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AN ALKALINE PROTEASE INHIBITOR FROM *HEVEA BRASILIENSIS* LATEX

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Key Word Index—*Hevea brasiliensis*; Euphorbiaceae; protease; inhibitor; subtilisin.

Abstract—A subtilisin inhibitor has been isolated from the latex of *Hevea brasiliensis* and characterized. This inhibitor (HP–In) is a protein (MW 11730) which readily dimerizes. Isoelectric focusing indicates the protein contains 95% of a single component of p*I* 4.15. HP–In reacts with subtilisin in equimolecular proportions, but shows little inhibitory activity towards trypsin, bromelain or papain. The amino acid analysis of HP–In bears little relationship to those of other known protease inhibitors including two which show high activity towards alkaline proteases. HP–In contains no cystine and only 1 mol of methionine. It is suggested that HP–In may act in a protective capacity in the tree and perhaps is only formed when the bark is cut, as in the normal tapping operation.

INTRODUCTION

The proteinase inhibitors found in a number of species of plants and micro-organisms have been reviewed by Richardson [1]. Most of the studies involving plants have concentrated on those parts (i.e. seeds or tubers) which are used for food. The vegetative parts of the plant have been less intensively investigated, but Chen and Mitchell [2] report some trypsin inhibitory activity in spinach, broccoli and brussels sprouts, whilst Wong *et al.* [3] have studied the inhibitor from callus tissues obtained from the stems of tobacco. Although it is very probable that protease inhibitors are widely distributed throughout the plant kingdom, the present paper appears to be the first report of such a compound either in a latex or in the trunk of a tropical tree.

Rubber occurs in some 2000 species of plants and micro-organisms [4] but it is obtained commercially almost exclusively from *Hevea brasiliensis*, in which latex forms the cytoplasm of a specialized vascular system contained in the inner bark [5, 6]. A large part of the protein, which is present in fresh latex, is adsorbed onto the surface of the rubber particles and is retained there on coagulation. For some industrial applications of rubber, this protein can adversely affect certain physical properties of the final product [7]. Several methods have been developed for the removal of protein from *Hevea* latex; these include treatment with surfactants followed by creaming of the latex [8] or digestion with proteolytic enzymes [9, 10]. However, these latter processes are of limited commercial use owing to the presence, in the main aqueous phase (or serum) of the latex, of a powerful inhibitor of subtilisin (EC 3.4.21.14) and other proteolytic enzymes [11]. The effect of the inhibitor is to increase the amount of enzyme required to such a degree as to make the process uneconomic, or alternatively to reduce the extent of deproteinization which can be attained in a reasonable time.

The object of the present work was to isolate and characterize the proteolytic enzyme inhibitor of *H. brasiliensis* (HP–In).

RESULTS AND DISCUSSION

Assay of proteolytic inhibitor

The concentration of inhibitor in any given solution was estimated from the volume needed to reduce the rate of enzymic hydrolysis of casein by a fixed proportion under standard conditions [11]. The measurement of the proteolytic activity of subtilisin in the presence of the aqueous serum phase of rubber latex obtained from *Hevea brasiliensis*, was rendered difficult by residual small rubber particles and other compounds, which interfered with the determination of protein degradation products, either by the UV absorption or Folin–Ciocalteu methods. The former technique was, however, used in some experiments on partially purified latex serum proteins, particularly at pH values near neutrality, when the autotitration technique described below failed to give satisfactory results.

The hydrolysis of casein was routinely followed by a method based on the autotitration technique of Ottesen and Svendsen [12]. The junction potential between the reference electrode and the solution in the titration cell was not sufficiently reproducible when the standard porous plug type of electrode supplied with the instrument was used. Very stable potentials were, however, obtained when a polyacrylamide gel plug containing saturated potassium chloride, as described in the Experimental, was used. The effect of adding increasing amounts of serum containing the inhibitor on the rate of hydrolysis of casein is shown in Fig. 1. The unit of inhibitory activity is defined as one-tenth of that amount which halves the initial rate of proton liberation under the stated conditions. As the system contains 20 nmol of subtilisin and evidence presented below indicates that 1 mol of subtilisin reacts with 1 mol of HP–In, 1 unit of inhibitor corresponds to 1 nmol (of MW 11 730).

