Prolyl Endopeptidase Inhibitors in Sake and Its Byproducts

Yoshiyuki Saito,* Shin Ohura, Akitsugu Kawato, and Koji Suginami

Research Institute, Gekkeikan Sake Co., Ltd., 24 Shimotoba Koyanagi-cho, Fushimi-ku, Kyoto 612, Japan

The purification of the inhibitory peptide for prolyl endopeptidase from sake (alcohol beverage in Japan) and its byproduct was studied. Prolyl endopeptidase resolves the antidiuretic hormone vasopressin, which is also involved in the maintenance of memory. After confirming the inhibitory activities in pepsin hydrolysate of sake cake (byproduct of sake) and sake concentrate, we separated them with several chromatographic procedures. Three inhibitory peptides were obtained from sake cake, and another three inhibitory peptides were obtained from sake. These peptides inhibit prolyl endopeptidase *in vitro*. These sequences exist in rice glutelin.

Keywords: *Proteinase inhibitor; peptide; anti-amnesia; rice; sake*

INTRODUCTION

Recent research reports that the food functions concerned with human health increase daily. In the field of alcoholic beverages, Marmot et al. (1981) reported that the mortality rate was lower in men reporting moderate alcohol intake than in either non-drinkers or heavy drinkers, and several investigators have noted the antioxidative effects of phenolic compounds present in wine (Renaud and de Lorgeril, 1992; Criqui and Ringel, 1994; Frankel et al., 1995).

We also have been studying the new functions of sake and its byproducts, rice bran and sake cake (lees of sake mash). Sake is a traditional and general alcoholic beverage in Japan. Sake is brewed from rice, shows an alcohol concentration of about 15%, has a characteristic fragrance, is slightly acidic, and has a sweet taste. There are two byproducts in the sake brewing process. The one is rice barn, and the other is sake cake. Rice grains are polished up to a weight loss of 30−60% to remove fats, proteins, and inorganic components present in large amounts in the outer layers of the grains before brewing. At the end of fermentation, matured mash is transferred to a filter cloth and sake is squeezed out from the sake cake, which is the lees of the sake mash. It has been an ecological problem that these byproducts have increased every year for the purpose of improvement of sake quality. So it is important to develop new uses of these byproducts.

In one of our previous studies, antihypertensive angiotensin I converting enzyme inhibitors from sake and its byproducts were isolated and identified as novel inhibitory peptides that had two to five amino acid residues (Saito et al., 1992, 1994a). We had reported that systolic blood pressure decreased significantly when these peptides were orally administered to spontaneously hypertensive rats as the hydrolysate of sake cake and concentrate of sake (Saito et al., 1994b).

Prolyl endopeptidase (PEP) exists in the hippocampus of the cerebrum and cuts off the peptide bond at the carboxyl site of proline residue (Yoshimoto et al., 1983). It is known that memory impediment or deterioration of learning occurs when the activity of PEP is in excess. In other words, the capacity of memory and learning is diminished when excess PEP inactivates bioactive peptides such as vasopressin and substance-P which are involved in memory and neural communication (Aoyagi et al., 1990; Atack et al., 1991; Yoshimoto et al., 1987). Indeed, it was confirmed that the PEP activity of amnestic patients was very high and vasopressin of these patients was unusually decomposed (De Wied et al., 1984). So it was expected that the prevention or treatment of amnesia and dementia may be possible by inhibition of PEP (Tsuru and Yoshimoto, 1987). In the field of medicine, a PEP inhibitor such as prolinal derivative (Tsuru and Yoshimoto, 1987; Yoshimoto et al., 1987) and thiazolidine family (Tsuru et al., 1988) has been investigated for practical use in anti-amnesia. Moreover in the field of foodstuffs, PEP inhibitor has been noticed as having a new function (Asano et al., 1991; Maruyama et al., 1992).

We studied the inhibitory activity to PEP of sake and its byproducts and confirmed significant inhibitory suppression. So we purified and identified the active component from sake and its byprocucts, and we describe the results in this paper.

