Angiotensin converting enzyme inhibitors from casein/Inhibidores de la enzima convertidora de angiotensina a partir de caseína

A JOURNEY FROM PORCINE PANCREATIN TO PRODUCTION OF ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITORS FROM CASEIN

L. Ozimek1, Sirinda Kusump2, Takuo Nakano1 and E.R. Silva3

1 Department of Agricultural, Food and Nutritional Science, University of Alberta. Edmonton, Alberta T6G 2P5, Canada email: lech.ozimek@ualberta.ca

2 Department of Food Science and Technology.Thammasat University. Tha Phra Cha Campus, Bangkok, Thailand

3 University of Veracruz, Xalapa, Veracruz, Mexico

SUMMARY

Angiotensin converting enzyme (E.C. 3.4.15.1) plays an important role in the regulation of blood pressure and pathophysiology of hypertension, which causes various diseases of the circulatory system including stroke, arteriosclerosis, and coronary heart disease. Angiotensin converting enzyme (ACE) can increase blood pressure by converting angiotensin I to a potent vasoconstrictor, angiotensin II, and by degrading an arteriolar vasodilator, bradykinin, into inactive peptides. Inhibition of ACE may exert an antihypertensive effect as a consequence of a decrease in angiotensin II activity and a concomitant increase in bradykinin activity.

The ACE inhibiting activity determined in the pancreatin hydrolysate of bovine casein was considerably high with its concentration of 50% inhibition (IC50) being 600 μg/mL. These results suggest that the pancreatin hydrolysate of bovine casein is a potential source of hydrophobic peptides with ACE inhibiting activity, and that chromatography on phenyl-agarose in 5 M NaCl is a useful technique to purify such peptides. Further studies to separate and characterize individual hydrophobic peptides are under progress in our laboratory.

Key words: ACE inhibitor, casein hydrolysate, hydrophobic peptide, hydrophobic interaction chromatography, phenyl-agarose

Short title: Angiotensin converting enzyme inhibitors from casein

UNA VIA A PARTIR DE LA PANCREATINA PORCINA PARA PRODUCIR INHIBIDORES DE LA ENZIMA CONVERTIDORA DE ANGIOTENSINA (ECA) PRESENTES EN LA CASEINA

RESUMEN

La enzima convertidora de angiotensina (E.C. 3.4.15.1) desempeña un papel importante en la regulación de la presión sanguínea y en la patofisiología de la hipertensión, que causa varias enfermedades del sistema circulatorio, incluyendo infarto, arterioesclerosis y enfermedades de las arterias coronarias. La enzima convertidora de angiotensina (ACE, acrónimo en inglés) puede incrementar la presión sanguínea mediante la conversión de angiotensina I en un potente vasoconstrictor, la angiotensina II, y por la degradación de un vasodilatador arterial, la bradiquinina, a péptidos inactivos. La Inhibición de la ACE puede ejercer un efecto antihipertensor como consecuencia de una disminución en la actividad de la angiotensina II y el aumento concomitante de la actividad de la bradiquinina.

La actividad inhibidora de la ACE en hidrolizados pancreáticos de caseína bovina fue considerablemente alta para su concentración con un 50% de inhibición (IC50) de 600 μg/mL. Estos resultados sugieren que el hidrolizado de pancreatina de caseína bovina es una fuente potencial de péptidos hidrofóbicos con actividad inhibidora de ACE, y que la cromatografía en fenil-agarosa en NaCl 5 M es una técnica útil para purificar tales péptidos. En nuestro laboratorio están en progreso más estudios para separar y caracterizar los péptidos hidrofóbicos individuales.

Palabras claves: inhibitor de ACE, hidrolizado de caseína, péptido hidrofóbico, cromatografía de interacción hidrofóbica, fenil-agarosa

Título corto: Inhibidores de la enzima convertidora de angiotensina a partir de caseína
INTRODUCTION

Food classified as functional are generally associated with an individual primary health concerns, with increasing cardiovascular disease, high blood pressure, stroke, high cholesterol and cancer. Hypertension or high blood pressure is a significant health problem worldwide (Ozimek 2010a,b).

