Note

Angiotensin I-Converting Enzyme Inhibitors in Fish Water Soluble Protein Hydrolyzates Prepared by Bioreactor

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Liquid seasoning was prepared from the wash water produced during cod surimi production, which is normally discarded, using an hydrolysis test plant. This solution had not only a good flavor but also showed inhibitory activity on the angiotensin I-converting enzyme (ACE). Inhibitors in the liquid seasoning showed a molecular weight distribution of about 200 to 500 by gel filtration chromatography. Two inhibitory peptides were isolated from the liquid seasoning using reverse-phase HPLC. The amino acid sequences of inhibitory peptides were Val-Trp and Ile-Trp. Peptide sequences of Val-Trp and Ile-Trp were found in the primary structure of actin. Val-Trp fragment exists also in myoglobin.

Keywords: angiotensin I-converting enzyme inhibitor, enzyme membrane reactor, surimi, waste water

From the viewpoint of resource recycling, the recovery of water-soluble protein from the wash water produced during surimi production has attracted attention (Miyata, 1984; Niki et al., 1985; Ninomiya et al., 1985). Previously, Handa showed that a liquid seasoning containing taste-related components such as amino acids, nucleic acids and sugar, could be continuously produced from the waste wash waters using a pilot plant scale hydrolysis system (treatment capacity: 3–4 t of wash water/day) (Handa, 1993). This test plant involving a free enzyme reactor with an ultrafiltration module enables the continuous removal of hydrolyzate from the reactor and the recycling of the enzymes. After proteolysis hydrolyzate were concentrated and a fishy odor removed by electrodialysis, the liquid seasoning obtained from this system was extensively evaluated for flavor and taste by a sensory test, and was found satisfactory. The beneficial health effects of food factors have recently attracted attention in the food science field, and this liquid seasoning contain an extremely large amount of taurine (1400 mg/100 ml) which possesses various physiological effects such as the hypcholesterolemic effect. We also found that this liquid seasoning had a significant inhibitory effect on the angiotensin I-converting enzyme (ACE). Potent inhibitors of this enzyme show an antihypertensive effect (Ondetti et al., 1977). This report describes the isolation and structural analysis of ACE inhibitors from this liquid seasoning.

Materials and Methods

The wash water of surimi production, containing nitrogen atoms at 140 mg/100 ml, was supplied by a fishing company (Hachinohe). The liquid seasoning was prepared by the hydrolysis test plant previously described (Handa, 1993). The wash water was adjusted to pH 8.5 with sodium hydroxide. Proteins in the water were pretreated with proteases (Deskin C and Prochin A3L, Daiwa Kasei Co., Osaka) at 45°C and 70°C successively for 1 h, respectively, and then filtered to remove the insoluble substances. The permeate, composed of pretreated protein and enzyme, was incubated at 70°C in a reactor tank with a continuous flow filtration system. The hydrolyzate was produced at a flow rate of 225 l/h. The liquid seasoning contained nitrogen atoms at 1370 mg/100 ml, and free amino acids at 5563 mg/100 ml. The ACE inhibitory activity was measured by the method described in our previous report (Wako et al., 1996). Each assay was done in 250 μl of reaction mixture containing 12.5 mM Hip-His-Leu, 2.5 milliunits of ACE, 400 mM NaCl, and 100 mM sodium borate buffer (pH 8.3). The IC50 value is the concentration of 50% ACE inhibition in the reaction mixture. The amount of total nitrogen was determined by an improved method of Pregl-Dumas’s technique with an N.C-analyzer (NC-80, Shimadzu Co., Kyoto) (Aoyagi et al., 1982). GPC analysis was performed by HPLC. The wash water and liquid seasoning were diluted ten-fold with 45% acetonitrile containing 0.1% trifluoroacetic acid. Ten microliters of each preparation was put on a TSK gel G2500PWXL column (7.8×300 mm, Tosco Co., Tokyo). The column was developed with 45% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. Absorbance was monitored at 215 nm. A half milliliter of each fraction was obtained and assayed for ACE inhibitory activity. The molecular weight distribution of the inhibitor in the liquid seasoning was estimated using mono, di, tri, tetra, and penta-alanins. Inhibitory peptides were isolated from the liquid seasoning using reverse-phase HPLC. Eighty milliliters of the liquid seasoning was put into an open
column (50×70 mm) packed with ODS (LP-60C18, Wako Pure Chemical Ind., Osaka). The column was washed with 500 ml of water before the adsorbed fraction was eluted with 40% (v/v) methanol solution. The eluate was concentrated and the crude peptides were purified by HPLC on a TSK gel ODS-120 column (7.8×300 mm, Tosoh Co.), which was developed at a flow rate of 1 ml/min by a linear gradient of acetonitrile (10 to 40% in 60 min) containing 0.1% trifluoroacetic acid. Each fraction was dried with a centrifugal concentrator and its inhibitory activity was measured. The active fraction was further purified on a Puresil C$_{18}$ column (4.6×250 mm, Waters Co., Tokyo) which was developed at a flow rate of 0.7 ml/min by a linear gradient of acetonitrile (10.4 to 32% in 55 min) in a 10 mM ammonium formate buffer (pH 6.2). Amino acids were determined by the method described by Knecht and Chang (1986). The amino acid sequences of the purified peptides were analyzed using a gas-phase protein sequencer (model PSQ-21, Shimadzu Co., Kyoto).

