Human Pancreatic Enzymes. Isolation and Properties of a Major Form of Chymotrypsin*

Michael H. Coan, Ronald C. Roberts, and James Travis†

ABSTRACT: The activation of acid extracts of human pancreas results in the formation of a single protease with properties similar to trypsin and two enzymes having properties like that of chymotrypsin. One of these enzymes, chymotrypsin II, has been purified to homogeneity by salt fractionation and ion-exchange chromatography on SE-Sephadex C-25. This protein has a molecular weight of 25,800, aggregates at alkaline pH, and is inactivated by both diisopropyl fluorophosphate and L-1-tosylamido-2-phenylethyl chloromethyl ketone. Amino- and carboxyl-terminal analyses of both the native and oxidized proteins indicate that the enzyme is composed of only two chains with amino terminals of half-cystine and isoleucine and carboxyl terminals of serine and asparagine.

From these data it is suggested that chymotrypsin II is analogous to bovine δ-chymotrypsin.

Previous studies of the proteolytic enzymes in human pancreatic juice indicated the presence of two forms of human trypsinogen (Keller and Allan, 1967). In activated extracts of human pancreatic juice, however, we have been able to detect only one enzyme, thus far, with properties similar to that of bovine trypsin (Travis and Roberts, 1969). One explanation for these observations may be that two trypsinogens are giving rise to a single enzyme. Such a system has been well characterized in experiments with sheep pancreatic tissue (Schyns *et al.*, 1968; Travis, 1968).

More recently it has been reported that human pancreatic juice contains three forms of chymotrypsinogen (Figarella *et al.*, 1969). A detailed examination of the chymotrypsin content of human pancreatic extracts after activation has confirmed the presence of at least two chymotrypsins. The present communication describes the purification and some of the properties of one of these components which is designated as chymotrypsin II.

Materials

Human pancreatic tissue was obtained at autopsy from either St. Joseph's Hospital, Marshfield, Wis., or Athens General Hospital, Athens, Ga. Only nonfibrotic tissues with a healthy appearance were utilized in these studies. Pipes,[1] Bz-L-ArgOEt, and *N*-Ac-L-TyrOEt were purchased from Calbiochem; DFP was bought from the Aldrich Chemical Co.; carboxypeptidase A was obtained from the Worthington Biochemical Co.; immunodiffusion plates (pattern B) were supplied by Hyland Laboratories. All other reagents were analytical grade obtained from various commercial sources.

Methods

Assay of Chymotryptic and Tryptic Activity. The esterolytic activity of chymotrypsin and trypsin were measured by the spectrophotometric method of Schwert and Takenaka (1955) using *N*-Ac-L-TyrOEt and Bz-L-ArgOEt as substrates, respectively. The enzyme and substrate were assayed at 25° in 0.1 M Tris-HCl (pH 8.0), containing 0.1 M $CaCl_2$. A unit of activity in both cases was defined as an absorbance change of 1 unit/min at 237 mμ for chymotrypsin and at 253 mμ for trypsin. Specific activity was calculated as units per milligram of protein. The concentration of protein was determined spectrophotometrically as described by Warburg and Christian (1942).

Polyacrylamide Electrophoresis. Analytical disc electrophoresis was performed using the method of Reisfeld *et al.* (1962) for basic proteins. The concentration of acrylamide in the small pore gel was 7.5%. Electrophoresis was also conducted at pH 2.3 with 7.5% and 13% gels, as described by Brewer and Ashworth (1969).

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed by the technique of Weber and Osborn (1969), except that the concentration of *N,N'*-methylenebisacrylamide was doubled to slow the migration rate, and the gels were polymerized with one-fourth the recommended amount of ammonium persulfate. The latter modification slowed the polymerization reaction so that cracking of the gels was prevented.

Ultracentrifugation Analysis. Analytical ultracentrifuge studies were carried out in a Beckman Spinco Model E ultracentrifuge equipped with an electronic speed control, an RTIC temperature control unit, and a schlieren interference optical system. The sedimentation velocity runs were carried out in the capillary-type synthetic boundary cell, and the sedimentation coefficients were calculated from measurements of the position of the maximum ordinate of the schlieren pattern (Schachman, 1967).

Sedimentation equilibrium measurements were made either in the six-channel cell designed by Yphantis (1964) or in a double-sector synthetic boundary cell. Sapphire windows were used in all experiments. Rayleigh interference patterns on Kodak spectroscopic plates type II-G were measured with a Nikon two-dimensional microcomparator. Corrections for

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† To whom correspondence should be addressed.

[1] Abbreviations used are: Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid) monosodium monohydrate; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; SE, sulfoethyl.

slight optical aberrations were made from interference patterns from blank runs on buffer solutions made under the same conditions as the equilibrium runs. All sedimentation equilibrium studies were performed at 20.0 ± 0.1°.

