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Angiotensin I-converting (ACE)-inhibitory and anti-inflammatory properties of commercially available Greek yoghurt made from bovine or ovine milk: A comparative study

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ABSTRACT

The objective of this study was to compare the presence of peptides with angiotensin I-converting (ACE)-inhibitory and immunomodulatory activities in commercial Greek yoghurt made from bovine or ovine milk. Water soluble extracts (WSE) in yoghurts made from ovine milk exhibited higher ACE-inhibitory activity than their bovine counterparts. Similarly, ovine yoghurt WSE inhibited more effectively the expression of two pro-inflammatory genes (inducible NO synthase and cyclooxygenase-2) by ovine monocytes. Following gel filtration of WSE by Sephadex G25, one fraction exhibited only ACE-inhibitory activity, two only immune inhibitory while one possessed both activities. Thus, selected peptidic fractions mimicked the original effects observed in WSE prior to fractionation.

1. Introduction

Several bioactive peptides are encrypted within the native milk proteins. These peptides are inactive within the sequence of the parent protein and they become active following their release by proteolysis. Proteolytic breakdown of milk proteins generates a number of peptides endowed with various biological properties (antimicrobial, antihypertensive, antithrombotic and immunomodulatory activities; Korhonen & Pihlanto, 2007). Among the various peptides, the angiotensin-converting enzyme inhibitor (ACE-I) family of peptides are the most widely studied. ACE inhibition leads to a decrease in the level of the vasoconstricting peptide, angiotensin II, and a corresponding increase in the level of the vasodilatory peptide, bradykinin, thus yielding an overall reduction in blood pressure. Peptides with ACE inhibitory activities have been detected in yoghurt (Chobert et al., 2005; Donkor, Henriksson, Singh, Vasiljevic, & Shah, 2007; Papadimitriou et al., 2007).

Yoghurt in Greece is manufactured with the use of bovine and/or ovine milk. To the best of our knowledge, there are no comparative studies concerning the existence of ACE inhibitory and immunomodulatory peptides in commercial yoghurt made from bovine or ovine milk in Greece. Thus, the objective of the present study was to compare the presence of peptides with ACE-inhibitory and immunomodulatory properties in commercially available yoghurt samples in the Greek market made from bovine or ovine milk. Furthermore, peptidic fractions isolated from water soluble extracts (WSE) obtained from both types of yoghurt by gel filtration were tested for their ACE-inhibitory and immunomodulatory effects.

2. Materials and methods

Unless otherwise stated all reagents were purchased from Sigma (St. Louis, MO, USA). A total of eight set type commercial Greek yoghurt brands were selected to participate in the present experiment. Four yoghurt brands were made using ovine and another four brands were made using bovine milk. All brands selected had comparable gross composition (fat 4.5%, protein 5.6% and total solids 15.8%). The protein composition of the four yoghurt brands made from bovine milk was elevated to the level of 5.6% by protein fortification. Yoghurt samples were stored for up to 18 days at 4 °C. All experiments were repeated three times.

Water soluble extracts (WSE) were obtained from all 8 brands at day 3, 8, 13 and 18 of storage using the method proposed by Kuchroo and Fox (1982). The determination of WSE protein concentration was performed using the Kjeldahl method (N × 6.38; AOAC, 1990).

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Sephadex G-25 resin (5 g) was soaked in deionised water overnight at 4 °C and then decanted to remove fine particles that did not settle [fractionation range, Mr = (1–5) × 10^3]. The hydrated resin was transferred to 0.075 mol L^{-1} of piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer, pH 5.7, for equilibration and transferred onto a column (diameter, 1 cm; length, 32 cm; Kontes® Flexcolumn chromatography column, VWR, Radnor, PA, USA). The flow rate was set at 0.5 mL min^{-1} (peristaltic pump EP-1 Econo Pump, Bio-Rad, Hercules, CA, USA). Water soluble extracts (0.25 mL) were applied to the column and were eluted with 0.075 mol L^{-1} PIPES, pH 5.7. Fractions of 1.0 mL were collected into tubes with the aid of a fraction collector (Model 328, Instrumentation Specialties, Sanford, FL, USA). The concentration of protein in the eluted fractions was monitored at 280 nm. Coenzyme B12 (MW = 1579 g mol^{-1}), was used as a standard to provide an estimate of the MW of the eluted fractions. Each fractionation was repeated twice. Fractions collected were stored in the refrigerator for up to 2 h and pooled before use.

The immunomodulatory activity of WSE was tested by measuring their ability to modulate the expression of iNOS and COX-2 by ovine monocytes. Monocytes were isolated using methods previously described by us (Politis, Theodorou, Lampidonis, Chronopoulou, & Baldi, 2012). The purity of monocytes ranged between 88 and 97%. Monocytes were cultured in cell culture medium (RPMI 1640 containing 2 mmol L^{-1} L-glutamine, 1.5 g L^{-1} HCO_3 and 10 mmol L^{-1} of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) supplemented with 5.5 mmol L^{-1} glucose, 1 mmol L^{-1} sodium pyruvate, 50 mg mL^{-1} penicillin and streptomycin and 10% foetal bovine serum) in the presence or absence of various amounts of WSE before fractionation or specific peptidic fractions after fractionation. After 3 h of incubation, cell culture medium was removed, cells were washed 3 times with Hank's Balanced Salt Solution (HBSS) containing 20 μmol L^{-1} HEPES and then phorbol myristate acetate (PMA, 81 nmol L^{-1}) was added to activate the cells. After incubation for 30 min, cells were washed again 3 times with HBSS and then processed for RNA isolation and Real-time quantitative PCR.