MATERIALS AND METHODS

Samples. We used sake brewed in our factory [Be, +3.0; Alc, 19.4 (v/v %); TA, 1.80 (mL), AA, 1.87 (mL)] and lyophilized sake cake from the process (Imayasu et al., 1989) using a liquefied product of rice slurry as the raw material (water content, 5%; crude protein, 81%; sugars, 12%; others, 2%).

Measurement of PEP Inhibitory Activity. Two methods of Yoshimoto et al. (1979) were used with some modifications. One is as follows (Figure 1a): 50 μL of sample solution with 100 μL of PEP (*Flavobacterium* sp. Seikagaku kogyo, Tokyo, Japan) solution (100 munits/mL in buffer solution) and 200 μL of phosphate buffer solution (pH 7.0, 100 mM) were preheated and incubated at 30 °C for 10 min with 100 μL of substrate solution [500 μM *N*-Cbz-glycyl-L-proline-*p*-nitroanilide (BACHEM Feinchemikalien, Bubendorf, Switzerland) in 40% dioxane]. The other is as follows (Figure 1b): 50 μL of sample solution with 50 μL of PEP solution (10 munits/mL in buffer solution) and 350 μL of Tris-HCl buffer solution (pH 7.0, 50 mM) were pre-heated and incubated at 30 °C for 10 min with 50 μL of substrate solution [50 mM *N*-Cbz-glycyl-L-proline-7-amido-4-methylcoumarin (BACHEM Feinchemikalien, Bubendorf, Switzerland) in distilled water]. We used the former for crude sample and the latter for the refined one. These assay procedures yield a linear relationship between activity and enzyme amount. PEP inhibitory activity was shown as the minimum sample amount that could inhibit 50% of the PEP activity.

Preparation of PEP Inhibitors. *PEP Inhibitory Activities of the Crude Sample.* The crude sample for PEP inhibitor

* Corresponding author [telephone +81 (0)75-623-2130; fax +81 (0)75-623-2132].

S0021-8561(96)00470-0 CCC: $14.00

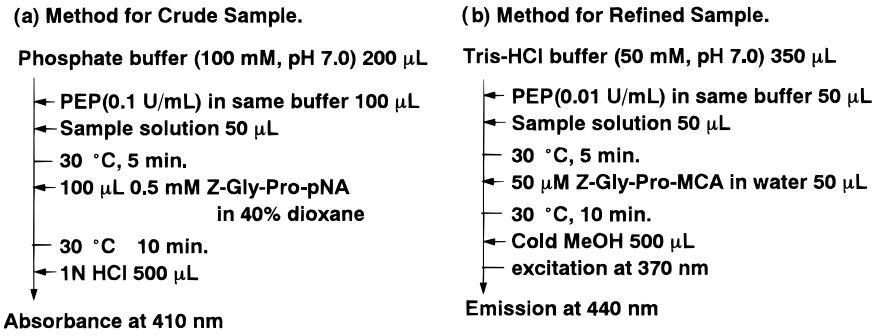

Figure 1. Measurement of PEP inhibitory activity for crude (a) and refined (b) samples.

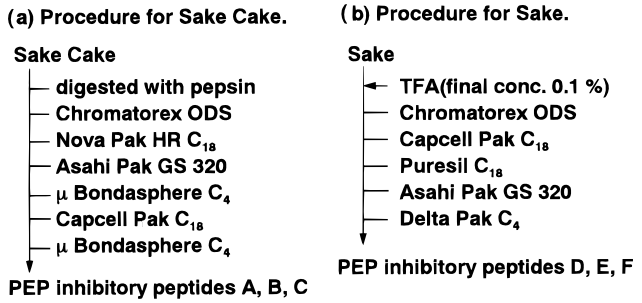

Figure 2. Purification procedure for PEP inhibitor for sake cake (a) and for sake (b).

was prepared as follows: lyophilized sake cake was added to water, adjusted to pH 1.5, and digested with pepsin (p7012, Sigma Chemical, St. Louis, MO) at 40 °C for 1 h. After neutralization, the reaction was terminated in boiling water and the mixture was centrifuged to obtain a clear solution. Hydrolysis was conducted under the condition that the substrate concentration was 5% in water (w/v) and the enzyme concentration was 0.5% of the substrate (w/w). Sake was condensed to one-tenth of the volume under reduced pressure. The inhibitory activities of these crude samples was then determined.

