Casein-derived peptides as an alternative ingredient for low-phenylalanine diets

Péptidos derivados de la caseína como ingrediente alternativo para las dietas bajas en fenilalanina

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Abstract

Introduction: casein-derived peptides can be liberated both in vivo via normal digestion of casein, as well as in vitro via enzymatic hydrolysis. These peptides were suggested to have biological activity.

Objectives: the aim of this study was to describe the production and characterization of casein peptides and to explore the potential of these peptides as an option for low-phenylalanine diets.

Methods: peptides were produced by tryptic hydrolysis of sodium caseinate and acid precipitation with HCl, followed by precipitation with ethanol or aggregation of CaCl2 or ZnSO4.

Results: the amino acid analysis revealed a significant reduction in the amount of phenylalanine from the original protein.

Conclusion: casein-derived peptides could be a future alternative of short chain peptides to low-phenylalanine formulations.

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INTRODUCTION

Caseins are the main milk proteins; they constitute a heterogeneous group of phosphoproteins present as stable calcium phosphate-protein complexes named micelles. Their biological functions include supplying phosphate (PO₄) and proteins to neonate, and the release of peptides with biological activity (1). Casein-derived peptides can be liberated both in vivo via normal digestion of casein, as well as in vitro via enzymatic hydrolysis. These peptides have been shown to enhance intestinal maturation in newborns with symptoms of infantile diarrhoea or necrotizing colitis (2). Additionally, they can stimulate insulin secretion and lower postprandial blood glucose levels, which demonstrates their relevance for diabetes 2 prevention and management (3). Moreover, some casein-derived peptides are high-energy phenylalanine-free food sources that can play a unique role on nourishing and preventing major neurocognitive deficits on patients with phenylketonuria (PKU) (4).

Phenylketonuria (PKU) is a hereditary metabolic disorder caused by a deficiency of hepatic phenylalanine hydroxylase, which converts the amino acid phenylalanine into tyrosine. Classical phenylketonuria has an international average incidence of 1:11,000 living newborns. If left untreated, the patient will present high phenylalanine concentrations in blood and tissues. This results in severe clinical manifestations such as mental retardation, epilepsy, and behavioral problems. Early detection and strict dietary control is thus essential for those patients (5-8).

The goal of a treatment with dietary restriction is to maintain blood phenylalanine concentrations within defined target limits, which may vary from country to country (7). The diet should be supplemented with formulas composed of mixtures of amino acids and/or derivatives of proteins in which phenylalanine has been reduced or excluded. Several phenylalanine-free formulas are available in the market. They are a mixture of amino acids plus carbohydrates, vitamins, minerals, and other ingredients, depending on the formula. Usually, they come in powder form and must be reconstituted in water according to the amount recommended by the health professional who monitors the patient. The treatments available may affect absorption of trace elements due to the fact that those diets are poor in animal protein and rich in plant fibers and phytates (9).

Therefore, the nutritional management of PKU would greatly benefit from new dietary options, in addition to new synthetic amino acids formulations that facilitate ingestion of a low-phenylalanine protein source throughout the day (10). Our objective is to explore the potential of casein-derived peptides as an option for low-phenylalanine diets and to describe the production and characterization of these peptides.

MATERIALS AND METHODS

MATERIALS

Casein-derived peptides were obtained from sodium caseinate (Lactonat HV, Lactoprot Deutschland GmbH), with the following composition: protein: 87.5%; moisture: 5.8%; ash: 5.3%; fat: 1.7%; lactose: 0.3%; and pH: 6.6. The Pancreatic Trypsin Novo (PTN 6.0S) enzyme was kindly provided by Novozymes (Novozymes A/S, Denmark).

CASEIN DIGESTION

Peptides were produced by three different methods, although under the same hydrolysis conditions. In the first process, sodium caseinate was hydrolyzed by trypsin with an enzyme to substrate ratio of 1:100, for 240 minutes, at pH 8 and at a temperature of 50 °C. Subsequently, the pH was adjusted to 4.64 with 2M HCl. The insoluble precipitate form was removed via centrifugation at 3,000 xg for ten minutes. This procedure aimed to remove the hydrophobic peptides from protein hydrolysate. CaCl₂ (1%) and ethanol (50%) were added to the supernatant; then the precipitate was collected by centrifugation at 4,000 xg for 15 minutes. These peptides were lyophilized and named peptide A (PA).

