Volume 305, number 3, 189–191 FEBS 11214 July 1992

Isolation of a new ligand-carrying casein fragment from bovine mammary gland microsomes

Ben Neuteboom, Maria Gabriella Giuffrida and Amedeo Conti

Centro Studio Alimentazione Animali, CNR, Torino, Italy

Received 22 April 1992

Whilst looking for components involved in retinol metabolism in secreting mammary gland cells, a 12 kDa protein was isolated. This protein had bound a ligand with characteristics of retinol. N-Terminal sequencing and amino acid analysis showed that this protein is highly homologous with an α-s1-casein fragment. No ligand was found for β-lactoglobulin, previously thought to be involved in retinol metabolism.

Mammary gland; β-Lactoglobulin; Casein; Retinol transport

1. INTRODUCTION

It has been known for a long time that serum retinol binding protein transports retinol from the liver to all parts of the body, including the mammary glands, and it has been suggested that β-lg is involved in these processes [1]. For a long time β-lg was thought to be exclusively present in ruminants' milk, but recently it has also been characterized in non-ruminant species, e.g. pig and kangaroo [2,3] and very recently it has been found in milk from primates [4]. β-lg binds a number of hydrophobic molecules, but has by far the highest affinity for retinol [5]; moreover, the structural similarity with serum retinol binding protein is striking [6] and therefore, retinol is thought to be a possible natural ligand. In milk, however, β-lg does not have bound retinol, but a variety of lipids, mainly as free fatty acids [7].

In order to determine a possible involvement of a protein carrier in retinol metabolism and/or secretion, secreting bovine mammary gland cells were fractionated and the protein contents of cytosol and microsomes were analysed for the presence of retinol.

2. MATERIALS AND METHODS

Bovine mammary glands were first cut into small pieces, then minced with an Ultra-turrax and washed thoroughly in 0.25 M sucrose to remove residual milk. Subsequently, the sample was homogenized in 0.25 M sucrose using a Potter-Elvehjem homogenizer with a tight-fitting pestle. The homogenate was centrifuged for 20 min at 10,000 × *g* to yield the post-mitochondrial supernatant. This supernatant was centrifuged at 100,000 × *g* for 120 min, to yield the microsomal pellet and the cytosolic supernatant. Pellets were resuspended in 0.25 M sucrose and spun down.

Washed pellets were resuspended in TKM-buffer (50 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, pH 7.6) and incubated with 0.1% sodium deoxycholate (Fluka, Buchs, Switzerland) at 20°C for 60 min. Insoluble material was spun down, and supernatants were lyophilized.

The lyophilized material was dissolved in 0.1% ammonium acetate, pH 6, and applied to a 21.5 × 600 mm TSK G2000 SWG HPLC-gel-filtration column (LKB, Bromma, Sweden). Fractions were pooled into 4 pools and lyophilized.

Pools from the gel-filtration were fractionated by reversed-phase HPLC on a Micropak Protein C18 column (4.0 × 300 mm; Varian, Palo Alto, CA, USA).

Polyacrylamide gel electrophoresis in the presence of SDS was performed as described [8]; the concentration of the gel was 12.5%. Analytical isoelectric focusing was performed on ready-to-use LKB PAG-plates, pH 4.0–6.5.

N-Terminal sequence analysis was carried out in an Applied Biosystems Model 470A Protein Sequencer. Amino acid analysis was performed on a Hewlett Packard 1090 Aminoquant analyzer after HCl gas-phase hydrolysis at 165°C for 2 h.

The in vitro retinol–β-lg complex was formed by adding 20-fold excess retinol dissolved in 66% ethanol to a 0.1 mM solution of β-lg, likewise dissolved in 66% ethanol. The reaction mixture was analyzed by HPLC chromatography, both reversed-phase (Micropak Protein C18) and gel-filtration (TSK G3000, SW, LKB), monitoring the effluent continuously between 220 and 370 nm.

3. RESULTS AND DISCUSSION

Samples from two mammary glands were analyzed. On fractionation of cytosolic proteins, measuring continuously the absorption spectrum between 220 and 370 nm, no absorption specific for retinol was found (330–350 nm).

