Chymotrypsins from the Deer (Cervidae) Family

ISOLATION, PARTIAL CHARACTERIZATION AND PRIMARY-STRUCTURE STUDIES OF CHYMOTRYPSINS A AND B FROM BOTH MOOSE (*ALCES ALCES*) AND ELK (*CERVUS ELAPHUS*) PANCREAS

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1. An anionic and a cationic chymotrypsin (EC 3.4.21.1) were isolated from the pancreas glands of the moose (*Alces alces*) and elk (*Cervus elaphus*). The A and B chymotrypsins from each species were purified to homogeneity by $(\text{NH}_4)_2\text{SO}_4$ fractionation, affinity chromatography on 4-phenylbutylamine-Sepharose and ion-exchange chromatography on DEAE- and CM-cellulose. 2. The molecular weight and pH optimum of each chymotrypsin were similar to those of the corresponding ox A and B chymotrypsins. 3. The substrate specificities of the chymotrypsins were investigated by digestion of glucagon and the oxidized B chain of insulin. The primary specificity of each chymotrypsin for aromatic amino acid residues was further established by determining the K_m and k_{cat} for the hydrolysis of a number of synthetic amino acid ester substrates. 4. The amino acid composition and total number of residues of moose and elk chymotrypsin A were similar to those of ox chymotrypsin A. An even greater similarity was observed among the B chymotrypsins of the three species. 5. The A chymotrypsins of moose and elk were fragmented to their constituent 'A', 'B' and 'C' polypeptide chains by succinylation (3-carboxypropionylation), reduction and alkylation of the native enzymes. In each case, the two major chains ('B' and 'C') were separated and isolated. By comparison of the amino acid compositions of moose, elk and ox 'B' and 'C' chains, a greater difference was observed among the three A chymotrypsins than was suggested by the amino acid compositions of the native enzymes alone. 6. Peptides were isolated from the disulphide bridge and active-site regions of the A and B chymotrypsins of moose and elk by diagonal peptide-'mapping' techniques. From the amino acid compositions of the isolated peptides (assuming maximum homology) and from a comparison of diagonal peptide 'maps', there was established a high degree of primary-structure identity among the moose, elk and ox chymotrypsins. Tentative sequences were deduced for the peptides isolated by diagonal peptide 'mapping'. 7. Details of the isolation procedures of the moose and elk chymotrypsins A and B and the amino acid analyses of some peptides obtained by diagonal peptide 'mapping' have been deposited as Supplementary Publication SUP 50064 (27 pages) at the British Library Lending Division, Boston Spa, Wetherby, W. Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1976) 153, 5.

As a family, the serine proteinases have been extensively studied, and the homology in their primary structures has been well established. Of all the serine proteinases, trypsin has received the greatest attention from a comparative viewpoint, and considerable progress is being made in the elucidation of the evolutionary history of trypsin-like enzymes (de Haen *et al.*, 1975). Although chymotrypsins have been isolated from a number of species, detailed

structural and functional studies of chymotrypsins have been restricted essentially to those obtained from ox and pig pancreas (Hartley, 1964; Hartley & Kauffman, 1966; Smillie *et al.*, 1968; Charles *et al.*, 1967). Other species from which chymotrypsins or their zymogens have been isolated and partially characterized include fin whale (Koide & Matsuoka, 1969, 1970), turtle (three species), cayman and tuna fish (Mockel & Barnard, 1969*a,b*; Bhargava & Barnard, 1973), African lung fish (Reeck *et al.*, 1970), spiny dogfish (Prah & Neurath, 1967; Lacko & Neurath, 1967), honey bee (Liebel *et al.*, 1971),

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sheep (Koide *et al.*, 1969), guinea pig (Tartakoff *et al.*, 1974), chicken (Ryan, 1965), human (Croston, 1965; Coan *et al.*, 1971), sea anemone (Gibson & Dixon, 1969; Stevenson *et al.*, 1974), hornet (Jany & Pfeiderer, 1974; Jany *et al.*, 1974) and the webbing clothes moth (Ward, 1975).

The present paper reports the isolation and characterization of the A and B chymotrypsins (EC 3.4.21.1) from the pancreas glands of two species of deer, the moose (*Alces alces*) and the Rocky Mountain elk or wapiti (*Cervus elaphus*). The isolation (Lievaart & Stevenson, 1974) and partial characterization (Stevenson & Voordouw, 1975) of moose trypsin and moose elastase have been reported. The Cervidae family, of which the moose and elk are members, is considered a relatively recent branch of the evolutionary line that led to the Bovidae (cattle, sheep, goats) (Romer, 1966). An older branch of this same line led to the Suidae (pig) family. In view of the information available on the primary structure of the ox and porcine chymotrypsins, the above evolutionary relationship indicated that a study of chymotrypsins from the deer family would provide additional and intermediate information on the evolutionary history of chymotrypsin-like enzymes.

Experimental

Materials

Glucagon (lot no. Y3843) and the oxidized B chain of insulin (lot no. Y1385) were obtained from Schwarz/Mann, Orangeburg, NY, U.S.A. The following substrates were obtained from Cyclo Chemical Co., Los Angeles, CA, U.S.A.: Ac-Tyr-OEt† (lot no. 030517), *N*-acetyl-L-tryptophan ethyl ester (lot no. 027035), *N*-acetyl-L-phenylalanine ethyl ester (lot no. R5278), *N*-benzoyl-L-leucine ethyl ester (lot no. K5108), *N*-benzoyl-L-tyrosine ethyl ester (lot no. R5769) and *N*-benzoyl-L-arginine ethyl ester (lot no. H4291). Hippuryl-DL- β -phenyl-lactic acid (lot no. 101193) was purchased from Calbiochem, Los Angeles, CA, U.S.A. Phenylmethanesulphonyl fluoride (lot no. 31C-2040), Tos-Phe-CH₂Cl (lot no. T-4376) and pepsin (lot no. 15B-1370-1) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Elastin Congo Red (lot no. 3845) and Tos-Lys-CH₂Cl (lot no. 7174) were obtained from Nutritional Biochemical Corp., Cleveland, OH, U.S.A. CM-cellulose (Whatman CM-23) and DEAE-cellulose (Whatman DEAE-51) were obtained from H. Reeve Angel and Co., Clifton, NJ, U.S.A. Methanesulphonic acid (4M), containing 0.2%

3-(2-aminoethyl)indole was purchased from Pierce Chemical Co., Rockford, IL, U.S.A. 1,3-Propane sultone (3-hydroxypropanesulphonic acid γ -sultone) was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A.

Pancreas glands from the moose (*Alces alces*) and the elk (*Cervus elaphus*) were obtained from Elk Island National Park, Alberta, Canada, through the generous assistance of the National and Historic Parks Branch of Canada and the Canadian Wildlife Service.

Methods

Enzyme activity assays. Chymotrypsin activity was assayed against Ac-Tyr-OEt as described by Enenkel & Smillie (1963a). Trypsin and carboxypeptidase A were similarly assayed against *N*-benzoyl-L-arginine ethyl ester and hippuryl-DL- β -phenyl-lactic acid (Snoke *et al.*, 1948) respectively. Elastolytic activity was assayed by the elastin Congo Red method (Gertler & Hofmann, 1967; Gertler & Birk, 1970).

Protein concentration. Protein concentration was determined by absorbance measurements at 280 nm. The approximate concentration of the chymotrypsins in impure preparations was calculated by using an $E_{1\text{cm}}^{1\%}$ value of 20.0, as reported for ox chymotrypsin A (Awad & Wilcox, 1963). However, $E_{1\text{cm}}^{1\%}$ values were determined at 280 nm for each purified chymotrypsin to be as follows: moose A, 18.9; moose B, 15.9; elk A, 19.2; elk B, 16.2.

Amino acid analyses. Amino acid analyses were performed on a Beckman 121 automatic amino acid analyser by the method of Spackman *et al.* (1958), as modified for accelerated analyses (Spackman, 1967). Triplicate analyses were carried out on each of the chymotrypsins hydrolysed in 6M-HCl at 110°C for 18, 24, 48 and 72 h. Half-cystine was determined as cysteic acid and methionine as methionine sulphone (Moore, 1963). Samples for tryptophan analyses were hydrolysed in 4M-methanesulphonic acid, containing 0.2% 3-(2-aminoethyl)indole, for 24, 48 and 72 h at 110°C (Liu & Chang, 1971). Tryptophan was corrected for 94% recovery based on results obtained for ox chymotrypsinogen A.

