material was sublimed and recrystallized from petroleum ether (40–65°) to give (11.9 g, 72.3 %) 3,4,5,6-tetrafluoro-*N*,*N'*-diphenyl-1,2-phenylenediamine (m.p. 95.5–97.5°). Characteristic infrared absorption bands in nujol were 3365(w), 3345(w), 1597(m), 1512(s), 1492(s), 1310(w), 1250(w), 1230(w), 1075(w), 1013(m), 980(s), 750(s), 695(m). (Found: C 65.24; H 4.02; mol.wt. 332. Calc. for $C_{18}H_{12}F_4N_2$: C 65.01; H 3.66; mol.wt. 332.3).

1,4-Difluorotetrakis (N-methylanilino)benzene. To a suspension of $LiNH_2$ (2.6 g) in THF (25 ml) at 0°, *N*-methylaniline (10.7 g, 0.1 mol) in THF (25 ml) was added over 50 min. 5 min later HMPT (30 ml) was added. Hexafluorobenzene (9.3 g, 0.05 mol) was added slowly over 25 min as an exothermic reaction took place. 35 min thereafter the 0° bath was removed. 1 h 45 min later the light brown mixture was cooled to 0° and hydrolyzed with 5 % HCl to acid reaction. A white precipitate which was present, was filtered off. The filtrate was separated, and the water layer treated twice with ether (30 ml). The collected organic layers were washed with water, dried with sodium sulphate, and concentrated under reduced pressure. The resultant solid was identical with the white precipitate, which was filtered off. Recrystallization from chloroform/petroleum ether (40–65°) gave (9.9 g, 74 %*) of 1,4-difluorotetrakis(*N*-methylanilino)benzene (m.p. 184–186°). Characteristic infrared absorption bands in nujol were 1590(s), 1568(w), 1489(s), 1475(s), 1352(m), 1292(w), 1278(m), 1199(w), 1181(w), 1150(m), 1105(m), 1026(w), 990(w), 969(m), 753(s), 738(w), 691(s). (Found: C 76.36; H 6.11; N 10.62; mol.wt. 534. Calc. for $C_{34}H_{32}F_2N_4$: C 76.38; H 6.03; N 10.48; mol.wt. 534.6).

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* Calculated on consumed *N*-methylaniline.

The C-Terminal Amino Acid Sequence of Bovine Pepsin

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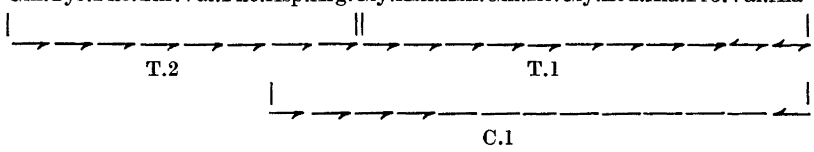
Previous studies have shown that the primary structure of calf chymosin (also called rennin), EC.3.4.4.3, shows a high degree of homology with the primary structure of gastric proteases from other species.[1–3] This has prompted us to start investigations on the primary structure of bovine pepsin in order to compare two acidic proteases produced in the same species, but on different stages of ontogeny. In this communication we report a C-terminal sequence of 19 amino acid residues from bovine pepsin. 10 of the residues are identical with similar residues in calf chymosin, but the C-terminal sequence of bovine pepsin seems more closely related to those of human and porcine pepsins than to that of calf chymosin.

Partly purified bovine pepsin was obtained as a gift from Chr. Hansen's Laboratory, Copenhagen. The enzyme was further purified, first by gel filtration on a column of Sephadex G-100, using 0.05 M sodium phosphate buffer of pH 5.5 as eluent, subsequently the fractions which contained milk clotting activity were pooled and subjected to ion exchange chromatography on a column of DEAE cellulose equilibrated with 0.05 M phosphate buffer of pH 5.5. Elution took place with a linear gradient of sodium chloride in the equilibration buffer. Under such conditions the pepsin was eluted at salt concentrations between 0.4 to 0.5 M sodium chloride. After dialysis the preparation was freeze-dried. 150 mg of the freeze-dried preparation was denatured as described by Tang and Hartley.[4] The enzyme was suspended in 3 ml of acetone and 3 M ammonia was added dropwise under constant stirring, until the total volume was about 12 ml. After stirring for 30 min at room temperature the solution was dialysed against 3 × 1 l of distilled water for a total of 24 h and subsequently freeze-dried. Digestion took place with 1.5 mg trypsin at pH 8.5, maintained constant by addition of 0.1 M NaOH. The digestion was brought to an end by lowering pH to 6 with addition of 0.1 M HCl. The peptide mixture was

