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Purification and Characterization of Bovine Gastricsin

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The results reported in the present paper and N-terminal sequence homologies established by other authors strongly support the assumption that the gastric protease previously called bovine pepsin I or bovine pepsin B belongs to the aspartate protease group and corresponds to gastricsin (or pepsin C) (EC 3.4.23.3) from other species.

Bovine gastricsin was prepared from commercial extracts of adult bovine vells by a procedure involving DEAE-cellulose chromatography, gel filtration on Sephacryl S-200 and a further DEAE-cellulose chromatography. The preparation thus obtained was shown to be free of chymosin and bovine pepsin A by immunodiffusion, selective inactivation in urea and isoelectric focusing. Its molecular weight was estimated by gel filtration to be 32800.

Bovine gastricsin displayed microheterogeneity on isoelectric focusing with pI values of the components ranging from 3.5 to 4.0. Chromatography of bovine gastricsin on hydroxyapatite separated three fractions, none of them being homogeneous by isoelectric focusing. Concanavalin-A–Sepharose 4B bound bovine gastricsin to some extent, but without any significant fractionation. Proteolytic activity could be detected directly on the isoelectric focusing gel for all the components of gastricsin and its fractions from hydroxyapatite and concanavalin-A–Sepharose 4B.

Bovine gastricsin and its fractions from hydroxyapatite have similar amino acid compositions, different from those of bovine chymosin and pepsin A but obviously related to those of human, simian and porcine gastricsins.

Bovine gastricsin which is inactivated by reaction with diazoacetyl-DL-norleucine methyl ester and with 1,2-epoxy-(*p*-nitrophenoxy)propane in a 1:1 and 1:2 stoichiometry, respectively, is able to hydrolyse a synthetic hexapeptide, Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe, used as reference substrate for aspartate proteases, and exhibits a low activity towards *N*-acetyl-L-phenylalanyl-L-diiodotyrosine. Its specific clotting activity with κ-casein as substrate is only half of that of chymosin and pepsin A.

Aspartate proteases (acid proteases) have a wide distribution in animal tissues and secretions. Cathepsin D, the main lysosomal protease, renin which is involved in the regulation of arterial pressure, a protease from the seminal plasma, and all gastric proteases seem to belong to this group.

Three different types of gastric proteases are found in mammals. They have been called 'major pepsins', 'minor pepsins' and 'foetal (or neonatal) pepsins' by Foltmann and Pedersen [1]. These three types have been detected in human, porcine and bovine stomachs.

Chymosin is a neonatal bovine pepsin, the secretion of which, as prochymosin, drops to a very low level at weaning [2]. The calf vell also secretes two pepsinogens which have been isolated by Antonini and Ribadeau Dumas [3] and designated pepsinogen I and II; pepsinogen II gives rise to pepsin II which corresponds to pepsin A. This enzyme, which is the predominant protease in the adult bovine vell, appears to be phosphorylated at different levels [4]. Only a few papers have been published on bovine pepsin I, also called pepsin B, which is a 'minor pepsin' in Foltmann and Pedersen's classification. Some sequence information, enzymic, electrophoretic and immunological properties have been reported [1,3,5]. From these reports it appears that bovine pepsin I might be homologous to gastricsin (also called pepsin C) isolated from human and porcine stomachs [6,7].

A more precise characterization of bovine pepsin I was undertaken in order to increase the basis for this assumption. In this paper we describe a method of preparation for this enzyme and we demonstrate that it belongs to the aspartate protease group by using two affinity labelling reagents. Further, we reveal its heterogeneity and determine some of its structural and enzymic properties. As the results of our investigations support the assumption that bovine pepsin I corresponds to gastricsin from other species, we use the name 'bovine gastricsin' throughout the paper.

MATERIALS AND METHODS

Enzymes

Liquid commercial bovine pepsin was kindly supplied by Boll (Chr. Hansen's Laboratory Ltd, France). Chymosin and bovine pepsin A were prepared as previously described [8]. Leucine aminopeptidase (105 U/mg) was obtained from Sigma Chemical Co (St Louis, MO, USA).

This work has been taken from a thesis to be submitted by Patrice Martin, to Paris-Sud University of Orsay, France, in partial fulfillment of the requirements for the degree of *Doctorat d'Etat*.

Abbreviations. AcPhe-Tyr(I_2), *N*-acetyl-L-phenylalanyl-L-diiodotyrosine; Temed, *N,N,N′,N′*-tetramethylethylene diamine.

Enzymes. Pepsin A (EC 3.4.23.1); pepsin C or gastricsin (EC 3.4.23.3); chymosin (EC 3.4.23.4); cathepsin D (EC 3.4.23.5); leucine aminopeptidase (EC 3.4.11.1).

Substrates

κA-casein was prepared according to Zittle and Custer [9], from the milk of a single cow (Française-Frisonne Pie Noire breed) homozygous at the κ Cn^A locus.

The hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe was obtained from Bachem (Marina del Rey, CA, USA). AcPhe-Tyr(I_2) was purchased from Sigma Chemical Co. (St Louis, MO, USA).

Chromatography and Electrofocusing Supports

Sephacryl S-200, concanavalin-A–Sepharose 4B and Sephadex G-25 were purchased from Pharmacia (Uppsala, Sweden); DEAE-cellulose (DE 32) from Whatman Ltd (Springfield, Kent, England); hydroxyapatite (Bio-Gel HTP) and ampholytes (Biolytes), technical quality (40% w/v), pH range 4–9, from Bio-Rad Laboratory (Richmond, CA, USA); silica gel thin layers 60 F_{245}, from Merck (Darmstadt, FRG), acrylamide (twice crystallized), *N,N'*-methylene bisacrylamide (twice crystallized) ammonium persulfate, from Serva Feinbiochemica (Heidelberg, FRG); Temed from Eastman (Rochester, NY, USA).

Other Products

Antisera against bovine chymosin, pepsin A and progastricsin (pepsinogen B) were supplied by Chr. Hansen's Laboratory Ltd (Copenhagen, Denmark). Agarose (Indubiose A_{37}) was purchased from IBF (Clichy, France).

