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ISOLATION OF BOVINE CARDIODILATIN BY FAST PROTEIN LIQUID CHROMATOGRAPHY AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Cardiodilatin (CDD), a polypeptide exhibiting vasorelaxant and diuretic natriuretic bioactivity, was isolated from bovine atria. The isolation procedure reported here is different from that originally used for the purification of porcine and bovine CDD. Instead of cation-exchange chromatography on Fractogel TSK-CM 650 S and several purification steps on different high-performance liquid chromatographic (HPLC) columns, it is now possible to obtain CDD-88 by an automated fast-protein liquid chromatography system for repeated injections and a motor valve as fraction collector and only one final step of reversed-phase HPLC on a TSK-ODS-120T column.

INTRODUCTION

Vasorelaxant and diuretic natriuretic polypeptides have been extracted from porcine[1] and bovine[2] atria during the past few years. It has been shown that in these species the cardiac polypeptide hormones are stored in their large molecular forms, denoted cardiodilatin-126 and cardiodilatin-88. Other research groups, in contrast, found mainly small-molecular forms, called cardionatrin[3], atrial natriuretic factor[4], atriopeptin[5], auriculin[6] or alpha-ANP[7]. The circulating form of this peptide family was isolated in our laboratory from human haemofiltrate and denoted CDD-28[8]; this circulating form of the atrial peptide is identical with alpha-ANP[7]. We have recently continued our studies on cardiac hormones, including bovine cardiodilatin, in a large-scale preparation in order to obtain the natural peptide for experimental cleavage studies. We also have simplified the purification method to obtain natural CDD in larger amounts for functional studies.

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EXPERIMENTAL

Crude material

A batch of 100 kg of bovine atria was used in this study and the crude peptide material was extracted according to Mutt[9]. The atria were removed from the hearts immediately after slaughter, boiled for 10 min and then frozen in liquid nitrogen. For the large-batch extraction, the atria were immersed in 0.5 *M* acetic acid (3 volumes) and stirred at room temperature overnight. The filtrate from this acetic extraction was adjusted to pH 2.7 and the peptides were adsorbed batchwise on alginic acid and eluted with 0.2 *M* hydrochloric acid. From this eluate the peptides were salted out with sodium chloride at pH 4.0. The salt precipitate was subjected to a fractionation with ethanol–water. The ethanol precipitate that appeared at −20°C was used in the purification by fast protein liquid chromatography (FPLC) and high-performance liquid chromatography (HPLC).

Ion-exchange FPLC

The instrument for ion-exchange FPLC consisted of two P-500 pumps, an LC-500 controller, an MV-7 valve as an injection system, a 50-ml superloop as sample reservoir, a UV monitor M and an MV-8 valve as fraction collector. All these components were supplied by Pharmacia (Uppsala, Sweden). The separation was carried out on a Pharmacia Mono S HR cation-exchange column (100 × 10 mm I.D.). Elution was performed with a gradient of ammonium hydrogencarbonate (pH 7.0) from 20 to 500 m*M*. The flow-rate was 4 ml/min and the absorbance of the eluent was recorded at 280 nm.

Reversed-phase HPLC

The apparatus, from LKB (Bromma, Sweden), was equipped with an LKB 2050 HPLC pump, an LKB 2152 controller, an LKB 2040 gradient-mixing valve, an LKB 2154 injector and an LKB 2151 HPLC variable-wavelength detector connected with an LKB 2210 recorder. The separation was performed on a TSK-ODS-120T column (300 × 7.8 mm I.D.). Solvent A was 0.01 *M* hydrochloric acid (Suprapur grade; Merck, Darmstadt, F.R.G.); solvent B was 0.01 *M* hydrochloric acid in 80% aqueous acetonitrile (HPLC-grade S; Rathburn Chemicals, Walkerburn, U.K.). All solvents were degassed by sparging with helium. The flow-rate was 1.5 ml/min and the effluent was monitored at 210 nm.

Bioassay

The purification steps during the separation procedure were followed by the determination of the vasorelaxant activity on norepinephrine-precontracted smooth muscle strips of the rabbit aorta[1].

Sequence analysis

Stepwise Edman degradation of the intact, highly purified peptide and the fragments derived from the enzymatic cleavage was performed on a gas-phase protein sequence (Model 470A) from Applied Biosystems (Foster City, CA, U.S.A.). The PTH-amino acids were identified by HPLC according to Lottspeich[10].