**Results and Discussion**

The ACE inhibitory activity was found in the liquid seasoning prepared from the wash water of surimi production (IC$_{50}$: 256 µg nitrogen/ml), while wash water alone showed no activity. Figure 1 shows the gel filtration profiles obtained from the wash water (a) and liquid seasoning (b). The digested materials in the liquid seasoning emerged later compared to the intact protein in the wash water which emerged near the void volume, demonstrating the hydrolysis of the protein to low molecular peptides by enzyme. The apparent molecular weight distribution of the ACE inhibitors in the liquid seasoning was then estimated (Fig. 1 b). Potent inhibitors appeared in tubes corresponding to the molecular weight range of about 200 to 500. These observations indicated that the ACE inhibitory substances may have been processed through proteolysis of the water soluble protein and the inhibitor seemed to be an oligopeptide. We then attempted to isolate the inhibitory substances from the liquid seasoning, and obtained two peptide preparations as examples. The liquid seasoning was put on a reverse-phase column containing ODS (LP-60C18, Wako Pure Chemical Ind.). The active crude peptide preparation was recovered in the adsorbed fraction, and was fractionated on the ODS column (TSK gel ODS-120 column 7.8×300 mm, Tosoh Co.) (Fig. 2). Three fractions indicated as A, B, and C had high ACE inhibitory activity (more than 85% inhibition). The active fractions were further purified on the ODS column (Puresil C$_{18}$ column 4.6×250 mm, Waters Co.). The peptide preparations of B-I and C-I were isolated from fraction B and C, respectively. However, we failed to isolate a peptide from fraction A. A protein sequencer was used to identify the primary structure of the purified peptides, the amino acid sequences of B-I and C-I being Val-Trp and Ile-Trp, respectively (Table 1). Val-Trp was isolated earlier from the hydrolysate of sake lees (Saito et al., 1992). These peptide fragments exist in muscle protein and/or sarcoplasmic

![Fig. 1. Gel filtration profile of wash water (a) and liquid seasoning (b) with TSK gel G2000PWx4. Wash water: the effluent from surimi production, liquid seasoning: the preparation from wash water by the hydrolysis test plant. 10 µl of sample was put on a column and developed with 45% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. Absorbance at 215 nm was monitored. The vertical bar represents the ACE inhibitory activity of each fraction. Arrows indicate the retention times of standard peptides.](image)

![Fig. 2. Purification of ACE inhibitory peptides by HPLC. The crude peptide preparation was put on a ODS-120 column (7.8×300 mm) and eluted with a linear gradient of 10 to 40% of acetonitrile containing 0.1% trifluoroacetic acid. The vertical bar represents the ACE inhibitory activity of each fraction. A, B, and C indicate the fractions with high activity.](image)

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Structure</th>
<th>IC$_{50}$ (µM)</th>
</tr>
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<tbody>
<tr>
<td>B-I</td>
<td>Val-Trp</td>
<td>1.4</td>
</tr>
<tr>
<td>C-I</td>
<td>Ile-Trp</td>
<td>2.0</td>
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</table>

*The data are from Cheung et al. (1980).*

![Table 1. ACE inhibitory peptides from fish water soluble protein hydrolysate and their synthetic peptides.](image)
protein which is found in fish meat. Peptide sequences of Val-Trp and Ile-Trp are found in the primary structure of actin. The Val-Trp fragment also exists in myoglobin. However, these dipeptide fragments can occasionally be found in other proteins, for example, sequences of Val-Trp are found in lactate dehydrogenase and prolactin, and sequences of Ile-Trp in glutamate dehydrogenase and endonuclease. The source of these peptides thus cannot be determined as a particular protein. To evaluate the IC_{50} values for these peptides, the inhibitory activity was measured. The precise concentrations of the peptides were also confirmed by an amino acid analysis after hydrolysis with hydrochloric acid. The IC_{50} value for Val-Trp and Ile-Trp were 1.4 μM and 2.0 μM (Table 1). Cheung et al. (1980) studied the ACE inhibitory activity of a series of synthetic dipeptide derivatives including Val-Trp and Ile-Trp. Val-Trp showed the most potent inhibitory activity among these dipeptides; Ile-Trp was also a very potent inhibitor. Seki et al. (1995) investigated the susceptibility of ACE inhibitory dipeptide to gastrointestinal peptidase. The dipeptides with NH2-terminal of Val or Ile and those with COOH-terminal of Trp or Tyr had a higher resistance to digestion than the other dipeptides. Consequently, Val-Trp and Ile-Trp may be stable in the digestive tract after oral administration. In fact, it has been reported that the oral administration of Val-Trp (100 mg/kg) decreases blood pressure in spontaneously hypertensive rat (SHR) (Saito et al., 1994).

In this study we demonstrated significant ACE inhibitory activity in the liquid seasoning prepared from the waste water of surimi production, which is normally discarded, using the hydrolysis test plant. However, the optimization of the seasoning preparation to give the most potent inhibitory activity awaits further study. While we obtained two potent ACE inhibitory peptide preparations, there must be many other potent peptides. To determine this liquid seasoning can be useful as a healthy foods, further study will be needed to isolate such peptides and to confirm their concentrations in the liquid seasoning.

References