Dansyl chloride, dansyl amino acids, insulin, ribonuclease A, myoglobin, bovine serum albumin, catalase, diazoacetyl-DL-norleucine methyl ester and 1,2-epoxy-(*p*-nitrophenoxy)propane were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Purification and Fractionation of Bovine Gastricsin

Conditions for chromatographic purification and fractionation of bovine gastricsin are given in the legends to Fig. 1–5.

Techniques for Identification and Characterization of Bovine Gastricsin

Urea Inactivation. Three sets of conditions for urea denaturation of chymosin and bovine pepsins were established by Douillard [12]. One of them gives very different first-order inactivation kinetics for chymosin, bovine pepsin A (or pepsin II) and bovine gastricsin (previously called bovine pepsin I). The inactivation is achieved in 0.05 M sodium citrate, 7.2 M urea buffer, pH 5.35 at 40 °C. Under these conditions, bovine gastricsin is 95% inactivated in less than 30 s.

Immunodiffusion. Double radial immunodiffusion according to Ouchterlony [13] was performed to assess possible contamination of gastricsin preparations by chymosin and pepsin A. The immunodiffusion was carried out in 1% (w/v) agarose containing 73 mM Tris, 24.5 mM barbital, 0.36 mM calcium lactate, 2 mM NaN_3, pH 8.6.

Electrofocusing. A stock solution of acrylamide (30%) and bisacrylamide (0.9%) (w/v) was prepared. To 2.7 ml of this solution 1.5 ml glycerol and 0.8 ml carrier ampholytes were added and the volume was adjusted to 16 ml with distilled water. After stirring and deaereting, 16.4 µl Temed and 390 µl 1% (w/v) ammonium persulfate solution were added. The mixture was poured after mild stirring between two glass plates. Polymerization occurred in less than an hour. Electrofocusing was achieved with a LKB multiphor apparatus, at constant power (4 W), under refrigeration (4 °C). After a 2-h pre-run, 5-µl samples (0.5% w/v) were placed on the gel and submitted to electrofocusing for 2 h. After electrofocusing, pH measurements at room temperature were achieved on the gel with an Ingold surface electrode. Finally the gel was stained as described by Bibring and Baxandall [14]. The proteolytic activity of the bands obtained by isoelectric focusing was revealed prior to staining by applying on the gel a strip of cellulose acetate which had been soaked in an 1% (w/v) whole casein solution at pH 3.5. The strip was then incubated at 37 °C for 45 min, shaken in 2% (w/v) trichloroacetic acid and stained with Coomassie brillant blue R-250 during 5 min and destained.

Molecular Weight Determination

Molecular weights were estimated by gel filtration [15] on a Sephacryl S-200 column (132 × 2.5 cm; $V_0 \approx 255$ ml) in 50 mM sodium citrate, 0.1 M NaCl, 0.2‰ (w/v) NaN_3 buffer, pH 5.6. Column calibration was achieved using the following proteins: catalase (M_r 250000), bovine serum albumin (M_r 66000), *Mucor miehei* acid protease (M_r 38200), *Endothia parasitica* acid protease (M_r 37500), bovine chymosin (M_r 35600), myoglobin (M_r 16700), ribonuclease A (M_r 13700) and insulin (M_r 5700).

Amino Acid Compositions

These were obtained with a Biocal BC 200 amino acid analyzer [16]. Samples ($\approx$ 0.8 mg) were hydrolysed in triplicate under vacuum with three-times-distilled 6 M HCl at 110 °C for 24, 48 and 96 h.

Cysteine and cystine were determined together as *S*-carboxymethylcysteine after treatment of the protein with iodoacetic acid [17]. Tryptophan was determined according to Edelhoch [18].

Determination of Organic Phosphate

The protein (5 mg) was dissolved in 1 ml 0.1 M sodium acetate buffer, pH 5.7, and dialysed first for 24 h against six 1-l portions of the same buffer and then for 10 h against bidistilled water, in order to remove any inorganic phosphate. The concentration of the solution thus obtained was determined from the absorbance at 277.5 nm, taking the scattered light into account. The absorption coefficient, 1.35 mg^{-1} ml cm^{-1} at 277.5 nm, was measured on whole gastricsin.

To a 1-ml sample, 50 µl concentrated H_2SO_4 were added. The solution was heated on a Bunsen burner for 2 h and cooled for a few minutes, 50 µl 72% $HClO_4$ were added and the solution was again heated until it became perfectly limpid. After cooling, 5 ml distilled water were added and the colorimetric determination was carried out according to Lowry et al. [19] with the adaptation to microdetermination of Chen et al. [20]; κ-casein, which contains 1 phosphate group/molecule, was used as a reference.

Determination of Sialic Acids

The method of Warren [21] was employed, using *N*-acetylneuraminic acid as a standard.

N-Terminal Amino Acid Determination

With Leucine Aminopeptidase [22]. 500 µl of a solution of *S*-carboxymethylated gastricsin (0.4 mg/ml) were dessicated under vacuum; 300 µl of 50 mM barbital, 2.5 mM $MgCl_2$ buffer, pH 8.5 and 15 µl of the leucine aminopeptidase solution were added. The mixture was placed at 37 °C for 2–3 h and dessicated. The solution obtained by adding 700 µl 0.2 M sodium citrate buffer, pH 2.2, was directly analyzed for amino acids. Proper blank determinations were carried out.

With Dansyl Chloride. About 3 nmol of gastricsin were dissolved in 500 µl 0.5 M sodium bicarbonate, 8 M urea buffer. After adding 500 µl of a dansyl chloride solution (2% w/v in acetone), the mixture was placed at 37 °C for 6 h, dialysed, dessicated under vacuum and hydrolyzed with 6 M HCl. The dansyl amino acids were identified by thin-layer chromatography on silica gel. The solvent employed (diethyl ether/methanol/acetic acid; 100/5/1, v/v/v) separates most of the dansyl amino acids. However dansyl-serine and dansyl-threonine cannot be clearly separated from dansyl-glutamic acid.