S-3-Sulphopropyl-L-cysteine (Ruegg & Rudinger, 1976) was eluted in the same position as cysteic acid (19 min) on the long column of the analyser, and the colour constant was 103% of that of aspartic acid, as determined from standards.

Polyacrylamide-gel electrophoresis. The purity of each of the isolated chymotrypsins was examined by electrophoresis in 7.5% (w/v) polyacrylamide gels by using the Tris/glycine system of Davis (1964). Gels, 8 cm in length, were stained with 1% (w/v) Amido Schwarz and destained by diffusion against 7% (v/v) acetic acid.

† Abbreviations: Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; Tos-Phe-CH₂Cl, 1-chloro-4-phenyl-3-L-tosylamidebutan-2-one; Tos-Lys-CH₂Cl, 7-amino-L-1-chloro-2-tosylamidoheptan-2-one; Bicine, *NN*-bis-(2-hydroxymethyl)glycine; Cys(O₃H), cysteic acid; Met(O₂), methionine sulphone.

High-voltage paper electrophoresis. The vertical-strip apparatus used for high-voltage paper electrophoresis was similar to that described by Ryle *et al.* (1955). The buffer systems and coolants were as previously described (Smillie & Hartley, 1966). Visual dye markers were used (Stevenson, 1971), and peptides were detected with the cadmium/ninhydrin reagent (Heilman *et al.*, 1957).

Specific detection reagents were used to indicate peptides containing tryptophan (Bailey, 1968a), histidine (Bailey, 1968b), tyrosine (Bailey, 1968b) and arginine (Yamada & Itano, 1966). The mobilities of peptides run at pH 6.5 ($m_{6.5}^{Asp}$) were calculated relative to aspartic acid, with measurements being taken from the migration point of neutral amino acids (Offord, 1966). Similarly, the mobilities of peptides run at pH 2.0 ($m_{2.0}^{Ser}$) were calculated relative to serine, with measurements being taken from the neutral point indicated by taurine. Purified peptides were eluted from paper strips with double-deionized water, taken to dryness and hydrolysed in 6M-HCl at 110°C for 18 h.

Enzyme specificity towards natural substrates. Glucagon and the oxidized B chain of insulin were digested with the purified A and B chymotrypsins of both moose and elk. The conditions for the digestions were similar to those described by Enenkel & Smillie (1963b). The molar ratio of polypeptide substrate to enzyme was approx. 250:1 (8–9 mg, approx. 2.5 μ mol) of substrate and 0.25 mg (approx. 10 nmol) of enzyme. The temperature was controlled at 37°C and the pH was maintained at 8.0 by the automatic addition of 0.1 M-NH₃. After 1 h, a further sample (0.25 mg, approx. 10 nmol) of enzyme was added, and 30 min later, when the addition of base had completely ceased, the reaction was terminated by lowering the pH to 3.0 with 1.0 M-acetic acid.

Enzyme specificity towards synthetic substrates. The studies on esterase specificity were performed with a Radiometer (Copenhagen) pH-stat consisting of an SBR 2 titrigraph, a TTT 11 titrator, a PHM 28c meter and an SBU 1a syringe burette. Substrates were prepared over the range 0.5–10 mM in 10 mM-Tris/HCl buffer, pH 8.0, containing 100 mM-KCl and 50 mM-CaCl₂ (Enenkel & Smillie, 1963a). In addition, the solutions were 15% (v/v) methanol for the *N*-acetyl amino acid esters and 30% (v/v) methanol with the *N*-benzoyl derivatives. For each assay a 3.0 ml portion of the substrate was pipetted into a polyethylene reaction vessel. The pH was first adjusted to 8.0, and then 25 μ l of enzyme was added. The pH was maintained at pH 8.0 by the automatic addition of 50 mM-NaOH, and the uptake of base was recorded against time. All assays were conducted in triplicate at 25°C $\pm$ 0.2°C.

With the more soluble substrates, the *N*-acetyl amino acid esters, Hofstee plots (Hofstee, 1959) were established from the initial velocities of the

hydrolysis reactions, determined from the initial linear portion of the titration curves. A correction was made for non-enzymic hydrolysis. Enzyme concentrations were determined from E_{280} measurements, by using $E_{1cm}^{1\%}$ values determined for each enzyme and an approximate mol.wt. of 25000. The K_m and k_{cat} were determined from the Hofstee plots.

For the less soluble *N*-benzoyl substrates an integrated Michaelis-Menten equation (eqn. 1) (Segel, 1968) was used to establish kinetic plots:

$$\frac{2.3}{t} \log \left(\frac{S_0}{S_0 - P} \right) = \frac{k_3 E_0}{K_m} - \left(\frac{1}{K_m} \cdot \frac{P}{t} \right) \quad (1)$$

where S_0 is the initial substrate concentration, P is the product concentration at time t , K_m is the Michaelis constant (apparent), E_0 is the enzyme concentration and $k_3 = k_{cat}$, the maximal turnover rate constant.

A plot of $(2.3/t) \log [S_0/(S_0 - P)]$ versus P/t gives a straight line of slope $-1/K_m$ with an intercept on the P/t axis of V_{max} .

For these assays, P values were determined on the non-linear part of the titration curve and were the amounts of base taken up after time t . The reactions were allowed to go to completion, and S_0 was determined from the total uptake of base.

Ultracentrifugation analyses. Sedimentation-equilibrium experiments were performed by the high-speed method of Yphantis (1964), in a Beckman model E analytical ultracentrifuge. The enzyme concentration was in the range 0.1–0.5 mg/ml, and before each experiment the enzyme solution was dialysed for 12 h against 0.1 M-glycine, adjusted to pH 2.3 with HCl, containing 0.1 M-NaCl. The runs were performed at 36000 rev./min, and equilibrium was always reached within 10 h. In calculating the molecular weight, an assumed partial specific volume of 0.725 g/ml was used. This was the mean of reported values for the ox chymotrypsins (Sober, 1968).

Enzyme purification. All operations were carried out at 4°C and, except where specifically mentioned, identical procedures were used for the isolation of an anionic and a cationic chymotrypsin from both moose and elk pancreas glands. Freeze-drying did not affect chymotryptic activity.

Step 1: acid extraction. Frozen pancreas (1 kg) was thawed in 3 litres of 0.125 M-H₂SO₄. Fat and connective tissue were removed and the remaining material was minced in an electric meat grinder. The minced pancreas was extracted twice with 0.125 M-H₂SO₄, first for 18 h in the solution in which the glands had been thawed and then for a further 6 h in 1 litre of fresh 0.125 M-H₂SO₄. In each case the suspension was stirred at 4°C, and after extraction the solid material was removed from the suspension by filtration through cheesecloth. The combined supernatants from the two extractions had a volume

of 4 litres and an E_{280} of 60–70 absorbance units, depending on the preparation.

Step 2: ammonium sulphate fractionation. Fractionation of the H_2SO_4 extract was made between 0 and 20%, 20 and 45% and 45 and 70% $(NH_4)_2SO_4$ saturation at 4°C, by using a nomogram (Dixon, 1953) to obtain the amounts of solid $(NH_4)_2SO_4$ to be added. The precipitate in each case was collected by centrifugation for 30 min at 11 000g. The precipitates from the 20–45%-satd. and 45–70%-satd. fractions were dialysed against 1 mM-HCl, filtered and freeze-dried. The yields of crude material from the moose pancreas extract were 15g/kg and 8g/kg for the 20–45%-satd.- and 45–70%-satd.- $(NH_4)_2SO_4$ fractions respectively. The corresponding yields from the elk pancreas extract were 17g/kg and 7g/kg respectively.

Step 3: affinity chromatography on 4-phenylbutylamine-Sepharose. The crude material (1g) from Step 2 was dissolved in 50ml of 50mM-Bicine buffer adjusted to pH8.0 with NaOH. Spontaneous activation of the chymotrypsinogens by the native trypsin present was obtained by gently stirring the above solution for 40 min at 0°C. The solution, now containing the active chymotrypsins, was subjected to affinity chromatography on 4-phenylbutylamine-Sepharose (Stevenson & Landman, 1971). Extraneous protein was washed from the column with 50mM-Bicine buffer adjusted to pH8.0 with NaOH, and the bound chymotrypsins were eluted with 0.1M-acetic acid. The fractions containing the chymotryptic activity were pooled, dialysed against 1mM-HCl and freeze-dried. The yields of purified chymotrypsins were 400mg/g and 100mg/g from the 45–70%-satd.- and 20–45%-satd.- $(NH_4)_2SO_4$ fractions respectively. The yields were similar for both moose and elk samples.

