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PROCEDURE FOR THE EVALUATION OF THE OPIOID ACTIVITY OF MILK PRODUCTS

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The aim of this work was to prepare the procedure for the evaluation of the opioid activity of milk products. Peptides from acidophilic milk were extracted with methanol/chloroform mixture (2:1 v/v) and from Gouda cheese with the same mixture in a ratio of 1:2 (v/v). The peptide extracts were desalted on Sephacryl S-100 HR column. The opioid activity of the peptide extracts was determined by the measurement of the motoric activity of isolated rabbit intestine (by Magnus method). The identification of the opioid peptides in the peptide extracts was made using thin layer chromatography. β-Casomorphin-5 was identified as opioid peptide in all peptide extracts. For the determination of the β-casomorphin--5 content in the peptide extracts, the standarization of the HPLC method was made. The contents of β-casomorphin-5 in the acidophilic milk and Gouda cheese peptide extracts were: 0.08% and 0.63%, respectively.

INTRODUCTION

Some peptides produced *in vitro* or *in vivo* by enzymatic hydrolysis of caseins and whey proteins can affect specific biological functions of the body and therefore they are called "bioactive peptides" [Tirelli *et al.*, 1999]. Milk proteins are a potential source of the opioid peptides – called casomorphins [Brantl *et al.*, 1979]. β-Casomorphins can be liberated from β-casein during the digestion in the gastrointestinal tract or they can be formed upon technological processes [Meisel, 1986; Jarmołowska *et al.*, 1999]. The presence of the opioid peptides in milk food products provokes an introduction of a new criterion of food quality, the so-called "opioid activity". This proposal is justified by various biological properties of β-casomorphins, including: induction of analgesic and sedative effects due to the action on the nervous system [Teschemacher, 1987], stimulation of the endocrine activity of the pancreas [Paroli, 1988; Zühlke *et al.*, 1994], gastrointestinal transit time amino acid absorption and water balance [Hahn *et al.*, 1994]. β-Casomorphins arise from human milk, pass through the mammary tissues and affect the release of prolactin and oxytocin [Bicknell, 1985]. It is necessary to note that literature provides scanty information on the determination of the content of the opioid peptides in cheeses and fermented milk products with the exception of previous own publications. Only β-casomorphin-5 was determined in human milk by the radioimmunoassay methods [Assargârd *et al.*, 1994]. From the analytical point of view, it is worth emphasising that the procedure of the isolation of peptides from human milk cannot be used for cheese and fermented milk products. For this reason we look for a new procedure.

The aim of this work was to prepare the chemical procedure enabling the evaluation of the opioid activity of milk products. Finally, on the basis of this method, an immunological method for the determination of the opioid activity of milk products will be prepared.

MATERIALS AND METHODS

Protocol for chemical and biological analyses

Material
(Acidophilic milk, Gouda cheese)
↓
Extraction of peptides
↓
Desalting of peptide extracts
↓
Testing of opioid activity of peptide extracts
(Magnus method with the use of the isolated rabbit ileum)
↓
Chromatographic identification of opioid peptides
(Thin layer chromatography, HPLC)
↓
Future investigations
↓
Production of polyclonal antibodies against opioid peptides
↓
Production of monoclonal antibodies against opioid peptides
↓
ELISA – quantitative determination of opioid peptides

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Extraction of peptides. Our earlier and present investigations proved that the universal solvent for the extraction of peptides from milk, fermented milk products and cheeses was the chloroform/methanol mixture [Harwalkar & Elliot, 1971]. This solvent mixture enables obtaining a defatted peptide extract with a low content of mineral components [Kostyra *et al.*, 1981]. The extraction of peptides proceeded as described below:

(a) Cheese: 100 g of ground cheese was homogenised with 800 cm^3 of a 2:1 v/v chloroform/methanol mixture for 15 min and filtered. The deposit was washed several times with the chloroform/methanol mixture. The supernatant was made biphasic by the addition of 0.2 volume of distilled water. The lower layer was discarded and the upper layer, containing mainly peptides and amino acids, was evaporated and lyophilised.

(b) Acidophilic milk: 100 cm^3 of chloroform/methanol mixture (1:2 v/v) was added to 100 cm^3 of acidophilic milk and mixed for 1 h at a room temperature. Then, the mixture was kept for 12 h at a temperature of 10°C. The two layers formed were separated. The lower layer was discarded. The upper layer was saturated with methanol to obtain the clear solution (the ratio of the methanol peptide extract to clear methanol was approximately 4:3 v/v). The mixture was rotated at 3000 g for 10 min at a temperature of 4°C. The supernatant was concentrated under diminished pressure at a temperature below 40°C. The extract was frozen and lyophilised.