Preparation of PEP Inhibitors from Sake Cake. Lyophilized pepsin digestate of sake cake was dissolved with buffer solution (20% acetonitrile, 0.1% trifluoroacetic acid; TFA), adsorbed to Chromatorex ODS (DM1020T, Fuji Silysia Chemical, Osaka, Japan), and washed with the same buffer solution (Figure 2a). The adsorbed fraction was then eluted with 60% of acetonitrile, and the non-adsorbed fraction was discarded.

The adsorbed fraction was fractionated with Prep Nova-Pak HR C_{18} (Waters, Milford, MA) (eluting conditions: 0.1% TFA, acetonitrile 20−35% 20 min, 5 mL/min). Second, active fractions were separated with Asahipak GS 320 (Shodex, Tokyo, Japan) (eluting conditions: distilled water, 5 mL/min). Active fractions were separated further with μ Bondasphere C_4 (Waters, Milford, MA) (eluting conditions: 0.1% TFA, acetonitrile 30−45%/20 min, 5 mL/min) and Capcell Pak C_{18} AG 120 A (Shiseido, Tokyo, Japan) [eluting conditions: 10 mM $(NH_4)_2CO_3$, acetonitrile 11−29%/20 min, 5 mL/min]. Finally, PEP inhibitory peptides were purified with μ Bondasphere C_4 (eluting conditions: 0.02% TFA, acetonitrile 40−45%/20 min, 5 mL/min).

Preparation of PEP Inhibitors from Sake. After TFA was added for a final concentration up to 0.1%, the sake was adsorbed directly to Chromatorex ODS and washed with 60% methanol (Figure 2b). The adsorbed fraction was then eluted with absolute methanol, and the non-adsorbed fraction was discarded.

The adsorbed fraction was fractionated with Capcell Pak C_{18}, AG 120 A [eluting conditions: 10 mM $(NH_4)_2CO_3$, acetonitrile 11−29%/20 min, 5 mL/min]. Second, active fractions were separated with Puresil C_{18} (Waters, Milford, MA) (eluting conditions: 0.1% TFA, acetonitrile 20−35%/20 min, 5 mL/min). Active fractions were further separated with Asahipak GS 320 (eluting conditions: distilled water, 5 mL/min). Finally, PEP inhibitory peptides were purified with Delta Pak C_4 (eluting conditions: 0.02% TFA, acetonitrile 30−60%/20 min, 5 mL/min).

Table 1. PEP Inhibitory Activity of Sake Cake and Sake

sample	IC_{50} (μg/L)
sake cake hydrolysate	74
sake concentrate	900

Deblocking of the N-Terminus Blocking Peptide. We tried three methods to deblock N-terminus blocking peptide according to the strategy of Hirano et al. (1992). (a) Deblocking of the *N*-formyl residue: Purified PEP inhibitory peptides from sake were incubated with 0.6 N HCl solution at 25 °C for 24 h. (b) Deblocking of the *N*-acetyl residue: These peptides were incubated 37 °C for 24 h with the reaction mixture [0.1 mM ditiothreitol; DTT, 50 milliunits of acylamino-acid-releasing enzyme (Takara Syuzo, Kyoto, Japan), 10 mM phosphate buffer, pH 7.2]. (c) Deblocking of *N*-pyrglutamyl residue: Inhibitory peptides were incubated with pyroglutamate aminopeptidase (Takara Syuzo, Kyoto, Japan) at 37 °C for 24 h with the reaction mixture (5 mM DTT, 10 mM EDTA, 0.5 milliunits of pyroglutamate aminopeptidase, 100 mM phosphate buffer pH 7.0). After the reaction mixture was lyophilized, N-terminus deblocked peptides were separated by reverse-phase HPLC, and then the sequences of the peptides were analyzed.