Step 4a: chromatography of moose chymotrypsins on DEAE-cellulose. The freeze-dried moose chymotrypsins (300mg) from Step 3 were dissolved in 20ml of 10mM-Tris solution adjusted to pH6.0 with acetic acid. The enzyme solution was applied to a column (45cm×2.5cm) of DEAE-cellulose equilibrated with 10mM-Tris adjusted to pH6.0 with acetic acid. The column was washed with 400ml of the starting solution and then a linear salt gradient was applied which consisted of equal volumes (175ml) of 10mM-Tris adjusted to pH6.0 with acetic acid with one solution containing 0.4M-NaCl. A major cationic protein with high chymotrypsin esterase activity, designated moose chymotrypsin A, was eluted at the void volume. Two further regions containing chymotrypsin activity were eluted with 0.11M- and 0.22M-NaCl and were designated moose chymotrypsin B and C respectively. Moose chymotrypsin C was in low yield and was not investigated further. The fractions under each protein peak were pooled, dialysed against 1mM-HCl and freeze-dried.

Step 4b: chromatography of elk chymotrypsins on DEAE-cellulose. The elk chymotrypsins obtained in Step 3 were chromatographed on DEAE-cellulose by a procedure essentially identical with that described in Step 4a. The major difference was that a 10mM-Tris buffer, adjusted to pH8.4 with acetic acid, was used. The elk chymotrypsins gave an elution profile on DEAE-cellulose column chromatography similar to that described for the moose chymotrypsins and these were designated elk chymotrypsin A, B and C respectively. The C enzyme was not investigated further.

Step 5a: chromatography of moose chymotrypsin A on CM-cellulose. The first protein peak (cationic moose chymotrypsin A), which emerged in the breakthrough on the DEAE-cellulose column in Step 4a, was rechromatographed on CM-cellulose. A solution of 300mg of enzyme in 20ml of 20mM-citric acid buffer adjusted to pH6.0 with NaOH was applied to a column (90cm×2.5cm) of CM-cellulose previously equilibrated with the starting buffer. The column was washed with 150ml of starting buffer and the bound enzyme was eluted with a linear gradient of equal volumes (350ml) of 20mM- and 200mM-citric acid buffer adjusted to pH6.0 with NaOH. Except for a small percentage of inactive protein eluted near the void volume, the sample was eluted as a single peak in the middle of the citrate buffer gradient. The fractions containing the major protein peak were pooled, dialysed against 1mM-HCl and freeze-dried.

Step 5b: chromatography of elk chymotrypsin A on CM-cellulose. The breakthrough peak (cationic elk chymotrypsin A) from DEAE-cellulose chromatography of elk chymotrypsins in Step 4b was rechromatographed on CM-cellulose. A solution of 200mg of enzyme in 20ml of 1mM-Tris adjusted to pH7.8 with HCl was applied to a column (90cm×2.5cm) of CM-cellulose previously equilibrated with the starting buffer. A linear gradient was used with equal volumes (400ml) of 1mM- and 250mM-Tris adjusted to pH7.8 with HCl. Elk chymotrypsin A was resolved into two active fractions in the mid-range of the Tris/HCl buffer gradient. The fractions containing the minor and major protein peaks were pooled, dialysed against 1mM-HCl and freeze-dried. The minor component, which was eluted first, possessed only half the specific activity of the major component. A single amino acid analysis of this minor component indicated that it was very similar to the major component. It was, therefore, assumed that the minor component might be either an activation intermediate between zymogen and native enzyme or, alternatively, a large active autolysis product.

Separation and isolation of the 'B' and 'C' polypeptide chains of moose and elk chymotrypsin A. The A chymotrypsins of moose and elk were

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of the A and B chymotrypsins of moose and elk

Electrophoresis was performed at pH9.5 in 7.5% (w/v) polyacrylamide gels with a current of 5 mA per tube for 40 min. All the enzyme samples were inactivated with phenylmethanesulphonyl fluoride before electrophoresis. The enzymes were: (a) moose chymotrypsin B; (b) moose chymotrypsin A; (c) elk chymotrypsin B; (d) elk chymotrypsin A. Migration was from cathode (top) to anode (bottom).

fragmented to their 'A', 'B' and 'C' chains by the method of Hapner & Wilcox (1970). The succinylated (3-carboxypropionylated) and reduced protein was alkylated with either iodoacetamide (Hapner & Wilcox, 1970) or 1,3-propane sultone (Ruegg & Rudinger, 1976).

In separate experiments, the succinyl-*S*-carboxyamidomethylated and succinyl-*S*-sulphopropylated proteins were chromatographed on Sephadex G-100 to separate the 'B' and 'C' polypeptide chains. The small 'A' chain of each enzyme was lost during dialysis. The fractions containing the 'B' and 'C' chains were pooled separately and dialysed against double-distilled water. The 'C' chain was concentrated by precipitation after acidification of the solution to pH 3.0 with 6M-HCl. The 'B' chain, remaining in solution, was concentrated by rotary evaporation.

Samples of both the carboxyamidomethylated and sulphopropylated chains were hydrolysed in 6M-HCl at 110°C for 24, 48 and 72 h and subjected to amino acid analyses.

Peptide 'maps'. Diagonal peptide 'maps' of peptic digests of moose and elk chymotrypsin A and B were prepared by high-voltage electrophoresis at pH 6.5 as described by Brown & Hartley (1963, 1966). Before running the second dimension of the diagonal peptide 'map', a side strip of the first dimension was exposed to performic acid vapour. Thus disulphide bridges were oxidized to cysteic acid residues and methionine to methionine sulphone. The diagonal peptide 'maps', after staining with cadmium/ninhydrin, served as the basis for the location of cystine peptides on the original pH 6.5 ionograms and for the subsequent isolation of cysteic acid peptides. These peptides were in most instances purified by a single high-voltage electrophoresis operation at pH 6.5. However, where necessary, further purification was achieved by high-voltage electrophoresis at pH 2.0 or by descending paper chromatography in a system of butan-1-ol/acetic acid/water (3:1:1, by vol.).

A modified diagonal peptide-'mapping' technique, as described by Stevenson (1974), was used to isolate selectively peptides encompassing the active-site histidine and active-site serine residue of each enzyme. Diagonal peptide 'maps' were prepared from peptic digests of reduced and cyanoethylated moose and elk chymotrypsins, which had been inactivated with Tos-Phe-CH₂Cl. With the absence of disulphide bridges, the effect of performic acid was directed towards a Baeyer-Villiger peracid rearrangement of the ketone moiety of Tos-Phe-CH₂Cl covalently attached to the *N*⁷-position of histidine-57 (Stevenson & Smillie, 1968). The transformation of the Tos-Phe-CH₂-histidinyl residue into *N*⁷-carboxymethylhistidinyl residue introduced an acidic group into the active histidine-57 peptide thus enabling it to migrate off the 'diagonal' during the second dimension of

high-voltage electrophoresis at pH 6.5. These 'maps' served as the basis for the location and selective isolation of peptides that contained *N*⁷-carboxymethylhistidine. In addition, however, peptides that contained *N*-terminal methionine sulphone or *N*-terminal *S*-cyanoethylcysteine sulphone were also observed just below the 'diagonal'.

Results and Discussion

Enzyme purification

The results of the purification procedures described under 'Methods' are summarized in Table 1. Approximately equal amounts (30–40%) of the original total activity were precipitated in the 20–45%-satd.- and 45–70%-satd.-(NH₄)₂SO₄ fractions. However, the recovery of esterase activity after affinity chromatography of the 20–45%-satd. fractions was very low (approx. 20%) compared with the recovery from the 45–70%-satd. fraction (approx. 90%). Under the conditions used, the 4-phenylbutylamine-Sepharose column was very selective for chymotrypsin. There was no trace of trypsin, carboxypeptidase A or elastase activity in the purified preparations of either moose or elk chymotrypsins.

Enzyme purity

Disc gel electrophoresis at pH 9.5 indicated a high degree of purity of the A and B chymotrypsins of both moose and elk, as is shown in Plate 1. This purity, however, could only be shown after enzymes had been inactivated with phenylmethanesulphonyl fluoride or Tos-Phe-CH₂Cl. The native enzymes, particularly the A chymotrypsins, autolysed rapidly under the conditions of sample preparation and electrophoresis in the Tris/glycine system of Davis (1964). Attempts to show purity at pH 2.7 or 4.5 with disc gel electrophoresis were unsuccessful, as the

Table 1. *Specific activities of moose and elk chymotrypsins after each step in the purification*

Specific activity was expressed as μmol of Ac-Tyr-OEt hydrolysed/min per ml divided by E_{280} .

