Immunological Cross-Reaction between Trypsin and Chymotrypsin as a Guide to Structural Homology*

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ABSTRACT: As a test for the conformational homology between bovine trypsin and chymotrypsin in solution, their immunological cross-reactivity was examined in order to identify the regions in chymotrypsin which are responsible for the cross-reaction with anti-trypsin. A 42-residue peptide from a peptic digest of chymotrypsin, including residues 131–141, 190–207, and 217–229 linked together by two disulfide bonds, has been isolated by complexing with anti-trypsin antibodies. The complex was precipitated with ammonium sulfate, dissociated in acid, and the peptides were separated from the antibodies on Sephadex. The 42-residue peptide binds both to anti-trypsin and anti-chymotrypsin, as do the two fragments of the peptide derived from cyanogen bromide cleavage at Met-192. A hexapeptide including residues 195–200 was also isolated by this method. A common feature of these peptides is the presence in each of one of the prolyl residues homologous in trypsin and chymotrypsin. These experiments provide evidence that common antigenic determinants can be present in structures homologous in the chemical sense.

Specific antibodies can be produced against most proteins. Usually they comprise a heterogeneous population of different species with specificity directed toward various determinants on the surface of the protein antigen. The exact features and structural requirements of antigenic determinants have been the subject of numerous studies with synthetic antigens (Sela, 1966) as well as protein conjugates (Boyd, 1962); however, little is known about determinants present in native proteins. Several studies directed toward this question have been reported including those by Crumpton (1967), Crumpton and Wilkinson (1965), and Atassi and Saplin (1968) on sperm whale myoglobin, by Shinka *et al.* (1962, 1967a,b) on egg-white lysozyme, and by Benjamini *et al.* (1964) on tobacco mosaic virus protein. These were carried out by the fractionation of an enzymatic digest of the particular protein and the isolation of the immunologically active fragments. In this way several determinants which are in part responsible for the antigenic specificity of these proteins were defined.

Immunological cross-reaction of proteins may be taken as an indication of structural resemblance between the proteins in question (Kabat, 1961) or, in other words, for the presence of similar antigenic determinants on the cross-reacting antigens. The implication is that these determinants have the capacity to react with antibodies elicited by either protein. Such cross-reactions have been demonstrated in the cases of several proteins, including hen and duck egg-white lysozyme (Fujio *et al.*, 1962), human and bovine serum albumin (Melcher *et al.*, 1953), and papain and chymopapain (Arnon and Shapira, 1968). However, no attempt has been made in any of these cases to identify the regions in the molecules that are responsible for the cross-reaction. Reported here is a study with another pair of immunologically related proteins, namely, trypsin and chymotrypsin.

The notion that bovine trypsin and chymotrypsin have very similar tertiary structures (Neurath *et al.*, 1967; Sigler *et al.*, 1968) is now generally accepted. The sequences of the enzymes have been shown to be 40% homologous (Walsh and Neurath, 1964; Hartley *et al.*, 1965; Mikeš *et al.*, 1966). Both proteins have a unique serine in an identical hexapeptide sequence that can be alkylated with DFP with concomitant loss of activity (Jansen and Balls, 1952). This same serine residue has been shown to be the site of the acylated intermediate in the catalytic mechanism (Oosterbaan *et al.*, 1962). A specific histidine located in a highly homologous seventeen amino acid disulfide loop has been indicated by Hartley (1964) and confirmed by Shaw *et al.* (1965) and Ong *et al.* (1964) to be a second component of the active site in both enzymes. Four of the disulfide bonds present in both zymogens are in homologous loci (Brown and Hartley, 1966; Kauffman, 1965). Further, the two disulfides present only in trypsin can be placed without significant distortion if the trypsin sequence is superimposed on the X-ray structure of chymotrypsin (Neurath *et al.*, 1967; Sigler *et al.*, 1968). Evidence for immunological similarity between these two proteins has been presented previously (Arnon and Schechter, 1966). Although direct cross-precipitation could not be demonstrated it was shown that antibodies against trypsin could inhibit the catalytic activity of chymotrypsin.

All this information prompted the study of the cross-reaction between bovine trypsin and chymotrypsin in an effort to define and characterize areas in chymotrypsin responsible for the similarity in structure between the two enzymes. To ensure the exclusive isolation of the cross-reacting peptide fragments, antibodies to trypsin were used

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for the selective binding of immunologically active fragments of chymotrypsin, and similarly antibodies against chymotrypsin were used for the binding of trypsin fragments. In this manner a selective isolation of peptides from chymotrypsin, containing the common antigenic determinants for the two enzymes, was achieved.

Materials

Bovine trypsin (twice crystallized, lot no. TR 7JA), bovine α-chymotrypsin (three-times crystallized, lot no. CD1 6164), and pepsin (twice crystallized, lot no. PM 691) were purchased from Worthington Biochemical Corp. Hammarsten quality casein was purchased from Mann Research Laboratories. Sephadex G-75, G-25, G-10, and G-100 were products of Pharmacia. All other chemicals used were reagent grade.