Sedimentation equilibrium experiments with low concentrations of chymotrypsin (0.05%) were carried out at pH 2.0 and 7.6 using the high-speed boundary depletion method of Yphantis (1964). A single low-speed equilibrium run was carried out at a higher concentration (0.3%) at pH 7.6 and evaluated by the fringe difference method of Nazarian (1968). The corresponding high concentration run at pH 2.0 was done in the double-sector synthetic boundary cell by a modification of the boundary depletion method of Chervenka (1970).

The enzyme preparations used were dialyzed against one of the following solutions prior to the ultracentrifugation experiments: 10^{-3} N HCl–0.1 M NaCl (pH 3.0), 10^{-2} N HCl–0.1 M NaCl (pH 2.0), and 0.0325 M K_2HPO_4–0.0026 M KH_2PO_4 (pH 7.6).

Amino Acid Analysis. The amino acid composition of human chymotrypsin was determined using a Beckman Model 120C amino acid analyzer according to the method of Spackman *et al.* (1958). All samples were hydrolyzed *in vacuo* with 6 N HCl for 22, 48, or 72 hr. Half-cystine and methionine were analyzed after performic acid oxidation (Hirs, 1956) and acid hydrolysis for 22 hr, as cysteic acid and methionine sulfone, respectively. Tryptophan was determined by amino acid analysis of samples of chymotrypsin hydrolyzed in the presence of 4% thioglycollic acid (Matsubara and Sasaki, 1969).

Amino- and Carboxyl-Terminal Analysis. Quantitative amino-terminal analysis of human chymotrypsin II was determined by the method of Stark and Smyth (1963) on samples of native and oxidized protein. Carboxyl-terminal amino acids were identified by hydrazinolysis of the native protein for 48 hr at 80° using the procedure of Braun and Schroeder (1967), or by digestion of the oxidized protein with carboxypeptidase A at 37° followed by removal of aliquots at timed intervals (Ambler, 1967). In all cases, quantitative identification of individual amino acids was performed with the use of the amino acid analyzer. In some experiments it was also necessary to use high-voltage paper electrophoresis for the unambiguous identification of serine, asparagine, and glutamine.

Immunodiffusion Studies. Antibodies to human trypsin and human chymotrypsin were prepared in rabbits by multiple subcutaneous injections of a total of 4 mg of protein emulsified in complete Freund's adjuvant. A booster injection containing 1 mg of protein was routinely given after 3 weeks. Double-diffusion experiments were performed on agar plates and developed for 18–24 hr.

Results

Purification of Human Trypsin and Chymotrypsin. Unless otherwise stated, all operations were performed at 4° and aqueous solutions were prepared with double-distilled water.

Preparation of Pancreatic Extracts. In a typical experiment 500 g of partially thawed pancreatic tissue was homogenized with three volumes of cold acetone (−15°) in a Waring blender. The precipitate obtained was washed with several volumes of cold acetone to remove lipid substances and the residue dried overnight at room temperature.

All of the acteone powder (20 g) was suspended in 300 ml of 0.01 N HCl and the mixture stirred for 16 hr. During the early phases of this extraction the pH tended to rise and 1 N HCl was added periodically to maintain the pH at 2.6. The suspension was centrifuged at 23,300*g* for 20 min and the residue reextracted with 200 ml of 0.01 N HCl for 30 min and centrifuged at 23,300*g*. The two supernatants were combined to yield 500 ml of cloudy yellow extract.

Ammonium Sulfate Fractionation. The acid extract from the previous step was brought to 0.2 saturation with solid ammonium sulfate, and, after thirty minutes it was centrifuged at 23,300*g* for 20 min. The precipitate was discarded and the supernatant brought to 0.8 saturation by the further addition of solid ammonium sulfate. The precipitate was collected by centrifugation at 23,300*g* and dissolved in a solution containing 0.1 M Tris-HCl (pH 8.0) and 0.05 M $CaCl_2$. The final volume was adjusted to 500 ml with this buffer.

Activation. The fraction obtained by ammonium sulfate precipitation was dialyzed for 2 days against several changes of 0.1 M Tris-HCl (pH 8.0), containing 0.05 M $CaCl_2$. During this time copious amounts of inactive precipitate formed which were removed by centrifugation at 23,300*g* for 30 min. The enzyme was precipitated from the supernatant solution (490 ml) by the addition of solid ammonium sulfate to 0.8 saturation. The precipitate was dissolved in 100 ml of 0.05 M Pipes (pH 6.5) and 0.025 M $CaCl_2$, and the solution dialyzed exhaustively against the same buffer. The final volume was 130 ml and the preparation at this stage contained about 1500 units of trypsin esterase activity and 3000 units of chymotrypsin esterase activity.