Analysis of Peptides. The purity of each peptide was confirmed by analytical HPLC using Purecil C_{18} (eluting conditions: 0.1% TFA, acetonitrile 10−60%/40 min, 1 mL/min). The amino acid composition of each peptide was analyzed with an L-8500 amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis with 6 N HCl (containing 5% mercaptoacetic acid) at 110 °C for 24 h under reduced pressure. The molecular weight of N-terminus blocked peptides was confirmed from their matrix-assisted laser desorption ionization time-of-flight mass spectrum (MALDI TOF-MS) obtained with a Shimazu-Krotos Kompact MALDI III (Shimazu, Kyoto, Japan). The sequence of the peptide was analyzed with a protein sequencer (470A; Perkin Elmer, Foster City, CA).

Peptide Synthesis. Solid-phase peptide synthesis was done with a peptide synthesizer (Pepticoupler 2200; Vega, Tucson, AZ).

RESULTS

PEP Inhibitory Activities of the Crude Sample. Concerning the hydrolysis of sake cake, we finally concluded that pepsin, an asparatic protease, was the most effective to obtain PEP inhibitor after we had compared the inhibitory activities with hydrolysate from serine protease, cysteine protease, asparatic proteinase, and metalloproteinase at various conditions (data not shown). Table 1 shows the PEP inhibitory activities of pepsin hydrolysate of sake cake and concentrate of sake. Inhibitory activities of sake cake hydrolysate was superior to that of sake concentrate. As significant inhibitory activities were observed from these samples, we tried to separate the pure active fraction from them.

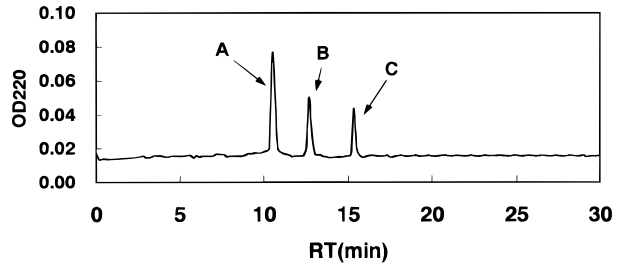

Figure 3. Reverse-phase HPLC of PEP inhibitory peptides in sake cake. Details are shown in Results ("Preparation of PEP Inhibitors from Sake Cake") and Table 2.

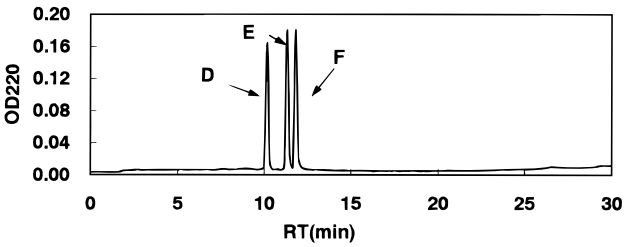

Figure 4. Reverse-phase HPLC of PEP inhibitory peptides in sake. Details are shown in Results ("Preparation of PEP Inhibitors from Sake") and Table 3.