Determination of Enzymic Activities

Clotting activity was measured at 30 °C on a κA-casein solution (0.2%, w/v) in 50 mM sodium citrate, 75 mM NaCl, 0.2 ‰ (w/v) NaN_3 buffer, pH 5.3 [23].

Proteolytic activity was estimated using as substrate the synthetic hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe as indicated elsewhere [8]. However the low stability of gastricsin made it necessary to prepare the enzyme solutions in a 50 mM sodium phosphate, 0.2% (w/v) bovine serum albumin buffer, pH 6.0. Proteolytic activities were expressed in nmol peptide hydrolyzed · s^{-1} · (mg protein)$^{-1}$ for 1 ml 0.2 mM peptide solution. It was checked by dansylation [24] and leucine aminopeptidase action [22] that gastricsin hydrolyzed only the Phe(NO_2)-Nle bond.

Hydrolysis of AcPhe-Tyr(I_2) at pH 2.0 was measured from the increase in free diiodotyrosine using the ninhydrin reaction as described by Tang [25]. Chymosin and bovine pepsin A were assayed on this substrate as references. Specific activity was expressed as change in absorbance at 570 nm ($A_{570\,nm}$)/mg of enzyme.

Reaction of Gastricsin with Specific Acid Protease Inhibitors

The reaction with diazoacetyl-DL-norleucine methyl ester was performed at a protein concentration of 26 µM with a 50-fold molar excess of inhibitor in 0.1 M sodium acetate buffer, pH 5.6 at 14 °C [26]. The molar ratio Cu(II):enzyme was 100:1. The reaction was stopped after 4 h by addition of an aqueous solution of EDTA in 10-fold molar excess over the cupric ions. The reaction mixture was then dialyzed against 0.01 M acetic acid and analysed for amino acid composition to determine the norleucine content of the enzyme.

The reaction with 1,2-epoxy-(*p*-nitrophenoxy)propane was performed at a protein concentration of 91.5 µM as described by Chang and Takahashi [26]. The number of 1,2-epoxy-(*p*-nitrophenoxy)propane molecules incorporated into the enzyme was estimated spectrophotometrically [27].

Diazo-modified and epoxy-modified gastricsin were treated with aqueous hydroxylamine and analysed as described by Chang and Takahashi [26].

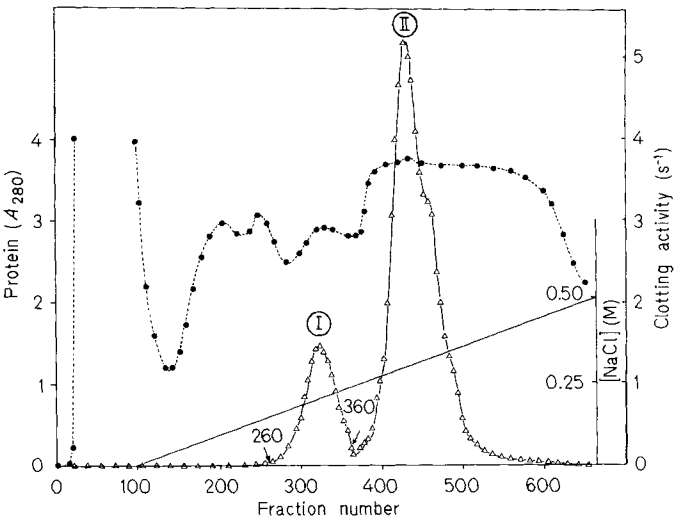

Fig. 1. *Elution profile of commercial bovine pepsin extract from DEAE-cellulose.* Chromatography of commercial extract (2 l concentrated and dialysed against starting buffer) was performed at 4 °C on a column (20 × 6 cm) of DEAE-cellulose (DE 32) as described by Garnot et al. [10]. Enzymes were eluted by a linear NaCl gradient: 0–0.5 M (2 × 3 l) in 25 mM piperazine/HCl buffer, pH 5.3. Flow rate was 120 ml/h and fraction size 12 ml. (Δ——Δ) Clotting activity on κ-casein; (●——●) protein

RESULTS

Preparation of Gastricsin

The elution pattern corresponding to the first chromatography on DEAE-cellulose is shown on Fig. 1. Fraction I (tubes 260–360), which is a mixture of chymosin and gastricsin, represents approximately 16% of the total activity put on the column. Fraction II contains only bovine pepsin A.

Although the first chromatographic step had removed a large amount of inactive material and all of the pepsin A, the mixture of chymosin and gastricsin still contained other products, some pigments in particular. Filtration on Sephacryl S-200 (Fig. 2) eliminated most of them. Furthermore, a partial separation of chymosin and gastricsin was observed. Fractions 75–94 contained about 70% gastricsin (see below) and fractions 95–125 about 75% chymosin. This observation was further confirmed during the calibration of the Sephacryl S-200 column for molecular weight estimation of gastricsin. Indeed, gastricsin which exhibits in our experimental conditions a distribution coefficient corresponding to an M_r of 32800 ± 2700, is eluted before chymosin, the M_r of which is 35652 [28]. Such a result appears rather inconsistent but is presumably due to the polar nature of Sephacryl S-200 which promotes protein-matrix charge interactions [29]. Chymosin, which is less acidic than gastricsin, is probably subjected to adsorption to some slight extent.

100 mg and 150 mg of material were obtained from these fractions, respectively, after dialysis and freeze-drying. Fractions 75–94 were contaminated with an inactive product of slight higher apparent molecular weight.

Finally, a second DEAE-cellulose column chromatography separated chymosin from gastricsin. The latter was eluted by a linear gradient of 0.1–0.3 M NaCl (Fig. 3) as shown by immunodiffusion (Fig. 6). Chymosin was totally eluted by 0.1 M NaCl; 60 mg gastricsin, free of chymosin, were recovered.

