Isolation and Characterization of Sheep Pepsin

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(Received 28 June 1976)

Sheep pepsin was isolated (approx. 120-fold purification) from aqueous abomasal homogenates by (1) pH fractionation, (2) chromatography on Sepharose 4B–poly-L-lysine columns and (3) gel filtration on Sephadex G-100. The enzyme had mol.wt. approx. 34000, *N*-terminal valine and *C*-terminal alanine. The amino acid composition of sheep pepsin was generally similar to that of pig and ox pepsins, with a very low content of basic residues and a high content of acidic and hydroxy-amino acids. The pH optimum for *NN*-dimethyl-casein and *NN*-dimethyl-haemoglobin as substrates was approx. 1.8. The K_m and k_{cat} for *NN*-dimethyl-haemoglobin were $46\mu\text{M}$ and 1100min^{-1} respectively, and for *NN*-dimethyl-casein the corresponding parameters were $50\mu\text{M}$ and 420min^{-1} . These values were generally similar to those for pig and ox pepsins. At the pH optimum of 4.6, the sheep pepsin was about 50% as active on benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-tryptophan ethyl ester as was pig pepsin. The pH optimum for the hydrolysis of *N*-acetyl-L-phenylalanyl-L-di-iodotyrosine by sheep, ox and pig pepsins was approx. 1.85.

The gastric enzymes, chymosins (rennins) and pepsins, are important members of the acid proteinase group (Hartley, 1960), but only pig pepsin (EC 3.4.23.1) and calf chymosin (EC 3.4.23.4) have been studied in detail. Northrop (1930, 1933) isolated and crystallized both pig and ox pepsins. Three minor isoenzymes of pig pepsin, B (EC 3.4.23.2), C (EC 3.4.23.3) and D, have been isolated and studied by Ryle & Porter (1959) and Ryle (1966). Rajagopalan *et al.* (1966) developed an improved method for the fractionation of commercial crystalline pig pepsin. The primary structure of pig pepsin has been the subject of a series of publications by Tang and co-workers (Tang *et al.*, 1973; Chen *et al.*, 1975; Marciszyn *et al.*, 1975; Sepulveda *et al.*, 1975).

Ox pepsinogen was isolated and characterized by Chow & Kassell (1968), and Fox (1969) reported the partial purification of ox pepsin. Meitner & Kassell (1971) and Lang & Kassell (1971) isolated and studied a number of minor ox pepsin isoenzymes. An affinity-chromatography system using poly-L-lysine covalently attached to Sepharose 4B was developed by Nevaldine & Kassell (1971) for the isolation of ox pepsinogen and pepsin. The amino acid composition and properties of ox pepsin differ considerably from those of pig pepsin (Lang & Kassell, 1971). O'Leary & Fox (1975) have developed a general method for the purification of pepsins and chymosins and reported briefly on some properties of sheep pepsin (O'Leary &

Fox, 1973). The microheterogeneity of human pepsin is well documented (Etherington & Taylor, 1969; Whitecross *et al.*, 1974), and some of the physico-chemical properties of human pepsin and human gastricsin (pepsin C) have been reported (Mills & Tang, 1967; Huang & Tang, 1970).

The present communication reports an isolation procedure for sheep pepsin and some chemical and kinetic characteristics of that enzyme.

Experimental

Isolation of pepsins

Extraction and activation. Sheep pepsinogen was extracted from the abomasa of adult sheep as described by O'Leary & Fox (1975). The zymogen in the extract was activated at pH 2.0 and 15°C for 30 min and the protein insoluble at pH 2.0 removed by centrifugation at 5000*g* for 30 min (O'Leary & Fox, 1975). The supernatant was adjusted to pH 6.0, dialysed overnight against water and freeze-dried.

Affinity chromatography. Poly-L-lysine (Sigma Chemical Co., St. Louis, MO, U.S.A.; type VIIIB, mol.wt. 30000–70000) was attached to Sepharose 4B (Pharmacia, Uppsala, Sweden) as described by Nevaldine & Kassell (1971). A 24 cm × 1.6 cm column of the derivative was used for chromatography.

The crude freeze-dried sheep pepsin was dissolved at 4% (w/v) in 0.15 M-NaCl in 0.01 M-sodium acetate buffer, pH 5.2, and applied to the poly-L-lysine–Sepharose 4B column. A 24 cm × 1.6 cm column com-

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pletely adsorbs the sheep pepsin in 5 g of crude powder. After application, the column was washed with 0.15 M-NaCl in 0.01 M-acetate buffer, pH 5.2, until the A_{280} of the eluate approached zero; 200 ml of buffer was usually required.

The enzyme was eluted by the application of a NaCl linear gradient, 0.15–1.2 M, in 0.01 M-acetate buffer, pH 5.2; 200 ml of each buffer was used to form the gradient. Throughout most of the run the flow rate was approx. 50 ml/h, but toward the end of the sample-application stage the rate decreased markedly because of the high protein concentration in the sample; the rate increased again after the unadsorbed protein had been washed out.