SE-Sephadex C-25 Chromatography. The activated solution was slowly pumped onto a column of SE-Sephadex C-25 which was previously equilibrated with 0.005 M Pipes buffer (pH 6.5), containing 0.025 M $CaCl_2$. A large quantity of protein, apparently devoid of tryptic and chymotryptic esterase activities, passed directly through the column. The column was washed with the buffer solution until an absorbancy at 280 nm of 0.010 was obtained. The concentration of $CaCl_2$ in the eluting solution was then increased to 0.05 M by the use of a linear gradient. The column was washed with Pipes buffer containing 0.05 M $CaCl_2$ until the absorbancy at 280 nm returned to 0.010. Finally, the column was eluted with Pipes buffer containing 0.05 M $CaCl_2$ and 0.1 M NaCl.

The results of a typical run are summarized in Figure 1. The data confirm our earlier observations that only a single fraction containing human trypsin activity was present. This component was almost completely resolved from the first chymotrypsin fraction (chymotrypsin I, 900 units).

The most interesting result, however, is the isolation of a second, major chymotrypsin component (chymotrypsin II, 2100 units). This fraction contains approximately two-thirds of the chymotrypsin esterase activity which is present in the activated pancreatic extracts. This enzyme, which is considerably more cationic in nature than chymotrypsin I, was undoubtedly retained on chromatographic columns in previous methods used to purify human trypsin.

Criteria of Homogeneity. Fractions 117–141 (trypsin) and 315–330 (chymotrypsin II) were pooled and the fraction containing trypsin activity was dialyzed against 10^{-3} N HCl and concentrated by ultrafiltration. The recovery of activity was 525 units with a specific activity of 10.5 units/mg.

Since chymotrypsin II was eluted by a stepwise increase in salt concentration, further purification was attempted using a gradient elution system. The pooled material, having a specific activity of 30.0 units/mg, was dialyzed against 0.05 M sodium citrate buffer (pH 3.0)–0.1 M NaCl. The sample was then passed onto a column of SE-Sephadex C-25 previously equilibrated against the same buffer and elution with a linear gradient from 0.1 to 0.9 M NaCl was initiated. The enzyme was

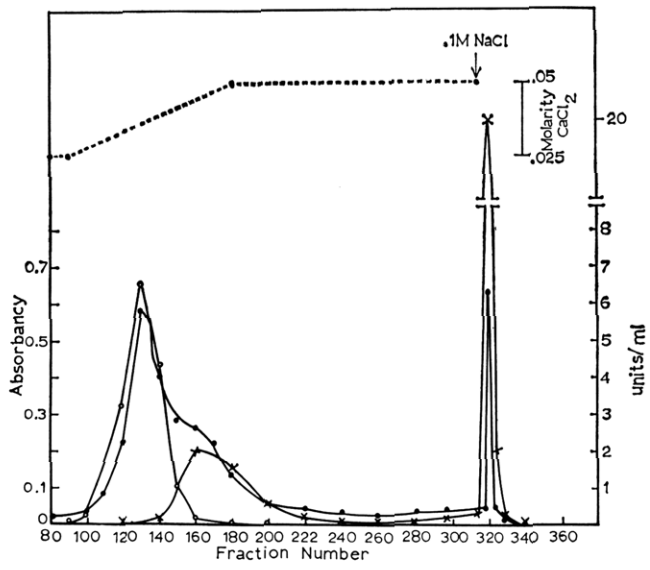

FIGURE 1: SE-Sephadex C-25 chromatography of activated human pancreatic enzymes. The column was equilibrated with 0.005 M Pipes buffer (pH 6.5), containing 0.025 M $CaCl_2$, and eluted with a linear gradient (800 ml) to 0.05 M $CaCl_2$ as indicated. The column was washed with the buffer containing 0.05 M $CaCl_2$, followed by the same buffer containing 0.1 M NaCl and 0.05 M $CaCl_2$. Column dimensions: 1.9 × 20 cm; flow rate, 18 ml/hr; fraction size, 9 ml. Curves are designated as follows: optical density at 280 nm, (●) left ordinate; activity against Bz-L-ArgOEt (○) and *N*-Ac-L-TyrOEt (×) right ordinate.

eluted in a single symmetrical peak (Figure 2) with constant specific activity in each fraction. This activity was identical with that of the preparation applied to the column which suggests the absence of any other contaminating proteins.

Additional evidence for the purity of this preparation was obtained by subjecting it to chromatography on a Sephadex G-75 column. In Figure 3 it can be seen that the preparation was again eluted as a single peak with a constant specific activity which was comparable to that of the applied enzyme.

