ANTIHYPERTENSIVE ACTIVITIES GENERATED FROM PORCINE SKELETAL MUSCLE PROTEINS BY LACTIC ACID BACTERIA

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Background

Many kinds of bioactive peptides are known to be generated from food proteins (Korhonen & Pihlanto, 2003). Especially from milk proteins, such peptides (*e.g.*, antihypertensive, antimicrobial and mineral binding peptides) have been discovered considerably. In some countries (*e.g.*, Japan), food industry has shown a great interest in bioactive peptides from food proteins for developing novel functional foods.

Angiotensin I-converting enzyme (ACE) inhibitory peptides are representative bioactive peptides generated from food proteins. ACE plays an important physiological role in regulating blood pressure (Figure 1). Several inhibitors of ACE have been found to be effective as antihypertensive pharmaceuticals. ACE inhibitory peptides derived from food proteins have been reported to create antihypertensive effects in spontaneously hypertensive rats by oral administration (Yamamoto *et al*., 1999). The antihypertensive effect of ACE inhibitory peptides derived from the casein of sour milk, was demonstrated in hypertensive human patients (Hata *et al*., 1996). This product has been developed into a new physiologically functional food in Japan. Research has also been conducted to characterize the ACE inhibitory activity derived from other foodstuffs, such as maize, eggs, gelatin, fish and fish products. However, little is still known about the derivation of such peptides from muscle proteins of domestic animals.

Recently, we have found that enzymatic hydrolysates of porcine skeletal muscle proteins exhibited potent ACE inhibitory activity (Arihara *et al*., 2001). Among muscle protein hydrolysates produced with eight different proteases, the digest of thermolysin showed the most potent inhibitory activity. ACE inhibitory peptides, named myopentapeptide A (Met-Asn-Pro-Pro-Lys) and B (Ile-Thr-Thr-Asn-Pro), were isolated from the thermolysin hydrolysate. Moreover, hydrolysates of porcine myosin as well as some peptides identical to a sequence of myosin showed antihypertensive activity in spontaneously hypertensive rats (Nakashima *et al*., 2002).

The results of these studies suggest that ACE inhibitory and antihypertensive peptides are easily generated from muscle proteins by enzymatic digestion. Thus, in meat products, such as fermented meat products with long-term ripening, these kinds of peptides may be generated. In fact, we have already detected ACE inhibitory activity in several commercial fermented meat products (unpublished data). Naturally occurring ACE inhibitory activity has also been detected in well-ripened cheese.

Objectives

In the present study, we investigated the generation of ACE inhibitory and antihypertensive activities in porcine skeletal muscle homogenates fermented with lactic acid bacteria. Efforts were also made to purify and identify the corresponding peptides from the homogenates. Such activities and substances could be utilized for producing new healthy meat products, which might open up a new market in the meat industry.

Materials and methods

Materials and Reagents

Fresh pork trim (ham) was obtained from a local packer. Hippuryl-L-histidyl-L-leucine (Hip-His-Leu) and ACE (from rabbit lung) were obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals were obtained from Wako Chemicals Co. (Tokyo, Japan).

Preparation of Fermented Homogenates

Fermented muscle homogenate was prepared as shown in Figure 2. Porcine skeletal muscle was homogenated in a Waring-type blender with four volumes of distilled water. Glucose (2%) and one of 5 strains (*Lactobacillus gasseri* JCM1131, *L. rhamnosus* FERM P-15120, *L. acidophilus* IAM12475, *L.*

helveticus JCM1554, *L. delbrueckii* subsp. *bulgaricus* NCFB2483) of lactic acid bacteria were added to the homogenate. These 5 strains of lactic acid bacteria were selected according to their proteolytic activities in our previous study (unpublished data). After 72 h of fermentation at 37°C, homogenates were heated at 98°C for 10 min to inactivate the bacterial and muscle protease activities. After removal of insoluble materials by centrifugation, the supernatants were utilized for further experiments.

