Heterogeneity of guinea-pig caseins synthesized and sequestered by cell-free protein-synthesizing systems

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1. Individual mRNA species encoding guinea-pig caseins A, B and C, and α -lactalbumin, were purified by hybridization to recombinant milk-protein plasmid DNA immobilized on diazobenzylloxymethyl-paper or diazobenzylloxymethyl-cellulose. Addition of the purified mRNA species to a reticulocyte-lysate cell-free system, in the presence or absence of a dog pancreas microsomal membrane fraction, established a precursor–product relationship between the primary translation products and those sequestered within microsomal vesicles, as determined by polyacrylamide-gel analysis in one and two dimensions. 2. Three sequestered variants of sequestered casein A were identified, but only single forms of sequestered casein B and α -lactalbumin. Sequestered variants of casein C proved to be unexpectedly basic, and did not focus on the pH gradient utilized. 3. Comparative analysis of milk proteins synthesized in the reticulocyte-lysate and wheat-germ cell-free systems by two-dimensional gel electrophoresis demonstrated both quantitative and qualitative differences. In particular, marked but variable heterogeneity was apparent within the primary translation products of casein A and casein B. Pre-casein C did not focus. Limited *N*-terminal processing of the primary translation products was also evident. These observations are discussed in relation to (i) unscheduled post-translational modifications by cell-free protein-synthesizing systems and (ii) multiplicity of signal sequences. 4. Overall we demonstrate that complex precursor–product relationships between primary translation products and their sequestered variants, programmed *in vitro* by a mixed mRNA population, may be readily analysed by using individual mRNA sequences purified by hybridization to immobilized cloned complementary-DNA sequences.

Guinea-pig milk contains four predominant proteins, namely the three caseins A, B and C and α -lactalbumin (Craig *et al.*, 1976). Partial characterization of the caseins shows that all are heavily phosphorylated, and to a lesser extent glycosylated (Craig *et al.*, 1978). Most interest in the biosynthesis of milk proteins from many different species stems from the manner in which intricate combinations of steroid and peptide hormones modulate their expression (see Banerjee, 1976). However, the way in which the mammary gland modifies and secretes these polypeptides also provides a fascinating system with which to study the mechanisms involved in the post-translational modification and secretion of proteins.

Abbreviations used: SDS, sodium dodecyl sulphate; cDNA, complementary DNA; DBM, diazobenzylloxymethyl.

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We have shown that the addition of mammary-gland poly(A)-containing RNA isolated from the lactating guinea pig to a variety of cell-free protein-synthesizing systems results in the synthesis of the primary milk protein translation products (Craig *et al.*, 1976, 1979*b*), and that these are partially processed at the level of the endoplasmic reticulum (Craig *et al.*, 1979*a,b*). This results in a change in mobility of the primary translation products on polyacrylamide gels, in part because of the removal of an *N*-terminal peptide, a co-translational event. However, the resulting peptides even then do not co-migrate with the mature proteins found in milk (Craig *et al.*, 1979*a,b*). We have always presumed that the sequestered caseins represent unmodified caseins A, B, and C in order of decreasing molecular weight. Unfortunately, since it has not been possible to separate by physical means the individual casein-mRNA sequences, it has proved difficult until now to determine with con-

fidence the identity of individual casein variants synthesized and sequestered by mRNA-directed protein-synthesizing systems or *Xenopus* oocytes (Colman & Morser, 1979; Lane *et al.*, 1979, 1980).

Here we separate individual milk-protein mRNA species by hybridization to cloned milk-protein cDNA sequences immobilized on DBM-paper or DBM-cellulose. Analysis by SDS/polyacrylamide-gel electrophoresis in one or two dimensions of the primary and sequestered [³⁵S]methionine-labelled polypeptides synthesized in mRNA-directed cell-free systems establishes precursor-product relationships which could not have been predicted by using a mixed milk-protein mRNA population. In particular, we establish the identity of one highly modified and two other variants of sequestered casein A, but only a single sequestered form of casein B. Furthermore, we show that unscheduled post-translational modifications to pre-caseins A and B occur in the wheat-germ and reticulocyte-lysate cell-free systems. We also provide evidence that both cell-free systems are capable of limited *N*-terminal processing. Overall, our experiments demonstrate that complex polypeptide populations synthesized and sequestered *in vitro* directed by a mixture of mRNA species may be effectively analysed by using mRNA species purified by hybridization to cloned cDNA sequences.