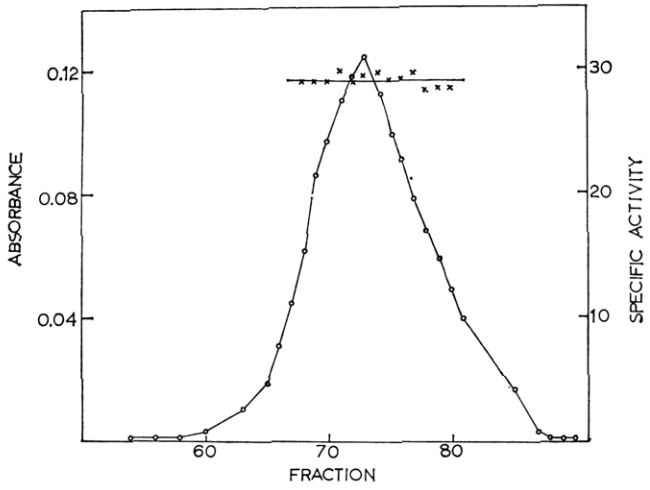

FIGURE 2: SE-Sephadex C-25 chromatography of purified chymotrypsin II. The column was equilibrated with 0.05 M sodium citrate buffer (pH 3.0), containing 0.1 M NaCl, and eluted with a linear gradient (800 ml) to 0.9 M NaCl. Column dimensions: 1.9 × 20 cm; flow rate, 20 ml/hr; fraction size, 10 ml. Curves are designated as follows: optical density at 280 nm (○) left ordinate; specific activity (×) right ordinate.

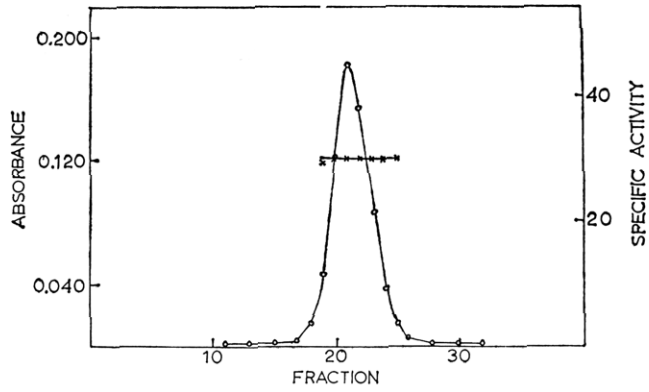

FIGURE 3: Sephadex G-75 chromatography of purified chymotrypsin II. The column was equilibrated and eluted with 0.05 M sodium citrate buffer (pH 3.0). Column dimensions: 1.9 × 50 cm; flow rate, 20 ml/hr; fraction size, 5 ml. Curves are designated as follows: optical density at 280 nm (○) left ordinate; specific activity (×) right ordinate.

Disc gel electrophoresis of chymotrypsin II was carried out at pH 4.3 in a 7.5% gel, and at pH 2.3 in both a 7.5% and a 13% gel. The sample moved as a sharp band (Figure 4) under all conditions. Less than 2% of an impurity was detectable only in the 13% gel by densitometer scanning.

Finally, the preparation of chymotrypsin II was found to be completely devoid of tryptic, elastolytic, carboxypeptidase A or B, and ribonuclease activities even when very large amounts of the enzyme was added to the assay mixtures.

Analytical Ultracentrifuge Studies. At pH 3.0 the purified preparation of chymotrypsin II sedimented as a single symmetrical peak with an $s_{20,w}$ of 2.4 S. At pH 7.6, in potassium phosphate buffer of 0.1 ionic strength, the sedimentation pattern was unsymmetrical and more difruse, showing skewing toward the meniscus. There was also some indication that material of a high molecular weight had accumulated at the bottom of the cell. The $s_{20,w}$ of the major component was 2.83 S in this case. These results indicate that human chymotrypsin II may aggregate at alkaline pH. The protein concentration in these runs was 3.0 mg/ml.

Meniscus depletion sedimentation equilibrium experiments

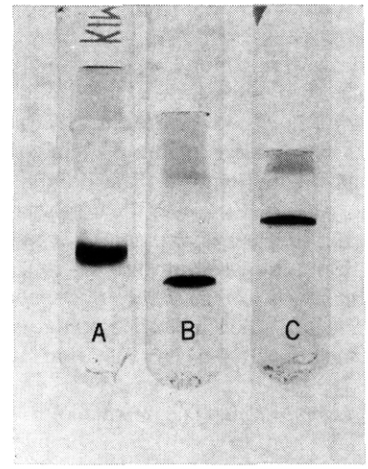

FIGURE 4: Polyacrylamide disc electrophoresis of chymotrypsin II (100 μg). Direction of migration is from anode (top) to cathode (bottom). Patterns were stained with 1% Amido Schwarz in 7.5% acetic acid. (A) pH 4.3, 7.5% gel; (B) pH 2.3, 7.5% gel; (C) pH 2.3, 13% gel.

