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EVIDENCE FOR THE EXISTENCE OF TWO ISOCOLIPASES IN HORSE PANCREAS.

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SUMMARY : The N-terminal amino acid sequences of two forms of colipase isolated from horse pancreas have been compared. Four sequence differences were found in the first 51 amino acids. This lead us to conclude that there are two distinct colipases in the horse pancreas.

INTRODUCTION.

Lipase (EC.3.1.1.3) and colipase are found in the pancreatic secretion of mammals. Colipase is a protein of low molecular weight (10 000 daltons) that activates lipase by enabling the lipolytic enzyme to bind to the triglyceride-water interface in the presence of high concentration of bile salt. Multiple active forms were identified in pancreatic extracts in studies on porcine colipase started several year ago in the groups of Desnuelle (1) and Borgstrom (2). Two forms of colipase, colipase I and II, each with a N-terminal glycine and having 94 and 84 amino acid residues, respectively, were first isolated (3). Comparison of their N-terminal sequence showed no difference in the first 21 residues of the proteins. Later, it was found that these molecules had lost a N-terminal pentapeptide (Val-Pro-Asp-Pro-Arg) by proteolytic cleavage during extraction and evidence was presented showing that porcine pancreas and pancreatic juice contained two colipases with

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similar amino acid composition (4,5). The primary structure of porcine colipase II has been established by Charles et al. (6,7).

We have recently describedthe isolation of colipase from pig and horse pancreatic tissue by a detergent method using Triton X 100 (8,9). The two forms of porcine colipase and the major form of the equine cofactor obtained by this method appear to be intact molecules since they possess a N-terminal valine. The sequence of the first 51 residues of the major form of horse colipase was determined and compared to the homologous part of porcine colipase II (9).

In the present communication we describe the isolation of two colipases from horse pancreas. From the comparison of the amino acid sequence of the amino terminal region of both proteins, it can be concluded that horse pancreas contains two distinct proteins with colipase activity (isocolipases).

METHODS AND MATERIALS.

Colipase and protein determination. Colipase was assayed at 25°C and pH 9 by measuring the rate of hydrolysis of emulsified triolein by pure horse lipase free of cofactor, in the presence of 10 mM sodium deoxycholate (Fluka). One colipase unit corresponds to one microequivalent fatty acid released per min by the activated enzyme as determined under the standard conditions (10). Protein concentration was determined by the method of Lowry or spectrophotometrically at 280 nm.

Amino acid analyses. Analyses were performed on a Biotronik (Model LC 2000) amino acid analyzer. Duplicate samples were hydrolyzed in 6 M HCl at 110°C for 24, 48 and 72 hrs. The usual extrapolations for serine and threonine were made. Values after 72 hrs hydrolysis were used for valine and isoleucine determination.

Amino terminal sequence. The N-terminal residue was determined by dansylation as previously described (9). Automated sequence analysis was carried out on 0.45 µmoles of reduced and carboxymethylated protein with a Beckman 890 C sequencer by using the 1 M Quadrol program (N° 122974) modified in order to have two cleavage steps at each cycle, the first one only being followed by butyl chloride extraction. Phenylthiohydantoin amino acids were identified on a High Pressure Liquid Chromatograph (Waters Associates) equipped with a Merck Hibar LiChrosorb RP-18 (7 µm, 250-4) column maintained at 40°C (curve n° 6 ; 13 min ; A : sodium acetate 10 mM ; B : methanol 20 to 45% ; 1.7 ml per min ; overall run time : 25 min).

Carboxy terminal residue. The C-terminal sequence was studied by following the rate of release of free amino acids during digestion by carboxypeptidase A and B (Boehringer). We followed the procedure described by Ambler (11). The digestion was carried out in 0.2 M N-ethylmorpholine acetate at pH 8 and 37°C. The amino acids released after 30, 60 and 80 min were analyzed on the amino acid analyzer.

RESULTS AND DISCUSSION.

