Removal of Adsorbed Elastase from Partially Digested Elastin

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Bovine ligamentum nuchae elastin that had been partially digested by porcine pancreatic elastase and then thoroughly washed with 0.2M-NaCl/0.05M-sodium borate solution continued to release peptide fragments in solution. Complete removal of elastase from the elastin surface required extraction with dilute alkali, a procedure that does not irreversibly inactivate the enzyme or hydrolyse elastin.

During the course of preparing specimens of partially elastase-digested elastin for comparisons with unaltered elastin with respect to surface composition, protein adsorption and thrombogenicity, we discovered that partially digested elastin would continue releasing peptides into solution even after thorough washing in NaCl/borate solution. This finding was unexpected, since it had been reported previously that elastase could be quantitatively removed from elastin by extraction with salt solutions (Gertler, 1971). Hence an investigation was undertaken to determine conditions for completely removing elastase from an elastin surface.

Materials and Methods

Particulate elastin from bovine ligamentum nuchae was prepared by hot-alkali extraction and chloroform/methanol delipidation by using published procedures (Lansing *et al.*, 1952; Kramsch *et al.*, 1971) modified as follows. The ligament (250g) was comminuted for 5 min in a Waring Blendor with 1.5 litres of distilled water. The suspension was brought to a final volume of 2.5 litres with water, autoclaved for 30 min at 120°C and filtered. The cake was dispersed in 2.5 litres of water with the Waring Blendor for 5 min and autoclaved again. The autoclaving and filtration steps were repeated until the liquid became completely clear and gave no precipitate with 5% (w/v) trichloroacetic acid, i.e. about 5 times. The cake was dispersed in 2.5 litres of 0.1M-NaOH, autoclaved for 20 min, filtered, washed with water, resuspended in 2.5 litres of water and neutralized with HCl. It was then washed with water by repeated 30 min autoclavings followed by filtration, until the liquid gave no reaction with AgNO₃. The purified elastin was dehydrated with 2×500ml of ethanol (for 4h each) and delipidated with 2×500ml of chloroform/methanol (1:1, v/v) (for 4h each). The product was filtered, rinsed with 100ml of ether, dried *in vacuo* and milled in an Analytical Micromill (TRI-R Instruments, Rockville Center, NY, U.S.A.) to pass through a sieve of 125 µm or smaller by using an ATM sonic sifter

(Fisher Scientific Co., Pittsburgh, PA, U.S.A.). Yield of particulate alkali-extracted chloroform/methanol-delipidated elastin was 70g.

Partially digested elastin was prepared as follows. Dry particulate alkali-extracted chloroform/methanol-delipidated elastin (5g) was suspended in 250ml of 0.2M-Tris/HCl, pH 8.8, by autoclaving for 15 min at 120°C, then by sonicating gently for several hours in a Disintegrator (Ultrasonic Industries, Lindenhurst, NY, U.S.A.). The suspension was left to settle overnight, decanted (175ml), warmed to 37°C and treated with a solution of 7.5 mg of porcine pancreatic elastase (EC 3.4.21.11; ESFF grade, Worthington Biochemical Corp., Freehold, NJ, U.S.A.) in 75 ml of 0.2M-Tris/HCl, pH 8.8, pre-warmed to 37°C. The reaction mixture was stirred gently for 13 min and the digestion was stopped by adding 115 ml of ice-cold 0.5M-sodium phosphate, pH 6.0 (Sachar *et al.*, 1955). The partially digested elastin was collected by centrifugation (20 min, 1300g). By using an extinction coefficient at 280 nm of about 0.2 absorbance unit/mg for solubilized elastin, digestion of elastin was estimated from the absorbance of the supernatant to be approx. 13%. The pellet was resuspended in 300 ml of ice-cold 0.5M-sodium phosphate, pH 6.0, by sonicating at 20 kHz for two 15 min periods in a Sonifier Cell Disrupter (Ultrasonic Industries), and cooling in ice/water. Elastin was collected by centrifugation and the washing process repeated. The final pellet was resuspended in approx. 100ml of water and dialysed [A. H. Thomas cellulose tubing with a 12000-mol.wt. cut-off, treated before use as described by Beers & Reich (1969)] for 3 days at 2°C against 2 litres of water, the fluid being changed daily. Freeze-drying yielded 4.3 g of partially digested elastin. A control elastin sample was treated in the same way, except that no enzyme was used.