Materials and methods

Materials

Bio-Lyte 3/10 ampholyte was obtained from Bio-Rad Laboratories, Watford, Herts., U.K., and L-[³⁵S]methionine (700–1300 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.; Whatman 540 paper and Whatman CC-41 cellulose powder were obtained from Reeve Angel Scientific, London SE1 6BD, U.K. All other chemicals and solvents were obtained from sources previously described (Craig *et al.*, 1976, 1979a,b, 1981).

Preparation of solutions and glassware

Preparation of buffers, scintillation fluid, and all recrystallization and distillation procedures were as described previously (Craig *et al.*, 1976, 1979b).

Cell-free protein synthesis and product analysis

Cell-free protein synthesis directed by mRNA was carried out either in the wheat-germ cell-free system as described by Craig *et al.* (1976) or in a nuclease-treated reticulocyte lysate as described by Pelham & Jackson (1976). When required, dog pancreas microsomal membranes were prepared as described by Katz *et al.* (1977). SDS/polyacrylamide-gel-electrophoretic analysis of [³⁵S]methionine-labelled cell-free products in one dimension was

performed on slab gels (Craig *et al.*, 1979b) in a Tris/glycine buffer system as described by Maizel (1972). Two-dimensional electrophoresis was as described by O'Farrell (1975). Routinely 10000–20000 c.p.m. were applied to each gel, which was then exposed for 2–4 weeks. Antibody-precipitation procedures were as described previously (Craig *et al.*, 1976), and fluorography followed the procedure of Bonner & Laskey (1974), except that dimethyl sulphoxide was replaced by acetic acid throughout (Burckhardt *et al.*, 1979).

Isolation of individual milk-protein mRNA species

mRNA species representative of caseins A, B and C and α -lactalbumin were separated from either a total post-nuclear RNA population or the post-nuclear poly(A)-containing RNA population isolated from the lactating guinea-pig mammary gland (Craig *et al.*, 1976, 1979a) by using recombinant milk-protein plasmids immobilized on paper or on cellulose (see Alwine *et al.*, 1977).

Plasmid DNA from the characterized recombinants pgp N-33 (casein A), pgp K-27 (casein B), pgp O-22 (casein C) and pgp K-9 (α -lactalbumin), previously transformed into *Escherichia coli* HB101, were cultured under conditions of good microbiological practice, in accordance with the guidelines laid down by the British Genetic Manipulation Advisory Group. Plasmid DNA was isolated from cleared cell lysates by CsCl-density-gradient centrifugation in the presence of ethidium bromide as described previously (Craig *et al.*, 1981). Plasmid DNA was then partially restricted with endonuclease *Hae*III, heat-denatured, and immobilized on either DBM-paper or DBM-cellulose. We have previously described our procedures for binding plasmid DNA to DBM-paper, and the stringent hybridization conditions used to isolate the individual mRNA species (Craig *et al.*, 1981). This procedure was used to isolate small amounts of mRNA (0.2 μ g/filter). Larger quantities of mRNA were isolated in the following manner. Cellulose powder (1 g) (3 g is the equivalent of a 14 cm $\times$ 25 cm area of Whatman 540 paper) was activated as described by Alwine *et al.* (1977). Endonuclease-*Hae*III-partially-restricted heat-denatured plasmid DNA (200 μ g) was then dissolved in 1 ml of 5 mM-potassium phosphate, pH 6.0, containing 80% (v/v) dimethyl sulphoxide. To this was added 100 mg of activated cellulose, and the mixture gently shaken overnight at 20°C. The resultant DNA-cellulose was recovered by centrifugation for 10 s in a Beckman Microfuge B, and then washed according to the procedure of Alwine *et al.* (1977). For each wash, the DNA-cellulose was resuspended in 1 ml of the appropriate solution, then recovered by centrifugation for 10 s in the Microfuge. The DNA-cellulose was then stored at 4°C until required in

