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ACE-inhibitory and antihypertensive properties of a bovine casein hydrolysate

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ABSTRACT

The aim of this study was to investigate the potential angiotensin converting enzyme (ACE)-inhibitory activity and the antihypertensive effect, after a single oral administration, of a pepsin hydrolysed bovine casein (HBC) and a fraction with molecular mass lower than 3000 Da (HBC < 3000). ACE-inhibitory activity was measured by spectrophotometric assay. These products were orally administered by gastric intubation. The systolic (SBP) and the diastolic blood pressure (DBP) were measured in spontaneously hypertensive rats by the tail cuff method before administration and also 2, 4, 6, 8, and 24 h post-administration. HBC showed a potent ACE-inhibitory activity. This activity was 10 times higher in HBC < 3000. HBC and HBC < 3000 decreased the arterial blood pressure of the rats. The decrease in the SBP observed for HBC (400 mg/kg) or HBC < 3000 (200 mg/kg) was less pronounced than that caused by 50 mg/kg of captopril (antihypertensive positive control). However, the maximal decreases in DBP caused by HBC or HBC < 3000 were as high as the maximum decrease observed for captopril. The antihypertensive effect of these products was transient and reverted 24 h after the administration. HBC and HBC < 3000 exert antihypertensive effect caused by small peptides with ACE-inhibitory activity.

1. Introduction

Inhibitors of angiotensin converting enzyme (ACE) are frequently used in therapy to reduce morbidity and mortality of patients with hypertension and other related diseases. Recently, an increasing number of studies have evidenced that food proteins have functions other than energetic and nutritional ones, and several peptides with potent ACE-inhibitory activity have been isolated from them (López-Fandiño, Otte, & van Camp, 2006; Miguel & Aleixandre, 2006; Murray & FitzGerald, 2007). These peptides are inactive within the protein sequence but may be released by hydrolysis, and, once released, they show biological activity. They can be generated *in vivo* by the action of gastrointestinal enzymes, and can also be obtained *in vitro* using specific enzymes, or can be produced during the manufacture of certain foods. Spectrophotometric, fluorimetric, chromatographic and capillary electrophoresis techniques have been used to isolate the active peptides and to measure their ability to inhibit ACE *in vitro*. However, it is only through *in vivo* studies that the antihypertensive effects of a given hydrolysate or peptide can be reliably assessed. Several studies have been performed to determine the antihypertensive effects of ACE-inhibitory peptides using spontaneously hypertensive rats (SHR) (Miguel, López-Fandiño, Ramos, & Aleixandre, 2005; Muguerza et al., 2006; Nakamura, Yamamoto, Sakai, & Takano, 1995; for a review see, FitzGerald, Murray, & Walsh, 2004, or López-Fandiño et al., 2006), but only few milk-derived peptides have been tested for their antihypertensive effect in humans (Hata et al., 1996; Mizuno et al., 2005; Jauhiainen et al., 2005, 2007; Seppo, Jauhiainen, Poussa, & Korpela, 2003).

In particular, milk proteins, both caseins and whey proteins, are a rich source of ACE-inhibitory products. For instance, bovine caseins (Maruyama & Suzuki, 1982; Maruyama et al., 1987) and human caseins (Kohmura et al., 1989) behave like ACE-inhibitors and, at the end of the 20th century, attempts were already being made to market several casein hydrolysates with antihypertensive effect. Thus, the study carried out by Sekiya, Kobayashi, Kita, Imamura, and Toyama (1992) made it possible to market a casein hydrolysate obtained by treating this protein with trypsin. Consumption of 20 g per day of this hydrolysate for 4 weeks caused a drop in arterial blood pressure in hypertensive patients (Sekiya et al., 1992). The product was marketed in Japan under the name "Casein DP Peptio Drink" (Sugai, 1998). Subsequently, in The Netherlands, another tryptic hydrolysate of casein went on sale, which also lowered arterial blood pressure in both animals and hypertensive patients (Cadee et al., 2007; Karaki et al., 1990; Townsend, McFadden, Ford, & Cadeé, 2004). The commercial name of this new hydrolysate was "C12 Peption".

