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A Chymotrypsin Inhibitor from the Parasitic Nematode, *Oesophagostomum radiatum*

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Abstract

An inhibitor of α-chymotrypsin was isolated from *O. radiatum* by affinity chromatography, as well as by ion-exchange and molecular sieve chromatography. The inhibitor was associated with the cuticle or musculature of the parasite. It completely inhibited the esterolytic activity of chymotrypsin. Molecular sieve chromatography gave an apparent molecular weight of 9700 for the inhibitor alone and 32000 for the complex with chymotrypsin. This suggests reaction in a 1:1 molar ratio. The inhibitor reacted with diisopropyl phosphoryl-chymotrypsin weakly, if at all.

Measurement of the dissociation constants for the enzyme–inhibitor complexes gave values of $2{\cdot}2\times10^{-9}$ M for the product of affinity chromatography but less than 2×10^{-10} M for all other preparations. Possible explanations of this are discussed.

Introduction

Despite the extensive literature on naturally occurring inhibitors of proteolytic enzymes, little attention has been paid to their occurrence in endoparasites. Although, from the few studies which have been undertaken, it is tempting to speculate that such inhibitors could have an important function as a defence against the host's digestive system, so little data are available that the question of this importance remains open (von Brand 1973). The only parasite in which inhibitors have been studied in detail is *Ascaris lumbricoides*. Several trypsin and chymotrypsin inhibitors have been well characterized (Kassell 1970) and the parasite contains four pepsin inhibitors as well (Abu-Erreish and Peansky 1974). The inhibition of chymotrypsin and trypsin by extracts of *Stephanurus dentatus* has also been observed, although the inhibitors have not been characterized (Rhoads and Romanowski 1974). Other reports indicate that such inhibitors may be widespread (Reichenbach-Klinke and Reichenback-Klinke 1970; Pappas and Read 1972*a*, 1972*b*). In general, however, this field has been curiously neglected.

One reason for this neglect is obvious. With the exception of *A. lumbricoides*, which is readily obtainable in kilogram quantities, the amount of parasite material available for study is often very small, making protein purifications difficult. Affinity chromatography, which is now commonly used for the purification of proteolytic enzyme inhibitors, would seem to have great advantages for such purifications since it normally gives high yields coupled with high purification factors and is readily adapted for small quantities of material. As this work will show, however, the method cannot be used uncritically with previously unstudied inhibitors. The present paper has two purposes. It reports the occurrence and partial characterization of a chymotrypsin inhibitor in *Oesophagostomum radiatum*, a parasitic nematode of

cattle. Secondly, during this investigation, it became apparent that there were slight but significant differences between inhibitors isolated by affinity chromatography and by more conventional techniques. A comparison is therefore given of three different preparations of the *O. radiatum* inhibitor.

Materials and Methods

Enzymes and chemicals were obtained from Sigma Chemical Corp. with the exception of the radiolabelled compounds, which were from the Radiochemical Centre, Amersham, England. Adult *O. radiatum* were obtained 6–7 weeks after infection of calves as described previously (Keith and Bremner 1973). The rearing of cattle under worm-free conditions, infection with *O. radiatum*, and subsequent collection of the worms took 3–4 months and produced about 1 g wet weight of material per calf.

Assay of Chymotrypsin and Chymotrypsin Inhibitor

Chymotrypsin was assayed at pH 7·2 in 0·1 M phosphate buffer, 10% (v/v) in dimethylformamide, containing $3{\cdot}7\times10^{-5}$ M *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester (L-CTN). The release of *p*-nitrophenol was measured at 400 nm and 25°C on the 0–0·1 absorbance scale of a Varian 635M spectrophotometer. The molarity of active enzyme was measured by titration of the enzyme with *p*-nitrophenyl acetate at pH 7·8 (Kézdy and Kaiser 1970).

In a typical inhibitor assay, a 5–100-μl aliquot of the solution to be tested was added to 100 μl of a solution of chymotrypsin in 0·1 M phosphate buffer, pH 7·5, containing bovine serum albumin (BSA) at a concentration of 1 mg/ml. The final volume was always adjusted to 200 μl by addition of 0·1 M phosphate buffer containing BSA. The chymotrypsin concentration in the final solution was usually $1–1{\cdot}5\times10^{-7}$ M. At this chymotrypsin concentration, the inhibition reaction was too rapid to be followed, but solutions were routinely left for at least 10 min before assaying a 25- or 50-μl aliquot for residual chymotryptic activity. The inhibitory activity was measured by the difference between the test assay and a control assay of chymotrypsin diluted in buffer only.