Desalting of peptide extracts. The peptide extracts were desalted on the column (0.42 x 30 cm) filled with Sephacryl S-100 HR. The column was equilibrated by 0.1 mol/L acetic acid. 50 mg of the peptide extract was dissolved in 10 cm^3 of 0.1 mol/L acetic acid and applied onto the column. The column was eluted with 0.1 mol/L acetic acid at the flow rate of 1.5 cm^3 /min. The efficiency of desalting was assessed by the reaction with silver nitrate. Absorbance of eluents (3 cm^3 fractions) was measured at 220 and 280 nm. The peptide fractions were concentrated under diminished pressure at a temperature below 40°C. Fraction obtained this wasy was lyophilised.

Thin layer chromatography. The peptide extracts were separated by analytical thin layer chromatography on Silufol silica gel (0.25 mm thick) plates (20 x 20 cm, Kivalier) in a chamber equilibrated with a freshly prepared solution of n-butanol/acetic acid/water (4:1:1 v/v/v) [Kostyra *et al.*, 1981; Jarmołowska *et al.*, 1999]. Aliquots of the peptide extracts (0.005 cm^3) and β-casomorphin-5 and 7 standard at a concentration of 5.0 mg/cm^3 were loaded on the gel. The standards were purchased from Sigma. The chromatogram was stained with ninhydrin. Comparing Rf with that of β-casomorphin-5 and 7, standards identified the opioid peptide.

HPLC chromatography. HPLC was carried out on a Schimadzu 10 A VP liquid chromatograph equipped with a Phenomenex Jupiter C-18 column (250x4.6 mm, 5μ), an LC-10 AD VP pump, and an SPD M-10 AVP diode detector. The fractions were detected at 210 nm. The peptide extracts were dissolved in the starting eluent (A) and cleaned by passing them through a nylon filter (0.45 μm). The sample (20 μL, concentration of 1 mg/mL) was added to the column. Flow rate of the eluent was 1 mL/min. The following solvent system was used as an eluent: Phase A: water/acetonitrile/TFA (95:5:0.1v/v), Phase B: acetonitrile/water/TFA (95:5:0.1 v/v); Gradient: 00–08 min, A:B (90:10), 08–14 min, A:B (74:26), 14–16 min, A:B (70:30), 16–20min, A:B (90:10).

Measurement of opioid activity. The opioid activity of the peptide extracts was determined by the Magnus method as described by Jeske [1955]. Contractions of the isolated intestine were registered with an apparatus for measuring the contractions of the isolated organs (Hugo-Sachs). The first spontaneous contractions were registered and then a solution of the peptide extract and naloxon was added successively to the vessel containing a fragment of intestine suspended in Tyrod's liquid. Next, the intestine was washed three times with Tyrod's solution. The solutions of the following concentrations were used: peptide extracts - 10 mg/cm^3 and naloxon - 0.2 mg/cm^3.

RESULTS AND DISCUSSION

The peptide extracts from the acidophilic milk and Gouda cheese were analysed biologically and chromatographically. At the beginning, the opioid activity of both extracts was determined by the measurement of the motoric activity of isolated rabbit intestine. The results are presented in Figures 1 and 2. The both peptide extracts caused an increase in the tone and amplitude of intestinal contractions, compared to the spontaneous ones. It is worth emphasising that the peptide extract from Gouda cheese caused more intense intestinal contractions than the peptide extract from the acidophilic milk. Therefore, naloxon, which is the antagonist of morphine and opioid peptides, softened the intestinal contractions caused by the peptide extracts. The obtained results proved that the peptide extracts from the acidophilic milk and Gouda cheese interact with opioid receptors of the intestine. The next step of the investigations was to identify the proper opioid peptide/peptides in the peptide extracts. For this purpose two chromatographic methods were used: thin layer chromatography and HPLC. The thin layer chromatography of both peptide extracts showed that they contained only one opioid peptide – β-casomorphin-5. In order to determine the content of β-casomorphin-5 in the peptide extracts the HPLC method was used. The optimal proper parameters for this analysis were established as follows: Phase A: water/acetonitrile/TFA (95–0.5–0.1 v/v), Phase B:acetonitrile-water-TFA (95–5.0–0.1 v/v), gradient: 00–08 min, A:B (90:10), 8–14 min, A:B (74:26), 14–16 min, A:B (70:30), 16–20 min, A:B (90:10). The retention time for β-casomorphin-5 is 12.88 min (Figure 1). So prepared HPLC was used for the separation of the peptide extracts of the acidophilic milk and Gouda cheese. The hromatograms of these separations are presented in Figures 3 and 4. The obtained chromatograms differed from each other. Five peaks were present on the chromatogram of the peptide extract from acidophilic milk and twelve ones on the chromatogram of the peptide extract from Gouda cheese. The contents of β-casomorphin-5 in the investigated peptide extracts were significantly different. The content of