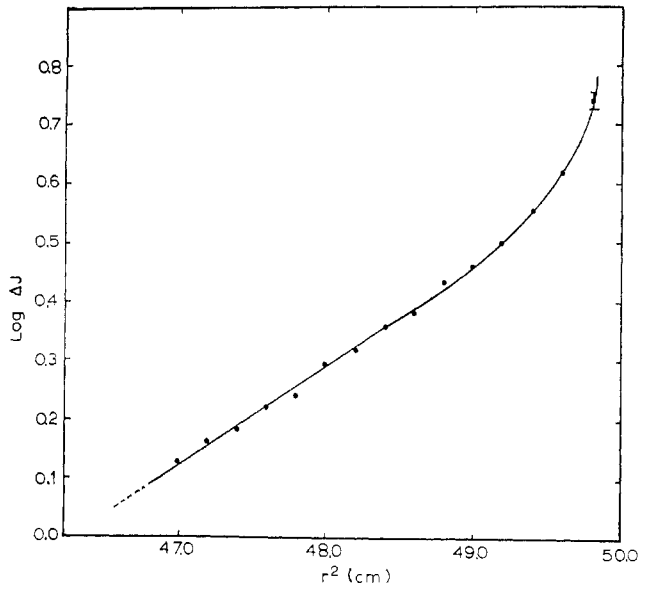

FIGURE 5: Plot of log ΔJ *vs.* r^2 for low-speed equilibrium centrifugation of human chymotrypsin at pH 7.6. The protein concentration was 3 mg/ml and the rotor speed was 24,000 rpm. Data were analyzed by the method of Nazarian (1968).

were carried out at pH 2.0 and 7.6 using initial protein concentrations of 3.0 mg/ml and 0.5 mg/ml. The ln C *vs.* r^2 plots for runs at both concentrations at pH 2.0 were linear. The data obtained in these experiments are summarized in Table I. The mean slope of the ln C *vs.* r^2 line was determined by averaging all possible point slopes obtained by a sliding linear least-squares analysis covering five points. The apparent molecular weights were calculated from the slopes, using a value of 0.77 for the partial specific volume which was calculated from the amino acid composition. The uncertainties in the apparent molecular weight values were estimated from the standard deviation of the mean slope, and they do not include any contribution arising from the partial specific volume term. Even though a slightly higher apparent molecular weight was obtained at the higher concentration at pH 2.0, no trend of increasing point weight-average molecular weights was observed toward the bottom of the cell, justifying the conclusion that within the limits of experimental accuracy, there was no detectable molecular aggregation at low pH.

At pH 7.6, however, definite signs of molecular heterogeneity were observed, especially at high concentrations of protein. The log ΔJ *vs.* r^2 plot (Nazarian, 1968), equivalent to log C *vs.* r^2 for a low-speed run, at a protein concentration of 3.0 mg/ml, is shown in Figure 5. The point weight-average molecular weight at $r^2 = 49.4$ (near the bottom of the solution column) was 51,800. The apparent Z-average molecular weight across the solution column was 83,700. However, the aggregation which was observed at low speeds (Figure 5) could not be detected when a sample containing 0.5 mg/ml of enzyme was examined by centrifugation at high speeds (Table I).

Amino Acid Composition. The results of the amino acid analysis of human chymotrypsin II are summarized in Table II. The number of residues of each amino acid present was based on an assumed value of 18 residues of leucine/mole of protein. A molecular weight of 24,909 was calculated from these data. This value is nearly the same as that determined by the ultracentrifugation studies described above. The composition of the human enzyme is also compared with those of porcine and bovine chymotrypsinogens in Table II.

TABLE I: Sedimentation Equilibrium Studies under Conditions of No Detectable Aggregation.

pH	Initial Protein Concn (mg/ml)	Rotor Speed	Mean Slope in C *vs.* r^2	Std Dev Mean Slope	App Mol Wt
2.0	0.5	40,000	1.0127	0.0489	24,800 ± 1200
2.0	3.0	24,000[a]	0.398	0.0469	27,100 ± 3191
7.6	0.5	40,000	1.0140	0.0331	24,800 ± 800

[a] Lower speed due to carrying out run by Chervenka's (1970) modification of the boundary depletion method.

Approximate extinction coefficients derived from amino acid analysis yielded values of 19.0 and 14.5 for human chymotrypsin II and human trypsin, respectively, for 1% solutions at 280 nm.

Effect of Inhibitors on Human Chymotrypsin II. DFP completely inhibited the enzyme in ten minutes at pH 8.0 when present at a molar ratio of 10:1. Similarly, TPCK, at a molar ratio of 100:1 completely inhibited human chymotrypsin II in less than 45 min at pH 7.0. As was expected, TLCK, a potent inhibitor of trypsin, was completely without effect even at a molar ratio of 100:1 at pH 8.0. These results give further credence to the classification of this enzyme as a member of the chymotrypsin family.

Amino- and Carboxyl-Terminal Amino Acids. Quantitative determination of the amino-terminal residues of chymotrypsin II yielded 0.9 mole of isoleucine (including alloisoleucine) and 0.6 mole of half-cystine (determined as cysteic acid) per mole of protein. All other amino acids were detected in quantities of less than 0.02 mole/mole of protein and consisted mainly of alanine and glycine.