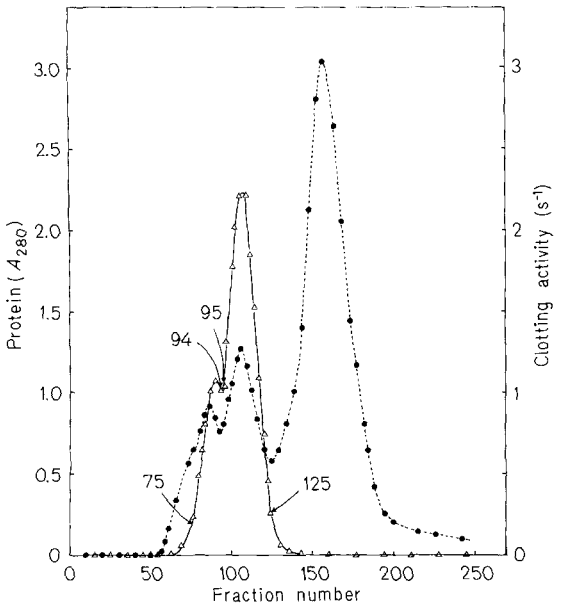

Fig. 2. *Elution profile of the mixture of gastricsin and chymosin (fraction I from DEAE-cellulose) from Sephacryl S-200.* The sample (800 mg) was applied as an 8 % (w/v) solution in eluent buffer on a column (135 × 2.5 cm) of Sephacryl S-200. Chromatography was performed at 4 °C with 50 mM sodium citrate, 0.1 M NaCl, 0.2‰ (w/v) NaN_3 buffer, pH 5.6. Flow rate was 50 ml/h; 5-ml fractions were collected. (Δ——Δ) Clotting activity on κ-casein; (●– – –●) protein

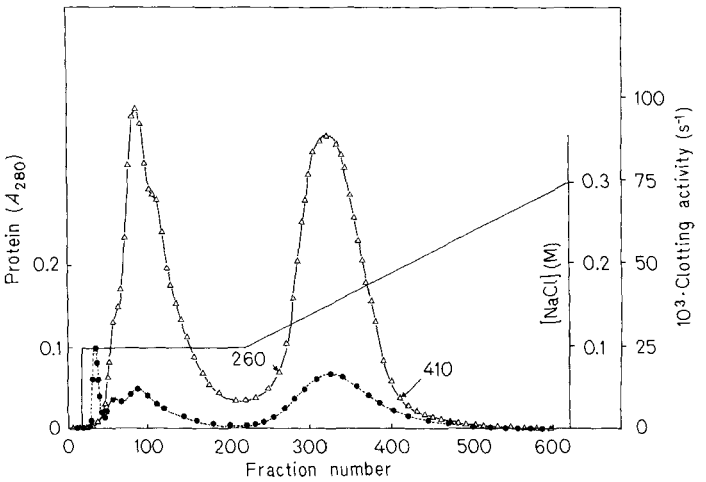

Fig. 3. *Elution profile of the mixture of gastricsin and chymosin (fractions 75–94 from Sephacryl S-200) from DEAE-cellulose.* The sample (100 mg) was applied as a 0.5 % (w/v) solution in 25 mM piperazine/HCl buffer, pH 5.3 (starting buffer) on a column (30 × 1.5 cm) of DEAE-cellulose (DE 32) equilibrated in the same buffer. The column was washed with 0.1 M NaCl in the starting buffer until A_{280} of the eluate was almost zero (after about 1 l buffer). Adsorbed protein was eluted by a linear NaCl gradient (2 × 1 l) 0.1–0.3 M in the starting buffer. Flow rate was 50 ml/h and fraction size 5 ml. (Δ——Δ) Clotting activity on κ-casein; (●– – –●) protein

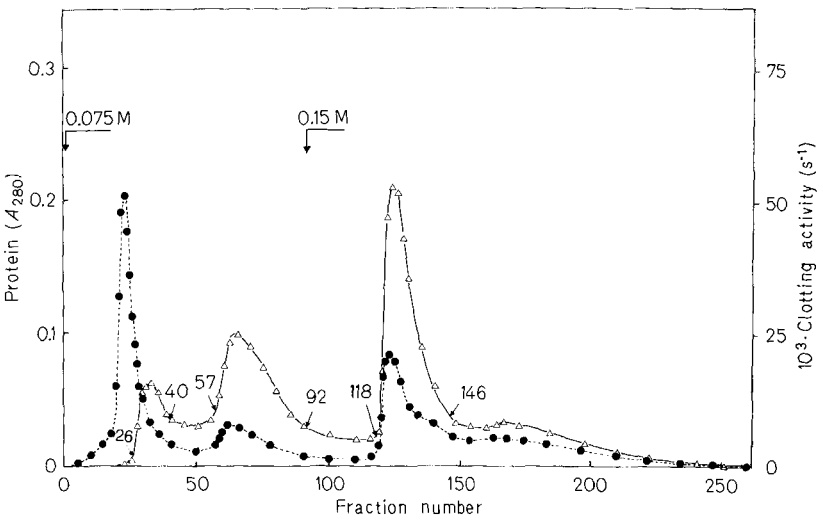

Fig. 4. *Elution profile of purified gastricsin from hydroxyapatite.* The sample (15 mg) was applied as a 0.75 % (w/v) solution in 30 mM sodium phosphate buffer, pH 5.7, on a column (30 × 1.5 cm) of hydroxyapatite. A stepwise elution was performed as described by Rajagopalan et al. [11]. Concentration changes in the buffer are indicated by arrows on the figure. Flow rate was 20 ml/h and fraction size 3 ml. (Δ——Δ) Clotting activity on κ-casein; (●– – –●) protein

Gastricsin Fractionation

Three main fractions, gastricsin 1, gastricsin 2 and gastricsin 3, were obtained from whole gastricsin by chromatography on hydroxyapatite (see Fig. 4). The first two products, gastricsins 1 and 2, were eluted by a 75 mM sodium phosphate buffer, pH 5.7, while gastricsin 3 was eluted at the same pH with a 0.15 M sodium phosphate concentration.

A fourth fraction was eluted after gastricsin 3 (tubes 150–200). However the recovered amount was too small to allow any thorough study. From 15 mg of whole gastricsin, 2 mg, 3.4 mg and 3.7 mg of gastricsins 1, 2 and 3 were obtained, respectively. As gastricsin 1 was still contaminated by a dark pigment, it was filtered again on Sephacryl S-200.