Table 2. Amino Acid Sequences and IC_{50} Values of PEP Inhibitors from Sake Cake

	sequence	IC_{50} (μM)
A:	Ser-Pro-Phe-Trp-Asn-Ile-Asn-Ala	42.8
B:	Leu-Ser-Pro-Phe-Trp-Asn-Ile-Asn-Ala	29.0
C:	Leu-Leu-Ser-Pro-Phe-Trp-Asn-Ile-Asn-Ala	24.3

Preparation of PEP Inhibitors from Sake Cake. We obtained 2.5 g of adsorbed and 23.2 g of non-adsorbed fractions, both by lyophilized weight, from 25 g of dried sake cake by pepsin treatment. We purified three PEP inhibitory peptides from pepsin hydrolysate of sake cake with a combination of four kinds of reverse-phase and gel filtration HPLC. Three components (A, B, and C) obtained here were examined for purity by analytical reverse-phase HPLC (Figure 3). These peaks were single and pure, and their retention times were 10.5, 12.7, and 15.3 min, respectively. The amino acid sequences of these peptides were determined by sequencing analysis. Table 2 shows the inhibitory activities and sequences of these peptides.

Preparation of PEP Inhibitors from Sake. We obtained 1.98 g of adsorbed and 68.4 g of non-adsorbed fractions from 5 L of sake by treatment with Chromatorex ODS. We purified another three PEP inhibitory peptides, all lyophilized weight, from this fraction by the series of HPLC. The purity of these three components (D, E, and F) obtained from sake was also confirmed by analytical HPLC (Figure 4). Retention times of these components were 10.2, 11.3, and 11.8 min, respectively, but we could not determine these sequences. We could not detect any peak on HPLC after performing the Edman degradation, ensuring adequate sample volume. So we concluded that N-terminus of these peptides were blocked with fatty acid, acyl, or alkyl residue.

Deblocking of N-Terminus Blocking Peptide. We then tried to deblock these peptides by three methods, deblocking *N*-formyl, *N*-acetyl, and *N*-pyroglutamyl residue, but only deblocking of *N*-pyroglutamyl residue was effective.

We examined the retention times of these peptides before and after this treatment. Retention times of peptides D, E, and F changed after enzyme treatment. After separation of deblocked peptides (D′, E′, and F′, respectively), the amino acid sequences of these peptides were determined by sequencing analysis as follows: D′, Leu-Phe-Asn-Pro-Ser-Thr-Asn-Pro-Trp-His-Ser-Pro; E′, Leu-Phe-Asn-Pro-Ser-Thr-Asn-Pro-Trp-His-Ser-Pro-Arg; F′, Leu-Phe-Gly-Pro-Asn-Val-Asn-Pro-Trp-His-Asn-Pro-Arg.

The $[M + H]^+$ of peptides D, E, and F appeared at *m*/*z* 1507, 1664, and 1661 in the MALDI-TOF-MS, respectively. The molecular weight of peptide D agreed with the sum of the molecular weight of peptide D′ and 111, the molecular weight of pyroglutamate residue. These relations are applicable between E and E′ and between F and F′, respectively. So we have determined the amino acid sequences of these peptides from these results. Table 3 shows the inhibitory activities and sequences of these peptides.

DISCUSSION

PEP inhibitors from sake and its byproducts were isolated, and we identified six new inhibitory peptides which have 8−14 amino acid residues.

We isolated peptides A, B, and C from sake cake. When one or two leucine residues were cleaved from N-terminus of peptide C, inhibitory activity gradually became weaker. These peptide sequences existed in rice