Carboxyl-terminal analysis of human chymotrypsin II by hydrazinolysis yielded 0.85 mole of serine/mole of enzyme and no other amino acids in significant quantities. When bovine α-chymotrypsin was used as a control, 0.9 mole of tyrosine and 0.95 mole of leucine could be detected per mole of protein. When oxidized human chymotrypsin II was digested with carboxypeptidase A for 30 min, 2 moles of amino acid with the elution time of serine, glutamine, or asparagine was detected with the amino acid analyzer. Since asparagine and glutamine were eluted in the same position as serine during a standard 55° run with the amino acid analyzer, the amino acids from a 30-min digestion mixture were separated by high-voltage paper electrophoresis at pH 2.0 for 60 min at 2000 V. Analysis of the pattern obtained after staining with ninhydrin indicated the presence of both serine and asparagine. Although the quantitation of asparagine was not made, the rapid early release of this amino acid by carboxypeptidase A, together with the results of hydrazinolysis, strongly indicate that serine and asparagine are the carboxyl-terminal amino acid residues of chymotrypsin II.

Identification of Peptide Chains. The results of the amino- and carboxyl-terminal analyses of human chymotrypsin II indicated that only two chains were present. When freshly prepared material was treated with DFP and subjected to disc electrophoresis in sodium dodecyl sulfate solutions after

TABLE II: Amino Acid Composition of Human Chymotrypsin II.

Amino Acids	Amino Acid Residues[a]							
	Human Chymotrypsin II[a]					Bovine Chymotrypsinogen A[e]	Bovine Chymotrypsinogen B[f]	Porcine Chymotrypsin C[g]
	22 hr[b]	48 hr[b]	72 hr[b]	Extrapolation or Av	Integral No.			
Tryptophan[c]	4.9			4.9	5	8	8	8
Lysine	16.1	15.8	16.1	16.0	16	14	11	7
Histidine	3.5	3.3	4.2	3.7	4	2	2	5
Arginine	6.3	7.0	8.0	8.0	8	4	5	7
Aspartic acid	23.9	23.3	22.5	23.2	23	23	20	22
Threonine	18.0	17.2	16.4	19	19	23	23	14
Serine	20.8	20.4	20.0	21	21	28	22	20
Glutamic acid	18.5	17.5	17.2	17.7	18	15	18	21
Proline	15.2	14.0	14.6	14.6	15	9	13	12
Glycine	25.8	24.9	23.8	24.8	25	23	23	25
Alanine	24.2	23.6	23.5	23.8	24	22	23	12
Half-cystine[d]	8.0			8.0	8	10	10	7
Valine	19.6	19.6	21.8	22	22	23	25	19
Methionine[d]	1.8			2	2	2	4	1
Isoleucine	9.1	10.6	10.8	11	11	10	9	12
Leucine	18.0	18.0	18.0	18.0	18	19	19	19
Tyrosine	2.6	2.7	2.7	2.7	3	4	3	6
Phenylalanine	6.9	7.7	7.6	7.4	7	6	7	4

[a] The number of residues were based on an assumed value of 18 leucine residues/mole of protein. [b] Average of two determinations. [c] Determined by method of Matsubara and Sasaki (1969). [d] Determined as cysteic acid and methionine sulfone. [e] Hartley (1964) and Hartley and Kauffman (1966). [f] Smillie and Hartley (1967) and Smillie *et al.* (1968). [g] Folk and Schirmer (1965).

either reduction or oxidation, only a single band was observed (Figure 6, gels 1 and 2). Using proteins of known molecular weight as standards in this system, the approximate weight of this component was calculated to be 25,000. Enzyme material not pretreated with DFP slowly lost activity, and this was reflected by the heterogenous pattern shown in Figure 6, gel 3. For comparison, the pattern obtained after reduction of bovine α-chymotrypsin is also given (Figure 6, gel 4).

Because of the slow loss of enzyme activity observed when chymotrypsin II was incubated at pH 8.0, attempts were made to determine whether this enzyme might be undergoing conversion to a less active form as occurs in the formation of bovine α-chymotrypsin from δ-chymotrypsin. When a sample of human chymotrypsin II was allowed to stand overnight at 4° in 0.05 M Tris-HCl (pH 8.0), a loss of two-thirds of the initial activity was observed. When this sample was chromatographed on a SE-Sephadex C-25 column using the purification scheme described above, only one enzymatically active fraction with the same elution properties of chymotrypsin II was found. This fraction had the same specific activity as the original sample prior to autolysis but only one-third of the total activity and protein. Thus, rather than being converted into an enzymatically active form similar to bovine α-chymotrypsin, human chymotrypsin II appears to undergo autolytic degradation.

Immunological Studies. In order to determine whether any structural relationships existed between human chymotrypsin II and human trypsin, antibodies to each enzyme were prepared. As can be seen in Figure 7A,B, both enzymes gave strong precipitin lines with their respective antibodies formed in rabbits. Furthermore, a precipitin line was also formed between anti-chymotrypsin II and human trypsin. No cross-reactions could be detected when identical concentrations of anti-trypsin and human chymotrypsin II were tested.

