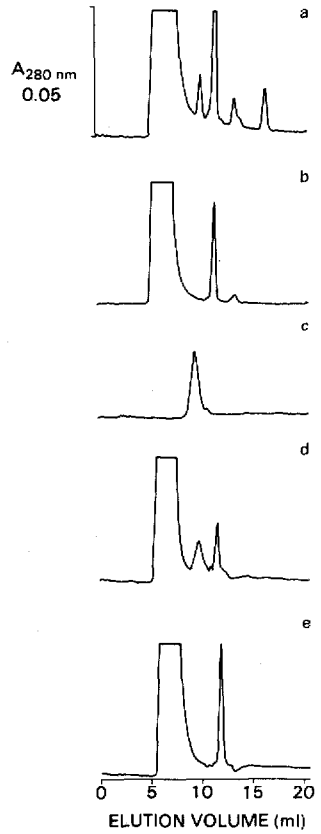

Fig. 1. HPSEC of human plasma, serum, porcine elastase and mixtures of serum and elastase. (a) 2.5 μl of plasma; (b) 1.0 μl serum; (c) 2 μg elastase, retention time 9.43 min; (d) 0.5 μl serum incubated with 2 μg of elastase for 15 min at 25°C; (e) 1.5 μl serum incubated with 2 μg of elastase. The samples were prepared for injection into the column as described in the Methods section.

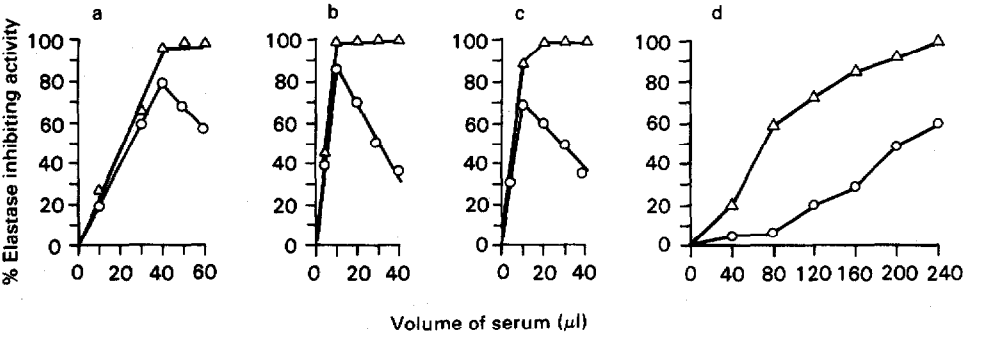

Fig. 2. Comparison of the spectrophotometric and HPSEC elastase inhibitory assays of human sera. Elastase (20 μg) was titrated with increasing volumes of (a) male serum; (b) serum from a pregnant woman, and (c) serum from a woman receiving oral contraceptives, and (d) male serum diluted to 10% with 0.1 *M* Tris—HCl buffer, pH 8.0. The elastase spectrophotometric (○——○) and HPSEC (△——△) inhibitory assays were performed as described in the Methods section. In (a) 2.5, (b) 0.50, (c) 0.55 and (d) 25 ml of serum inhibited 1 mg of elastase, respectively as calculated from the HPSEC assays.

The inability of inactivated chymotrypsin to form a complex with α_1-PI

In order to study further the application of HPSEC in the interaction of α_1-PI with proteinases, the subsequent experiments described below were performed. The chymotrypsin-inhibitory capacity of α_1-PI (Fig. 3a) against native chymotrypsin (Fig. 3b) was 98.9% (Fig. 3d). When complex formation occurred, the retention time of 6.53 min for α_1-PI (Fig. 3a) was shortened to 6.32 min (Fig. 3d). However, inactive chymotrypsin (Fig. 3c) failed to form a complex with α_1-PI as evidenced by the fact that the area of chymotrypsin did not decrease or disappear (Fig. 3e). Furthermore, both the area and retention time of α_1-PI were altered (Fig. 3e). It should be noted that when chymotrypsin was inactivated by phenylmethanesulfonyl fluoride, a 39% decrease in its area occurred (Fig. 3c).

