ACE-inhibitory and antihypertensive properties of a bovine casein hydrolysate

M. Miguel[1], M.M. Contreras[2], I. Recio[2], A. Aleixandre[1*]

[1]*Instituto de Farmacología y Toxicología (CSIC), Departamento de Farmacología, Facultad de Medicina, U. Complutense, Madrid. Spain.*

[2]*Instituto de Fermentaciones Industriales (CSIC), Madrid, Spain.*

***Correspondence should be sent to:**

Dra. M.A. Aleixandre

Dpto. de Farmacología

Facultad de Medicina

Universidad Complutense

28040 Madrid

Spain

Telephone: 34-91-3941475

Fax: 34-91-3941463

E-mail: amaya@med.ucm.es

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 hours post-administration. HBC showed a potent ACE-inhibitory activity. This activity was ten 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 ~~a~~ good antihypertensive effect caused by small peptides with ACE-inhibitory activity.

Key words: ACE-inhibitory peptides, antihypertensive activity, milk proteins

Abbreviation key:

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

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 hypotensive effects of a given hydrolysate or peptide can be reliably assessed. Several studies have been performed to determine the hypotensive effects of ACE-inhibitory peptides using spontaneously hypertensive rats (SHR) (Nakamura, Yamamoto, Sakai & Takano, 1995; Miguel, López-Fandiño, Ramos & Aleixandre, 2005; Muguerza et al., 2006; for a review see, FitzGerald, Murray & Walsh, 2004, or López-Fandiño, Otte & van Camp, 2006), but only few milk-derived peptides have been tested for their antihypertensive effect in humans (Hata, Yamamoto, Ohni, Nakajim, Nakamur & Takano, 1996; Seppo, Jauhiainen, Poussa & Korpela, 2003; Mizuno et al., 2005. Jauhiainen, Vapaatalo, Poussa, Kyrönpalo, Rasmussen & Korpela, 2005; Jauhiainen, Ronnback, Vapaatalo, Wuolle, Kautiainen & Korpela, 2007).

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, Mitachi, Awaya, Kurono, Tomizuka & Suzuki, 1987) and human caseins (Kohmura, Nio, Kubo, Minishima, Munekata & Ariyoshi, 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 et al., (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, Kobayashi, Kita, Imamura & Toyama, 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 (Karaki et al., 1990; Townsend, McFadden, Ford & Cadeé, 2004; Cadee, Chang, Chen, Huang, Chen & Wang, 2007). The commercial name of this new hydrolysate was "C12 Peption".

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

101 was washed three times with acidulated water at pH 4.6. The remaining fat in the casein
102 precipitate was removed by washing with dichlomethane-acidulated water (1:1, v/v).
103 The final casein precipitate was lyophilised. This product is referred to as bovine casein
104 (BC).

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

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

116

117 *2.2. ACE-inhibitory activity*

118 ACE-inhibitory activity was measured by the spectrophotometric assay of
119 Cushman & Cheung (1971) with some modifications. Each sample (40 µl) was
120 incubated with 100 µl of 0.1 M borate buffer (pH 8.3) containing 0.3 M NaCl, and 5
121 mM hippuryl-histidyl-leucine (Sigma Chemical, St. Louis, MO, USA). ACE (2 mU)
122 (EC 3.4.15.1, 5.1 U/mg, Sigma) was added and the reaction mixture was incubated at
123 37ºC for 30 min. The reaction was stopped with 150 µl of 1 M HCl. The hippuric acid
124 formed was extracted with ethyl acetate (1000 µl) and, after removal of ethyl acetate by
125 heat evaporation, hippuric acid was redissolved in distilled water (800 µl) and measured

126 spectrophotometrically at 228 nm. The activity of each sample was tested in triplicate.
127 Inhibitory activity was expressed as the protein concentration required to inhibit the
128 original ACE activity by 50% (IC_{50}) and one unit of ACE-inhibitory activity was
129 expressed as the potency showing 50% ACE inhibition under these conditions. A non-
130 linear adjustment of the data obtained was performed to calculate the IC_{50} values with
131 the programme PRISM version 4.02 for Windows (GraphPad Software, Inc. San Diego,
132 CA, USA). This programme gives, as result, the estimated value of the IC_{50}, together
133 with the standard error. For this purpose, the protein concentration of the water-soluble
134 extracts was determined by the Kjeldahl method.
135
136 *2.3. Antihypertensive activity*
137 We have used 17-20-week-old male SHR weighing 314 ± 3 g, to evaluate the
138 antihypertensive activity of the following products: BC (400 mg/kg), HBC (400
139 mg/kg) and HBC<3000 (200 mg/kg). These animals were obtained from Charles River
140 Laboratories, España S.A. They remained at a temperature of 23º C with 12 hour
141 light/dark cycles, and consumed tap water and a standard diet for rats (A04 Panlab,
142 Barcelona, Spain) *ad libitum* during the experiments. All the above-mentioned products
143 were orally administered by gastric intubation, between 9 and 10 a.m., to the rats.
144 Distilled water served as negative control, and captopril (50 mg/kg) (Sigma, USA), a
145 known ACE inhibitor, served as positive control. We always administered water (1
146 ml/rat) and, when a compound was orally given, 1 ml/rat of an appropriate solution of
147 this compound was also administered. We measured the systolic blood pressure (SBP)
148 and the diastolic blood pressure (DBP) of the rats by the tail cuff method before
149 administration and also 2, 4, 6, 8, and 24 hours post-administration. Before the
150 measurement, the rats were kept at 30ºC for 10 minutes to make the pulsations of the