Methods

Enzymatic Digestion. Trypsin or chymotrypsin at a concentration of 10 mg/ml in 5% formic acid was digested at 37° with pepsin at a 1:20 weight ratio. After 8 hr another aliquot of pepsin in a 1:60 weight ratio was added and the digestion was continued overnight. The digest was lyophilized and stored as a powder.

Radioactive Labeling. Trace labeling of tyrosine residues with ^{125}I (Mallinckrodt) was carried out as described by Talmadge and Claman (1967). In the case of peptide labeling 0.5–1.0 μmole of peptide was reacted with 0.1 mCi of ^{125}I in a total volume of 1.0 ml and the products were separated on a 1.5 × 30 cm column of Sephadex G-10 equilibrated with 1% acetic acid.

^{14}C-Acetylated peptide was prepared by reacting a 10-fold molar excess of [^{14}C]acetic anhydride (New England Nuclear) with the peptide in 1.0 ml of 0.05 M potassium phosphate buffer (pH 8.0) with stirring at room temperature for 2 hr (Fraenkel-Conrat and Colloms, 1967). The reaction was stopped by acidification to pH 2 with glacial acetic acid and the peptide was separated from the reagents on a 1.5 × 30 cm column of Sephadex G-10 equilibrated in 1% acetic acid.

Cleavage with Cyanogen Bromide. Peptides (at 0.5 μmole/ml) were cleaved at the methionyl residue using 0.18 M CNBr at room temperature in 70% formic acid for 18 hr according to the method of Gross and Witkop (1962). The reaction was stopped by freezing and the reagents were removed by lyophilization.

End-Group Determinations. Subtractive Edman degradation of peptides was carried out according to the modification of the method of Konigsberg and Hill (1962) described by Shearer *et al.* (1967). Digestion with carboxypeptidase A was carried out at room temperature in 0.05 M Tris (pH 8) using a 1:1 molar ratio of enzyme to peptide. In cases where a time course of hydrolysis was necessary the aliquots were removed at 15 min, 45 min, and 3 hr and diluted into pH 2.2 citrate buffer (Spackman *et al.*, 1958).

High-Voltage Paper Electrophoresis. Analytical high-voltage paper electrophoresis was carried out at 40 V/cm at either pH 6.5 (10 ml of pyridine and 0.4 ml of acetic acid diluted to 100 ml with water) or pH 3.75 (1.0 ml of pyridine and 10 ml of acetic acid diluted to 100 ml with water). Preparative paper electrophoresis was done on Whatman No. 3MM paper that had been washed for 72 hr in the appropriate electrophoresis buffer and dried before the sample was applied.

Enzymatic Assays. Proteolytic activity of trypsin and chymotrypsin was measured using casein as substrate by the method of Kunitz (1947). Inhibition of enzymatic activity by the antibodies was measured by preincubating the enzyme with increasing amounts of antibody under conditions of antigen excess at 37° for 30 min preceding the addition of casein to start the assay. Per cent inhibition was calculated by referring to a standard curve prepared the same day using

$$\% \text{ inhibition} = \frac{(\text{act. standard} - \text{act. remaining}) \times 100}{\text{act. standard}}$$

Immunization Procedure. Rabbits were immunized by multiple intradermal injections using 10 mg of trypsin or chymotrypsin in complete Freund's Adjuvant (Difco). Commencing 10 days after injection 40 ml was bled from the marginal ear vein once a week. The sera thus obtained were pooled and frozen. Titers of these antisera were approximately 0.5 mg/ml as measured by the precipitin test.

Preparation of IgG[1] Fraction. This fraction was prepared from whole sera by five successive precipitations with 33% saturated $(NH_4)_2SO_4$. The precipitates were dissolved in deionized water after each step and after the final precipitation the water solution was dialyzed against three changes of 0.15 M NaCl at 4°.

Antibody Purification. Anti-chymotrypsin and anti-trypsin were purified from whole sera using specific immunoadsorbents according to the procedure of Robbins *et al.* (1967).

Quantitative Precipitin Tests. These were carried out by reacting a constant amount of antiserum (or IgG, or purified antibody) with varying amounts of antigen. After 1-hr incubation at 37° and 18 hr at 4° the precipitates were centrifuged and washed twice with cold 0.15 M NaCl and dissolved in 0.1 N NaOH. The optical density was read at 280 nm within 10 min.

Inhibition of the precipitin test was measured by preincubating the antibodies with increasing amounts of a peptic digest of chymotrypsin or trypsin for 30 min at 37° before adding the amount of antigen corresponding to the equivalence zone. The reaction mixtures were then treated as in the quantitative precipitin test.

Binding of Radioactive Antigen to Antibodies. This was measured by incubating labeled protein or peptides with the antibodies at 37° for 1 hr. The antigen–antibody complexes were cooled to 4° and precipitated by addition of saturated $(NH_4)_2SO_4$ at 4° to 40%. The precipitates were centrifuged, washed twice with 40% saturated $(NH_4)_2SO_4$, and counted. Controls were done by measuring the amount of nonspecific binding to normal serum, or IgG fractions prepared from normal serum, containing the same amount of protein as the antibody fraction. The nonspecific binding was subtracted from the values obtained with antibody fractions.

