Pigment Cell Res 1996; 9:235–239
Printed in the United States of America - all rights reserved.

Copyright © Munksgaard 1996
PIGMENT CELL RESEARCH
ISSN 0893-5785

κ-Casein Suppresses Melanogenesis in Cultured Pigment Cells

MITSUNARI NAKAJIMA, ICHIZO SHINODA, YOSHIKO SAMEJIMA, HIROFUMI MIYAUCHI, YASUO FUKUWATARI, AND HIROTOSHI HAYASAWA
Nutritional Science Laboratory, Morinaga Milk Industry Co., Ltd.,
5-1-83, Higashihara, Zama, Kanagawa 228, Japan

The effects of bovine milk proteins on melanogenesis in B16 cells were examined. Both whey protein isolate and casein exhibited depigmenting properties. Among the major protein components of milk—including β-lactoglobulin, α-lactalbumin, α-, β-, and κ-casein—only κ-casein exhibited the depigmenting effect. However, the carboxyl terminal peptide of κ-casein, glycomacropeptide, did not show this activity. Also, κ-casein promoted the proliferation of the cells and inhibited the activity of tyrosinase in the cells. These results indicate that κ-casein acts as a melanogenesis-suppressing modulator.

Key Words: Casein, Whey, Depigmentation, Melanin, Melanoma

INTRODUCTION

Bovine milk proteins are used as an industrial source of high-quality proteins. These are roughly classified into casein and whey proteins (Eigel et al., 1984). Casein is defined as phosphoproteins that precipitate from raw skim milk following acidification to pH 4.6 at 20°C, and casein consists of α-(α_{s1}- and α_{s2}-), β-, and κ-casein. The term "whey proteins" is used to describe the group of milk proteins that remain soluble in whey after precipitation of casein; β-lactoglobulin, α-lactalbumin, and serum albumin are the major components of this group. Whereas the biological activities of several minor proteins in milk including lactoferrin (Nichols et al., 1987), epidermal growth factor (Hagiwara et al., 1995), transforming growth factor-β (Sato and Rifkin, 1989), and immunoglobulins (Underdown and Schiff, 1986) have been investigated, there are few reports concerning the biological functions of the major proteins. Although bovine casein and whey proteins are used as cosmetic materials in Japan, the influence of these proteins on skin pigmentation has not been reported. In the present study, the effects of bovine milk proteins on the pigmentation of melanocytes have been assessed.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Grand Island, New York). Fetal calf serum (FCS) was obtained from HyClone (Logan, Utah). Aminophylline ([theophylline]$_2$ · ethylenediamine]), bovine β-lactoglobulin, α-lactalbumin, α-, β-, and κ-caseins were obtained from Sigma (St. Louis, Missouri). Bovine glycomacropeptide (GMP) was from the New Zealand Dairy Board (New Zealand). Bovine whey protein isolate (WPI) was from Milei GmbH (Germany). Bovine casein was from Nacalai Tesque (Kyoto, Japan).

Cell Culture

B16 4A5 mouse melanoma cells (RCB No. RCB0557) were obtained from the RIKEN Cell Bank (Tsukuba, Japan). They were maintained in DMEM containing 10% FCS and antibiotics at 37°C in a humidified incubator with 5% CO_2 in air.

Cell-Blotting Assay

For evaluation of pigmentation, we used a new cell-blotting assay, which will be described in detail elsewhere. Briefly, B16 melanoma cells were plated onto plastic 24-well plates (2 cm^2) at a density of 0.75×10^4 cells/cm^2. After one day of culture, the medium was changed to a fresh medium containing 2 mM theophylline, an inducer of pigmentation (Imokawa, 1990), and various concentrations of milk proteins; the cells were then cultured for an additional three days. Following this, the cells in each well were collected by treatment with Mg^{2+}-, Ca^{2+}-free phosphate-buffered saline (PBS[-]) containing trypsin (0.05%) and ethylenediaminetetraacetic acid (EDTA; 0.44 mM), blotted onto nitrocellulose membranes (0.45 μm pore size, Pharmacia, Sweden) using a slot-blotting apparatus (Bio-Dot SF; BIO-RAD, Hercules, California), and fixed by air drying. The image of each blotted membrane was scanned by gray scale and incorporated into a personal computer using the Epson GT-6000 system with EpScan Mac software (Seiko-Epson, Japan), and the density of cell blots was analyzed with NIH Image software programmed by Dr. Wayne Ras-

Address reprint requests to M. Nakajima, Nutritional Science Laboratory, Morinaga Milk Industry Co., Ltd., 5-1-83, Higashihara, Zama, Kanagawa, 228, Japan.