The relationship between the two chymotrypsins present in activated extracts of pancreas was of interest because of the

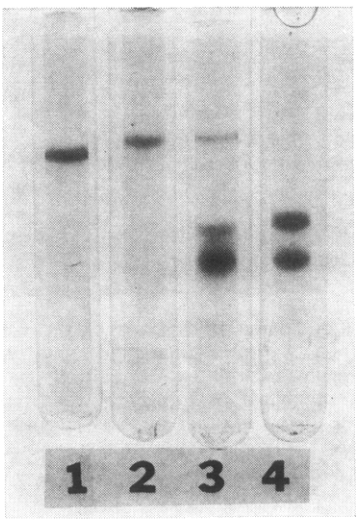

FIGURE 6: Polyacrylamide disc electrophoresis of chymotrypsin II in sodium dodecyl sulfate solution. Direction of migration is from cathode (top) to anode (bottom). Patterns were stained with 0.25% coomassie brilliant blue. (1) DIP–human chymotrypsin II (reduced); (2) DIP–human chymotrypsin II (oxidized); (3) human chymotrypsin II (reduced); (4) bovine α-chymotrypsin (reduced).

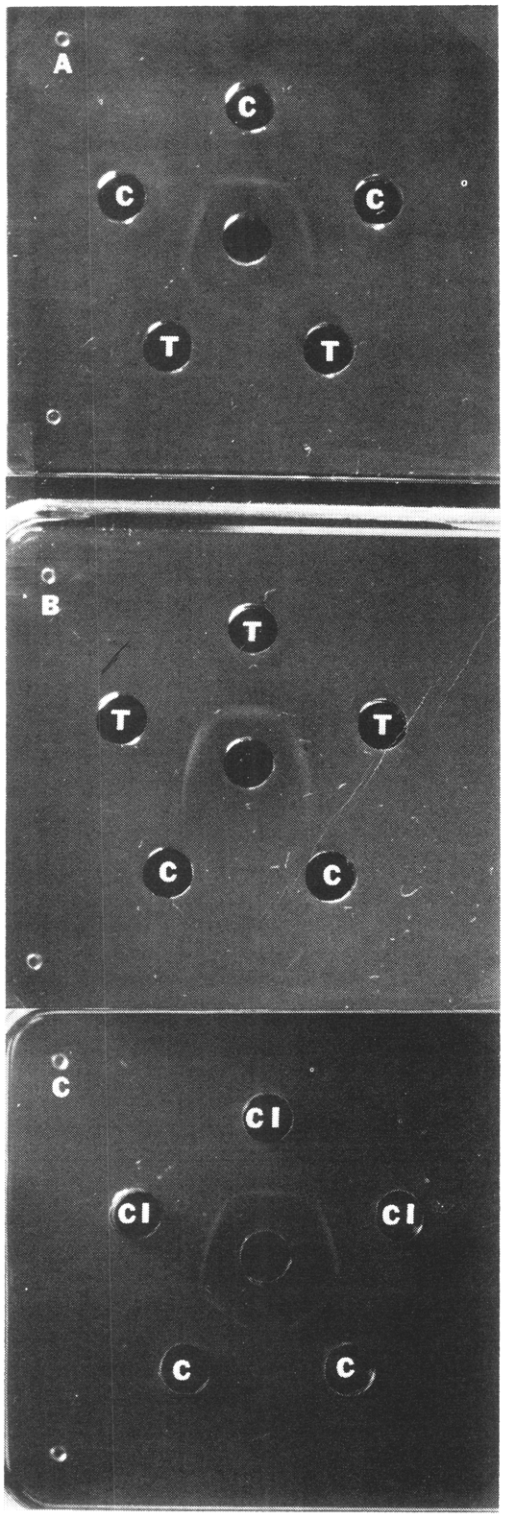

FIGURE 7: Immunodiffusion of human trypsin, chymotrypsin I, and chymotrypsin II. (A) Antiserum to human chymotrypsin II in center well; human trypsin (T) and chymotrypsin II (C) in outer wells. (B) Antiserum to human trypsin in center well; human trypsin (T) and chymotrypsin II (C) in outer wells. (C) Antiserum to chymotrypsin II in center well; human chymotrypsin I (CI) and human chymotrypsin II (C) in outer wells.

possibility that one was derived from the other. Incubation of either fraction at 4° for 48 hr at pH 6.5, in Pipes (0.005 M) buffer, followed by rechromatography, caused no change in the elution profile. This would suggest that each of these enzymes was derived from a separate zymogen. Nevertheless, when immunodiffusion experiments were performed with partially purified chymotrypsin I and anti-chymotrypsin II, complete lines of identity were obtained (Figure 7C). Further studies on the purification and properties of chymotrypsin I are in progress.