For the second process, the same procedures were performed as described for PA, but after the addition of CaCl₂, the solution was ultrafiltered through a 10 kDa membrane. The pH of the retentate was reduced to 3.55. The product was submitted to diafiltration (1 kDa) with water and concentrated. Thereafter, the pH was raised to 7. The product was lyophilized and named peptide B (PB).

For the third process, the initial steps, which correspond to an acid precipitation, were similar to the previous ones, however 1.1% ZnSO₄ was added in order to promote the aggregation of peptide to zinc. The solution was ultrafiltered through a 10 kDa membrane, diafiltered (1 kDa) and concentrated. The product was then lyophilized and named peptide C (PC).

CHARACTERIZATION OF CASEIN PEPTIDES

Composition

Samples were analyzed for nitrogen content by micro-Kjeldahl. Protein content was calculated by multiplying the nitrogen content by 6.25. Ashes were measured via incineration method at 450 °C. Phosphorus content was determined by the molybdovanadate colorimetric method, procedures 22.042-2045 (11).

Amino acids

For the determination of amino acids, samples were hydrolyzed at 110 °C in the presence of 6M HCl for 22 hours. By the end of this period, the acid had evaporated; the pH was raised to 8 and the volume to 20 ml. Before the analysis, samples were filtered through 0.22 µm membranes. The analysis was performed by a PNA 402 (Protein and Nucleic Acid Analyzer, Applied Biosystems), with borate buffer at pH 9 at 15 °C. The detection was made on 400 nm/500 nm with a 10 mW laser and 500 KHz.
Predicted protein efficiency ratio

Predicted protein efficiency ratio (PER) was calculated according to Alsmeyer, Cunningham and Happich (12).

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P-PER = -0.468 + 0.454 \times \text{leucine} - 0.105 \times \text{tyrosine}
\]

RESULTS AND DISCUSSION

The chemical analysis of the products (Table I) revealed that the protein content of PC is about 84% similar to the content of sodium caseinate, indicating that only a few peptides were removed from the original protein. PA and PB showed higher content of ashes (19-29%) and lower content of proteins (64-67%).

The association of ions to molecules of casein was reviewed by Gaucheron et al. (13). The authors evaluated the binding of different cations (calcium, manganese, zinc, copper and iron) to caseinate and the stability of these associations under different physicochemical conditions.

Some patents suggested the possibility of purification of casein-derived peptides with the use of any divalent or trivalent minerals. Calcium chloride was the preferential base used, but using ferric chloride or zinc sulfate is also possible (14-16).

Sample PB, obtained with the use of calcium chloride, presented high level of ashes, indicating high concentration of the mineral calcium due to the fact that it was not dissociated from the product by the end of the process (in the diafiltration).

The production of peptides from the digestion of casein increases the bioavailability of calcium (17). Ingestion of casein and β-casein produces casein phosphopeptides in vivo in the intestinal tract of rats and is associated with a higher concentration of soluble calcium in the distal ileum lumen (18).

Studies of bone mineral status of children and adults with phenylketonuria showed delayed maturation of the skeleton and osteoporosis (19,20). Thus, casein-derived peptides could be used to improve the profile of bone mineralization of those patients by increasing calcium intake (21).

AMINO ACIDS

Table II shows the values of amino acid composition of peptides obtained. The amino acid composition of sodium caseinate showed high levels of phenylalanine. The comparison of the amino acid profile of peptides obtained and sodium caseinate demonstrated a significant reduction in the levels of phenylalanine.

Tryptophan was not determined because this amino acid is destroyed during the acid hydrolysis used in the preparation of the samples.

The advantage of using casein-derived peptides, instead of hydrolysates treated for the elimination of phenylalanine, is that casein-derived peptides do not suffer high losses of amino acids that could compromise the nutritional value of these compounds.

Bernardi et al. (22) found that the use of a vacuum evaporator to remove HCl promotes loss of threonine and serine, primarily related to destruction by heat. The neutralization with citrate/NaOH reduces these losses. However, the inclusion of salts interferes with the determination by capillary electrophoresis, and for this reason, neutralization was not adopted.