Abbreviations: ACE, angiotensin converting enzyme; BC, bovine casein by enzymatic treatment with pepsin; DBP, diastolic blood pressure; HBC, hydrolysed bovine casein; HBC < 3000, fraction with molecular mass lower than 3000 Da of HBC; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats.

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The aim of this study was to investigate the potential ACE-inhibitory activity and the possible antihypertensive effect after a single oral administration in hypertensive rats of a hydrolysed bovine casein (HBC) obtained in our laboratory from bovine casein (BC) by enzymatic treatment with pepsin. We also investigate the ability of the fraction with molecular mass lower than 3000 Da (HBC < 3000) to inhibit ACE *in vitro* and to lower the arterial blood pressure.

2. Material and methods

2.1. Studied products

Isoelectric casein was prepared by precipitation from whole milk by adding 2 M HCl to pH 4.6, followed by centrifugation at 4500 *g* for 15 min. The casein precipitate was washed three times with acidulated water at pH 4.6. The remaining fat in the casein precipitate was removed by washing with dichloromethane-acidulated water (1:1, v/v). The final casein precipitate was lyophilised. This product is referred to as bovine casein (BC).

The hydrolysate of bovine casein was prepared by dissolving isoelectric casein at 0.5% (w/v) in water, the pH was adjusted to 3.0 with 1 M HCl and casein was digested with 3.7% (w/w of substrate) porcine pepsin A (E.C. 3.4.23.1., 570 U/mg protein, Sigma, St. Louis, MO, USA) for 3 h at 37 °C. The reaction was terminated by heating at 80 °C for 15 min and the pH was adjusted to 7.0 by addition of 1 M NaOH. The digest was centrifuged at 16.000 *g* for 15 min and the supernatant was lyophilised prior to use. This sample is designated as hydrolysed bovine casein (HBC).

The supernatant of the hydrolysate was subjected to ultrafiltration through a hydrophilic 3000 Da cut-off membrane (Centripep, Amicon, Inc., Beverly, MA, USA). The 3000 Da permeate (termed HBC < 3000), and retentate (HBC > 3000) were freeze-dried and kept at −20 °C prior to use.

2.2. ACE-inhibitory activity

ACE-inhibitory activity was measured by the spectrophotometric assay of Cushman and Cheung (1971) with some modifications. Each sample (40 μl) was incubated with 100 μl of 0.1 M borate buffer (pH 8.3) containing 0.3 M NaCl, and 5 mM hippuryl–histidyl–leucine (Sigma Chemical, St. Louis, MO, USA). ACE (2 mU) (EC 3.4.15.1, 5.1 U/mg, Sigma) was added and the reaction mixture was incubated at 37 °C for 30 min. The reaction was stopped with 150 μl of 1 M HCl. The hippuric acid formed was extracted with ethyl acetate (1000 μl) and, after removal of ethyl acetate by heat evaporation, hippuric acid was redissolved in distilled water (800 μl) and measured spectrophotometrically at 228 nm. The activity of each sample was tested in triplicate. Inhibitory activity was expressed as the protein concentration required to inhibit the original ACE activity by 50% (IC_{50}) and one unit of ACE-inhibitory activity was expressed as the potency showing 50% ACE inhibition under these conditions. A non-linear adjustment of the data obtained was performed to calculate the IC_{50} values with the programme PRISM version 4.02 for Windows (GraphPad Software, Inc. San Diego, CA, USA). This programme gives, as result, the estimated value of the IC_{50}, together with the standard error. For this purpose, the protein concentration of the water-soluble extracts was determined by the Kjeldahl method.