Assay of ACE Inhibitory Activity

The ACE inhibitory activity was measured by a spectrophotometric assay according to Cushman and Cheung (1971) with modification by Nakamura *et al*. (1995). This assay is based on the liberation of hippuric acid from Hip-His-Leu catalyzed by ACE. The method was slightly modified in the present study. A sample solution of peptides (15 µl) was mixed with 125 µl of 100 mM sodium borate buffer (pH8.3) containing 7.6 mM Hip-His-Leu and 608 mM NaCl and then preincubated for 5 min at 37°C. The reaction was initiated with the addition of 50 µl of ACE dissolved in distilled water (50 m units/ml), and the mixture was incubated for 30 min at 37°C. The reaction was stopped by adding 125 µl of 1N HCl. The hippuric acid liberated by ACE was photometrically determined at 228 nm after ethyl acetate extraction. The concentration of ACE inhibitors needed to inhibit 50% of ACE was defined as the IC_{50} value.

Antihypertensive Effect in Spontaneously Hypertensive Rats

Male spontaneously hypertensive rats (SHR), 10 week old, were purchased from Charles River Japan Inc. (Yokohama, Japan). The SHR were housed in cages on a cycle of 12 h of light and 12 h darkness. The temperature and humidity in the cages were maintained at 24°C and 50 to 60%, respectively. The SHR were fed a standard laboratory diet (CE-2; Clea Japan, Inc., Tokyo, Japan), and tap water was freely available. A solution containing peptides was adjusted to 50 mg of solid material per ml, and was administered on the rats at a dose of 1 ml ie., 150 mg/kg of body weight. The *in vivo* antihypertensive activity was measured by monitoring the systolic blood pressure (SBP) of the SHR, from 15 to 28 week old (280 to 390 g in body weight). Rats given the sample solution via a gastric metal zonde were put in a thermostatted box at 40°C for 15 min, and the SBP was measured with a tail cuff equipped with a programmed electrosphygmomanometer (BP-98; Sftron Co., Tokyo, Japan, Figure 4). Phosphate-buffered saline (PBS) was used as a control in SHR.

Purification of ACE inhibitory Peptide

The supernatant solution of fermented muscle homogenate was fractionated by high-performance liquid chromatography (HPLC) with reversed-phase mode (column: CAPCELL PAK C_{18} UG120 4.6 x 150 mm; Shiseido, Tokyo, Japan) as described previously (Arihara *et al.*, 2001). Elution was performed with a linear gradient system from solvent A (0.1% trifluoroacetic acid in CH_3CN) at a flow rate of 1m/min, and absorbance was detected at 215 nm (first HPLC run). The active fraction was lyophilized, dissolved with distilled water, and rechromatographed under the same conditions as described above (second HPLC run). The peptide samples were further purified by HPLC with the same system except for the elution solution (third HPLC run). Elution was performed with a linear gradient (solvent A: 0.015% ammonia in distilled water, solvent B: 0.015% ammonia in CH_3CN).

Analysis of Peptide

The molecular formula of the peptide was confirmed from its fast atom bombardment mass spectrum (FAB-MS) obtained using an HX-110 spectrometer (JEOL Ltd, Tokyo, Japan). The sequence of the peptide was analyzed by automated Edman degradation using a 470A Protein Sequencer (Applied Biosystems, Inc., Forster City, CA).

Synthesis of Peptide

The synthesized peptide used in this study was prepared by the solid phase method with a 430A Peptide Synthesizer (Applied Biosystems, Inc.). Hydrogen fluoride was used for removing the side chain-protecting groups and for cleaving the peptide from their solid support. The synthesized peptides were purified by HPLC on a reversed-phase column (CAPCELL PAK C_{18} UG120 4.6 x 150 mm; Shiseido, Tokyo) with a linear gradient of CH_3CN (0 to 20%) in 0.1% trifluoroacetic acid.