Comparing the amino acids profile of the obtained peptides (Table II) with the Food and Agriculture Organization/World Health Organization (FAO/WHO) (23) recommendations, an average value of 10 g of protein per day is the recommended protein intake for a three-year-old child weighing approximately 15 kg (0.66 g/kg/day). Essential amino acids analyzed in samples PB and PC were sufficient to meet the amino acid requirements for preschool children and adults (23). PA, PB and PC could contribute significantly to the proper daily intake of non-essential amino acids such as serine, glycine, glutamic acid, alanine, aspartic acid, and arginine.

Amino acid analysis revealed a significant reduction in the amount of phenylalanine (Phe) in the PA. The value for this product represented a reduction of 72.33% compared to sodium caseinate, and of 51.89% and 26.61% for other studies which developed hydrolysates with low phenylalanine (5,24). These studies used charcoal in the adsorption of amino acid, which may cause loss of essential amino acids due to the low specificity of coal.

Peptides PA, PB and PC resulted in products with 12.70 mg, 21.60 mg and 31.90 mg of Phe per gram of protein, respectively. Phenylalanine tolerance is variable among phenylketonurics, depending on their residual enzyme activity. This tolerance ranges between 200 mg/day and 2,000 mg/day. The majority of patients with severe PKU tolerate less than 500 mg/day of phenylalanine. Vegetables and cereals are the main sources of protein in their diets (25). Supposing that the recommendation of daily protein intake for a three-year-old child is 0.66 g/kg/day (23), a formulation that uses the PA peptide will respect the acceptable daily intake of phenylalanine to children with phenylketonuria.

Protein efficiency ratio (PER) is an indicator of protein quality in food. The predicted PER value obtained for sodium-caseinate

| Table I. Composition of sodium caseinate and casein peptides |
|---------------------------------|----------------|----------------|
| **Protein (%)** | **Ash (%)** | **Phosphorus (%)** |
| Na-caseinate | 87.5 ± 0.9 | 5.77 ± 0.07 | - |
| PA | 64.28 ± 0.60 | 19.06 ± 0.03 | 2.89 ± 0.15 |
| PB | 67.59 ± 0.38 | 29.23 ± 0.14 | 1.45 ± 0.08 |
| PC | 84.85 ± 0.56 | 16.14 ± 0.0003 | 1.05 ± 0.03 |

Each value is the average ± standard deviation of triplicate analysis.
and the different casein derived peptides accessed based on amino acid analysis were of 2.8 for sodium-caseinate, 2.1 for PA, 2.9 for PB and 2.6 for PC. PER value greater than 2 are well correlated with a protein’s adequate capacity to promote physical growth and development. Therefore, all casein-derived peptides seem to be well recommended to patients within various life stages, including those with greater nutritional demand (12,26).

The use of protein hydrolysates containing short-chain peptides is highly desired in dietary formulas. The absorption of short-chain peptides is considered to be more efficient compared to an equivalent amount of free amino acids. This is due to the availability and speed of peptides’ specific transport system in the enterocyte, and to their subsequent break into amino acids, caused by the action of cytoplasmic peptidases before transportation to the blood circulation. Moreover, the transport of free amino acids is easily saturable and competitive, decreasing the speed and the rate of absorption (27).

Furthermore, peptides are less hypertonic mixtures of amino acids, facilitating the absorption of other dietary components, thus reducing osmotic problems. Due to chemical instability and insolubility in water, some amino acids, such as glutamine, tyrosine, and cysteine cannot be easily administered in free form (27).

Additionally, peptides derived from proteins of bovine milk have the potential of exerting beneficial physiological effects, hence why they are called bioactive peptides. Anti-hypertensive, anti-inflammatory, antioxidant, immunomodulatory, antimicrobial, opioid agonist, opioid antagonist, and antiproliferative activities are the main functions that can be performed by bioactive casein-derived peptides in different organism systems (28).

A dietary treatment option for PKU patients is the glycomacropeptide (GMP), a protein derived from k-casein via the action of chymosin. This protein is palatable, rich in branched chain amino acids, and it does not present aromatic amino acids (phenylalanine, tryptophan and tyrosine) in its natural composition (29). GMP contains limited amounts of histidine, indispensible for individuals with PKU (30). Commercial highly-purified GMP contains less than 2.0 mg phenylalanine per gram of protein. However, to provide a complete source of protein for individuals with PKU, GMP must be supplemented with arginine, histidine, leucine, tyrosine, and tryptophan (31).