2.3. Antihypertensive activity

We have used 17–20-week-old male SHR weighing 314 ± 3 g, to evaluate the antihypertensive activity of the following products: BC (400 mg/kg), HBC (400 mg/kg) and HBC < 3000 (200 mg/kg). These animals were obtained from Charles River Laboratories, España S.A. They remained at a temperature of 23° C with 12 h light/dark cycles, and consumed tap water and a standard diet for rats (A04 Panlab, Barcelona, Spain) *ad libitum* during the experiments. All the above-mentioned products were orally administered by gastric intubation, between 9 and 10 a.m., to the rats. Distilled water served as negative control, and captopril (50 mg/kg) (Sigma, USA), a known ACE inhibitor, served as positive control. We always administered water (1 ml/rat) and, when a compound was orally given, 1 ml/rat of an appropriate solution of this compound was also administered. We measured the systolic blood pressure (SBP) and the diastolic blood pressure (DBP) of the rats by the tail cuff method before administration and also 2, 4, 6, 8, and 24 h post-administration. Before the measurement, the rats were kept at 30 °C for 10 min to make the pulsations of the tail artery detectable. The original method for measuring arterial blood pressure using the tail cuff provides only SBP values (Buñag, 1973), but the equipment used in this study, LE 5001 (Letica, Hospitalet, Barcelona, Spain), has a high sensitivity pulse transducer coupled with an accurate microprocessor programme, and allows us to distinguish between SBP and DBP. To establish the values of SBP and DBP, five measurements were taken, and the average of all of them was obtained. To minimize stress-induced variations in blood pressure, all measurements were taken by the same person in the same peaceful environment. Moreover, to guarantee the reliability of the measurements, we established a training period of two weeks before the actual trial time, and during this period the rats were accustomed to the procedure.

All the above-mentioned experiments were performed as authorized for scientific research (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food).

The results are expressed as mean values ± SEM for 5–7 experiments, and were analyzed by a two-way ANOVA, using the GraphPad Prism 4 software. In addition, in order to compare the different treatments and to assess the effect of time within each treatment, some data were also analyzed by a one-way ANOVA. Differences between the groups were assessed by the Bonferroni test and we always consider the differences between the means to be significant when $P < 0.05$.

3. Results and discussion

Enzymatic proteolysis can release bioactive peptides from milk protein precursors, and digestive enzymes are often used for this purpose. In our study, we have used pepsin to perform the hydrolysis of BC. The resulting HBC showed a potent ACE-inhibitory activity, with an IC_{50} value of 52.8 μg/ml. In order to measure the contributions of small peptides to the ACE-inhibitory activity, the HBC was filtered through a 3000 Da cut-off membrane. Table 1 shows the *in vitro* ACE-inhibitory activity of the different studied products. The activity of the HBC < 3000 (IC_{50} = 5.5 μg/ml) was 10 times higher than that found in the HBC and more than 40 times

Table 1
Angiotensin converting enzyme (ACE)-inhibitory activity *in vitro* of bovine casein (BC), its hydrolysate (HBC), the permeate (HBC < 3000) and the retentate fraction (HBC > 3000) after ultrafiltration through a 3000 Da membrane

	IC_{50} (μg/ml)%
BC	>1000
HBC	52.8 ± 2.6
HBC < 3000	5.5 ± 0.4
HBC > 3000	242 ± 34.9

IC_{50} = Concentration needed to inhibit 50% of ACE activity (expressed as the mean coefficient ± SEM).

higher than that measured in the retentate. This suggests that ACE inhibition was mainly attributable to peptide components with molecular masses lower than 3000 Da and, therefore, ultrafiltration through membranes with 3000 Da of molecular mass cut-off could be used to obtain a product enriched in ACE-inhibitory peptides. This idea had previously been proposed by our own and other research groups (Mullally, Meisel, & Fitzgerald, 1997; Fujita, Yamagam, & Ohshima, 2001; Miguel, Recio, Gómez-Ruiz, Ramos, & López-Fandiño, 2004; Quirós et al., 2007). The IC_{50} value of BC obtained in this study was, on the other hand, very high (>1000 μg/ml), and this confirms that the hydrolysis is necessary to obtain ACE-inhibitory products.