Received June 17, 1996; accepted September 2, 1996.

band. Data in each experiment were normalized by the control value, and expressed as a percentage of the control mean ± standard error (SE) (n=3).

Measurement of Melanin Content

Melanin content in cultured B16 cells was measured by the method of Hosoi et al. (1985). Synthetic melanin (Sigma) was used as standard. The absorbance was read at 470 nm, and the melanin content was expressed as μg/mg protein. Protein content was measured with a BCA Protein Assay kit (Pierce; Rockford, Illinois).

Cell-Pellet Assay

B16 cells were plated into culture flasks (75 cm^2) at an initial density of 0.75×10^4 cells/cm^2, and cultured as in the experiments that employed the cell-blotting assay. After trypsin treatment, each cell suspension was pelleted in a tube by centrifugation.

Assay of Tyrosinase

The activity of tyrosinase in each culture well was assessed by the method described by Tomita et al. (1992). B16 melanoma cells were plated at a density of 2,100 cells per well in 70 μl of medium in 96-well plates. After one day, the medium was changed to a fresh medium containing various concentrations of milk proteins. The cells were incubated for an additional three days, and then washed with PBS(-) and lysed with 1% Triton-X/PBS(-) (45 μl per well). After mixing the lysates by vibration, 5 μl of 10 mM L-DOPA was added to each well. Following incubation of the plates at 37°C for 30 min, the absorbance was measured at 475 nm. Mushroom tyrosinase (Sigma) was used as a standard. The standardized values were expressed as a percentage of the mean of the total activity of all cells in the control wells.

RESULTS

Depigmenting Effects of Casein and κ-Casein on B16 Melanocytes

The effects of bovine milk proteins on melanogenesis in cultured B16 melanocytes were examined using the cell-blotting assay. As shown in Figure 1A, the densities of the blotted cells cultured with either WPI or casein decreased in a dose-dependent manner in each instance. We then assessed the effects of major proteins present in WPI or casein: β-lactoglobulin, α-lactalbumin; α-, β-, and κ-casein (Fig. 2A). Among these proteins, only κ-casein exhibited the depigmenting effect at concentrations of 10–1000 μg/ml. However, the carboxylterminal peptide of κ-casein produced by chymosin (EC 3.4.23.4) hydrolysis, GMP, did not show this activity.

Because cell numbers in cultures supplemented with casein or κ-casein slightly increased (Fig. 1B and Fig. 2B), the decrease in the densities of cell blots seemed to be due to a decreased melanin content in the cells. To confirm this point, we measured the melanin content of cultured melanocytes by the classical biochemical method. As shown in Figure 3, decreases in melanin content relative to protein content were observed in the cases of casein and κ-casein. The density of pellets of B16 cells also decreased following treatment with casein or κ-casein (Fig. 4).

Decreased Activity of Tyrosinase in B16 cells Cultured With Casein or κ-Casein

To explore the mechanisms responsible for the decreases of melanin content induced by casein or κ-casein, the ef-