The protein concentration of the eluate was measured by its A_{280} , assuming $A_{280}^{1\%} = 15$ (Lang & Kassell, 1971; Chow & Kassell, 1968), and its enzymic content by milk-clotting activity; the A_{260} of the eluate was also monitored. The column was reactivated by washing with 500 ml of 0.1 M-NaHCO₃, followed by 500 ml of 0.15 M-NaCl in 0.01 M-acetate buffer, pH 5.2. After every five fractionations the Sepharose-polylysine was removed from the column washed several times with 0.1 M-NaHCO₃ and the column repacked. To date, the column has been used for about 30 fractionations, with no diminution of capacity.

Gel filtration. Active fractions from the Sepharose-polylysine column were pooled, dialysed against 2×20 vol. of water for 24 h at 2°C and freeze-dried.

The freeze-dried powder (approx. 50 mg) was dissolved in 2 ml of 0.02 M-sodium phosphate buffer, pH 6.3, applied to the top of an 80 cm $\times$ 2.5 cm column (V_0 approx. 100 ml) of Sephadex G-100 (Pharmacia) equilibrated with 0.02 M-phosphate buffer and eluted with the same buffer at 2°C at a flow rate of approx. 20 ml/h. The protein content of the eluate was monitored by A_{280} and A_{260} measurements and enzymic activity by milk-clotting ability.

Active fractions from the Sephadex columns were pooled, dialysed against deionized water for 48 h and freeze-dried.

The isolation may be shortened by dialysing the active fraction from Sepharose-polylysine chromatography only sufficiently to permit freeze-drying and by performing gel filtration in deionized water, where the collected active fractions may be freeze-dried directly without dialysis. This modification lessens the risk of autolysis, but the gel-filtration profile is quite diffuse.

Ox pepsin. This was isolated from the abomasa of adult cattle by the same procedure as used for sheep pepsin.

Pig pepsin. This was purified from commercial crude pig pepsin (Nutritional Biochemicals Corp., Cleveland, OH, U.S.A.) by chromatography on Sepharose-polylysine and Sephadex G-100, as described for sheep pepsin.

Characterization

Gel electrophoresis. Homogeneity of the pepsin preparations was assessed by disc electrophoresis in polyacrylamide gels at pH 3.8 (7.5% acrylamide), at pH 7.5 (7.5% acrylamide) and at pH 8.9 (10% acrylamide) as described by Gabriel (1971). The pepsins were dissolved in running buffer for application to the gels at the amount of 50 μ g of protein per tube.

Electrophoresis was run at about 20°C, 2 mA per tube for 30 min and 4 mA per tube thereafter (90 min) being used. The gels were fixed with 12.5% (w/v) trichloroacetic acid and stained for 1 h in a 1:20 dilution of Coomassie Brilliant Blue R250 (Colab Laboratories, Chicago Heights, IL, U.S.A.) in 12.5% trichloroacetic acid. After staining, the gels were washed and stored in 7% (w/v) acetic acid.

Amino acid analysis. Amino acid analyses were carried out essentially as described by Moore & Stain (1963). Samples of protein (approx. 1 mg) were hydrolysed in 5.7 M-HCl at 110°C for 24, 48 and 72 h. Ion-exchange chromatography of the hydrolysates was performed in duplicate on a Technicon Autoanalyser. Peak areas were computed by a Beckman Computing Integrator and converted into amino acid concentration by using colour factors determined on standard amino acid mixtures (Technicon standard). The tryptophan content of sheep pepsin was determined as described by Edelhoch (1967).

Determination of organic phosphate. The organic phosphate content of sheep, pig and ox pepsins was determined as described by Meitner & Kassell (1971).

N-Terminal analysis. The N-terminal residues of 5 mg samples of pig, ox and sheep pepsins were treated with 1-fluoro-2,4-dinitrobenzene (Eastman Chemicals, Rochester, NY, U.S.A.) as described by Narita (1970). The Dnp*-proteins were hydrolysed in 5.7 M-HCl at 110°C for 16 h and the Dnp-amino acids extracted and prepared for chromatography as described by Narita (1970). Chromatography was performed on thin-layer plates of silica gel G (Sigma) developed with benzene/pyridine/acetic acid (40:20:1, by vol.) Standard Dnp-amino acids (Nutritional Biochemicals) were run simultaneously.

C-Terminal analysis. Carboxypeptidase A (Sigma; treated with di-isopropyl phosphorofluoridate) was prepared as described by Ambler (1967). Pig, ox and sheep pepsins were dissolved at a concentration of 20 mg/ml in 0.2 M-N-ethylmorpholine (Eastman) adjusted to pH 8.5 with acetic acid. Carboxypeptidase A was added at an enzyme/substrate ratio of 1:80 (w/w) to 0.25 ml portions of the pepsin solutions and the solutions were incubated at 37°C for 0, 10, 20 and

* Abbreviations: Dnp-, dinitrophenyl; CbzHis-Phe-TrpOEt, benzylloxycarbonyl-L-histidyl-L-phenylalanyl-L-tryptophan ethyl ester; AcPhe-TyrI₂, N-acetyl-L-phenylalanyl-L-di-iodotyrosine.