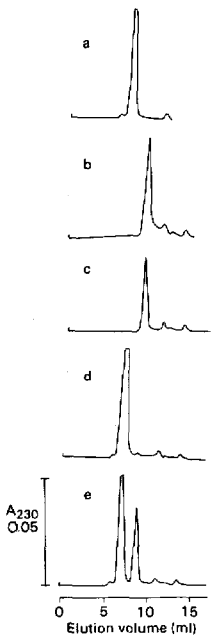

Fig. 3. The inability of inactivated chymotrypsin to form a complex with α_1-PI. Active site inactivated and native chymotrypsin were reacted with α_1-PI as described in the Methods section and 20 μl were applied to HPSEC. (a) 2.8 μg of α_1-PI control, retention time 6.53 min; (b) 1.0 μg of chymotrypsin control, retention time 8.31 min; (c) 1.0 μg of modified chymotrypsin, retention time 8.24 min; (d) 1.15:1 molar mixture of α_1-PI and native chymotrypsin, retention time of complex 6.32 min; (e) 1.15:1 molar mixture of α_1-PI and modified chymotrypsin; 2.8 μg of α_1-PI and 1.0 μg of modified chymotrypsin; retention time of α_1-PI 6.53 min; retention time of modified chymotrypsin 8.24 min. The percent elastase-inhibitory activity of (d) was 98.9%, while that of (e) was 0%.

Dissociation of the α_1-PI—elastase complex by exposure at pH 12

A 1:1 molar ratio of α_1-PI (Fig. 4a) and elastase (Fig. 4b) was incubated and HPSEC analysis showed that 95% of the elastase was inhibited by α_1-PI (Fig. 4c). The retention time of the complex was 6.38 min. After 15 min at pH 12, 65.6% of the elastase was recovered (Fig. 4d) and the retention time of the complex (6.38 min) changed to 6.49 min, indicating the formation

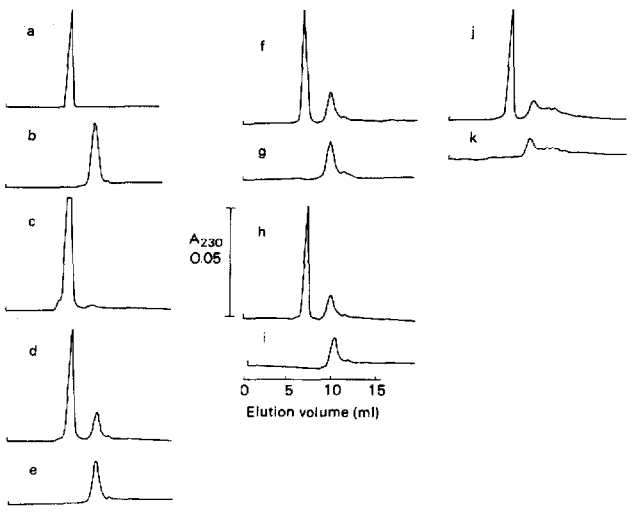

Fig. 4. Dissociation of the α_1-PI—elastase complex by exposure at pH 12. A 1:1 molar mixture of α_1-PI and elastase was incubated under standard conditions (25°C, pH 8.0, 15 min), at which point the pH was adjusted to 12, as described in the Methods section. (a) 2.5 μg of α_1-PI control; retention time 6.55 min; (b) 1.0 μg of elastase control, retention time 9.51 min; (c) incubation mixture after 15 min, and before adjustment to pH 12: 3.5 μg of complex, retention time 6.38 min; (d—k) incubation mixtures (3.5 μg total protein) and elastase controls (1.0 μg) after treatment at pH 12 for various time periods; (d) pH 12 for 15 min, retention time of complex and dissociated α_1-PI 6.49 min; retention time of elastase 9.44 min; (e) elastase control, pH 12 for 15 min, retention time 9.44 min; (f) pH 12 for 45 min, retention time of complex and dissociated α_1-PI 6.50 min; retention time of elastase 9.37 min; (g) elastase control, pH 12 for 45 min, retention time 9.38 min; (h) pH 12 for 133 min, retention time of complex and dissociated α_1-PI 6.51 min; retention time of elastase 9.29 min; (i) elastase control, pH 12 for 133 min, retention time 9.29 min; (j) pH 12 for 22 h, retention time of complex and dissociated α_1-PI 6.54 min; retention time of elastase 9.23 min; (k) elastase control, pH 12 for 22 h, retention time 9.12 min. The percent elastase liberated from the complex upon treatment at pH 12 was 65.6%, 78.1%, 81.5%, and 98% for (d), (f), (h), and (j), respectively.