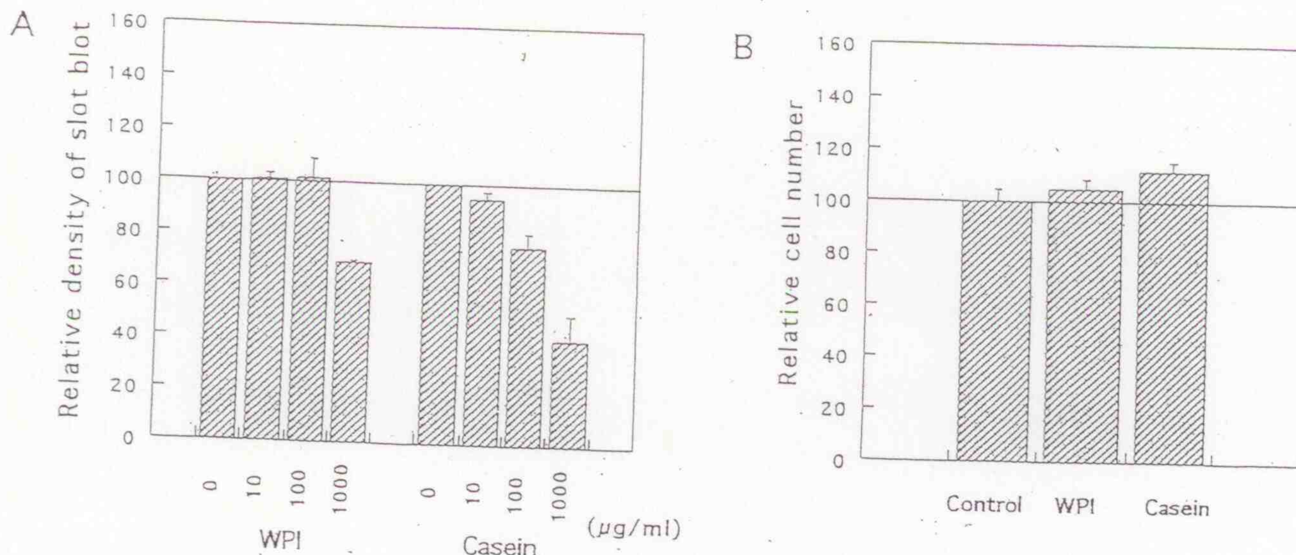
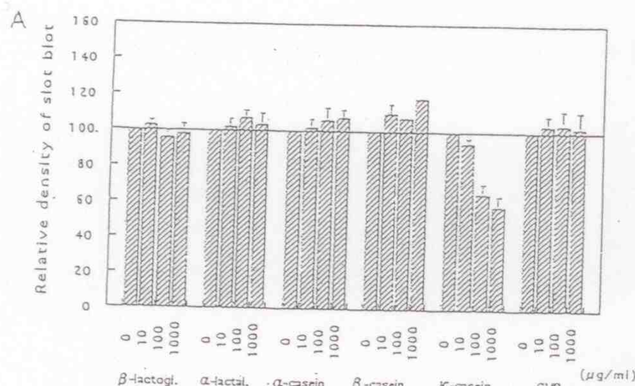


Fig. 1. Effects of WPI and casein on pigmentation and cell growth of B16 melanocytes. A: B16 melanocytes were cultured with WPI or casein in the presence of 2 mM theophylline, and examined by the cell-blotting assay. B: B16 melanocytes were plated onto 6-well plates (9 cm^2) at an initial density of 0.75×10^4 cells/cm^2, and cultured with WPI or casein (1 mg/ml) in the presence of theophylline for 3 days. The number of cells was counted with a hemocytometer after trypsin treatment. Values are expressed as a percentage of the control mean ± SE of 2 independent experiments (n=6).



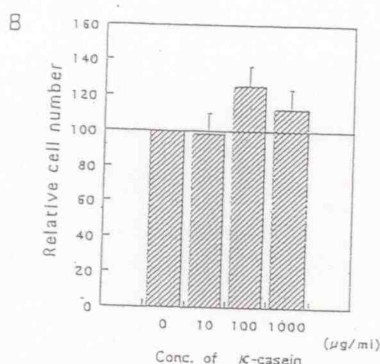


Fig. 2. Depigmenting and growth-promoting effects of κ-casein on B16 melanocytes. A: B16 melanocytes were cultured with major milk proteins in the presence of 2 mM theophylline, and examined by the cell-blotting assay. B: B16 melanocytes were cultured with κ-casein, and the cell number was assessed as described in the legend of Figure 1B.