40 min. An incubation period of 10 min was adequate to release the C-terminal residue fully.

After hydrolysis the samples were placed in a boiling-water bath and evaporated to dryness in a stream of air. The dried residues were extracted with 40 μ l of water, and 25 μ l of the extracts was applied to silica-gel G plates. A series of standard amino acids (Mann Research Laboratories, New York, NY, U.S.A.) were also applied and the plates were developed in phenol/water (7:3, v/v). The chromatograms were sprayed with ninhydrin reagent [0.1% in butan-1-ol made to 3% (v/v) with acetic acid] to locate the amino acids.

Preparation of substrates

NN-Dimethyl-casein. The *NN*-dimethyl derivative of sodium caseinate (Nutritional Biochemicals) was prepared as described by Lin *et al.* (1969). After derivative formation, the casein was precipitated from solution at pH 4.6 and 35°C, washed twice with water and the cycle repeated. The modified casein was exhaustively dialysed against deionized water and freeze-dried. More than 98% of the ϵ -amino groups of lysine were modified by this procedure.

For determination of the pH optima for proteolysis, the *NN*-dimethyl-casein was prepared at 0.5% (w/v) in HCl/KCl buffers (Gomori, 1955). Because of relatively low solubility of the modified casein in the desired pH range it was found convenient first to dissolve the casein at 5.0% (w/v) in deionized water and then to dilute the stock solution 1:9 (v/v) with the relevant buffer. It was not possible to dissolve the freeze-dried *NN*-dimethyl-casein directly in these low-pH buffers and it was best to add the stock casein solution to the buffer with stirring. The solutions were kept overnight at 2°C and their pH values were measured before and after assay with a Radiometer model 22 pH-meter.

Solutions of *NN*-dimethyl-casein for K_m determinations were prepared by diluting a 1% (w/v) solution of the protein in HCl/KCl buffer, pH 1.82, with the same buffer to give solutions of concentration ranging from 0.033 to 1.0% (w/v) (0.014–0.4 mM, assuming the mol.wt. of *NN*-dimethyl-casein to be 25000). These solutions were dialysed overnight with stirring at 2°C against 40 vol. of HCl/KCl buffer, pH 1.82. Before use, the pH of the casein solutions was checked and their protein concentration determined by A_{280} measurement, assuming an $A_{280}^{1\%}$ of 10.0 (Herskovits, 1966).

NN-Dimethyl-haemoglobin. The *NN*-dimethyl derivative of haemoglobin (ox, twice crystallized; Mann Research Laboratories) was prepared essentially as described by Lin *et al.* (1969). The haemoglobin was first bleached by adding 3% (v/v) of 30% (v/v) H_2O_2 (Mallinckrodt, St. Louis, MO, U.S.A.) to the haemoglobin solution in 0.1 M-sodium borate buffer, pH 9.0 at 50°C. This treatment decreased the $A_{420}^{1\%}$ of the

solution to 0.25. The concentrations of $NaBH_4$ and formaldehyde used during derivative formation were twice those used by Lin *et al.* (1969). After reaction the modified haemoglobin was exhaustively dialysed against deionized water and freeze-dried. The $A_{420}^{1\%}$ of the resulting product after reaction with trinitrobenzenesulphonic acid was 0.7.

Solutions for the determination of pH optima were prepared by dissolving *NN*-dimethyl-haemoglobin at 1% (w/v) directly in HCl/KCl buffers (Gomori, 1955). The solutions were kept overnight at 2°C and their pH values then determined before and after assay.

Solutions of *NN*-dimethyl-haemoglobin for determination of K_m were prepared by appropriately diluting a 1% (w/v) solution of the protein in HCl/KCl buffer at pH 1.82 with HCl/KCl buffer, pH 1.82, to a range of concentrations from 0.03 to 1.0% (w/v) (0.0046–0.154 mM, assuming the mol.wt. of *NN*-dimethyl-haemoglobin to be 65000). These solutions were dialysed with stirring at 2°C overnight against this buffer.

CbzHis-Phe-TrpOEt. The substrate (chromatographically pure in three solvents; Cyclo Chemicals, Los Angeles, CA, U.S.A.) was dissolved initially in 95% (v/v) ethanol at 20 mM and diluted 1:50 with a series of 40 mM-sodium citrate buffers, pH 2.25 to 5.00, giving final solutions containing 2% (v/v) ethanol and 0.4 mM substrate. The substrate was sparingly soluble at pH 5.0.

Because of very low activity of ox and sheep pepsins on this substrate the influence of substrate concentration was not measured.

AcPhe-TyrI₂. This substrate (Cyclo Chemicals; chromatographically pure in three solvents) was dissolved in two equivalents of alkali (9.3 mg in 300 μ l of 0.1 M-NaOH) and diluted to 3 ml with methanol, giving a 5 mM stock solution. Substrate solutions for the determination of pH optima were prepared by diluting the stock solution 1:33 with HCl/KCl buffers of pH between 0.5 and 2.5 (Gomori, 1955), giving solutions 0.15 mM with respect to substrate. The pH of the solutions was checked after preparation of substrate solutions.

