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Evidence That Cocoonase and Trypsin Interact with Soybean Trypsin Inhibitor at the Same Reactive Site*

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ABSTRACT: Incubation of soybean trypsin inhibitor with catalytic quantities of cocoonase leads to the appearance of a new band in disc gel electrophoresis suggesting cleavage of one peptide bond. Sephadex chromatography of the reduced, carboxymethylated product allows separation into three components whose elution volumes and amino acid analyses correspond to reduced, carboxymethylated virgin inhibitor and to reduced carboxymethylated large fragment and reduced carboxymethylated small fragment of trypsin-modified inhibitor. At neutral pH cocoonase catalyzes the establishment of the equilibrium between trypsin-modified soybean trypsin inhibitor and virgin soybean trypsin inhibitor. Cocoonase-modified soybean trypsin inhibitor is quantitatively converted into virgin soybean trypsin inhibitor by formation of a complex with trypsin and subsequent rapid dissociation of this complex. Cocoonase, like trypsin, is inhibited by virgin soybean trypsin inhibitor but not by des-Arg(64)-modified soybean trypsin inhibitor. On these bases it is concluded that cocoonase interacts with soybean trypsin inhibitor at the same reactive site as does trypsin.

Kafatos *et al.* (1967a,b) have isolated an interesting new proteolytic enzyme, cocoonase, from the mouth parts of silk moths. On the basis of the substrate specificity (for Arg and Lys bonds) they have characterized this enzyme as trypsin-like. Inhibition by diisopropyl phosphofluoridate shows it to be a serine esterase. The analogy to trypsin was extended further since cocoonase, like trypsin, is inactivated by tosyllysine chloromethyl ketone and is moderately strongly inhibited by soybean trypsin inhibitor ($K_{assoc} = 1 \times 10^7$). This association constant is intermediate between that for the interaction of the inhibitor with chymotrypsin and with trypsin.

The interaction of cocoonase with soybean trypsin inhibitor was of considerable interest to us since we have previously shown that incubation of soybean trypsin inhibitor with catalytic quantities of trypsin leads to cleavage of a single Arg (64)–Ile peptide bond (Figure 1) and that this bond is the reactive site involved in the trypsin–inhibitor combination (Finkenstadt and Laskowski, 1965; Ozawa and Laskowski, 1966). A gift of a small quantity of cocoonase offered us an opportunity to determine whether cocoonase also cleaves a single reactive-site peptide bond in STI and whether the reactive sites for trypsin and for cocoonase are the same.

Experimental Section

Materials and Methods. Virgin soybean trypsin inhibitor (Kunitz, 1947), special grade, lot B 7303 (selected after extensive purity testing of commercially available lots), was obtained from Gallard–Schlesinger Chemical Corp.; 5 mg of cocoonase (*Antheraea polyphemus*) was a gift of Dr. John H. Law of the University of Chicago. Bovine trypsin (EC 3.4.4.4), lot TRL71C was obtained from Worthington Biochemical Corp. Glycine, acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and naphthol blue black were purchased from Eastman Organic Chemicals. Sephadex G-200 and G-75 were obtained from Pharmacia Fine Chemicals. Tris(hydroxymethyl)aminomethane, primary standard, was purchased from Fisher Scientific, and 2-mercaptoethanol and iodoacetic acid were purchased from Matheson Coleman & Bell. *p*-Tosyl-L-arginine methyl ester was a product of Mann Research Laboratories. All other chemicals were reagent grade.

All pH measurements were made using a Radiometer pH

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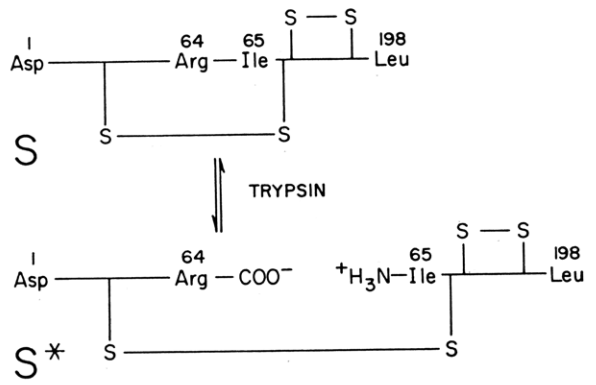

FIGURE 1: Chemical event occurring on tryptic conversion of virgin into modified soybean trypsin inhibitor according to Ozawa and Laskowski (1966).

