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Inactivation of Human α_1 Proteinase Inhibitor by Thiol Proteinases

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Human plasma α_1 proteinase inhibitor is the body's principal modulator of serine proteinases (such as those released from phagocytic cells). Cysteine-active-site proteinases, which are not inhibited, have now been found to inactivate this important inhibitor by proteolytic cleavage of a scissile peptide bond. Papain carries out this inactivation catalytically, whereas cathepsin B1 acts stoicheiometrically. Thus thiol proteinases could easily disrupt the delicately regulated balance between serine proteinases and α_1 proteinase inhibitor.

The regulation of proteolytic activity in blood and other tissues is believed to be controlled by the several proteinase inhibitors present in blood plasma (Heimburger *et al.*, 1971). The two major proteins in this class, α_2-macroglobulin and α_1 proteinase inhibitor, also known as α_1-antitrypsin, account for most of the proteinase-inhibitor activity in plasma, and deficiency of the latter protein in the circulation can be readily correlated with the development of pulmonary emphysema (Laurell & Erikson, 1963).

Although results obtained with partially purified α_1 proteinase inhibitor (hereafter called 'the inhibitor') suggest a broader specificity (Cooreman *et al.*, 1975), it is generally believed that the inhibitor forms complexes specifically with serine proteinases (Vogel *et al.*, 1968) and does not affect the activity of thiol proteinases such as cathepsin B1 (Starkey & Barrett, 1973) or papain (Mortorana & Share, 1976). For this reason the latter enzyme has been commonly used to induce the development of emphysema in animal model systems (Goldring *et al.*, 1960; Gross *et al.*, 1964), the presumption being that papain acts specifically by degrading the alveolar structural proteins, particularly elastin.

We have made a careful study of the interaction of the thiol proteinases papain and cathepsin B1 with the inhibitor. The results obtained suggest that these enzymes could act not only by digesting lung structural proteins directly but also by enzymically inactivating the inhibitor, thereby lowering its protective concentration in and around lung tissue.

Materials and Methods

Human α_1 proteinase inhibitor (mol.wt. 52000) was purified from plasma as described earlier (Pannell *et al.*, 1974). Papain (mol.wt. 21000) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Human liver cathepsin B1 was a gift from Dr. A. J. Barrett (Strangeways Research Laboratory). The enzyme migrated as two barely separable bands on gel electrophoresis, indicative of microheterogeneity as originally described (Barrett, 1973). Pig trypsin was prepared by affinity chromatography (Johnson & Travis, 1976).

Papain and cathepsin B1 activities were measured in 0.1 M-phosphate buffer, pH 6.0. Both 1% azocasein and *N*-benzoyl-DL-arginine 2-naphthylamide (Barrett, 1973) were used to measure the proteinase and esterase activities (respectively) of cathepsin B1. Papain esterase activity was measured with benzoyl-L-arginine ethyl ester as substrate. Activation of papain was obtained by adding cysteine (final concn. 5 mM), whereas cathepsin B1 was activated with dithiothreitol (final concn. 5 mM). Measurement of the residual activity of the inhibitor after incubation with either of the thiol proteinases was made by adjustment of the pH of the mixture to 8.0, followed by addition of a standard quantity of pig trypsin. After 1 min the solution was assayed for residual trypsin activity. Trypsin esterase activity was determined by using benzoyl-L-arginine ethyl ester as substrate (Schwert & Takenaka, 1955). The inhibitor's activity was calculated by measuring loss of esterase activity of a standard pig trypsin preparation after incubation with the inhibitor (Pannell *et al.*, 1974).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis depicted in Plates 1 and 2 (gel length 7 cm) was performed by adaptation of the procedure of Laemmli (1970) to the pulsed-power Ortec electrophoresis system. The composition of the gel was as previously reported (Baugh & Travis, 1976), and electrophoresis was carried out a constant 260 V. The electrophoresis system used in the experiment described in Plate 2 (gel length 5 cm) was that of Starkey & Barrett (1975). Sequence analysis of proteins was determined with a Beckman model 890C protein sequencer (Edman & Begg, 1967), by using the 0.1 M-Quadrol program (Brauer *et al.*, 1975). The amino acid phenylthiohydantoins were

identified by amino acid analysis after back-hydrolysis (Mendes & Lai, 1975).