Enzyme assays

Enzyme solutions. For the proteolysis of *NN*-dimethyl-casein and *NN*-dimethyl-haemoglobin 20 μ l of approx. 2 μ M enzyme stock solutions was used per ml of substrate, i.e. enzyme concentration in the reaction solution was 0.04 μ M. The stock solutions of enzymes were prepared from freeze-dried powders of known protein content; the mol.wt. of the pepsins was assumed to be 34000. The milk-clotting activity of these enzyme solutions at pH 6.5 and 36°C was 270 s at a ratio of 10 μ l of stock enzyme/ml of milk.

A much higher amount of enzyme was required for the peptide substrates to give measurable amounts of proteolysis: 10 μ M stock enzyme solutions were used at a ratio of 50 μ l of stock enzyme/ml (0.5 μ M in reaction mixture), with considerably longer incubation periods. These enzyme solutions had milk-clotting activities at pH 6.5 and 36°C of approx. 70s at a ratio of 5 μ l of stock enzyme/ml of milk.

Milk-clotting activity. Milk-clotting activity was measured at 36°C on 10% (w/v) low-heat skim-milk powder (Carnation, Los Angeles, CA, U.S.A.) dissolved in 0.01 M-CaCl₂ and adjusted to pH 6.5. The substrate (2ml) was placed in screw-capped test tubes, which were clipped (submerged) to a rocking platform in a water bath. After temperature equilibration, the enzyme solution was added by micro-syringe normally at a concentration necessary to give coagulation in 1–5 min. The end point was detected by the appearance of particles of curd in the flowing milk. Enzymic activity was expressed in rennin units (R.U.), defined as the amount of enzyme (approx. 25 μ g) required to coagulate 10ml of substrate in 100s at pH 6.5 and 36°C (Berridge, 1945).

Proteolytic and peptidase activity. The proteolytic activity of sheep pepsin at 30°C as a function of pH and substrate concentration was determined on *NN*-dimethyl-haemoglobin and *NN*-dimethyl-casein, and the peptidase activity as a function of pH on CbzHis-Phe-TrpOEt and AcPhe-TyrI₂ and compared with the activities of pig and ox pepsins determined simultaneously. Proteolysis was measured by the trinitrobenzenesulphonate method (Fields, 1972); A_{420} was measured by using a Beckman DU spectrophotometer equipped with a Gilford attachment. Initial velocities (rates of liberation of amino groups) for K_m determinations on *NN*-dimethyl-casein and *NN*-dimethyl-haemoglobin were obtained from plots of the extent of proteolysis in samples incubated for three different lengths of time, the longest giving not more than 1% hydrolysis. With *NN*-dimethyl-haemoglobin as substrate the plot of proteolysis versus time was linear over the incubation period, but for *NN*-dimethyl-casein the plot was slightly curved and initial velocities were calculated from a tangent to the curve drawn through the origin.

pH-stability of the pepsins

The stability of sheep, ox and pig pepsins in the pH range 0.5–4.5 was determined by mixing equal volumes of pepsin solutions, standardized to equal milk-clotting activity at pH 6.5 and HCl/KCl buffers (Gomori, 1955). The reaction mixtures were maintained at 20°C and samples withdrawn and assayed for milk-clotting activity after various incubation times ranging from 0 to 24h. The pH of the mixtures was measured before and after incubation.

The stability of the three pepsins and that of crystal-

line calf chymosin (Miles-Seravac, Maidenhead, Berks., U.K.) at pH 7.5, 20°C, was determined by mixing equal volumes of 0.5 M-Tris/HCl buffer, pH 7.5, and standardized solutions of the enzymes. Samples were taken at intervals up to 20 min and assayed for milk-clotting activity. The decrease in activity was taken as a measure of denaturation.

Results

Affinity chromatography

A typical elution pattern of crude sheep pepsin preparation on a column of Sepharose 4B-poly-L-lysine is shown in Fig. 1. In this experiment, 2g of crude powder was applied to the column; 98–99% of the material was not adsorbed and was washed through with starting buffer. Enzymic activity was eluted as a relatively small peak, with an A_{280}/A_{260} ratio approx. 1.5, at approx. 0.6 M-NaCl. The fractions containing most of the activity were pooled, dialysed against water and freeze-dried. Disc gel electrophoresis at pH 8.9 of a 50 μ g sample showed very little difference in the electrophoretograms across the peak, and the total peak was therefore normally pooled, dialysed and freeze-dried. In this experiment, which is typical, 25mg of protein was recovered from the activity peak.

Recovery of enzymic activity from Sepharose-polylysine columns was always excellent. In the experiment in Fig. 1, about 2500 R.U. was applied

Fig. 1. Elution profile of crude sheep pepsin extract from Sepharose 4B-poly-L-lysine

●, A_{280} ; ○, milk-clotting activity; △, NaCl concentration. The sample (2g) was applied as a 4% (w/v) solution in 0.15 M-NaCl in 0.1 M-acetate buffer, pH 5.2, and the column washed with the same buffer until A_{280} of the eluate was approx. 0. Adsorbed protein was eluted by a linear NaCl gradient (0.15–1.2 M) in 0.1 M-acetate buffer, pH 5.2. Flow rate was 50 ml/h, fraction size 6 ml and temperature, 2°C.