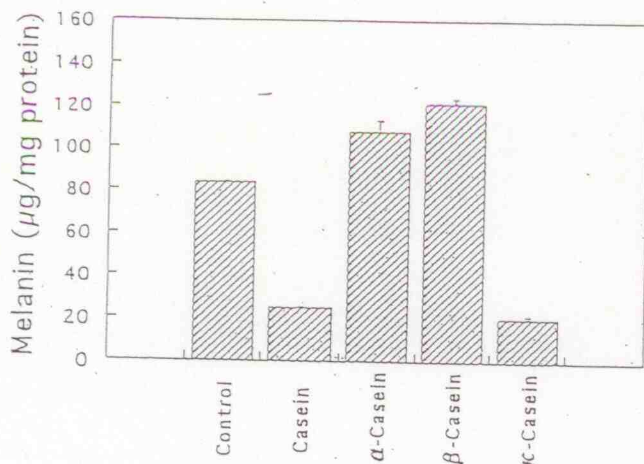


Fig. 3. Decrease of melanin content in B16 cells following treatment with casein or κ-casein. B16 melanocytes were plated onto 100 mm dishes (60 cm^2) at an initial density of 0.75×10^4 cells/cm^2, cultured with samples (1 mg/ml) in the presence of theophylline for three days, and harvested for measurement of melanin content. Values are the mean ± SE (n=3).

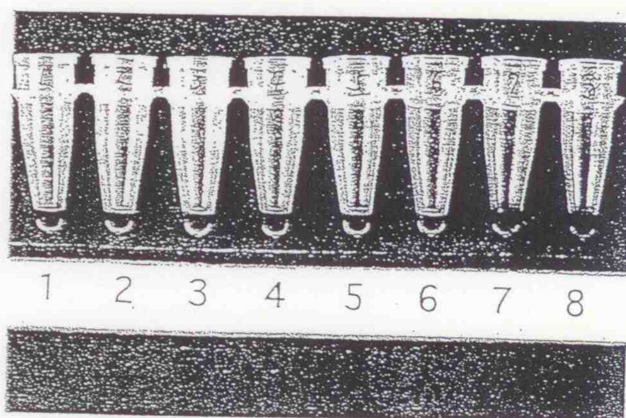


Fig. 4. Photograph of pellets of B16 cells after treatment with caseins. B16 cells were plated into culture flasks (75 cm^2) at an initial density of 0.75×10^4 cells/cm^2, and cultured with samples in the presence of theophylline for three days. After trypsin treatment, each cell suspension was pelleted in a tube by centrifugation. 1: without caseins; 2: 100 μg/ml of casein; 3: 1,000 μg/ml of casein; 4: 10 μg/ml of κ-casein; 5: 100 μg/ml of κ-casein; 6: 1,000 μg/ml of κ-casein; 7: 1,000 μg/ml of α-casein; and 8: 1,000 μg/ml of β-casein.

fects of these proteins were examined on the activity of tyrosinase in cultured melanocytes. Tyrosinase is known to be a key enzyme in the processes of melanogenesis. As shown in Figure 5, the activity of tyrosinase decreased in the presence of casein or κ-casein, whereas tyrosinase activity increased in the presence of α-lactalbumin, α-casein, or β-casein.

DISCUSSION

In the present study, we have demonstrated that κ-casein has a depigmenting effect on cultured B16 melanocytes. Because κ-casein promoted the proliferation of the cells, the observed depigmenting effect could not be attributed to the induction of a decrease in cell number. Depigmentation was confirmed by the cell-pellet assay and the direct measurement of melanin content in proportion to the protein content of the cells. Furthermore, our findings suggest that the melanogenesis-suppressing activity of κ-casein is mediated by the inhibition of tyrosinase activity.

In contrast to κ-casein, the other components of casein such as α- and β-caseins did not promote melanogenesis in B16 cells. Casein, composed of α-, β-, and κ-casein, exhibited the same depigmenting effect as κ-casein did. These observations may be influenced by the structural features of casein: α- and β-caseins exist inside the casein micelles, in contrast with κ-casein which exists on the surface of the micelles, although the exact state of these caseins under the culture conditions used is not clear.