Borate ions at a concentration of 0.01 M were found to interfere with the autotitration method of assay, as they appeared to inhibit the release of protons from casein in the presence of subtilisin. The rate at which HP–In reacted

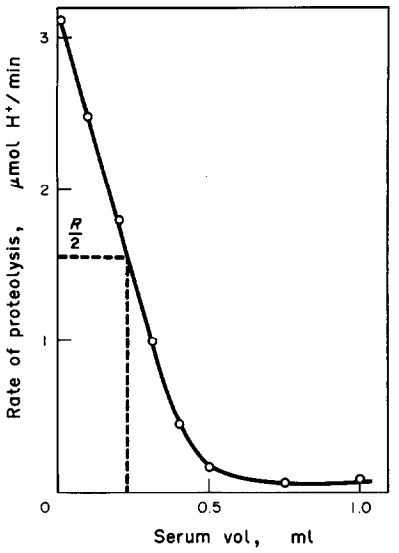

Fig. 1. Effect of latex serum on the rate of proteolysis of casein by subtilisin. Initial rates of hydrolysis measured by autotitrimetry on 20-ml samples of 1% casein at pH 10.0 and 25° in the presence of 20 nmol subtilisin.

with the proteolytic enzyme was found to depend on the pH and the particular enzyme used. For subtilisin at pH 10 the interaction was fully complete in less than 1 min, which was adopted as the standard time; however, with papain, bromelain, etc. 10 min was routinely used.

The aqueous serum obtained by centrifugation of *H. brasiliensis* latex was shown to contain significant quantities of protease inhibitor. None was detected in the lutoid or 'bottom fraction' [13] which, however, is known to contain proteolytic and other degradative enzymes [14]. The inhibitor was itself apparently proteinaceous, but the activity was not associated with any of the known proteins which have been isolated from *Hevea* latex [15–17]. The serum phase of latex corresponds to the cytosol of the latex vessel [6], whereas the lutoids constitute a polydisperse vacuome [18]. The above observations, therefore, indicate a cytoplasmic [19] rather than a vacuolar [20] locus for HP–In. Freeze-dried serum, obtained by centrifuging fresh latex, was a stable source of the inhibitor, but its concentration appeared to be dependent on the clone of trees from which the latex had been obtained (Table 1). As the solids contained *ca* 18% protein [21] the inhibitor accounts for 1.1–12% of the total protein present in the serum. These figures may be compared with the 5–10% reported for the protease inhibitor content of the water-soluble proteins from cereal grains [22]. Latex serum contains, on average, 6.2% solid matter; the concentration of HP–In in the latex as tapped from the tree ranged from 0.01 to 0.14%, for the five clones studied. If this wide variation in inhibitor content is in fact a reproducible clonal characteristic, then it would appear that latex from certain clones was more suitable for deproteinization than that from others.

Isolation of inhibitor

The separation of HP–In from a solution of latex serum solids is outlined in Table 2 and resulted in at least a 43-fold purification. Acidification of the serum to pH 4 removed most of the small rubber particles, together with inactive protein, without any loss of activity. However, it

Table 1. Protease inhibitor contents of freeze-dried serum from clonal latices

Clone	Inhibitor concn	
	units/g	mg/g*
RRIM 600	1940	22.7
Tj 1	1490	17.4
PB 86	830	9.7
RRIM 701	250	3.0
RRIM 501	170	2.0

* Assuming 1 unit of inhibitor represents 1 nmol of MW 11 730 (see Results and Discussion).