Partially digested elastin was washed with saline buffer (Gertler, 1971) at room temperature (25°C) as follows. The elastin (20mg) was dispersed in 0.5ml of 0.2M-NaCl in 0.05M-sodium borate, pH 8.8, and centrifuged at 700g for 10 min. The pellet was resuspended in 0.25ml of this solution and centrifuged as

above; it was washed twice more in the same way and once with 1 ml of the NaCl/borate buffer. The final pellet was resuspended in 2 ml of this buffer to monitor peptide release (see below) as a function of time. The combined 0.2 M-NaCl/0.05 M-sodium borate washings were dialysed overnight against 500 ml of ice-cold 0.05 M-sodium borate, pH 8.8, and incubated at room temperature with 20 mg of elastin that had not been previously exposed to elastase, and checked for elastase activity.

Partially digested elastin was washed with dilute alkali at room temperature as follows. The elastin (20 mg) was dispersed in 0.5 ml of 0.2 M-Tris/0.01 M-NaOH and centrifuged at 700g for 10 min. The pellet was resuspended in 0.25 ml of this solution and centrifuged as above; it was washed twice more in the same way and once with 1 ml of the alkaline solution. The final pellet was resuspended in 2 ml of 0.2 M-Tris/HCl (pH 8.8)/0.01 M-NaCl, for monitoring peptide release in the liquid phase as a function of time (see below). The combined alkaline washings were adjusted to pH 8.8 with HCl for assessment of the elastase activity eluted from partially digested elastin, by using elastin that had never been previously exposed to elastase.

Kinetics of solubilization of elastin by elastase were monitored as follows. The elastin (20 mg) was dispersed in 2 ml of solution at room temperature. At time-intervals, the mixture was centrifuged at 700g for 10 min, and the E_{280} of the supernatant (Grant & Robbins, 1957) was determined; the pellet was resuspended in its supernatant and maintained at room temperature until the next reading. This method of operation was possible since the solubilization of elastin under the conditions of the present work (low concentrations of elastase, room temperature) took place over a period of several days, and was virtually undisturbed by measurements that lasted only a few minutes. When the absorbance of the liquid phase was greater than 1, a tenfold dilution of a 0.2 ml portion of the reaction mixture with the appropriate buffer was centrifuged as above, and the E_{280} of the supernatant determined. Elastin that had not been previously exposed to elastase, and partially digested elastin that had been autoclaved and washed twice in buffer, were used as controls.

Results

Fig. 1 shows that partially elastase-digested elastin, when dispersed in buffer, dissolved within 48 h, although its preparation had included two washings with 0.5 M-sodium phosphate, pH 6.0, and sonication at 20 kHz. Partially digested elastin, which had been autoclaved and, like elastin, never previously exposed to elastase, released less than 1% of its total protein over a 4-day incubation period. After extraction with 0.2 M-NaCl/0.05 M-sodium borate, pH 8.8,

Fig. 1. Kinetics of solubilization of elastin in 0.05 M-sodium borate, pH 8.8, at room temperature

■, Elastin not previously exposed to elastase. Partially elastase-digested elastin: before NaCl/borate extraction (○) and after extraction with 0.2 M-NaCl/0.05 M-sodium borate, pH 8.8 (●). □, Elastin, not previously exposed to elastase, in combined 0.2 M-NaCl/borate extracts from partially digested elastin, dialysed against 0.05 M-sodium borate, pH 8.8.

over half of the washed partially digested elastin dissolved within 4 days. When control elastin (not previously exposed to elastase) was dispersed in the combined 0.2 M-NaCl/0.05 M-sodium borate extracts, which had been dialysed, it too dissolved, but within 3 days, i.e. more slowly than partially digested elastin that had not been washed with NaCl/borate buffer. Thus salt extraction of elastase, strongly adsorbed on elastin, occurred but was incomplete.