of free α_1-PI (Fig. 4d). After 45 min, 133 min, and 22 h a recovery of 78.1%, 81.5% and 98% (Fig. 4f, h and j, respectively) of the elastase originally bound to α_1-PI (Fig. 4c) was observed. After 22 h, the retention time of the complex (6.38 min) had returned to that of α_1-PI (6.54 min, Fig. 4a and j) showing complete dissociation. Furthermore, only after 22 h were any low molecular weight peptides observed for either the dissociated mixture (Fig. 4j) or the elastase control (Fig. 4k), indicating that elastase was stable at pH 12 for at least 133 min. It is very possible that the low molecular weight peptides formed at 22 h incubation are autocatalytic products of elastase and not from α_1-PI. α_1-PI alone remained intact for 22 h under the same incubation condition (Fig. 4j).

HPSEC preparative elution profiles of oxidized and nitrated α_1-PI

Fig. 5 represents a typical preparative elution profile of (a) 10 mg of NCS-oxidized α_1-PI and (b) 10 mg of TNM-nitrated α_1-PI. In the case of the oxidized α_1-PI, the peak (Fig. 5a) as measured at 280 nm, was symmetrical, indicating

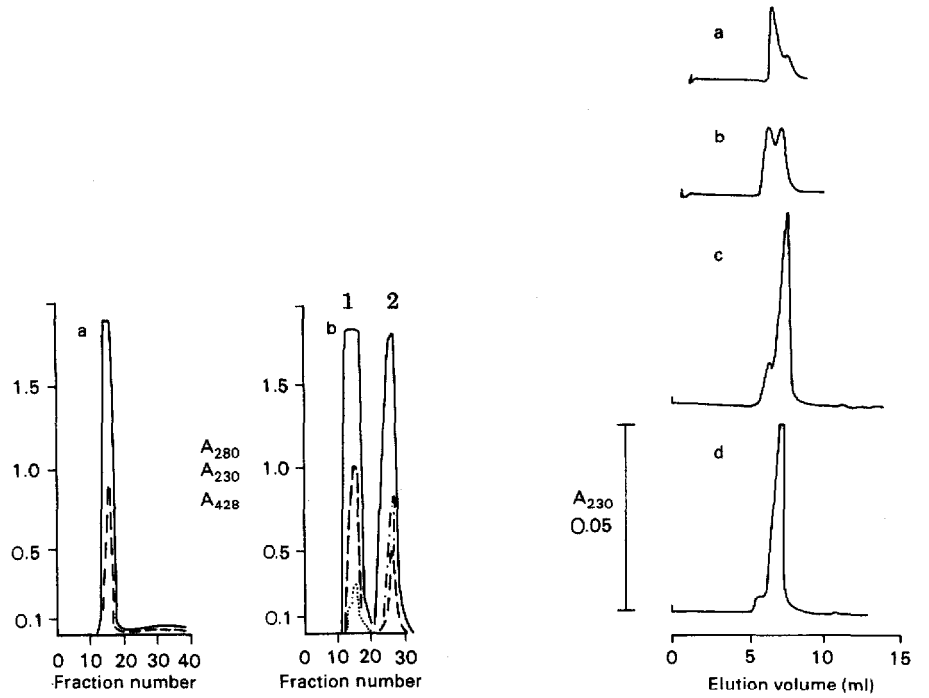

Fig. 5. HPSEC elution profiles of oxidized or nitrated α_1-PI. (a) α_1-PI (10 mg, 18.5 nmol) was oxidized with NCS as described in the Methods section. At the end of the reaction, the mixture was lyophilized, redissolved in 500 μl of distilled water, and subsequently subjected to HPSEC. The protein was eluted and fractions were collected. After the addition of 2.0 ml of 0.1 *M* sodium phosphate buffer, pH 6.5, the protein was detected by its absorbance at 230 nm (——) and 280 nm (— — —). Fractions 15—18 were pooled and the pH was adjusted to 8.0 with 4 *N* sodium hydroxide. (b) α_1-PI (10 mg, 18.5 nmol) was nitrated as described in the Methods section. The mixture was prepared, subjected to HPSEC, and processed as described in (a). In addition to 230 nm (——) and 280 nm (— — —), the nitrated inhibitor (b), peak 1 was detected by the absorbance of its nitrotyrosine residues at 428 nm (. . . .). Fractions 15—18 were pooled and the pH was adjusted to 8.0 with 4 *N* sodium hydroxide. Peak 2 represented the other product of the nitration reaction, namely, nitroformate.

