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Specific Enzymatic Release of an A-casein-exorphin from Milk Casein

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Abstract: The specific enzymatic generation of an αs1-casein-exorphin from milk casein was achieved by using the Staphylococcus Aureus strain V8 protease. The kinetic of proteolysis of the substrate was studied with the free and the immobilized form of the enzyme. The continuous process with the immobilized enzyme appeared highly effective for the purpose in term of yield, giving the maximal rate of bioactive peptide which could be expected. The hydrolysate was partially purified and tested for its opioid potency in the rat vas deferens bioassay system.

Key words: α-casein-exorphin, peptide, enzymatic release

INTRODUCTION

In addition to their classical uses in the food industry, due to their nutritional quality, protein molecules and particularly caseins, could give rise to other non-conventional uses because of their functional properties and the physiological roles associated with peptides whose sequences are encrypted in the primary structure of the protein mother. In these fields, the occurrence of polar and non polar side chains in the aminoacids, forming their polymeric chain, makes protein molecules surface active so that they adsorb at interfaces. This property allows the stabilization of colloidal dispersion and awards to proteins a key role in the stabilization of foam, emulsions, and composite systems in food products and in cosmetics[1].

Besides, milk proteins are believed to be the main sources of biologically active peptides such as opioid peptides, mineral-binding peptides, immunomodulating peptides, antithrombotic peptides, angiotensine-I-converting inhibitory peptides and antimicrobial peptides[2-4].

For the above biological potencies it is evident that several possibilities for the design of dietary products and natural drugs were considered, especially from an industrial point of view.

For example, Phosphopeptides, originated from bovine caseins have already found interesting applications both as dietary supplements and as pharmaceutical preparations[5].

In the particular case of the casein-opiate-like acting peptides, the main sources of these bioactive peptides are β and α s1-caseins. Different peptidic fragments were purified and characterized. They have been obtained after non specific protease digestion of caseins and named β-casomorphins[6] and α-casein-exorphins[7] owing to their exogenous origin and morphine-like activity. However, since their finding and structure elucidation, knowledge about the identity and the biological potencies of these opioid-agonist peptides was obtained subsequently by the use of synthetic peptides, and, up to now, no enzymatic-based process has been developed for their production from a natural source at a preparative scale. This could be explained by the lack of specific hydrolysis sites and consequently by the need of a multi-enzymatic system to attack casein sequences and to liberate bioactive peptides[8].

Since the aim of this study was to develop an enzymatic process for the generation of the αs1-casein exorphin corresponding to the 90-96 fragment, the protease of Staphylococcus Aureus strain V8 and the bovine α-casein, were chosen. This enzyme cleaves specifically peptide bonds on the carboxyl side of glutamic and aspartic aminoacid residues[9]. The occurrence of glutamyl residues in 89 and 95 positions of the primary structure of αs1-casein makes it suitable for the purpose.

MATERIALS AND METHODS

Preparation of α-s casein: Whole casein was obtained from skim-milk by isoelectric precipitation at pH 4.6 and 20°C. The α-s casein (a mixture of αs1 and αs) was purified from whole casein by ion exchange chromatography in the presence of urea and 2-mercaptoethanol according to the procedure originally described by Thompson[10]. Briefly, the natriumcaseinate (250 mg) was dissolved in 10 mL of equilibration buffer (10 mM phosphate, pH 7, 3.3 M urea, 0.83% (v/v) 2-mercaptoethanol) and stirred overnight at 4°C. This solution was then chromatographied on a column of DEAE trisacryl

(Pharmacia) (10x1 cm). Elution was performed in the equilibration buffer using a gradient from 0 to 0.25 M NaCl at a flow rate of 1 mL min^{-1}. The different peaks were dialysed and lyophilized. Their purity was controlled by Sodium Dodecyl Sulfate polyacrylamid gel electrophoresis.

Enzyme source: The Staphylococcus Aureus strain V8 protease (EC.3.4.21.19) was purchased from Sigma (France). One enzyme unit (U) was defined as the quantity of enzyme catalysing the hydrolysis of 1 mM of N-t-Boc-L-glutamic acid-α-phenylester per minute at 37°C and pH 7.8.