Purification step	Specific activity	
	Moose	Elk
Crude extract	17	37
45–70%-satd.-(NH ₄) ₂ SO ₄ fraction	165	336
Affinity chromatography	230	590
DEAE-cellulose		
Chymotrypsin A	293	600
Chymotrypsin B	265	200
CM-cellulose		
Chymotrypsin A	336	630

Fig. 1. Peptides produced on digestion of glucagon and the oxidized B chain of insulin with moose and elk chymotrypsin A.

The digestion conditions and purification of the digestion products were as described in the text. The horizontal lines depict the peptide fragments identified by amino acid analyses.

Fig. 2. Peptides produced on digestion of glucagon and the oxidized B chain of insulin with moose and elk chymotrypsin B

The digestion conditions and purification of the digestion products were as described in the text. The horizontal lines depict the peptide fragments identified by amino acid analyses.

moose and elk chymotrypsins showed no appreciable mobility at either pH.

The purity of each chymotrypsin was further supported by gel-filtration experiments on a column of Sephadex G-75. Each enzyme preparation was eluted essentially as a single peak with a constant specific activity. This was comparable with the specific activity of the applied enzyme. A varying amount of low-molecular-weight material was eluted after the major peak and was doubtless autolysis products formed during sample preparation and even during the experiment.

Molecular-weight determinations

Sedimentation analyses of moose chymotrypsin A and B gave linear plots of the logarithm of the fringe displacement versus r^2 over the entire cell. In contrast, similar plots for both elk chymotrypsin A and B exhibited a slight upward curvature at the cell bottom. This suggested that, even at pH 2.3, elk chymotrypsin A or B may dimerize to some extent. Dimerization of ox chymotrypsin A(α) in the acid region has been well documented (Egan *et al.*, 1957; Aune & Timasheff, 1971), but this occurs between pH 2.5 and 5.5 with a maximum around pH 4.0.

The mol.wts. calculated for each of the chymotrypsins from the above plots were: 24 700, moose A; 26 800, moose B; 28 300, elk A; 28 700, elk B. The mol.wts. of the moose chymotrypsins are similar to those of the corresponding ox chymotrypsins (25 200), as calculated from their sequences (Dayhoff, 1972) within the limits of experimental error.

pH optima and inactivation

The pH optimum range for moose and elk chymotrypsin A towards the substrate Ac-Tyr-OEt was pH 7.5–8.0. The corresponding pH optimum range for the B enzymes was slightly lower at pH 7.3 to 7.7. The chymotrypsins were completely inactivated with phenylmethanesulphonyl fluoride in less than 40 min at pH 8.0 when the inhibitor was present at a molar ratio of 100:1. Similarly, Tos-Phe-CH₂Cl, at a molar ratio of 10:1, completely inactivated all the chymotrypsin within 3–5 h. As was expected, the potent trypsin inhibitor Tos-Lys-CH₂Cl was without effect, even at a molar ratio of 100:1 at pH 8.0. These results give support to the classification of these enzymes as members of the chymotrypsin family.

Enzyme specificity towards natural substrates

The peptides depicted by solid lines in Figs. 1 and 2 were produced on digestion of glucagon and the oxidized B chain of insulin by moose and elk chymotrypsin A and B respectively. The sequence and alignment of each peptide was deduced from the amino acid composition, electrophoretic mobility and specific stain data compiled for each peptide.

The primary specificity of moose and elk chymotrypsin A and B for aromatic residues is supported by cleavages occurring on the C-terminal side of tyrosine, tryptophan and phenylalanine residues in both polypeptide hormones. The extent of cleavage at a particular aromatic residue was partially dependent on the residues located *N*-terminally to the aromatic residue.

The cleavage of glucagon on the C-terminal side of arginine-17 and -18 by moose and elk chymotrypsins A was not typical of chymotrypsin-like proteinases. Initially, this was assumed to be the result of trypsin contamination, but pretreatment of the enzyme preparations with Tos-Lys-CH₂Cl in a 100:1 molar excess failed to eliminate this cleavage at arginine. In addition, when the enzyme preparations were inactivated with Tos-Phe-CH₂Cl before the digestion, no hydrolysis of glucagon took place. These two observations suggest that moose and elk chymotrypsin A are themselves capable of hydrolysing glucagon on the C-terminal side of its two arginine residues. In contrast, hydrolysis did not occur on the C-terminal side of arginine-23 of the oxidized B chain of insulin. It is possible that the cleavages on the C-terminal side of arginine in glucagon represent a special case. It is, however, not representative of the true specificity of the moose and elk chymotrypsins.

Comparing Figs. 1 and 2, the B chymotrypsins of moose and elk have a slightly broader specificity than the A chymotrypsins of the same species. The partial specificity of the B enzymes for leucine and to some extent methionine residues is similar to the reported specificity difference between ox chymotrypsins B and A (Enenkel & Smillie, 1963*b*; Bromer *et al.*, 1957). The B chymotrypsins were also found to hydrolyse glucagon on the C-terminal side of arginine-17 and -18. In addition, the B enzymes cleaved the oxidized B chain of insulin on the C-terminal side of its sole arginine residue at position 22. Pretreatment of the B chymotrypsins with either Tos-Lys-CH₂Cl or Tos-Phe-CH₂Cl failed to substantiate that these cleavages on the C-terminal side of arginine residues were due to trypsin contamination.

Enzyme specificity towards synthetic substrates

The kinetic constants, K_m and k_{cat} , determined for five ester substrates of moose and elk chymotrypsins A and B, are listed in Table 2. These values are compared with the reported values for ox chymotrypsin A and B, obtained under similar conditions.

The substrate *N*-benzoyl-L-leucine ethyl ester was very slowly hydrolysed by the A chymotrypsins of moose and elk, whereas the corresponding B chymotrypsins hydrolysed this substrate at a rate comparable with that reported for ox chymotrypsin B (Folk & Schirmer, 1965). The difference in the rate of

Table 2. Kinetic constants determined for some ester substrates of moose and elk chymotrypsins

Substrate	Chymotrypsin A						Chymotrypsin B					
	Moose			Elk			Moose			Elk		
	K_m (mM)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)
Ac-Tyr-OEt	2.1±0.04	256±5	1.7±0.04	432±12	0.7	193	1.5±0.03	210±4	2.5±0.04	183±4	—	—
Ac-Phe-OEt	2.3±0.05	64±2	1.9±0.04	116±3	1.2	173	2.8±0.05	117±3	1.6±0.04	78±2	—	—
Ac-Trp-OEt	1.5±0.04	95±3	2.5±0.07	33±1	0.09	47	1.8±0.04	12±0.4	2.3±0.07	7±0.2	2.1	0.71
Bz-Tyr-OEt†	0.6±0.003	51±2	1.4±0.06	75±3	2.6	43	6.3±0.2	13±0.5	1.8±0.07	23±1	1.0	63
Bz-Leu-OEt†	—	—	—	—	12.5	0.07	0.15±0.006	1.5±0.06	49±2	0.1±0.004	72	0.11

* Data compiled from Folk & Schirmer (1965) and Cunningham (1965).
† The abbreviation Bz- represents benzoyl.

hydrolysis of the leucine substrate by the A and B chymotrypsins was in agreement with the observed differences in the specificity of each enzyme towards leucine residues in the polypeptide substrates.

The trypsin substrate *N*-benzoyl-L-arginine ethyl ester was not hydrolysed by either the A or B chymotrypsins of moose or elk, even at high enzyme concentration. This indicates that there was no significant contamination of any of the purified preparations with trypsin. Further, this gives support to the contention that the hydrolysis of glucagon on the C-terminal side of arginine-17 and -18 by moose and elk chymotrypsins is attributable to the chymotrypsins.