Fig. 2. Elution pattern of purified sheep pepsin from Sephadex G-100

Chromatography of purified pepsin (50mg dissolved in 2ml of eluent buffer) was performed at 2°C on an 80cm×2.5cm column (V_0 approx. 100ml) of Sephadex G-100. The chromatogram was developed with 0.02M-sodium phosphate buffer, pH 6.3, at a flow rate of 20ml/h; 5ml fractions were collected. ●, A_{280} ; ○, milk-clotting activity.

to the column (1.25 R.U./mg); 80% of the activity was recovered after discarding the leading and trailing edges of the activity peak. The specific activity at the centre of the peak was 60 R.U./mg of protein, i.e. about 50-fold purification.

Gel filtration

Although chromatography on Sepharose-polylysine gave excellent recovery of enzyme with a high purification factor, it was obvious from electrophoretograms and from gel filtration that proteins other than pepsin were adsorbed. These contaminating proteins moved slowly in 10% polyacrylamide gels at pH 8.9 and were effectively removed by filtration on Sephadex G-100 (Fig. 2), indicating that the contaminants had higher molecular weights than pepsin. The contaminants were eluted in a small peak, with an A_{280}/A_{260} ratio at approx. 1.5, close to the void volume of the column, whereas pepsin was eluted as a symmetrical peak in which the protein and activity profiles were superimposed; the specific activity across the peak varied between 63 and 70 R.U./mg of protein. At least 90% of the enzymic activity applied to the column was recovered in the main peak. The electrophoretograms of samples taken from the centre of the pepsin peak showed

that the pepsin was homogeneous by electrophoresis in 10% polyacrylamide at pH 8.9, in 7.5% polyacrylamide at pH 7.5 and in 7.5% acrylamide at pH 3.8.

Molecular weight

The elution volumes of sheep, ox and pig pepsins of Sephadex G-100 were indistinguishable. Assuming that the enzyme molecules from the three species have the same general shape and behave similarly on gel filtration, the mol.wt. of sheep pepsin should be about 34000 (Rajagopalan *et al.*, 1966).

Amino acid analysis and organic phosphate content

The amino acid analyses of sheep pepsin, in most cases the average of duplicate determinations on 24, 48 and 72h hydrolysates, are presented in Table 1. Phenylalanine gave very reproducible results, and, since it is present in both pig and ox pepsins at 14 residues/molecule, the residues of various amino acids per molecule were normalized relative to phenylalanine at 14 residues per molecule of protein. The tryptophan content of sheep pepsin, assayed spectrophotometrically (Edelhoc, 1967), was 5 residues per molecule, giving a molecule with a total of 323 residues. The amino acid compositions of pig, ox and human pepsins, as determined in this study or as reported in the literature, are included in Table 1 for comparative purposes.

Analysis of sheep pepsin for organic phosphorus showed it to contain (to the nearest integer) 1 atom of phosphorus per molecule. A similar value was found for the ox and pig pepsins isolated in this study.

N-Terminal residue

T.l.c. of a diethyl ether extract of a 5.7M-HCl hydrolysate of pig, ox and sheep Dnp-pepsins showed that the only Dnp-amino acids present in detectable amounts were isoleucine (or leucine), valine and valine respectively, indicating that sheep pepsin has the same N-terminal residue, valine, as ox pepsin (Meitner & Kassell, 1971). The solvent system used (benzene/pyridine/acetic acid, 40:10:1, by vol.) unequivocally resolves Dnp-valine from all other Dnp-amino acids, but fails to resolve leucine and isoleucine. Isoleucine is well established as the predominant N-terminal residue of pig pepsin (Tang *et al.*, 1973).

C-Terminal residue

Alanine was the only amino acid liberated by carboxypeptidase A from pig, ox and sheep pepsins during the early stages of reaction, indicating that, like pig and ox pepsins, the C-terminal residue of sheep pepsin is alanine.

Table 1. *Amino acid composition of pig, ox, sheep and human pepsins*

Results are expressed as residues/molecule to the nearest integer, relative to phenylalanine taken as 14 residues/molecule.

Amino acid	Pig pepsin			Ox pepsin		Sheep pepsin	Human pepsin
	This study*	Rajagopalan <i>et al.</i> (1966)	Tang <i>et al.</i> (1973)	This study	Lang & Kassell (1971)	This study	Mills & Tang (1967)
Asp	41	40	42	38	36	37	40
Thr	26	25	27	26	25	30	27
Ser	45	43	44	49	44	51	43
Glu	28	26	26	34	28	30	31
Pro	16	16	15	15	15	15	19
Gly	36	34	35	33	33	33	35
Ala	16	16	16	15	14	15	18
Val	21	20	22	22	23	22	27
Cys	6	6	6	6	6	6	6
Met	4	4	4	3	3	3	5
Ile	23	23	25	27	28	24	25
Leu	27	28	27	19	19	19	22
Tyr	16	16	15	14	16	15	15
Phe	14	14	14	14	14	14	15
Lys	1	1	1	1	0	1	0
His	1	1	1	1	1	1	1
Arg	2	2	2	3	3	2	3
Trp	5	5-6	5	5	5	5	5
P (atoms/molecule)	1	—	—	1	1-3†	1	—

* The values for this study are the average of 24, 48 and 72 h hydrolysates, except for isoleucine and valine, which are the average of 72 h hydrolysates, and serine and tyrosine, which were estimated by extrapolation to zero-time hydrolysis. Tryptophan was determined by spectrophotometric assay (Edelhoch, 1967).