Fig. 6. Polymerization of α_1-PI upon nitration. Samples of 60 μl were taken from fractions 13—16 isolated from the HPSEC fractionation of nitro-α_1-PI (Fig. 5b). The samples in μg amounts were subjected to HPSEC. (a) Fraction No. 13, retention time 5.31 min; (b) fraction No. 14, retention time 5.43 and 6.31 min; (c) fraction No. 15, retention time 5.58 and 6.44 min; (d) fraction No. 16, retention time 6.48 min.

that no polymerization occurred. Although polymerization of the nitrated α_1-PI was not clearly evident (Fig. 4b, first peak), the absorbance at either 280 nm or 428 nm showed a shoulder at fraction No. 14. The second peak (fractions 21—30), absorbing at 428 nm, represented the other product of reaction, namely nitroformate. The inability of the TSK-2000 column to separate the polymer from the monomer was due to overloading. When aliquots were removed from fractions 13—16 and microgram amounts were chromatographed, polymerization was evident (Fig. 6). Fraction No. 13 contained mostly polymer (Fig. 6a), while fractions 14, 15 and 16 (Figs. 6b, c, d) con-

tained increasing amounts of the nitrated monomer. The area of the polymer in Fig. 6a was approximately 15% of the area of the nitrated monomer (Fig. 6d), which agrees well with the values obtained after column chromatography on Sephadex G-100. To minimize the amount of polymer, fractions 16—18 were pooled for subsequent studies (Fig. 7).

The same HPSEC studies were performed with the NCS-oxidized α_1-PI. The results (not shown) revealed that only the monomeric form of the material existed.

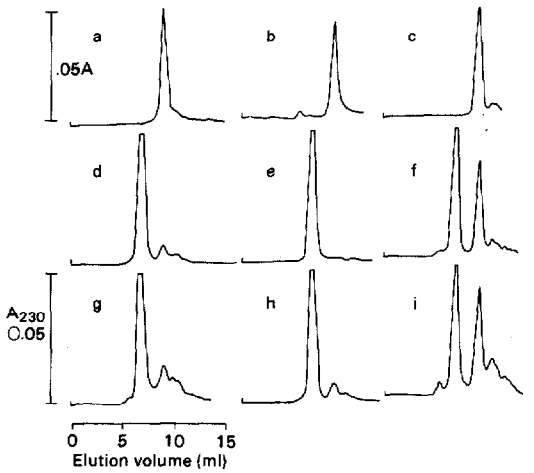

Fig. 7. Loss of complex formation between elastase and nitrated or oxidized α_1-PI. Samples of nitrated and oxidized α_1-PI (Fig. 5) were tested for their ability to form a complex with trypsin, chymotrypsin, and elastase. The HPSEC inhibitory assays were performed as described in the Methods section. (a—c) Amounts of 1 μg of trypsin, chymotrypsin and elastase, respectively, retention times (a) 8.59 min, (b) 8.37 min, (c) 8.80 min; (d, e) complex of oxidized α_1-PI with trypsin and chymotrypsin, respectively; 3.8 μg total protein, retention times (d) 6.39 min, (e) 6.35 min; (f) incubation mixture of oxidized α_1-PI with elastase, 2.8 μg of oxidized α_1-PI and 1.0 μg of elastase; retention time of oxidized α_1-PI, 6.55 min; retention time of elastase, 8.82 min; (g, h) complexes of nitrated α_1-PI monomer with trypsin and chymotrypsin, respectively, 3.8 μg total protein, retention time (g) 6.39 min, (h) 6.36 min; (i) incubation mixture of nitrated α_1-PI monomer with elastase; 2.8 μg of nitrated α_1-PI and 1.0 μg of elastase; retention time of nitrated α_1-PI 6.53 min, retention time of elastase 8.80 min. The complex formation of oxidized α_1-PI with trypsin (d), chymotrypsin (e), and elastase (f), was 85%, 98%, and 11%, respectively. The complex formation of nitrated α_1-PI with trypsin (g), chymotrypsin (h), and elastase (i) was 80%, 85%, and 0%, respectively.

