A novel whey protein synthesized only in late lactation by the mammary gland from the tammar (*Macropus eugenii*)

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A major whey protein which appears in milk from the tammar wallaby (*Macropus eugenii*) only during the second half of lactation (late lactation protein-A, LLP-A) was purified to apparent homogeneity by ion-exchange chromatography and gel filtration. An M_r of $21\,600 \pm 2000$ was calculated from its amino acid composition. A computer-based comparison of the sequence of the first 69 amino acid residues with the Atlas of Protein Sequence data base showed no significant homology with known proteins. Antiserum to LLP-A was prepared in rabbits, and single radial immunodiffusion was used to measure the amounts of LLP-A in milk during the first 40 weeks of lactation. LLP-A was first detected at 26 weeks; thereafter its concentration increased abruptly, to reach a maximum of 26 g/l at approx. 36 weeks of lactation. Explants prepared from mammary gland biopsies at 20 and 35 weeks of lactation were exposed to [3 H]amino acids for 8 h; immunoprecipitation of tissue extracts showed that, whereas the rate of casein synthesis was the same at both stages of lactation, LLP-A was synthesized only by the 35-week mammary gland.

INTRODUCTION

It is now well established that, in several species of marsupial, marked changes take place during the course of lactation in the composition and production of the milk (Gross & Bolliger, 1959; Jenness *et al.*, 1964; Lemon & Barker, 1967; Griffiths *et al.*, 1972; Messer & Green, 1979; Green *et al.*, 1980, 1983; Green, 1984). One of these changes, which has been observed electrophoretically in red (*Macropus rufus*) and eastern grey (*Macropus giganteus*) kangaroos, in the tammar wallaby (*Macropus eugenii*) and in the quokka (*Setonix brachyurus*), is the appearance of specific whey proteins at late stages of lactation (Bailey & Lemon, 1966; Lemon & Bailey, 1966; Jordan & Morgan, 1968; Lemon & Poole, 1969; Bell *et al.*, 1980; Green & Renfree, 1982). Very little is known about these proteins, although two of them have been isolated from the milk of red and grey kangaroos and their M_r values and amino acid compositions determined (McKenzie *et al.*, 1983).

Studies in our laboratories are aimed at elucidating the mechanism by which marsupials change from synthesizing 'early' to 'late' lactation milk. In the tammar wallaby the major changes occur at 26–30 weeks *post partum* and accompany exit from the pouch and accelerated physiological development of the young (Bentley & Shield, 1962; Murphy & Smith, 1970; Setchell, 1974; Call *et al.*, 1980; Paton & Janssens, 1981; Wilkes & Janssens, 1986). Should a second young be born after the first leaves the pouch, it will suckle from an adjacent mammary gland and receive 'early' lactation milk, while the older animal at heel receives 'late' lactation milk (Messer & Green, 1979). The question arises of whether this transformation is directed by changes in circulating concentrations of milk-stimulating

factors or changes in the way the epithelium responds to these factors. Alternatively, the tissue may respond to an intrinsic factor which is synthesized and acts locally in the mammary gland. The isolation and characterization of appropriate specific markers is of value to study this question. We report here on the isolation and partial characterization of a whey protein obtained from late-lactation milk of the tammar (late lactation protein-A, LLP-A) and show that its synthesis is induced in the mammary gland only from animals which have lactated for at least 26 weeks.