Table 2. Isolation of *Hevea* proteinase inhibitor from freeze-dried serum

	Inhibitor activity		Protein content			
	Units* ($\times 10^{-3}$)	Yield	g†	EV‡ ($\times 10^{-2}$)	Yield	Sp. act.‖
Soln of freeze-dried serum	114	100	30.0	(300)§	100	3.8
pH 4 supernatant	115	101	11.7	(129)§	39	8.9
35–55% satd $(NH_4)_2SO_4$ ppt	62.1	54	3.9	42.6	13	14.6
Chromatography on Ultrogel AcA-54	51.2	45	—	10.6	3.2	48.3
Chromatography on QAE–Sephadex A-25	37.9	33	—	2.91	0.88	130
Concn by $(NH_4)_2SO_4$ pptn and Sephadex G-25 chromatography	24.9	22	—	1.68	0.51	148
Isoelectric focusing	13.3	12	—	0.88	0.27	151

*For definition of inhibitor unit see Results and Discussion.
†Protein N precipitable with tannic acid × 6.3.
‡EV = $E^{1\,cm}_{280\,nm}$ × vol. (ml).
§Calculated assuming $E^{1\%}_{1\,cm} = 11$.
‖Inhibitor activity units/EV.
For other details see Experimental.

was noted that the active precipitate, which formed on further lowering of the pH, was only soluble with difficulty at high pH values. The last traces of rubber were removed when ammonium sulphate was added to the solution. Chromatography on Ultrogel AcA-54 (Fig. 2) was much superior to that using Sephadex G-100. By calibrating the column with ribonuclease (MW 12 600), α-lactalbumin (MW 15 500) and chymotrypsin (MW 23 200) an approximate MW, for the inhibitor, of 12 000 was estimated. The amino acid analysis (Table 3) indicates a MW of 11 730 which is of the same order of magnitude as a number of other macromolecular proteinase inhibitors from plants [1]. Furthermore, HP-In readily dimerizes [11], as does the protease inhibitor from potato (MW of monomer 10 500) [23], and that from *Streptomyces albogriseolus* (MW of monomer 11 500) [24].

Since freeze-drying was found to denature the inhibitor,

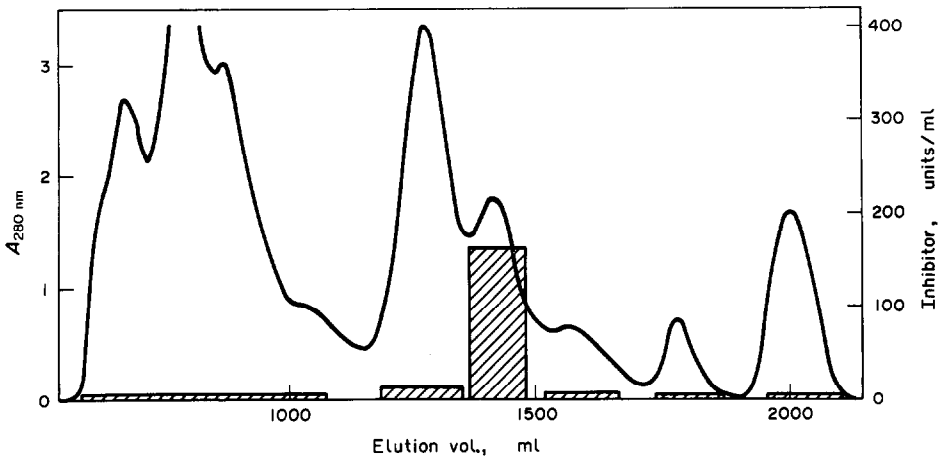

Fig. 2. Gel-chromatography of protease inhibitor from latex serum. $(NH_4)_2SO_4$ fraction of serum chromatographed on Ultrogel AcA-54 at 2° and eluted with 0.145 M Na_2HPO_4 buffer plus 0.01 M cysteine at pH 7. (—)UV absorbance; (▨) inhibitor activity.

