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Simultaneous purification of biotin-binding proteins-I and -II from chicken egg yolk and their characterization

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Chicken egg yolk biotin-binding protein-I (BBP-I) has been purified to homogeneity along with the tetrameric BBP-II by a common protocol. The purification includes delipidation of egg yolk by butanol extraction, DEAE-Sephacel chromatography, treatment with guanidinium chloride and biotin–aminohexyl–Sepharose affinity chromatography. The identity of purified BBP-I was ascertained by its physicochemical properties as well as by its immunological cross-reactivity and precursor–product relationship with BBP-II.

INTRODUCTION

Most of the biotin in chicken egg is associated with the yolk tightly yet non-covalently bound to protein [1,2]. It has been shown that biotin deposition in the yolk of the avian oocyte is mediated by a high-affinity biotin-binding protein (BBP) sequestered from the maternal circulation through the oocyte plasma membrane [3]. BBP is a trace protein constituting about 0.03% of the total protein in an egg yolk [4]. More recently, investigations have revealed that there are two types of BBP (I and II) in chicken egg yolk which differ in their thermal stability [5]. A general transport function has been attributed to the less thermolabile BBP-I whereas BBP-II seems to be involved in vitamin deposition in the yolk of developing oocyte [5]. BBP-II is a tetramer with a subunit M_r of 18200 and is stable up to a temperature of 45 °C, whereas BBP-I, a monomer with an apparent M_r of 68000, is stable even at 65 °C [6,7].

Experimental evidence suggests a precursor–product relationship between the two vitamin binders involving an unusual processing pathway in which four tandemly repeated biotin-binding domains of BBP-I become the subunits of BBP-II after limited proteolysis [7]. We predicted such a phenomenon previously [8] on the basis of the fact that BBPs isolated from pregnant rat serum [9] and chicken egg white [8] have an approximate M_r of 67000 which is similar to that of chicken yolk BBP-I. Up until now, BBP-I [7] and BBP-II [4,6,10] have been purified individually from chicken egg yolk and characterized by different procedures. The present paper describes the simultaneous purification of BBP-I and BBP-II by a common protocol involving a minimal number of steps. In addition, experimental evidence for their identities including their precursor–product relationship is described.

MATERIALS AND METHODS

Materials

DEAE-Sephacel and low-M_r calibration kit for proteins were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Guanidinium chloride (GdmCl), Protein A and trypsin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. [^{3}H]Biotin (specific radioactivity 41 Ci/mmol), carrier-free $Na^{125}I$ and Amplify solution were from Amersham International, Amersham, Bucks., U.K. Other chemicals and reagents were of analytical grade and obtained locally.

Purification of BBP-I and BBP-II from chicken egg yolk

BBPs from chicken egg yolk were purified to homogeneity by the procedure described previously [10] with appropriate modifications. The two forms of the protein were isolated by a common procedure involving sequentially delipidation of egg yolk by butanol extraction, DEAE-Sephacel column chromatography and biotin–aminohexyl (AH)–Sepharose affinity chromatography. Briefly, yolks from 200 eggs were homogenized and the yolk lipids were extracted with butan-1-ol [4]; the aqueous protein layer (95 g of protein; 3 litres) was dialysed extensively against 50 mM sodium phosphate buffer, pH 7.0, and loaded on a DEAE-Sephacel column (4 cm × 30 cm) pre-equilibrated with the same buffer. Bound proteins (57 g; 350 ml) were eluted with 1 M NaCl and treated with GdmCl to a final concentration of 6 M to release the bound ligand. The dialysed protein (37 g; 150 ml) was passed through a biotin–AH–Sepharose [11] column (1 cm × 5 cm) pre-equilibrated with 10 mM sodium phosphate, pH 7.4, containing 1 M NaCl at a flow rate of 10 ml/h. Elution of the bound proteins was carried out in the following order: (i) 30 ml of 1 M GdmCl in water; (ii) 30 ml of 6 M GdmCl in water. Fractions of volume 1 ml were collected and the absorbance monitored at 280 nm. The two eluates were pooled separately and dialysed against PBS at 4 °C. All the above steps were carried out at 4 °C with the solutions invariably containing 0.02% (w/v) NaN_3 to avoid microbial contamination.

