

Digestion Pattern of Antihypertensive Angiotensin I-Converting Enzyme Inhibitory Peptides from *Saccharomyces cerevisiae* in a Successive Simulated Gastric-intestinal Bioreactor

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A cell-free extract of *Saccharomyces cerevisiae* containing the angiotensin I-converting enzyme (ACE) inhibitory peptide was treated in a successive simulated gastric-intestinal bioreactor (step 1: amylase digestion, step 2: gastric fluid digestion, step 3: intestinal fluid digestion) to illustrate the absorption pattern of antihypertensive ACE inhibitory peptide, and the ACE inhibitory activities of each step were determined. Total ACE inhibitory activities of step 1, step 2, and step 3 were 55.96%, 80.09%, and 76.77%, respectively. The peptide sequence of each steps was analyzed by MS/MS spectrophotometry. Eleven kinds of representative peptide sequences were conserved in each step, and representative new peptides including RLPTES-VPEPK were identified in step 3.

KEYWORDS : Angiotensin I-converting enzyme inhibitory peptides, Digestion pattern, Simulated gastric-intestinal bioreactor

Angiotensin I-converting enzyme (dipeptidyl carboxy peptidase I, kinase II, E.C 3.4.15.1, ACE) is the key enzyme in the rennin-angiotensin system [1], which catalyzes production of the active hypertensive hormone angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) from the inactive pro-hormone angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) and also inactivates the hypotensive peptide bradykinin [2, 3]. This conversion endows ACE with a very important role in regulating blood pressure through the direct action of angiotensin II on blood vessels, sympathetic nerves, and adrenal glands [2, 3]. ACE inhibitors have long been very useful because they inhibit both ACE in the rennin-angiotensin system and the kallikrein-kinin system [4]. Although some ACE inhibitors have been produced and characterized from several natural sources [5], few are used commercially because of their low antihypertensive action and poor effectiveness in the gastric and intestinal systems. In a previous study [6], we purified and characterized an ACE inhibitor from *Saccharomyces cerevisiae*. The present study describes the absorption pattern of a novel ACE inhibitory peptide from alcohol fermented *S. cerevisiae*.

The purified ACE inhibitor from *S. cerevisiae* characterized in a previous paper with an IC_{50} of 0.07 mg [6] was used in this study. ACE was extracted from rabbit lung acetone powder with 100 mM sodium borate buffer (pH 8.3) containing 300 mM NaCl. The extract was maintained overnight at 4°C and its activity was determined using hippuric acid-histidine-leucine (Hip-His-Leu) as a substrate [1]. The rabbit lung acetone powder, pepsin (4,150 unit/

mg), trypsin (1,200 units/mg), protease N (185 units/mg), and the Hip-His-Leu were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Simulated gastric and intestinal fluid solutions were prepared according to United States Pharmacopeia specifications [7] as follows. For the simulated gastric fluid (SGF), 0.2% sodium chloride and 0.32% pepsin (from porcine stomach mucosa) were dissolved in 7.0 mL hydrochloric acid and a sufficient volume of water to make 1 L (pH 1.2). The purified peptide (1 mg/mL) was added to the SGF and incubated in a shaking incubator (37°C, 120 rpm) for 2 hr. Digestion was stopped by raising the pH to 7.5 using 1 N NaOH. For the simulated intestinal fluid (SIF), 0.68% monobasic potassium phosphate was dissolved in 250 mL water. To this, 77 mL of 0.2 N sodium hydroxide and 500 mL water were added and mixed along with 1% pancreatin (from porcine mucosa) (pH 6.8). The SIF solution was adjusted to pH 6.8 with 0.2 N HCl and then diluted with water to 1 L.

The SIF was added to the digests obtained from the SGF reaction and incubated for 2 hr. Then, these solutions were incubated in a shaking incubator (37°C, 120 rpm) for 10 hr. The final SGF and SIF digested samples were lyophilized immediately and used for measuring ACE inhibitory activity (IC_{50}). The stability of the purified peptide in SGF and SIF was analyzed by reverse phase high performance liquid chromatography. A simulated gastric-intestinal (SGI) bioreactor was designed using the gastric and intestinal fluids as shown in Fig. 1.