† Meitner & Kassell (1971).

Fig. 3. Hydrolysis of *NN*-dimethyl-casein (■) and *NN*-dimethyl-haemoglobin (□) by sheep pepsin

Both proteins were dissolved at 1% (w/v) in HCl/KCl buffers at pH 1.82, mixed with enzyme (0.04 μM) and incubated at 30°C. Proteolysis was assayed by the formation of free amino groups as measured at 420 nm by reaction with trinitrobenzenesulphonic acid.

Kinetic studies on *NN*-dimethyl-haemoglobin

Haemoglobin is a very commonly used substrate for proteolytic assays, but proteolytic activity has traditionally been measured by changes in substrate

solubility which, because of its complexity, makes the assay rather imprecise. Lin *et al.* (1969) recommended the use of *NN*-dimethylated haemoglobin as a substrate for proteolytic enzymes, with activity being measured by the hydrolysis of peptide bonds (formation of free amino groups) as assayed by reaction with trinitrobenzenesulphonate (Fields, 1972), but it does not appear to have been widely used.

NN-Dimethyl-haemoglobin proved to be an excellent substrate for measurement of peptic activity. The data in Fig. 3 for the hydrolysis of 1% (w/v) substrate, pH 1.8, as a function of time show that hydrolysis was linear for at least 30 min.

The influence of reaction pH on the proteolytic activity of sheep pepsin on 1% *NN*-dimethyl-haemoglobin at 30°C is shown in Fig. 4. Proteolysis was optimal at approx. pH 1.8. A similar value was found for pig and ox pepsins. The pH-activity curve was somewhat asymmetrical, being very sharp on the acid side of the pH optimum but tailing slightly on the alkaline side. No activity was detected under the assay conditions at pH 4.1 or higher.

The equation for the Lineweaver-Burk plot for the influence of substrate concentration in the proteolytic activity of sheep pepsin at pH 1.82 and 30°C was $1/v = 0.001(1/s) + 0.024$, from which a K_m of 46 μM

Fig. 4. Influence of reaction pH on the proteolytic activity of sheep pepsin on *NN*-dimethyl-haemoglobin

The substrate was dissolved in HCl/KCl buffers at 1% (w/v) and incubated at 30°C with sheep pepsin at a concentration of 0.04 μM. The extent of proteolysis was determined after 30 min incubation by reaction of the free amino groups formed with trinitrobenzenesulphonic acid and expressed as μmol of NH₂ formed/min per μmol of enzyme.

Table 2. Kinetic constants for pig, ox and sheep pepsins on *NN*-dimethyl-haemoglobin at 30°C, pH 1.82

See the Experimental section for details. K_m and k_{cat} were derived from Lineweaver-Burk plots for which the equations are given in the Results section.

Enzyme	pH optimum	K_m (μM)	k_{cat} (min ⁻¹)
Pig	1.8	37 ± 2	850 ± 60
Ox	1.8	34 ± 3	960 ± 40
Sheep	1.8	46 ± 3	1040 ± 60

(0.30%, w/v, assuming the mol.wt. of *NN*-dimethyl-haemoglobin is 65000) and $k_{cat} = 1040 \text{ min}^{-1}$ were calculated. The corresponding constants for pig and ox pepsins were very similar to those for sheep pepsin (Table 2).

Kinetic studies on *NN*-dimethyl-casein

NN-Dimethyl-casein has been recommended by Lin *et al.* (1969) as a good substrate for the assay of

Fig. 5. Influence of reaction pH on the proteolytic activity of sheep pepsin on *NN*-dimethyl-casein

The substrate was dissolved in HCl/KCl buffers at 0.5% (w/v) and incubated at 30°C with sheep pepsin at a concentration of 0.04 μM. The extent of proteolysis was determined after 15 min incubation by reaction of the free amino groups formed with trinitrobenzenesulphonic acid and expressed as μmol of NH₂ formed/min per μmol of enzyme.

proteinases with pH optima above 6.0, but not for pepsins, because of its relatively low solubility at pH values below its isoelectric point. However, if prepared as described, it is possible to obtain solutions of *NN*-dimethyl-casein at a concentration of 1% (w/v), which is well above K_m , at pH values below 4.0. Therefore *NN*-dimethyl-casein is quite suitable as a substrate for the pepsins, although its hydrolysis as a function of time was not as linear as that of *NN*-dimethyl-haemoglobin under the same assay conditions (Fig. 3).