Fig. 1 shows the fractionation by gel-filtration of peripheral microsomal proteins from bovine mammary gland, extracted from the microsomes in 0.1% sodium deoxycholate. Reversed-phase HPLC of pool 3 from the gel-filtration resulted in the isolation of pure β-lg (Fig. 2A, peak 2). No differences were found with the 18 kDa

Abbreviations: β-lg, β-lactoglobulin; TFA, trifluoroacetic acid; SDS, sodium dodecylsulfate.

Correspondence address: A. Conti, CNR, via P. Giuria 7, 10125 Torino, Italy.

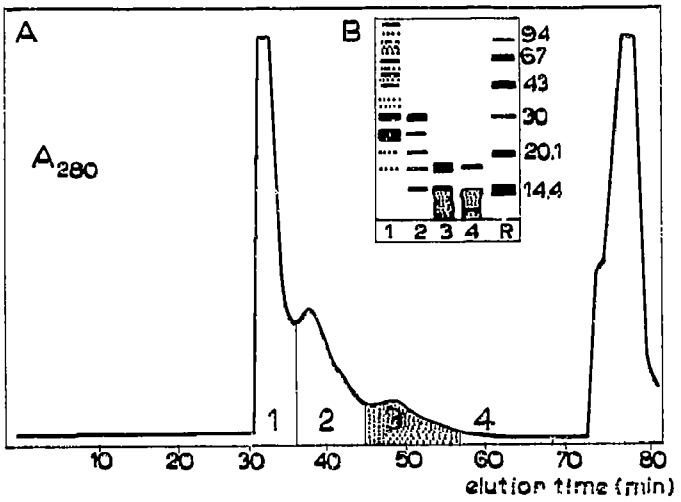

Fig. 1. (A) Chromatography on TSK G2000 SWG of peripheral microsomal proteins from bovine mammary gland. Elution was performed with 0.1% ammonium acetate, pH 6.0; flow rate 2.5 ml/min. The eluent was monitored at 280 nm. Fractions were pooled into four pools as indicated. (B) Schematic representation of SDS-PAGE of the four pools from Fig. 1A. Reference proteins: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa).

β-lg from milk. Moreover, no absorption at 350 nm was observed for this peak, indicating that no retinol was bound.

In order to exclude the possibility that the retinol–β-lg complex is not resistent to the chromatographic conditions used for fractionation of microsomal proteins, the in vitro complex was subjected to the same conditions. In contrast to the experiments described by Fugate and Song [9], the complex was only obtained in partly organic solutions, probably because of poor solubility of retinol in aqueous solutions. Once formed, the complex, easily recognized by its spectrum [9,10], could be separated from its components by reversed-phase HPLC, indicating that it is resistant to these conditions (Fig. 3). It can be concluded that β-lg is not involved in the binding and transport of retinol in mammary gland cells.

Absorption at 350 nm was observed for another protein from fractionation of microsomal proteins, present in very small quantities, eluting much earlier than β-lg

Table I

N-Terminal sequence of the ligand-carrying protein

	1	17
lc	F-V-A-P-F-P-E-V-F-G-K-E-K-V-N-E-L	
	24	40
hs	F-V-A-P-F-P-Q-V-F-G-K-E-K-V-N-E-L	

lc = ligand-carrying protein; hs = human α-s1-casein. On alignment with α-s1-casein sequence 24–40, one difference is found (pos. 7 Glu → Gln).