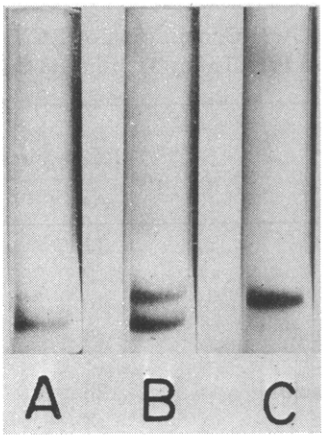

FIGURE 2: Polyacrylamide disc gel electrophoresis patterns of: (A) trypsin-modified soybean trypsini nhibitor; (B) cocoonase-modified soybean trypsin inhibitor (this reaction clearly has not proceeded to equilibrium; see text; (C) cocoonase-modified soybean trypsin inhibitor in which the cleaved reactive site bond has been resynthesized by trypsin according to the procedure of Finkenstadt and Laskowski (1967) (see text).

meter, Model TTT1A. All protein concentrations were determined with a Hitachi–Perkin–Elmer spectrophotometer. Optical factors (at 280 mμ) used were 0.651 mg ml^{-1} (optical density unit)$^{-1}$ for trypsin (Worthington, 1967) and 1.10 mg ml^{-1} optical density unit)$^{-1}$ for virgin and modified inhibitors (Kunitz, 1947). All proteins were dissolved in 0.5 M KCl and 0.05 M $CaCl_2$ solution. The buffer solution used for gel analysis was 0.025 M Tris and 0.2 M glycine (pH 8.4). Molecular weights used were 22,000 for soybean trypsin inhibitor[1] and 24,000 for trypsin.

Gel Analysis. Small pore polyacrylamide gels were prepared according to the formulation of Davis (1964). The analytical gel procedure of Niekamp *et al.* (1969) was used to detect the appearance of modified inhibitor and to determine the relative amounts of virgin and modified inhibitors (Figure 2).

Preparation of Modified Inhibitors. Equilibrium mixtures of virgin and modified inhibitor were prepared by incubation of virgin inhibitor with a catalytic amount of trypsin according to the method of Ozawa and Laskowski (1966). Mixtures of virgin and modified inhibitor were also prepared using very small amounts (1 wt %) of cocoonase. Screening of incubation pH values indicated that about pH 6 was the optimum region for cocoonase-catalyzed conversion of virgin into modified inhibitor. Below pH 6 the rate of conversion of virgin inhibitor into modified inhibitor increased but cocoonase stability decreased more rapidly. This pH dependence of stability is in accord with the observations of Kafatos *et al.* (1967b). Above pH 6 the rate of conversion was significantly slower. Even under optimum conditions a conversion into about 30% modified inhibitor was the best that could be achieved in a 6-week incubation at room temperature before cocoonase activity disappeared. Des-Arg(64)-modified STI was prepared by carboxypeptidase B treatment of modified inhibitor according to the method of Sealock and Laskowski (1969).

Substrate Activation and Inhibition Studies. Cocoonase hydrolyses of *p*-tosyl-L-arginine methyl ester were performed on a Radiometer TTT1 automatic titrator coupled with an Ole Dich syringe drive recorder. Reactions were pH-stated under a nitrogen atmosphere using 0.02 M KOH as a titrant. Typical reaction volumes were 7 ml. Inhibition studies were made using a pH-Stat method suggested by Green (1953). First, a normal cocoonase hydrolysis of *p*-tosyl-L-arginine methyl ester was performed. Then the same amount of cocoonase was injected into an identical *p*-tosyl-L-arginine methyl ester sample to which 1 ml of 1 mg/ml inhibitor solution had been added. The time course inhibition was then observed.

Preparation of RCM Proteins and RCM Protein Fragments. Reduction and carboxymethylation of inhibitor mixtures were carried out according to the procedures described by Crestfield *et al.* (1963). These RCM mixtures were then chromatographed on a column of Sephadex G-75 (2.5 × 110 cm) similar to that used by the above authors. The sample was not driven in with air but was allowed to flow into the column under 140-cm pressure head. To obtain good separations, slow flow rates, 6–8 ml/hr, were used and small fractions (80 drops) were collected.