Amino acid analysis of moose and elk chymotrypsin A and B

The amino acid compositions determined for the A and B chymotrypsins of moose and elk are presented in Table 3 and compared with the correspond-

Fig. 3. Separation of the 'B' and 'C' chains of moose chymotrypsin A by gel filtration on Sephadex G-100

Sample preparation was as described under 'Methods'. —, Elution profile of a sample of a solution of the succinylated and carboxyamidomethylated chains which had been dialysed after sample preparation. On acidification of the solution to pH 3.0, half of the protein precipitated and half remained in solution. The precipitate was washed twice with 3 mM-HCl and then dissolved in 10 mM-ammonium acetate. Gel filtration of this sample gave rise to the elution profile indicated by —. The protein that remained in solution after acidification was concentrated by rotary evaporation and dialysed against 10 mM-ammonium acetate. The elution profile obtained on gel filtration of this sample is indicated by the open circles (○). Gel-filtration experiments were carried out on a column (85cm×1.5cm) of Sephadex G-100. All samples were 3.0ml, and elution was with 10 mM-ammonium acetate. Fractions of volume 2.4ml were collected.

ing ox chymotrypsin. Calculation of the number of residues of each amino acid was based on an assumed value for the number of residues of alanine per mole of protein (Bergsetz *et al.*, 1963). With these assumptions, the total number of residues of each of the moose and elk chymotrypsins was very similar to, if not identical with, the total number of residues of the corresponding ox chymotrypsin. This was in agreement with the similarity found in the molecular weights of these enzymes.

Separation, isolation and amino acid analyses of the 'B' and 'C' chains of moose and elk chymotrypsin A

Chromatography of the carboxyamidomethylated 'B' and 'C' chains of moose chymotrypsin A on Sephadex G-100 is depicted by the broken line in Fig. 3. The 'C' chain of moose chymotrypsin A was eluted well ahead of the larger 'B' chain in contrast with the separation of the 'B' and 'C' chains of ox chymotrypsin A (Hapner & Wilcox, 1970). The two chains could also be separated by fractional precipi-

tation (cf. Fig. 3). Amino acid analyses established that the precipitate was the 'C' chain, whereas the 'B' chain remained in solution. Essentially identical data were obtained with moose or elk chymotrypsin A after reduction and alkylation with propane sulfone.

The amino acid compositions of the reduced and carboxymethylated 'B' and 'C' chains of moose and elk chymotrypsin A are presented in Table 4. The total amino acid residues of the 'C' chains of moose, elk and ox chymotrypsin A were identical, and the total amino acid residues of the 'B' chains of these three enzymes were very similar.

Further, the amino acid compositions indicated a greater difference among the primary structures of these three chymotrypsins than is evident from a comparison of the amino acid compositions of the native enzymes alone.

When propane sulfone was substituted for iodoacetamide in the alkylation of the 'B' and 'C' chains, the data in Table 4 were essentially duplicated. However, the quantification of half-cystine residues

Table 3. Amino acid compositions of the A and B chymotrypsins of moose and elk

The value for alanine was arbitrarily set at 22.0 residues for moose and elk chymotrypsin A, 20.0 residues for moose chymotrypsin B and 21.0 residues for elk chymotrypsin B. The data for ox chymotrypsin A were reported by Hartley (1964) and Hartley & Kauffman (1966). The data for chymotrypsin B were reported by Smillie & Hartley (1967) and Smillie *et al.* (1968).

	Chymotrypsin A					Ox	Chymotrypsin B					Ox
	Moose		Elk		Moose		Elk					
	No. of residues	Integral values	No. of residues	Integral values	No. of residues		Integral values	No. of residues	Integral values			
Lysine	9.6	10	8.3	8	14	11.4	11	11.8	12	11		
Histidine	1.8	2	1.8	2	2	1.9	2	2.3	2	2		
Arginine	3.3	3	4.3	4	3	3.0	3	3.7	4	4		
Tryptophan*	7.9	8	7.8	8	8	7.6	8	8.1	8	8		
Aspartic acid	21.1	21	23.9	24	22	19.2	19	20.2	20	19		
Threonine*	21.2	21	24.1	24	22	23.2	23	21.9	22	23		
Serine*	31.0	31	29.9	30	27	23.2	23	20.9	21	22		
Glutamic acid	14.6	15	14.5	15	15	19.4	19	19.3	19	18		
Proline	8.6	9	8.6	9	9	14.2	14	12.2	12	13		
Glycine	22.7	23	23.7	24	23	22.8	23	23.2	23	23		
Alanine	22.0	22	22.0	22	22	20.0	20	21.0	21	21		
Half-cystine†	9.8	10	9.8	10	10	9.7	10	9.8	10	10		
Valine‡	22.1	22	23.4	23	23	25.3	25	25.4	25	25		
Methionine§	3.8	4	1.8	2	2	3.9	4	3.8	4	4		
Isoleucine‡	10.0	10	8.9	9	10	8.2	8	8.2	8	8		
Leucine	18.6	19	18.2	18	19	18.7	19	18.6	19	18		
Tyrosine	3.9	4	2.7	3	4	3.3	3	3.2	3	3		
Phenylalanine	6.1	6	5.2	5	6	7.2	7	7.1	7	7		
Total no. of residues	240		240		241	241		240		240		

* Extrapolated to zero time.

† Determined as cysteic acid.

‡ Extrapolated to 100 h.

§ Determined as methionine sulphone.

Table 4. Amino acid compositions of the 'B' and 'C' chains of moose and elk chymotrypsin A

The value for alanine was arbitrarily set at 11.0 residues for the 'B' chain of moose and elk chymotrypsin A. The value for glycine was arbitrarily set at 12.0 residues and 11.0 residues for the 'C' chain of chymotrypsin A from moose and elk respectively. The data for the 'B' and 'C' chains of ox chymotrypsin A were reported by Hapner & Wilcox (1970). CM-cysteine refers to S-carboxymethylcysteine. Tryptophan was not determined.

	'B' chain of chymotrypsin A					'C' chain of chymotrypsin A				
	Moose		Elk		Ox	Moose		Elk		Ox
	No. of residues	Integral values	No. of residues	Integral values		No. of residues	Integral values	No. of residues	Integral values	
Lysine*	5.6	6	4.3	4	8	3.9	4	3.9	4	6
Histidine	1.8	2	1.8	2	2	0.2	0	0	0	0
Arginine	1.5	2	1.6	2	1	1.5	2	2.1	2	2
CM-cysteine	4.2	4	3.6	4	4	4.4	5	4.4	5	5
Aspartic acid	12.9	13	15.4	15	13	7.6	8	8.4	8	9
Threonine†	9.7	10	10.6	11	13	11.1	11	11.2	11	9
Serine†	19.2	19	17.2	17	15	10.9	11	12.1	12	11
Glutamic acid	9.9	10	12.3	12	10	3.6	4	3.8	4	4
Proline	7.3	7	3.0	3	3	3.3	3	3.1	3	4
Glycine	12.3	12	13.1	13	11	12.0	12	11.0	11	10
Alanine	11.0	11	11.0	11	11	8.8	9	9.4	9	10
Valine‡	12.2	12	11.7	12	13	9.1	9	9.2	9	8
Methionine	0.6	1	0	0	0	2.5	3	1.6	2	2
Isoleucine	5.1	5	5.8	6	6	3.0	3	2.8	3	3
Leucine	10.1	10	8.8	9	9	6.1	6	7.4	7	8
Tyrosine	1.5	2	1.1	1	2	1.5	2	1.7	2	2
Phenylalanine	3.8	4	4.2	4	6	0.6	1	0.8	1	0
Total no. of residues	130		126		127	93		93		93

* Recovery from 72h hydrolysate.
† Extrapolated to zero time.
‡ Extrapolated to 100h.

Fig. 4. Diagonal peptide 'maps' of pepsin digests of moose (a) and elk (b) chymotrypsin A and ox chymotrypsin A (c) High-voltage electrophoresis was conducted at pH6.5, 60V/cm, for approx. 1h in both dimensions with intervening per-formic acid treatment. The first dimension is shown by the dashed segment. OG denotes the visual dye marker Orange G.

as S-sulphopropylcysteine residues was better than the data obtained from quantification of half-cysteine as S-carboxymethylcysteine. The following values were obtained for S-sulphopropylcysteine (expected

values in parentheses): 3.8 (4) and 4.9 (5) for the 'B' and 'C' chains respectively of moose chymotrypsin A; 3.9 (4) and 4.9 (5) for the 'B' and 'C' chains respectively of elk chymotrypsin A.

Table 5. Amino acid compositions and alignments of peptides isolated by diagonal peptide 'mapping' from pepsin digests of moose and elk chymotrypsin A and B

The following peptides containing cysteic acid, isolated from moose and elk chymotrypsin A and B, were not identical in size or amino acid composition with the corresponding peptides isolated from ox chymotrypsin A and B.