Angiotensin converting enzyme, the peptidyl dipeptide hydrolase (E.C. 3.4.15.1), commonly named ACE (see for example, Coates 2003), is an exopeptidase which cleaves dipeptides from the C-terminal of various peptide substrates. This enzyme plays an important role in the regulation of blood pressure and pathophysiology of hypertension, which causes various diseases of the circulatory system including stroke, arteriosclerosis, and coronary heart disease. Angiotensin converting enzyme can increase blood pressure by converting the inactive decapeptide angiotensin I to a potent vasoconstrictor, angiotensin II (Ondetti and Cushman 1982; Matsui et al 1999), and by degrading an arteriolar vasodilator, bradykinin (Ferreira et al 1970), into inactive peptides. A schematic diagram describing the mode of action of ACE in presented in figure 1. Inhibition of ACE may exert an antihypertensive effect as a consequence of a decrease in angiotensin II activity and a concomitant decrease in angiotensin II, which is a strong vasoconstrictor, angiotensin II (Ondetti and Cushman 1982; Matsui et al 1999), and by degrading an arteriolar vasodilator, bradykinin (Ferreira et al 1970), into inactive peptides.

Biologically active peptides are of particular interest in food science and nutrition because they have been shown to play physiological roles. Hidden or inactive in the amino acid sequence of native dairy proteins, they can be released or activated in vivo during gastrointestinal digestion, or upstream during food processing through enzymatic proteolysis, for example during cheese ripening or yogurt manufacturing. Bioactive peptides derived from milk proteins that inhibit ACE in the cardiovascular system can contribute to the prevention and treatment of hypertension. These ACE inhibitory peptides can be derived from many food proteins.

Recently, numerous researchers have confirmed that food proteins are precursors of many different biologically active peptides such as opiate, antithrombotic, antihypertensive, immunomodulating and antibacterial (Kitts and Weiler 2003; Korhonen and Pihlanto 2003, 2006; Hartmann and Meisel 2007), as it was previously claimed (Ariyoshi 1993).

It is well known that milk protein derived peptides do have several physiological activities (Meisel 1998, 2005). In this connection, several studies have demonstrated ACE inhibiting activities in peptides released after enzymatic hydrolysis of food proteins including milk proteins (Kusump 2006; Kusump and Ozimek 2007; Kusump et al 2007). These peptides are known to contain between two and 25 amino acid residues, and the majority are hydrophobic in nature (Bouhallab et al 1992, 1993). In this connection, there is limited information available concerning ACE inhibitors from caseins treated with pancreatin, a mixture of pancreatic enzymes.

The objective of this study was, therefore, to determine the ACE inhibiting activity in a pancreatin hydrolysate of casein by fractionating using hydrophobic interaction chromatography on phenyl-agarose. This medium was used because most ACE inhibitors are known to be hydrophobic peptides. A preliminary report concerning this subject was already done (Ozimek et al 2010).

MATERIALS AND METHODS

A sample of bovine casein hydrolysate was prepared by hydrolysis with pig pancreatin. Pancreatin:substrate ratio was 1:100. Pancreatin was added to an aqueous solution of 6% casein adjusting the medium to pH 7.6 by adding 1 N NaOH. Hydrolysis was undertaken at 37°C for 8 hours (Ozimek et al 1993; Kusump 2006). The mixture containing the casein hydrolysate was adjusted to pH 4.6 with 1 N HCl and was centrifuged at 12,000 g during 15 minutes, at 20°C. The resulting supernatant from each sample was fractionated using an ultrafiltration system at 20 psi of pressure with 2000 Da molecular weight cut-off membrane to give permeate and retentate. The obtained impregnate was lyophilized and stored at -20°C for subsequent purifications.

A portion of hydrolysate was dissolved in 0.01M sodium phosphate, pH 6.8 containing 5 M NaCl (solution A). This preparation was applied to a 1.5 x 6.5 cm column of phenyl-agarose (Sigma Chemical Co) equilibrated with solution A, and materials retained in the column were eluted with water. Fractions (2 mL) collected at a flow rate of 7.5 mL/hour were monitored for peptide contents by measuring the absorbance at 210 nm (Stoschek 1990) using serum bovine albumin as protein standard, and for ACE inhibiting activities.