The IC_{50} values of HBC and HBC < 3000 were low and lay within the concentration range reported in the literature for compounds with antihypertensive activity (Gobbetti, Ferranti, Smacchi, & Goffredi, 2000; Miguel et al., 2004, 2006). Therefore, in this study, we have administered (by gastric intubation) these products to SHR. Captopril, a potent ACE inhibitor with an IC_{50} value of 0.002 μM (Fujita & Yoshikawa, 1999) was also administered to the SHR as positive control. Just before the experiments, the animals showed SBP and DBP values of 175 ± 5.2 mm Hg, and 125.3 ± 7.8 mm Hg respectively, and Figs. 1 and 2 show the decreases of the SBP and DBP obtained, at different moments, after the oral administration of the different products. The administration of HBC (400 mg/kg) produced a significant decrease in the SBP and DBP in the SHR ($p < 0.05$). The administration of HBC < 3000 (200 mg/kg) produced a blood pressure lowering effect similar to that of HBC ($p > 0.05$), and the decreases in both variables, SBP and DBP, were maximum 2 h after the administration of these products. The decrease in the SBP observed for HBC or HBC < 3000 was less pronounced than that caused by 50 mg/kg of captopril ($p < 0.05$). However, the maximal decreases in DBP caused by the hydrolysate or its permeate were as high as the maximum decrease measured for captopril ($p > 0.05$). Moreover, the maximal effect in the DBP was obtained before when we administered HBC or HBC < 3000 than when we administered captopril (between 4 and 6 h post-administration). The antihypertensive effect of these products was transient and reverted 24 h after the administration. At that moment, the values of the SBP and the DBP of the SHR were, therefore, similar to the initial values. It is also important to note that the administration of BC, as expected, produced only a slight and non-significant decrease in the SBP and DBP of the SHR.

In conclusion, this report shows the preparation of a peptic casein hydrolysate with antihypertensive properties. The results obtained in this study indicate that both bovine casein hydrolysed with pepsin and its 3000-Da permeate contain ACE-inhibitory and antihypertensive peptides. Moreover, our study suggests that the activity is caused by small peptides contained in the permeate fraction.

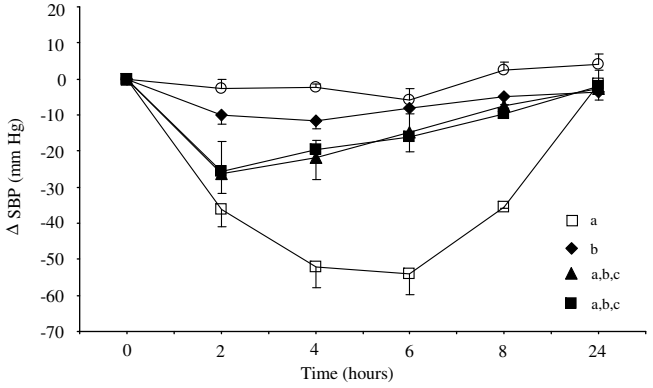

Fig. 1. Decrease in systolic blood pressure (SBP) caused in spontaneously hypertensive rats by the administration of water (○), captopril (50 mg/kg) (□), BC (400 mg/kg) (♦), HBC (400 mg/kg) (▲), and HBC < 3000 (200 mg/kg) (■). The data represent the mean values ± SEM for 5–7 rats. *P* estimated by a two-way analysis of variance. [a]$P < 0.05$ vs. water; [b]$P < 0.05$ vs. captopril; [c]$P < 0.05$ vs. BC.

