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FURTHER CHARACTERIZATION OF THE ESTROGEN BINDING MACROMOLECULE IN HUMAN PANCREAS

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Summary—The estrogen binding protein in human pancreas has been purified from pancreatic cytosol by chromatography on Concanavalin-A–Sepharose and hydroxyl-apatite followed by ion-exchange chromatography carried out using a fast-protein liquid chromatography apparatus (FPLC). The purified protein, still able to bind labelled [^{3}H]estradiol, appeared as one single band corresponding to 31 K in SDS-gel electrophoresis. Total amino acid analysis revealed high levels of histidine, glutamic acid and leucine. The capacity of the purified protein to bind estrogens could be increased more than 4-fold by addition of a cytosolic factor, probably being a small peptide, that is present in crude cytosol, but lost during the purification procedure. The iodinated protein does not bind to DNA-cellulose or phosphocellulose, and shows no similarities to estrogen receptor proteins.

INTRODUCTION

Human pancreatic tissue contains large amounts (2–4% of the total cytosol protein content) of a protein which binds estrogens with medium affinity ($K_d \sim 10^{-7}$ M) and high capacity. Its highly specific estrogen binding may indicate a role in a possible interaction between sex steroids and the pancreatic gland [1, 2]. We have recently described the purification and partial characterization of this protein [1]. In the original procedure, final purity was achieved by polyacrylamide gel electrophoresis. However, this technique did not yield sufficient amounts of protein for immunization and for further characterization. The present communication describes a modified purification procedure for the estrogen binding protein in human pancreas (hEBP), allowing the preparation of large amounts of this protein. Data are also provided on the further characterization of this estrogen binding macromolecule, including the demonstration of a low molecular weight cytosolic factor necessary for the binding of estradiol to EBP, analogously with the corresponding accessory factor shown by Boctor *et al.* for the rat EBP [3].

EXPERIMENTAL

Steroids

[2,4,6,7-^{3}H]Estradiol (91.3 Ci/mmol) was purchased from New England Nuclear Chemicals, GmbH, Dreieichenhain, West Germany.

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Preparation of cytosol

Human pancreatic glands were collected from corpses 24–28 h after death, or in a few cases parts of normal pancreatic tissues were removed in connection with surgery. It was assured that the patients before death not had suffered from any diagnosed pancreatic disorder. Following removal the tissue was immediately frozen at −20°C. For preparation of cytosol pancreatic tissue was thawed and homogenized in 3 vol of ice-cold buffer A (50 mM Tris; 1 mM EDTA; 50 mM NaCl, pH 7.4) using an Ultra-Turrax homogenizer. The homogenate was centrifuged for 60 min at 105,000 *g*. After removal of the floating lipid layer, the cytosol was decanted and immediately frozen at −20°C.

Purification of hEBP

A pool of 400 ml of cytosol was prepared and frozen in 10 ml aliquots. Forty ml were thawed on ice and divided into two 20 ml portions. In order to enable monitoring of the purification, the cytosol was incubated with 50,000 dpm [^{3}H]estradiol per ml for 1 h at 0°C. Each 20 ml portion was chromatographed on a 40 ml Concanavalin-A column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in Buffer A. After application of the sample, the column was washed with Buffer A until no further protein (measured at 280 nm) was eluted. The washing was repeated using Buffer A: 1 M KCl and hEBP was eluted using Buffer A: 1 M KCl: 0.1 M alfa-D-mannose–glycosidic acid. The latter eluate was collected in 2 ml fractions. Fractions containing radioactivity were pooled from 2 columns, and the pooled eluate was applied to a 20 ml hydroxyapatite column. The column was washed using Buffer A: 1 M KCl