The described fractionating method is a consequence of employing techniques of column chromatography separation developed in our laboratory (Nakano and Ozimek 2001; Nakano et al 2009).
Assays of ACE inhibiting activity were carried out using hippuryl-L-histidyl-L-leucine as substrate, following Cushman and Cheung (1971) recommendations, as modified by Nakamura et al (1995). ACE was obtained from rabbit lungs. Briefly, 80 µL of each sample were added to 200 µL of 0.1 mol/L potassium phosphate containing 0.3 mol/L NaCl and 5 mmol/L hippuryl-L-histidyl-L-leucine, pH 8.3. The reaction was stopped by adding 250 µL of 1 N HCl. The content of hippuric acid was released from the substrate by ACE hydrolysis, by extraction with ethyl acetate, heat evaporated at 95°C for 10 min, dissolved in distilled water. The absorbance of the extract was spectrophotometrically measured at the wavelength of 228 nm.

The inhibition activity was calculated using the following equation:

\[
\text{Inhibition activity, %} = 100 \times \left( \frac{(A - B) - (C - D)}{A - B} \right)
\]

where A is the absorbance of the solution containing ACE, but without the sample, B is the absorbance of a solution with ACE previously inactivate by adding HCl and without the sample, C is the absorbance in the presence of ACE and sample, and D is the absorbance with ACE previously inactivated with HCl and containing the sample.

The inhibitory activity of the hydrolysates or collected fractions was expressed as percentage of ACE inhibition at a given protein concentration. IC\(_{50}\) was defined as the concentration of an ACE inhibitor, in µg peptide/mL needed to inhibit 50% of ACE activity.

Data are presented as means and standard deviation of mean (Steel and Torrie 1980) whereas anova was conducted according to a one-way classification when required.

**RESULTS AND DISCUSSION**

The ACE inhibiting activity determined in the pancreatin hydrolysate of bovine casein was considerably high with its concentration of 50% inhibition (IC\(_{50}\)) being 600 µg/mL. The value was as high as the IC\(_{50}\) found in β-casomorphin, an ACE inhibiting peptide isolated from enzymatic digest of β-casein (see for example, Yannakis 1997).

The inhibitory activity of the hydrolysates or collected fractions was expressed as percentage of ACE inhibition at a given protein concentration. IC\(_{50}\) was defined as the concentration of an ACE inhibitor, in µg peptide/mL needed to inhibit 50% of ACE activity.

Further studies to separate and characterize individual hydrophobic peptides are under progress in our laboratory.

**ACKNOWLEDGEMENTS**

The authors gratefully acknowledge the facilitation activity of the librarians, Swine Research Institute, Havana, and to Professor W. Sauer for revising the final draft.

**REFERENCES**

Ariyoshi, J. 1993. Angiotensin-converting enzyme inhibitors derived from food proteins. Trends in Food Science and Technology, 4:139-144


---

**Table 1. Inhibitory activity of ACE in fractions of the pancreatin hydrolysate of casein as obtained by phenyl-agarose chromatography**

<table>
<thead>
<tr>
<th>Phosphate buffer</th>
<th>NaCl</th>
<th>ACE inhibition, %</th>
<th>IC(_{50}), µg peptide/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.0</td>
<td>4 M</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>3 M</td>
<td>4</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>2 M</td>
<td>3</td>
<td>nd</td>
</tr>
<tr>
<td>H(_2)O</td>
<td>-</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>SD ±</td>
<td></td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>pH 6.8</td>
<td>4 M</td>
<td>14</td>
<td>&gt;1 000</td>
</tr>
<tr>
<td></td>
<td>3 M</td>
<td>11</td>
<td>&gt;1 000</td>
</tr>
<tr>
<td></td>
<td>1 M</td>
<td>4</td>
<td>&gt;1 000</td>
</tr>
<tr>
<td>H(_2)O</td>
<td>-</td>
<td>7</td>
<td>&gt;1 000</td>
</tr>
<tr>
<td>SD ±</td>
<td></td>
<td>3.8</td>
<td></td>
</tr>
</tbody>
</table>

*nd express not determined*


Ozimek, L. 2010a. Food protein derived peptides as potential remedy for hypertension. In: Fourth International Congress in Food Science and Food Biotechnology in Developing Countries. Boca del Río (Veracruz), electronic version available in compact disc


