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ISOLATION FROM FETAL BOVINE SERUM OF A PEPTIDE SIMILAR TO THE ALPHA CHAIN OF THROMBIN BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

During the preparation of erythrotropin from fetal bovine serum, a group of peptides co-eluted with this erythroid cell stimulating factor on semi-preparative reversed-phase high-performance liquid chromatography. They could be subsequently separated by a combination of reversed-phase high-performance liquid chromatography in the presence of heptafluorobutyric acid as ion-pairing reagent and gel permeation high-performance liquid chromatography. One of these peptides has been extensively purified. Partial amino acid sequence analysis indicated that fourteen of the seventeen N-terminal amino acids are identical with the N-terminal sequence of the alpha chain of bovine thrombin. The same isolation procedure could be useful for the identification of other major peptides of fetal bovine serum.

INTRODUCTION

Although fetal bovine serum is the most widely used serum supplement for animal cell cultures, very little is known about the main components of this complex mixture of essential nutrients, hormones, transport proteins and growth-promoting factors. A promising new approach for the separation and characterization of the serum components is to take advantage of the hydrophobic character of proteins and peptides with the use of hydrophobic interaction or reversed-phase high-performance liquid chromatography (HPLC). Albumin and gamma globulins can be separated and purified by hydrophobic interaction chromatography[1]. Small peptides such as the erythroid cell stimulating factor of fetal bovine serum (erythrotropin) can be isolated by reversed-phase HPLC[2,3]. In this paper we describe how the same procedure of reversed-phase HPLC can been used to isolate successfully and perform partial sequence analysis of other major peptide of fetal calf serum, which seems to be identical with the alpha chain of bovine thrombin.

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MATERIALS AND METHODS

Fetal bovine serum was obtained from Flow laboratories (Rockville, MD, U.S.A.). Thrombin was purchased from Parke-Davis Canada (Scarborough, Canada) and coproporphyrin I was from Sigma (St. Louis, MO, U.S.A.). Heptafluorobutyric acid (HPLC grade) was from Pierce (Rockford, IL, U.S.A.). [^{3}H]Thymidine was from ICN Canada (Montreal, Canada) and had specific activities of 42–50 Ci/mmol. The HPLC equipment used here was a Milton Roy minipump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) used together with a second pump for gradient preparation[4]. We used two μBondapak C_{18} columns (Waters, Milford, MA, U.S.A.) attached in series for reversed-phase HPLC, and two I-125 columns (Waters) for gel permeation HPLC[2,3].

Before reversed-phase HPLC of fetal bovine serum[3], a group of acid-soluble, hydrophobic peptides including erythrotropin was separated from other serum components with a short heat denaturation procedure[2]. All the chromatographic procedures were identical with those used for the purification of erythrotropin[2,3]. The thymidine-incorporation bioassay for erythrotropin using bovine fetal liver cells has been described in detail elsewhere[3]. Sequence analysis was done using the Applied Biosystems gas phase protein sequencer (Foster City, CA, U.S.A.).

RESULTS

Fig. 1A shows the elution profile of 10 mg of reversed-phase extract from fetal

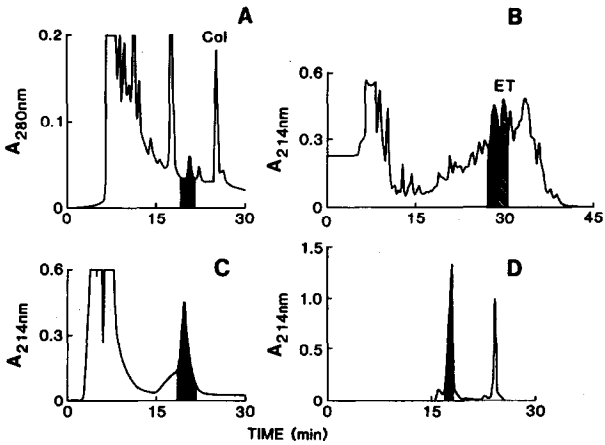

Fig. 1. Purification of a serum peptide co-eluting with bovine serum erythrotropin. (A) 10 mg of a reversed-phase extract from fetal bovine serum and 10 μg of coproporphyrin I (CoI) were applied to two μBondapak C_{18} columns attached in series and eluted with a linear gradient at 1.5 ml/min from 28 to 42% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid in water for 40 min. The fractions indicated with the black bar were pooled. (B) The pooled fractions of Fig. 1A coming from 400 ml of serum were then applied to the same columns but this time they were eluted with a linear gradient from 30 to 55% acetonitrile in 0.13% heptafluorobutyric acid in water for 60 min. ET = Elution position of erythrotropin. (C) The fractions indicated with the black bar in Fig. 1B were further purified using identical chromatographic conditions as indicated for Fig. 1A. This step was repeated until a single absorbance peak was obtained. (D) The purified peptide was applied to two gel-filtration columns attached in series and eluted in 40% acetonitrile and 0.1% trifluoroacetic acid in water at 1 ml/min. A_{214} = Absorbance at 214 nm.