The pH for optimal hydrolysis of *NN*-dimethyl-casein was approx. 1.8 (Fig. 5), but significant hydrolysis occurred at pH 4.9, the highest pH at which the casein was sufficiently soluble, whereas no hydrolysis of *NN*-dimethyl-haemoglobin occurred at pH 4.1. Optimal hydrolysis of *NN*-dimethyl-casein by ox

Table 3. Kinetic constants for pig, ox and sheep pepsins on *NN*-dimethyl-casein at 30°C

See the Experimental section for details. K_m and k_{cat} were derived from Lineweaver-Burk plots for which the equations are given in the Results section.

Enzyme	pH optimum	K_m (μM)	k_{cat} (min^{-1})
Pig	1.6	23 ± 3	180 ± 10
Ox	1.8	31 ± 4	420 ± 26
Sheep	1.8	52 ± 5	440 ± 28

pepsin also occurred at approx. pH 1.8, but pig pepsin showed optimal activity at pH 1.6.

The equation for the Lineweaver-Burk plot for the proteolysis of *NN*-dimethyl-casein by sheep pepsin at pH 1.82 and 30°C was $1/v = 0.003(1/s) + 0.0568$, from which a K_m of $52 \mu M$ (0.13%, w/v, assuming the mol.wt. of *NN*-dimethyl-casein is 25000) and $k_{cat} = 440 \text{ min}^{-1}$ were calculated. The corresponding constants for pig and ox pepsins are shown in Table 3.

Hydrolysis of CbzHis-Phe-TrpOEt

CbzHis-Phe-TrpOEt is considered a good synthetic substrate for pig pepsin (Inouye *et al.*, 1966), but Lang & Kassell (1971) found that ox pepsin had only 10% the activity of pig pepsin on this substrate. The lower activity of ox pepsin on CbzHis-Phe-TrpOEt (at 0.4 mM) has been confirmed in the present study, with ox pepsin showing approx. 20% of the activity of pig pepsin at their respective pH optima. Sheep pepsin was somewhat more active than ox pepsin, with about 50% of the activity of pig pepsin. The pH optimum for hydrolysis by pig pepsin of CbzHis-Phe-TrpOEt was 4.2, in agreement with Lang & Kassell (1971), but the optima for ox and sheep pepsins were pH 4.6.

When the pH of the solutions was raised during the reaction with trinitrobenzenesulphonic acid, considerable turbidity developed. The A_{420} reading was corrected for turbidity by subtracting from it the A_{600} value, because the reaction product had negligible absorption at 600 nm.

Hydrolysis of AcPhe-TyrI₂

The pH optima for the hydrolysis of AcPhe-TyrI₂ (at 0.15 mM) by pig, ox and sheep pepsins were 1.85, in agreement with Lang & Kassell (1971). At pH 1.85 the relative activities of pig/ox/sheep pepsins on this peptide were 2.5:1.7:1 respectively. Pig and sheep pepsins showed distinct shoulders on the curve at pH 2.2–2.5, a feature not present in the curve for ox pepsin.

Fig. 6. Stability of pig pepsin (○), sheep pepsin (■) and ox pepsin and calf chymosin (●) at pH 7.5, 20°C

Solutions (2 ml) of each of the enzymes standardized to equal milk-clotting activity at pH 6.5 and 30°C were mixed with 2 ml of Tris/HCl buffer, pH 7.5, 20°C. Samples were taken after various incubation times at 20°C and assayed for milk-clotting activity at pH 6.5 and 30°C; decrease in milk-clotting activity was taken as a measure of denaturation.

pH-stability

Sheep, ox and pig pepsins showed no significant loss of activity over a 24 h period at 20°C in the pH range 1.0–4.5. However, at pH 7.5 at 20°C, pig pepsin was completely denatured within 1 min, sheep pepsin was somewhat more stable, but ox pepsin and calf chymosin showed no loss of activity during a 20 min incubation period (Fig. 6). At pH 7.5, 40°C, pig and sheep pepsins were completely denatured in less than 30 s, whereas ox pepsin lost about 90% of its activity in 5 min and calf chymosin lost only about 30% of its activity in 5 min.

Discussion

The procedure described for the isolation of sheep pepsin is rapid and quite reproducible. The extraction, activation and pH-fractionation steps can be readily performed in 1 day. It is not necessary to dialyse or freeze-dry the preparation after pH fractionation, but it is convenient to do so, especially if one starts with large quantities of material. pH fractionation gives approximately a 2-fold purification and the extract may be applied directly to the Sepharose-polylysine column.

Sepharose-polylysine has been described by Nevaldine & Kassell (1971) as an affinity-chromatographic medium for pepsins. In our experience it is not a true affinity system, but adsorbs other proteins of molecular weight considerably higher than the pepsins.