Enzyme immobilization: Sepharose 4B-CNBr activated was from Pharmacia. The resin 0.5 g, was suspended in 25 mL of 1 mM HCl for 20 min and rinsed with the same solution. The protease (400 U) was dissolved in 2 mL of coupling buffer (0.1 M carbonate/bicarbonate, pH 8.2, 0.5 M NaCl). The resin was washed once with the coupling buffer and immediately transferred to the protease solution. The coupling takes place in 2 h at room temperature under gentle stirring. The excess of reactive groups was blocked by adding 5 mL of 0.2 M glycin buffer, pH 8. Two hours later the excess of buffer was removed and the bead washed first with the coupling buffer followed by 0.1 M acetate buffer, pH 4, 0.5 M NaCl and finally with the coupling buffer. The coupled bead was then mixed with an equal volume of sephadex G50 and storred at 4°C in 30 mM phosphate buffer, pH 7.8 containing 0.2% of natriumazid.

α-s casein hydrolysis: Hydrolysis of α-s casein was performed in 30 mM phosphate buffer pH 7.8 and at 37°C. The substrate solution was treated either with the free enzyme at different enzyme to substrate ratios or with the immobilized form of the protease. α-casein, 50 mg in 10 mL 30 mM phosphate buffer was applied to a thermostated column filled with the coupled enzyme and connected to a feeding reservoir. The closed circuit circulation of substrate solution was achieved by means of peristaltic pump at 0.1 mL min^{-1}.

Analytical procedures: The hydrolysis rate of α-casein with the staphyloccal protease was checked by 17% sodium-dodecyl-sulfat polyacrylamid gel electrophoresis.

The time course of peptide generation was followed by TLC using 0.2 mm thick silica gel 60 plates with n-butanol/acetic acid/water (80:20:20, by vol.) as mobile phase and the 90-96 αs1-casein-exorphin synthetic peptide (from SIGMA) as standard. At required times, aliquots of incubation mixture were withdrawn, run on silica gel plates and the reaction products were revealed by ninhydrin spray and drying the plates at 120°C. Quantitative estimation of α-casein exorphin was carried out by TLC. Chromatograms were scanned and analysed using Gel Pro 32 analyser software (from Media Cybernetics).

The partial purification of hydrolysate was achieved by gel filtration. Total hydrolysate was chromatographied on Sephadex G 25 column (90x1.5 cm) equilibrated with 30 mM phosphate buffer pH 7.8. Fractions corresponding to the pic with the same elution volume than synthetic peptide were pooled and lyophilized.

Determination of opioid activity: Opioid activity was determined in the isolated vas deferens of rat (RVD)[11] . *Vasa deferencia* was discarded out. The semen was gently expressed from the lumen and the tissue mounted in 5 mL organ bath. All measurements were done at 37°C in Krebs solution aerated with 95%O_2 and 5% CO_2. The intramural nerves were excited by electric pulses applied through platinum electrodes placed on either side of the vas. The samples were tested for an inhibitory effect on the electrically induced contractions of organ preparation. The inhibition was considered as an opioid effect if it could be antagonised by Naloxone (a specific opiate antagonist).

RESULTS

Hydrolysis of α-casein with free protease: Preliminary trials for the hydrolysis conditions performed over 3 hours with a fixed enzyme to substrate ratio or by mixing increasing units of protease with a constant amount of α-casein, showed that the electrophoretic pattern of the hydrolysate remained evolutionary.

Fig.1 shows the time course of a batch process performed at 37°C and pH 7.8 with an enzyme to substrate ratio of 50 U mg^{-1}. The substrate appears to be completely transformed after 120 and 180 min (lanes 8 and 9), while new bands could still be generated by adding 100 U of enzyme per mg of α-casein (lane 6). Since the specific activity of the staphylococcal protease was weak, it was impossible to perform experiments with so high ratios. Subsequently, the hydrolysis of α-casein was carried out with lower enzyme rates and at prolonged time to establish the highest proteolytic rate of substrate that could be reached. A ratio of 10 U mg^{-1} of enzyme to substrate (1.36% w/w) and 30 hours of incubation time were necessary for the complete hydrolysis of α-casein.