Table 3. Amino acid composition of *Hevea* proteinase inhibitor

Amino acid	Mol %	Nearest integer	Other subtilisin inhibitors		
			Streptomyces albogriseolus [44]	Barley [27]	Barley SP II A [28]
Asp	17.3	17	9	17	8
Thr	7.1	7	8	8	4
Ser	3.8	4	9	10	3
Glu	14.7	15	6	13	11
Pro	7.4	7	8	15	5
Gly	26.2	26	11	18	6
Cys	0	0	4	4	0
Ala	10.4	10	18	15	4
Val	12.3	12	13	13	12
Met	0.9	1	3	2	1
Ileu	6.6	7	0	7	6
Leu	6.0	6	9	10	6
Tyr	0	0	3	7	1
Phe	2.2	2	3	7	1
Try	1.2* 1.3†	1	1	3	1
Lys	4.7	5	2	6	8
His	0	0	2	9	1
Arg	4.6	5	4	13	5
Total		125	113	177	83
MW by calculation		11 730	11 483	19 528	9245

*UV method of Beaven and Holiday [42].
†UV method of Bencze and Schmid [43].

precipitation with ammonium sulphate followed by removal of the salt on a Sephadex G-25 column, was adopted as the standard procedure for the concentration of dilute solutions; as an alternative, concentration with Biogel P-6 was effective. Several ion-exchange resins were tested for further purification of the inhibitor and the best results (Fig. 3) were obtained with the cation exchanger QAE–Sephadex operated with a linear salt gradient (0–0.5 M NaCl), which was stopped automatically at 0.23 M as soon as the active peak started to emerge from the column. No further UV-absorbing material was eluted after the gradient was resumed. HP–In was recovered from the active fractions by precipitation with ammonium sulphate and desalting on a Sephadex G-25 column as before.

In the final stage of purification, in which a modification of the preparative electrofocusing method of Radola [25] was used, the dyed print taken from the surface of the Sephadex gel-bed after electrofocusing showed that the material from the ion-exchange chromatography stage consisted of a major active component of p*I* *ca* 4.2 and two other minor components (p*I* = 3.7 and 4.7) of very low inhibitory activity, together with several other small and inactive components. The slight increase in specific activity of the HP–In observed in this stage is indicative of a relatively high state of purity for the inhibitor protein. From the inhibitory activities of the original dried serum and of the final solution, an overall yield of 11% of the activity was estimated.

Properties of the purified inhibitor

Analysis by isoelectric focusing. Isoelectric focusing in polyacrylamide gel layers over the range pH 3.5–9.5, showed a single main component isoelectric at pH *ca* 4.1 and two small components of p*I* 3.7 and 5.0; no other components stainable with Coomassie blue could be detected. Repeat experiments over the range of pH from 2.5 to 5.0 confirmed the p*I* of HP–In to be 4.15 ± 0.02 (pH measured at 22°). The purity of the inhibitor sample was estimated by extracting the colour from electrofocused gels which had been stained by bromophenol blue. Assuming that the impurities and HP–In have the same affinities for the dye, a figure of 95% for the purity of the product was estimated.

Element analysis. Considerable difficulties were experienced in preparing a sample of dry HP–In which was free from inorganic sulphur. Two passes through a Sephadex G-25 column using water as the solvent, followed by freeze-drying, gave a product containing 8% sulphur, and when 0.01 M ammonium hydroxide was substituted as the solvent, 4.6% sulphur (of which at least 80% was present as SO_4^{2-}) remained in the freeze-dried product. Further treatment of this material on a column of Dowex AG1-X10 (200–400 mesh), which had been regenerated and converted to the formate form immediately before use, was only able to reduce the sulphur content to 2.6%. Estimations of the thiol content of the dodecylsulphate-denatured HP–In, using Ellmans reagent [26] before and after reduction with sodium borohydride, gave 0.015 ± 0.005% and 0.189 ± 0.010%, respectively. These figures correspond to 0.05 and 0.7 mol sulphur/mol protein of MW 12000, and are consistent with the presence of 1 mol methionine/mol protein (see below). It is probable that the high sulphur contents given above are due to impurities tightly bound to the HP–In. Other analyses carried out on the purified dry HP–In gave C, 52.3; H, 7.5; N, 16.2%.