Superose 12 gel filtration

The purified forms of BBP-I and BBP-II (100 µg) were individually subjected to analytical gel filtration using a Superose 12 column attached to an FPLC system (Pharmacia) at a flow rate of 0.5 ml/min, and 1 ml fractions were collected using either PBS alone or PBS/6 M GdmCl as the elution buffer. The elution volumes of different proteins of known M_r used for calibration were also determined by injecting 100 µg of M_r marker proteins (Blue Dextran 2000000; BSA 66000; carbonic anhydrase 29000; cytochrome *c* 12400; aprotinin 6500) (Sigma).

Abbreviations used: BBP-I and BBP-II, biotin-binding proteins-I and -II; GdmCl, guanidinium chloride; AH, aminohexyl.

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SDS/PAGE

This was carried out by the procedure of Laemmli [12].

Western blotting

After SDS/PAGE, the proteins were electroblotted on to nitrocellulose membrane by the procedure of Towbin et al. [13]. The membrane was incubated with anti-BBP-II polyclonal antibody (raised in rabbits) followed by ^{125}I-labelled Protein A before autoradiography.

Thermal stability of ligand-binding characteristics of BBP-I and BBP-II

To determine the influence of temperature on the ligand-binding characteristics of the purified proteins, equivalent amounts (40 μg) of BBP-I and BBP-II were individually incubated with [^{3}H]biotin (approx. 1×10^5 c.p.m.) at 65 °C. A similar amount of BBP-II was also treated separately with [^{3}H]biotin at 45 °C. These three samples with bound radioactive biotin as well as apo forms of BBP-I and BBP-II were individually resolved on a 7.5 % alkaline polyacrylamide gel [14]. The lanes containing the proteins without the labelled biotin were stained with Coomassie Brilliant Blue and the remaining gel was fixed in methanol/acetic acid/water (40:10:50, by vol.) for 2 h. It was then soaked in Amplify solution (Amersham) for 20 min with shaking in the dark. The gel was dried and exposed to an X-ray film (Indu, Ooty, India) in a cassette (Kodak) equipped with intensifying screens at −70 °C. The autoradiogram was developed after 10 days of exposure.

Conversion of BBP-I into BBP-II by limited proteolysis

To 240 μg of BBP-I in 300 μl of 4 % (w/v) NH_4HCO_3, pH 8.0, was added TosPheCHCl_2-treated trypsin at an enzyme/substrate molar ratio of 1:50 [7]. Aliquots of the reaction mixture (30 μl) were removed at 0, 10, 30 and 360 min and separated by SDS/PAGE (10 % gel).

Characterization of BBP-II obtained by fragmentation of BBP-I

A portion of the trypsinolysis reaction mixture of BBP-I was subjected to preparative gel filtration using a Superose 12 HR 16/50 column attached to an FPLC system (Pharmacia) at a flow rate of 1 ml/min, and 1 ml fractions were collected using PBS/6 M GdmCl as the elution buffer. Fractions corresponding to M_r 19000 were pooled and dialysed against PBS. The ability of this protein species to bind biotin was assessed by fluorography as described above.

Miscellaneous methods

Protein A was radioiodinated by the Iodogen method of Fraker and Speck [15]. Protein concentration was determined as described by Lowry et al. [16] with BSA as standard. Polyclonal antiserum to the subunit form of BBP-II was raised in rabbits and characterized as described previously [10].