Other Methods. Optical densities were determined in a Zeiss PMQ II spectrophotometer in 1-cm quartz cells. ^{125}I was counted in a Packard Tri-Carb scintillation counter equipped with a γ well. ^{14}C was counted in a Packard Tri-

[1] IgG = immunoglobulin G.

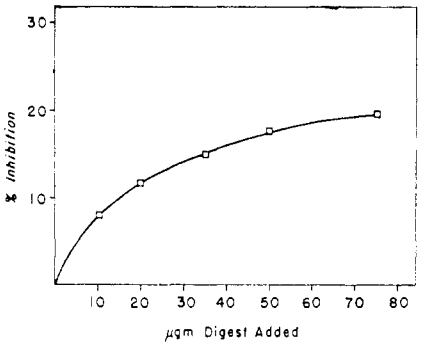

FIGURE 1: Inhibition of the precipitin reaction between trypsin and antitrypsin IgG by a peptic digest of chymotrypsin. Anti-trypsin IgG (2.1 mg) and an increasing amount of peptic digest of chymotrypsin were preincubated for 30 min at 37° before adding 50 μg of trypsin and proceeding with a precipitin test.

Carb scintillation counter after dissolving the precipitates in distilled water and transferring 0.5-ml aliquots to scintillant prepared as 12.5% naphthalene and 8.9% Omnifluor (New England Nuclear) in *p*-dioxane (Baker). Amino acid analyses were performed according to the method of Spackman *et al.* (1958) on a Beckman Model 120B analyzer.

Results

Immunological Cross-Reaction between Trypsin and Chymotrypsin. In the precipitin test chymotrypsin does not precipitate at all with anti-trypsin and trypsin precipitates only to a small extent with anti-chymotrypsin. In order to determine whether a cross-reaction occurs between the two enzymes, both trypsin and chymotrypsin were labeled with ^{125}I and binding of each was measured directly with both homologous and heterologous antibodies. Using this system it was found that anti-trypsin IgG bound 20.2% as much chymotrypsin as trypsin (Table I). The extent of binding of ^{125}I-labeled trypsin to anti-chymotrypsin IgG is about the same.

This cross-reactivity between trypsin and chymotrypsin was also observed with peptide fragments of chymotrypsin. A peptic digest of α-chymotrypsin (the digest was completely soluble at both neutral and acid pH) was tested for its capacity to inhibit the precipitin reaction in the trypsin–anti-trypsin system (Figure 1). As shown, the peptic digest inhibited the heterologous antigen–antibody precipitation to a final extent of 20%. In order to isolate the immunologically cross-reactive peptide fragments the whole digest was allowed to react with anti-trypsin antibodies and the bound peptides were isolated from the antibody–peptide complex after acid dissociation.

TABLE I: Cross-Reaction between Trypsin and Chymotrypsin and Their Heterologous Antibodies.[a]

Antibody	μmoles of [^{125}I]-Chymotrypsin Bound × 10^3	μmoles of [^{125}I]-Trypsin Bound × 10^3	% Cross-Reaction
Anti-trypsin	0.28	1.36	20.2
Anti-chymotrypsin	0.70	0.14	19.6

[a] ^{125}I-Labeled trypsin (4 μg) or chymotrypsin (4 μg) was incubated for 1 hr at 37° with 2 mg of IgG fractions of homologous and heterologous antibodies. After cooling to 4°, $(NH_4)_2SO_4$ was added to 40% saturation to precipitate the IgG fraction. After two washes with 40% saturated $(NH_4)_2SO_4$ the precipitates were counted in a γ well.

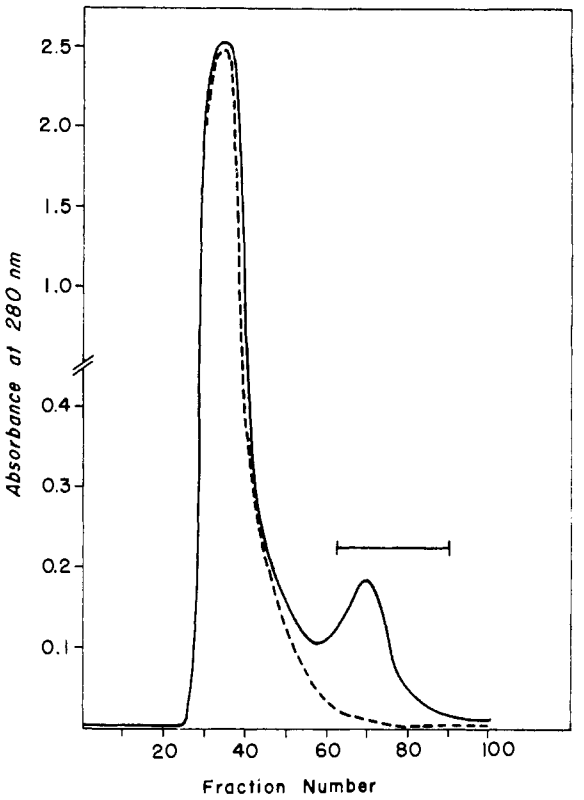

FIGURE 2: Sephadex G-75 gel filtration of acid-dissociated complex of chymotrypsin peptides and anti-trypsin antibodies. (——) Elution profile for dissociated complex of chymotrypsin peptides and anti-trypsin antibodies. (-----) Control without peptides added to the antibodies; 1.5 × 90 cm column in 0.1 N acetic acid, 4°, 1.5 ml/fraction, 10 ml/hr flow rate. A 3.0-ml sample was applied. Peptide fraction was pooled as indicated.

