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OPIOID ACTIVITY OF HIGH-PROTEIN SUPPLEMENT FOR SPORTSMEN

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Recent investigations have suggested that special food should be evalueted not only from the nutritional but also from the physiological point of view. Opioid peptides derived from milk proteins belong to compounds whose physiological importance has not been fully recognized. Two opioid peptides with agonistic activity (β-casomorphin-5 and -7) were found in a peptide extract from the high-protein supplement (Ultimate Protein) and its pepsin-trypsin hydrolysate. It was concluded that the presence of β-casomorphin-5 and -7 in the peptide extract from high-protein supplement is probably caused by hydrolysis of milk proteins by native enzymes of milk and technological processing. In conclusion, a question arises about consequences of a high intake of food proteins being the precursors of opioid peptides.

INTRODUCTION

The progress of civilization creates new attitude to life. One of these attitudes is the cult of an athletic figure of human. People try to achieve this athletic figure by physical exercises and special supplements. For this reason, an increasing interest is being observed in high-protein supplements. These supplements are mainly prepared on the basis of milk and soy proteins. From the nutritional point of view, an excessive intake of such supplements can be detrimental to health. This opinion seems to be justified when taking into consideration the biological activity of peptides derived from food proteins. An example of these proteins are milk proteins. The following proteins and peptides are classified in this group: hormones from the anterior pituitary gland (*e.g.* prolactin and somatotropin), the hypothalamus (*e.g.* somatotropin-releasing hormone and somatostin), and the gut (*e.g.* vasoactive intestinal peptide, gastrin and substance P). Milk also contains a number of growth factors and a variety of other bioactive peptides, including insulin-like growth factor I and II (IGF), IGF binding proteins, epidermal growth factor (EGF), transforming growth factors, prostaglandin $F_{2\alpha}$ and E, lactofferin and transfferin [Boland *et al*., 2001; Bos *et al*., 2000; Brantl *et al*., 1979; Donovan *et al*., 1994; Kostyra *et al*., 2004; Meisel & Schlimme, 1990; Pihlanto-Leppälä, 2001; Zinn, 1997]. A specific group of biologically-active milk peptides are the opioid peptides. Opioid peptides are generated *in vivo, in vitro* and during food processing. They are largely found in milk, fermented milk and cheeses. Proteolytic enzymes, naturally-occuring in milk and enzymes from lactic acid bacteria or from exogenous sources contribute to the generation of opioid peptides. Dairy processing conditions, such as cheese ripening, are also relevant [Samacchi & Gobetti, 2000]. The precursors of opioid peptides derived from milk proteins are: bovine β-casein, α-casein, β-lactoglobulin, serum and κ-casein. The source of β-casomorphin-5 and –7 is bovine β-casein. The biological activity of β-casomorphins varies. They can influence gastrointestinal motility and food intake as well as induce ananalgesic and sedative effects. Moreover, they can modify respiratory, cardiovascular, immunomodulatory and allergic action [Kostyra *et al.,* 2004].

MATERIAL AND METODS

Experimental material

Investigations were carried out on a commercial high-protein supplement (Ultimate Protein produced by the Trec Nutrition Company, Poland). The composition of protein was a mixture of 80% concentrate of whey protein and 85% of milk protein. The proportion of these proteins was not declared.

Isolation and purification of peptide fraction

Peptides were extracted from the supplement or its pepsin-trypsin hydrolysate as follows: 20 g of the supplement were suspended in 100 mL of water and mixed with 200 mL of a 2:1 (v/v) methanol-chloroform mixture for 1 h. The mixture was kept at room temperature for separation into double layer. The upper layer, containing mainly peptides, was again treated with 100 mL of methanol and centrifuged at 5500×g (Eppendorf Centrifuge 5804R, Hamburg, Germany) for 20 min at 4°C. The supernatant was evaporated at 40°C and lyophilized. Peptide extracts were purified with SPE method using a STRATA C-18T column (140 Å, 50 μm) (Phenomenex, Torrance, CA, USA). The peptide extract was injected into a counterbalance column with 0.1% trifluoroacetic acid (TFA)

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and washed with a mixture of 10% acetonitrile and 0.1% TFA. Peptides were eluted from the column using a mixture of 50% acetonitrile and 0.1% TFA. The elute was lyophilized.