RESULTS AND DISCUSSION

With the discovery of BBP-I in the sera of egg-laying hens and the egg yolk [5], it was considered desirable to purify both the biotin binders in reasonable amounts by employing a common procedure involving a limited number of steps. Both BBPs have been purified previously but by two different multistep protocols [6,7]. In the present study, BBP-I and BBP-II were purified in their apoprotein forms in yields of 60 μg and 1 mg respectively from 200 chicken eggs by a single relatively simpler procedure. Effective removal of the majority of the lipids and lipoproteins was achieved by butanol extraction. The acidic proteins were concentrated by DEAE-Sephacel column chromatography. Both BBP-I and BBP-II, being of yolk origin, have endogenously bound biotin; treatment with the chaotrope GdmCl facilitated the removal of biotin as previously observed during purification of BBP-II by ligand-affinity chromatography [10]. Affinity chromatography on biotin–AH–Sepharose was performed in the presence of high salt concentrations to minimize non-specific adsorption of protein. The matrix-bound proteins were eluted successively with 1 M and 6 M GdmCl (Figure 1). Proteins present in the two eluates were analysed by SDS/PAGE (Figure 2). Under denaturing conditions, single bands corresponding to

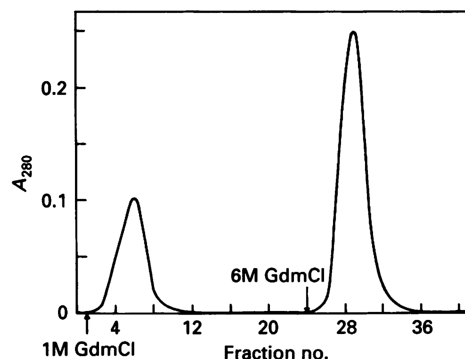

Figure 1 Biotin–AH–Sepharose affinity chromatography of BBP-I and BBP-II

GdmCl-treated DEAE-Sephacel eluate was dialysed against 10 mM sodium phosphate, pH 7.4, and applied to a column of biotin–AH–Sepharose equilibrated with the above buffer containing 1 M NaCl. After extensive washing, the bound proteins were eluted sequentially with (i) 1 M GdmCl in water and (ii) 6 M GdmCl in water. Fractions of volume 1 ml were collected at a flow rate of 10 ml/h. The 1 M GdmCl eluate was found to contain BBP-I and the 6 M GdmCl eluate to contain BBP-II.

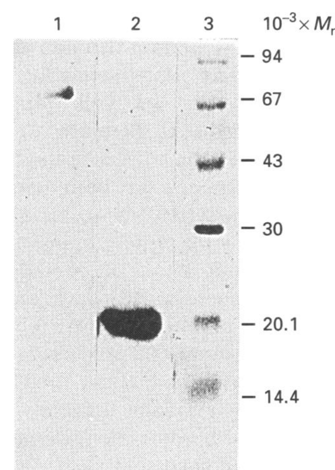

Figure 2 Assessment of purity of BBP-I and BBP-II by Coomassie Brilliant Blue R-250 staining of SDS/10% polyacrylamide gel

Lane 1, 10 μg of BBP-I; lane 2, 40 μg of BBP-II; lane 3, M_r markers: phosphorylase *b* (94000), BSA (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100) and α-lactalbumin (14400).