Isolation of horse colipase A and B. The method was adapted from the previously described procedure (9) with several modifications a) during purification, DFP* was omitted and replaced by PMSF* b) after precipitation with ethanol, the pellets were solubilized with 250 ml of 10 mM acetate buffer pH 4.2 containing 0.05% Triton X 100, 2 mM benzamidine and 1 mM PMSF. The colipase solution was dialyzed overnight against 20 liters of the same buffer and chromatographed on SP-Sephadex C 25 equilibrated in 100 mM acetate buffer containing 0.05% Triton X 100 and 50 mM NaCl and no protease inhibitor c) fractions of the two peaks of colipase eluted from the SP-Sephadex column (designated as colipase A and B according to their order of elution) were separately pooled and brought to pH 3.5. Each colipase sample was chromatographed on SP-Sephadex at pH 3.5 in 100 mM acetate buffer containing 150 mM NaCl.

From the 2.5 10^6 units of colipase originally present in the pancreatic extract, 208 000 units of colipase A (14 mg) and 560 000 units of colipase B (37 mg) were obtained. Both preparations have a specific activity around 15 000 units mg^{-1} under conditions of the cofactor assay. Analysis by polyacrylamide gel electrophoresis revealed the presence of a single band in both preparations. The two forms of horse colipase have distinct electrophoresis migration at pH 8.4 and pH 4.5.

* DFP : diisopropylphosphofluoridate.
PMSF: phenylmethylsulfonyl fluoride.

Table I - Amino acid composition of horse colipase A and B.

Amino acid	Colipase A	Colipase B
Ala	8	8
Arg	3	4
Asx	8	10-11
Cys	10	10
Glx	10	9
Gly	6	6
His	2	1
Ile	5	5
Leu	6	6
Lys	4	5
Met	1-2	2
Phe	2	2
Pro	4	3-4
Ser	9	8-9
Thr	5	4
Trp	1	1
Tyr	3	3
Val	6	6
Total	93-94	93-96

N- and C-terminal residues. Valine was identified by dansylation as N-terminal residue in both proteins. Only alanine (0.85 mole/mole of colipase) was liberated after digestion of native colipase A or B by carboxypeptidase A or a mixture of carboxypeptidase A and B. The removal of the C-terminal residue of alanine did not affect the capacity of horse colipases to activate lipase under the standard assay conditions.

Amino acid compositions. The amino acid compositions of colipases A and B are almost identical as indicated in Table I. It appears from the analysis that the two proteins contain about 94 amino acid residues. NMR studies on horse colipase A and B clearly indicate that ring resonances corresponding to one tryptophan

residue are present in both proteins. At acidic pH, compared analysis of the low-field region of the spectra of colipases confirms the difference in the histidine content of the proteins (P.J. Cozzone and P. Canioni, personal communication).

N-terminal sequences. Amino terminal sequence analyses were performed on reduced and carboxymethylated colipase A and B. The sequences were compared to that of the major form of horse colipase established in our previous studies (9).

Analyses of colipase A provided the first 55 residues of the protein. By comparing this sequence with the homologous sequence previously established, four substitutions were found. Analyses of colipase B provided the first 25 residues of the polypeptide chain. From the sequence, it was possible to identify this protein with the major form of horse colipase previously studied. Actually, these two proteins had the same chromatographic behaviour on SP-Sephadex at pH 4.2.

The amino acid sequence of the N-terminal regions of horse colipase A and B are presented in Table II with the homologous sequence of porcine colipase (5,6) and the partial sequence of human colipase (12). The sequences reported in Table II indicate that there exists a high degree of homology between the two horse colipases since only four differences are found in the first 51 residues. Differences occur at position 18 (Leu → Met), 22 (Gln → Glu), 29 (His → Thr) and 30 (Gln → Arg). Residues at position 44 and 47 in colipase A were identified as serine. By comparing the partial structure of colipase from the different species it can be noticed that variations at residue 7 (Leu → Val), 12 (Glu → Asp), 16 (Ile → Leu) and 3 (Glu → Asp) are highly conservative. Furthermore the leucine and methionine difference found at position 18 in pig

Table II - Amino terminal sequence of horse colipase A and B. Comparison to the homologous sequence of porcine (Ref. 5-6) and human (ref. 12) colipase.