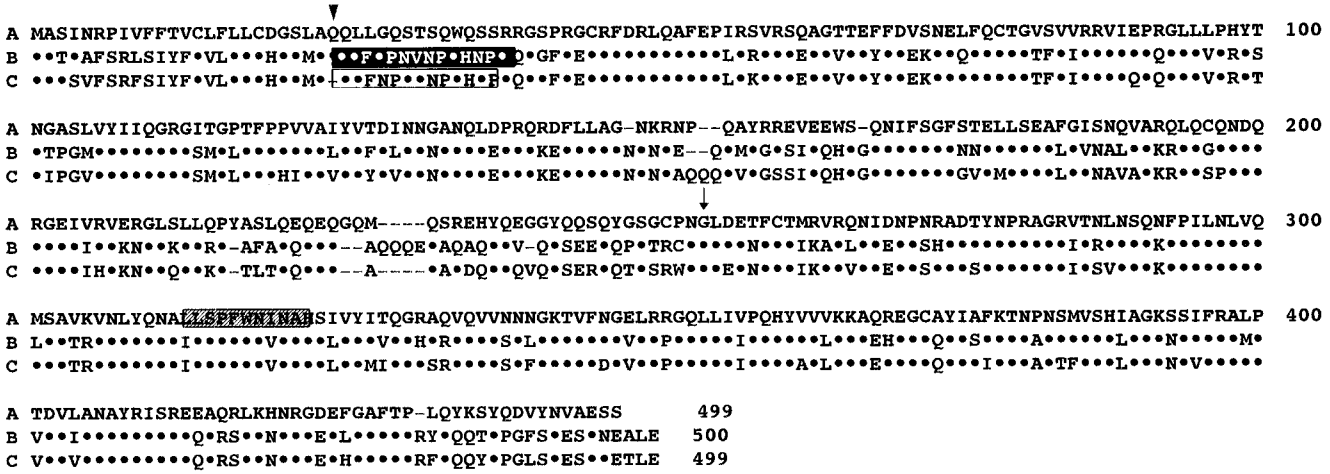

Figure 5. Amino acid sequences of PEP inhibitory peptides in glutelin: pREE77 (a); λRG 21 (b); pREE K1 (c). The shaded boxes show the PEP inhibitory peptide. The arrowhead indicates the cleavage site of signal peptide, and the short arrow indicates the cleavage site between the acidic and basic subunits. Identical amino acids are omitted.

Table 3. Amino Acid Sequences and IC_{50} Value of PEP Inhibitors from Sake

	sequence	IC_{50} (μM)
D:	pGlu-Leu-Phe-Asn-Pro-Ser-Thr-Asn-Pro-Trp-His-Ser-Pro	24.3
E:	pGlu-Leu-Phe-Asn-Pro-Ser-Thr-Asn-Pro-Trp-His-Ser-Pro-Arg	14.1
F:	pGlu-Leu-Phe-Gly-Pro-Asn-Val-Asn-Pro-Trp-His-Asn-Pro-Arg	11.8

protein. Protein sequences of pre-proglutelin, the principal protein of rice, are shown in Figure 5. Our peptide C corresponds to the sequence of amino acid residues contained in pre-proglutelin, indicated by the hatched box in Figure 5 (Takaiwa et al., 1986, 1987; Okita et al., 1989). Moreover, the Phe-Trp-Asn sequences in our PEP inhibitory peptide were already isolated and identified by us as ACE inhibitory peptides (Saito et al., 1992). So these peptides have been expected to be multifunctional peptides which have not only antihypertensive but also anti-amnestic effects.

We isolated peptides D, E, and F from sake. These peptides could not be sequenced directly by Edman degradation. In those cases the amino acid of the N-terminus happened to be blocked. So we tried to deblock these peptides by three methods. After treatment with pyroglutamate aminopeptidase, the amino acid sequences of these peptides could be determined. From the results of enzyme treatment, MALDI-TOF-MS, and sequencing analysis, we finally determined the amino acid sequences of these peptides as described in Table 3. Inhibitory activities of these peptides are stronger than those of peptides from sake cake.

These peptide sequences also exist in rice protein (pre-proglutelin); namely, our peptide E is equal to the amino acid residues of pre-proglutelin, indicated by the box in the Figure 5 (Takaiwa et al., 1989), and peptide D is shorter by one amino acid residue from the C-terminus than peptide E. Peptide F was also equal to the amino acid residue of another type of pre-proglutelin, indicated by black box in the Figure 5 (Masumura et al., 1989). Rice protein (Pre-proglutelin) is so microheterogeneous that at least four types of polypeptides exist (Masumura and Tanaka, 1993). There are two types which polymerize to high molecular weight glutelin (Types I and II), one type which remains a heterodimer (Table III), and another type which does not split into two subunits (Type IV). We believe that peptides A, B, and C remained in sake cake because they exist as Type I glutelin; on the other hand, peptides D and E dissolved in the sake because they exist as Type III glutelin, which does not polymerize and has a low molecular weight. We believe that peptide F also originates from sake, like peptides D and E, although it exists as Type II glutelin because peptide F is the N-terminus of mature glutelins such as peptides D and E.