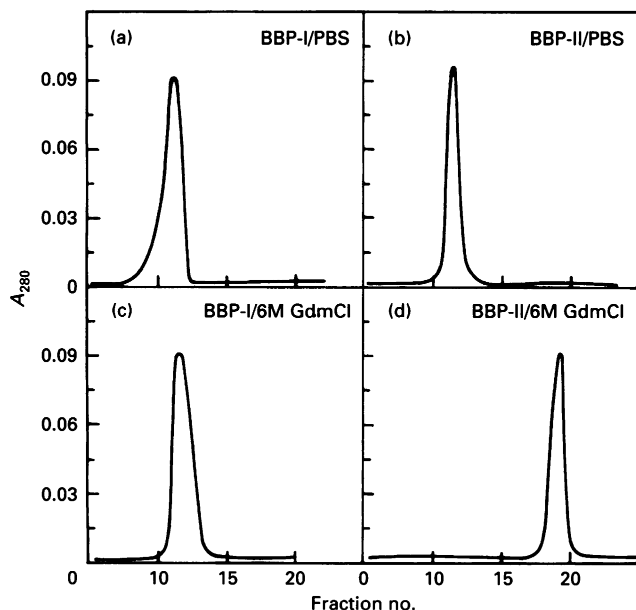

Figure 3 FPLC profile of BBPs

BBP-I (**a**, **c**) and BBP-II (**b**, **d**) (both 100 μg) were subjected to Superose 12 analytical gel filtration at a flow rate of 0.5 ml/min with PBS (**a** and **b**) and PBS/6 M GdmCl (**c** and **d**) as the eluting buffer. Fractions of volume 1 ml were collected.

an M_r of 68000 for protein eluted by 1 M GdmCl (i.e. monomeric BBP-I) and a subunit M_r of 19000 for protein eluted by 6 M GdmCl, i.e. tetrameric BBP-II [6,10]) were recognized on staining with the dye.

Further evidence for the difference in the molecular characteristics of the two biotin binders stems from gel-filtration column chromatography of the proteins in the 1 M and 6 M GdmCl eluates on Superose 12 with or without GdmCl (Figure 3). The 1 M GdmCl-eluted protein, BBP-I, emerged at the position corresponding to an M_r of 70000 whether or not 6 M GdmCl was used for the elution. In contrast, the 6 M GdmCl-eluted protein BBP-II, exhibited elution characteristics of a protein of M_r 70000 when PBS alone was used as the eluting buffer but under denaturing conditions (i.e. with 6 M GdmCl), there was a shift to a position corresponding to M_r 19000. The finding that both the proteins specifically bind to immobilized biotin but their size characteristics markedly differ under denaturing conditions (Figures 3c and 3d) lend credence to the premise that the protein eluted by 1 M GdmCl is indeed BBP-I.

Additional evidence for the identities of the isolated yolk proteins BBP-I and -II comes from a comparison of their physicochemical characteristics and immunological relationships [7]. For example, it was reported previously that BBP-I can bind biotin even at 65 °C whereas BBP-II is unstable at this temperature, although at 45 °C, its ligand-binding ability is appreciable. Therefore the ligand-binding characteristics of both BBP-I and BBP-II using [^{3}H]biotin for ligand saturation was investigated. BBP-II bound biotin only at 45 °C but not at 65 °C (Figure 4). In contrast, BBP-I interacted with biotin significantly when incubated with the ligand at 65 °C. The relative mobilities of these radiolabelled samples were similar to the corresponding species in a dye-stained gel.

Despite these subtle differences in thermal sensitivity in terms of interaction with biotin, the two biotin binders appear to be immunologically related. This was illustrated by Western-blot

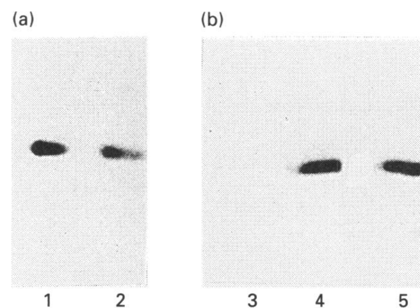

Figure 4 Effect of temperature on binding of [^{3}H]biotin to BBP-I and BBP-II

(**a**) BBP-I (lane 1) and BBP-II (lane 2) were run along with radiolabelled samples, and this portion of the gel was stained with Coomassie Brilliant Blue R-250. (**b**) Fluorogram of a native gel containing BBP-II incubated with [^{3}H]biotin at 65 °C (lane 3), BBP-II incubated with [^{3}H]biotin at 45 °C (lane 4) and BBP-I incubated with [^{3}H]biotin at 65 °C (lane 5).