Chromatography on concanavalin-A–Sepharose 4B separated gastricsin into two components. The first one was eluted in the starting buffer and the second at a 30 mM methyl α-D-glucoside concentration (Fig. 5). These two components gave quite similar elution profiles in hydroxyapatite, close to that obtained with whole gastricsin (Fig. 4).

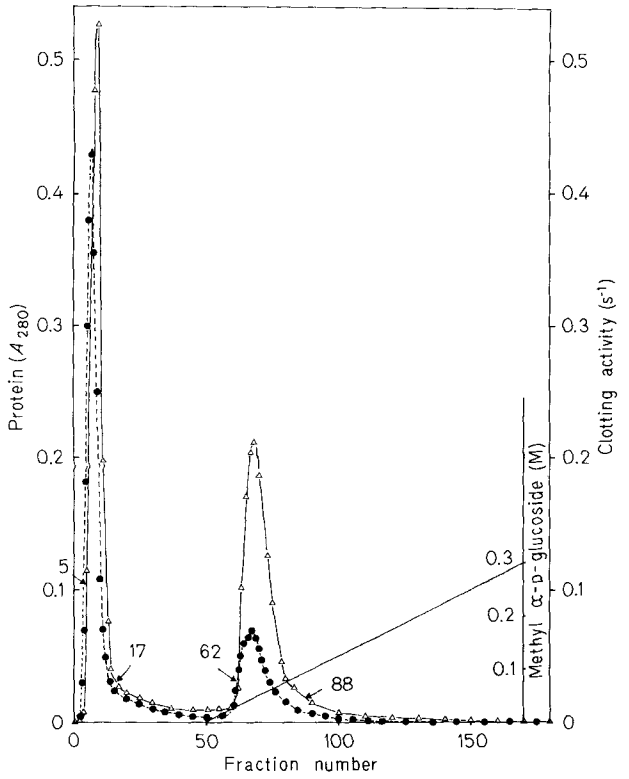

Fig. 5. *Elution profile of whole gastricsin from concanavalin-A – Sepharose 4B.* The sample (50 mg) was applied as a 1.67% (w/v) solution in 0.1 M sodium acetate, 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.2‰ (w/v) NaN_3 buffer, pH 5.6 (starting buffer), on a column (12 × 1.5 cm) of concanavalin-A – Sepharose 4B. The column was washed with the same buffer until A_{280} of the eluate was almost zero. Adsorbed protein was eluted by a linear gradient of methyl α-D-glucoside (2 × 250 ml) 0 – 0.5 M in the starting buffer. Flow rate was 10.5 ml/h and fraction size 3 ml. (△——△) Clotting activity on κ-casein; (●- - - -●) protein

Urea Inactivation

Inactivation kinetics by 7.2 M urea obtained with the different gastricsin preparations, as well as with reference chymosin and bovine pepsin A, were identical to those reported by Douillard [12]. Fraction 75 – 94 from Sephacryl S-200 gave second-order inactivation kinetics, with two slopes, probably corresponding to the inactivation of gastricsin and chymosin, respectively. Therefore it appears that this fraction is a mixture of these two proteases. Their proportions are approximately 70% gastricsin and 30% chymosin.

Immunodiffusion

As no precipitation lines were observed between gastricsins 1 – 3 and whole gastricsin, on the one hand, and anti-chymosin or antipepsin A, on the other hand, all gastricsin preparations appear to be free of chymosin and pepsin A (Fig. 6a, b, g). Furthermore, Fig. 6c, f, h and i confirm that the whole gastricsin preparation and its three individual components which give positive reactions with antiprogastricsin, immunologically correspond to authentic gastricsin. This is in accordance with the kinetics of urea denaturation.

Isoelectric Focusing

Under our working conditions, a linear pH gradient was obtained between pH 3.0 and pH 9.5. Chymosin isoelectric pH seems to be between 4.5 and 5.0, while that of bovine pepsin A is very likely below 3.0. Gastricsin gives several bands with isoelectric pH between 3.5 and 4.0 (Fig. 7).

The four gastricsin samples (whole gastricsin and its fractions from hydroxyapatite) give similar patterns, all containing two main bands between pH 3.5 and pH 3.7. However the band with the lowest mobility towards the anode is much weaker in gastricsin 2, when compared with gastricsin 1. On the other hand it is difficult to see any difference between gastricsin 3 and gastricsin 1. Electrofocusing shows again the absence of chymosin and pepsin A in all the preparations of gastricsin.

The two fractions obtained from concanavalin-A – Sepharose 4B show identical patterns.

Revealing the proteolytic activity after focusing shows activity on whole casein for all the bands obtained from gastricsin and its fractions.

Amino Acid Composition, Content in Phosphate and Sialic Acid

The amino acid compositions of gastricsins 1 – 3 are given in Table 1, together with those of bovine pepsin A, chymosin, human, simian and porcine gastricsins. From the similar composition of the three fractions (gastricsins 1 – 3), an average composition of whole gastricsin is also given. The amino acid compositions are calculated on the basis of an apparent M_r of 32800.

Gastricsins 1 – 3 differ in their organic phosphate content (see Table 1) which ranges over 0.07 – 1.83 mol/mol enzyme. The two fractions of gastricsin obtained from concanavalin-A – Sepharose 4B and gastricsin 1 from hydroxyapatite contain approximately 5 mol sialic acid/mol whereas gastricsins 2 and 3 have only 1 mol/mol.

Identification of the N-Terminal Amino Acid

Together with other amino acids, mainly glycine an alanine, serine is released in the largest amounts by leucine aminopeptidase from the three gastricsin fractions from hydroxyapatite. The number of seryl residues liberated from each of them is fairly large (2.9 – 3.7 residues/molecule of enzyme). The result is qualitatively confirmed by dansylation of the three fractions.

Enzymic Activities

Table 2 compares the activities of whole gastricsin and its fractions with those of chymosin and pepsin A on several substrates. Gastricsin 1 has the highest activity on κ-casein as well as on the hexapeptide. However, the ratio clotting activity/proteolytic activity is identical for all the three fractions. Proteolytic activity of gastricsin 1 is quite similar to that of bovine pepsin A, whereas its clotting activity is only half. For the same proteolytic activity, gastricsin 1 is approximately 50-fold less active on κ-casein than chymosin.