Isolation of Cross-Reacting Peptides from a Peptic Digest of Chymotrypsin. A peptic digest of 1 μmole of chymotrypsin was incubated with 40–50 mg of purified anti-trypsin antibodies in 0.15 M NaCl at 37° for 1 hr. The reaction mixture was cooled to 4° and saturated ammonium sulfate at pH 7 and 4° was added to 40% saturation. The precipitate was allowed to form overnight at 4°; it was then centrifuged and washed twice with 40% saturated $(NH_4)_2SO_4$. The precipitated complex was dissolved in 3.0 ml of 0.1 M acetic acid, the pH was adjusted to 2 with 6 N HCl and the solution was applied directly to a G-75 Sephadex column equilibrated with 0.1 N acetic acid.

A typical elution profile (Figure 2) from the G-75 Sephadex column showed the presence of a low molecular weight fraction absorbing at 280 nm separated from the high molecular weight antibodies. Analytical high-voltage paper elec-

TABLE II: Amino Acid Compositions of Peptic Peptides from Chymotrypsin Isolated by Binding to Anti-trypsin Antibodies.[a]

	42-Residue Peptide (131–141; 190–2072; 17–222)			CNBr Fragment I (131–141; 193–207)			CNBr Fragment II (190–192; 217–229)		Peak Ia Residues 195–200	
Residue	Peak I a	Theory b	Peak I[b] c	Obtained d	Theory e	One Turn of Edman f	Obtained g	Theory h	Obtained i	Theory j
Asp	2.00	2.0	2.00	2.00	2.0	2.00				
Thr	7.04	7.0	5.96	4.07	4.0	4.22	3.35	3.0		
Ser	5.89	6.0	4.76	1.66	1.0	1.35	4.91	5.0	1.08	1.0
Glu										
Pro	1.98	2.0	2.02	1.08	1.0	1.14	1.01	1.0	1.00	1.0
Gly	8.30	8.0	8.60	6.61	6.0	5.83	2.20	2.0	1.79	2.0
Ala	2.72	3.0	2.73	2.87	3.0	2.05				
Cys/2	3.01	4.0	3.33	1.79	2.0	1.42	1.68	2.0		
Val	2.66	3.0	2.92	1.90	2.0	2.02	1.00	1.0	1.10	1.0
Met	0.89	1.0	0.93							
Ile										
Leu	1.26	1.0	1.38	1.10	1.0	1.37			0.92	1.0
Tyr	0.47	1.0	0.73				0.72	1.0		
Phe										
Lys	1.95	2.0	1.89	2.01	2.0	*c*				
His										
Arg										
Trp	*d*	2.0	*d*	*d*	2.0	*d*				
Hse[e]							1.04	1.0		

[a] Peaks I and Ia are derived from the experiment illustrated in Figure 3. CNBr fragments of the 42-residue peptide (peak I) were isolated as described in the text. Residue numbers are taken from Figure 4, semicolons indicate sequence groups linked by disulfide bonds. The column headings a–j serve to refer to the text. [b] This preparation of the 42-residue peptide was isolated directly from a peptic digest of chymotrypsin without the use of anti-trypsin. [c] Lysine is lost because of reaction with phenyl isothiocyanate. [d] Tryptophan was present on analysis but not calculated. [e] Homoserine and homoserine lactone added together.

trophoresis of the low molecular weight material at pH 3.75 showed several ninhydrin-positive components. A control experiment (dashed line, Figure 2), where no digest was added to the antibodies, but all other conditions were kept as outlined above, showed no ninhydrin-positive or 280-nm-absorbing material in the low molecular weight region, indicating that no low molecular weight material was present in the antibody preparation, nor was it generated by the procedure.

Repetition of the peptide absorption six times on the same scale as above yielded a sufficient quantity of peptide material for further purification and identification of the components. Gel filtration on G-25 Sephadex in 1% acetic acid (Figure 3) resolved three major components. Peak I from this column migrated as one band on analytical high-voltage paper electrophoresis at pH 6.5 and pH 3.75. The amount of peptide in peak I was 0.54 μmole and the amino acid composition of peak I is shown in Table II (column a). Peak Ia contained a mixture of peptides which were resolved on preparative high-voltage electrophoresis at pH 3.75. The major component eluted from the paper was neutral and had the amino acid composition shown in Table II (column i). Peak II contained a complex mixture of acidic and neutral peptides in low yield and no further work was done on it.