UV spectra. Figures found are at pH 7, λ_{max} 282 nm ($E^{1\%}_{1cm}$ 5.73), λ_{min} 250 nm ($E^{1\%}_{1cm}$ 2.62) and in 0.1 M sodium hydroxide λ_{max} 280 nm ($E^{1\%}_{1cm}$ 5.97), λ_{min} 256 nm ($E^{1\%}_{1cm}$ 5.42).

Amino acid analysis. The results of the amino acid analysis carried out on the hydrochloric acid hydrolysate of the purified HP–In are recorded in Table 3. Cysteine, hydroxyproline, histidine, ornithine and tyrosine were not detected. A MW calculated from the amino acid composition gives 11730 as compared with the approximate value of 12000 given above, which was based on gel-permeation chromatography.

The amino acid analysis of HP–In bears no relation to those of a wide range of plant protease inhibitors which frequently have strictly homologous regions in their molecules [1]. In particular, HP–In contains 21 mol %

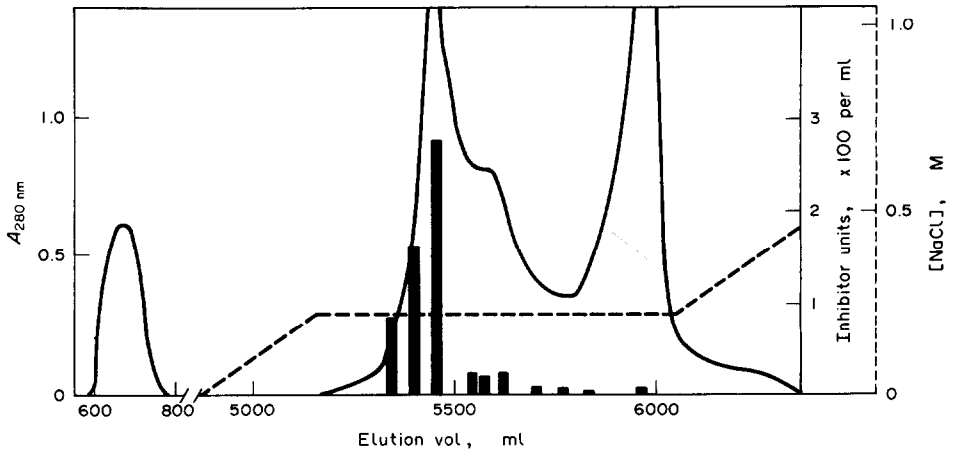

Fig. 3. Ion-exchange chromatography of protease inhibitor from latex serum. The active fraction from Fig. 2, desalted on Sephadex G-25, was applied to a column of QAE–Sephadex A-25 equilibrated with 0.05 M Tris–HCl buffer plus 0.01 M cysteine at pH 7.6. The active fraction was eluted with the NaCl gradient, shown by the dashed line.

glycine, whereas this is usually a minor component of many inhibitors. On the other hand, although the trypsin inhibitors from several species of legume contain some 19 mol % cysteine, none has been detected in HP–In. The analyses of three other macromolecular protease inhibitors, with a marked specificity towards subtilisin, have been reported so far, and are included in Table 3 for comparative purposes. There appears to be, in general, a better correlation between the compositions of those inhibitors which are mainly specific towards subtilisin than there is between HP–In and the other groups of inhibitors.

Although HP–In and the inhibitor (SP IIA) isolated from barley by Svendsen *et al.* [28], seem to be unique amongst the low MW inhibitors reported so far in not containing any cysteine or cystine residues, there are several differences in the amino acid composition (particularly with respect to glycine) of these two molecules. Further work is clearly needed in order to show if the homology [28] between SP IIA and the protease inhibitor, isolated from potato by Melville and Ryan [29], may be extended to cover HP–In. The absence of cystine bridges in the HP–In molecule probably accounts for its greater ease of denaturation compared with most of those previously reported. In respect of its not containing cysteine residues, HP–In appears to be more comparable with the α-serum trypsin and chymotryspin inhibitors, although these compounds are of higher MW (54 000–62 000) [30].