Results and discussion

All muscle homogenates fermented with any of the *Lactobacillus* strains (*L. gasseri* JCM1131, *L. rhamnosus* FERM P-15120, *L. acidophilus* IAM12475, *L. helveticus* JCM1554, *L. delbrueckii* subsp. *bulgaricus* NCFB2483) showed ACE inhibitory activities higher than non-fermented muscle homogenate (Figure 4).

Among these 5 strains, the homogenate fermented with *L. rhamnosus* showed the highest inhibitory activity. Further experiments were then carried out on the muscle homogenate fermented with *L. rhamnosus*. The single oral administration of this homogenate to SHR significantly decreased their systolic blood pressure (Figure 5). The peptide responsible for the ACE inhibitory activity was purified from the homogenate by the combination of HPLC with reversed-phase mode. Octapeptide with the ACE inhibitory activity was purified and its amino acid sequence was determined (Val-Phe-Pro-Met-Asn-Pro-Pro-Lys). A search for sequence homology in databases revealed that the same sequence existed in the primary structure of the porcine skeletal muscle myosin heavy chain. The ACE inhibitory activity (IC_{50}) against the synthesized octapeptide was determined as 66.0 uM.

In the dairy industry, many physiologically functional foods have been developed. Asic studies on the tertiary function of milk components and on physiologically functional dairy prdoducts have been extensively conducted (Arai, 1996). However, there have been few such studies on meat products. Although many low-fat and low-salt meat products have been developed, there have been no efforts to introduce physiologically functional properties into meat products. We have reported recently that the concept of probiotics (cultures of live microorganisms that benefit the host by improving properties of indigenous microflora) has great potential in the meat industry (Arihara *et al*., 1998; Sameshima *et al*., 1998). *L. rhamnosus* FERM P-15120, the strain selected in this study, is a probiotic lactic acid bacteria isolated from human intestinal tract (Sameshima *et al*., 1998). Therfore, fermented meat products prepared with this strain are expected to have both probiotic and antihypertensive properties.

By using bioactive components, properties having potential health benefits can be introduced into meat products, thus improving the nutritional value of the products. Utilization of ACE inhibitory activity and substances from meat proteins may lead to the development of new healthy meat products.

Conclusions

This study demonstrated that peptides with ACE inhibitory and antihypertensive activity may be generated from meat by bacterial fermentation. Furthermore, fermentation of meat by lactic acid bacteria resulted in the generation of the ACE inhibitory peptide. The results of this study suggest that ACE inhibitory and antihypertensive activities generated by fermentation could be utilized to develop physiologically functional foods. Although bioactive peptides, such as ACE inhibitors, have not yet been utilized in the meat industry, meat products with such acitivity could open up a new market in the near future. It is expected that increasing interest will be shown in basic research and potential applications of bioactive peptides for meat products.

References

Arai, S. 1996. Review. Studies on functional foods in Japan. *Bioscience, Biotechnology and Biochemistry*, **60**: 9-15.

Arihara, K., Ota, H., Itoh, M., Kondo, Y., Sameshima, T., Yamanaka, H., Akimoto, M., Kanai, S., & Miki, T. 1998. *Lactobacillus acidophilus* group lactic acid bacteria applied to meat fermentation. *Journal of Food Science*, **63**: 544-547.

Arihara, K., Nakashima, Y., Mukai, T., Ishikawa, S., and Itoh, M. 2001. Peptide inhibitors for angiotensin I-converting enzyme from enzymatic hydrolysates of porcine skeletal muscle proteins. *Meat Science*, **57**: 319-324.

Cushman, D. W. and Cheung, H. S. 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochemical Pharmacology*, **20**: 1637-1648.

Hata, Y., Yamamoto, M., Ohni, M., Nakajima, K., Nakamura, Y., and 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.

Korhonen, H., & Pihlanto, A. 2003. Food-derived bioactive peptides: opportunities for designing future foods. *Current Pharmaceutical Design*, **9**: 1297-1308.

Nakashima, Y., Arihara, K., Sasaki, A., Mio, H., Ishikawa, S., and Itoh, M. 2002. Antihypertensive activities of peptides derived from porcine skeletal muscle myosin in spontaneously hypertensive rats. *Journal of Food Science*, **67**: 434-437.