Enzymatic digestion

Enzymatic hydrolysis was performed as follows: 1.3 g of supplement was suspended in 50 mL of 0.1 mol/L acetic acid and boiled for 10 min. The cooled suspension was incubated with 10 mg of pepsin (Sigma P-6887, 4550 U/mg) at 37°C for 4 h. Pepsin was inactivated by increasing pH to 7.8 with 2 mol/L NaOH. Thereafter, 10 mg of trypsin (T-8003, Sigma) were added and hydrolysis was continued for 4 h at 37°C. To inactivate trypsin the solution was heated for 5 min at 95°C. The peptides were isolated the same way as described above.

RP-HPLC chromatography of a peptide extract

Separation of the peptide extract by reversed phase high-performance liquid chromatography (RP-HPLC) was performed using a C-18 Phenomenex Jupiter Proteo column (250 x 4.6 mm, 4 μm, 90 Å), fitted to a Shimadzu system (Japan) composed of a LC-10AD pump, SCL 10A system controller, and SPD M 10 A dual wavelength detector. Samples (1 mg/mL) were dissolved in 0.1% TFA and filterd through a 0.45 μm cellulose acetate filter (Sartorius AG, Germany). Peptides were separated in a gradient system: 100% A (0.1% v/v, TFA in deionized water) for 5 min, then from 0% to 30% B (0.1% v/v, TFA in acetonitrile) over 45 min. Opioid peptides in the extract were identified by comparison of their retention times with that of standard: β-casomorphin-5 and -7 (C5900, C5147-5MG, Sigma-Aldrich, Poznań, Poland). Additionally, the presence of opioid peptides was confirmed using the internal standard method.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein supplement and pepsin-trypsin hydrolysates were analysed by SDS-PAGE following the general procedure of Laemmeli using 15% acrylamide gel [Laemmeli, 1970]; the marker of molecular weight of proteins and peptides was obtained from Sigma.

UV spectra

Ultraviolet spectra of an aqueous peptide extract from the protein supplement and its pepsin-trypsin hydrolysate solutions (concentration of 1 mg/mL) were recorded using a Beckman (USA) DU 7500 spectrophotometer.

Thin layer chromatography

For the thin layer chromatography there were used the Kiesegel 60 F_{254} plates (Merck). Chromatographic analysis was carried out on 0.5 μL of investigated solutions (concentration of 1%). Chromatogram was developed by a solvent mixture: n-butanol: acetic acid: water (3:1:1, v/v) at room temperature. The chromatogram was visualized with a ninhydrin reagent.

RESULTS AND DISCUSSION

The amounts of opioid peptides in the peptide extracts obtained from the Ultimate Protein supplement (Ups) and its pepsin-trypsin hydrolysate are presented in Figures 1 and 2. One opioid peptide, β-casomorphin-5, was identified in the peptide extract obtained from the Ups. While, two opoid peptides, β-casomorphin-5 and -7 were identified in the pepsin-trypsin hydrolysate obtained from the Ups. Both of these peptides belonged to the agonist. The content of these opioid peptides in both peptide extracts were differentiated (Table 1). Contents of these peptides in the peptide extract obtained from the Ups and its pepsin-trypsin hydrolysate expressed in mg/g of the Ups were as follows: 0.004 and 3.260 in the case of β-casomorphin-5 and 0.000 and 2.320 in the case of β-casomorphin-7, respectively. Generally, the content of opioid peptides in the pepsin-trypsin hydrolysate obtained from the Ups was a few dozen times higher. The presence of opioid peptides in the Ups and its pepsin-trypsin hydrolysate requires explanation. This explanation should begin from the fact that