The composition of the peptide in peak I was similar to that reported by Brown and Hartley (1966) for a 42-amino acid fragment of chymotrypsin containing two disulfide bonds and the active-site Ser-195. According to their work the structure of this fragment is as shown in Figure 4 and its predicted amino acid composition is shown for comparison with peak I in Table II (column b). The residues in parentheses in Figure 4 are the two extra residues contained in a larger fragment (which includes Gly-Leu, residues 142–143) and which occurs to the extent of about 50% in the peptide fractions isolated. This is indicated by the values for Leu and Gly which are consistently higher than expected.

To prove that the structure of the isolated peptide was identical with the known structure the peptide was treated with cyanogen bromide to cleave at the methioninyl residue. The cleavage products were separated on a 1.5 × 90 cm column of G-25 Sephadex in 1% acetic acid and their amino acid compositions, obtained and predicted, are shown in Table II (columns d, e, g, and h). The larger piece (CNBr fragment I, underlined in Figure 4), including residues 131–141 (or 143) linked by a disulfide bond to residues 193–207, was recovered in 83% yield. CNBr fragment II (residues 190–192 linked by a disulfide bond to 217–229) was found in 60% yield. The loss was apparently sustained on the G-25 Sephadex column as the products of the cleavage were completely soluble.

CNBr fragment I was subjected to Edman degradation

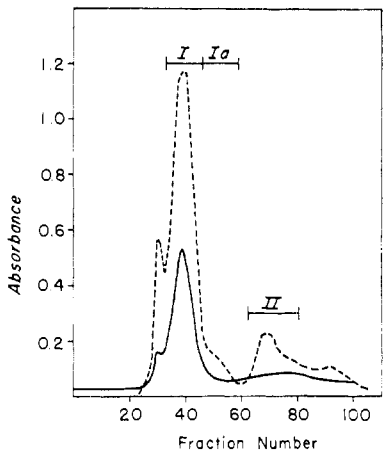

FIGURE 3: G-25 Sephadex gel filtration of peptides from chymotrypsin isolated by binding with anti-trypsin; 1.5 × 90 cm column of Sephadex G-25 in 1% acetic acid, room temperature, 1.2 ml/fraction, 9.6 ml/hr flow rate, sample applied in 1.0 ml. (——) Absorbance at 280 nm; (-----) absorbance at 230 nm.

and carboxypeptidase A digestion to further confirm the identification of this peptide. Results of the amino acid analysis after one turn of the subtractive Edman degradation are shown in column f of Table II. Glycine (0.82 residue mole) and alanine (0.82 residue mole) were lost as expected. Carboxypeptidase A digestion produced tryptophan and leucine in 70 and 60% of the expected yields, respectively.

The major peptide isolated from peak Ia of Figure 3 had the composition expected for residues 195–200 in chymotrypsin (Table II, columns i and j) which also includes the active-site Ser-195. The structure of that fragment according to Hartley *et al.* (1965) is as follows: Ser-Gly-Gly-Pro-Leu-Val. The amount recovered was 0.18 μmole. Because of the antepenultimate prolyl residue carboxypeptidase A digestion would be expected to yield only valine. The peptide was therefore subjected to a 3-hr carboxypeptidase digestion. This treatment yielded valine in 89% yield and no other amino acids.

Peptide Binding to the Antibodies. Brown and Hartley (1966) had shown that the 42-amino acid peptide (corresponding to the one isolated by complexing with the antibodies) could be obtained in a nearly pure form directly from a whole peptic

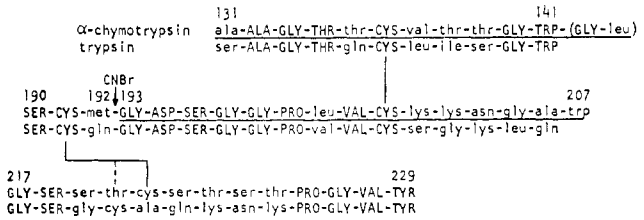

FIGURE 4: Structure of the 42-residue peptide from bovine chymotrypsin compared with the comparable region of the sequence of bovine trypsin. Capital letters denote identical amino acids in homologous positions. The numbering system is that used for chymotrypsinogen (Hartley, 1964). Parentheses denote contamination by a larger peptide containing two extra residues. The underlined portion denotes CNBr-I obtained from cyanogen bromide cleavage.