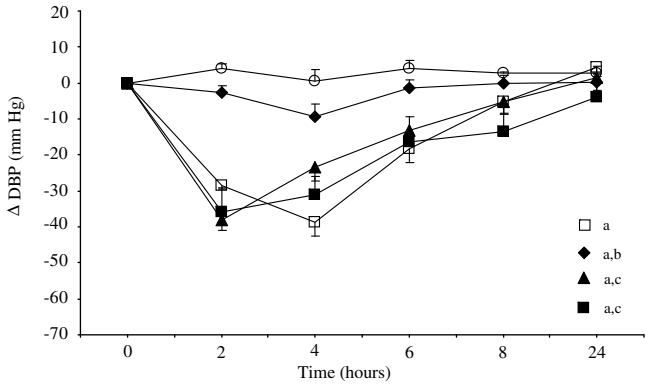

Fig. 2. Decrease in diastolic blood pressure (DBP) caused in spontaneously hypertensive rats by the administration of water (○), captopril (50 mg/kg) (□), BC (400 mg/kg) (♦), HBC (400 mg/kg) (▲), and HBC < 3000 (200 mg/kg) (■). The data represent the mean values ± SEM for 5–7 rats. *P* estimated by a two-way analysis of variance. [a]$P < 0.05$ vs. water; [b]$P < 0.05$ vs. captopril; [c]$P < 0.05$ vs. BC.

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References

Buñag, R. D. (1973). Validation in awake rats of a tail-cuff method for measuring systolic pressure. *Journal of Applied Physiology, 34*, 279–282.

Cadee, J. A., Chang, C. Y., Chen, C. W., Huang, C. N., Chen, S. L., & Wang, C. K. (2007). Bovine casein hydrolysate (c12 peptide) reduces blood pressure in prehypertensive subjects. *American Journal of Hypertension, 20*(1), 1–5.

Cushman, D. W., & Cheung, H. S. (1971). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochemical Pharmacology, 20*, 637–1648.

FitzGerald, R. J., Murray, B. A., & Walsh, G. J. (2004). Hypotensive peptides from milk proteins. *Journal of Nutrition, 134*, 980S–988S.

Fujita, H., & Yoshikawa, M. (1999). LKPNM: A prodrug-type ACE-inhibitory peptide derived from fish protein. *Immunopharmacology, 44*, 123–127.

Fujita, H., Yamagam, T., & Ohshima, K. (2001). Effects of an ACE-inhibitory agent, katsuobushi oligopeptide, in the spontaneously hypertensive rat and in borderline and mildly hypertensive subjects. *Nutrition Research, 21*, 1149–1158.

Gobbetti, M., Ferranti, P., Smacchi, E., & Goffredi, F. (2000). Production of angiotensin I-converting peptides in fermented milks started by *Lactobacillus delbruckii subsp. bulgaricus* SS1 and *Lactococcus lactis subsp. cremoris* FT5. *Applied and Environmental Microbiology, 66*, 3898–3904.

Hata, Y., Yamamoto, M., Ohni, M., Nakajim, K., Nakamura, Y., & Takano, T. (1996). A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *American Journal of Clinical Nutrition, 64*, 767–771.

Jauhiainen, T., Vapaatalo, H., Poussa, T., Kyrönpalo, S., Rasmussen, M., & Korpela, R. (2005). *Lactobacillus helveticus* fermented milk lowers blood pressure in hypertensive subjects in 24-h ambulatory blood pressure measurement. *American Journal of Hypertension, 18*, 1600–1605.

Jauhiainen, T., Ronnback, M., Vapaatalo, H., Wuolle, K., Kautiainen, H., & Korpela, R. (2007). *Lactobacillus helveticus* fermented milk reduces arterial stiffness in hypertensive subjects. *International Dairy Journal, 17*, 1209–1211.

Karaki, H., Doi, K., Sugano, S., Uchiya, H., Sugai, R., & Murakami, U., et al. (1990). Antihypertensive effect of tryptic hydrolysate of milk casein in spontaneously hypertensive rats. *Comparative Biochemistry and Physiology, 96C*, 367–371.