WPI has been shown to have a depigmenting effect on B16 cells, but its effectiveness is less than that of casein. Since β-lactoglobulin and α-lactalbumin, the major proteins in WPI, did not show this effect, it appears that minor components of WPI are responsible for its ability to suppress

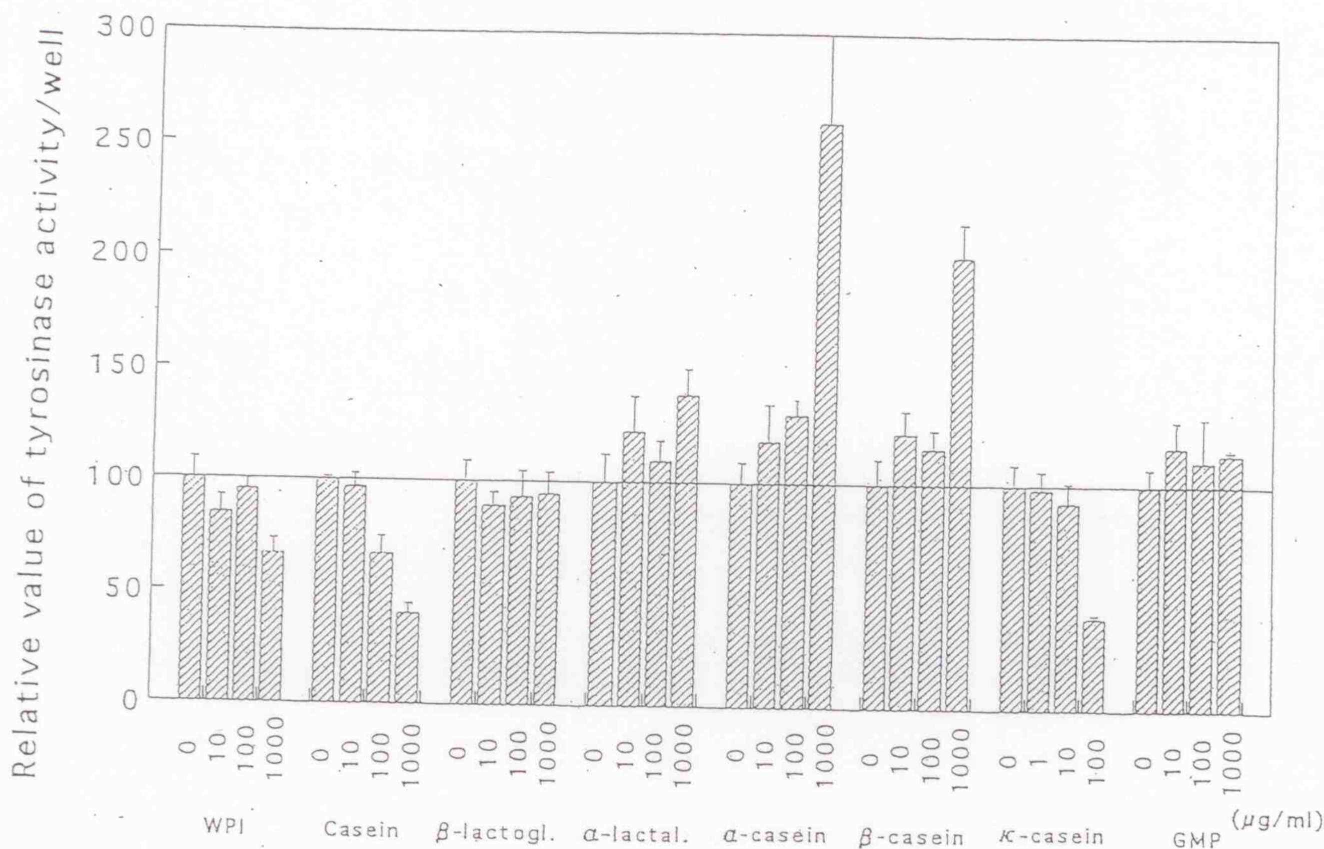


Fig. 5. Effects of major milk proteins on tyrosinase activity in B16 melanocytes. B16 melanocytes were cultured with major milk proteins, and assessed for tyrosinase activity as described in the text. Data are expressed as a percentage of the control mean ± SE (n=3).

melanogenesis. Lactoferrin, one of the minor proteins in WPI, has this depigmenting effect (data not shown); however, the depigmenting effect of WPI could not be attributed to lactoferrin only, because the effective dose of lactoferrin is within the same range as WPI. Therefore, it seems that WPI contains other components that can modulate melanogenesis.