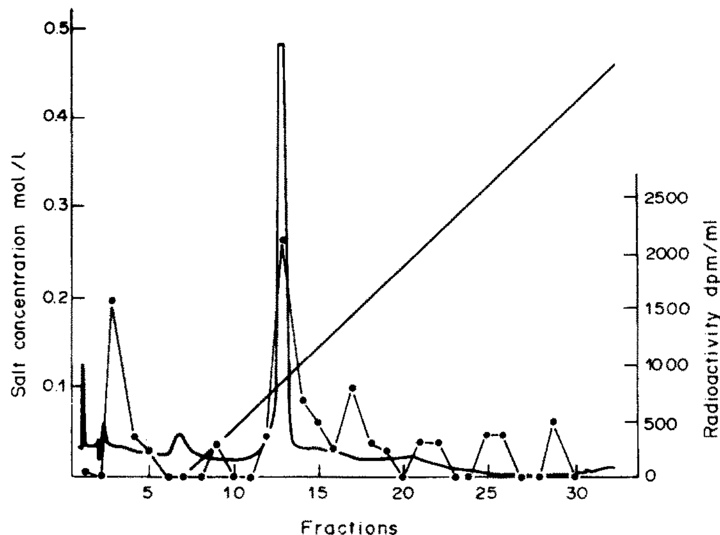

Fig. 1. Ion-exchange chromatography using FPLC of partially purified estrogen-binding protein. Following chromatography of 40 ml cytosol on Concanavalin-A–Sepharose and hydroxyl-apatite the eluate was dialyzed and incubated with 2.5×10^6 dpm [^{3}H]estradiol/ml before applied to the FPLC-apparatus. After washing the proteins were eluted using a salt gradient and aliquots of collected fractions were taken for analyzes of radioactivity and proteins (polyacrylamide gel electrophoresis). The single fraction corresponding to salt concentration 0.12 M contained almost purified estrogen binding protein. O.D. 280 nm ——; Radioactivity ●—●

until no further radioactivity or protein was eluted. The hEBP was then eluted using Buffer A: 1 M KCl: 0.5 M K_2PO_4. The sharp radioactivity peak (about 14 ml) was collected and dialyzed against Buffer A for 12–16 h. The eluate was incubated at 0°C using 2.5×10^6 dpm [^{3}H]estradiol (5 nM) per ml for 16 h and further purified by ion exchange chromatography using an FPLC apparatus (Fast Protein Liquid Chromatography) equipped with a Mono Q column. The eluate was added to the column and following washing eluted with a 30 ml linear 0–0.5 M NaCl salt gradient. Fractions (0.5 ml) were collected and scanned for radioactivity and absorbance at 280 nm. One fraction correpsonding to 0.12 M contained the radioactive material and was taken for analysis of purity (Fig. 1).

Analytical polyacrylamide gel electrophoresis

The electrophoresis was performed using a 10% cross-linked gel and electrode buffer pH 8.6. For estimation of molecular weight sodium dodecyl sulfate was included and standard protein kits (Pharmacia Fine Chemicals Uppsala, Sweden) were used as references. In some cases gels were analyzed by protein staining and radioactivity measurement. In these cases, after finishing the electrophoresis, the gel was cut along the tracks and one piece was cut into 1.2 mm thick slices subjected to radioactivity counting or extraction as described earlier [1] (Figs 2, 3).

Analysis of amino acid composition

To achieve complete removal of Tris, the buffer was changed to 0.01 M sodium phosphate at pH 7.4 by repeated dialysis. The macromolecular fraction

