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Isolation and some effects of functional, low-phenylalanine κ-casein expressed in the milk of transgenic rabbits

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Abstract

Patients suffering certain metabolic diseases (e.g. phenylketonuria) need a low-phenylalanine diet throughout their lives. Transgenic rabbits were created to express low-phenylalanine κ-casein in their milk. The aim was to demonstrate for the first time the feasibility of producing a modified milk protein in addition to normal milk proteins. A gene construct containing the coding region of the rabbit κ-casein gene was modified by site-specific oligonucleotide directed mutagenesis. Four of the five phenylalanine amino acids present in the mature protein were mutated and the gene construct was used to create two transgenic rabbit lines. The transgenic rabbits produced the recombinant κ-casein at a high level in their milk causing a reduction in the average size of the casein micelles. The low-phenylalanine κ-casein was digestible with chymosin and it was separated from its native counterpart and from the other milk proteins by a one-step HPLC method on a reversed-phase column. In the future, low-phenylalanine casein produced in transgenic animals could be used as dietary replacements to meet the special requirements of certain consumer groups.

Keywords: Altered milk composition; Transgenic rabbit; Low-phenylalanine κ-casein; Casein micelle size; Phenylketonuria

1. Introduction

Phenylketonuria (PKU) is a genetic inborn error of metabolism, which results in an inability to metabolize the amino acid phenylalanine. A low protein diet (10 mg kg^{-1} body weight phenylalanine/tyrosine (Phe/Tyr) per day) is recommended for individuals with PKU. For most PKU sufferers the reduction of Phe ingestion to this level is sufficient to avoid mental retardation. However, to avoid elevated PKU levels, high protein foods including milk, cheese, eggs, meat, fish and other high protein foods are not allowed. The observation that low-Phe and Tyr diets limit tumour growth

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in animal models of primary and metastatic melanoma, lung, hepatocarcinoma, leukaemic and mammary adenocarcinoma suggested a novel cancer therapy (Lorincz et al., 1969; Pine, 1978; Meadows et al., 1982; Fu et al., 1997). However, when low protein diets were used with advanced cancer patients, they could not tolerate the low-Phe and low-Tyr protein powder formulated from crystalline amino acids (Harvie et al., 2002). Premature babies fed on high casein formulas have higher plasma Phe and Tyr concentrations than those fed on mother's milk (Darling et al., 2004), which again demonstrates that there is a demand for low-Phe milk and milk products. While the availability and quality of low-Phe diet has improved in the last decade it is still unusual, and there is poor compliance by the patients. Our ultimate aim is to develop a technology to produce a low-Phe milk product as an alternative of the currently existing non-animal protein based diets. As a first step towards that aim the feasibility of creating transgenic livestock expressing low-Phe κ-casein was examined. The rabbit was chosen for this work because it can produce much more milk than smaller laboratory animals and has a shorter gestation time and lower maintenance costs than larger livestock species. Other lines of transgenic rabbits expressing high levels of biologically active proteins in their milk have been created and characterized (for review Bősze et al., 2003). Here we report the creation and phenotypic analysis of transgenic rabbits expressing a high concentration of low-Phe κ-casein in their milk, its effect on the viscosity of the milk and on the average size of the casein micelles. We also describe a convenient method for the chromatographic separation of the mutated κ-casein from its native form and from the other native milk proteins. Projects employing transgenic animals to produce foods with improved nutritional value for people suffering from certain chronic diseases may help to change the currently unfavourable attitude of the public towards transgenic technology.

2. Materials and methods

2.1. The rabbit Phe-substituted κ-casein construct and generation of transgenic rabbits

Construction of transgene based on the rWAP promoter and the native rκ-casein gene has been previously described (Hiripi et al., 2000). In the current study, targeted mutation of selected Phe coding base triplets were based on the alignment of the published kappa-casein amino acid sequences (Fig. 1A). Seven Phe residues are present in the κ-casein protein sequence. Two of them (at positions 14 and 18) are in the signal peptide, which is not present in the mature protein, thus we did not need to deal with them. One of the remaining five Phes (at position 126) is necessary for chymosin/rennin digestion. To preserve this vital function of the protein we left this Phe unchanged. To minimize the alterations in the secondary structure of the mature κ-casein the four remaining Phes were modified to Tyr, valine (Val) and serine (Ser), which are present at the same positions in the human or mouse/rat κ-casein sequences: Phe-39-Tyr, Phe-76-Tyr, Phe-88-Val, Phe-125-Ser. To create the mutated κ-casein the oligonucleotide directed mutagenesis method was applied (Quick Change Site-Directed Mutagenesis Kit, STRATAGENE). Specific primers used for mutagenesis are described in Fig. 1B. The altered base triplets in the injected rWAP-mκ-casein construct were verified by DNA sequencing.

The generation of two transgenic rabbit lines and determination of transgene copy numbers has been published recently (Devinoy et al., 2005). Preliminary analysis of the recombinant protein by Western analysis revealed that the mutant protein was expressed at different levels in the two transgenic lines (#63 and #82) and that the level of expression was higher in the homozygote rabbit milk samples (Devinoy et al., 2005).

Transgenic experiments were approved by the Hungarian Animal Care and Ethics Committee (Ref. no. 767/001/2003) and complied with the Hungarian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2. Analysis of low-Phe κ-casein expression by RT-PCR

Total RNA was extracted using RNAzol B (Tel-Test Inc., Texas) from frozen mammary gland biopsy samples taken on various days during the first 36 weeks of lactation. One lactating female from each transgenic line (#63 and #82) was sacrificed on the 3rd week of lactation and RNA was extracted from tissue samples collected from the brain, skin, mammary gland, muscle, spleen, liver, salivary gland, heart, lung and kidney.