Hydrolysis of α-casein with immobilized enzyme: In this attempt, a column, filled with the protease immobilized on a Sepharose bed supplied with feeding reservoir

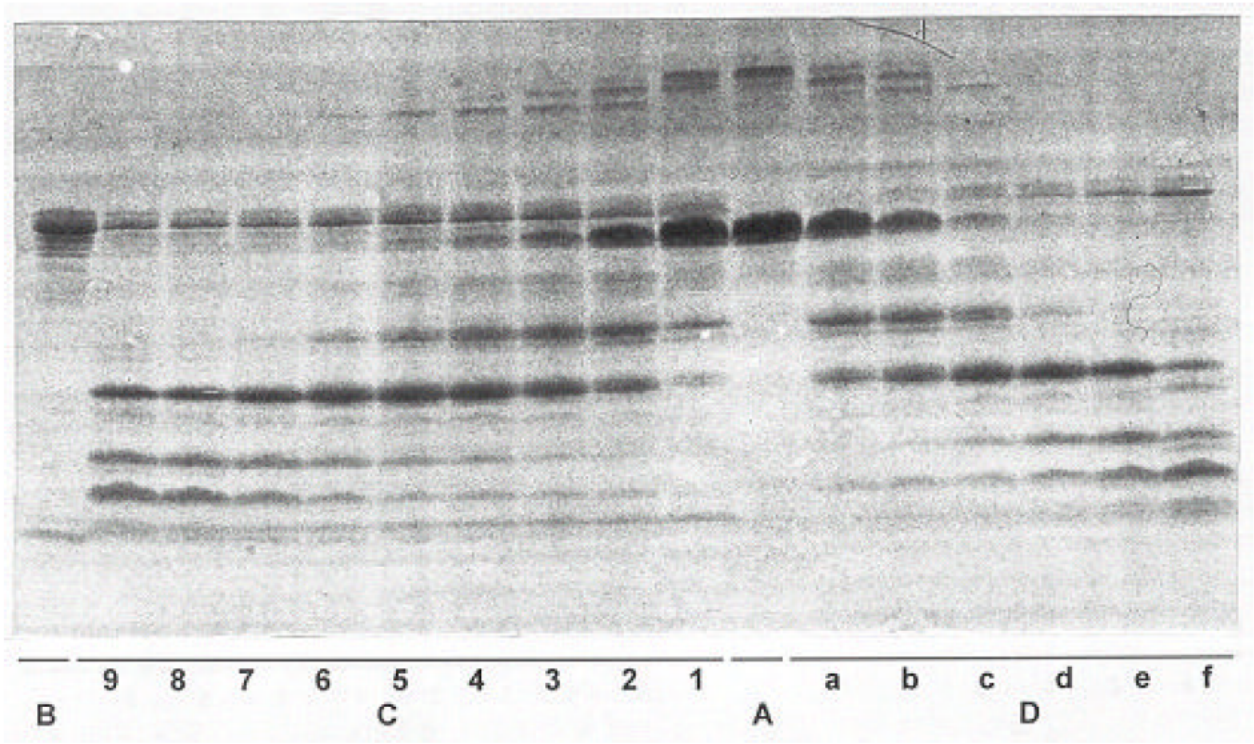

Fig.1: Sodium-dodecyl-sulfat polyacrylamid gel electrophoresis (17%) pattern of (A) α-casein, (B) protease from staphylococcus Aureus, (C, D) α-casein hydrolysed with protease.α-casein with protease at fixed enzyme to substrate ratio (50 U mg^{-1}): 1 min (lane 1), 5 min. (lane 2), 10 min (lane 3), 15 min (lane 4), 20 min. (lane 5), 30 min (lane 6), 60 min (lane 7), 120 min (lane 8) and 180 min (lane 9) α-casein with variable enzyme to substrate ratios (U mg^{-1} of substrate) at fixed time (180 min.): 3.12 U (lane a), 6.24 U (lane b), 12.5 U (lane c), 25 U (lane d), 50 U (lane e) and 100 U (lane f)

Table 1. Production of α-casein-exorphin: Comparison between the batch process with the free enzyme and the closed circuit process with the immobilized enzyme

		α-casein-exorphin (mg) Hydrolysis time (h)		
Hydrolysis process	α-casein (mg)	15	20	30
free enzyme[a]	100	1.84	2.67	3.43
Immobilized enzyme[b]	100	3.66	3.72	3.81

[a] The hydrolysis process using the free enzyme was carried out at 37°C with enzyme to substrate ratio of 10 U mg^{-1} (1.36% w/w). [b]The continuous process with the immobilized enzyme (400 U) was carried out at 37°C. α-casein, 50 mg in 5 mL, was treated in closed circuit system

and pump in a closed circuit system, was used to carry out a continuous process. When the feeding reservoir was connected to the column, α-casein began to be hydrolysed in small amounts and a gradient of preferential hydrolysis sites was created. Aliquots of incubation mixture were withdrawn for checking the hydrolysis rate. A detectable amount of the peptide was already observed after 15 h from the beginning of the proteolysis process and remained unchanged over 30 h (Table 1).