For an accurate measurement of inhibitor concentration, increasing volumes of the test solution were mixed with a constant amount of chymotrypsin, and the plot of residual activity was extrapolated to complete inhibition. A typical result for the assay of a crude worm extract is shown in Fig. 1. The amount of inhibitor which just completely inhibits a given amount of enzyme will be referred to as one equivalent. A unit of inhibitor was taken as the amount necessary to inhibit 1 nmol of active chymotrypsin. The specific activity is given as the inhibitor concentration divided by the absorbance at 280 nm of the solution.

Protein concentrations were measured by the absorbance at 280 nm. Amounts of protein are given as A_{280} units, that is as the product of the volume of the solution by its absorbance at 280 nm in a 1-cm cell.

Preparation of [^3H]Dinitrophenyl (DNP)-chymotrypsin

1-Fluoro-2,4-dinitro-3,5[^{3}H]benzene with a specific activity of 19 Ci/mmol was supplied as a solution 0·5 mCi/ml in benzene. A 100-μl aliquot in a small tube was dried very slowly on a warm water-bath, then 1·5 mg of chymotrypsinogen in 200-μl of 1 M sodium bicarbonate was added and the solution incubated for 12 h at 37°C. After addition of 1 ml of 0·1 M tris buffer, pH 7·7, the solution was dialysed against the tris buffer, made 10 mM in calcium ion and cooled to 4°C. The incorporation of tritium into non-dialysable material at this stage was 58%. Trypsin, 80 μg, was added and after 2 h, when the activation was complete, the solution was dialysed against 1 mM HCl to give a product with a specific activity of $3{\cdot}9\times10^6$ cpm per milligram protein. These are the conditions for the rapid activation of chymotrypsinogen (Wilcox 1970). Radioactivity was measured in a Packard 3320 liquid scintillation spectrometer.

Preparation of Partially Inhibited [^3H]Diisopropyl Phosphoryl (DIP)-Chymotrypsin

Partially inhibited chymotrypsin was prepared by reacting the enzyme with less than a stoichiometric amount of diisopropyl phosphorofluoridate (DFP). [^{3}H]DFP, 0·11 μmol containing 0·48 mCi of tritium, was added to 20 mg of chymotrypsin in 10 ml 0·05 M phosphate buffer, pH 7·5. After 80 min, when approximately 12% of the enzymic activity had been lost, the solution was dialysed against cold buffer to give a product with a specific activity of $9{\cdot}4\times10^6$ cpm per milligram protein.

Preparation of DIP-Chymotrypsin

Chymotrypsin, 2 mg/ml in 0·1 M phosphate buffer, pH 7·5, was inactivated by addition of 10% (v/v) of an 11 mM solution of DFP in ethanol. After 30 min at 25°C, there was less than 0·1% activity remaining. Dialysis to remove excess DFP was carried out without detectable recovery of enzymic activity.

Preparation of Chymotrypsin–Sepharose 4B

The insolubilized enzyme derivative was prepared using cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions, except that a 1 M ethanolamine buffer, pH 8·5, was used to inactivate unreacted groups on the Sepharose. The initial coupling reaction was carried out for 3 h with 2 g of the Sepharose preparation in 10 ml of sodium bicarbonate solution, adding 30 mg of chymotrypsin together with 0·3 mg of [^{3}H]DIP-chymotrypsin as a tracer. The product, after being washed to remove non-covalently bound protein, showed the coupling of 81% of the initial protein present to give an enzyme concentration of 4·7 mg/ml of gel.

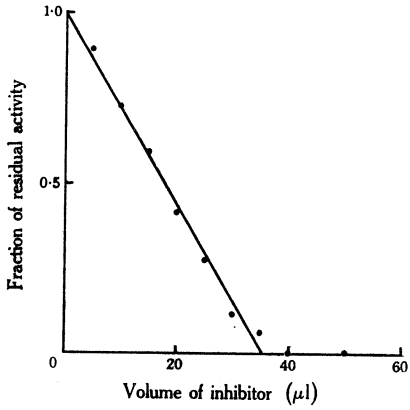

Fig. 1. Inhibition of α-chymotrypsin by a 1:4 dilution of a crude extract of *O. radiatum*.