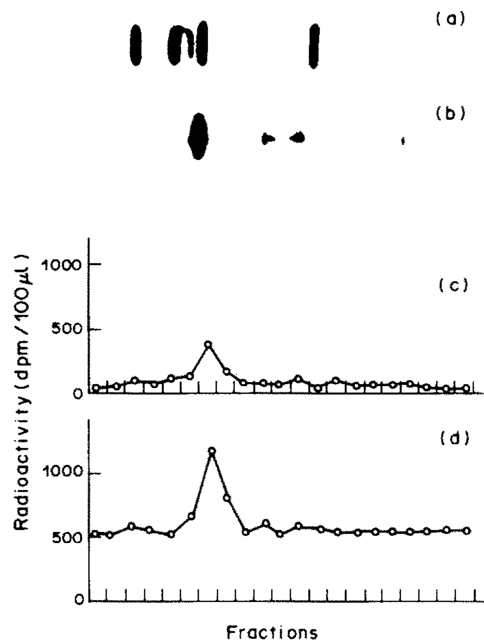

Fig. 2. Polyacrylamide gel electrophoresis of (A) standard proteins (B) and (C) purified [^{3}H]estradiol-estrogen binding protein. Following electrophoresis the gel was cut in two tracks along the gel. One part (A and B) was stained by Coomassie blue while the other was cut in 1.2 mm thick slices and extracted using 0.5 ml buffer A. Fig. 1 C shows the radioactivity recovered from this extraction and lane D shows bound radioactivity following incubation with [^{3}H]estradiol of fractions extracted from lane C in the presence of the cytosolic factor as described in the Experimental section. Standard proteins (lane A) used as references were phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase and soybean trypsin inhibitor.

was analyzed for amino acid composition at the Central Amino Acid Analysis Laboratory, Institute of Biochemistry, Uppsala, Sweden. Acid hydrolysis for 24 h was used and values for threonine and serine were extrapolated to 0 hydrolysis time to correct for destruction or incomplete hydrolysis. Methionine and cysteine were determined after oxidation with performic acid.

Preparation of ^{125}I-labelled hEBP

Radioiodination of purified protein (hEBP fraction from FPLC, about 95% pure) was performed by using the chloramine-T technique [4]. This procedure yielded preparations with a specific radioactivity of 180–260 Ci/g of protein.

Chromatography on DNA-cellulose-, phosphocellulose- and cellulose columns

Following iodination of purified hEBP, the interaction of this compound with DNA-cellulose, phosphocellulose and cellulose was studied [5]. DNA-cellulose was prepared according to Albert and Herrick [6] using calf thymus DNA. Portions of DNA-cellulose suspension containing approx 1 mg DNA/g cellulose (dry weight) were used to prepare columns with a bed volume of about 1 ml. Phosphocellulose and cellulose was purchased from Bio-Rad Laboratories, Richmond, CA, and columns (1 ml) were prepared according to the manufacturers recommendations. The columns were finally equilibrated in Buffer A. Aliquots of iodinated hEBP (about 10^6 dpm) were than added to each column and eluted with 10 ml of buffer A. It was assured that the last fraction did not contain more than background level of radioactivity. The columns were then eluted with 10 ml buffer A: 1 M NaCl that was collected in 1 ml fractions and radioactivity was measured in each fraction.

Preparation of cytosolic factor

Preparation of cytosolic factor was essentially performed as described by Boctor *et al.* for the rat estradiol binding protein [3]. Human pancreatic cytosol (5 ml) was mixed with 24 ml of ethanol and incubated for 30 min at 0°C. Following centrifugation at 3000 *g* for 30 min the supernatant was decanted, evaporated to dryness, and dissolved in 2.5 ml of buffer A.

Assay for binding of [^3H]estradiol

Generally 100 μl sample containing hEBP was mixed with 50 μl buffer A containing [^{3}H]estradiol and 50 μl buffer A or cytosolic factor preparation. Following incubation for 1 h at 20°C, 0.3 ml of DCC mixture [0.5% (w/v) charcoal and 0.05% (w/v) dextran in buffer A] was added and incubation continued for 30 min at 0°C. The dextran coated charcoal was spun down at 2000 *g* for 30 min and 100 μl of the supernatant was taken for measurement of radioactivity.