Papain-modified α_1 proteinase inhibitor was prepared by treating 50mg of the inhibitor with 40μg of papain (500mol of inhibitor/mol of papain) in 10mM-NH_4HCO_3 buffer, pH6.5, containing 1mM-EDTA and 2mM-mercaptoethanol for 2h at 23°C, followed by freeze-drying. The modified inhibitor was recovered from the reaction mixture by chromatography on Sephadex G-50. The modified inhibitor, which was homogeneous by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, was dialysed against water and freeze-dried before sequencing.

Results

(a) Interaction of the inhibitor with papain

When the inhibitor and papain were mixed in a molar ratio of 200:1 (inhibitor/papain) in a buffer containing 0.03M-sodium phosphate, pH6.5, and 5mM-cysteine, and the solution assayed at various time intervals for trypsin-inhibitory activity, a rapid decrease in the activity of the inhibitor was noted (Fig. 1). Within 10min, over 90% of the inhibitor was inactivated. Examination of samples of the incubation mixture at the same time intervals by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated a shift in the mol.wt. of inhibitor from 53000 to 47000, indicating peptide-bond cleavage during the incubation without the formation of a stable complex (Plate 1). The *N*-terminal sequence of this modified inhibitor was found to be Thr-Ile-Pro-Pro-Glx-Val-..., in contrast with that of normal inhibitor, which is Gln-Asp-Pro-Glu-Gly-Asn-....

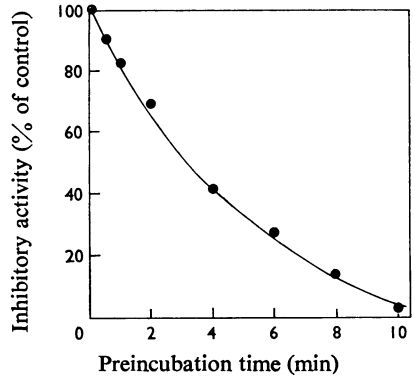


Fig. 1. *Inactivation of α_1 proteinase inhibitor by papain*
Samples of the inhibitor (200mol/mol of papain) were incubated with papain at 23°C, and samples were removed at the time points indicated and assayed for residual inhibitory activity towards pig trypsin.

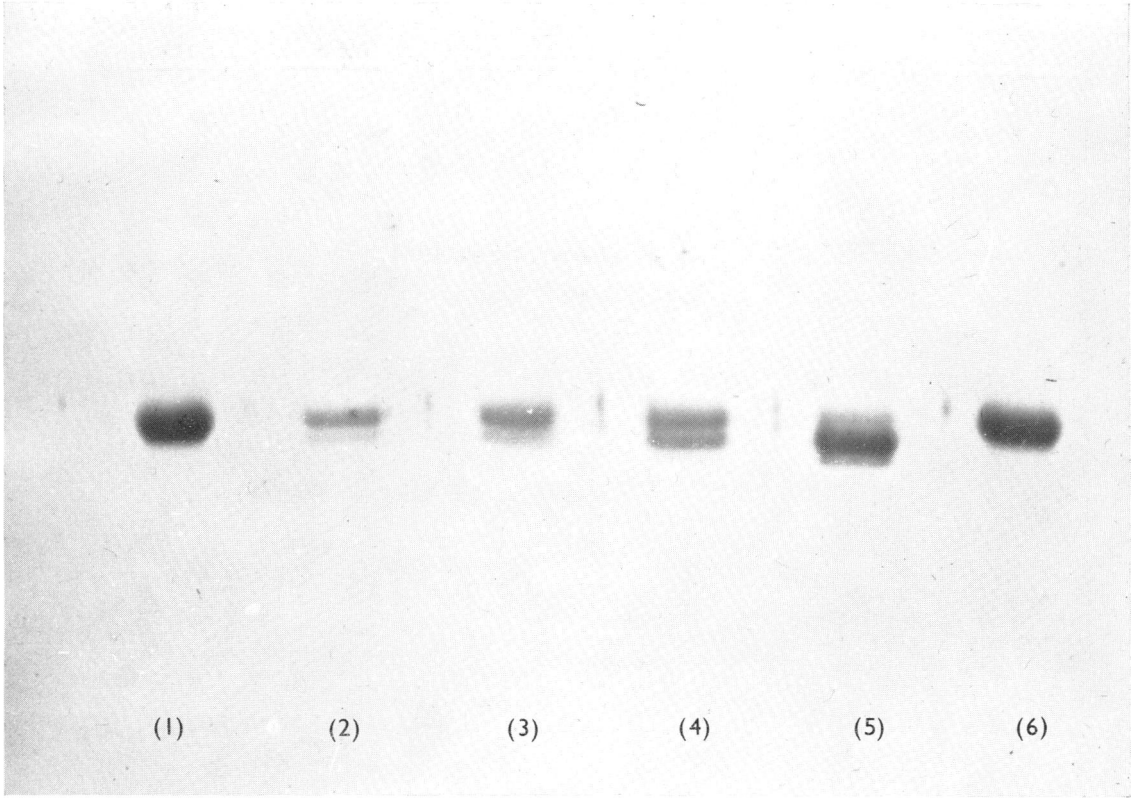
(b) Interaction of the inhibitor with cathepsin B1