Measurement of Molecular Weights by Gel Filtration

Molecular weights were determined as described by Andrews (1970), using a 0·9 by 26 cm column of Sephadex G100 Superfine in 0·1 M phosphate buffer, pH 7·5. Protein samples and Blue Dextran, in a total volume of 0·3 ml, were chromatographed at 4°C. The ratio of elution to void volume, V_e/V_0, was used in the calculations. Proteins used to calibrate the column were BSA, ovalbumin, bovine α-chymotrypsin, sperm whale myoglobin, and horse cytochrome *c*.

Results

Preparation of Chymotrypsin Inhibitor without Affinity Chromatography

Preparation A

O. radiatum, 1·36 g wet weight, were ground in a glass homogenizer, using 2 ml of 0·05 M phosphate buffer, pH 7·5, per gram of worms, centrifuged, and the extraction repeated with 1 ml per gram. The supernatants were combined, dialysed against the extraction buffer and chromatographed on a 2·5 by 34 cm column of Sephadex G200 in the same buffer. The fractions with the highest inhibitory activity were pooled and concentrated on an Amicon UM10 membrane. All steps in this and the following preparations were carried out at 4°C. A summary of the preparation is given in Table 1.

Preparation B

O. radiatum, 1·03 g, were homogenized in 10 ml of 0·05 M acetate buffer, pH 3·8, per gram of worms, centrifuged, and loaded onto a 2·5 by 10 cm column of carboxymethyl (CM)-cellulose equilibrated in the same buffer. After the column was washed

a 200-ml linear gradient, 0–0·5 M in sodium chloride in the pH 3·8 acetate buffer, was applied. The inhibitor was eluted at a salt concentration of 0·1 M. The most active fractions were pooled, concentrated on an Amicon UM05 membrane after addition of pH 7·5 phosphate buffer to a final concentration of 0·05 M, then chromatographed on a 2·5 by 36 cm column of Sephadex G50 Medium in 0·1 M phosphate buffer, pH 7·5. The most active fractions were once again concentrated on a UM05 membrane. The preparation is summarized in Table 1.

Table 1. Preparation of chymotrypsin inhibitor from *O. radiatum*

Purification step	Activity (A_{280} units)	Protein units	Specific activity	Purification factor	Yield (%)
Preparation A					
Crude extract	14·7	82	0·18	1	100
Final product	6·2	3·5	1·8	9·8	42
Preparation B					
Crude extract	15·8	78	0·20	1	100
CM cellulose	5·2	2·1	2·4	12	33
Sephadex chromatography	1·7	0·35	4·3	21	11
Affinity chromatography					
Crude extract	23·5	132	0·18	1	100
Final product	8·9	0·31	28	161	38

Preparation of Chymotrypsin Inhibitor by Affinity Chromatography

O. radiatum were extracted with 0·1 M phosphate buffer, pH 7·5, as described for preparation A to give 7 ml of solution from 1·3 g of worms. This was washed through a 2-ml column of chymotrypsin–Sepharose 4B, followed by 0·1 M phosphate buffer, pH 7·5. After the absorbance at 280 nm had fallen to a low value (0·04) the eluting buffer was changed to 0·1 M phosphate, pH 2·85.

Fractions collected through the loading and washing procedure were assayed for chymotrypsin inhibitor. The results are shown in Fig. 2 and Table 1. Of the inhibitor applied to the chymotrypsin–Sepharose column, 52% was eluted in the active fractions, the yield being reduced to 38% by pooling only the tubes containing the most activity. The product was adjusted to pH 6·8 and stored at 4°C.

Disc gel electrophoresis was carried out in 10% polyacrylamide using the system suggested by Gabriel (1971). The product of the affinity chromatography gave a single band after staining with Naphthalene Black 10B.

The three preparations listed in Table 1 involved a total of four chromatographic steps—two on Sephadex, one on ion-exchange cellulose, and one on an affinity column. In all of these, only a single peak of inhibitor was found, suggesting that only one active species is present.

Apparent Molecular Weight of the Inhibitor

The inhibitor was chromatographed on a standardized Sephadex G100 column as described in Materials and Methods. Two samples were used, 0·028 A_{280} units of preparation A and 0·009 A_{280} units of the inhibitor from the affinity chromatography. The former gave a V_e/V_0 ratio of 2·60 and the latter 2·64. These values are not significantly different. As an indication of the reproducibility of the results, six determinations of V_e/V_0 for chymotrypsin gave values ranging from 1·94 to 1·99

with an average of 1·96. The apparent molecular weight of the chymotrypsin inhibitor was calculated to be 9700.