MATERIALS AND METHODS

Materials

Sheep prolactin (NIH-P-16) was provided by the Hormone Distribution Program, NIADDK, NIH, Bethesda, MD, U.S.A. Pig insulin (154-YB-9) was a gift from Eli Lilly Co., Indianapolis, IN, U.S.A. Cortisol and phenylmethanesulphonyl fluoride were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Medium 199 containing Hanks' salts was from Grand Island Biological Co., Grand Island, NY, U.S.A. DEAE-Sephacel was from Pharmacia, Uppsala, Sweden, and Ultrogel AcA 44 from LKB, Bromma, Sweden. All reagents for SDS/polyacrylamide-gel electrophoresis were from Bio-Rad, Richmond, CA, U.S.A. Freund's complete adjuvant was purchased from CSL, Crows Nest, N.S.W., Australia, and oxytocin (Syntocinon) from Australian Pharmaceutical Industries, Alexandria, N.S.W., Australia. Sodium methohexitone (Brietal) was from Eli Lilly Co., Sydney, N.S.W., Australia. [3 H]Amino acid mixture was from Amersham International, Amersham, Bucks., U.K.

Abbreviations used: LLP-A, late lactation protein-A.

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Animals

Tammar were obtained from a breeding colony at the Division of Wildlife and Rangelands Research, CSIRO, Canberra. Animals were kept in open grassy yards with lucerne, hay, oats and water available *ad libitum*. The stage of lactation was determined from known birth dates or by measurement of the head length of pouch young.

Milk collection

Samples for determination of the concentration of LLP-A were collected from 50 different wallabies during 1981 and 1982 (Green *et al.*, 1980). Milk for protein purification was collected from tammar at 30–40 weeks of lactation.

Purification of LLP-A

Milk samples were thawed, pooled and centrifuged at 3000 *g* for 15 min at 4 °C. The skim milk was centrifuged at 50000 *g* for 1 h at 4 °C. The pellet (casein) was discarded. The supernatant was dialysed overnight against several changes of distilled water and freeze-dried. A fraction of the whey proteins (about 200 mg) was dissolved in 8 ml of 0.020 M-Tris/0.017 M-HCl, pH 7.4 (at 20 °C)/0.01 M-NaCl and chromatographed on a column (1.5 cm × 17.0 cm) of DEAE-Sephacel equilibrated with the same buffer. Protein was eluted with a linear gradient of 280 ml each of 0.02 M-Tris/HCl, pH 7.4, containing 0.01 M- and 0.125 M-NaCl. The flow rate was 20 ml/h, and 3 ml fractions were collected. The protein and α -lactalbumin contents of fractions were monitored at 280 nm and by the lactose synthetase reaction (Fitzgerald *et al.*, 1970), respectively. The protein corresponding to LLP-A was dialysed overnight at 4 °C against distilled water, freeze-dried and then gel-filtered on a column (1.5 cm × 120.0 cm) of Ultrogel AcA 44 or Sephadex G-50 (superfine). The column was equilibrated with 0.020 M-Tris/0.017 M-HCl, pH 7.4 (at 20 °C), and 2.6 ml fractions collected at 15 ml/h. The LLP-A was dialysed overnight at 4 °C against distilled water and freeze-dried.

Amino acid analysis and sequencing of LLP-A

The methods used were essentially the same as described previously (Kaminogawa *et al.*, 1984; McKenzie & Shaw, 1985).

Preparation of antiserum and measurement of LLP-A in milk

Two rabbits were each injected intradermally, at multiple sites along the back, with freeze-dried protein (1 mg) dissolved in a mixture of 0.9% NaCl (0.5 ml) and complete Freund's adjuvant (0.5 ml). Subsequent injections were intramuscular, with protein (0.5 mg) in a mixture of 0.9% NaCl (0.5 ml) and incomplete Freund's adjuvant (0.5 ml). These injections were done at intervals of 2–3 weeks until satisfactory antiserum (tested by Ouchterlony double immunodiffusion) was obtained (10–12 weeks after the first injection). The specificity of the antiserum was established by double immunodiffusion by using a range of dilutions of purified late lactation protein and of tammar wallaby milk. The concentration of the protein in milk was measured by single radial immunodiffusion (Mancini *et al.*, 1965).