Total RNA extraction from approximately 5 × 10^6 monocytes, subsequent cDNA synthesis and quantitative PCR were performed as described previously by our group (Politis et al., 2012). Relative levels of mRNA were quantified for inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and b-actin (housekeeping gene). Primers were designed using PERLprimer software (Marshall, 2004) (iNOS: F 5′TGAGAATGGCAGCTACTGG3′, R 5′CTTCC ACTTGTTGTAATCTGAGG3′, COX-2: F 5′TTTGAGGAACTTACAGGAGA G3′, R 5′CAGTACTCAGGAGAACATATAGG3′, b-actin: F 5′AAGACCTC TACGCCAACAC3′, R 5′ACTCGTCGTACTCCTGCT3′). Each sample was measured in duplicates. The Pfaffl normalisation method (Pfaffl, 2001) was used for normalisation against the housekeeping gene.

ACE inhibitory activity was determined by the method of Nakamura et al. (1995). ACE-inhibitory activity was expressed as the protein concentration needed to inhibit 50% of the original ACE activity (IC50).

Statistical analysis was performed using the PASW Statistics 18 release (SPSS Inc.) program. Data represent the means and standard errors of at least three independent experiments. Within each experiment, each treatment or sample was performed in triplicate. Data were analysed either by ANOVA using Fisher's Least Significant Difference (LSD) post-hoc test or with the use of the unpaired student's t-test depending on the number of groups compared. Means were considered to be significantly different at the 95% confidence interval.

3. Results and discussion

Changes in peptide concentration of WSE and pH during the 18 days storage period are presented in Fig. 1. As expected, there was an increase in total protein concentration in WSE obtained from yoghurts made from bovine or ovine milk with advancing storage time. Peptide concentration was inversely related to changes in the pH. There were no differences in total protein concentrations between WSE obtained from yoghurts made from bovine or ovine milk throughout the storage period. Furthermore, WSE from storage day 18 that had the highest concentration of the peptides was selected for the determination of immunomodulatory and ACE-inhibitory activity (see below).

The ability of WSE obtained from yoghurts made from bovine or ovine milk at day 18 of storage to modulate the expression of iNOS and COX-2 by ovine monocytes was examined and the results are presented in Fig. 2. Data indicate that inhibition of expression of iNOS by approximately 50% (45–54%) was achieved when monocytes were cultured in the presence of 0.25 and 0.12 mg mL^{-1} protein concentration in WSE obtained from bovine and ovine milk, respectively. In a similar manner, inhibition of expression of COX-2 by approximately 50% (46–48%) was achieved when monocytes were cultured in the presence of 0.38 and 0.20 mg mL^{-1} of protein concentration in WSE obtained from bovine and ovine milk, respectively.

A comparison of the ACE-inhibitory activity of the WSE obtained from yoghurts made from bovine or ovine milk at day 18 of storage indicates the existence of peptides with ACE-inhibitory activity in both WSE obtained from yoghurts made with bovine or ovine milk. However, WSE obtained from ovine milk had higher inhibitory potency as the concentration needed to reduce ACE activity by 50% was statistically significantly lower ($P < 0.05$) (IC50 = 1.51 ± 0.04 mg mL^{-1}), when compared with the corresponding concentration in WSE obtained from yoghurts made from bovine milk (IC50 = 1.28 ± 0.04 mg mL^{-1}).

The first novel finding emerging from the present study is that yoghurts made from ovine milk exhibited higher immunomodulatory and ACE-inhibitory activities than the corresponding yoghurts made from bovine milk. The reason behind this puzzling observation suggesting that the origin of milk plays a crucial role is not known with certainty. The most logical explanation is that ovine milk is more prone to a specific pattern of proteolysis generating more peptides capable of inhibiting immune and the ACE-system. We believe a factor that may have contributed to the observation of higher activities in ovine milk is differences in milk quality between ovine and bovine milk. Despite the efforts to improve milk quality, it is general knowledge that ovine milk in Greece is of inferior quality and characterised by high total bacteria counts and milk SCC. We consider it possible that the differences could be related to differences in starter cultures. Against this notion is the consistency of our results. In other words, 8 different yoghurt brands, sampled at various time points in an experiment repeated three times, there was not even a single case that did not follow the general pattern.

In an attempt to explain the effect of milk origin on inhibitory potency of WSE obtained from yoghurts made from bovine and ovine milk, Sephadex G-25 column fractionation was performed and the results are presented in Fig. 3. The fractionation profiles were in general similar in both types of yoghurt. There were two main peaks at 16 and 52 min. The elution time of the first peak corresponds to elution time of peptides of the void volume and it most likely corresponds to peptides with MW higher than 5000 g mol^{-1} since the fractionation range of Sephadex G-25 is 1000–5000 g mol^{-1}. Coenzyme B12 with a MW of 1579 g mol^{-1} which eluted at 34 min was used to provide a relative indication of the MW of the eluted fractions. Thus, fractions 16–34 had MW in the range of 1600–5000 g mol^{-1} and the remaining fractions had MW lower than 1600 g mol^{-1}. One difference in the peptide fractionation profiles is that the protein concentration of peptides after