hybridization buffer, which consisted of 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.6, containing 0.6 M-NaCl, 1 mM-EDTA, 0.2% (w/v) SDS, poly(A) (50 µg/ml) and 60% (v/v) formamide. For mRNA isolation, 100 mg of DNA-cellulose was suspended in 1 ml of hybridization buffer containing 200 µg of total post-nuclear RNA isolated from the lactating guinea-pig mammary gland, and incubated for 18 h at 25°C. The DNA-cellulose was then sedimented as described above, the supernatant discarded, and the DNA-cellulose washed with 4 × 1 ml of 20 mM-NaCl/8 mM-sodium citrate (pH 7.6)/0.2% (w/v) SDS containing 60% (v/v) formamide at 25°C. Hybridized RNA was then eluted by incubation at 60°C for 2 min in 200 µl of 20 mM-Hepes, pH 7.6, containing 1 mM-EDTA, 0.2% (w/v) SDS and 90% (v/v) formamide. The mixture was then centrifuged for 10 s in a Beckman Microfuge B, the supernatant removed, and the elution procedure repeated twice. The pooled supernatants were made 0.1 M with respect to NaCl, 7.5 µg of wheat-germ tRNA was added as a co-precipitant, and the RNA precipitated overnight at -20°C in a Beckman SW56 centrifuge tube after the addition of 2.5 vol. of ethanol. The precipitated RNA was then removed by centrifugation at 90 000 g_{av} for 30 min at -5°C, washed once in 70% (v/v) ethanol containing 40 mM-NaCl, once in ethanol, then dissolved in sterile double-distilled water and stored at -70°C until required. This procedure yielded 1–2 µg of purified mRNA. The DNA-cellulose was then recycled for further use, precisely as described by Alwine *et al.* (1977), and stored in hybridization buffer.

Results

The translation of post-nuclear poly(A)-containing RNA in a reticulocyte-lysate cell-free system, followed by separation of the 35 S-labelled polypeptides by using band-sharpening SDS/polyacrylamide-gel electrophoresis (Maizel, 1972), demonstrated that a vast spectrum of polypeptides was synthesized (Fig. 1). The pre-caseins A and B (which co-electrophorese) and pre-casein C were particularly prominent, and pre- α -lactalbumin was represented by a diffuse band co-electrophoresing with globin (Fig. 1, lane 1). One other polypeptide, of 33 000 mol.wt., was also prominent. The addition of a microsomal-membrane preparation immediately after the onset of protein synthesis resulted in significant changes in the distribution of the predominant polypeptides. A polypeptide of apparent mol.wt. 64 000 was now present, and there was also a redistribution of the polypeptides in the caseins-A and -B region, and a decrease in the apparent

Fig. 1. Sequestration of mRNA-directed guinea-pig milk proteins synthesized by a rabbit reticulocyte lysate in the presence of a dog pancreas microsomal membrane fraction

Total post-nuclear-poly(A)-containing RNA (0.4 µg) from the lactating guinea-pig mammary gland was added to a 50 µl nuclease-treated reticulocyte-lysate cell-free protein-synthesizing system using [35 S]-methionine (800 µCi/ml) as the radiolabelled amino acid. After a 2 min incubation, a dog pancreas microsomal-membrane (DPM) preparation was added to the appropriate assays (1 µl of a 25A₂₈₀ units/ml suspension to each 50 µl assay; see lanes 4, 5 and 6) and incubation continued for a further 2 h. Samples (10 µl) were then either analysed directly by single-dimension SDS/polyacrylamide-gel electrophoresis, followed by fluorography as described in the Materials and methods section (see lanes 1 and 4), or incubated for a further 2 h at 0°C in the presence of 200 µg of proteinase K/ml (see lanes 2 and 5), then analysed, or made 1% (v/v) with respect to Triton X-100 before incubation with proteinase K (see lanes 3 and 6), then analysed. Molecular-weight estimations were based on the comparative mobility of Coomassie Blue-stained marker proteins (see Craig *et al.*, 1976) electrophoresed in parallel with unprocessed and processed mRNA-directed milk proteins (i.e. lanes 1 and 4) on a separate gel. Arrows (i) denote the relative positions on the gel of the mature guinea-pig milk proteins isolated from milk (caseins A, B and C, and α-lactalbumin), and (ii) indicate the identity of the primary milk-protein translation products synthesized *in vitro* (pre-caseins A, B and C, and pre-α-lactalbumin). Abbreviation: BSA, bovine serum albumin.