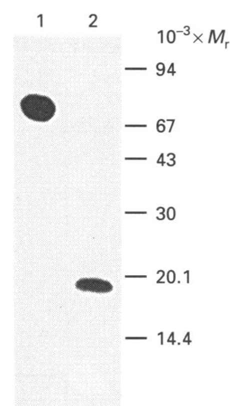

Figure 5 Immunological similarity between BBP-I and BBP-II

The proteins BBP-I (lane 1) and BBP-II (lane 2) were resolved by SDS/PAGE (12.5% gel), transferred to a nitrocellulose filter, probed with rabbit anti-BBP-II polyclonal antibody (1:200 dilution) followed by ^{125}I-labelled Protein A (approx. 1×10^6 c.p.m./ml) and an autoradiogram was developed. Migration of M_r markers is also indicated.

analysis in which the proteins were resolved under denaturing conditions and the blots probed with rabbit anti-BBP-II polyclonal antibody. The results in Figure 5 reveal that BBP-II antiserum could unequivocally bind BBP-I in addition to BBP-II.

In order to confirm the proposed precursor–product relationship between the two biotin binders [7,8], limited proteolytic digestion of BBP-I was performed. Controlled tryptic digests of BBP-I at different time points were resolved by SDS/PAGE. Figure 6 shows a Coomassie Blue-stained gel illustrating the pattern of cleavage of BBP-I as a function of time. With time, a smaller protein species with an M_r corresponding to that of BBP-II subunit appeared along with intermediate species of apparent M_r 34000 and 51000, i.e. multiples of the M_r of BBP-II subunit.

To ascertain that the protein species corresponding to the M_r of BBP-II subunit obtained from tryptic digestion of BBP-I is in fact capable of assuming a quarternary structure similar to the native BBP-II tetramer, the digestion products obtained from trypsinolysis of BBP-I were subjected to preparative Superose 12 gel filtration in the presence of 6 M GdmCl to disrupt non-

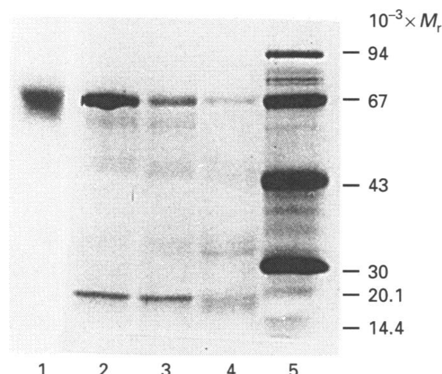

Figure 6 Conversion of BBP-I into BBP-II by limited proteolysis

To BBP-I was added TosPheCHCl$_2$-treated trypsin at a molar ratio of 1:50. Aliquots (30 μl) of the reaction mixture were removed at 0 min (lane 1), 10 min (lane 2), 30 min (lane 3) and 360 min (lane 4) and mixed with an equal volume of SDS/PAGE sample buffer, boiled and subjected to SDS/PAGE (10% gel). Lane 5 contains M_r markers. The gel was stained with Coomassie Brilliant Blue R-250.