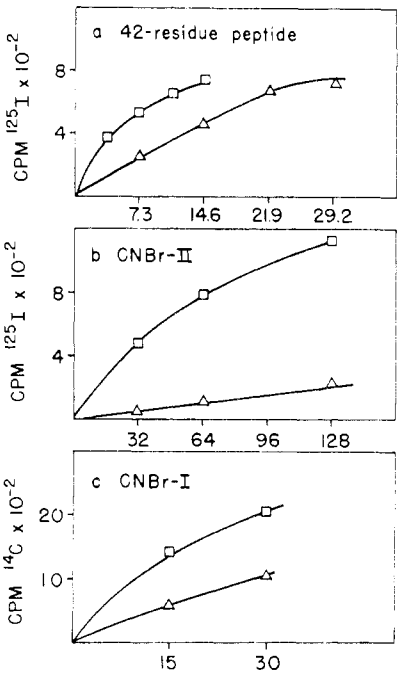

FIGURE 5: Binding of 42-residue peptides from peak I and CNBr fragments I and II to anti-trypsin and anti-chymotrypsin antibodies. The specific radioactivities of the labeled peptides were: [^{14}C]acetyl-CNBr-I, 1.3 × 10^{7} cpm/μmole; ^{125}I-labeled CNBr-II, 8.5 × 10^{6} cpm/μmole; ^{125}I-labeled 42-residue peptide, 5.6 × 10^{6} cpm/μmole. (a) Binding of ^{125}I-42-residue peptide to □, anti-chymotrypsin (2.5 × 10^{-4} μmole of antibody in each sample) and △, anti-trypsin (3.1 × 10^{-4} μmole of antibody in each sample). Samples containing 8.5 × 10^{-5} μmole of antibody were used in binding experiments for CNBr-I and CNBr-II. (b) Binding of [^{125}I]CNBr-II to □, anti-chymotrypsin, and △, anti-trypsin. (c) Binding of [^{14}C]acetyl-CNBr-I to △, anti-trypsin and □, anti-chymotrypsin. The number of micromoles of antibody present was determined by measuring the number of micromoles of ^{125}I-labeled homologous antigen bound under identical conditions.

digest of chymotrypsin by high-voltage electrophoresis at pH 6.5. In order to isolate large quantities of the peptide sufficient for binding studies with both the homologous and heterologous antibodies, Brown and Hartley's procedure was used and was modified to include passage of the peptic digest of chymotrypsin through G-25 Sephadex (1.5 × 90 cm) in 1% acetic acid. The peak fractions running in the same position as peak I in Figure 3 were pooled and subjected to preparative high-voltage electrophoresis at pH 6.5. As expected, the peptide was pure after elution from the paper. Overall yield was 52%. Amino acid analysis (column c of Table II) indicated that it was indeed identical with the material that was isolated by complexing the peptic digest of chymotrypsin with the anti-trypsin antibodies. This observation was further confirmed by cyanogen bromide fragmentation which gave results analogous to those described above.

The purified 42-residue peptide was iodinated with ^{125}I and the interaction of the ^{125}I-labeled peptide with both anti-trypsin and anti-chymotrypsin IgG fractions was then measured by direct binding experiments. Results of these experiments are shown in Figure 5a for anti-trypsin IgG and anti-chymotrypsin IgG. Binding of the peptide to anti-trypsin IgG

TABLE III: Effect of 42-Residue Peptide on Antibody-Neutralizing Activity.[a]

Enzyme	Inhibn by Antibody (%)	Inhibn in Presence of 25-fold Molar Excess Peptide (%)	Inhibn in Presence of 50-fold Molar Excess Peptide (%)
Chymotrypsin	40	43	45
Trypsin	49	48	48

[a] Inhibition was measured by preincubating the homologous antigen and antibody together under conditions of antigen excess for 30 min at 37° before adding casein as described in Methods. The effect of the 42-residue peptide was measured by incubating the indicated molar excess of peptide over antibody equivalence point with the antibody for 30 min at 37° before adding the enzyme for a second preincubation and proceeding as outlined above. Trypsin (10 μg) and chymotrypsin (5 μg) were incubated with 3 and 2 mg of anti-trypsin and anti-chymotrypsin IgG fractions, respectively.

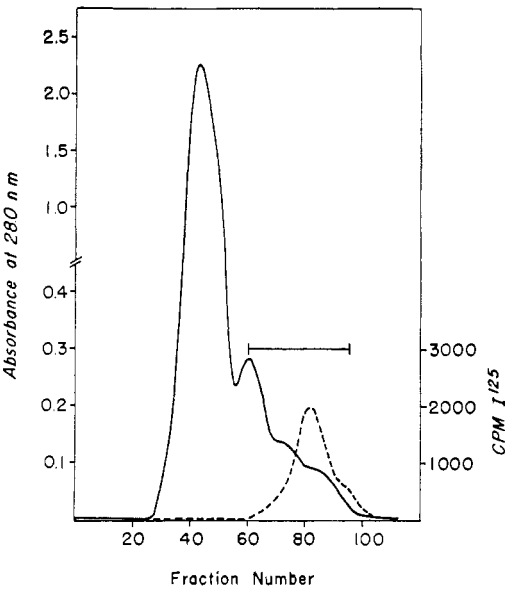

FIGURE 6: Sephadex G-100 gel filtration of acid-dissociated complex between trypsin peptides and anti-chymotrypsin antibodies. (——) Absorbance at 280 nm. (-----) ^{125}I label (cpm) on tyrosine-containing peptides; 1.5 × 90 cm column in 0.1 N acetic acid; 4°; 1.5-ml fraction; 8 ml/hr flow rate; sample applied in 3.0 ml.

is not as strong as binding to anti-chymotrypsin IgG, as might be expected. Further experiments were conducted in order to define the regions in the peptide which are responsible for its binding capacity. For this purpose the two fragments obtained after CNBr cleavage were prepared with a radioactive label and the binding of each to both anti-trypsin and antichymotrypsin sera was measured. ^{125}I-Labeled CNBr fragment II was prepared by CNBr cleavage of the ^{125}I-labeled 42-residue peptide. The binding of [^{125}I]CNBr-II to anti-chymotrypsin and anti-trypsin serum pools is shown in Figure 5b. The affinity of [^{125}I]CNBr-II for anti-chymotrypsin was much stronger than for anti-trypsin, but the binding with anti-trypsin, although low, was consistent and reproducible.