**CONCLUSION**

The protein efficiency ratio of the obtained peptides demonstrated that they can be used as a main protein source. Therefore, the most important finding of this study was that casein-derived peptides could be used in the future as an important alternative in the preparation of low-phenylalanine formulations.

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REFERENCES

1. Rasmussen LK, Johnsen LB, Tørrilår A, Sorensen ES, Thomesen, JK, Nielsen NC, et al. Disulphide-linked caseins and micelles. Int Dairy J 1999;6(3):215-8. DOI: 10.1016/S0958-6946(98)00063-1

2. Cossais F, Clavin-Râdecker I, Lorenzen PC, Klempt M. Tryptic β-casein hydrolysate modules enteric nervous system development in primary culture (short communication). J Dairy Sci 2017;100(5):3396-403. DOI: 10.3168/jds.2016-11440

3. Jonker J, Wijngaarden M, Kloeck J, Groeneveld Y, Gerhardt C, Brand R, et al. Effects of low doses of casein hydrolysate on post-challenge glucose and insulin levels. Eur J Int Med 2011;22(3):245-6. DOI: 10.1016/j.ejim.2010.12.015

4. Daly A, Evans S, Chahal S, Santra S, MacDonald A. Glycomacropeptide in children with phenylketonuria: does its phenylalanine content affect blood phenylalanine control? J Hum Nutr Diet 2017;30:515-23. DOI: 10.1111/jhn.12376

5. López-Bajonero LJ, Iara-Calderon P, Gálvez-Mariscal A, Velásquez-Arellano A, López-Munguia A. Enzymatic production of a low-phenylalanine product from skim powder and caseinate. J Food Sci 1991;56(4):938-42. DOI: 10.1111/j.1365-2621.1991.tb14610.x

6. Fishberg RM, Silva-Fernandes ME, Fishberg M, Schmidt BJ. Plasma zinc, copper, and erythrocyte superoxide dismutase in children with phenylketonuria. Nutrition 1999;15(8):449-52. DOI: 10.1016/S0899-9007(99)00022-9

7. Ahring K, Quintana AB, Dokoupil K, Ozel HG, Lammardo AM, Mac Donald A, et al. Dietary management practices in phenylketonuria across European centres. Clin Nutr 2009;28(3):231-6. DOI: 10.1016/j.clnut.2009.03.004

8. Ahring K, Quintana AB, Dokoupil K, Ozel HG, Lammardo AM, Mac Donald A, et al. Blood phenylalanine control in children with phenylketonuria: a survey of 10 European centres. Eur J Clin Nutr 2010;65(2):275-8. DOI: 10.1038/ejcn.2010.258

9. Rijn MV, Hoeksma M, Sauer P, Szczerbak B, Gross M, Reijngoud DJ, et al. Protein metabolism in adult patients with phenylketonuria. Nutrition 2007;23(6):445-53. DOI: 10.1016/j.nut.2007.03.009

10. MacLeod EL, Clayton MK, Calcar SCV, Ney DM. Breakfast with glycomacropeptide compared with amino acids. Am J Clin Nutr 2009;89:1068-77. DOI: 10.3945/ajcn.2008.27280

11. Association of Official Analytical Chemists International. Official methods of analysis of ADAC International. 15th ed. Arlington; 1990.

12. Alsmeyer RH, Cunningham AE, Happich ML. Equations predict PER from amino acid analysis. Food Technol 1974;28:34-8.

13. Gaucheron F, Graet YL, Groeneveld Y, Gerhardt C, Brand R, et al. Effects of low doses of casein hydrolysate on post-challenge glucose and insulin levels. Eur J Int Med 2011;22(3):245-6. DOI: 10.1016/j.ejim.2010.12.015

14. Reynolds EC. Production of glycomacropeptide from casein. United States Patent n° US 6448374. 2002.

15. Kitts DD, Yuan YY, Nagasawa T, Moriama Y. Effects of casein, casein phosphopeptide and calcium intake on ileal 45Ca disappearance and postprandial systolic blood pressure in spontaneously hypertensive rats. Br J Nutr 1992;68(3):765-81. DOI: 10.1079/BJN19920132