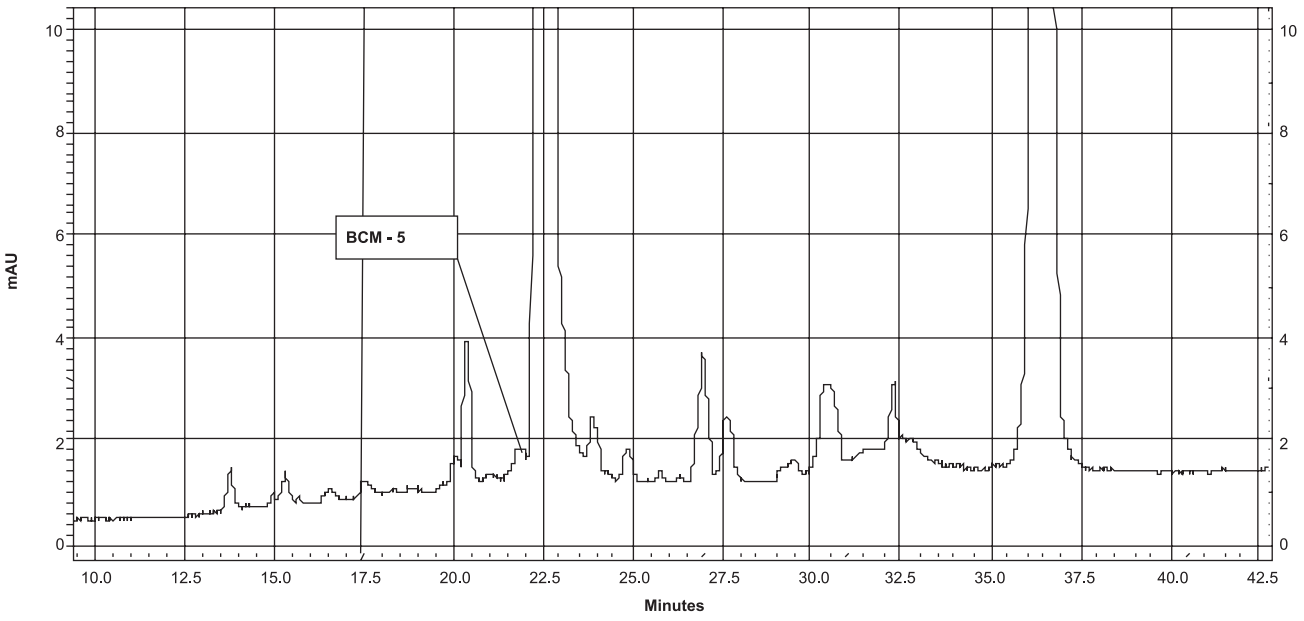

FIGURE 1. RP-HPLC chromatogram of a peptide extract from supplement (BCM-5: β-casomorphin-5).

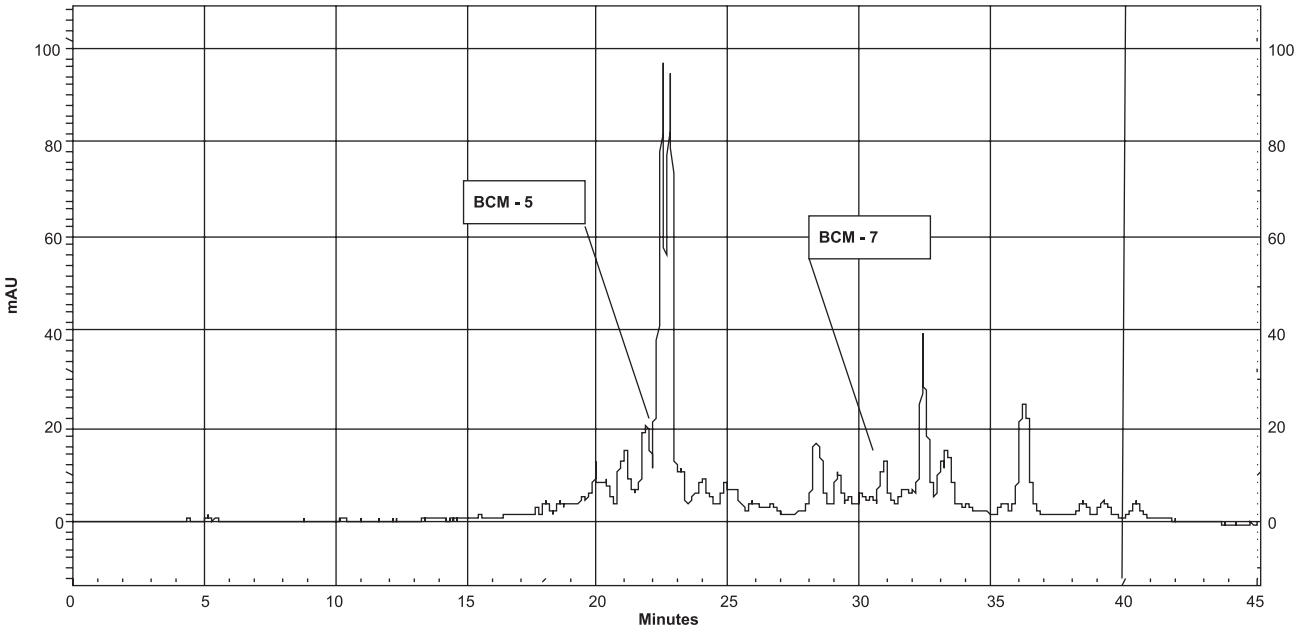

FIGURE 2. RP-HPLC chromatogram of a peptide extract from pepsin-trypsin hydrolysate of supplement (BCM-5: β-casomorphin-5; BCM-7: β-casomorphin-7).

TABLE 1. Content of casomorphins in a peptide extract from supplement and its pepsin-trypsin hydrolysate.