The evidence that elastase can be inactivated and removed from partially digested elastin with dilute alkali, and then reactivated at pH 8.8, is presented in Fig. 2. First, it is shown that partially digested elastin released about 3% of its weight of elastin peptides immediately on exposure to 0.01 M-NaOH/0.2 M-Tris at room temperature, but no further peptides were released over a 4-day incubation period. Secondly, after extraction with dilute alkali and resuspension in 0.2 M-Tris/HCl (pH 8.8)/0.01 M-NaCl, at most 2% of total releasable peptides were released after 4 days. Thirdly, when the combined alkaline washings were adjusted to pH 8.8 with HCl and incubated with control elastin (not previously exposed to

Fig. 2. Kinetics of solubilization of partially digested elastin at room temperature before and after extraction with dilute alkali

Partially digested elastin in 0.2M-Tris/0.01M-NaOH (●), in 0.2M-Tris/HCl (pH 8.8)/0.01M-NaCl before extraction with dilute alkali (○) and in 0.2M-Tris/HCl (pH 8.8)/0.01M-NaCl after extraction with 0.2M-Tris/0.01M-NaOH (▲). Elastin not previously exposed to elastase, in combined alkaline extracts from partially digested elastin, adjusted to pH 8.8 (■).

elastase), the latter was digested at a rate only slightly lower than that of partially digested elastin with elastase still present; on incubation with alkali-washed partially digested elastin instead, the full digestion rate was recovered. The stability of elastase in cold pH 12 solutions has been noted before (Lewis *et al.*, 1956).

Parallel studies of solubilization behaviour at lower and higher pH values showed that even at pH 11 there was still some peptide release from elastase-containing elastin, and at pH values above 13 (e.g. 0.25M-NaOH) there could be detected a slow base-catalysed hydrolysis of either the partially digested elastin or elastin not exposed previously to elastase.

Discussion

Gertler (1971), reporting on the adsorption of porcine pancreatic elastase and other proteins on elastin, pointed out that 'in all cases, the activities adsorbed

on elastin could be quantitatively recovered by extracting with 0.2M-NaCl in 0.05M-sodium borate, pH 8.8'. Our results, however, demonstrate the following: (a) residual elastase activity remains strongly bound to partially digested elastin even after washing with 0.5M-sodium phosphate, pH 6.0 (Sachar *et al.*, 1955), and sonication; (b) further extraction of partially digested elastin with 0.2M-NaCl in borate buffer, pH 8.8, removes some but not all of the residual elastase activity; (c) this activity can be removed completely by washing with dilute alkali, and recovered. The main difference between Gertler's (1971) determination of elastase activity and ours lies in the fact that, for the determination of small amounts of adsorbed elastase, monitoring the elastase activity in the liquid phase (Gertler, 1971) is not as sensitive as the method reported here, in which one monitors the solubilization of elastin by the adsorbed elastase. Depending on the initial amount of adsorbed elastase, the recovery of enzyme as determined by Gertler (1971) could well have been close to 100%, since only a few μ g of adsorbed elastase per g of elastin could account for the slow digestion of elastin that we monitored over several days.

Our results showed that pH values as high as 11 were inadequate to abolish this enzyme activity, and that above pH 13 base-catalysed hydrolysis commenced. At pH 12, however, although there was an immediate elution of a small amount of presumably adsorbed peptides, there was no continuing release. Thus this condition represents the minimal strength of alkali needed to stop enzymic hydrolysis completely and to extract elastase from the elastin but without base-catalysed attack on the elastin or denaturation of the enzyme.

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