End-group analysis

The C-terminal amino acids were determined by cleavage of the peptide with carboxypeptidase A (Boehringer, Mannheim, F.R.G.) and analysed in a Waters (Waters Chromatography Division, Saint Quentin en Yvelines, France) amino acid analysis system[11].

Amino acid analysis

The total composition was determined after acid hydrolysis on a Waters amino acid analysis system.

RESULTS

Isolation of cardiodilatin-88

From 100 kg of bovine atria we obtained about 20 g of starting material. Batches of 200 mg of crude material were dissolved in 5 ml of 250 m*M* ammonium hydrogencarbonate (pH 7.0) and subjected to ion-exchange FPLC (Fig. 1). Fractions of 2 ml (*i.e.*, 0.5 min) were collected and assayed for bioactivity. All vasorelaxant peptides were eluted in the hatched area of Fig. 1. On the basis of this chromatogram it was possible to automate the FPLC system. A schematic diagram of the automated FPLC system for repetitive injections and an MV-8 valve as fraction collector, used for this study, are depicted in Fig. 2. A total amount of 2 g of crude material was then chromatographed on the cation-exchange FPLC column. All ten individual chromatograms showed an identical elution pattern.

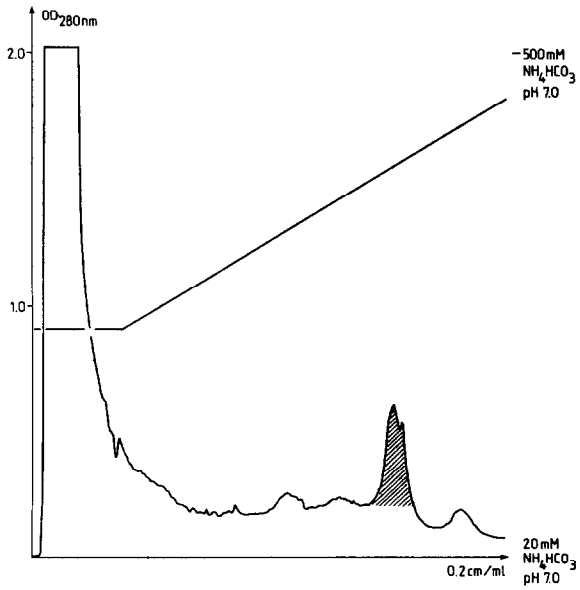

Fig. 1. Elution diagram of the starting material by cation-exchange chromatography in the FPLC system. Column, Mono S HR (100 × 10 mm I.D.); solvent system, A = 0.02 *M* ammonium hydrogencarbonate (pH 7.0), B = 0.5 *M* ammonium hydrogencarbonate (pH 7.0); flow-rate, 4 ml/min. The gradient profile is shown by a continuous line. All vasorelaxant peptides are eluted in the hatched area.

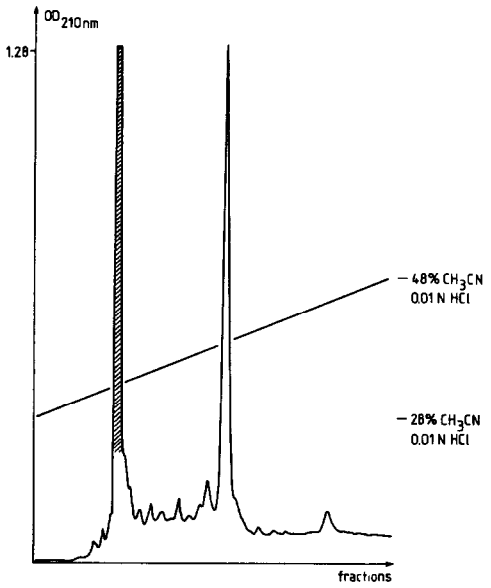

Fig. 2. Final purification of cardiodilatin-88 by reversed-phase HPLC. Load, 10% of the fraction containing the bioactive material, diluted with two volumes of water, from ten FPLC runs was repeatedly injected with a 2-ml loop until the entire fraction was applied. Column, TSK-ODS-120T (300 × 7.8 mm I.D.). Solvent system, A = 0.01 *M* hydrochloric acid, B = 0.01 *M* hydrochloric acid in 80% aqueous acetonitrile; flow-rate, 1.5 ml/min. The gradient profile is shown by the continuous line. Cardiodilatin-88 is found in the early eluted peak, indicated by the hatched area.