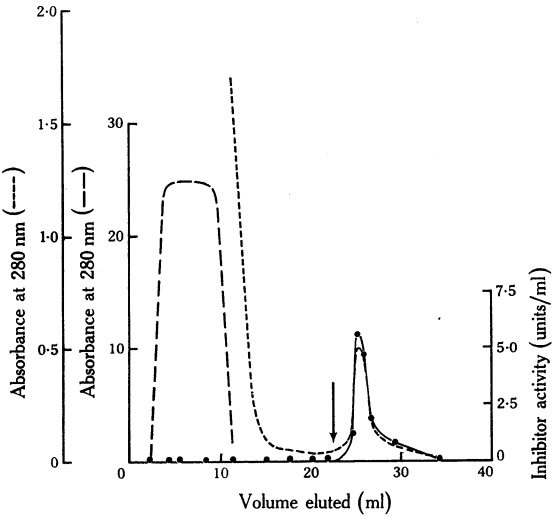

Fig. 2. Purification of *O. radiatum* chymotrypsin inhibitor by affinity chromatography on chymotrypsin–Sepharose. The vertical arrow shows the point at which a pH 7·5 phosphate buffer was replaced by one at pH 2·8. Absorbance at 280 nm on a scale of 0–20 (—— ——) and on a scale of 0–1 (-------); inhibitor activity (——●——).

Stoichiometry of the Reaction of Inhibitor with Chymotrypsin

A 0·78-μg sample of [^{3}H]DNP-chymotrypsin was reacted with 1·09 equivalents of preparation A. This was then chromatographed on G100 Superfine as described for the molecular weight determinations. Aliquots from each of the fractions were counted, and the results are shown in Fig. 3. The leading peak corresponds

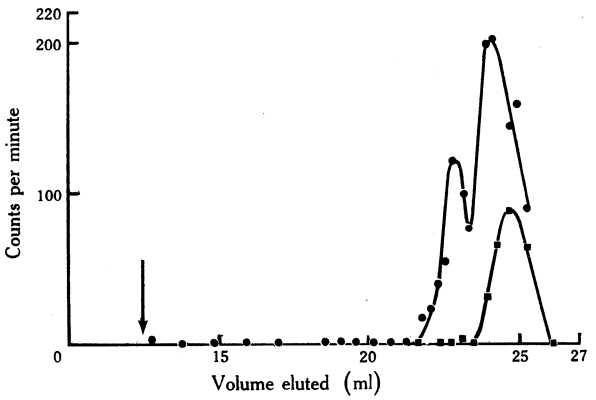

Fig. 3. Chromatography of [^{3}H]DNP-chymotrypsin alone (■) and of a mixture of [^{3}H]DNP-chymotrypsin and preparation A of the inhibitor (●) on Sephadex G100. The void volume is shown by the vertical arrow.

to a V_e/V_0 ratio of 1·82, and the second peak to a ratio of 1·92. The same amount of chymotrypsin, chromatographed alone, gave a single, symmetrical peak of radioactivity with V_e/V_0 ratios in duplicate determinations of 1·97 and 2·00. The experiment was repeated, using 1·16 equivalents of the inhibitor from affinity chromatography and 2·3 μg of [^{3}H]DNP-chymotrypsin. The result was essentially the same, the V_e/V_0 ratios being 1·84 and 1·92. The slower-eluting tritiated material, which is probably free chymotrypsin, may arise either by slow dissociation of the inhibitor–enzyme complex, or from tritiated protein which is incapable of reacting

with inhibitor, or by a combination of both effects. The fact that the leading peak is found only in the presence of inhibitor suggests it is the enzyme–inhibitor complex. The apparent molecular weight, 32 000, is in reasonable agreement with that expected for formation of a 1:1 molar complex—34 200.

Reaction of Inhibitor with DIP-Chymotrypsin

Chymotrypsin, partially tritiated and 12% inhibited, was prepared as described in Materials and Methods. The amount of inhibitor necessary to inhibit the unphosphorylated enzyme was measured as usual. Then 3·5 μg of chymotrypsin and 1·05 equivalents of preparation A were mixed and chromatographed on Sephadex G100 as described for the molecular weight determinations. In duplicate experiments, the tritium label was eluted as a single peak with V_e/V_0 ratios of 1·94 and 1·99. No measurable enzymic activity was recovered. Chromatography of the same amount of chymotrypsin in the absence of inhibitor gave V_e/V_0 ratios of 1·94 and 1·96, the enzymic activity and tritium label being, as expected, coincident.