	¹²⁰	¹²²	¹³⁰
Ox A	Ala - Val - Cys - Leu - Pro - Ser - Ala - Ser - Asp - Asp - Phe		
Moose A	Val,Cys(O ₃ H)Leu, (0.9) (0.9) (1.0) (1.1) (3.9)	Pro, Ser, Ser, Asp (1.0)*	
Elk A	Ala, Val,Cys(O ₃ H)Leu, (0.7) (1.0) (0.8) (0.8) (0.3) (2.6)	Pro, Ser, Ser, Ser, Asp, Asp, Phe, (2.0)*	
Ox B	Ala - Val - Cys - Leu - Pro - Ser - Ala - Asp - Glu - Asp - Phe		
Moose B	Val,Cys(O ₃ H)Leu, (0.9) (1.2) (1.0) (1.1) (1.9) (1.0)*	Pro, Ser, Gly, Ser, Glu, Asp (1.2) (0.9)	
Elk B	Val,Cys(O ₃ H)Leu, (1.0) (1.1) (0.8) (1.0) (0.9) (1.2) (2.0)*	Pro, Ser, Ala, Asp, Glu, Asp, Phe (1.8)	
Ox A	¹³² Ala - Gly - Thr - Thr - Cys - Val - Thr - Thr - Gly - Trp - Gly	¹³⁶	¹⁴²
Moose A (i)	Ala, Gly, Met(O ₂), Ser, Cys(O ₃ H)Ala, (2.0)* (2.1) (0.6) (1.3) (1.0) (1.7)	Thr, Thr, Thr, Gly	
Moose A (ii)	Cys(O ₃ H)Ala, Thr, Gly (0.8) (1.0)*	(1.7) (1.2)	
Elk A (i)	Ala, Gly, (Thr, Ser)Cys(O ₃ H)Val (1.0)* (1.4) (0.9) (0.8) (0.7) (0.5)		
Elk A (ii)	Gly, (Thr, Ser)Cys(O ₃ H)Val, (2.0)* (1.9) (1.6) (0.8) (0.8)	(Thr, Ser), Gly	
Ox B	Ala - Gly - Met - Leu - Cys - Ala - Thr - Thr - Gly - Trp - Gly - Lys - Thr - Lys		
Moose B	Cys(O ₃ H)Ala, Thr, Thr, (0.7) (1.3) (2.4)	Gly, Trp, Gly, Lys, Thr, Lys (2.0)*(n.d) [†] (1.6)	
Elk B	Ala, Gly, Met(O ₂), Leu, Cys(O ₃ H)Ala, (2.1) (2.0)* (0.6) (0.8) (0.8)	Thr, Thr, Gly (1.8)	

Ox A	162									
	Leu	-	Leu	-	Ser	-	Asn	-	Thr	-
Moose A	168									
	Ser,	Asn,	Thr,	Asn,Cys(O ₃ H)	Lys	-	Lys	-	Tyr	-
Elk A (i)	173									
	Ile,	Leu,	Thr,	Asn,	Ser,	Asn,Cys(O ₃ H)	Lys	-	Trp	-
Elk A (ii)										
	Asn,	Ser,	Asn,Cys(O ₃ H)	Lys,	Lys,	Tyr,	Phe	-		
Ox B										
	Gln	-	Ala	-	Thr	-	Leu	-	Pro	-
Moose B										
	Gln,	Ala,	Thr,	Leu,	Pro,	Gly,	Val,	Ser,	Asn,	Thr,
Elk B										
	Ile,	Val,	Ser,	Asn,	Thr,	Asp,Cys(O ₃ H)	Arg,	Lys,	Tyr,	Gly
Ox A										
	Gly	-	Ser	-	Thr	-	Ser	-	Thr	-
Moose A										
	Gly,	Ser,	Ser,	Thr,Cys(O ₃ H)	Ser,	Thr,	Ser,	Thr,	Pro,	Gly,
Elk A										
	Gly,	Ser,	Ser,	Thr,Cys(O ₃ H)	Ser,	Thr,	Ser,	Thr,	Pro,	Gly,
Ox B										
	Gly,	Ser,	Ser,	Thr,Cys(O ₃ H)	Ser,	Thr,	Ser,	Thr,	Pro,	Gly,
Moose B										
	Gly,	Ser,	Ser,	Thr,Cys(O ₃ H)	Ser,	Thr,	Ser,	Thr,	Pro,	Gly,
Elk B										
	Gly,	Ser,	Ser,	Thr,Cys(O ₃ H)	Ser,	Thr,	Ser,	Thr,	Pro,	Gly,

* Arbitrarily taken as an integral number of residues with recoveries of other amino acids relative to this amino acid.
† Not determined.

Fig. 5. Diagonal peptide 'maps' of pepsin digests of moose (a) and elk (b) chymotrypsin B and ox chymotrypsin B (c). High-voltage electrophoresis was conducted at pH 6.5, 60V/cm, for approx. 1 h in both dimensions with intervening performic acid treatment. The first dimension is shown by the dashed segment. OG denotes the visual dye marker Orange G.

Diagonal peptide 'mapping'

The diagonal peptide 'maps' obtained from pepsin digests of moose and elk chymotrypsin A and B are illustrated in Figs. 4 and 5 and are compared with that reported for ox chymotrypsinogen A and B respectively (Brown & Hartley, 1966; Smillie & Hartley, 1967). The numbers opposite each peptide lying below the diagonal refer to the sequential position of the half-cystine residue present in that peptide. The numbering system is based on the sequence of ox chymotrypsin A (Hartley, 1964). The general similarity of these 'maps' suggested that there was probably a high degree of identity in the sequences of moose, elk and ox chymotrypsin A and B, at least in the regions of their disulphide bridges. In four cases (Cys¹-Gly-Val-Pro-Ala-Ile-Gln; His-Phe-Cys⁴²-Gly-Gly-Ser-Leu; Ala-Ala-His-Cys⁵⁸-Gly-Val-Thr-Thr-Ser-Asp; Met-Ile-Cys¹⁸²-Ala-Gly-Ala-Ser-Gly-Val-Ser) the amino acid compositions of the equivalent cysteic acid peptide from moose and elk chymotrypsin A and B were virtually identical. On the basis of the amino acid composition, electrophoretic mobility, cadmium/ninhydrin colour and specific stain data compiled for these peptides, it is proposed that the sequences of moose, elk and ox chymotrypsin A and B are identical, assuming maximum homology, in the regions of half-cystine residues 1, 42, 58 and 182.

The amino acid compositions and proposed alignments of cysteic acid peptides which account for half-cystine residues 122, 136, 168 and 220 of moose and elk chymotrypsins A and B are presented in Table 5. In the region of any one of these half-cystine residues the corresponding cysteic acid peptides isolated from the A and B chymotrypsins of moose

and elk were not identical. In some instances the peptide fragments differed in amino acid composition. However, the amino acid compositions of the cysteic acid peptides isolated from moose and elk chymotrypsin A were sufficiently similar to corresponding fragments of ox chymotrypsin A that it was possible, assuming homology, to deduce tentative sequences from these amino acid compositions. In the peptide sequences proposed for moose chymotrypsin A there were only five positions out of 36 where the amino acid residue was different from the corresponding residue in the sequence of ox chymotrypsin A. Similarly, elk chymotrypsin A differed in only seven out of 43 positions with the sequence of ox chymotrypsin A.

The tentative sequences proposed around half-cystine residues 122, 136, 168 and 220 of the B chymotrypsins from moose and elk are very similar to corresponding portions of the sequence of ox chymotrypsin B (Smillie *et al.*, 1968). Elk chymotrypsin B was identical in 43 positions with the corresponding sequence of ox chymotrypsin B. Moose chymotrypsin B, on the other hand, was found to differ in at least four out of 38 positions from the sequence of ox chymotrypsin B.

The peptide sequences in the regions of half-cystine residues 1, 42, 58 and 182, and those shown in Table 5, for moose and elk chymotrypsins A and B, are tentative as they are based only on amino acid composition, electrophoretic mobility, cadmium/ninhydrin colour and specific stain data compiled for each cysteic acid peptide. They also assume maximum homology. However, the agreement of these proposed sequences with the known sequences of the ox chymotrypsins is suggestive of a high degree of identity

among the sequences of moose, elk and ox chymotrypsins A or B in the regions of their disulphide bridges.

Although the cysteic acid diagonal peptide-'mapping' technique proved very useful, it failed to yield suitable fragments from the moose and elk chymotrypsins equivalent to the peptide regions around half-cystine residues 191 and 201 of ox chymotrypsin A. The sequence between half-cystine residues 191 and 201 is of particular interest as it encompasses the active-site serine residue, serine-195, of the ox chymotrypsins. The inadequacy in the cysteic acid diagonal peptide-'mapping' technique was overcome by using a modified diagonal peptide-'mapping' technique (Stevenson, 1974). The diagonal

peptide 'maps' obtained by this method from pepsin digests of reduced and cyanoethylated moose and elk chymotrypsins, inhibited with Tos-Phe-CH₂Cl, are illustrated in Fig. 6. The peptides indicated by Roman numerals on these 'maps' were isolated and the amino acid compositions of the peptides are presented in Table 6.