molecular weight of casein C (Fig. 1, lane 4). We have established elsewhere (Craig *et al.*, 1979b; Lane *et al.*, 1979) that such changes result from the interaction of the nascent milk-protein peptides with membrane receptors, culminating in the segregation of partially processed milk proteins within membrane vesicles. Consequently, the addition of proteinase K, in the absence of detergent, to a cell-free system containing such segregated polypeptides demonstrated that the caseins, α -lactalbumin, the 64 000-mol.wt. protein and several other peptides were protected from proteolytic digestion (Fig. 1, lane 5). The addition of proteinase K in the presence of detergent resulted in proteolytic degradation of all the peptides (Fig. 1, lane 6).

We have always presumed, on the basis of antibody-precipitation data (Craig *et al.*, 1976), that the processed polypeptides represent partially modified or unmodified variants of caseins A, B and C, in order of decreasing molecular weight. Furthermore, we have been unable to determine whether the appearance of the 64 000-mol.wt. polypeptide results from the processing of the 33 000-mol.wt. polypeptide, or any other of the more prominent higher-molecular-weight polypeptides (Fig. 1, lane 1).

Positive identification of each processed casein polypeptide was obtained in the following manner. Individual milk-protein mRNA sequences were separated by using plasmid DNA immobilized on DBM-paper or DBM-cellulose. These were then used to direct the synthesis of each milk protein in a nuclease-treated reticulocyte-lysate cell-free system, in both the presence and absence of a microsomal

Fig. 2. Identification of processed variants of guinea-pig milk proteins

Individual mRNA sequences which direct the synthesis of the primary translation products of guinea-pig caseins (Cas) A, B and C and α -lactalbumin (α LA) were isolated by using cloned cDNA sequences immobilized on DBM-paper. The purified mRNA sequences (0.05–0.1 μ g) were then added to a nuclease-treated reticulocyte-lysate cell-free system in either the presence or absence of dog pancreas microsomal membranes (DPM), as described in the legend to Fig. 1. After 2 h incubation, 5 μ l samples of the [35 S]methionine-labelled polypeptides were analysed by SDS/polyacrylamide-gel electrophoresis in a single dimension, followed by fluorography. Arrows indicate the relative electrophoretic mobilities of caseins A, B and C, and α -lactalbumin isolated from guinea-pig milk.

Fig. 3. Identification of pre- α -lactalbumin and α -lactalbumin

Purified α -lactalbumin mRNA was added to a nuclease-treated reticulocyte lysate in the absence (a) and presence (b) of dog pancreas microsomal membranes as described in the legend to Fig. 2. The [35 S]methionine-labelled polypeptide(s) were then analysed by two-dimensional polyacrylamide-gel electrophoresis as described by O'Farrell (1975), followed by fluorography. The pH gradient of the isoelectric-focusing dimension was determined by slicing a gel run in parallel. The ampholyte was then eluted from each slice into 1 ml of water, and the pH measured.

membrane preparation. The resulting polypeptides were then resolved by electrophoresis in a band-sharpening SDS/polyacrylamide-gel system (Maizel, 1972). This revealed interesting differences (see Fig. 2). The primary translation product of casein A electrophoresed as a diffuse band, consistent with the presence of several polypeptides. The addition of microsomal membranes resulted in processing and the redistribution of these bands. Two prominent bands were now apparent, one of slightly decreased electrophoretic mobility, and the other in the position of the 64 000-mol.wt. band, identifying this as a modified variant of casein A. Analysis of pre-casein B also revealed several polypeptides, in this instance two major and two minor bands. Processing resulted in a redistribution to the expected position of the processed casein B. Analysis of pre-casein C revealed a single band, which when processed remained as a single band and showed a slightly increased electrophoretic mobility. Pre- α -lactalbumin was also resolved as a single polypeptide, and was appropriately processed to α -lactalbumin. This was demonstrated by two-dimensional electrophoresis (see Fig. 3) in order to resolve the α -lactalbumin from the endogenous globin.

A separate analysis of the casein primary and processed translation products synthesized in the wheat-germ system produced slightly different results. The multiple pre-casein B polypeptides all showed slightly different mobilities when compared with those synthesized in the reticulocyte lysate. In addition, heterogeneity of casein C was occasionally observed, but the highly modified casein A variant was never observed, in spite of using precisely the same dog pancreas microsomal preparation shown to be active in the reticulocyte lysate (results not shown).