Amino Acid Analysis of Protein Hydrolysates. The pooled fractions from the RCM separation were dried by lyophilization. The dried samples were dissolved in 2 ml of 6 N hydrochloric acid, evacuated, sealed, and hydrolyzed for 20 hr at 110°. The hydrolysates were then analyzed on the Beckman Model 120B analyzer. Replicate analyses were made. The small amount of samples made it impossible to vary hydrolysis times and to correct for incomplete hydrolysis or for destruction during hydrolysis. Calculations for each sample were made on the basis of the alanine values for each fragment determined by Ozawa and Laskowski (1966).

Results

Incubation of soybean trypsin inhibitor with catalytic quantities of cocoonase at pH 6 leads to a slow appearance of a new, faster moving band on disc gel electrophoresis (Figure 2). This band was indistinguishable from that of trypsin-modified soybean trypsin inhibitor (Arg(64)–Ile bond cleaved). However, since the basis of separation is presumably the difference in charge arising from peptide bond cleavage-(modified inhibitor is more negative at high pH since the newly

[1] Abbreviations used are: STI, soybean trypsin inhibitor, RCM, reduced, carboxymethylated.

TABLE I: Amino Acid Composition of RCM Virgin Soybean Trypsin Inhibitor and of RCM Fragments of Modified Soybean Trypsin Inhibitor Prepared by Trypsin and Cocoonase Incubation.

	RCM Virgin Inhibitor		RCM Small Fragment		RCM Large Fragment	
	A[a]	B[b]	Trypsin Modified[a]	Cocoonase Modified[a]	Trypsin Modified[a]	Cocoonase Modified[b]
Lysine	11	10.2	1	1.32	10	9.95
Histidine	2	2.49	0	0.13	2	2.54
Arginine	10	8.78	4	4.23	6	5.88
Carboxymethyl-cysteine	4	4.26	1	1.77	3	3.90
Aspartic acid	28	30.6	9	9.33	19	22.5
Threonine	8	8.3	5	4.40	3	2.75
Serine	12	12.2	4	3.76	8	8.10
Glutamic acid	21	22.6	6	5.70	15	18.6
Proline	11	10.2	4	4.11	7	7.67
Glycine	18	18.6	8	7.55	10	10.0
Alanine	9	9	3	3	6	6
Valine	15	11.7	3	2.34	12	9.54
Methionine	2	2.15	0	0.21	2	2.63
Isoleucine	15	13.6	6	4.27	9	8.61
Leucine	16	16.1	5	4.84	11	12.3
Tyrosine	4	4.3	3	2.32	1	1.38
Phenylalanine	9	9.4	2	2.02	7	7.78

[a] Ozawa and Laskowski (1966). [b] Analyses of peaks I, II, and III of Figure 3.

formed COO^- charge is no longer neutralized by the corresponding NH_3^+ charge) the same result would have been expected if any one peptide bond were cleaved. Indeed, Frattali and Steiner (1969) have shown that trypsin- and chymotrypsin-modified Bowman–Birk soybean inhibitors are electrophoretically indistinguishable under similar conditions even though the reactive sites for trypsin and chymotrypsin are different (Birk *et al.*, 1967). In order to show that peptide bond cleavage did take place and to roughly locate the split peptide bond, 100 mg of soybean trypsin inhibitor was incubated with catalytic quantity (~1 wt %) of cocoonase for 6 weeks, and showed 30% conversion into modified form. This mixture was reduced and carboxymethylated and the products were separated on a Sephadex column. A preparation containing 70% virgin and 30% trypsin-modified inhibitor was similarly treated. The elution patterns (Figure 3) are strikingly similar and show three major peaks: the first corresponds to RCM virgin inhibitor and the last to RCM small fragment of modified inhibitor. The amino acid analyses of the peaks obtained from the cocoonase incubation are compared with the results of Ozawa and Laskowski (1966) for trypsin incubation (Table I). The fair agreement suggests that cleavage took place at the same Arg(64)–Ile bond.