Fig. 1. SDS/polyacrylamide-gel electrophoresis of tammar LLP-A and total whey protein from early and late lactation

Whey protein obtained from tammar at 35 weeks (lane 1) and 17 weeks (lane 2) of lactation and purified LLP-A (lane 3) were electrophoresed on a SDS/polyacrylamide (20%) gel by the method of Laemmli (1970). Major protein bands (A and B) found only in late lactation are arrowed.

Protein synthesis in mammary-gland explants

Mammary-gland tissue was biopsied aseptically from anaesthetized lactating tammar (20 and 35 weeks). Explants were prepared and incubated in 35 mm-diam. culture dishes containing medium 199 as described previously (Nicholas & Tyndale-Biscoe, 1985). Mammary explants from biopsy tissue were incubated for 8 h in 1.5 ml of medium with [³H]amino acid mixture (30 μ Ci/ml). They were homogenized (5%, w/v) in phosphate-buffered saline containing 1% (w/v) Triton X-100 and phenylmethanesulphonyl fluoride (25 μ g/ml), and centrifuged at 100000 *g* for 1 h at 4 °C. The supernatant was used as a tissue extract for the specific immunoprecipitation of LLP-A and casein. Samples of extract were incubated overnight with rabbit antisera directed against either tammar LLP-A or casein, and then 10 μ g of each protein was added as a carrier. Normal rabbit serum was added to control tubes.

Fig. 2. Purification of LLP-A from late-lactation whey proteins

(a) Whey protein (200 mg) was applied to a column (1.5 cm $\times$ 17 cm) of DEAE-Sephacel equilibrated with 0.02 M-Tris/HCl (pH 7.4)/0.01 M-NaCl. Protein (●) was eluted with a linear gradient of 280 ml of each of 0.02 M-Tris/HCl (pH 7.4), containing 0.01 M- and 0.125 M-NaCl (-----). Fractions (3 ml) were collected at 25 ml/h. α -lactalbumin (○) was detected as lactose synthetase activity in peak 5. Fractions from peak 4 were pooled (as indicated by the bar). (b) Peak 4 was applied to a column (1.5 cm $\times$ 120 cm) of Ultrogel AcA 44 in 0.02 M-Tris/HCl, pH 7.4. Protein (●) was determined in 2.6 ml fractions collected at 15 ml/h. Fractions constituting the LLP-A peak (as indicated by the bar) were pooled.

Samples were incubated for an additional 6 h and the immunoprecipitates washed three times with phosphate-buffered saline/1% Triton X-100. Either the precipitates were dissolved in 0.2 ml of Protosol and their radioactivity was determined in 5 ml of Econofluor, or they were dissolved in 10 mM-sodium phosphate, pH 7.0, containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol and subjected to SDS/polyacrylamide (15%)-gel electrophoresis (Weber & Osborn, 1969). These gels were sliced (1.1 mm), dissolved in 1 ml of 30% H_2O_2 , and their radioactivity was determined in 5 ml of Picofluor-30.

Total protein synthesis was measured in samples of tissue extract as described previously (Ono & Oka, 1980).

RESULTS

Electrophoresis of whey protein from milk collected early (17 weeks) and late (35 weeks) in lactation revealed several differences in their protein content (Fig. 1). Two major proteins (arrowed A and B) were present only in

the late-lactation milk. The faint protein band from early-lactation whey with a mobility slightly greater than band A (Fig. 1) was seen in all samples examined.

Chromatography of whey protein from late tammar milk on DEAE-Sephacel resolved the proteins into five major peaks (Fig. 2a). Peak 5 demonstrated α -lactalbumin activity in the lactose synthetase reaction. Peak 4, which was not present in whey from milk collected before 24 weeks of lactation, was gel-filtered (Fig. 2b) on Ultrogel AcA 44, to yield a single major protein peak. Electrophoresis (Fig. 1) showed a homogeneous protein with the same mobility as band A in late-lactation milk. This protein has been named late lactation protein-A (LLP-A).