κ-Casein exhibited cell growth–promoting activity as well as the melanogenesis-suppressing effect, suggesting that its mechanism of action may involve the triggering of signal transduction pathways via cell-surface receptors. There are several reports indicating the involvement of intracellular signaling molecules in melanogenesis: Decreased melanization occurs in the presence of a calcium ionophore or phorbol esters (Fuller, 1987; Mufson et al., 1979), which are activators of protein kinase C; and increased melanization occurs in the presence of theophylline and melanocyte-stimulating hormone (Hu et al., 1982), which induce elevated intracellular cAMP levels. Considering that cell proliferation is also known to be modulated by these signaling molecules, κ-casein might act to trigger intracellular signaling. Identification of the signals induced by κ-casein will be the next object of our studies.

Treatment of κ-casein with a hydrolytic enzyme, chymosin, divides this protein molecule into two fragments: para-κ-casein, which consists of the region of residues 1–105; and GMP, which consists of residues 106–169 including phosphorylation and glycosylation sites (Eigel et al., 1984). We assessed only the activity of GMP, since the other fragment was not available to us. The fact that GMP showed no depigmenting activity suggests that the para-κ-casein portion rather than GMP is responsible for triggering the action. Alternatively, the intact κ-casein molecule might be required for expression of this activity.

It is conceivable that casein and κ-casein used as cosmetic materials may contribute to the healing of wounded skin tissue. Since casein and κ-casein promote the proliferation and depigmentation of cultured B16 cells, application of these proteins at sites of skin damage might stimulate the growth of melanocytes without inducing increases in pigmentation of the tissue. Further investigations will be required to support such use in a clinical application.

REFERENCES

Eigel, W.N., J.E. Butler, C.A. Ernstrom, H.M. Farrell, Jr., V.R. Harwalkar, R. Jenness, and R.McL. Whitney (1984) Nomenclature of proteins of cow's milk: fifth revision. J. Dairy Sci. 67:1599–1631.

Fuller, B.B. (1987) Inhibition of tyrosinase activity and protein synthesis in melanoma cells by calcium ionophore A23187. Pigment Cell Res. 1:176–180.

Hagiwara, T., I. Shinoda, Y. Fukuwatari, and S. Shimamura (1995)

Effects of lactoferrin and its peptides on proliferation of rat intestinal epithelial cell line, IEC-18, in the presence of epidermal growth factor. Biosci. Biotech. Biochem. 59:1875–1881.
Hosoi, J., E. Abe, T. Suda, and T. Kuroki (1985) Regulation of melanin synthesis of B16 mouse melanoma cells by 1α,25-dihydroxyvitamin D3 and retinoic acid. Cancer Res 45:1474–1478.
Hu, F., K. Mar, and D.F. Teramura (1982) Electron microscopic and cytochemical observations of theophylline and melanocyte stimulating hormone effects on melanoma cells in culture. Cancer Res. 42:2786–2791.
Imokawa, G. (1990) Analysis of carbohydrate properties essential for melanogenesis in tyrosinases of cultured malignant melanoma cells by differential carbohydrate processing inhibition. J. Invest. Dermatol. 95:39–49.
Mufson, R.A., P.B. Fisher, and I.B. Weinstein (1979) Effect of phorbol ester tumor promoters on the expression of melanogenesis in B-16 melanoma cells. Cancer Res. 39:3915–3919.
Nichols, B.L., K.S. McKee, J.F. Henry, and M. Putman (1987) Human lactoferrin stimulates thymidine incorporation into DNA of rat crypt cells. Pediatr. Res. 21:563–567.
Sato, Y., and D.B. Rifkin (1989) Inhibition of endothelial cell movement by pericytes and smooth muscle cells: Activation of a latent transforming growth factor-β1-like molecule by plasmin during co-culture. J. Cell Biol. 109:309–315.
Tomita, Y., K. Maeda, and H. Tagami (1992) Melanocyte-stimulating properties of arachidonic acid metabolites: Possible role in postinflammatory pigmentation. Pigment Cell Res. 5:357–361.
Underdown, B.J., and J.M. Schiff (1986) Immunoglobulin A: Strategic defense initiative at the mucosal surface. Ann. Rev. Immunol. 4:389–417.