Several further tests for the identity of the reactive sites are also possible and are in fact more convincing than the product isolation and analysis. Virgin and trypsin-modified (Arg(64)–Ile-bond cleaved) inhibitors were shown to exist in an enzyme-catalyzed equilibrium (Niekamp *et al.*, 1969). Therefore, any enzyme which is capable of specifically cleaving this bond must also be capable of resynthesis of this bond in mixtures where this is required to restore equilibrium. At pH 3.75 the equilibrium fraction of virgin inhibitor is 14 ± 2%, but at pH 6 it is 25 ± 2% (Niekamp *et al.*, 1969). Therefore, a trypsin-modified inhibitor equilibrium mixture was prepared at pH 3.75 and was shown to contain 14 ± 2% of virgin inhibitor by disc gel electrophoresis. Trypsin was removed as trypsin–inhibitor complex by the method of Ozawa and Laskowski (1966). The completeness of trypsin removal was tested by incubating the mixture at pH 6 for 14 days without any change in the virgin: modified inhibitor ratio. To this sample a small quantity (~4 wt %) of cocoonase was added and after only 4 additional days of incubation the mixture had attained the expected 25% virgin, 75% modified composition and remained at this equilibrium composition (Table II). Thus cocoonase is clearly capable of resynthesis of the Arg(64)–Ile bond. If cocoonase were cleaving another peptide bond then the fraction of modified inhibitor should increase rather than decrease as observed here. It does at first seem surprising that equilibrium was attained so rapidly in this case, while addition of cocoonase to virgin inhibitor led to only 30% conversion into modified inhibitor (far from attainment of equilibrium) in 6 weeks. These observations become easier to understand when we recall that trypsin catalyzes the modified → virgin inhibitor conversion about an order of magnitude faster than the virgin → modified conversion at pH 4 (Niekamp *et al.*, 1969) and that this ratio becomes even greater at higher pH values (unpublished results from our laboratory). Evidently cocoonase shares with trypsin this faster rate of synthesis than of hydrolysis of the Arg(64)–Ile bond.

Finkenstadt and Laskowski (1967) have shown that trypsin can resynthesize the Arg(64)–Ile bond completely when *equimolar* quantities of trypsin are added to modified inhibitor to

TABLE II: Resynthesis of the Cleaved Reactive Site Bond of Trypsin-Modified Soybean Trypsin Inhibitor by Cocoonase at pH 6.0. Summary of Polyacrylamide Disc Gel Electrophoresis Analyses.

Time of Incubation (Days)	Fraction S
0	0.14
14	0.14
18 (4 days after cocoonase addition)	0.25
46	0.24

form trypsin–inhibitor complex and when the resultant complex is rapidly dissociated at strongly acidic solution (pH 2). Note that this is a very unusual reaction leading to a nonequilibrium distribution of virgin and modified inhibitors. It seems extremely unlikely that any other cleaved bond in soybean trypsin inhibitor could be resynthesized by trypsin in this manner. Therefore, an equimolar quantity of trypsin was added to a cocoonase-modified sample of soybean trypsin inhibitor (tube B in Figure 2) at pH 8. The pH was then rapidly lowered to 2 by addition of HCl and the inhibitor precipitated with $(NH_4)_2SO_4$. The isolated inhibitor was predominantly virgin (tube C, Figure 2) showing that the resynthesis took place and thus strongly indicating again that the cocoonase-cleaved bond was the same bond as is cleaved by trypsin.

A reciprocal experiment–resynthesis of the trypsin-cleaved bond by an equimolar quantity of cocoonase would clearly be interesting. However, it was not possible to carry it out because of exhaustion of the limited cocoonase supply.