Opioid peptide	Content in peptide extract from	
	supplement (mg/g)	pepsin-trypsin hydrolysate of supplement (mg/g)
β-Casomorphin-5	0.004	3.26
β-Casomorphin-7	-	2.32

neither pepsin nor trypsin are able to liberate β-casomorphin-5 and -7 from cow milk β-casein. Pepsin and trypsin are able to liberate β-casomorphin-6 (Tyr-Pro-Val-Gln-Pro-Phe) from cow's milk β-casein, the sequence of which significantly differs from the sequence of β-casomorphin-7 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) [Jinsmaa & Yoshikawa, 1999; Jarmołowska *et al.,* 1999]. The presence of opioid peptides in dairy products may result from food processing, pH and proteolytic enzymes naturally-occurring in milk [Kostyra *et al*. 2004]. This fact seems to be a very reasonable explanation of the presence of β-casomorphin-5 in the peptide extract obtained from the Ups. The explanation of the presence of β-casomorphin-7 in the pepsin-trypsin hydrolysate obtained from the Ups is more difficult. RP-HPLC chromatogram of the peptide extract obtained from the Ups shows a wide peak appearing at the range of the retention time of β-casomorphin-7. It is likely that in this peak there occur β-casomorphin-7 and other peptides. The pepsin-trypsin hydrolysis of the Ups evoked the hydrolysis of these peptides, except β-casomorphin-5 in the peptide extract obtained from Ups. For this reason, the peak corresponding to the retention time of β-casomorphin-7 is present on the chromatogram of the pepsin-trypsin hydrolysate obtained from the Ups.

The additional confirmation of the presence of β-casomorphin-5 and -7 in the peptide extract can be their UV spectra, SDS-PAGE electrophoresis and thin layer chromatography. UV spectra (Figure 3) characterising the absorption maximum were at about 280 nm. It proves that peptides containing aromatic amino acids occur in the peptide extract. It should be emphasized that in the sequence of β-casomorphin-5 and -7 there occur two aromatic amino acids, Tyr and Phe. Figure 4 shows the electrophoretic separation of supplement proteins and its pepsin-trypsin hydrolysate. The molecular weight of supplement proteins ranged from about 66.0 to 14.2 kDa. After pepsin-trypsin hydrolysis there have been observed not completely hydrolysed protein fractions with molecular weight of about 14.2, 18.0 and 45-60 kDa. It is necessary to note that peptides with molecular weight under 1.5 kDa are not visulised with Commassie Blue reagent. For this reason, peptide extracts from the supplement and its pepsin-trypsin hydrolysate were subjected to thin layer chromatography analysis (Figure 5). Four fractions were identified in the peptide extract from supplement and seven others from its pepsin-trypsin hydrolysate. These results proved that the peptide extracts from the supplement and its pepsin-trypsin hydrolysate contained low

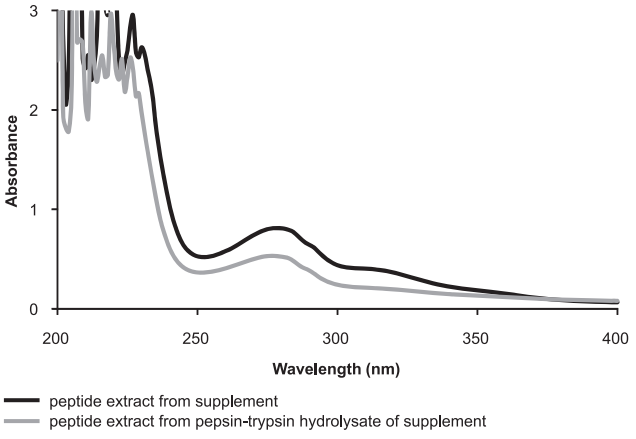

FIGURE 3. UV spectra of peptide extract from supplement and its pepsin-trypsin hydrolysate.

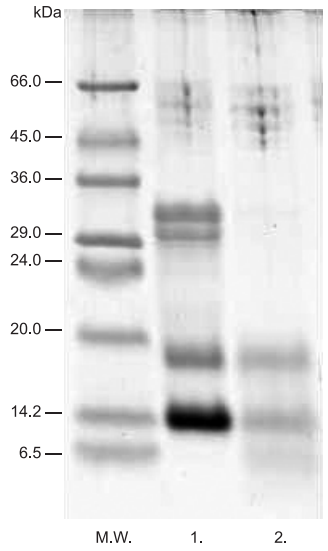

FIGURE 4. Electrophoretic (SDS-PAGE) separation of supplement proteins (1) and their pepsin-trypsin hydrolysate (2) (M.W.-molecular weight marker).