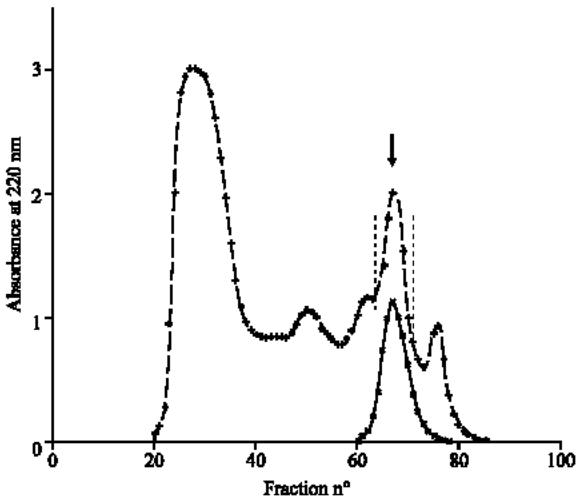

Fig.2: Gel filtration chromatography on sephadex G25 of (+): 20 h digest of α-casein by the Staphylococcus Aureus protease, (x): Synthetic peptide.The position of α-casein-exorphin is indicated by an arrow. Pooling is indicated by hatched lines.

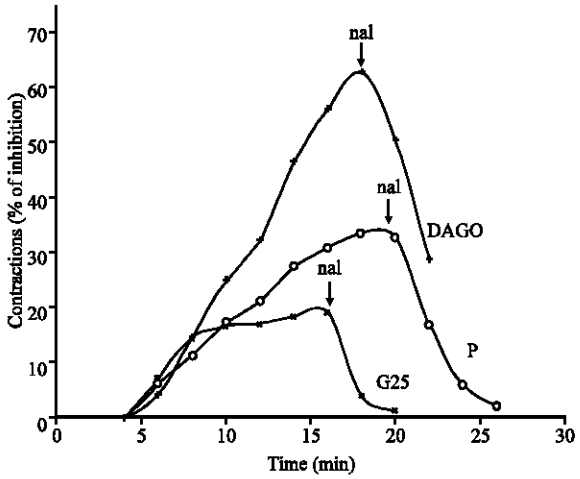

Fig.3: Comparative inhibitory effect produced by: The partially purified hydrolysate of α-casein on Sephadex G25 (100 μg), the synthetic peptide P (100 μg) and DAGO (40 μg) on the electrically stimulated contractions of rat vas deferens. Add: Denotes the addition of the tested substance. Nal: Denotes the addition of 0.25 mM naloxone.

Opiate-like activity: The opiate-like activity of the partially purified enzymatic hydrolysate was made *in vitro* on RVD bioassay system. After concentration, the pic with the same elution volume as the synthetic peptide (Fig. 2) was concentrated and assayed for its biologic effect. An inhibition of the electrically induced contractions of the tissue was obtained by 100 μg. This inhibition was regarded as an opioid-like effect since it was reversed by Naloxone.

Results are presented in Fig. 3 comparatively to those obtained by the synthetic α-casein-exorphin (100 μg) and DAGO (D-Ala^2-N-Me-Phe^4-Gly^5-ol-Enkephalin) (40 μg) an opioid agonist substance[10].

DISCUSSION

In this study, as the target was to select the best operative conditions for high αs1-exorphin production and maximal recovery of enzyme activity, it appeared of interest to test enzyme activity in its free and immobilized forms. In fact, a comparison among different processes represents an important aspect when an enzymatic process has to be developed[12,13]. The hydrolysis of αs1-casein was carried out in batch with the enzyme in its free form and in continuous operative conditions on column with the immobilized protease on a sepharose bed. The hydrolysis of the substrate in a closed circuit system with immobilized enzyme allowed the maximal yield in a shorter time and avoided both enzyme auto-destruction and its presence in the final hydrolysate. To analyse the possibility of using the immobilized enzyme for new α-casein-exorphin production, the enzyme activity was tested 2 months later. No decrease of its activity has been noted.