Kohmura, M., Nio, N., Kubo, K., Minishima, Y., Munekata, E., & Ariyoshi, Y. (1989). Inhibition of angiotensin-converting enzyme by synthetic peptides of human β-casein. *Agricultural and Biological Chemistry, 53*, 2107–2114.

López-Fandiño, R., Otte, J., & van Camp, J. (2006). Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. *International Dairy Journal, 16*, 1277–1293.

Maruyama, S., Mitachi, H., Awaya, J., Kurono, M., Tomizuka, N., & Suzuki, H. (1987). Angiotensin I-converting enzyme inhibitory activity of the C-terminal hexapeptide of αs_1-casein. *Agricultural and Biological Chemistry, 51*, 2557–2561.

Maruyama, S., & Suzuki, H. (1982). A peptide inhibitor of angiotensin-I converting enzyme in the tryptic hydrolyzate of casein. *Agricultural and Biological Chemistry, 46*, 1393–1394.

Miguel, M., & Aleixandre, A. (2006). Antihypertensive peptides derived from egg proteins. *Journal of Nutrition, 136*, 1457–1460.

Miguel, M., López-Fandiño, R., Ramos, M., & Aleixandre, M. A. (2005). Short-term effect of egg white hydrolysate products on the arterial blood pressure of hypertensive rats. *British Journal of Nutrition, 94*, 731–737.

Miguel, M., Recio, I., Gómez-Ruiz, J. A., Ramos, M., & López-Fandiño, R. (2004). Angiotensin I-converting enzyme inhibitory activity of peptides derived from egg white proteins by enzymatic hydrolysis. *Journal of Food Protection, 67*, 1914–1920.

Mizuno, S., Matsuura, K., Gotou, T., Nishimura, S., Kajimoto, O., & Yabune, M., et al. (2005). Antihypertensive effect of casein hydrolysate in a placebo-controlled study in subjects with high-normal blood pressure and mild hypertension. *British Journal of Nutrition, 94*, 84–91.

Muguerza, B., Ramos, M., Sánchez, E., Manso, M. A., Miguel, M., & Aleixandre, A., et al. (2006). Antihypertensive activity of milks fermented by *Enterococcus faecalis* strains isolated from raw milk. *International Dairy Journal, 16*, 61–69.

Mullally, M. M., Meisel, H., & Fitzgerald, R. J. (1997). Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to tryptic fragment of bovine β-lactoglobulin. *FEBS Letters, 402*, 99–101.

Murray, B. A., & FitzGerald, R. J. (2007). Angiotensin converting enzyme inhibitory peptides derived from food proteins: biochemistry, bioactivity and production. *Currents Pharmaceutical Design, 13*, 773–791.

Nakamura, Y., Yamamoto, N., Sakai, K. Y., & Takano, T. (1995). Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I converting enzyme. *Journal of Dairy Science, 78*, 1253–1257.

Quirós, A., Ramos, M., Muguerza, B., Delgado, M. A., Miguel, M., & Aleixandre, A., et al. (2007). Identification of novel antihypertensive peptides in milk fermented with *Enterococus faecalis*. *International Dairy Journal, 17*, 33–41.

Sekiya, S., Kobayashi, Y., Kita, E., Imamura, Y., & Toyama, S. (1992). Antihypertensive effects of tryptic hydrolysate of casein on normotensive and hypertensive volunteers. *Journal of Nutrition and Food Sciences, 45*, 513–517.

Seppo, L., Jauhiainen, T., Poussa, T., & Korpela, R. (2003). A fermented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects. *American Journal of Clinical Nutrition, 77*, 326–330.

Sugai, R. (1998). ACE inhibitors and functional foods. *Bulletin of the IDF, 336*, 17–20.

Townsend, R. R., McFadden, C. B., Ford, V., & Cadeé, J. A. (2004). A randomized, doubled-blind, placebo controlled trial of casein protein hydrolysate (C12 peptide) in human essential hypertension. *American Journal of Hypertension, 17*, 1056–1058.