Table 1. The C-terminal amino acid sequence of bovine pepsin compared to the C-termini of other gastric proteases. Symbols used: — indicates the quantitative amino acid composition of the peptide, —→ location of the residue is established by Edman degradation and dansylation, ←— the residue is identified after hydrolysis with carboxypeptidase A.

Human gastricsin [3]	Gln.Phe.Tyr.Thr.Val.Phe.Asp.Arg.Ala.Asn.Asn.Lys.Glu.Gly.Leu.Ala.Pro.Val.Ala
Human pepsin [3]	Gln.Phe.Tyr.Thr.Val.Phe.Asp.Arg.Ala.Asn.Asn.Gln.Val.Gly.Leu.Ala.Pro.Val.Ala
Porcine pepsin [3]	Gln.Tyr.Tyr.Thr.Val.Phe.Asp.Arg.Ala.Asn.Asn.Lys.Val.Gly.Leu.Ala.Pro.Val.Ala
Chymosin [2]	Glu.Tyr.Tyr. Ser.Val.Phe.Asp.Arg.Ala.Asn.Asn.Leu.Val.Gly.Leu.Ala.Lys.Ala.Ile
Bovine pepsin	Gln.Tyr.Phe.Thr.Val.Phe.Asp.Arg.Gly.Asn.Asn.Gln.Ile.Gly.Leu.Ala.Pro.Val.Ala



subjected to the gel filtration on a column (2 × 100 cm) of Sephadex G-25 equilibrated with 0.05 M NH_3.

The low molecular weight peptides were further purified by high voltage paper electrophoresis. Only two such peptides were obtained in significant yields (10 % of the theoretical which is acceptable considering the methods of purification). The peptides were designated T.1 and T.2; both behaved neutral at pH 6.5, the peptides were further purified by paper electrophoresis at pH 2. Under such conditions the mobilities relative to glycine were 0.3 for T.1 and 0.6 for T.2. The peptides were analysed for quantitative amino acid composition and sequence by sequential Edman-degradation/dansylation (Gray [5]). The analyses of these peptides are summarized in Table 1. The absence of basic amino acid residues in the neutral peptide T.1 shows that all the dicarboxylic amino acids must be present as amides; in T.2 the presence of glutamine was established by the observation that the peptide remained neutral after removal of the N-terminal residue. This was further substantiated as the neutral peptide Asp-Arg was isolated from a chymotryptic digest of T.2.

A peptide which provides an overlap between T.1 and T.2 was subsequently obtained from a chymotryptic digest of another part of the freeze-dried pepsin. The peptides of this digest were fractionated by combinations of high voltage paper electrophoresis and chromatography. Arginine-containing peptides were looked for by specific staining according to Sakaguchi as modified by Acher and Crocker.[6] Eventually the peptide C.1 was purified and analysed as shown in Table 1. The recovery in high yields of the tryptic peptide T.1 with the C-terminal sequence Val-Ala definitely suggests that this peptide in fact represents the C-terminal sequence of the original protein. Since it is generally assumed that the activation of gastric proteases takes place by cleaving off a peptide from the N-terminal part of the zymogen, the above result is consistent with the observation of Meitner and Kassell [7] who recently reported the same sequence as C-terminal in the bovine pepsinogen.

In Table 1 the results of this investigation are compared to the C-termini of other gastric proteases. It is obvious that a high degree of homology exists in the C-terminal areas of these enzymes, and most of the substitutions are typically conservative substitutions. However, although a high degree of homology exists between bovine pepsin and calf chymosin, bovine pepsin seems more related to the pepsin from other species than to the calf chymosin.

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