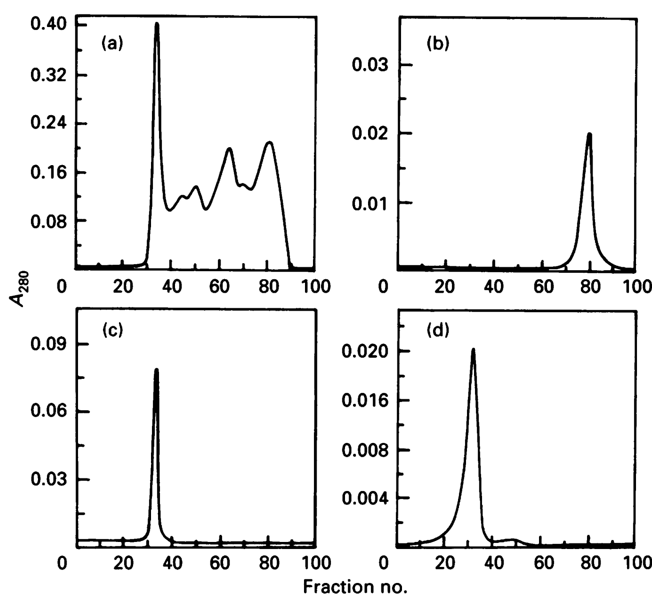

Figure 7 Isolation of monomeric BBP-II and reconstitution to tetrameric BBP-II

(**a**) FPLC gel-filtration pattern of tryptic digest of BBP-I. A volume of 180 μl containing 140 μg of protein at the end of 6 h of protease digestion of BBP-I was injected on to a Superose 12 preparative gel-filtration column using PBS/6 M GdmCl for elution. Fractions 80–90 were pooled. (**b**) Pooled fractions from (**a**) were rechromatographed under identical conditions. (**c**) Authentic BBP-II (100 μg) was subjected to gel-filtration chromatography using PBS as the elution buffer. (**d**) The pooled fractions from (**a**) were dialysed against PBS and the dialysate was chromatographed using PBS for elution under identical conditions. Gel-filtration M_r marker proteins (see the Materials and methods section) were used for calibration.

covalent interactions (Figure 7a). Separation of the protein moiety corresponding to an M_r of 19000 could be achieved under denaturing conditions without appreciable contamination with other M_r species (Figure 7b). On extensive dialysis against PBS, this preparation of monomeric BBP-II obtained under denaturing conditions oligomerized to tetrameric BBP-II (Figure 7d) corresponding to the approximate M_r (70000) of native BBP-II

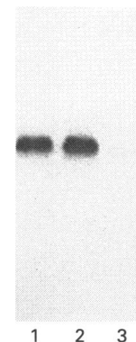

Figure 8 Assessment of biotin binding to reconstituted BBP-II

Fluorogram of a native 7.5% polyacrylamide gel containing authentic BBP-II incubated with [^{3}H]biotin at 45 °C (lane 1), reconstituted BBP-II incubated with [^{3}H]biotin at 45 °C (lane 2) and reconstituted BBP-II incubated with [^{3}H]biotin at 65 °C (lane 3).

(Figure 7c), as shown by gel-filtration chromatography. Moreover, the reconstituted BBP-II was similar to the native BBP-II in terms of thermal sensitivity of ligand binding, as it could bind biotin at 45 °C but not at 65 °C (Figure 8).

The ability of the purified larger biotin binder (M_r 68000) from the 1 M GdmCl eluate to bind biotin at 65 °C, its estimated molecular size and immunological similarity to BBP-II, and its proteolytic cleavage to the subunit form of BBP-II all clearly demonstrate that the stable monomeric form of yolk biotin binder (BBP-I) has been isolated using the purification procedure described above. However, the chemical basis for the differential elution of BBP-I and BBP-II with different concentrations of the chaotrope is not currently known. One possibility is that BBP-I has lower affinity for the immobilized ligand possibly because of steric factors dictated by the covalent nature of the connecting links between four tandemly repeating biotin-binding domains along the protein chain. This is unlike the situation in BBP-II and other biotin binders which have a non-covalent quarternary structure with four dissociable subunits each capable of binding one molecule of the vitamin. It is pertinent to mention at this juncture that the two other tetrameric biotin binders, i.e. avidin and streptavidin, exist in the form of subunits that assume quarternary structure subsequent to biosynthesis [17,18]. It is clear from the data presented above and elsewhere [7–9] that BBP-II differs in this respect as it arises from limited proteolysis of its multidomain precursor BBP-I.

The tetrameric BBP-II species with all the subunits held together purely by non-covalent interactions is more abundant in yolk than in plasma, in contrast with BBP-I, and is more efficiently deposited in the yolk [5]. The predominant occurrence of BBP-II in yolk could be explained by the fact that biotin can be removed more readily from BBP-II at 42 °C (chicken body temperature) and therefore a continuous supply of vitamin for the developing embryo can be easily achieved. It is likely that BBP-I, which is less abundant in the yolk, acts as a store for biotin for use at later stages of embryonic development. Further studies along these lines should help us to understand the significance of two protein-bound forms of the vitamin in avian embryonic nutrition.

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