CNBr fragment I was labeled by acetylation with [^{14}C]-acetic anhydride. Prior to the acetylation it was shown by amino acid analysis to be free of contamination by CNBr fragment II. Its binding to anti-trypsin and anti-chymotrypsin sera was then measured and the results are shown in Figure 5c. Again, binding to anti-chymotrypsin is stronger than to anti-trypsin, but in this case the binding strength differs approximately by a factor of two. A similar difference in affinity for the two antibodies had been seen with the whole 42-residue peptide.

Since the 42-amino acid peptide isolated from chymotrypsin contains the active-site serine, it was of interest to determine whether the peptide would relieve the inhibition of proteolytic activity of either chymotrypsin or trypsin by their respective antibodies. To this end, the peptide was preincubated with anti-chymotrypsin in 25- and 50-fold molar excess over the equivalence zone at 37° for 1 hr. Chymotrypsin was then added and the inhibition of chymotryptic activity by anti-chymotrypsin was assayed as usual. Although the peptide concentrations were such that maximum binding to the antibodies would be achieved, no relief of the anti-chymotrypsin inhibition of casein hydrolysis by chymotrypsin was observed (Table III). The same experiment was attempted in the trypsin–anti-trypsin system with the same results.

Cross-Reacting Peptides in a Peptic Digest of Trypsin. A preliminary experiment was done using the method outlined above to see whether cross-reacting peptides from a peptic digest of [^{125}I]trypsin could be isolated by complexing with anti-chymotrypsin antibodies. The elution profile of the acid-dissociated complex from a 1.5 × 90 cm G-100 Sephadex column is shown in Figure 6. The low molecular weight fraction contained both ^{125}I label and 280-nm-absorbing material. Sufficient material for further characterization has not yet been accumulated.

Discussion

In discussing the homology of proteins distinction should be made between chemical and conformational homology (Neurath *et al.*, 1969). Whereas chemical homology refers to similarity in amino acid sequence (Neurath *et al.*, 1967) conformational homology implies similarity in three-dimensional features. The well-documented chemical homology of bovine trypsin and chymotrypsin is based on their similarity in primary sequence (Walsh and Neurath, 1964; Hartley *et al.*, 1965; Mikeš *et al.*, 1966) and on the similarity of the functional components of their active sites (Cunningham, 1965; Bender and Kaiser, 1962; Bender, 1962). Conformational homology is implied by the hypothesis that the information for three-dimensional structure is coded directly in the amino acid sequence (Epstein *et al.*, 1963) and is supported by experimental evidence as reviewed in the introduction.

Previous studies (Arnon and Schechter, 1966) have indicated that trypsin and chymotrypsin are immunologically related, but this conclusion was based merely on the capacity of anti-trypsin antibodies to inhibit partially the catalytic

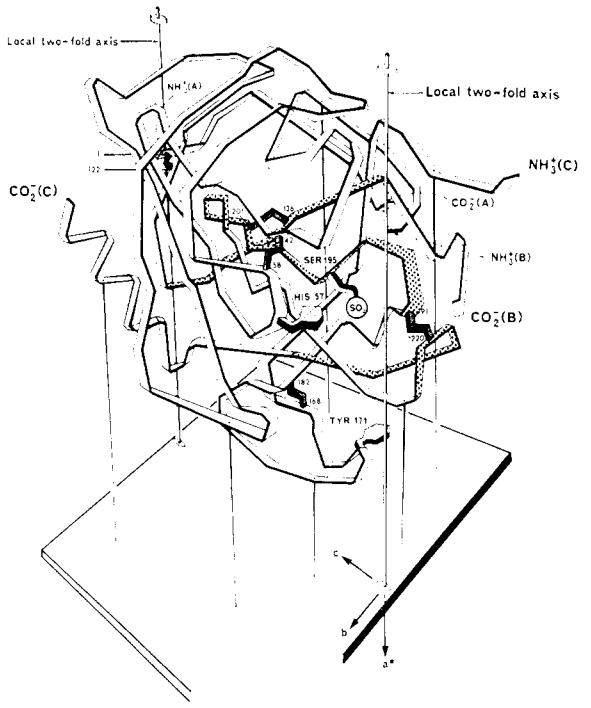

FIGURE 7: Drawing of model of α-chymotrypsin X-ray structure determined by Matthews *et al.* (1967). Shaded area denotes 42-residue peptide isolated by binding with antitrypsin antibodies. This photograph was kindly provided by Dr. D. M. Blow.

activity of chymotrypsin. This present work demonstrates that common antigenic determinants are indeed present on both enzymes.