There is some inconsistency between the sequences of preproglutelin and our inhibitory peptide. The N-termini of peptides D, E, and F are pyroglutamate, but amino acid residues of pre-proglutelin, which corresponds to our peptide, are glutamine. Mature glutelin is made from pre-proglutelin after the signal peptide is cleaved and divided into acidic and basic subunits, which then combine with each other. The signal peptide of glutelin is formed by residues 1−24 of pre-proglutelin, so amino acid residue 25, the N-terminus of peptides D, E, and F, is the N-terminus of mature glutelin. It generally happens that the glutamine residue at the N-terminus of peptide easily encircles itself and becomes pyroglutamate residue. The rate of pyroglutamate formation depends on the amino acid sequences and increases when leucine, asparagine, or glycine is the C-terminus end of glutamine (Orlowska et al., 1987). Takaiwa et al. (1986) and Okita et al. (1989) stated that the N-terminus amino acid of mature glutelin acidic subunit was glutamine and that glutamine cyclized to pyroglutamate.

Recently many pyroglutamyl peptides, such as THR and LH-RH, were found in the neuro system and attention is being drawn to their roles. These Pyr-peptides are comparatively stable in the living body because they are resistant to aminopeptidase. So our peptides also have been expected for the PEP inhibitory effect *in vivo*. PEP inhibitory peptides from β-casein, zein, and proline-rich protein of soy are known to be derived from foodstuffs (Asano et al., 1991; Maruyama et al., 1992), but the constructions of our peptides are different from these peptides. In the future we intend to study relationships between structures and activities of these peptides and anti-amnestic effects on living bodies.

LITERATURE CITED

Aoyagi, T.; Wada, T.; Nagai, M.; Kojima, F.; Harada, S.; Takeuchi, T. Deficiency of kallikrein-like enzyme activities in cerebral tissue of patients with alzheimer's disease. *Experientia* **1990**, *46*, 94−97.

Asano, M.; Nio, N.; Ariyoshi, Y. Inhibition of prolyl endopeptidase by synthetic peptide fragments of human β-casein. *Agric. Biol. Chem*. **1991**, *55*, 825−828.

Atack, J. R.; Suman-Chauman, N.; Dawson, G.; Kulagowski, J. J. *In vitro in vivo* inhibition of prolyl endopeptidase. *Eur. J. Pharmacol*. **1991**, *205*, 157−163.

Criqui, M. H.; Ringel, B. L. Does diet or alcohol explain the French paradox? *Lancet* **1994**, *344*, 1719−1723.

De wied, D.; Gaffori, O.; Van Ree, J. M.; De Jong, W. Central target for the behavioural effects of vasopressin neuropeptides. *Nature* **1984**, *308*, 276−278.

Frankel, E. N.; Waterhouse, A. L.; Teissedre, P. L. Principal phenolic phytochemicals in selected california wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins. *J. Agric. Food Chem*. **1995**, *43*, 890−894.

Hirano, H.; Komatsu, S.; Takakura, H.; Sakiyama, F.; Tsunasawa, S. *J. Biochem*. **1992**, *111*, 754−757.

Imayasu, S.; Hata, Y.; Ohishi, K.; Kawato, A.; Suginami, K. Sake brewing using the liquefied product of rice slurry as raw material. *Nippon Nogeikagaku Kaishi* **1989**, *63*, 971−979.

Marmot, M. G.; Rose, G.; Shipley, M. J.; Thomas, B. J. Alcohol and mortality: A U-shaped curve. *Lancet* **1981**, *14*, 580−583.