bovine serum applied to two reversed-phase columns attached in series. Because of the amount applied and the use of two columns this run can be considered at a semi-preparative level and the separation is therefore less effective than a regular analytical run using the same columns at the microgram level. Coproporphyrin I (10 μg) was added as a marker (CoI). We have found that the material eluting before CoI as indicated with the black bar contains erythrotropin[2] together with many other different compounds. The nature of this mixture could be better appreciated after the separation of the main components using reversed-phase HPLC in the presence of heptafluorobutyric acid as a ion-pairing reagent (Fig. 1B). We were particularly interested in all the peptides with similar retention times to that of erythrotropin, because they may be structurally related to this erythroid cell stimulating factor. In this paper we analyzed the material eluting just before erythrotropin (ET), as identified with the black bar of Fig. 1B. The position of erythrotropin was identified with the thymidine-incorporation bioassay (results not shown). The fractions indicated with the solid black bar in Fig. 1B were further purified using reversed-phase HPLC in the presence of trifluoroacetic acid as an ion-pairing reagent (Fig. 1C). There were still some contaminants, but the main absorbance peak (labeled black) could be further purified using the same chromatographic method of Fig. 1C until a single symmetrical absorbance peak was obtained (results not shown). The purified HPLC fraction was finally subjected to gel permeation HPLC (Fig. 1D) in order to remove some minor contaminants of different molecular weight. The isolated peptide had the same elution position as erythrotropin on gel permeation HPLC, indicating a very similar apparent molecular weight of *ca.* 3500 daltons. However, we know that the real molecular weight of erythrotropin is large (*ca.* 5600 daltons) when the disulfide bridges are reduced. This could also be the case with the peptide shown on Fig. 1D.

Sequence analysis of the isolated peptide (Fig. 2) indicated that the N-terminal amino acid sequence was almost identical with the alpha chain of bovine thrombin[5], as identified using the Protein Identification Resource of the National Biomedical Research Foundation, Georgetown University, Washington, DC, U.S.A. The thrombin-like peptide (TLP) shows only two amino acid differences when compared with the alpha chain of thrombin at the residues 9 (Ser) and 14 (Lys). The amino acid residue number 10 could not be identified with certainty.

It was important to test the biological activity of thrombin in our *in vitro* bioassay of thymidine incorporation into liver erythroid cells (Fig. 3), because throm-

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
TLP	Thr	Ser	Glu	Asp	His	Phe	Gln	Pro	Ser	xxx	Asn	Glu	Lys	Lys	Phe	Gly	Ala	---
THROMBIN [ALPHA CHAIN]	Thr	Ser	Glu	Asp	His	Phe	Gln	Pro	Phe	Phe	Asn	Glu	Lys	Thr	Phe	Gly	Ala	Gly

	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
	Glu	Ala	Asp	Cys	Gly	Leu	Arg	Pro	Leu	Phe	Glu	Lys	Lys	Gln	Val	Gln	Asp	Glu

	37	38	39	40	41	42	43	44	45	46	47	48	49
	Thr	Gln	Lys	Glu	Leu	Phe	Glu	Ser	Tyr	Ile	Glu	Gly	Arg

Fig. 2. N-terminal amino acid analysis of the thrombin-like peptide (TLP) isolated in Fig. 1. The similarity with the alpha chain of thrombin[5] was found using the facilities of the National Biomedical Research Foundation, Georgetown, Washington, DC, U.S.A. xxx = Unknown amino acid.

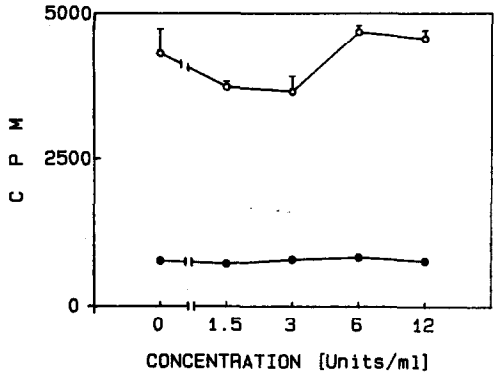

Fig. 3. Effect of thrombin on [^{3}H]thymidine incorporation in fetal liver erythroid cells. Different concentrations of thrombin (abscissa) were added to cell cultures of bovine fetal liver cells in the presence (open circles) or absence (closed circles) of 0.1 U/ml sheep plasma erythrotropin. After 20-h incubation thymidine incorporation was measured as previously described[3]. The results are cpm per 10^5 cells. The values are the mean and standard deviations of three determinations.

bin has been recognized as an important growth factor for different cell types[6]. Fig. 3 shows that thrombin did not share two main properties of erythrotropin. First, it did not stimulate thymidine incorporation at concentrations known to be effective in other cell types[7]. And second, it did not potentiate the effect of erythropoietin, as has been shown for erythrotropin in calf and rat liver erythroid cells[8]. As far as the isolated thrombin-like peptide was concerned, we tested two different preparations using the thymidine-incorporation assay. One preparation was completely inactive, and the second one had some activity, probably due to a contamination with erythrotropin.