Stability. The activity of the inhibitor was maintained fully when the freeze-dried latex serum was stored at −20° for at least 18 months, but solutions of the serum lost 14%, on average, of their activity when kept for 10 days under the normal conditions used for the storage of commercial rubber latex (25° with added ammonia at pH 10). Solutions of a partially purified sample of the inhibitor when stored at 2° and pH 6.9 lost some 11% of their activity in 24 hr. A thiol compound such as cysteine or 2-mercaptoethanol appeared to be necessary to maintain activity, but dithiothreitol [31] was apparently less effective as a preservative. In the absence of thiol compounds, HP–In was found to dimerize to give an apparent MW of 23 000, without any major loss of inhibitory activity. In contrast to many of the protease inhibitors isolated from plants [1], HP–In was deactivated to *ca* 23% by heating the serum to 100° for 10 min.

Reaction stoichiometry. Assuming a MW for the HP–In of 11 730, it was estimated that 1 mol of subtilisin (MW 27 500) reacted with 1.2 mol of inhibitor; a 1:1 stoichiometry was, therefore, inferred. The affinity of HP–In for subtilisin was measured using bovine serum albumin in place of casein as the substrate, on account of the albumin being obtainable in a purer form with a more accurately known MW. The substrate concentration was varied over the range 74–625 μM. The results when plotted according to the Lineweaver–Burk method indicated competitive inhibition, with K_i *ca* 4 μM. Under the experimental conditions, the affinity of the inhibitor for the subtilisin appears to be some 90-times that of the substrate for the enzyme. The reaction of HP–In with the enzyme was complete within *ca* 30 sec at pH 7–10, and the activity of a given solution of HP–In was not significantly altered over this range.

Enzyme specificity. HP–In had no detectable effect on the rate of hydrolysis of casein by trypsin (EC 3.4.21.4) at pH 7 when measured by the TCA precipitation method, even when a 15-fold molar excess of the inhibitor was present. Under similar conditions with bromelain (EC 3.4.22.4), a two-fold excess of the inhibitor caused only a very small decrease in the proteolytic activity of the enzyme, and when the bromelain-inhibitor reaction was allowed to proceed for a longer period before adding the substrate, an even smaller effect on the rate of hydrolysis of casein was observed. With papain (EC 3.4.22.2) a slight increase in hydrolysis rate was noted when the enzyme was preincubated with HP–In, and this effect was shown not to be due to any residual proteinase activity being present in the inhibitor sample. It is possible that there are present in *Hevea* latex, other protease inhibitors such as, for example, the low MW inhibitor reported in a species of *Streptomyces* by Murao and Watanabe [32]. Compounds of this type might easily have been removed during the preparative procedure used in the present work.

Function of the protease inhibitor in H. brasiliensis

So far no alkaline proteases have been detected in *Hevea* latex and it is thus unlikely that the physiological function of HP–In is in controlling the activity of such enzymes. Three physiological functions for plant protease inhibitors have been suggested, including acting as a storage protein, controlling metabolism and acting in a defensive role [1]. Although no evidence has, as yet, been obtained for, or against, any of these three mechanisms in *H. brasiliensis*, it may be that HP–In protects the proteins in latex from degradation by the acid protease(s) known to occur in the very labile lutoid particles [14]. Alternatively, the inhibitor may help to protect the tree from attack by outside organisms, as suggested for other systems by Birk and Gertler [33]. It is also possible that the formation of HP–In is only induced in the latex vessel by the tapping operation itself, and that it plays a part in restoring the rubber lost to the tree on tapping. If this were so, it would be expected that a 'protease inhibitor inducing factor' similar to that discovered in tomato leaves by Green and Ryan [34] would be detectable in *H. brasiliensis* tissues after tapping, but not in young trees in which the bark was uncut.