The preceding experiments show that the cocoonase preparation cleaves in soybean trypsin inhibitor the Arg(64)–Ile peptide bond and no other bonds. However, a possible objection remains that the cleavage was caused not by the bulk of the cocoonase but by a minor contaminant present in this preparation. Two lines of argument refute this objection. Trypsin converts virgin into modified STI more rapidly at pH 4 than at pH 6. However, at pH 4 cocoonase rapidly looses activity (Kafatos *et al.*, 1967b). We have found that at pH 4 cocoonase initially starts to convert virgin into modified inhibitor rapidly but then the conversion ceases (cocoonase inactivation). This is in contrast to the trypsin-catalyzed conversion at pH 4 which continues to proceed until equilibrium is attained. Thus the enzyme responsible for the Arg(64)–Ile bond cleavage in cocoonase preparation shares with the bulk of cocoonase its loss of activity at low pH. Secondly, trypsin is inhibited by virgin trypsin soybean trypsin inhibitor but not by des-Arg-(64)-modified inhibitor. Therefore, we have studied the inhibition of cocoonase by virgin and des-Arg(64)-modified inhibitor. When cocoonase was added to 0.001 M *p*-tosyl-L-arginine methyl ester solution at pH 7 a constant rate of hydrolysis (zero order) was observed. On the other hand when cocoonase was added to 0.007 M *p*-tosyl-L-arginine methyl ester containing 0.1 mg/ml of virgin STI the same initial rate of hydrolysis indicated that virgin STI combines with cocoonase even in the presence of *p*-tosyl-L-arginine methyl ester. On the other hand when des-Arg(64)-modified inhibitor (~1 mg/ml) was added to 0.001 M *p*-tosyl-L-arginine methyl ester the rate

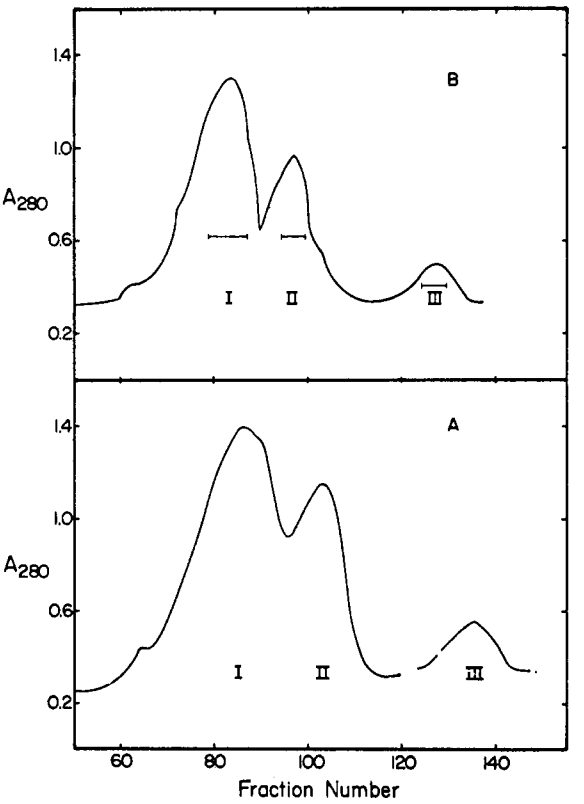

FIGURE 3: Chromatographic elution patterns of RCM mixtures of virgin and of modified soybean trypsin inhibitor. Effluent absorbance was monitored at 280 nm and 80 drop fractions were collected. (A) Mixture containing 70 mg of virgin and 30 mg of trypsin-modified inhibitor; (B) mixture containing 70 mg of virgin and 30 mg of cocoonase-modified inhibitor. Peaks I, II, and III are the virgin inhibitor, large fragment, and small fragment of Table I, respectively

of hydrolysis remained constant indicating no interaction with this inhibitor derivative. Finally, Hruska *et al.* (1969) have recently shown that the preparation of cocoonase employed by us contains only one kinetically distinguishable species capable of reacting with the specific trypsin acylating agent *p*-nitrophenyl-*p'*-guanidinobenzoate. This species is cocoonase itself and, therefore, the presence of other trypsin-like contaminants is highly unlikely.

Discussion

The striking similarity between cocoonase and bovine trypsin, clearly recognized by Kafatos *et al.* (1967a), is further extended by the observations reported in this paper. Cocoonase and bovine trypsin both interact with soybean trypsin inhibitor at the same reactive site (Arg(64)–Ile in the inhibitor molecule). Both cocoonase and trypsin, when added in catalytic amounts to solutions of virgin trypsin inhibitor, hydrolyze the Arg(64)–Ile bond. In view of the fact that cleaving the bond is reversible (Niekamp *et al.*, 1969), both enzymes must be capable of resynthesizing this bond in mixtures where this is necessary to restore equilibrium and both have been shown to do so. Both are inhibited by virgin inhibitor and neither is inhibited by des-Arg(64)-modified inhibitor. Thus the very close similarity extends to details of the inhibition mechanism.