151 tail artery detectable. The original method for measuring arterial blood pressure using
152 the tail cuff provides only SBP values (Buñag, 1973), but the equipment used in this
153 study, LE 5001 (Letica, Hospitalet, Barcelona, Spain), has a high sensitivity pulse
154 transducer coupled with an accurate microprocessor programme, and allows us to
155 distinguish between SBP and DBP. To establish the values of SBP and DBP, five
156 measurements were taken, and the average of all of them was obtained. To minimize
157 stress-induced variations in blood pressure, all measurements were taken by the same
158 person in the same peaceful environment. Moreover, to guarantee the reliability of the
159 measurements, we established a training period of two weeks before the actual trial
160 time, and during this period the rats were accustomed to the procedure.

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

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

170

171 **3. Results and discussion**

172 Enzymatic proteolysis can release bioactive peptides from milk protein
173 precursors, and digestive enzymes are often used for this purpose. In our study, we have
174 used pepsin to perform the hydrolysis of BC. The resulting HBC showed a potent ACE-
175 inhibitory activity, with an IC_{50} value of 52.8 μg/ml. In order to measure the

176 contributions of small peptides to the ACE-inhibitory activity, the HBC was filtered
177 through a 3000 Da cut-off membrane. Table 1 shows the *in vitro* ACE inhibitory
178 activity of the different studied products. The activity of the HBC<3000 (IC_{50} = 5.5
179 μg/ml) was ten times higher than that found in the HBC and more than 40 times higher
180 than that measured in the retentate. This suggests that ACE inhibition was mainly
181 attributable to peptide components with molecular masses lower than 3000 Da and,
182 therefore, ultrafiltration through membranes with 3000 Da of molecular-mass cut-off
183 could be used to obtain a product enriched in ACE inhibitory peptides. This idea had
184 previously been proposed by our own and other research groups (Mullally, Meisel &
185 Fitzgerald, 1997; Fujita, Yamagama & Ohshima, 2001; Miguel, Recio, Gómez-Ruiz,
186 Ramos & López-Fandiño, 2004; Quirós et al., 2007). The IC_{50} value of BC obtained in
187 this study was, on the otherhand, very high (>1000 μg/ml), and this confirms that the
188 hydrolysis is necessary to obtain ACE inhibitory products.
189 The IC_{50} values of HBC and HBC<3000 were low and lay within the
190 concentration range reported in the literature for compounds with antihypertensive
191 activity (Gobbetti, Ferranti, Smacchi & Goffredi, 2000; Miguel et al., 2004; Muguerza et
192 al., 2006). Therefore, in this study, we have administered (by gastric intubation) these
193 products to SHR. Captopril, a potent ACE inhibitor with an IC_{50} value of 0.002 μM
194 (Fujita & Yoshikawa, 1999) was also administered to the SHR as positive control. Just
195 before the experiments, the animals showed SBP and DBP values of 175 ± 5.2 mm Hg,
196 and 125.3 ± 7.8 mm Hg respectively, and Figures 1 and 2 show the decreases of the
197 SBP and DBP obtained, at different moments, after the oral administration of the
198 different products. The administration of HBC (400 mg/kg) produced a significant
199 decrease in the SBP and DBP in the SHR ($p<0.05$). The administration of HBC<3000
200 (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 hours 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 hours 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.

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307 **Figure captions**

308

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

314

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316

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

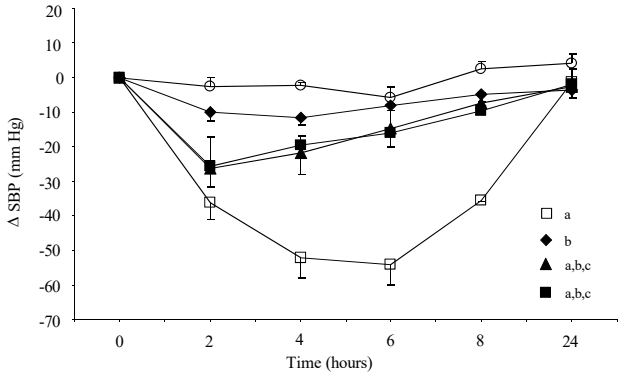
322

323 Table 1 Angiotensin converting-enzyme (ACE)-inhibitory activity *in vitro* of bovine
324 casein (BC), its hydrolysate (HBC), the permeate (HBC<3000) and the retentate
325 fraction (HBC>3000) after ultrafiltration through a 3000 Da membrane
326

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

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

Miguel et al., Figure 1



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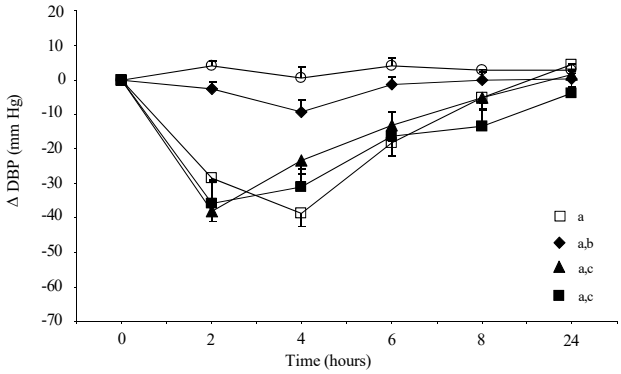
Miguel et al., Figure 2



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