The modified diagonal peptide-'mapping' technique of Stevenson (1974) was designed to isolate selectively peptides encompassing the active-site histidine residue of ox chymotrypsin A (histidine-57) and other chymotrypsin-like enzymes. The active-site histidine peptides isolated from moose and elk chymotrypsins A and B by the above technique had amino acid compositions (Table 6a) virtually

Fig. 6. Diagonal peptide 'maps' of pepsin digests of the reduced and cyanoethylated A and B chymotrypsins of moose (a) and (b) and elk (c) and (d)

The enzymes had previously been inhibited with Tos-Phe-CH₂Cl. High-voltage electrophoresis was conducted at pH 6.5, 60 V/cm, for approx. 1 h in both dimensions, with intervening performic acid treatment. The first dimension is shown by the dashed segment. OG denotes the visual dye marker Orange G.

Table 6. *Amino acid composition of peptides isolated by diagonal peptide 'mapping' from pepsin digests of reduced and cyanoethylated moose and elk chymotrypsin A and B inhibited with Tos-Phe-CH₂Cl*

The amino acid sequence of ox chymotrypsin A adjacent to the active-site histidine is Ala⁵⁵-Ala-His-Cys⁵⁸-Gly-Val-Thr-Thr-Ser-Asp⁶⁴ and the active-site serine is Cys¹⁹¹-Met-Gly-Asp-Ser¹⁹⁵-Gly-Gly-Pro-Leu-Val²⁰⁰. The Roman numerals refer to the numbering of peptides on the diagonal peptide 'maps' in Fig. 5. Histidine was quantified as *N*-carboxymethylhistidine, half-cystine as *S*-carboxyethylcysteine sulphone and methionine as methionine sulphone.

(a) Active-site histidine peptides from moose and elk chymotrypsins

Chymotrypsin	Amino acid composition									
	Ala	Ala	His	Cys	Gly	Val	Thr	Thr	Ser	Asp
Moose A (I)	2.0*		0.9	0.8	1.2	1.1	1.0	—	—	—
Moose A (II)	2.0*		0.6	0.8	2.3	1.2	1.9	—	1.4	0.8
Elk A (I)	—	1.0*	0.6	0.5	0.9	0.4	0.5	—	—	—
Elk A (II)	—	—	0.9	1.1	1.2	1.0*	1.6	—	0.8	0.9
Moose B (IA)	2.0*		0.9	0.6	0.7	—	—	—	—	—
Moose B (IB)	2.0*		0.9	0.5	1.2	0.8	0.9	—	—	—
Moose B (II)	2.0*		0.7	0.4	1.4	1.0	1.6	—	0.8	0.6
Elk B (I)	2.0*		0.8	0.6	0.9	—	—	—	—	—
Elk B (II)	2.0*		0.7	0.5	1.0	0.8	0.9	—	—	—
Elk B (III)	—	1.0*	0.9	0.7	1.1	0.8	1.8	—	0.8	1.0

(b) Active-site serine peptides from moose and elk chymotrypsins

Amino acid	Chymotrypsin					
	Ox A	Moose A (III)	Elk A (III)	Ox B	Moose B (III)	Elk B (IV)
<i>S</i> -Carboxyethylcysteine sulphone	1	0.67	0.46	1	0.45	0.53
Aspartic acid	1	1.00*	1.71	1	1.00*	1.00*
Methionine sulphone	1	0.85	0.76	1	0.83	0.82
Threonine		0.15				
Serine	1	0.86	0.86	1	0.95	1.23
Glutamic acid		0.10	0.25		0.21	0.33
Proline	1	1.01	0.87	1	1.07	0.95
Glycine	3	3.12	3.00*	3	3.03	3.00
Valine	1	0.70	0.79	1	0.48	0.72
Leucine	1	0.89	0.78	1	0.85	0.81

* Arbitrarily taken as an integral number of residues with the recoveries of other amino acids relative to this amino acid.

identical with those of corresponding fragments of ox chymotrypsin A isolated by this method. The major weakness in the amino acid analyses of these active-site histidine peptides was the low recoveries of *S*-carboxyethylcysteine sulphone. This has been attributed to the instability, to performic acid oxidation, of the intermediate *S*-cyanoethylcysteinyl residue in the peptides in an analogous manner to that observed with *S*-pyridylethylcysteinyl residues (Stevenson, 1973). The tentative sequences deduced from the amino acid compositions of these active-site histidine peptides, shown in Table 6(a), are identical with the corresponding sequences of the ox chymotrypsins.

In addition to the selective isolation of active-site histidine peptides, the modified diagonal peptide-'mapping' technique also permitted the isolation of a peptide encompassing the active-site serine residue of ox chymotrypsin A (Stevenson, 1974). This

peptide possessed an *N*-terminal *S*-cyanoethylcysteine residue which was oxidized to its sulphone during performic acid treatment. Selective isolation of the active serine peptides of moose and elk chymotrypsins occurred as a result of the change in electrophoretic mobility at pH 6.5, owing to the depression of the *pK* of the *N*-terminal amino group by the inductive effect of the sulphonyl group. The amino acid compositions of these active-site serine peptides, presented in Table 6(b), were identical. On the basis of the amino acid composition, electrophoretic mobility and cadmium/ninhydrin colour of each peptide, it is proposed that the peptides shown in Table 6(b) are identical with the region Cys¹⁹¹-Val²⁰⁰ of ox chymotrypsin A and B.

General comparison

The major differences and similarities between either the moose or elk chymotrypsins and the

Table 7. *A summary of the 'qualitative' differences in the primary structures of mouse and elk chymotrypsins when compared with the corresponding ox A or B chymotrypsins*

	Chymotrypsin			
	Moose A	Elk A	Moose B	Elk B
Total difference in amino acid composition of the native enzymes relative to ox chymotrypsin A or B	13 (~5%)	19 (~8%)	7 ~3%	8 (~3%)
Total difference in amino acid composition of the 'B' and 'C' chains of the enzyme relative to the 'B' and 'C' chains of ox chymotrypsin A	30 (~13%)	31 (~13%)	—	—
Total number of sequence positions for which a possible sequence was proposed	80	87	82	87
(i) Sequence positions proposed as identical with those of the corresponding ox chymotrypsin	75	80	78	87
(ii) Sequence positions proposed as different from those of the corresponding ox chymotrypsin	5	7	4	0

corresponding ox chymotrypsins are summarized in Table 7. The difference between the amino acid compositions of either moose or elk chymotrypsin B and ox chymotrypsin B was so small as to be within the limits of experimental accuracy. This did not, of course, include any differences between asparagine/aspartic acid or glutamine/glutamic acid content, as these were not determined. A more substantial difference was observed between the amino acid compositions of either moose or elk chymotrypsin A and ox chymotrypsin A. However, these differences were still quite small as a percentage of the total amino acid content of the enzymes.

Comparison of the amino acid compositions of the 'B' and 'C' chains of moose, elk and ox chymotrypsin A indicated a greater qualitative difference among the primary structures of these enzymes than was apparent from a comparison of the amino acid compositions of the native enzymes. The values given in Table 7 probably represent only the minimum differences, as the content of asparagine, glutamine and tryptophan in these chains was not determined.

By diagonal peptide-'mapping' techniques, peptides were isolated around the disulphide bridges and active-site regions of moose and elk chymotrypsins A and B. In total these peptides accounted for about one-third (80-90 residues) of the sequence of each enzyme. Although the sequences of these peptides were deduced, assuming maximum homology, from amino acid compositions and related data rather than from sequence-determining procedures, a very high degree of identity was found among the A and B chymotrypsins of the moose, elk and ox. The range of identity over 87 positions was from complete, for elk and ox chymotrypsin B, to about 90%, for elk and ox chymotrypsin A.

Complete sequence data would be necessary before exact comparisons could be made among the primary structures of moose, elk and ox chymotrypsins. However, from the data reported it is

apparent that there are at least minor differences in the primary structures of the A or B chymotrypsins of moose, elk and ox. It is also apparent that most of the primary-structure differences occur in regions of the sequences removed from either the active-site or disulphide bridges of these enzymes.