At pH 2.0, chymosin and gastricsin exhibit a low activity toward AcPhe-Tyr(I_2) (3.56 and 3.66 $A_{570\,nm}$ units/mg of enzyme, respectively) compared to that of bovine pepsin A (13.56 $A_{570\,nm}$ units/mg).

Inactivation of Gastricsin with Acid-Protease-Specific Inhibitors

Gastricsin is rapidly inactivated by diazo-DL-norleucine methyl ester (see Fig. 8). The activity is lost almost com-

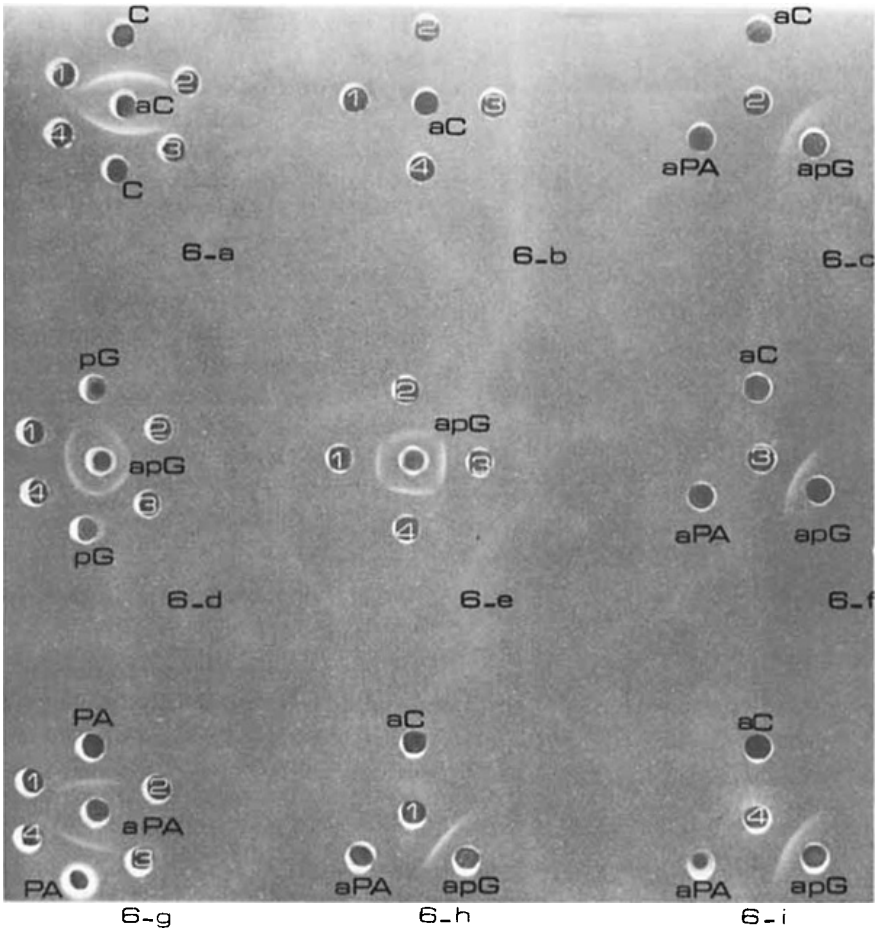

Fig. 6. *Immunodiffusion analysis of gastricsin, versus antichymosin (6a, 6b), antipepsin A (6g) and antiprogastricsin (6c, 6f, 6h, 6i).* aPA, apG, aC represent antipepsin A, antiprogastricsin and antichymosin, respectively; PA, pG and C represent bovine pepsin A, progastricsin and chymosin references, respectively. Samples: (1) whole gastricsin; (2) gastricsin 1; (3) gastricsin 2; (4) gastricsin 3

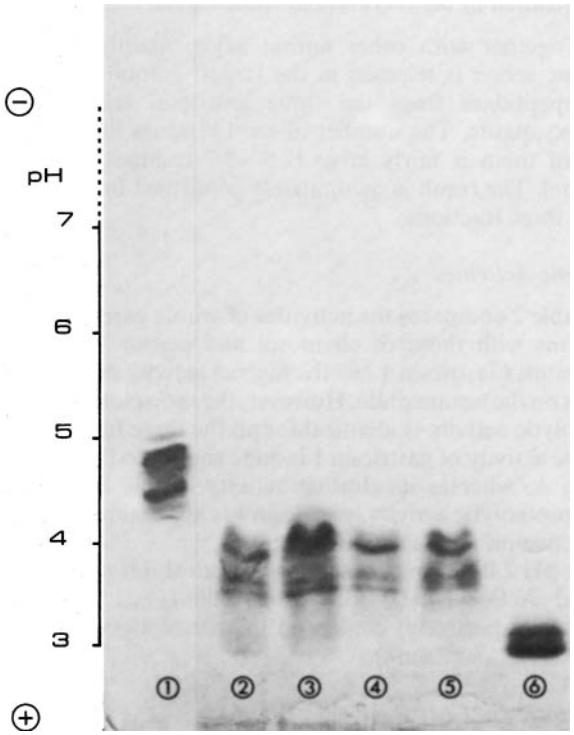

Fig. 7. *Isoelectric focusing of bovine gastric proteases in the pH range 4 – 9.* Samples: (1) crystalline chymosin; (2) whole gastricsin; (3) gastricsin 1; (4) gastricsin 2; (5) gastricsin 3; (6) bovine pepsin A

pletely after 2 min at 14 °C in the conditions in which the reaction was performed.

From amino acid analysis of the acid hydrolysate of diazo-inactivated gastricsin, we have determined that 0.91 mol norleucine was incorporated/mol enzyme, which is easily removed by treatment with hydroxylamine.

Under the reaction conditions used, the rate of reaction of gastricsin with 1,2-epoxy-(*p*-nitrophenoxy)propane was very slow: 5% enzymic activity still remained after 43 h at 25 °C (see Fig. 8). The amount of 1,2-epoxy-(*p*-nitrophenoxy)-propane incorporated was calculated to be 1.87 mol/mol gastricsin. After treatment with hydroxylamine, 1 mol 1,2-epoxy-(*p*-nitrophenoxy)-propane/mol still remained bound to gastricsin.