In a second experiment, 0·007 A_{280} unit samples of chymotrypsin inhibitor, preparation A, were incubated at 25°C with aliquots of 0·3–6·5 μg of non-radioactive but enzymically inactive DIP-chymotrypsin, in a total volume of 200 μl of 0·1 M phosphate buffer, pH 7·5. After 30 min, 10 μl of active chymotrypsin solution containing 0·83 μg of enzyme was added to each sample. After 20–40 min, there was no more than 1% activity in any sample. The amount of inhibitor used in this experiment was sufficient to completely inhibit 1·04 times the amount of active chymotrypsin finally added. Clearly, pre-incubation of the inhibitor with DIP-chymotrypsin did not markedly reduce its ability to neutralize native enzyme.

These two experiments demonstrate that the reaction of the inhibitor with DIP-chymotrypsin, if it occurs at all, must be much more freely reversible than that with the native enzyme.

Estimation of Equilibrium Constants for the Reaction of Chymotrypsin with Various Inhibitors

The most sensitive way of detecting differences between inhibitor preparations is by measuring the dissociation constants of the enzyme–inhibitor complexes. To do this, three pieces of information are necessary—the stoichiometry of the reaction, the concentration of enzyme, and the concentration of the inhibitor. The first, from the chromatography on Sephadex, is known to be a 1:1 molar ratio of inhibitor to enzyme. The second was measured, as before, by titration of chymotrypsin, and the third was measured, as described in Materials and Methods, by mixing inhibitor and enzyme at sufficiently high concentration so that the reaction effectively goes to completion.

The concentration of each inhibitor was measured by mixing aliquots of inhibitor with chymotrypsin at a final concentration of $1{\cdot}45\text{–}1{\cdot}5 \times 10^{-7}$ M. The enzyme was then diluted to approximately $1{\cdot}0 \times 10^{-8}$ M and incubated with a range of inhibitor concentrations for between 10 min and 1 h at 24°C. All solutions were in 0·1 M phosphate buffer, pH 7·2, containing BSA at 1 mg/ml. Residual activity was then measured with 200-μl aliquots in the normal assay system. The residual activity was independent of the time of incubation in the range above. Theoretical curves for the equilibrium were calculated assuming formation of a 1:1 molar complex between enzyme and inhibitor in a freely reversible reaction. The results for the three

preparations of inhibitor are shown in Fig. 4. As can be seen, if such a reaction occurs for preparations A and B, the dissociation constant must be less than 2×10^{-10} M, whilst for the material from affinity chromatography, it is approximately $2{\cdot}2 \times 10^{-9}$ M. To show that there was a genuine equilibrium in the latter case, chymotrypsin was mixed with 1·25 equivalents of inhibitor in the above buffer to give a final (inhibited) enzyme concentration of $1{\cdot}5 \times 10^{-7}$ M. Then 50-μl aliquots were diluted into 2 ml of the pH 7·2 buffer at 25°C, and the active enzyme measured subsequently by adding 15 μl of 3 mM L-CTN solution. The initial activity was less than 3% of that of the uninhibited enzyme. After 8 min, it had reached a stable value of 42%. The value predicted from a dissociation constant of $2{\cdot}2 \times 10^{-9}$ M is 46%.

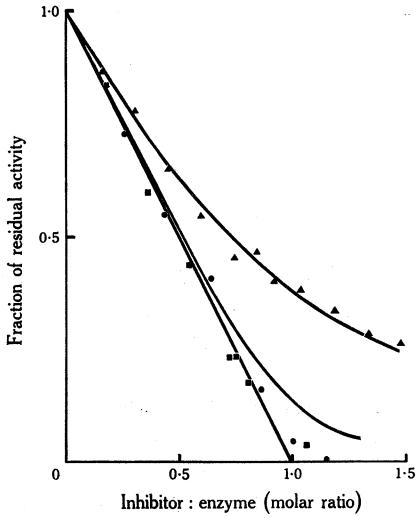

Fig. 4. Measurement of equilibrium constants for the reaction of chymotrypsin inhibitor with enzyme. ■ Preparation A. ● Preparation B. ▲ Product of affinity chromatography. The lines shown are, in order of decreasing curvature, calculated for a dissociation constant of $2{\cdot}2 \times 10^{-9}$ M for the product of affinity chromatography, for a dissociation constant of 2×10^{-10} M for preparation B, and for a dissociation constant of zero.