β-casomorphin-5 was 0.08% in acidophilic milk and 0.63% in Gouda cheese peptide extracts. It is difficult to evaluate the content of β-casomorphin-5 in the investigated milk products because similar investigations were not found in the literature. It can be said that the quantities of β-casomorphin-5 in Gouda cheese and acidophilic milk were far higher than those in milk of the lactating women [Assargard *et al.*, 1994]. It seems rational because the woman milk is the albumin milk while the cow milk is the casein milk. The second very important difference is the composition of the microflora in women and acidophilic milk and cheese. This heterogenous microflora is the reason of the quantitative differences in the contents of β-casomorphin-5 in Gouda cheese and acidophilic milk. The presence of the opioid peptides in the milk and milk products is still a very interesting problem from the physiological point of view and should be taken into consideration as an indicator of food biological activity.

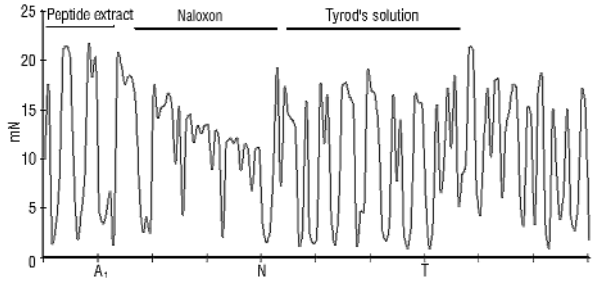

FIGURE 1. Effect of peptide extract from acidophilic milk on peristalsis of isolated rabbit intestine. A – peptide extract from acidophilic milk; N – naloxon; T – washing with Tyrod's solution.

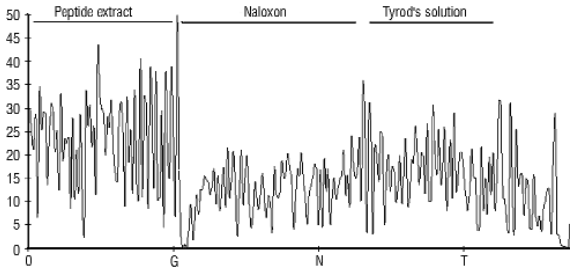

FIGURE 2. Effect of peptide extract from Gouda cheese on peristalsis of isolated rabbit intestine. G – peptide extract from acidophilic milk; N – naloxon; T – washing with Tyrod's solution.

The investigations are continued and focus on preparing an immunometric method for the determination of opioid peptides in milk products.

CONCLUSIONS

1. The peptide extracts of acidophilic milk and Gouda cheese showed the opioid activity. The source of the opioid activity of these extracts was β-casomorphin-5.

2. The quantitative differences in the contents of β-casomorphin-5 in Gouda and acidophilic milk are probably cased by the heterogenous microflora present in these products.

3. The opioid activity of milk and milk products can be a new indicator of the biological activity of food.

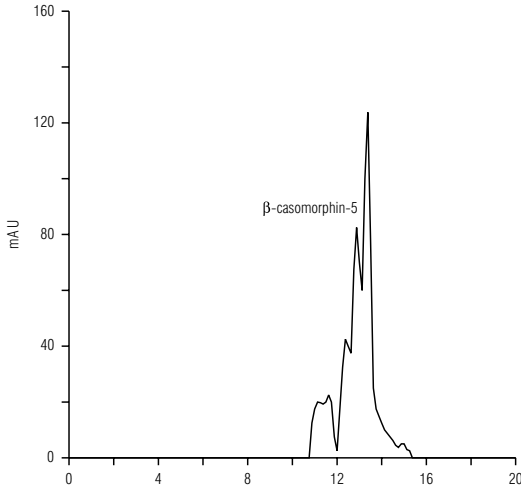

FIGURE 3. HPLC chromatogram of peptide extract from acidophilic milk.

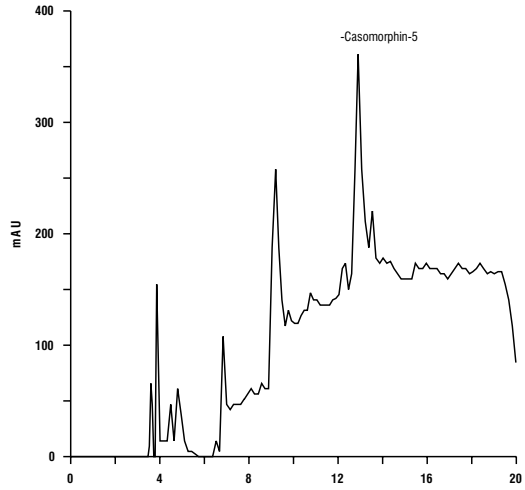

FIGURE 4. HPLC chromatogram of peptide extract from Gouda cheese.

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