	1	5	10	15
Horse A	Val-Pro-Asp-Pro-Arg-Gly-*Val*-Ile-Ile-Asn-Leu-*Glu*-*Ala*-Gly-Glu-			
Horse B	Val-Pro-Asp-Pro-Arg-Gly-*Val*-Ile-Ile-Asn-Leu-*Glu*-*Ala*-Gly-Glu-			
Pig	Val-Pro-Asp-Pro-Arg-Gly-*Ile*-Ile-Ile-Asn-Leu-*Asp*-*Glu*-Gly-Glu-			
Man	Gly-*Ile*-Ile-Ile-Asn-Leu-*Glu*-*Asn*-Gly-Glu-			

	16	20	25	30
Horse A	*Ile*-*Cys*-*Leu*-Asn-Ser-Ala-*Gln*-Cys-Lys-Ser-*Glu*-Cys-Cys-*His*-*Gln*-			
Horse B	*Ile*-*Cys*-*Met*-Asn-Ser-Ala-*Glu*-Cys-Lys-Ser-*Glu*-Cys-Cys-*Thr*-*Arg*-			
Pig	*Leu*-*Cys*-*Leu*-Asn-Ser-Ala-*Gln*-Cys-Lys-Ser-*Asn*-Cys-Cys-*Gln*-*His*-			
Man	*Leu*-(*)-*Met*-Asn-Ser-Ala-*Gln*-			

	31	35	40	45
Horse A	*Glu*- X -Ser-Leu-Ser-Leu-Ala-Arg-Cys-Ala-*Ala*-Lys-Ala-*Ser*-Glu-			
Horse B	*Glu*-*Ser*-Ser-Leu-Ser-Leu-Ala-Arg-Cys-Ala-*Ala*-Lys-Ala- X -Glu-			
Pig	*Asp*-*Thr*-Ser-Leu-Ser-Leu-----Arg-Cys-Ala-*Leu*-Lys-Ala-*Arg*-Glu-			

	46	50	55
Horse A	Asn-Ser-Glu-Cys- X -Ala-*Trp*-Thr-Leu-Tyr-		
Horse B	Asn- X -Glu-Cys- X -Ala-		
Pig	Asn-Ser-Glu-Cys-----Ala-*Phe*-Thr-Leu-Tyr-		

The numbering of residues is based on the sequence of horse colipase. Residues designated X have not been definitely identified. They probably are Serine residues. The dotted lines indicate deletions. Italics show residues which differ.

*In man colipase, residue 17 is Asp or Glu (Ref. 12).

and in man is also represented in the two forms of horse colipase. The only tryptophane residue of horse colipase is located at position 52 in a peptide sequence Cys-Ser-Ala-Trp-Thr-Leu. Alignement of this peptide with the homologous sequence in porcine colipase (Table II) suggests that phenylalanine at position 52 in the porcine protein is the corresponding aromatic residue. We also note that insertion of alanine occurs at position 37 in both colipase A and B and that residues 29 and 30 in horse colipases are also found in the porcine protein, but in a reverse order.

Up to now, most of the structural and conformational studies on pancreatic colipase have been carried out on the porcine cofactor (13-15). However, it is not known yet if the multiple forms of porcine colipase are isoproteins with amino acid differences. This communication presents evidence showing that two isocolipases exist in horse pancreas. Elucidation of the sequence of the first half of both polypeptides has revealed four amino acid substitutions. This finding confirms that secretory pancreatic proteins might, in some cases, exist in the form of isoproteins as illustrated by a recent report (16) showing that two isophospholipases A_2 differing by four substitutions are found in the pig. Horse colipases have been prepared from pancreatic glands obtained from several animals. No investigation was made to know if the two isocolipases occur either separately or together in any individual animal.

Separation and characterization of horse colipases will help in future investigations of the structure-function relationships in this protein.

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