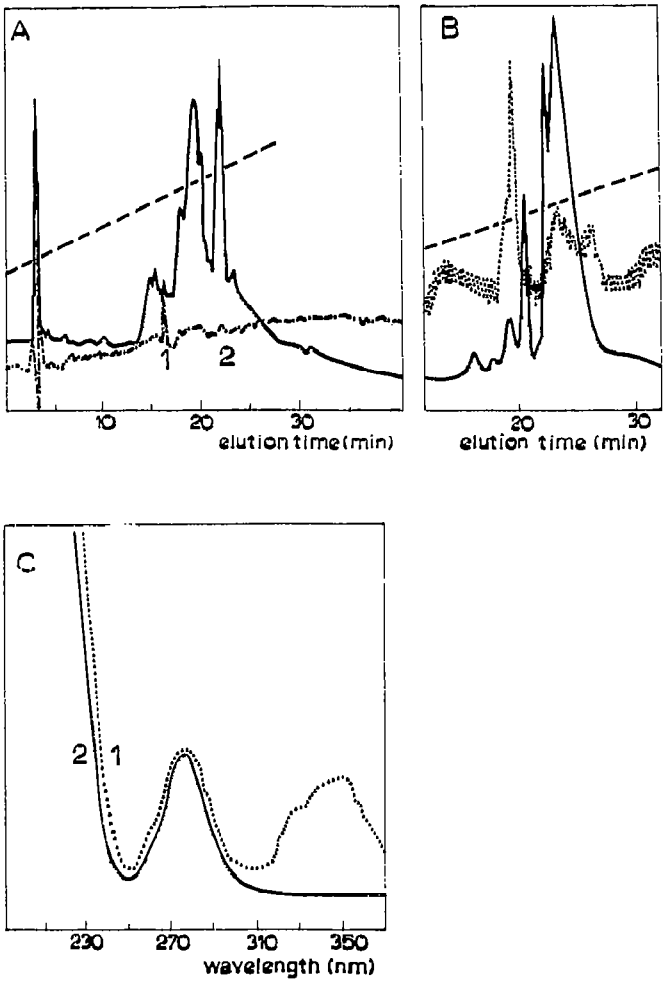

Fig. 2. (A) Chromatography on reversed-phase HPLC of the β-lactoglobulin-containing pool 3 (Fig. 1A). Eluent: 0.1% TFA in a gradient of 20–75% acetonitrile in 40 min. Flow rate 1 ml/min. (—) $A_{280\ nm}$; (–.–) A_{350nm}. (B) Re-purification of peak 1 from Fig. 2A with 0.1% ammonium acetate in a gradient of 20–75% acetonitrile in 40 min. Flow rate 1 ml/min. (—) A_{220nm}; (.....) A_{350nm}. (C) Absorption spectra of peaks 1 and 2 from Fig. 2A.

(Fig. 2A, peak 1). This protein was further purified (Fig. 2B). The absorption at 350 nm was gradually lost during subsequent purifications, indicating that a loosely bound ligand caused this absorption. The spectrum showed retinol–protein characteristics (Fig. 2C) and SDS-PAGE revealed a band at 12 kDa. N-Terminal sequencing showed that this protein is almost identical with an internal part of α-s1-casein [11] (Table I). Amino acid analysis showed a very low tyrosine content, in contrast to α-s1-casein, which has 8 tyrosine residues concentrated in an internal strech of 30 residues. This stretch is clearly absent in the 12 kDa protein. The best fit was found with α-s1-casein sequences 24–101 + 174–199 (data not shown). The apparent protein–retinol complex is much less hydrophobic than the β-lg–retinol complex; this suggests that in the new 12 kDa protein, retinol is bound in a central cavity, where

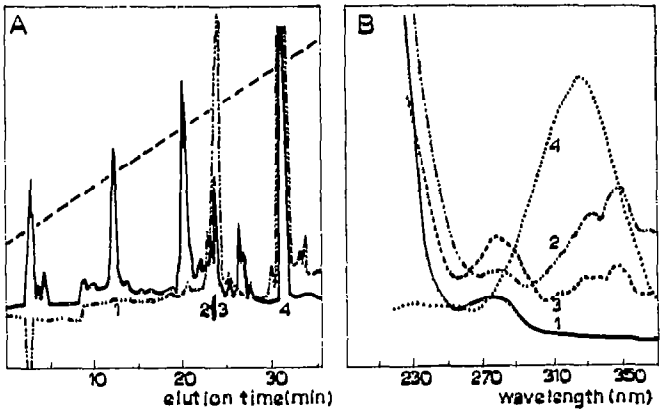

Fig. 3. (A) Fractionation of the in vitro retinol–β-lactoglobulin complex by RP-HPLC in 0.1% TFA in a gradient 20–100% acetonitrile over 40 min. Flow rate 1 ml/min. (—) A_{280nm} A; (-·-·-) A_{350nm}. (1) β-lactoglobulin; (2) and (3) protein–retinol complexes; (4) retinol. (B) Absorption spectra of peaks 1, 2, 3, 4 from Fig. 3A.

it does not much effect the hydrophobicity. The role of this protein in transport of retinol in secreting mammary gland cells remains to be determined as well as the right biological role of the various casein components.

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