Supplementary information

Detailed evidence has been deposited with the British Library Lending Division, Boston Spa, Wetherby, W. Yorkshire LS23 7QB, U.K., for storage on microfiche (Supplementary Publication SUP 50064). The information comprises: (1) details and figures of the purification procedures used to isolate the moose and elk chymotrypsin A and B; (2) amino acid analyses and electrophoretic mobilities of peptides purified from the digest of glucagon and the oxidized B chain of insulin by moose and elk chymotrypsins A and B; (3) amino acid analyses of peptides (Cys¹-Gly-Val-Pro-Ala-Ile-Gln; His-Phe-Cys⁴²-Gly-Gly-Ser-Leu; Ala-Ala-His-Cys⁵⁸-Gly-Val-Thr-Thr-Ser-Asp; Met-Ile-Cys¹⁸²-Ala-Gly-Ala-Ser-Gly-Val-Ser) obtained by diagonal peptide 'mapping' of moose and elk chymotrypsins A and B which were identical with the amino acid analyses of the corresponding peptides from ox chymotrypsin A and B.

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References

- Aune, K. C. & Timasheff, S. N. (1971) *Biochemistry* **10**, 1609-1617

- Awad, W. M. & Wilcox, P. E. (1963) *Biochim. Biophys. Acta* **73**, 285–292
- Bailey, J. L. (1968a) *Techniques in Protein Chemistry*, 2nd edn., p. 21, Elsevier, London
- Bailey, J. L. (1968b) *Techniques in Protein Chemistry*, 2nd edn., p. 23, Elsevier, London
- Bargetzi, J. P., Sampath Kumar, K. S. U., Cox, D. J., Walsh, K. A. & Neurath, H. (1963) *Biochemistry* **2**, 1468–1474
- Bhargava, A. K. & Barnard, E. A. (1973) *J. Mol. Evol.* **2**, 187–198
- Bromer, W. W., Sihm, L. G. & Behrens, O. D. (1957) *J. Am. Chem. Soc.* **74**, 2798–2801
- Brown, J. R. & Hartley, B. S. (1963) *Biochem. J.* **89**, 59P–60P
- Brown, J. R. & Hartley, B. S. (1966) *Biochem. J.* **101**, 214–228
- Charles, M., Gratecos, D., Rovey, M. & Desnuelle, P. (1967) *Biochim. Biophys. Acta* **140**, 395–409
- Coan, M. H., Roberts, R. C. & Travis, J. (1971) *Biochemistry* **10**, 2711–2717
- Croston, C. B. (1965) *Arch. Biochem. Biophys.* **112**, 219–223
- Cunningham, L. (1965) *Compr. Biochem.* **16**, 85–188
- Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404–427
- Dayhoff, M. O. (1972) *Atlas of Protein Sequence and Structure*, vol. 5, pp. D107–D108, The National Biomedical Research Foundation, Silver Spring, MD
- de Haen, C., Neurath, H. & Teller, D. C. (1975) *J. Mol. Biol.* **92**, 225–229
- Dixon, M. (1953) *Biochem. J.* **54**, 457–458
- Egan, R., Michel, H. O., Schlueter, R. & Jandorf, B. J. (1957) *Arch. Biochem. Biophys.* **66**, 366–373
- Enenkel, A. G. & Smillie, L. B. (1963a) *Biochemistry* **2**, 1445–1448
- Enenkel, A. G. & Smillie, L. B. (1963b) *Biochemistry* **2**, 1449–1454
- Folk, J. E. & Schirmer, E. W. (1965) *J. Biol. Chem.* **240**, 181–192
- Gertler, A. & Birk, Y. (1970) *Eur. J. Biochem.* **12**, 170–176
- Gertler, A. & Hofmann, T. (1967) *J. Biol. Chem.* **242**, 2522–2527
- Gibson, D. & Dixon, G. H. (1969) *Nature (London)* **222**, 753–756
- Hapner, K. O. & Wilcox, P. E. (1970) *Biochemistry* **9**, 4470–4480
- Hartley, B. S. (1964) *Nature (London)* **201**, 1284–1287
- Hartley, B. S. & Kauffman, D. L. (1966) *Biochem. J.* **101**, 229–231
- Heilman, J., Barollier, J. & Watzke, E. (1957) *Hoppe-Seyler's Z. Physiol. Chem.* **309**, 219–220
- Hofstee, B. H. J. (1959) *Nature (London)* **184**, 1296–1298
- Jany, K. D. & Pfeleiderer, G. (1974) *Eur. J. Biochem.* **42**, 419–428
- Jany, K. D., Tabatabai, M. S. & Pfeleiderer, G. (1974) *FEBS Lett.* **48**, 53–55
- Koide, A. & Matsuoka, Y. (1969) *J. Biochem. (Tokyo)* **66**, 541–548
- Koide, A. & Matsuoka, Y. (1970) *J. Biochem. (Tokyo)* **68**, 1–7
- Koide, A., Kataoka, T. & Matsuoka, Y. (1969) *J. Biochem. (Tokyo)* **65**, 475–477
- Lacko, A. G. & Neurath, H. (1967) *Biochem. Biophys. Res. Commun.* **26**, 272–277
- Liebel, W., Zwillling, R. & Pfeleiderer, G. (1971) *Comp. Biochem. Physiol.* **B38**, 197–210
- Lievaart, P. A. & Stevenson, K. J. (1974) *Can. J. Biochem.* **52**, 637–644
- Liu, T.-Y. & Chang, Y. H. (1971) *J. Biol. Chem.* **246**, 2842–2848
- Mockel, W. & Barnard, E. A. (1969a) *Biochim. Biophys. Acta* **178**, 354–363
- Mockel, W. & Barnard, E. A. (1969b) *Biochim. Biophys. Acta* **191**, 370–378
- Moore, S. (1963) *J. Biol. Chem.* **238**, 235–237
- Offord, R. E. (1966) *Nature (London)* **211**, 591–598
- Prahl, W. P. & Neurath, H. (1967) *Biochem. Biophys. Res. Commun.* **26**, 272–277
- Reeck, G. R., Winter, W. P. & Neurath, H. (1970) *Biochemistry* **9**, 1398–1403
- Romer, A. S. (1966) *Vertebrate Paleontology*, 3rd edn, p. 274, University of Chicago Press, Chicago
- Ruegg, U. T. & Rudinger, J. (1976) *Int. J. Pept. Protein Res.* in the press
- Ryan, C. A. (1965) *Arch. Biochem. Biophys.* **110**, 169–174
- Ryle, A. R., Sanger, F., Smith, L. F. & Kitai, R. (1955) *Biochem. J.* **60**, 541–556
- Segel, I. H. (1968) *Biochemical Calculations*, pp. 393–395, John Wiley and Sons, New York
- Smillie, L. B. & Hartley, B. S. (1966) *Biochem. J.* **101**, 232–241
- Smillie, L. B. & Hartley, B. S. (1967) *Biochem. J.* **105**, 1125–1133
- Smillie, L. B., Furka, A., Nagabhushan, H., Stevenson, K. J. & Parkes, C. O. (1968) *Nature (London)* **218**, 343–346
- Snoke, J. E., Schwert, G. W. & Neurath, H. (1948) *J. Biol. Chem.* **175**, 7–13
- Sober, H. A. (1968) *Handbook of Biochemistry Selected Data for Molecular Biology*, p. C-10, Chemical Rubber Co., Cleveland
- Spackman, D. H. (1967) *Methods Enzymol.* **11**, 3–15
- Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190–1206
- Stevenson, K. J. (1971) *Anal. Biochem.* **40**, 29–34
- Stevenson, K. J. (1973) *Anal. Biochem.* **56**, 450–459
- Stevenson, K. J. (1974) *Biochem. J.* **139**, 215–220
- Stevenson, K. J. & Landman, A. (1971) *Can. J. Biochem.* **49**, 119–126
- Stevenson, K. J. & Smillie, L. B. (1968) *Can. J. Biochem.* **46**, 1357–1370
- Stevenson, K. J. & Voordouw, J. K. (1975) *Biochim. Biophys. Acta* **386**, 324–331
- Stevenson, K. J., Gibson, D. & Dixon, G. H. (1974) *Can. J. Biochem.* **52**, 93–100
- Tartakoff, A., Greene, L. J. & Palade, G. E. (1974) *J. Biol. Chem.* **249**, 7420–7431
- Ward, C. W. (1975) *Aust. J. Biol. Sci.* **28**, 1–23
- Yamada, S. & Itano, H. A. (1966) *Biochim. Biophys. Acta* **130**, 538–540
- Yphantis, D. A. (1964) *Biochemistry* **3**, 297–317