Examination of the primary milk-protein cell-free translation products of total milk-protein poly(A)-containing mRNA by two-dimensional polyacrylamide-gel electrophoresis (Fig. 4) confirmed: (i) the heterogeneous nature of the primary translation products of caseins A and B, and (ii) that quantitative and qualitative differences existed between the wheat-germ and reticulocyte-lysate primary translation products. An additional prominent polypeptide was common to both cell-free systems. On the basis of mobility in the second dimension it seems likely that this represents the 33 000-mol.wt. polypeptide described above (see Fig. 1). Individual polypeptides representing variants of caseins A and B were identified by similar analysis of the cell-free translation products directed by the purified mRNA species. In this manner, pre-casein A synthesized in the wheat-germ cell-free system was resolved into four major variants, and pre-casein B into two major and several minor variants (detailed analysis not shown, but variants identified in Fig. 4a). In the

Fig. 4. *Heterogeneity within the primary translation products of guinea-pig caseins*

Total post-nuclear poly(A)-containing RNA (0.2 μ g) from the lactating guinea-pig mammary gland was translated in either a wheat-germ (a) or a nuclease-treated reticulocyte-lysate (b) cell-free system, in the presence of [35 S]methionine. The individual translation products were then analysed by two-dimensional polyacrylamide-gel electrophoresis and fluorography. Arrows indicate the identity of the major polypeptides determined in parallel experiments using purified mRNA sequences (see also Fig. 5). Abbreviations: α -LA, α -lactalbumin; 33K, 33 000-mol.wt. polypeptide.

reticulocyte lysate, pre-casein A was resolved into two or possibly three variants, and pre-casein B into three major and several minor variants (see Fig. 5, also arrowed in Fig. 4b). Pre-casein C was not resolved in the isoelectric-focusing dimension owing

Fig. 5. Identification of sequestered variants of caseins A and B by two-dimensional polyacrylamide-gel electrophoresis. Purified mRNA encoding casein A or casein B was translated in a nuclease-treated reticulocyte lysate in either the presence or absence of dog pancreas microsomal membranes. Peptides synthesized in the absence of added membranes were analysed directly by two-dimensional polyacrylamide-gel electrophoresis, and those synthesized in the presence of membranes were similarly analysed after digestion with proteinase K as described in the legend to Fig. 1. (a) +Casein A mRNA; (b) +casein A mRNA + membranes + proteinase K; (c) +casein B mRNA; (d) +casein B mRNA + membranes + proteinase K.

to the unexpected basic nature of this peptide (J. C. Pascall & A. P. Boulton, unpublished work).

The analysis in two dimensions of the processed milk-protein translation products synthesized in the reticulocyte cell-free system in the presence of a dog pancreas microsomal membrane preparation, and then treated with proteinase K to remove residual unprocessed polypeptides, produced simplified patterns (see Fig. 5). In this instance, although the highly modified variant of sequestered casein A, and sequestered casein C were not resolved in the isoelectric-focusing dimension, sequestered casein A was resolved into two variants, and sequestered casein B into a single species.

Discussion

The combination of purified mRNA, heterologous cell-free protein synthesis and polyacrylamide-gel electrophoresis in two dimensions has enabled us to establish a precursor-product relationship between the primary milk-protein translation products synthesized in the absence of

microsomal membranes, and those partially processed and sequestered when synthesized in the presence of such a membrane fraction. The results extend our previous knowledge, based on antibody-precipitation data alone (Craig *et al.*, 1976), and justify our assessments of precursor-product relationships in subsequent publications (Craig *et al.*, 1979*a,b*; Colman & Morser, 1979; Lane *et al.*, 1979, 1980; Colman *et al.*, 1981). Moreover, the approach also provides novel data regarding the primary casein translation products and their partially processed and sequestered counterparts.