Sameshima, T., Magome, C., Takeshita, K., Arihara, K., Itoh, M., & Kondo, Y. 1998. Effect of intestinal *Lactobacillus* starter cultures on the behaviour of *Staphylococcus aureus* in fermented sausage. *International Journal of Food Microbiology*, **41**: 1-7.

Yamamoto, N., Maeno, M., and Takano, T. 1999. Purification and characterization of an antihypertensive peptide from yogurt-like products fermented by *Lactobacillus helveticus* CPN4. *Journal of Dairy Science*, **82**: 1388-1393.

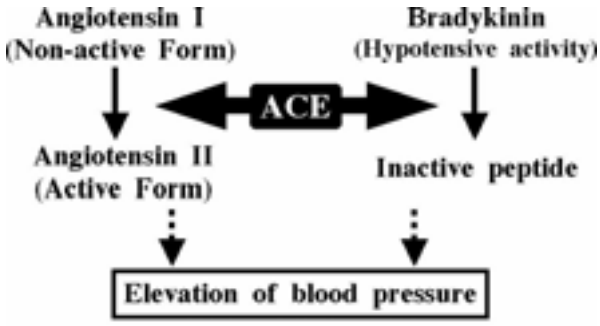

Figure 1. Role of angiotensin I-converting enzyme

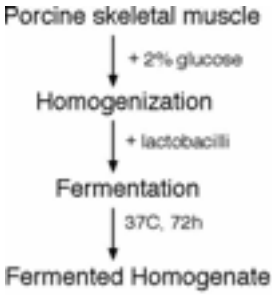

Figure 2. Preparation of fermented muscle homogenates.

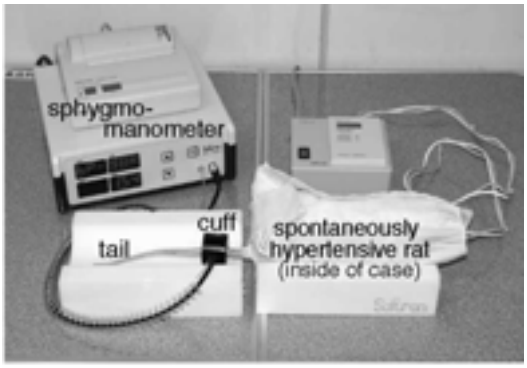

Figure 3. Electrosphygmomanometer used for the measurement of SBP.

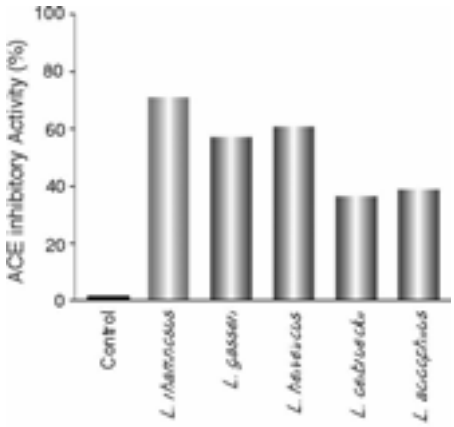

Figure 4. ACE inhibitory activities of fermented muscle homogenates.

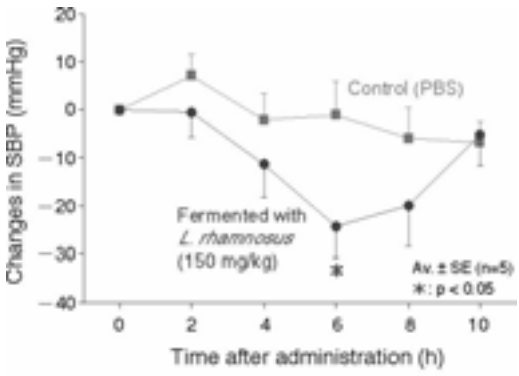

Figure 5. Antihypertensive activities of fermented muscle homogenates given to SHR in single oral administration.