ACE inhibitory activity was assayed by the modified method of Cushman and Cheung [8]. A mixture containing 100 mM sodium borate buffer (pH 8.3), 300 mM NaCl, 3 units of ACE from rabbit lung, and an appropri-

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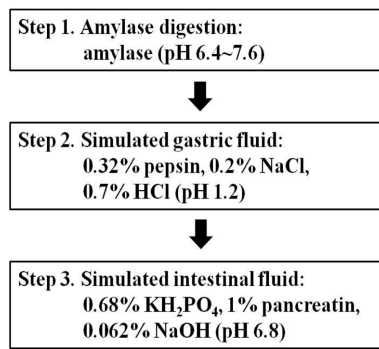


Fig. 1. Flow chart of digestion in the simulated gastric-intestinal bioreactor.

Table 1. Angiotensin I-converting enzyme (ACE) inhibitory activity of the purified ACE inhibitor after digestion in a simulated gastric-intestinal bioreactor

Steps	Control	Step 1	Step 2	Step 3
ACE inhibitory activity (%)	67.6 ± 0.46	56.0 ± 0.66	80.1 ± 0.42	76.8 ± 0.19

Control, without treatment; Step 1, mouth digestion; Step 2, stomach digestion; Step 3, small intestinal digestion.

ate amount of the inhibitor solution was preincubated for 10 min at 37°C. The reaction was initiated by adding 50 µL of Hip-His-Leu at a final concentration of 5 mM and was terminated after a 30-min incubation by adding 250 µL of 1.0 M HCl. The hippuric acid liberated was extracted with 1 mL of ethyl acetate, and 0.8 mL of the extract was evaporated with a Speed Vac concentrator (Eyela Co., Tokyo, Japan). Then, the residue was dissolved in 1 mL of sodium borate buffer. Absorbance at 228 nm was measured to estimate ACE inhibitory activity. The ACE inhibitor concentration required to inhibit 50% of the ACE activity under the assay conditions was defined as the IC₅₀. The amino acid composition of the ACE inhibitor from *S. cerevisiae* was analyzed with a fluorometric analysis system (SLM-Aminoco, Urbana, IL, USA) after hydrolysis for 24 hr in 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole at 110°C [9]. The amino acid sequence was determined by the Edman method [4], using an Applied Biosystems 491A automatic protein sequencer (Applied Biosystems, Foster City, CA, USA) [10].

We determined the inhibitory activity of the purified ACE inhibitor from *S. cerevisiae* after a digestion with gastric and intestinal fluids in the SGI bioreactor (Table 1). The original ACE inhibitory activity (67.6%) of the purified ACE inhibitor decreased to 56.0% after mouth digestion (step 1) and then increased to 80.1% after stomach digestion (step 2) and again decreased to 76.8% after digestion by small intestinal fluids (step 3). These changes in inhibitory activity suggest that some ACE inhibitory

Table 2. Peptides sequence of the control (non-treatment), step 1 (mouth digestion), step 2 (stomach digestion), and step 3 (small intestine digestion) hydrolysates