The amino acid composition of LLP-A, calculated on the assumption that it has a single methionine residue, was as follows; Asx₂₄, Thr₁₆, Ser₈, Glx₃₀, Pro₁₀₋₁₁, Gly₅₋₆, Ala₈, $\frac{1}{2}$ -Cys₃₋₄, Val₆, Met₁, Ile₁₂₋₁₃, Leu₁₀, Tyr₅₋₆, Phe₁₁, His₄, Lys₁₂, Arg₁₁₋₁₂, Trp₁. The minimum M_r from this would be 21600. However, the integrated value for

Asp-Asp-Phe-Ala-Phe-Ser-Glu-Phe-Lys-Pro-Ser-Glu-Gly-Thr-Tyr-Tyr-
 Val-Gln-Val-Ile-Ala-Val-Asp-Lys-Glu-Phe-Pro-Glu-Asp-Glu-Ile-Pro-
 Arg-Asp-Ile-Ser-Pro-Leu-Thr-Ile-Ser-Tyr-Leu-Asn-Asn-Gly-Lys-
 Met-Glu-Ala-Lys-Phe-Thr-Val-Lys-Lys-Asp-Asn-Asn-Cys-Glu-Glu-Ile-
 Asn-Leu-Thr-Leu-Glu-Lys-Xaa-----Ile-Leu-Ser-Cys-Val-Arg-
 Xaa-Xaa-Gln-Gly-Ile-Gln-Ile-Arg-Glu-Ala-----

Fig. 3. Partial amino acid sequence of tammar LLP-A

Xaa indicates that the residue has not been identified. The position of the smaller 16 residue sequence is not known.

Fig. 4. Progressive changes in concentration of LLP-A in tammar milk during lactation

Milk samples were collected from tammars under Brietal anaesthesia by removal of pouch young (0.5–3.0 h) and administration of 1.0 i.u. of syntocin. The concentration of LLP-A was determined by single radial immunodiffusion (Mancini *et al.*, 1965). Each value represents mean $\pm$ S.E.M., with the number of animals in parentheses.

methionine can be affected by partial oxidation and by its position on the chromatogram (methionine eluted after a buffer baseline change), resulting in an error of $\pm 10\%$.

Automated amino acid sequencing of 10 nmol of the native protein gave the sequence of the *N*-terminal 54 residues (Fig. 3), which includes a residue of methionine located at position 48. When a sample was cleaved with (CNBr and the mixture of fragments subjected to sequencing for five cycles we found an extra sequence, Glu-Ala-Lys-Phe-Thr, which corresponded to residues 49–53 and which therefore resulted from cleavage after the single methionine residue. Partial resolution of the fragments was achieved by their relative solubility in pyridine acetate buffer, pH 3.1. Sequencing of the less-soluble fraction allowed extension of the sequence to residue 69. A similar experiment using BNPS-skatole for cleavage of the protein after typtophan showed that there

was also only a single residue of tryptophan, and the sequence of a further 16 residues, which did not overlap with the first 69 residues, was established with little ambiguity. A computer-based comparison of overlapping 20 residues of the 69-residue sequence, with the Atlas of Protein Sequence data base (National Biomedical Research Foundation, Washington, DC, U.S.A.), did not show any significant homology with known proteins.

Antiserum prepared against LLP-A was used to measure the concentration of this protein in milk samples collected throughout lactation (Fig. 4). LLP-A was absent from milk before 24 weeks of lactation, but after this time its concentration increased rapidly, reaching a maximum of 26 g/l at 36 weeks *post partum*.

To establish that LLP-A is synthesized by the mammary gland, explants were prepared from mammary tissue obtained by biopsy at either 20 or 35 weeks of lactation. The explants were cultured for 8 h in media containing a mixture of [3 H]amino acids, and a tissue extract was then immunoprecipitated with antisera directed against either tammar LLP-A or casein. Fig. 5 shows the radioactivity profile of LLP-A immunoprecipitates that had been subjected to SDS/polyacrylamide (15%) gel electrophoresis and sliced.