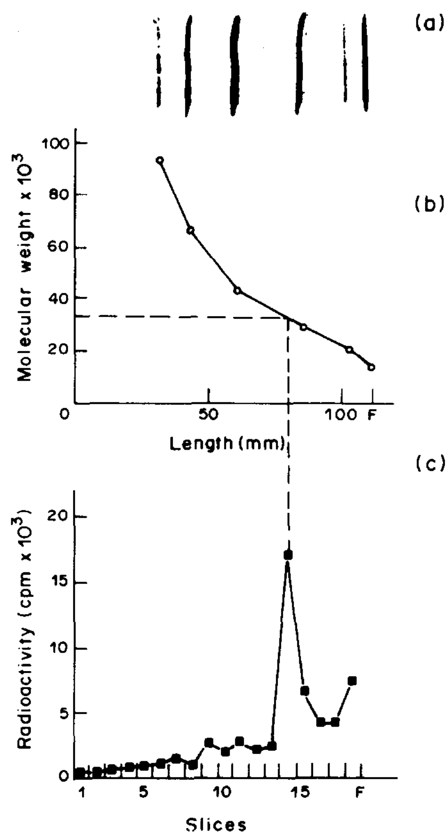

Fig. 3. Polyacrylamide gel electrophoresis of purified [^{125}I]estrogen binding protein and reference compounds in the presence of SDS. Following electrophoresis the gel was cut in two tracks. One lane containing reference compounds was subjected to protein dying and the other part containing labelled protein was cut in 1.2 mm thick slices and radioactivity was measured in a γ-counter. Lane A shows the reference compounds, lane B the plotted standard proteins and Fig. C shows the calculated radioactivity. The estrogen binding protein was found to have a molecular weight of 31 K. Standard proteins used were: Phosphorylase b (mol. wt 94,000), bovine serum albumin (mol. wt 67,000), ovalbumin (mol. wt 43,000), carbonic anhydrase (mol. wt 30,000), Soybean Trypsin Inhibitor (mol. wt 20,100) and α-lactalbumin (mol. wt 14,400).

RESULTS

Purification of hEBP

Compared to the earlier purification procedure higher yields were obtained as a total recovery of about 20% was achieved by changing the preparative polyacrylamide gel electrophoresis to ion exchange chromatography performed by a FPLC-apparatus. Starting with 40 ml of cytosol containing about 400 mg of protein, the recovery and purity following the first steps were as earlier reported [1]. The last step (FPLC) allowed 90% recovery which meant that about 80 mg of protein was purified each time. Using this protocol the protein was purified to about 95% homogeneity (Figs 2 and 3) and when analyzed with polyacrylamide gel electrophoresis without SDS, the

Table 1. Amino acid composition of the estrogen binding protein in human pancreas

Amino acid	nmol/100 nmol protein
Asparatic acid	8.6
Threonine	5.0
Serine	7.8
Glutamic acid	10.8
Proline	5.0
Glycine	9.5
Alanine	6.9
Half cystine	1.5
Valine	6.7
Methionine	1.1
Isoleucine	3.6
Leucine	8.2
Norleucine	0.0
Tyrosine	2.7
Phenylalanine	3.4
Glucosamine	0.0
Histidine	11.0
Lysine	4.1
Arginine	4.4
Total	100.3

purified [^{3}H]estradiol-hEBP moved to the same position as protein purified according to the former protocol [1] (Fig. 2).

Determination of molecular weight

Determination of mol. wt using SDS-polyacrylamide gel electrophoresis revealed a 31 K macromolecule (Fig. 3). For these studies iodinated hEBP was used as this compound later was used for further characterization. The iodinated protein moved to the same position as [^{3}H]estradiol-hEBP when analyzed with polyacrylamide gel electrophoresis in the absence of SDS (data not shown).

Further characterization of hEBP

The total amino acid composition of hEBP is shown in Table 1. When [^{125}I] hEBP was subjected to chromatography on DNA cellulose, phosphocellulose and cellulose, no specific binding to the former column material was detected (data not shown).