EXPERIMENTAL

Materials. H. brasiliensis latex was obtained from trees grown at the Rubber Research Institute of Malaysia Experiment Station, Sungei Buloh, Selangor, W. Malaysia and tapped regularly on a half-spiral alternate-day tapping system. The first runnings were rejected and the latex was then collected into glass vessels surrounded by ice, for *ca* 30 min. The sq. serum was prepared by centrifuging the latex, as described by Karunakaran *et al.* [35] and the freeze-dried serum air-mailed to England where it was stored at −20°. Subtilisin A was obtained from Novo Industri A/S, Copenhagen; bromelain as a suspension in $(NH_4)_2SO_4$ came from Boehringer-Mannheim, whilst trypsin and papain were supplied as twice crystallized materials by Sigma, London.

Assay of proteolytic activity. For expts with subtilisin at pH 10.0, the potentiometric technique of Ottensen and Svendsen [12] was used with radiometer equipment set at pH 10.00. As the standard calomel reference half-cell supplied with the apparatus was found not to be sufficiently stable, the porous-plug junction was replaced by a polyacrylamide gel plug 12 × 5 mm diameter formed at the lower end of a glass tube containing saturated KCl soln, into which the calomel element

was fitted. The polymer was produced from a soln containing acrylamide (10%) in satd aq. KCl and crosslinked with diallyltartardiamide (2%) [36]. Catalyst and other impurities were removed from the gel plugs by electrolysis before installing them in the titration apparatus. The 0.25-ml burette was filled with 0.1 M NaOH, and the stirred titration cell, which was thermostatically controlled at 25 ± 0.01°, contained 17 ml 1.18% (w/w) casein (Hammarsten), adjusted before use to pH 10.00, as the substrate. The reactions were started by the rapid injection of the subtilisin soln (20 nmol in 3 ml, pH 10.0). After allowing a few sec for pH equilibrium to be regained, a nearly linear trace was obtained on the recorder, from which the initial rate of hydrolysis of the casein was estimated graphically. Preliminary expts established that the rate of proton formation was a linear function of the enzyme concn, at least up to that routinely used (1 μM).

Assay of inhibitor. To estimate the inhibitory activity of a given soln, increasing vols. (0–2 ml) were premixed with the subtilisin soln (1 ml, 20 nmol, pH 10.0) in a stoppered syringe for 1 min at 25° before adding to the 17 ml casein in the titration cell. The vol. of inhibitor soln needed to reduce the uninhibited rate of hydrolysis of casein by a factor of two was determined graphically (see Fig. 1) from which the inhibitor concn was estimated.

Activity of proteases other than subtilisin. Unfortunately the above technique was not satisfactory at pH 7, which was chosen as a single suitable pH at which to investigate the inhibition of several other proteolytic enzymes. In these cases the method of Kunitz [37] was used with casein as the substrate. The unhydrolysed protein was ppted with buffered TCA as recommended by Hagihara *et al.* [38] and the *A* of the supernatants measured at 280 nm. For this reason the presence of rubber particles or UV-absorbing compounds was precluded from incubations of this type.

Isolation of inhibitor. Freeze-dried serum (150 g) was dispersed in 1400 ml air-free H_2O and the pH reduced to 4.0 with 1 M HCl. Inactive protein, containing some small particulate rubber left behind by the original centrifugation of the latex, was removed and the pH of the supernatant raised to 7.0 before adding $(NH_4)_2SO_4$ to give 35% satn and a further ppt of inactive protein. The fraction ppted between 35 and 55% satd $(NH_4)_2SO_4$ was recovered and dissolved in 0.01 M cysteine soln at pH 7 for chromatography.

Gel-filtration chromatography. Columns of Ultrogel AcA-54 (100 × 5 cm) were equilibrated with 0.145 M Na_2HPO_4 plus 0.01 M cysteine adjusted to pH 7 before use. All chromatography was carried out at 2° and the effluents were monitored continuously at 280 nm. The protein was recovered from the active fraction by $(NH_4)_2SO_4$ pptn, with subsequent removal of the salt by Sephadex G-25 chromatography using 0.01 M cysteine at pH 7 as elutant.