16. Reynolds EC. Production of glycomacropeptide from casein. United States Patent n° US 6448374. 2002.

17. Kitts DD, Yuan YY, Nagasawa T, Moriama Y. Effects of casein, casein phosphopeptide and calcium intake on ileal 45Ca disappearance and postprandial systolic blood pressure in spontaneously hypertensive rats. Br J Nutr 1992;68(3):765-81. DOI: 10.1079/BJN19920132

18. Al-Qadreh A, SchulpisKH, Athanasopoulou H, Mengrelli C, Skarpalezou A, Zoskaki I. Bone mineral status in children with phenylketonuria under treatment. Acta Paediatr 1998;87(11):1162-6. DOI: 10.1111/j.1651-2227.1998. tt00924.x

19. C. C. H. Krüger et al. Disulphide-linked caseins and micelles. Int Dairy J 1999;6(3):215-8. DOI: 10.1016/S0958-6946(98)00063-1

20. Groot MJ, Hoeksma M, Rijn MV, Sart RJ, Sprossen FJ. Relationships between lumbar bone mineral density and biochemical parameters in phenylketonuria patients. Mol Genet Metab 2012;105(4):566-70. DOI: 10.1016/j.ymgme.2012.01.006

21. Modan-Moses D, Vered I, Schwatz G, Anikster Y, Abraham S, Segrev R, et al. Peak bone mass in patients with phenylketonuria. J Inherit Metab Dis 2007;30:202-8. DOI: 10.1007/s10545-007-0462-9

22. Bernardi CR, Luiz MTB, Zanotto DL, Guidoni AL. Preparo de hidrolisados proteicos para a análise de aminoácidos. Ciência Tecnol Aliment 2003;3(33):37-22. DOI: 10.1590/S0101-206120030300004

23. Food and Agriculture Organization (FAO); World Health Organization (WHO). Evaluation on protein and amino acid requirements in human nutrition. Report of the joint FAO/WHO/UNU. Expert consultation on protein and amino acid requirement in human nutrition. United Nation University. WHO Technical Report Series nº 935. Geneva; 2007.

24. Monteiro JLB. Desenvolvimento de uma formulação proteica com teor reduzido de fenilalanina [Dissertation]. Curitiba: Federal University of Paraná; 2003.

25. Lammardo AM, Robert M, Rocha JC, Van Rijn M, Ahring K, Bélanger-Quintana A, et al. Main issues in micronutrients supplementation in phenylketonuria. Mol Genet Metab 2013;110:1-5. DOI: 10.1016/j.ymgme.2013.08.008

26. Ayalew Y, Retta N, Dessie G, Mohammed A, Mellesse A. Amino acid profile and protein quality in tuber and leaf of Coccnia abyssinica (Lam.) (Cogn.) accessions of Ethiopia. Food Sci Nutr 2017;5:722-9. DOI: 10.1002/fsn3.452

27. Clement A. Enzymatic protein hydrolysates in human nutrition. Trends Food Sci Technol 2000;11(7):254-62. DOI: 10.1016/S0924-2244(01)00007-3

28. Zoskaki I. Bone mineral status in children with phenylketonuria under treatment. Acta Paediatr 1998;87(11):1162-6. DOI: 10.1111/j.1651-2227.1998. tt00924.x

29. Strisciuglio P, Concolino D. New strategies for the treatment of phenylketonuria. Nutr Metab 2007;4:63. DOI: 10.1002/nm.1237

30. Hernández-Ledesma B, García-Nebot MJ, Fernández-Tomé S, Amigo L, Recio I. Dairy protein hydrolysates: peptides for health benefits. Int Dairy J 2013;10:111-5. DOI: 10.1016/j.idairyj.2013.11.004

31. Van Calcar SC, MacLeod EL, Gleason ST, Etzel MR, Clayton MK, Wolff JA, et al. Improved nutritional management phenylketonuria by using a diet containing glycomacropeptide compared with amino acids. Am J Clin Nutr 2009;89:1068-77. DOI: 10.3944/ajcn.2008.27280

32. Reynolds EC. Production of glycomacropeptide from casein. United States Patent n° US 6448374. 2002.