The presence of a low molecular weight cytosolic factor prepared from the pancreatic cytosol greatly increased the estradiol binding capacity of purified hEBP preparations, but did not increase this capacity to any greater degree in crude preparations (Table 2). The cytosolic factor itself did not show any interaction with [^{3}H]estradiol (data not shown). Binding studies performed in the absence and presence of cytosolic factor indicated that the number of binding sites but not the apparent K_d was affected by the cytosolic factor (data not shown).

DISCUSSION

Compared to the earlier procedure [1], the modified purification technique, described in the present paper, increased the yield of hEBP from 2 to 20% without changing the characteristics of the protein obtained. The modified procedure is therefore well suited to large scale preparations of hEBP, necessary for the further biochemical and biological characterization of the protein, including the raising of specific antibodies. The partial characterization of the purified protein revealed a rather small macromolecule having a glycoprotein structure, as judged from the binding of hEBP to Concanavalin A. As earlier pointed out this protein shows no similarities to the estrogen receptor [1, 11]. Our previous work to purify and characterize hEBP has in part been hampered by loss of the estrogen binding capacity during the purification procedures. However, Boctor and coworkers [3] recently demonstrated that an accessory factor, probably a small peptide, is necessary for the estrogen binding capacity of rEBP. This accessory factor is lost in the purification procedure. Our present results demonstrate that a similar accessory factor also is necessary for the estrogen binding capacity of the human EBP, and is also present in human pancreatic tissue. However when comparing our results with human pancreas with those obtained using rat preparations [3], it seems that the estrogen binding of hEBP is not as dependent upon the accessory factor as is the binding of rEBP. Nevertheless, we have previously succeeded in purifying hEBP [1] as well as rEBP [7] without knowledge of this factor, although addition of accessory factor to the EBP preparations obtained after the different purification steps greatly facilitated the monitoring of the purification procedure.

The biological role of the "medium affinity" high capacity estrogen binding proteins in the pancreas is not known. Their high concentration in the pan-

Table 2. Binding of [^{3}H]estradiol to human pancreatic estrogen binding protein preparations of different purity with and without cytosolic factor added

	Bound estradiol dpm/0.1 ml		
Preparation	Without cytosolic factor	With cytosolic factor	Relative increase of estradiol binding (%)
Crude cytosol	325	309	0
Eluate from Con-A	88	208	136
Eluate from FPLC	389	1480	280

The relative increase of estradiol binding was calculated as $\frac{\text{Bound estradiol with cytosolic factor}}{\text{Bound estradiol without cytosolic factor}} \times 100\text{–}100$

creatic tissue may however explain the high uptake and retention of estrogens in the pancreas, shown already in 1963 by Ullberg and Bengtsson[8]. Estrogen binding macromolecules in the pancreas have also previously been described by Sandberg and co-workers [9, 10]. Furthermore the high specific estrogen retention in the pancreas may indicate some kind of interaction between estrogens and pancreatic function. It is known that estrogens affect the pancreatic function in rat as well as in humans in different ways (for review see ref. 11). However the presence of classical estrogen receptors in the pancreas is doubtful, and what previously has been described as estrogen receptors may well has been the estrogen binding protein described in the present paper. If so, one may speculate over an action of estrogens upon the pancreas not involving classical receptors, but rather a new type of "medium affinity" high capacity estrogen binding protein. On the other hand estrogen receptors may be present in the pancreatic tissue, but their detection may be disturbed by high protease activity or interaction with the EBP. If so, it may be speculated that EBP participates in some kind of concentration mechanism for the estrogens in the pancreatic gland. In such a mechanism, the estrogens bound in high concentration to the medium affinity binding protein EBP will be transported and bound to the high affinity estrogen receptor protein. However extensive further studies, including e.g. tissue specificity are necessary to elucidate the biological function of the pancreatic estrogen binding protein.

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