The weak inhibitory effect caused by the partially purified hydrolysate comparatively to the synthetic peptide could be correlated to the degree of purity reached by the technique used. Assuming that αs1-casein (MW 23 000, 199 aminoacids) represents 75% of α-casein mixture (αs1 and αs2) (Schmidt 1980) and 90-96 peptidic sequence (MW 1360) represents 5.9% of the protein mother, the amount of bioactive peptide obtained from α-casein corresponded to a recovery yield of 85%.

CONCLUSION

In conclusion, the development of the continuous process with the immobilized enzyme for the production of α-casein exorphin was useful since: 1 it allowed the increase of the hydrolysis time and the use of high amount of enzyme, 2 avoided the self-destruction of enzyme and the loss of enzyme activity and 3 gave the possibility of using the same enzyme preparation for new peptide productions.The developed process did not required sophisticated or expensive equipments and appeared compatible with a preparative scale production.

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REFERENCES

1. Brash, J.L. and P.W. Wojciechowski, 1996. Interfacial phenomena and bioproducts. Marcel Dekker, New York.
2. Meisel, H. and W. Bockelmann, 1999. Bioactive peptides encrypted in milk proteins: Proteolytic activation and thropho-functionnal properties. Antonie Van Leeuwenhoek 76:207-215. [online]http: //www.ncbi.nlm.nih. gov/entrez/query. fcgi? cmd=Retrieve&db=pubmed[PMID 10532380 consulted 21/6/2002]
3. Meisel, H. and R.J. Fitzgerald, 2000. Opioid peptides encrypted in intact milk proteins sequences. Br. J. Nutr., 84: S27-31.[online]http://www.ncbi.nlm.nih. gov/entrez/query.fcgi?cmd=Retrieve&db=pubmed [PMID 11242443 consulted 21/6/2002]
4. Silva, S.V. and X. Malcata, 2005. Caseins as source of bioactive peptides. Intl. Dairy J., 15: 1-15.

5. Brulé, G., L. Roger, J. Fauquant and M. Pitot, 1980. Procédé de traitement d'une matière à base de caséine contenant des phosphocaséinates de cations monovalents et leurs dérivés. Produits obtenus et applications. Brevet Fr. n° 80 022 81.
6. V. Brantl, H. Teschemacher, A. Henschen and F. Lottspiech, 1979. Novel opioid peptides derived from casein (β-casomorphins). I: Isolation from casein peptone. Hoppe-seyler's Z. Physiol. Chem, 360 : 1211-1216.
7. Zioudrou, C., R.A. Streaty and W.A. Klee, 1979. Opioid peptides derived from food proteins. The exorphins. J. Biol. Chem., 254: 2446-2449.
8. Yunden, J. and Y. Masaaki, 1999. Enzymatic release of neocasomorphins from bovine β-casein. Peptides. 20 Issue 8: 957-962.
9. Drapeau, G.R., 1977. Cleavage at glutamic acid with staphylococcal protease. Meth. Enzymol., 47: 189-191.
10. Thompson, M.P., 1966. DEAE cellulose urea chormatography of casein in the presence of 2-mercaptoethanol. J. Dairy Sci., 49: 792-795.
11. Leslie, F.M., 1987. Methods used for the study of opioid receptors. Pharmacological. Rev., 39, N°3: 197-249.
12. Alfani, F., M. Cantarella, N. Cutarella, A. Gallifuoco, P. Golini and D. Bianchi, 1997. enzymatic conversion of cephalosporin C into glutaryl 7-amino cephalos poranic acid. A study in different reactor configurations. Biotechnol. Lett., 19: 175-178.
13. Borg, P., M. Girardin, B. Rovel and D. Barth, 2000. Comparison between two processes for the enzymaticsynthesis of tridocosahexaenoylglycerol in solvent-free medium.Biotechnol. Lett., 22: 777-781.
14. Schmidt, D.G., 1980. Colloïdal aspects of casein. Neth. Milk and Dairy. J., 34: 42-64../