Incubation of α_1 proteinase inhibitor with cathepsin was essentially under the same conditions as that with papain, except that the pH was lowered to 6.0. Again, activity of the inhibitor towards trypsin was lost with no apparent effect on the esterolytic or proteolytic activity of cathepsin B1. However, the reaction was significantly different in that the interaction, cleavage of peptide bond(s) and inactivation of the inhibitor were stoicheiometric. The reaction between the two proteins, measured as a function of time, was complete in less than 1min, as judged by loss of inhibitor activity. However, several incubations were made for longer time periods (up to 24h) at various molar ratios of cathepsin B1 to inhibitor. In all cases no further decrease in inhibitory activity could be detected over that which occurred initially, again confirming the ability of cathepsin B1 to catalytically inactivate the inhibitor. As with papain, the inhibitor was converted into a form with a slightly lower molecular weight (Plate 2), without apparent complex-formation. Owing to the quantity of cathepsin B1 which would have been required to inactivate enough inhibitor for sequence studies, this type of experiment was not attempted.

Discussion

The inactivation of thiol proteinases by the inhibitor was originally noted with bromelain and crude preparations of the inhibitor (Cooreman *et al.*, 1975). However, examination by this same group of workers of more purified inhibitor after bromelain treatment indicated that it had been modified, although complex-formation was never detected and there was no loss in the enzymic activity of this thiol proteinase. The results for bromelain are thus consistent with those obtained in the present study. The disappearance of bromelain activity in crude preparations was therefore probably due to other contaminating proteins. By contrast, it was readily shown that human liver cathepsin B1 could only be inactivated by α_2-macroglobulin and that the inhibitor had no effect on its activity (Starkey & Barrett, 1973).

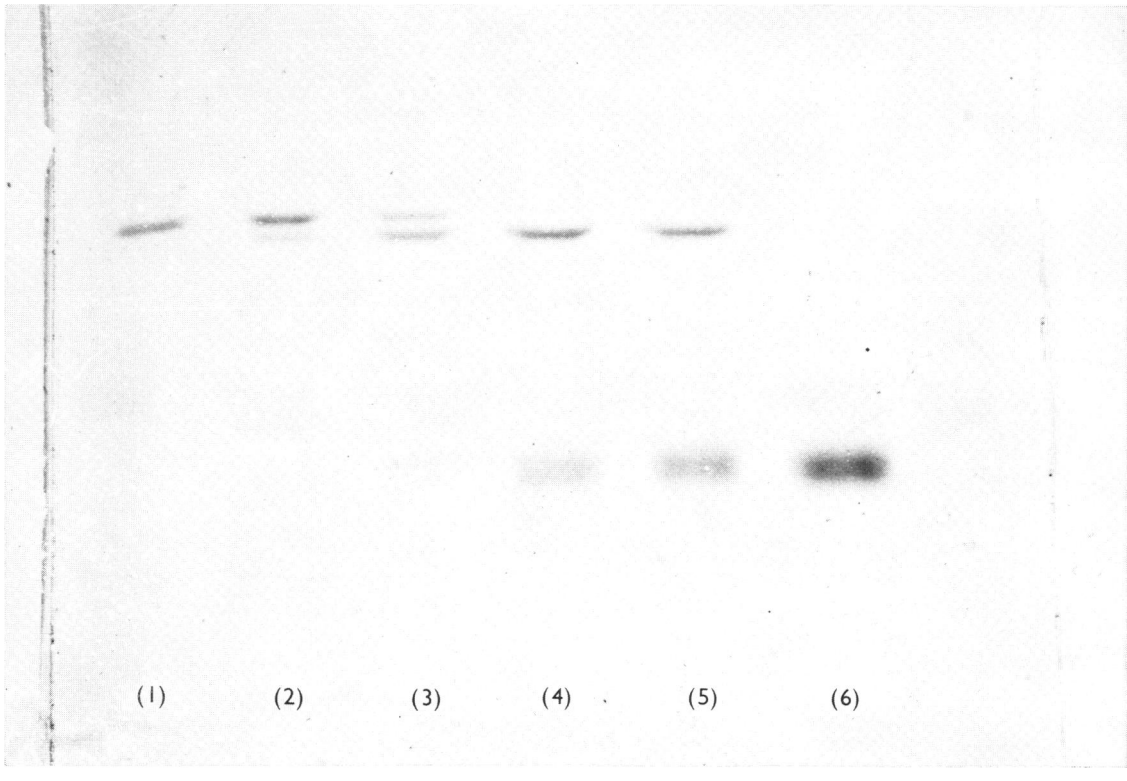
The present results indicate that both papain and cathepsin B1 inactivate the inhibitor by specific peptide-bond hydrolysis. That the same peptide bond was cleaved during the inhibition of trypsin by the inhibitor was suggested by the observation that both the trypsin- and the papain-modified inhibitor have the same *N*-terminal sequence, namely Thr-Ile-Pro-Pro-Glx-Val-... (Johnson & Travis, 1976). Residue 3, originally described as threonine, has now been confirmed as proline. Although peptide-bond cleavage by serine proteinases eventually results in a very stable complex, thiol proteinases presumably hydrolyse the same bond but without formation of a stable