Many protein inhibitors of proteolytic enzymes are capable of inhibiting more than one, and sometimes a great many, enzymes (*e.g.*, Vogel *et al.*, 1968). This inhibition clearly does not always take place at the same reactive site. Many cases are known where an enzyme A can be inhibited by a complex of the inhibitor with another enzyme B (and which is already saturated with respect to enzyme B). Such inhibitors have been named by Feeney "doubleheaded" (Rhodes *et al.*, 1960). The case which is probably most relevant to this paper is that of Bowman–Birk soybean inhibitor, which inhibits both trypsin and chymotrypsin on separate nonoverlapping reactive sites (Birk, 1968). This inhibitor can be trypsin modified by cleavage of a single peptide bond, which leads to alteration in the rate of reaction with trypsin (Birk *et al.*, 1967; Frattali and Steiner, 1969). Subsequent treatment with carboxypeptidase B eliminates the interaction with trypsin but both the trypsin-modified and the trypsin-modified, carboxypeptidase B-treated inhibitors retain their normal interaction with chymotrypsin (Birk *et al.*, 1967). Similarily modification with chymotrypsin leads to a striking reduction in the rate of chymotrypsin inhibition but leaves the interaction with trypsin unaltered.

On the other hand several inhibitors are also known (*e.g.*, Vogel *et al.*, 1968) where inhibition of one enzyme eliminates the ability to inhibit other enzymes, *i.e.*, cases in which the enzymes compete for the inhibitor molecule. This will be necessarily true when both enzymes interact with the inhibitor at the same reactive site but competitive behavior could also be observed when the two enzymes employ different reactive sites due to, for example, steric overlap (less likely but also possible are strong charge repulsions between the two enzymes or an important conformational change in the inhibitor molecule). It appears that it is experimentally very much easier to show that two enzymes utilize two distinct reactive sites on an inhibitor molecule than that they utilize the same one. Noncompetitive inhibition or unequal response to chemical modification of the inhibitor in its ability to bind the two enzymes clearly show that the reactive sites are distinct. On the other hand competitive inhibition or simultaneous loss of activity toward both enzymes as a result of chemical modification is indicative but not sufficient to prove that the same site is employed. In our opinion only the cleavage of a single, reactive site peptide bond by both enzymes and a demonstration that the same bond was cleaved by both offer sufficient proof.

The great success of various laboratories in obtaining new purified proteolytic enzymes from a variety of biological sources leads to an interesting but very complex classification and nomenclature problem. Obviously, a variety of approaches must be used to solve it, for example, the large number used by Kafatos *et al.* (1967a) in characterizing cocoonase. Among these approaches inhibition or lack of inhibition by both small specific organic inhibitors and natural protein inhibitors plays a major role. However, most of these approaches do not lend themselves to a simple yes/no type of answer but rather rely on relative k_{cat} and K_m values for various substrates or association rates and binding constants for the inhibitors. This can give rise to confusing results. Thus the association constant of cocoonase–STI system is closer to that of bovine α-chymotrypsin–STI than to that of bovine trypsin–STI system. Yet cocoonase and trypsin share the same substrate specificity and the same reactive site in STI. Determination of the reactive site for a particular enzyme–inhibitor interaction seems at the present stage to be a yes/no type characterization of specificity and, therefore, a valuable method for classification of specificity of proteolytic enzymes in those cases where specific cleavage of the inhibitor may be achieved.

After the cleavage is obtained with two enzymes, proof of identity or nonidentity of the two modified inhibitors is required. The reduction, carboxymethylation, separation, and analysis of fragments are relatively laborious procedures requiring rather large quantities of inhibitor and especially of enzyme. Furthermore, in order to positively prove that the cleavage occurred at the same site rather than at a closely adjacent one very accurate analytical data are required. Certainly, the data presented in Table I do not have the required accuracy. However, such an analysis is probably needless. We feel that the strongest proofs for identity of the cocoonase and trypsin reactive sites in STI presented in this paper are the resynthesis by catalytic quantities of cocoonase of the trypsin-cleaved bond in STI and the resynthesis of the cocoonase-cleaved bond by combination with equimolar quantity of trypsin. We further feel that these proofs would be sufficient had they been presented alone. Since such proofs are relatively easy to obtain and because the great sensitivity of disc gel electrophoresis requires small quantities of material they should be preferred.

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