The fractions showing cardiodilatin bioactivity (hatched zone in Fig. 1) were pooled, diluted with 2 volumes of highly purified water (Millipore/Waters Chromatography Division) and 10% of the total volume was subjected directly to reversed-phase HPLC. The HPLC elution profile is shown in Fig. 2. The peaks were collected manually and tested for cardiodilatin bioactivity. With this purification procedure, a total of about 3 mg of the peptide were obtained from ten successive HPLC runs.

Amino acid analysis

The results of the amino acid determination are summarized in Table I. The values obtained after 24 h of acid hydrolysis and determination in a Waters amino acid analysis system after derivatization with phenyl isothiocyanate according to Bidlingmeyer *et al.*[11] confirm the amino acid sequence (see below and Fig. 4).

Enzyme digestion

The purified CDD-88 was subjected to enzyme digestion with endoproteinase Arg C, Glu C and Lys C, using 50 n*M* for each experiment. The fragments were separated by reversed-phase HPLC (see Fig. 3). The conditions for enzyme digestion and separation are described in detail in the caption. The structure of the distinct fragments was again determined with the gas-phase protein sequence. In addition,

TABLE I

ACID HYDROLYSIS OF CARDIODILATIN-88

Values are molar ratios without correction for destruction, incomplete hydrolysis, or impurity.

Residue	*Acid hydrolysis*	*Sum of sequence*	*Residue*	*Acid hydrolysis*	*Sum of sequence*
Glx	9.7	10	Val	2.3	2
Ser	11.7	12	Met	2.7	3
Gly	10.8	11	Cys	1.8	2
His	0.2	–	Ile	1.2	1
Arg	9.9	10	Leu	9.9	10
Thr	1.2	1	Phe	1.9	2
Ala	7.2	7	Lys	2.0	2
Por	7.1	7	Trp	N.D.	2
Tyr	0.8	1			
			Total	86	88

the primary structure of the fragments was confirmed by the sequence analysis of 5 n*M* of the undigested polypeptide of each corresponding experimental batch. The primary structure of the entire CDD molecule and the unequivocally analysed fragments are shown in Fig. 4. Further, the C-terminal amino acid residue was identified after enzymatic cleavage with carboxypeptidase A and subsequent HPLC separation. After 60 min, only tyrosine was found. Prolongation of the digestion to 180 min resulted also in the cleavage of arginine, which is the second C-terminal residue (Fig. 4).