Caution should be used in interpreting these experiments because of the possibility that cross-contamination is responsible, at least in part, for this cross-reaction. Trypsin preparations are known to have a low level of the substrate specificity characteristic of chymotrypsin. However, this has been shown to be a property of trypsin itself rather than due to contamination by chymotrypsin (Inagami and Sturtevant, 1960). In addition, the particular lot of trypsin used in these experiments has been shown by disc gel electrophoresis to be free of detectable contamination by chymotrypsin-like material (M. M. Sanders, unpublished results). This evidence, considered with the observation that trypsin and chymotrypsin are approximately equally weak immunogens, indicates that the cross-reaction found here is indeed real.

The 42-residue peptide of chymotrypsin, isolated from a peptic digest of the enzyme by direct binding to antibodies against trypsin, contains at least two antigenic determinants common to trypsin and chymotrypsin. This is concluded from the evidence that two fragments of the peptide, CNBr-I and CNBr-II, are each independently capable of binding (though to different extents) both anti-trypsin and anti-chymotrypsin (Figure 5).

The 42-residue peptide of chymotrypsin is over 50% homologous with the corresponding region in the trypsin sequence (Figure 4) whereas the overall homology between the two proteins is about 40% (Walsh and Neurath, 1964). More important than the average homology is probably the fact that one of the homologous regions contains only two replacements in twelve amino acids and a second region has a sequence of four identical residues. CNBr fragment I contains a sequence of nine amino acids which is identical in eight of the positions in both enzymes. This region also encompasses the hexapeptide containing residues 195–200 which had been isolated independently from the antibody-bound peptide fraction (Figure 3). This fact suggests that this region of CNBr-I contributes to the binding capacity of CNBr-I to anti-trypsin, but it may not necessarily be the only immunologically active region in the fragment.

Assuming that the 42-residue peptide from chymotrypsin contains a determinant which is common to trypsin and chymotrypsin one would expect the presence of a structurally similar peptide in trypsin. This assumption was tested by performing a parallel experiment using anti-chymotrypsin antibodies for the isolation of immunologically active fragments from a digest of trypsin. Preliminary results (Figure 6) indicate that this procedure may yield a peptide-containing fraction and that the search for common antigen determinants of trypsin and chymotrypsin can be pursued using the trypsin molecule as well.

The method used in this work for isolating cross-reacting determinant-containing peptides directly from a whole digest has distinct advantages over previously used techniques of screening the effluent of peptide separation columns for immunologically active fragments. First, it is a direct method for separating in one step the immunologically active peptides from those that do not bind to the antibodies at all. Separation and purification of the components in the simpler mixture of determinant-containing peptides is technically an easier problem in principle than the purification of components of a complete digest. The method also is adequate for the isolation of sufficient material for characterization studies, as shown by the fact that, respectively, 0.5 and 0.19 μmole of the 42- and 6-residue peptides from chymotrypsin were isolated in this work. Obviously the method will be even more powerful for the isolation of determinant-containing peptides from homologous antigen–antibody systems than it has been for a cross-reacting system because the affinity of major immunologically active peptides for their homologous antibodies would tend to be higher. This method cannot be expected to yield all of the antigenic determinants on any protein, however, because of the fact that some of the determinants are undoubtedly destroyed by denaturation and by the digestion process, as evidenced by the fact that no digest of a protein reported to date has completely inhibited reaction of that protein with its antibodies.

A common feature of the immunologically active fragments isolated in this study, namely, CNBr-I, CNBr-II, and the hexapeptide including residues 195–200, is the presence in each of one of the proline residues that are homologous in trypsin and chymotrypsin. Survey of the literature shows that immunologically active peptide fragments from other proteins have also contained proline. Atassi and Saplin (1968) isolated five immunologically active tryptic peptides from sperm whale myoglobin that contained three of the four proline residues in myoglobin. The peptic peptide from hen egg white lysozyme isolated by Shinka *et al.* (1962, 1967a,b) and by Arnon (1968) contains both residues of proline present in that molecule. And tryptic peptide 8 from TMV protein isolated and studied by Benjamini *et al.* (1965) contains a proline adjacent to but not included in the most immunologically

active region of this eicosapeptide. These separate observations raise the question of whether a proline residue, known to occur between helical sections in helix-containing proteins, can represent a feature on the surface of a protein molecule that can constitute a recognition point for the immune system. This is consistent with the results of Crumpton and Wilkinson (1965) and Atassi and Saplin (1968) who have observed that several peptides from myoglobin possessing immunological activity include the corner regions between helix segments seen in the X-ray structure. The peptide isolated in the present study also occupies corners of the polypeptide chain, as can be seen in Figure 7, which appear from this drawing of the chymotrypsin model (Sigler *et al.*, 1968) to be on the surface of the molecule. Moreover, the knowledge that the same antigenic determinants must be present on trypsin as well allows the prediction that the corresponding amino acid sequence regions in trypsin will occupy similar positions on its molecular surface. These points will be clarified by the continuation of this study to include the detailed identification of all the immunologically active peptides of both trypsin and chymotrypsin.

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