The loss of complex formation between elastase and nitrated or oxidized α_1-PI

When oxidized (Fig. 7d, e, f) and nitrated α_1-PI (Fig. 7g, h, i) were reacted separately with trypsin (Fig. 7a), chymotrypsin (Fig. 7b) and elastase (Fig. 7c), the percent complex formation of the modified derivatives with the enzymes agreed well with the values previously obtained with the corresponding spectrophotometric inhibitory assays. The complex formation of oxidized α_1-PI with trypsin (Fig. 7d), chymotrypsin (Fig. 7e), and elastase (Fig. 7f) was 85%, 98%, and 11%, respectively. The complex formation of nitrated α_1-PI with trypsin (Fig. 7g), chymotrypsin (Fig. 7h), and elastase (Fig. 7i)

was 80%, 85% and 0%, respectively. Not only did both derivatives fail to inhibit elastase but they also underwent proteolysis with elastase as demonstrated by the appearance of new peptide peaks (Fig. 7f and i).

Degradation (proteolysis) of oxidized and nitrated α_1-PI by elastase

Fig. 8 represents the elution profile of mg amounts of oxidized or nitrated α_1-PI (6.8 mg) after 1.0 min of incubation with elastase (2.7 mg). When compared to unreacted controls (Fig. 8a and b, nitrated and oxidized α_1-PI, respectively), it is immediately apparent that the modified derivatives underwent proteolysis by elastase. Furthermore, in the case of the nitrated α_1-PI (Fig. 8c), the appearance of peptides containing 3-nitrotyrosine residues was observed. These peptides are now being purified for sequence determination.

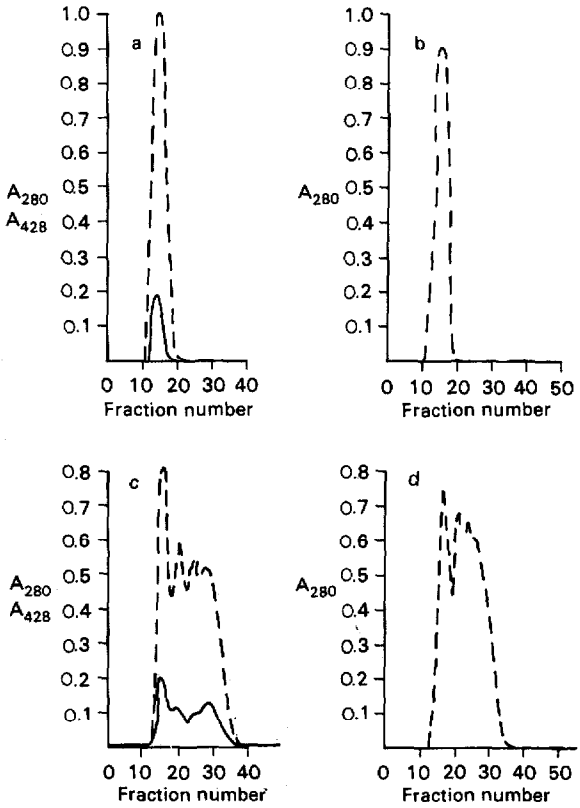

Fig. 8. Digestion of nitrated or oxidized α_1-PI by eleastase. Aliquots of 125 nmol (6.8 mg) of (c) nitrated monomer and (d) oxidized α_1-PI were reacted separately with a 1:1 molar ratio of elastase (2.7 mg) for 1.0 min at 25°C in a total of 6.2 ml of 0.05 *M* Tris—HCl buffer, pH 8.0. After 1.0 min, a 100-fold molar excess of phenylmethanesulfonyl fluoride was added to each and the mixtures were frozen and lyophilized. The samples were redissolved in 500 µl of distilled water and subjected to HPSEC. Fractions of 500 µl were collected and 100 µl of 0.1 *M* sodium phosphate buffer were added to each fraction. (a) and (b) represent undigested (no elastase added) nitrated and oxidized α_1-PI, respectively. The absorbance of each fraction at 280 nm (— — —) was measured, as well as the absorbance at 428 nm (——) for the nitrated α_1-PI—elastase mixture.