Ion-exchange chromatography. Carried out on a column of quaternary aminoethyl Sephadex A-25 (42 × 5 cm) with 0.05 M Tris–HCl buffer, pH 7.6, containing 0.01 M cysteine. A linear gradient up to 0.5 M NaCl was supplied by a Gilson Mixograd.

Preparative isoelectric focusing. Carried out in a horizontal bed (245 × 110 × 4 mm) of Sephadex G-75 (superfine), at pH 3.5–5.0, in an LKB Ampholine electrofocusing kit, according to the manufacturers instructions. The catholyte and anolyte were, respectively, 0.1 M NaOH and 0.05 M DL-glutaric acid [39], and the pH gradient was established along the length of the tray at 2° by applying an initial potential of 200 V increasing to 480 V over 2.5 hr, and then left to equilibrate overnight at this potential. An area of gel across the width of the bed, between 4 and 8 cm from the cathode, was removed and replaced by a mixture of 1 g dry Sephadex G-75, 20–23 ml of inhibitor soln from the previous stage and 1 ml Ampholine buffer soln (pH 3.5–5.0). The voltage was applied for a further 24 hr and two 1 cm strips of dry filter paper pressed onto the surface of the gel along the two sides. The positions of the protein bands were then established by staining the strips with Coomassie blue and washing in MeOH–H_2O–HOAc (5:5:1). The gel-bed was then cut up into 30 segments using the fractionating grid supplied with the apparatus, and the inhibitor extracted with 0.005 M cysteine at pH 7. The soln was finally concd under N_2 and the Ampholine removed on a column of Sephadex G-25 with 0.01 M cysteine at pH 7 as elutant.

Analytical electrofocusing. Carried out in polyacrylamide gel sheets containing Ampholine buffers. Commercial PAG-plates (LKB, 120 × 110 × 1 mm) were used for the pH range 3.5–9.5, and for narrow range work (pH 2.5–5.0) 115 × 250 × 1 mm sheets of 5% w/v polyacrylamide, 3% crosslinked with the aid of riboflavin, were prepared in the laboratory [40]. The samples of protein soln to be analysed were applied to the gel surface in pieces of Whatman 3 MM filter paper (10 × 5 mm) using ovalbumin and bovine serum albumin as standards. Electrophoresis was carried out overnight in a refrigerator with initial and final voltages of 200 and 500 V. The temp., as measured in the gel at the end of the expt using a thermocouple, was 5°. The pH gradient throughout the gel was determined by cutting sections along the side and determining the pH with a glass electrode on the samples dispersed in a small vol. of CO_2-free H_2O at room temp. Staining of the protein bands in the focused gels was normally carried out using Coomassie blue [41], but for the purpose of estimating the purity of the final inhibitor, bromophenol blue (1% in EtOH–HOAc–H_2O, 15:4:21) was used, as the Coomassie blue proved difficult to extract from the gel using 0.1 M NaOH; the blue colour was measured at 575 nm.

Element analysis. C, H and N were measured on a Perkin-Elmer Analyser, Model 240, and S was estimated by oxygen flask combustion followed by $Ba(ClO_4)_2$ titration.

UV analysis. Samples of HP–In were dissolved in 0.1 M NaOH or 1.4 mM mercaptoethanol soln adjusted to pH 7, and the spectrum measured from 230 to 325 nm in 1 cm cells. The protein concn of the solns was measured by Kjeldahl N using a factor of 6.17.

Amino acid analysis. The purified HP–In was hydrolysed in 6 M HCl at 105° for 20 hr and analysed on a Durrum D-500 instrument. The tyrosine and tryptophan contents were calculated from the spectral data obtained in 0.1 M NaOH soln by the methods of Beaven and Holiday [42] and Bencze and Schmid [43].

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