Discussion

In our previous attempts to purify human trypsin, difficulties were encountered due to the presence of a chymotrypsin contaminant (chymotrypsin I). The procedure described in this paper involves the introduction of a buffer which can be used in the presence of calcium ions at pH values near neutrality (Good *et al.*, 1966) as well as the replacement of a stepwise gradient by a more complex system. Thus, it now becomes possible to isolate both human trypsin and human chymotrypsin (chymotrypsin II) in one chromatographic step.

Many of the properties of human chymotrypsin II are identical with those of bovine δ-chymotrypsin. These include a high specific activity relative to bovine α-chymotrypsin, aggregation at alkaline pH, a structure derived from two polypeptide chains joined together through disulfide linkages, and identical amino-terminal residues. The mechanism by which this enzyme is activated is, however, unknown. Certainly it does not arise as an artifact of isolation since in other experiments we have observed that ion-exchange chromatography of human duodenal juice yields a major chymotryptic component which is eluted in the same position as human chymotrypsin II. Also, the presence of a carboxyl-terminal serine would suggest an unusual activation mechanism unless this residue is also the carboxyl terminal of the human chymotrypsinogen from which it is derived.

The relationship between human chymotrypsin I and human chymotrypsin II is still unclear. Although there is a great deal of immunological similarity we have been unable to interconvert either form. Thus, we have no direct evidence to indicate whether both proteins are derived from either the same or separate zymogens. In this respect, the detection of only two chymotrypsins in these experiments cannot be directly related to the observations of Keller and Allan (1967) who detected a single chymotrypsinogen or to those of Figarella *et al.* (1969) who found three chymotrypsinogens. Attempts to detect other forms of chymotrypsin using other extraction procedures have so far been unsuccessful. It seems likely, then, that differences in isolation techniques resulting in the destruction of certain chymotrypsin species (Gratecos *et al.*, 1969) are apparently not involved.

Allelomorphism at the bovine carboxypeptidase A locus has been observed (Petra *et al.*, 1969) and may also be responsible for differences in the structure of two sheep trypsinogens (Schyns *et al.*, 1968). Whether or not a similar situation is present in the human chymotrypsinogen system cannot, as yet, be determined. However, such an occurrence could explain the differences between our results and those of others.

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Demonstration of a Change in the Rate-Determining Step in Papain- and Ficin-Catalyzed Acyl-Transfer Reactions*

Patricia M. Hinkle† and Jack F. Kirsch‡

ABSTRACT: Advantage was taken of the sensitivity of the rates of deacylation of acyl-papains and ficins to increases effected by added nucleophiles in order to change the rate-determining steps in the reactions of several esters from deacylation to acylation and thereby permit the direct study of the acylation step under steady-state conditions. The change in the rate-limiting step was demonstrated by measuring the initial rates of release of alcohol from ester substrates in the presence of increasing concentrations of amine. The observed velocities increased to a plateau level as the concentration of amine was raised. At the plateau level the rate constant for deacylation of the acyl-enzyme intermediate exceeds that for acyl-enzyme formation. The plateau velocity was independent of the nucleophile used to change the rate-determining step and it was dependent on a group with an apparent pK_a of 8.65, as expected for rate-determining acylation. At high concentrations of amine where the acylation reaction is rate determining, the constants derived from the Michaelis–Menten equation under steady-state conditions are the rate constant for acylation and the enzyme–substrate dissociation constant. These parameters were obtained for the acylation of papain and ficin by *p*-nitrophenyl esters of hippuric acid and carbobenzoxyglycine; the apparent dissociation constants of the enzyme–substrates complexes were between 0.2 and 0.9 mM. In contrast to the results obtained with the *p*-nitrophenyl ester of carbobenzoxyglycine, the dissociation constant of the complex between papain and the *o*-nitrophenyl ester was immeasurably high (>6 mM). The present data together with those in the literature suggest that the principal mode for the binding of *p*-nitrophenyl esters of *N*-acylamino acids to papain and ficin is nonproductive.

The evidence that the papain-catalyzed hydrolysis of esters proceeds through the two-step mechanism involving an intermediate acyl-enzyme is substantial and has been discussed extensively (*e.g.*, Lowe, 1970). A minimal mechanism is shown in Scheme I, where K_s is the dissociation constant of the enzyme–substrate complex, k_2 the acylation rate constant, and k_3' ($= k_3[H_2O]$) the rate constant for the reaction of the acyl-enzyme with water. For most esters so far examined the rate-determining step has been shown to be deacylation of the acyl-enzyme, *i.e.*, $k_2 \gg k_3'$ (Henry and Kirsch, 1967; Fink and Bender, 1969). The exceptions are α-*N*-benzoylarginine ethyl ester for which the acylation rate is only about five times that of deacylation (Whitaker, 1969), isopropyl hippurate for which the rate-determining step is probably acylation (Lucas and Williams, 1969), and *p*-nitrophenyl hippurate for which it has been suggested that there is a kinetically significant step

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† Present address: Pharmacology Department, Harvard School of Dental Medicine, Boston, Mass.

‡ To whom correspondence should be addressed.