DISCUSSION

Reversed-phase HPLC is a powerful technique for the isolation of peptides[9]. In this paper we show how this technique could be used for the isolation and characterization of unknown peptides present in the complex mixture of fetal bovine serum. We have decided to characterize first those peptides which have similar chromatographic properties to bovine serum erythrotropin, but in principle the same method could be used for the isolation and characterization of many of the major peptides present in serum. We have found that crude reversed-phase extracts of fetal calf serum contain many peptides as analyzed by reversed-phase HPLC (Fig. 1A) and that the thymidine-incorporation stimulating activity in fetal erythroid cells is localized in a region which can be easily identified using coproporphyrin I as a chromatographic marker[3]. The fractions defined according to their elution position relative to coproporphyrin I[3] can be further separated using heptafluorobutyric acid as an ion-pairing reagent[10]. The peptide erythrotropin (ET in Fig. 1B) is contaminated with many other substances. The different fractions eluted from the column can then be individually purified. However, further purification should take into consideration that the recoveries of peptides isolated by reversed-phase HPLC and other techniques can be low during the last stages of purification, because contaminating peptides and

proteins which protest against loss are eliminated[9]. It is therefore of interest to perform all the final purification steps (Fig. 1C and D) as soon as possible to prevent losses due to storage, freezing, lyophylization and adsorption to glass tubes and other materials of containers[9]. A fast purification of the different fractions surrounding ET in Fig. 1B can be done simply by following and collecting the fractions having the maximum absorbance at 214 nm (Fig. 1C and D). Aliquots of the samples are taken for a subsequent bioassay in order to identify the material having thymidine-incorporation stimulating activity. Partial amino acid sequence of the peptide chosen for purification in Fig. 1 indicated that it is practically identical with the N-terminal portion of the alpha chain of bovine thrombin (Fig. 2). However, there are two amino acids of this thrombin-like peptide which are different from the thrombin sequence. It is not known if this may be characteristic of the particular batch of fetal calf serum used for the purification. The peptide could represent a fetal type of thrombin or an allele of the thrombin gene. Alternatively, it could be the N-terminal portion of another protein. It remains to be seen if this peptide is a product of the reduction of a thrombin-like protein followed by a dissociation of the alpha and beta chains during the isolation procedure, or if it is present in fetal bovine serum before acid extraction.

Although thrombin, a serine protease, acts as a growth-promoting factor in a variety of cells[6], it did not have any activity similar to erythrotropin on thymidine incorporation in fetal calf liver cells (Fig. 3). Furthermore, the growth-promoting activity of thrombin requires the intact catalytic site, which is localized in the beta chain of the enzyme[11]. It is therefore very unlikely that the small alpha-subunit or a structurally similar peptide could have any growth-promoting activity by itself. Therefore the similar elution position of the isolated thrombin-like peptide and erythrotropin does not imply a common biological function.

In principle the retention times of peptides on reversed-phase HPLC are a function the amino acid composition[12,13]. It is possible that peptides having different amino acid compositions may elute together depending on the balance between hydrophobic and hydrophilic amino acids. However, the probability that the same peptides may have similar retention times in the presence of trifluoroacetic acid and (at the same time) heptafluorobutyric acid is very small[12].

It is then reasonable to assume that erythrotropin, the thrombin-like peptide isolated here and the 11 000-dalton erythrotropin-like peptide isolated from fetal bovine serum[14] may have a hydrophobic structure or domain which is important for the interaction with the reversed-phase column independent of the different amino acid sequence. Cases of dissimilar proteins having the same secondary structure and similar protein domains are known[15]. The peptides erythrotropin (a growth factor), erythrotropin-like peptide (similar to the low density lipoprotein receptor) and the thrombin-like peptide (similar to a serine protease) have in fact different amino acid sequences at their N-terminal sections. Nevertheless, they have in common not only their similar elution behavior on reversed-phase HPLC, but also their sequence homology to proteins which interact or are integral part of cell membranes. They may share a protein domain which recognizes certain membrane structures. The presence of a similar protein domain in otherwise dissimilar proteins may be the result of "protein domain shuffling"[15], a recent evolutionary event which has been proposed for sections of the epidermal growth factor precursor, the low density lipoprotein receptor and several serine proteases[15,16].

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