The peak of [3 H]protein obtained from immunoprecipitation of tissue extract with anti-LLP-A was obtained only in tissue from the tammar at 35 weeks of lactation. In contrast with LLP-A, the casein synthesis was the same in tissue from animals at 20 weeks and 36 weeks of lactation. Furthermore, the incorporation of [3 H]amino acids into trichloroacetic acid-precipitable protein was the same in explants from early (45212 ± 2778 d.p.m./mg of tissue; mean $\pm$ S.E.M., $n = 3$) and late (47363 ± 2734 d.p.m./mg of tissue) lactation. The secretion of [3 H]protein into the medium was not determined.

DISCUSSION

Marsupials differ markedly from most eutherians by their capacity to change the composition and production of milk during lactation (Green, 1984). In order to study the mechanism of control of this process, we are attempting to purify various milk-specific proteins, which change in amount during the cross-over from early

Fig. 5. Synthesis of LLP-A and casein by mammary explants from tammars in early and late lactation

Mammary tissue was obtained by biopsy from anaesthetized animals at 20 and 35 weeks of lactation. Mammary explants were cultured for 8 h in medium 199 containing [³H]amino acid mixture (30 μ Ci/ml). Tissue extracts were immunoprecipitated with antiserum to LLP-A and casein. The LLP-A immunoprecipitate was electrophoresed on SDS/polyacrylamide (15%) gels (Weber & Osborn, 1969), sliced (1.1 mm), and radioactivity was determined. The profile for LLP-A synthesized in early (○) and late (●) lactation is shown. The insert shows the rate of casein synthesis in the same mammary tissue samples (mean $\pm$ S.E.M., $n = 3$).

to late lactation milk. In this paper we report on a whey protein (LLP-A) which is not present in the milk before 26 weeks of lactation, but which then appears at concentrations that are similar to those observed for casein (Green & Renfree, 1982). In contrast with casein, which is synthesized by the mammary gland throughout lactation, LLP-A was synthesized in mammary-gland biopsy samples only at 35 weeks of lactation and not at 20 weeks.

Green *et al.* (1980) have shown that towards the end of lactation there is an increase in the total protein concentration of tammar wallaby milk, amounting to about 44 g/l, between 24 and 40 weeks *post partum*. Most if not all of this increase is due to the whey proteins, since these increased in concentration from a mean of 32.5 to 75.5 g/l over this period (Green & Renfree, 1982). The present results, which give a value of about 26 g/l for the concentration of LLP-A at 36–40 weeks, therefore suggest that over half of the increase in milk protein concentration that occurs towards the end of lactation may be due to this one protein. Fig. 1 shows that two low- M_r proteins which are present in early lactation are absent in late lactation, but these proteins appear to be minor components in terms of their contribution to the total protein content.

Comparison of the partial sequence of LLP-A with the Atlas of Protein Sequence data base did not reveal homology with the sequence of any known protein. However, comparison of our results with those of McKenzie *et al.* (1983) suggests that the LLP-A of tammar wallaby milk may be homologous with the Zone C proteins of red- and grey-kangaroo milk. Their M_r values and amino acid compositions are similar, and so are the time of first appearance in the milk of LLP-A, at 26 weeks *post partum*, and of the Zone C protein of the red kangaroo, at 28 weeks. In the grey kangaroo, in which the young have a longer pouch life, Lemon & Poole (1969) found that a protein designated p2, which is probably identical with the Zone C protein found by McKenzie *et al.* (1983), first appeared at 33–35 weeks. A function for LLP-A has yet to be determined. The high concentration of this protein in milk suggests that it does not have an enzymic, hormonal or growth-factor role, but rather may fulfil an unknown nutritional requirement. Alternatively, LLP-A may have a role to play in the gut as the young leaves the pouch and changes from an exclusive diet of milk to an herbivorous diet.

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