DISCUSSION

The results presented in this manuscript clearly demonstrate the usefulness of HPSEC on Spherogel as a means of assaying proteinase inhibitory activity of whole serum, as well as of purified α_1-PI as reported previously [6]. The assay is based on the quantitative disappearance of the elastase, trypsin or chymotrypsin peak when each of the enzymes was incubated with increasing amounts of serum or pure α_1-PI. Of serum or plasma, only the former can be used because the latter contains a highly absorbing peak that elutes at the same position as the enzymes. Adding increasing amounts of serum eventually interferes with the spectrophotometric inhibitory assay presumably due to the increased absorbance of serum at the wavelength employed to measure the hydrolytic products of the substrates; this is not the case for the HPSEC assay since no substrate is necessary. The HPSEC assay can be employed not only for normal male serum, but also in other conditions, whereby α_1-PI concentration is increased, such as in pregnancy and women receiving oral contraceptives [9], as well as, perhaps, in a genetic deficient state [10] as simulated by the normal serum diluted to 10%.

In contrast to the standard spectrophotometric inhibitory assay which requires about 10 μg of enzyme and 25 μg of pure α_1-PI or 25 μl of undiluted serum the present method may require only 1/10 of the above materials and, therefore, is more economical. Very often and especially in the case of the elastase spectrophotometric assay, the substrate N-*tert.*-butyloxycarbonyl-L-alanine-*p*-nitrophenylester (NBA) is relatively unstable under the assay conditions and, therefore, difficult to obtain stable baseline readings. This problem is clearly obviated when HPSEC is used, since, as already mentioned, no enzyme substrate is needed.

The versatile and rapid technique of HPSEC on the Spherogel column can be employed also, as shown in the present communication, in studies dealing with the capacity of the native α_1-PI to form complexes with native proteinases, the inability of the nitrated or oxidized α_1-PI to inhibit or form a complex with elastase, although retaining the capacity to inactivate trypsin or chymotrypsin as evidenced by complex formation with these enzymes, the loss of the capacity of the inactivated proteinases to form a complex with α_1-PI, and to detect the presence or absence of aggregation when α_1-PI is chemically modified. Thus, aggregation was shown during nitration with TNM but not oxidation with NCS.

Morii et al. [8] had observed the release of a peptide from human α_1-PI with an approximate molecular weight of 3600, when the native inhibitor formed a complex (0.05 *M* Tris—HCl buffer, pH 8.0, 25°C, 5 min) with native bovine chymotrypsin. The peptide was isolated by heating the reaction solution at 100°C for 2 min in the presence of 1% sodium dodecyl sulfate and followed by polyacrylamide gel electrophoresis. Peptides of the same molecular weight were also formed during the interaction of α_1-PI and bovine trypsin or porcine elastase [8]. Carrell et al. [11] incubated a 1:1 molar ratio of human α_1-PI and bovine trypsin in 0.025 *M* Tris—HCl, pH 7.3, at 25°C for 30 sec, and then formic acid and 95% ethanol were added to precipitate the protein. The ethanol supernatant was dried in vacuo and subjected

to two-dimensional high-voltage and conventional paper electrophoresis, whereby small peptides were observed in the chromatogram. After amino acid sequence determination, the peptides identified were to have arisen from the 37 residues in the C-terminal region of the native α_1-PI.

The above experiments were carefully repeated with the exception that HPSEC was utilized for the detection of the peptides released instead of polyacrylamide gel electrophoresis or high-voltage paper electrophoresis. Unfortunately, we were unable to detect by this method any peptide(s) released even after repeated attempts. In these experiments, the chromatographic time was extended for as long as 60 min. Standard amino acids such as lysine, glutamic acid or leucine were each eluted in 12 min, while bovine insulin (molecular weight 6000) in 10 min. In some instances, in order to decrease the possibility that the sensitivity of the technique was inadequate, the amount of the reactants, i.e., α_1-PI and enzymes, injected to the column was increased five times the amount normally detectable by this method. However, as with the other experiments, no peptide peak was observed. Moreover, dissociation of the α_1-PI—elastase complex at alkaline pH did not show any release of small peptide either. We have no explanation at this time for our negative results, although we believe that if the peptide(s) were in fact released during the interaction of α_1-PI and a proteinase, HPSEC as presently used should have been able to detect them.

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