EXPLANATION OF PLATE 1

Gel-slab electrophoresis of an α_1-proteinase-inhibitor/papain incubation mixture

Electrophoresis was carried out with 7.5% (w/v) gels, and the running time was 40min. Samples were removed after 20, 40, 60 and 80% inactivation of the inhibitor (see Fig. 1), immediately incubated at 100°C with β-mercaptoethanol/sodium dodecyl sulphate and subjected to vertical polyacrylamide-slab electrophoresis. Gels (1) and (6), inhibitor control samples; gel (2), 20% inactivation; gel (3), 40% inactivation; gel (4), 60% inactivation; gel (5), 80% inactivation.



EXPLANATION OF PLATE 2

Gel-slab electrophoresis of an α_1-proteinase-inhibitor/cathepsin B1 incubation mixture

Electrophoresis was carried out with 10% (w/v) gels, and the running time was 2h. In this experiment, samples of the inhibitor were incubated for 30min with increasing concentrations of cathepsin B1, samples were assayed for trypsin-inhibitory activity, and the remainder of the sample treated as for the inhibitor/papain incubation mixtute (Plate 1) Gel (1), inhibitor control; gel (2), 20% inactivated inhibitor/cathepsin B (1:0.2); gel (3), 60% inactivation (1:0.6); gel (4), 90% inactivation (1:0.8); gel (5), 100% inactivation (1:1); gel (6), cathepsin B1 control.

complex, indicating that the serine hydroxyl group plays an important role in complex-formation and stabilization.

We have not yet determined why papain, acts catalytically and cathepsin B1 acts stoicheiometrically during the conversion of the inhibitor into a modified form. Certainly, gel-filtration experiments do not indicate any type of complex-formation between the inhibitor and cathepsin B1. It may be that the low-molecular-weight fragment(s) which is cleaved during the interaction with cathepsin B1 remains weakly bound to this enzyme, preventing further interaction with native inhibitor, but causing no interference with its normal function as a proteinase. Papain, however, cleaves this fragment into several components, either during or after the conversion of the inhibitor into its inactive form (D. Johnson & J. Travis, unpublished work).

As suggested elsewhere (Martorana & Share, 1976), proteolytic enzymes from granulocytes and possibly macrophages, which accumulate in lungs after papain exposure, may be liberated and contribute to the development of papain-induced emphysema, particularly if the tissue concentrations of the inhibitor are depleted by the catalytic action of this thiol proteinase. We have no plausible explanation at present, however, as to why peptide-bond cleavage by papain inactivates the inhibitor, yet this same reaction apparently occurs during complex-formation with trypsin.

These results suggest a new pathway by which active-inhibitor concentrations in tissues may be substantially decreased so that endogenous proteolysis may occur. That is, any type of reaction which might activate thiol proteinases could result in the inactivation of normally protective proteinase inhibitors. For example, HCN, a major component of cigarette smoke, readily activates thiol proteinases and could therefore play a major role in the induction of proteolysis in lung tissue and the concomitant development of lung diseases. Although cathepsin B1 is an intracellular enzyme, it is well known that the inhibitor can be detected in cells (i.e. macrophages, leucocytes). Thus cathepsin B1 and other acid proteinases could readily decrease the protective concentration of this inhibitor in tissues.

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