It appears to act in part as an ion-exchange medium rather than specific affinity system. Nevertheless, it provides an excellent means of giving approximately 50-fold purification of the crude pepsin extract (i.e. a 100-fold purification over aqueous abomasal extract). In our experience, Sepharose-polylysine gives approximately the same degree of purification as DEAE-cellulose chromatography, but is very much easier to operate in that flow rates are considerably faster and it is not necessary to break down and repack the column between fractionations. Sheep, ox and pig pepsins, homogeneous on disc gel electrophoresis at pH 3.8, 7.5 and 8.9, were obtained by chromatography of the pooled active fractions from Sepharose-polylysine on Sephadex G-100. The isolation procedure described is simpler than that used by Nevaldine & Kassell (1971), in that $(\text{NH}_4)_2\text{SO}_4$ fractionation and chromatography on CM-cellulose were not necessary. The same procedure, without modification, was used to isolate pig and ox pepsins. Sepharose-polylysine bound chymosins and pepsins from a wide range of species, but without exception proteins other than the proteinases were also adsorbed.

The amino acid compositions of sheep, ox and pig pepsins were generally similar: all had a low content of basic residues and a high content of acidic and hydroxy residues. The amino acid composition of the pig pepsin isolated and analysed in the present study agrees very closely with those of Rajagopalan *et al.* (1966) and of Tang *et al.* (1973), based on amino acid sequence. Lang & Kassell (1971) detected only traces of lysine in ox pepsin, but the ox pepsin isolated in the present study contained 1 mol of lysine/mol of enzyme. The sheep pepsin also contained 1 mol of lysine/mol of enzyme. Lang & Kassell (1971) reported that ox pepsin contained considerably less leucine than did pig pepsin; the present analyses show that both sheep and ox pepsins have a relatively low content of leucine. Sheep pepsin had a higher content of serine and threonine than did the ox pepsin analysed, and these values in turn were higher than the corresponding values reported by Lang & Kassell (1971).

The three pepsins contained 1 atom of phosphorus per molecule and had the same C-terminus, alanine; sheep pepsin had the same N-terminal residue, valine, as ox pepsin, but pig pepsin had an isoleucine residue at the N-terminus.

Sheep and ox pepsins were more effective than pig pepsin in hydrolysing *NN*-dimethyl-casein, but the three enzymes were about equally effective on *NN*-dimethyl-haemoglobin. The sheep and ox enzymes appear to be more effective on *NN*-dimethyl-haemoglobin than on unmodified haemoglobin (Lang & Kassell, 1971; P. A. O'Leary & P. F. Fox, unpublished work). Both *NN*-dimethyl-casein and *NN*-dimethyl-haemoglobin are very good substrates for

peptic action, especially when proteolysis is monitored by formation of amino groups as measured by reaction with trinitrobenzenesulphonate. This latter method is much more specific than measurement of changes in substrate solubility and gives very reproducible results. The solubility of *NN*-dimethyl-casein at acid pH values is adequate provided that the procedure described is followed.

Hydrolysis of *NN*-dimethyl-haemoglobin was linear over a considerable period of time, but the hydrolysis of *NN*-dimethyl-casein was non-linear. The non-linearity of the hydrolysis of the latter may reflect the fact that casein contains a few peptide bonds particularly susceptible to proteolysis: Phe-Met residues (105–106) of κ -casein is several orders of magnitude more susceptible to proteolysis than any other peptide bond in casein (Delfour *et al.*, 1965). The bond Ala-Phe (residues 189–190) of β -casein is 60–200 times (depending on reaction temperature) more susceptible to proteolysis by chymosin than any other bond in β -casein (Creamer *et al.*, 1971; Creamer, 1975), and the bonds Phe-Phe (residues 23–24) (Hill *et al.*, 1974) and Phe-Val (residues 24–25) (Creamer & Richardson, 1974) are the most susceptible bonds of α_1 -casein. Although these specificity studies were made at higher pH values (5.5–7.0), it is probable that similar disparities in susceptibility also exist at low pH values.

The three pepsins studied were very much less effective in hydrolysing synthetic peptides than proteins. In fact, the very low activity of the ox and sheep enzymes on the two peptides studied made accurate assay difficult. In agreement with Lang & Kassell (1971), CbzHis-Phe-TrpOEt was found to be a very poor substrate for ox pepsin and only slightly better for sheep pepsin. This, coupled with the low solubility of the peptide at pH values above 5.0 and the development of turbidity during assay with trinitrobenzenesulphonate, makes this peptide a very poor substrate for studies on ox and sheep pepsins. It may be useful in distinguishing between and classifying pepsins.

As discussed by Inouye *et al.* (1966) for the hydrolysis of peptides by pig pepsin, the presence of a free carboxyl group adjacent to the peptide bond results in a lower pH optimum for hydrolysis than when the carboxyl group is esterified. Although only a limited range of substrates was used, a similar effect was observed in this study with ox and sheep pepsins, both of which hydrolysed CbzHis-Phe-TrpOEt optimally at pH 4.6, but had optimum activity at pH 1.85 on AcPhe-TyrI₂.

Pig pepsin is more susceptible than calf chymosin to denaturation at alkaline pH values (de Koning *et al.*, 1969; Showers *et al.*, 1972; Lim & Dinesen, 1973; O'Leary & Fox, 1974). The data in Fig. 6 confirm these earlier findings and show that in its stability at slightly alkaline pH values sheep pepsin resembles pig pepsin more closely than ox pepsin.

The advice of Professor M. A. Moran, Department of Statistics, University College, Cork, on statistical matters is gratefully acknowledged.

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