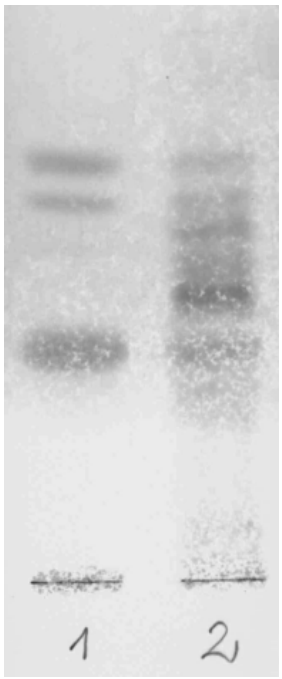

FIGURE 5. Thin layer chromatography of a peptide extract from supplement (1) and its pepsin-trypsin hydrolysate (2).

molecular peptides, *i.e.* casomorphin-5 and 7. The presence of β-casomorphins in dairy products seems to be the natural phenomenon. However, the problem concerns the amount of these peptides in an everyday diet of humans [Jarmołowska *et al*., 2007]. A man with medium daily physical activity should intake about 0.8 g of protein per each kilogramme of body mass. In turn, a sportsman should intake about 1.4 to 2.4 g of protein per each kilogramme of body mass [Hoffman & Falvo, 2004; Lemon, 1995]. Generally, a question arises about the consequences of so high intake of proteins being the precursors of opioid peptides, which can play alike positive and negative physiological functions.

CONCLUSIONS

1. β-Casomorphin-5 and a peptide fraction eluted during HPLC analysis at retention time similar to that of β-casomorphin-7 were identified in the peptide extract from the high-protein supplement.

2. β-Casomorphin-5 and -7 were identified in the peptide extract from pepsin-trypsin hydrolysate of the high-protein supplement.

3. The presence of β-casomorphin-5 and -7 in the peptide extract from the high-protein supplement is probably caused by hydrolysis of milk proteins by native proteolytic enzymes of milk and technological processing.

REFERENCES

1. Boland M., MacGibbon A., Hill J., Desiner milks for the new millennium. Livest. Prod. Sci., 2001, 72, 99–109.
2. Bos C., Gaudichon C., Tomé D., Nutritional and physiological criteria in the assessment of milk protein quality for humans. J. Am. Coll. Nutr., 2000, 19, 191S–205S.
3. Brantl V., Teschemacher H., Hemschem A., Novel opioid peptides derived from casein (β-casomorphins). Z. Physiol. Chem., 1979, 360, 1211–1216.
4. Donovan S.M., Odle J., Growth factors of infant development. Ann. Rev., 1994, 14, 147–167.
5. Hoffman J.R., Falvo M.J., Protein – which is best? J. Sport Sci. Med., 2004, 3, 118–130.
6. Jarmołowska B., Kostyra E., Krawczuk S., Kostyra H., β-Casomorphin-7 isolated from Brie cheese. J. Sci. Food Agric., 1999, 79, 1788–1792.
7. Jarmołowska B., Szłapka-Sienkiewicz E., Kostyra E., Kostyra H., Mierzejewska D., Marcinkiewicz-Darmochwał K., Opioid activity of Humana formula for newborns. J. Sci. Food Agric., 2007, 87, 2247–2250.
8. Jinsmaa Y., Yoshikawa M., Enzymatic release of neocasomorphin and β-casomorphin from bovine β-casein. Peptides, 1999, 20, 957–962.
9. Kostyra E., Sienkiewicz-Szłapka E., Jarmołowska B., Krawczuk S., Kostyra H., Opioid peptides derived from milk proteins. Pol. J. Food Nutr. Sci., 2004, 13/54, SI 1, 25–35.
10. Laemmli U.K., Clevage of structural proteins during the assembly of the head of bacteriophage T-4. Nature, 1970, 227, 680–685.
11. Lemon P.W.R., Do athletes need more dietary protein and amino acids? Int. J. Sport Nutr., 1995, 5, 39–61.
12. Meisel H., Schlimme E., Milk proteins precursors of bioactive peptides. Trends Food Sci. Technol., 1990, 1, 41–43.
13. Pihlanto-Leppälä A., Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. Trends Food Sci. Technol., 2001, 11, 347–356.
14. Smacchi E., Gobetti M., Bioactive peptides in dairy products: synthesis and interaction with proteolytic enzymes. Food Microbiol., 2000, 17, 129–141.
15. Zinn S.A., Bioactive components in milk. Livest. Product. Sci., 1997, 50, 101–103.

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