DISCUSSION

We have described a procedure for separating gastricsin from chymosin, using bovine stomach extracts as starting material. A microheterogeneity of our preparation was observed using isoelectric focusing. Although chromatography of gastricsin on hydroxyapatite and concanavalin-A – Sepharose 4B gives several fractions, none of them was homogeneous in isoelectric focusing. Amino acid analyses and determinations of N-terminal residues did not allow discrimination between the components. However three N-terminal residues per molecule of enzyme indicate that some non-specific cleavage may have occurred (cf. [1]) and the enzyme properties (Table 2) suggest differences in the sub-

Table 1. *Amino acid composition of bovine gastricsins, compared to that of chymosin and some mammalian gastricsins and pepsins*
The values for bovine gastricsin were calculated assuming the molecular weight to be 32800. Cysteine was determined as *S*-carboxymethyl-cysteine; values for threonine, serine and tyrosine were obtained by extrapolating to zero time of hydrolysis. Tryptophan was determined spectrophotometrically [18]. The values for whole gastricsin are nearest integers of the average of gastricsin 1, gastricsin 2 and gastricsin 3. Except for Trp, Thr, Ser and Tyr, each value is an average of 24-h, 48-h and 96-h hydrolysates. Values in parentheses were not taken into account for this calculation since a high scattering (± 3.0 to ± 6.2 mol/mol) in the estimation of these three amino acid residues was observed, due to the occurrence of unidentified peaks. The 0.95 confidence limits for the other determinations range from ± 0.2 to ± 0.6 mol/mol. n.d. = not determined

Amino acid	Gastricsin 1	Gastricsin 2	Gastricsin 3	Whole gastricsin	Bovine pepsin A [39]	Chymosin [28]	Simian pepsin C [40]	Human gastricsin [25]	Porcine gastricsin [25]
	mol/mol protein								
Asx	27.4	26.2	25.3	26	36	36	21	26	26
Thr	27.1	24.5	21.0	24	25	23	17	21	23
Ser	24.0	28.2	23.7	25	44	31	25	32	32
Glx	37.8	34.7	34.3	36	28	33	33	39	39
Pro	19.9	19.0	20.0	20	15	15	16	17	15
Gly	27.0	31.2	33.2	30	33	28	27	33	31
Ala	16.4	15.6	14.3	15	14	15	15	18	19
Val	19.6	17.6	14.9	17	23	26	18	23	19
Cys	5.3	6.3	6.0	6	6	6	6	6	6
Met	3.6	2.5	1.7	3	3	8	4	5	4
Ile	11.4	11.1	11.1	11	28	19	12	13	13
Leu	23.4	20.4	(14.9)	22	19	23	20	25	30
Tyr	16.3	15.7	(19.5)	16	16	19	12	17	16
Phe	14.8	11.3	(8.8)	13	14	17	14	15	19
Lys	3.6	4.3	4.0	4	0	9	1	0	4
His	2.1	3.1	2.7	3	1	5	1	1	1
Arg	3.3	4.2	3.7	4	3	6	3	3	4
Trp	4.3	3.9	4.2	4	5	4	3	4	3
Total				279	313	323	248	298	304
P	0.07	1.02	1.83		0–3		n.d.		0
Sialic acids	5.27	1.12	1.06				0		

Table 2. *Specific activities of bovine gastric proteases on various substrates*
Experiments were carried out at 30 °C in 0.1 M sodium acetate buffer (pH 4.7) using the hexapeptide as substrate, in 0.05 M sodium citrate, 0.075 M NaCl buffer (pH 5.3) using κ-casein as substrate, and at 37 °C (pH 2.0) using AcPhe-Tyr(I_2) as substrate. Activity on the hexapeptide was assayed as hydrolysed peptide (μmol mg^{-1} s^{-1}) as described in Materials and Methods. Activity on κ-casein is reciprocal of the mass of enzyme which gives a clotting time of 100 s at 30 °C with 1 ml 0.2% κ-casein solution. Activity on AcPhe-Tyr(I_2) is the absorbance at 570 nm produced/h from the substrate by 1 mg of enzyme. Activities were estimated by linear regression analysis on the basis of five (in duplicate) different enzyme concentrations. Numbers in parentheses indicate the 0.95 confidence limits

Protease	Specific activity on			10^3 B/A
	AcPhe-Tyr(I_2)	Hexapeptide (A)	κ-casein (B)	
	A_{570} units · mg^{-1}	μmol mg^{-1} s^{-1}	μg^{-1}	
Whole gastricsin	3.66 ± 0.09	1577.2 ± 39.7	0.82 ± 0.02	0.52
Gastricsin 1		2327.7 ± 47.0	1.24 ± 0.01	0.53
Gastricsin 2		1090.5 ± 15.4	0.56 ± 0.01	0.51
Gastricsin 3		1491.9 ± 13.2	0.68 ± 0.01	0.46
Pepsin A[a]	13.56 ± 0.31	2540	2.6	1.0
Chymosin A[a]	3.56 ± 0.17[b]	95	2.3	24.2
Chymosin B[a]	3.56 ± 0.17[b]	93	2.1	22.3

[a] Data from Martin et al. [8].
[b] Result obtained with crystalline chymosin.

strate binding area. Further it should be observed that Foltmann and Pedersen [1] found an N-terminal isoleucyl residue for whole gastricsin. Nevertheless, the N-terminal residues of human and porcine gastricsins were reported to be seryl or valyl [30] and seryl, leucyl or isoleucyl [31], respectively.

Meitner and Kassell [4] and Rajagopalan et al. [11] have succeeded in fractionating bovine and porcine pepsin A, respectively, on hydroxyapatite, according to their phosphate content. The observed heterogeneity of gastricsin might be due in part to different levels of phosphorylation. The phosphate content of gastricsins 1–3 is in accordance with their behavior on hydroxyapatite.