Two explanations might account for the multiple forms of the primary translation products of caseins A and B. Firstly, since heterogeneity is absent after *N*-terminal processing and sequestration of casein B, the possibility exists that the multiplicity of primary translation products results from heterogeneity within the signal sequence because of either different casein B genes or the addition of different signal sequences to a single gene transcript during mRNA maturation. These explanations, though attractive, seem unlikely. Hydrophobic leader sequences pres-

ent in the primary translation products of a variety of different milk proteins from several species have been sequenced (Gaye *et al.*, 1977; Mercier *et al.*, 1978a,b), and some were shown to be correctly processed by microsomal membranes (Gaye *et al.*, 1979). In no instance has heterogeneity within the leader sequences been reported. Furthermore, our own restriction analysis of a series of recombinant DNA plasmids containing the guinea-pig casein B cDNA sequence has so far failed to reveal any differences (D. Parker & R. K. Craig, unpublished work), and size analysis of the individual milk-protein mRNA sequences similarly failed to provide any evidence for mRNA heterogeneity (Craig *et al.*, 1981). Similar explanations may also apply to casein A variants, though seem less likely, as in this instance the degree of heterogeneity after sequestration and processing is still considerable. However, until we have established whether the three sequestered casein A polypeptides represent varying degrees of post-translational modification of a common peptide chain, or alternatively whether there exist three different peptide chains, each encoded by mRNA species differing only slightly in nucleotide sequence, the possibility of heterogeneity within the signal sequence cannot be excluded.

The second explanation for the heterogeneity concerns the possibility of unscheduled peptide modifications by the wheat-germ and reticulocyte cell-free systems. We have established that processing and sequestration of the milk proteins is a co-translational event (Craig *et al.*, 1979a; Lane *et al.*, 1979). In the absence of microsomal membranes the proposed unscheduled modifications may well occur on amino acid residues not normally exposed to cytosolic enzymes. Whether this includes modification to the signal peptide remains supposition, though it seems unlikely, since no multiple forms of pre- α -lactalbumin were observed. However, as qualitative and quantitative differences in the products of the same mRNA preparations were seen after translation in different cell-free systems, we conclude that at least some of the heterogeneity observed must result from differential modification of the pre-caseins A and B polypeptides by the cell-free systems. In addition, it was also apparent from comparison of the two-dimensional gel-electrophoretic patterns of pre-caseins A and B (see Figs. 4 and 5) that minor peptides were present, which electrophoresed in the expected positions of the sequestered and processed peptides. This suggests that the wheat-germ and reticulocyte-lysate cell-free systems are capable of a limited degree of processing activity, a result consistent with observations reported by others using the wheat-germ cell-free system (Peeters *et al.*, 1979).

Analysis of the sequestered milk proteins provides data which can be related to previous

less-definitive studies, in particular to the highly modified variant of casein A. It is likely that this represents the peptide designated M-54, described by Colman & Morser (1979) during studies on the rate of secretion of foreign proteins by *Xenopus* oocytes, in this instance as a result of micro-injected poly(A)-containing RNA from the lactating guinea-pig mammary gland. We have yet to establish the nature of this modification, though the casein A variant, in common with the remaining processed casein polypeptides, whether synthesized *in vitro* or in *Xenopus* oocytes, will not bind to concanavalin A-Sepharose (see Colman *et al.*, 1981). A peptide of similar electrophoretic mobility represents a minor component of guinea-pig milk and co-purifies with casein A (D. Parker & R. K. Craig, unpublished work). A secreted peptide of similar electrophoretic mobility also represents a small proportion of the [³⁵S]methionine-labelled peptides secreted by explants of the lactating guinea-pig mammary gland in culture (see Burditt *et al.*, 1981).

We conclude that the combination of mRNA-directed cell-free protein synthesis using highly purified individual mRNA species, and one- and two-dimensional SDS/polyacrylamide-gel electrophoresis, permits the identification of precursor-product relationships not possible when using a mixed milk-protein mRNA population. In particular, three different sequestered variants of casein A have been identified, but only a single polypeptide representative of sequestered casein B and α -lactalbumin. Definitive evidence for or against variants of sequestered casein C was not obtained. Recent experiments using explants of lactating guinea-pig mammary gland in organ culture, and mRNA-directed protein synthesis in the presence of microsomal membranes isolated from the lactating guinea-pig mammary gland, provide evidence that all the sequestered milk-protein variants described here represent true intermediates in the biosynthesis of the fully processed and modified proteins isolated from guinea-pig milk (J. C. Pascall & A. P. Boulton, unpublished work).

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