The different behaviour of the material from affinity chromatography could be due either to the effect of exposure to low pH, or to some variability in batches of *O. radiatum*. To test the effect of exposure to low pH, preparation A was kept in 0·1 M phosphate buffer, pH 3·1, for 91 h at 4°C, a longer time than that used in affinity chromatography. The dissociation constant measured subsequently was unchanged. In case there were variations between batches of *O. radiatum*, the crude extract from the affinity preparation was used, as above, to measure the dissociation constant of the inhibitor complex. It was again less than 2×10^{-10} M. Thus neither of these possibilities accounts for the dissociation constant of the affinity product.

Tissue Distribution of Inhibitory Activity

Twelve worms were dissected into heads, excretory glands, intestines, gonads, and a fifth fraction containing the residual material. Each fraction was homogenized in a total of 1·5 ml 0·1 M phosphate buffer, pH 7·5, centrifuged to remove particulate material and assayed for inhibitory activity. The results are listed in Table 2.

The inhibitor occurred mainly in the 'residue', a fraction containing the cuticle, hypodermis, and musculature. The distribution of inhibitor is thus similar to that found in *Ascaris suum* (Rhodes *et al.* 1973) but different from that in *Stephanurus dentatus* (Rhoads and Romanowski 1974) where the inhibitor is chiefly in the excretory glands.

Discussion

If a particular biochemical activity is found in a range of organisms, it is reasonable to consider that that activity is functionally important. The fact that *O. radiatum* contains a low-molecular-weight, stable, specific chymotrypsin inhibitor adds it to the short list of parasites for which such activity has been reliably demonstrated. The point is of interest in view of the belief that it is the impermeability of the cuticle of such parasites, rather than the presence of enzyme inhibitors, which protects them from digestion (von Brand 1973).

Table 2. Tissue distribution of inhibitory activity

Tissue	Total inhibitor in this tissue (%)	Specific activity
Heads	4	0·30
Excretory glands	0	0
Gonads	14	0·50
Intestines	3	0·09
Residue	79	1·82

The comparison of three inhibitor preparations was an interesting comment on purification procedures. There would seem to be compelling reasons for using affinity chromatography in the purification of inhibitors such as the one examined here. The procedure used—attachment of the inhibitor to an insolubilized enzyme followed by elution at low pH—has been used by a number of authors, for example Fritz *et al.* (1967, 1969), Turková *et al.* (1973), and Feinstein *et al.* (1974). In the present case the inhibitor prepared by affinity chromatography has been compared with the material isolated in more conventional ways. Preparation A is the least purified one, but the simplicity and avoidance of low pH in its isolation mean it should be unmodified by the isolation procedure. Preparation B is the result of a conventional purification and could clearly be purified further, though the small amount of material available is disadvantageous. The inhibitor prepared by affinity chromatography, although indistinguishable from these by the size of either the inhibitor alone or the complex formed with chymotrypsin, is different as shown by measurement of the equilibrium constants for dissociation of the chymotrypsin–inhibitor complex. Two possible explanations for the difference have been tested and discarded. The difference cannot be due to the presence of more than one inhibitor. Since 52% of the inhibitory activity is recovered from the affinity column, measurement of the dissociation constant with a crude extract would have to show the presence of lower affinity material, if it occurred. This is not found. Consequently it is suggested that the inhibitor is irreversibly modified on formation of the complex with chymotrypsin.

Two kinds of similar behaviour have been reported. In inhibitors with independent trypsin- and chymotrypsin-reactive sites, one may be modified but not the other (Belew and Eaker 1976). Alternatively, temporary inhibition may occur (Tschesche 1967; Fritz *et al.* 1968; Tschesche and Klein 1968). In the present case, however, the change in the inhibitor is minimal and is seen as a decreased affinity for the enzyme which would not be detected in a normal assay. An analogous isolation from pig pancreas of a modified trypsin inhibitor with reduced, but still high, affinity for enzyme has been reported, but its formation was transient (Tschesche and Klein 1968).

The present report suggests that retention of activity in inhibitors isolated by affinity chromatography is not, in itself, evidence that the isolated form is the native one.

Acknowledgments

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