Maruyama, S.; Miyoshi, S.; Osa, T.; Tanaka, H. Prolyl endopeptidase inhibitory activity of peptides in the repeated sequence of various proline-rich proteins. *J. Ferment. Bioeng*. **1992**, *74*, 145−148.

Masumura, T.; Kidzu, K.; Sugiyama, Y.; Mitsukawa, N.; Hibino, T.; Tanaka, K.; Fujii, S. Nucleotide sequence of a cDNA encoding a major rice glutelin. *Plant Mol. Biol*. **1989**, *12*, 723−725.

Masumura, T.; Tanaka, K. Biosynthesis and accumlation of rice storage proteins. *J. Brew. Soc. Jpn*. **1993**, *88*, 414−420.

Okita, T. W.; Hwang, Y. S.; Hnilo, J.; Kim, W. T.; Aryan, A. P.; Larson, R.; Krishnan, H. B. Structure and expression of rice glutelin multigene family. *J. Biol. Chem*. **1989**, *264*, 12573−12581.

Orlowska, A.; Witkowska, E.; Izdebski, J. Sequence dependence in the formation of pyroglutamyl peptides in solid phase peptide synthesis. *Int. J. Pept. Protein Res.* **1987**, *30*, 141−144.

Renaud, S.; de Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523−1526.

Saito, Y.; Nakamura, K.; Kawato, A.; Imayasu, S. Angiotensin I converting enzyme inhibitors in sake and its by-products. *Nippon Nogeikagaku Kaishi* **1992**, *66*, 1081−1087.

Saito, Y.; Wanezaki, K.; Kawato, A.; Imayasu, S. Structure and activity of angiotensin I converting enzyme inhibitory peptides from sake and sake lees. *Biosci. Biotechnol. Biochem.* **1994a**, *58*, 1767−1771.

Saito, Y.; Wanezaki, K.; Kawato, A.; Imayasu, S. Antihypertensive effects of peptide in sake and its by-products on spontaneously hypertensive rats. *Biosci. Biotechnol. Biochem.* **1994b**, *58*, 812−816.

Takaiwa, F.; Kikuchi, S.; Oono, K. The structure of rice storage protein glutelin precursor deduced from cDNA. *FEBS Lett.* **1986**, *206*, 33−35.

Takaiwa, F.; Kikuchi, S.; Oono, K. A rice glutelin gene family-A major type of glutelin mRNAs can be divided into two classes. *Mol. Gen. Genet.* **1987**, *208*, 15−22.

Takaiwa, F.; Kikuchi, S.; Oono, K. The complete nucleotide sequence of new type cDNA coding for rice storage protein glutelin. *Nucleic Acids Res.* **1989**, *17*, 3289.

Turu, D.; Yoshimoto, T.; Koriyama, N.; Furukawa, S. Thiazolidine derivatives as potent inhibitors specific for prolyl endopeptidase. *J. Biochem.* **1988**, *104*, 580−586.

Turu, D.; Yoshimoto, T. Exploitation of anti-amnestic drug. *BioIndustry* **1987**, *4*, 788−796.

Yoshimoto, T.; Ogita, K.; Walter, R.; Koida, M.; Turu, D. Post-proline cleaving enzyme. *Biochem. Biophys. Acta.* **1979**, *569*, 184−192.

Yoshimoto, T.; Nishimura, T.; Kita, T.; Turu, D. Post-proline cleaving enzyme (prolyl endopeptidase) from bovine brain. *J. Biochem.* **1983**, *94*, 1179−1190.

Yoshimoto, T.; Kado, K.; Matubara, F.; Koriyama, N.; Kaneto, H.; Turu, D. Specific inhibitors for prolyl endopeptidase and their anti-amnesic effect. *J. Pharmacobio-Dyn.* **1987**, *10*, 730−735.

Received June 28, 1996. Revised manuscript received December 16, 1996. Accepted January 2, 1997.[⊗]

JF9604706

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1997.