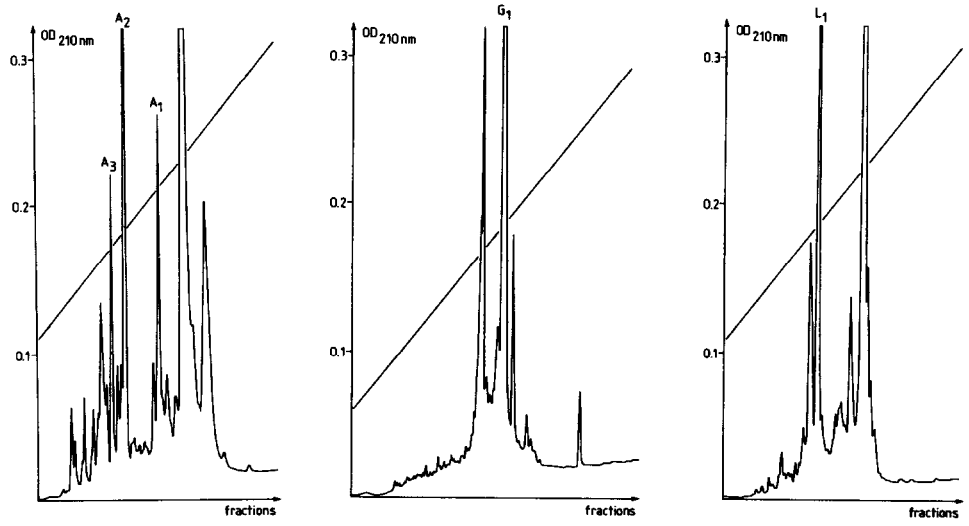

Fig. 3. Separation of the enzymatically cleaved fragments of cardiodilatin-88 by reversed-phase HPLC on a TSK-ODS-120T column (250 × 4.8 mm I.D.). Solvent system as in Fig. 3; flow-rate, 0.8 ml/min. The gradient profile is shown by the continuous line. The enzymatic degradation was performed at 37°C for 3 h. The protein: enzyme ratio was 50:1. (a) Enzymatic degradation with endoproteinase Arg C; (b) enzymatic degradation with endoproteinase Glu C; (c) enzymatic degradation with endoproteinase Lys C. The amino acid sequence of fragments A_1, A_2, A_3, G_1 and L_1 was determined as illustrated in Fig. 4.

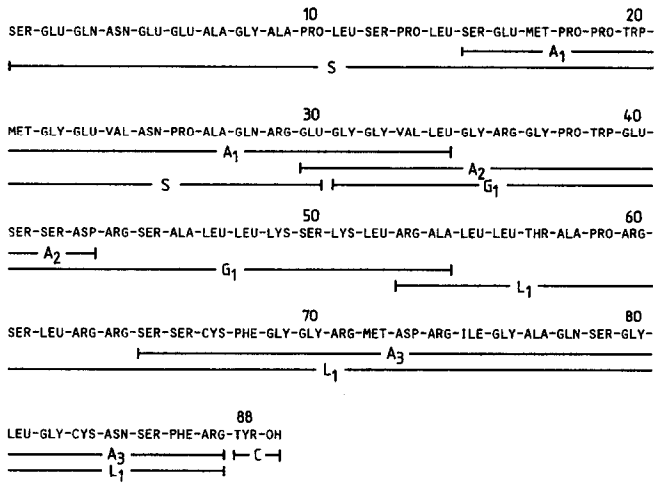

Fig. 4. Complete amino acid sequence of bovine cardiodilatin-88. Stepwise Edman degradation of the intact peptide (S) and the fragments (A_1, A_2, A_3, G_1, L_1) from the enzymatic cleavage was performed on the 420A gas-phase protein sequence. The PTH-amino acids were identified by HPLC. The C-terminal tyrosine (C) was identified by enzymatic cleavage with carboxypeptidase A.

DISCUSSION

This work was carried out to develop a simple method for large-scale extractions of the cardiac polypeptide prohormones of the CDD/ANP family. From 100 kg of atrial tissue, a crude peptide extract of 20 g of ethanol–water precipitate was separated, containing most of the vasorelaxant activity. In two further chromatographic steps, using an FPLC ion-exchange system followed by reversed-phase HPLC, almost 3.0 mg of CDD-88 can be isolated from 2 g of crude extract. This is a yield of 0.3 mg of CDD-88 from 1 kg of bovine cardiac atria. The method is particularly suitable for large-scale extractions of bovine cardiodilation, and probably also for the homologous polypeptides of other species, owing to its molecular characteristics, such as isoelectric point and basicity. Further advantages of the method are that only one intermediate lyophilization is necessary to obtain the crude peptide material and that the fractions collected from the ion-exchange FPLC can be directly injected into the HPLC system after dilution with two volumes of water. According to the sequence study and further analytical determinations, the purification achieved is high, routinely over 90%.

CDD-88 was first isolated by our research group[2,12] and preliminary sequence data have been published[13,14]. In the meantime, extraction studies by Ong *et al.*[15] resulted in the isolation of the 28-amino acid residue containing a C-terminus, which, in contrast to our studies, is present in atrial extract only in negligible amounts. However, the 28-amino acid C-terminus is the post-translationally processed cardiac hormone[16], circulating in the bloodstream, as identified in rats[17,18] and man[8]. We presume that our method omits the cleavage of the CDD/ANP polypeptides during extraction, because many other research groups have isolated mainly C-terminal frag-

ments[16]. Recently, genetic studies have also been carried out which are in complete agreement with our results[19].

In our enzymatic cleavage experiments an interesting finding was that the biological activity of the vasorelaxant substance is observed in both the CDD-88 molecule and a fragment in which the entire C-terminus of at least 28 amino acid residues is conserved. Hence it is obvious that the biologically important vasorelaxant part of the molecule is located in a part where a complete sequence homology for human, porcine and bovine cardiac polypeptides is now well established[16].

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