On the other hand, gastricsin fractions seem to differ in their sialic acid content. Foltmann and Pedersen [1] report that they have never been able to detect the occurrence of any sugar in bovine gastric proteases. The presence of some sialic-acid-rich contaminant in our preparations cannot be excluded. Nevertheless, the behavior of gastricsin on concanavalin-A–Sepharose 4B strongly supports the occurrence of carbohydrate attached to the enzyme but does not give any indication as far as the involvement of these carbohydrates in the microheterogeneity is concerned. Concanavalin-A–Sepharose 4B does not bind chymosin nor bovine pepsin A (results not shown).

The average amino acid composition of gastricsin (Table 1) shows that, like most aspartate proteases, gastricsin has a high content in dicarboxylic amino acids and their amides (≈ 22%) but a low proportion of basic amino acids (≈ 4%).

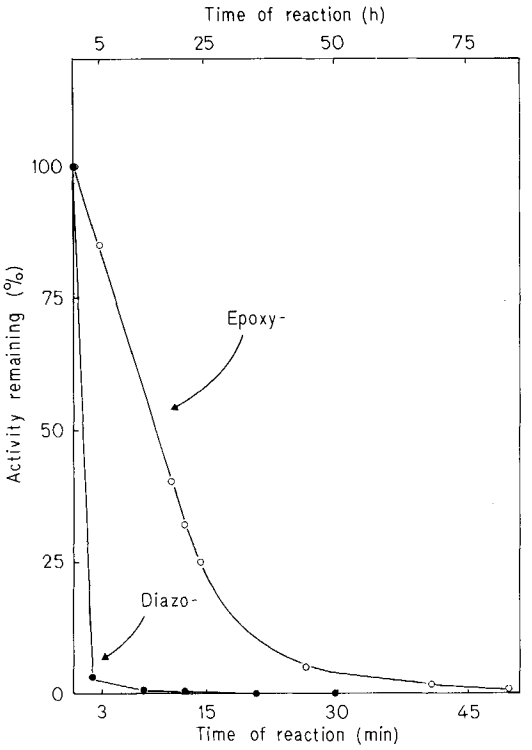

Fig. 8. *Inactivation of gastricsin by diazoacetyl-DL-norleucine methyl ester and by 1,2-epoxy-3-(p-nitrophenoxy)propane.* The reaction with the diazo compound (●——●), lower abscissa) was performed at a protein concentration of 26 μM with a 50-fold excess of diazoacetyl-DL-norleucine methyl ester in 0.1 M sodium acetate buffer at pH 5.6 and 14 °C. The molar ratio of Cu(II) to the enzyme was 100. The reaction with the epoxy compound (○——○), upper abscissa) was performed at a protein concentration of 91.5 μM with a 200-fold molar excess of 1,2-epoxy-3-(*p*-nitrophenoxy)propane, in 0.1 M sodium citrate buffer at pH 4.6 and 25 °C

Table 3. *Molar ratios Ile/Leu and Asx/Glx observed for the main components (pepsins A) and for the minor components (gastricsins or pepsins C) of the pepsin group in the human, porcine, simian and bovine species*

Protease	Reference	Ile/Leu	Asx/Glx
Porcine pepsin A	[25]	0.82	1.54
Bovine pepsin A	[39]	1.47	1.29
Human pepsin A	[25]	1.14	1.29
Simian pepsin A (III_3)	[40]	1.09	1.21
Porcine gastricsin	[25]	0.43	0.67
Bovine gastricsin		0.50	0.72
Human gastricsin	[25]	0.52	0.67
Simian pepsin C	[40]	0.60	0.64

Compared with bovine pepsin A, gastricsin has a higher basic amino acid content (four lysines residue instead of none in particular). But the most significant difference appears to be in the Ile/Leu (0.5 vs 1.5) and Asx/Glx (0.7 vs 1.3) ratios. The inversion of these ratios is also observed in human, porcine and simian species, when pepsins A are compared with gastricsins within each species (Table 3). The ratios Ile/Leu and Asx/Glx (0.41 and 0.70 respectively) for the acid protease of the seminal plasma [32] are quite comparable to those of the minor pepsins, the Ile/Leu ratios of porcine renin [33] and human liver cathepsin D [34] (0.5–0.6) are also comparable.

Tang [35] has suggested that the origin of the gastric aspartate proteases was rooted in the lysosomal cathepsin D. Following this line of reasoning, and from the above observations, one might consider that gastricsins (or homologous proteases) may be transitory material between cathepsin D and the major gastric aspartate proteases (pepsins A). Such an assumption would be supported if the occurrence of carbohydrate attached to gastricsin peptide chain was confirmed, since it is clear that almost all lysosomal enzymes are glycoproteins [36–38].

Gastricsin appears to have a similar molecular weight (32800) to that of porcine (32500) and human (31400) gastricsins [25]. As well as human and porcine gastricsins, its activity towards AcPhe-Tyr(I_2) is weak compared to that of the major pepsin component (see Results), but strong towards Cbz-Tyr-Ala [1]. The hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe, which is used as a reference substrate for most of the milk-clotting enzymes, seems to be an excellent substrate for gastricsin.

Gastricsin was inactivated by reaction with diazoacetyl-DL-norleucine methyl ester and 1,2-epoxy-(*p*-nitrophenoxy)-propane in a 1:1 and 1:2 stoichiometry, respectively. The incorporated norleucine residue and one of the two 1,2-epoxy-(*p*-nitrophenoxy)propane molecules incorporated per molecule of enzyme are easily removed from the protein by treatment with aqueous hydroxylamine, suggesting that there are two essential carboxyl groups at the active site of gastricsin, as has previously been shown for all the gastric and microbial aspartate proteases so far tested. As expected, bovine gastricsin belongs to the asparate protease group, and the results reported in the present paper, together with those of other authors such as (a) N-terminal amino acid sequence homologies with human gastricsin (or pepsin C) [1]; (b) high activity towards carbobenzoxy-tyrosyl-alanine [1] and (c) optimal pH close to 3.0, using hemoglobin as substrate [3], strongly support the assumption that bovine pepsin B corresponds to human and porcine gastricsins.

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