Peptide sequences	
Control	DDELDISWGIFTPLLK EIKLQILLLLEIIGLNDLDWNFR SFYMILPYM*VADLSGVLHNPR GQLGEM*AKC#LDNPDQGISMCR PYIAVGTISITKGEDIAVR KYSESPWLLVDEAQLVISYAK SC#LLPNVSSKMAVMM*AGLLGSLVIR VGLVQYHPTAANVLASSADNTIK ITVEKALPQLEMLGGLVACTEK LSGNPILGDGKSDNQNMSIPYIR DDELDISWGIFTPLLK
Step 1	LDVNTSVDEFFQGCFLNKLC#ISDK LNLQMPPIQTM*VRWGHQYAPR YIAM*DC#EFVGVGPEGKESALAR FGSQEEGLSLALSHYFDHNSGTSISK GQLGEM*AKC#LDNPDQGISMCR FLVNAC#M*NPVTAILDC#VNGEMK TDDDEDNSHLLVHHIPTIIRK QLSMM*LM*LAQSNPQLFALMGTR LCDFGVSGNLVASISK SVSKDANIMVVINAACHIQAM*AK LDVNTSVDEFFQGCFLNKLC#ISDK
Step 2	MLTGSQIVTM*EPENERTM*CSSAHMK ILKTELTIGNHIESQK FKFTPCGFC#FIIYSCPDEALNALK ILC#NLC#NVVPGGM*VCFLPSYEYLR GQLGEM*AKC#LDNPDQGISMCR DTHNAYSNDVYFLNLLSLKWYK LVNEVENVLNKEGVLOK LLEELQRLSGLSHK NPVYLTSTQSSSDYVFDTSCLK PAHPSTM*DENEIVHEEDVK MLTGSQIVTM*EPENERTM*CSSAHMK
Step 3	RLPTESVPEPK KETLGNPLK GLEFVRINNYFTTHDLQSFK TRNPLTHSTPK VGLVQYHPTAANVLASSADNTIK VVVDNQPPQPK PNYLQYRIIGGIVDLYIYVGGTK KYVSELLSNSWLSNLSETQVPYDPK DLAQQFGINLHMFSQLSDM*EFVMK LPNCIFLSDEMNHASM*INGIRNSR
Conserved peptide sequences ^a	GLEFVRINNYFTTHDLQSFK GQLGEM*AKC#LDNPDQGISMCR LISHGKISSNNVPGHSGNLNHYHR DFTRDVSJLSTVM*K PNYLQYRIIGGIVDLYIYVGGTK LSLAQYYGLTLEAADLC#LM*NFFQK TNSMSSSGLGSPNRSSPSIICM*PK IHARGPMGLDGLTIYK NPDLGFSDYAAAQLR EDNNDAVNSIMALK GLEFVRINNYFTTHDLQSFK

^aConserved peptides were present in every step including the control.

Table 3. Angiotensin I-converting enzyme (ACE) inhibitory activity of the 5-kDa cut-off filtrate extracts from the intestinal fluid hydrolysis following C18 solid phase chromatography (step 3)

C18 SPE								
0%	5%	25%	35%	45%	55%	65%	85%	100%
n.d	4.43 ± 0.86	39.95 ± 0.12	69.93 ± 0.07	74.15 ± 0.37	63.29 ± 0.39	20.29 ± 0.34	9.22 ± 0.40	n.d

n.d, not detected.

peptides were produced newly or decomposed by digestion with pepsin (stomach) and pancreatin (intestinal). Furthermore, the peptide sequences of the step 1, 2, and 3 hydrolysates were also analyzed by MS/MS spectrophotometry (Table 2). Eleven kinds of peptide sequences were conserved and ten kinds of new peptide sequences including RLPTESVPEPK were produced in step 3. It was assumed that these conserved and new peptide sequences were very important for antihypertensive action. Therefore, further determination of the ACE inhibitory activities of these conserved peptide sequences and new peptide sequences is required.

Several functional peptides are absorbed in the small intestine. Therefore, the step-3 hydrolysates were ultrafiltered with a 5 kDa cut-off filter, and the filtrates were subjected to C18 solid phase extraction chromatography. As shown in Table 3, 45% of the C18 solid phase extract showed the highest ACE inhibitory activity of 74.2%, and 35% of the C18 solid phase extracts and 55% of the solid phase extract also showed high ACE inhibitory activities of 69.9% and 63.3%, respectively. Further purification of the ACE inhibitor in the 45% fraction from the C18 solid phase extraction chromatography and a comparison of its inhibitory activities with the purified ACE inhibitor and the identified peptide sequences from SGI bioreactor are also required.

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