Study on uric acid lowering peptides in proteolytic digest of shark cartilage.

サメ軟骨のプロテアーゼ分解物に含まれる

尿酸値低下ペプチドに関する研究

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CONTENTS

Chapter 1. General introduction

Utilization of shark for foods, cosmetics and medicines-history and recent trend.

Shark belongs to the subclass *Elasmobranchii* of the class *Chondrichthyes*, with the branchial clefts opening on the sides of the body. About 500 species are widely distributed in the oceans of the earth, and some are known to inhabit brackish water and fresh water.

The first record of shark meat as food was observed in *Engi Shiki* in Japan. It was prepared and paid to government as tax in Heian era (Narita et al.; 2001). Since sharks use urea for the regulation of the osmotic pressure of body fluids, urea accumulates in their muscle. As shark meat emits the smell of ammonia, when freshness is lost. Frequently, shark meat are ground into paste and used as *Surimi* for *Kamaboko* and *Hanpen*. On the other hand, transparent fibers on the both side of shark fins cartilage are used for high grade Chinese cuisine. In some cases, shark is discarded to sea except for fins. Shark has cartilage as endskeletal and large liver and has unique compounds in body. Shark has，therefore, attracted attention as source for pharmaceutical and functional food ingredients. Shark liver oil is rich in squalene. squalane, diacyl glyceride and so on. Squalene and squalen have been demonstrated to exert beneficial effects such as

maintaining skin functions and improving the liver and immune functions. Diacyl glyceryl ethers have also been reported to enhance immune system, inhibit cancer progress, maintain skin functions (Nobuto et al.;1988, Nobuto et al.; 1987, Brohult et al.; 1970, Homma et al.;1990, Nishikawa et al.; 2011), and attenuate the damages by radiation (Brohult et al.; 1958, Brohult et al.; 1954, Alexander et al.; 1959).

In addition to liver oil, shark cartilage has been used to produce beneficial ingredients for foods, cosmetics and medicines. The skeleton of the shark, a cartilaginous fish, is made up entirely of cartilage, which primarily consists of calcium, their salt, collagen and proteoglycans. Proteoglycan is a complex of non-collagenous core protein and mucopolysaccharides. Chondroitin sulfate, hyaluronic acid, and keratan sulfate are main mucopolysaccharide in proteoglycans in shark cartilage. Shark cartilage has been used as a folk medicine to moderate rheumatoid arthritis, osteroarthritis. It has been also used for enhancement of the wound healing and the treatment of non-tumorous chronic inflammatory diseases since the 1950s to 1990s. Recently, highly purified chondroitin sulfate or chondroitin sulfate sodium is used for the treatment of low back pain, arthralgia, shoulder periarthritis by injection (Hiroaki et al.; 1994, Jackson et al.; 1953, Hochberg et al.; 2008), and for the protection of the superficial layer of the cornea as eye drops (Embriano; 1989). Animal experiments have also demonstrated potential beneficial

activities of shark cartilage compounds. Moses et al. discovered that shark cartilage has an anti-angiogenic action in the 1990s (Moses et al.; 1990), and reported the possibility that this action is derived from the inhibition of matrix metalloproteinases (MMPs)(Moses et al.; 1991, Moses et al.; 1992, Folknan; 1995), which are involved in tumors proliferation and metastasis (Hidalgo et al.; 2001, Gonzalez et al.;2001). To confirm the antitumor effect of crude shark cartilage powder and their extract, clinical studies have been initiated (Marwick et al.; 2001, Ernst; 1998, Miller et al.; 1998, Tsukasa et al.; 2012, Tsukasa et al.; 2006). However, controversial results have been reported. Some studies showed disappointed results possibly due to the difficulty in administration of effective dose of shark cartilage for cancer patient (Murata et al.; 2002). Then, efforts have been made to extract active components from shark cartilage to reduce dosage necessary for the beneficial activities. After crushing of dried shark cartilage to 20 μm by freeze fracturing mill. Significant amounts of proteoglycan and other compounds can be extracted with water (Murota et al.; 2014). It has been demonstrated that the water extract of shark cartilage increases serum inhibitory activities against MMP-2 and MMP-9 and suppress progression of pancreatic duct cancer in animal model. These effects were not observed in a water extract of bovine cartilage prepared by the same method (Tsukasa et al.; 2012, Tsukasa et al.; 2006). These reports suggest that the

anticancer component contained in shark cartilage can be extracted under moderate conditions using water, which can reduce dose compared to shark cartilage powder.

Recently in Japan, a field of medicine referred to as complementary and alternative medicine (Suzuki; 2004), characterized by combining treatments of Western medicine and traditional treatments including those of oriental medicine, has rapidly emerged. Concerning cancer treatment, the Japanese Society for Palliative Medicine prepared the "Guidelines for Complementary and Alternative Treatments for Cancer" as a project sponsored by the Cancer Research Subsidy of the Ministry of Health, Labor and Welfare. Shark cartilage is positive mentioned in the guidelines (Sumiyoshi; 2006, Suzuki; 2006), and the results reported by Murata may accelerate this approach.

Background of the present study-Gout and Anti-hyperuricemic activity of protease digest of shark cartilage.

The present study originated from the research aimed to identify novel pharmacological effects of edible chondroitin sulfate, which is a food-grade crude protease digest of shark cartilage. Blood biochemical parameters were checked after two weeks administration of the food-grade crude protease digest of shark cartilage in rat

(1g/kg body weight). Fall tendency of serum uric acid was observed, which initiated the present study.

Uric acid tends to crystallize in blood more than 7 mg/dl (Nuget et al.;1959). The crystal deposits in joints, tendons, and surrounding tissues, which induce sever inflammation, namely gout. Gout is a medical condition usually characterized by recurrent attacks of acute inflammatory arthritis – red, tender, hot, swollen joint. The metatarsal – phalangeal joint at the base of the big toe is most commonly affected. Formation of uric acid crystal and the conversely dissolution depend on the concentration of uric acid, pH, sodium concentration, and temperature. Hyperuricemia is, therefore, one of important risk factors for the onset of gout (Hall et al.; 1967, Campion et al.; 1987, Terkeltaub; 2010, Weaver; 2008). To treat gout, chemically synthesized drugs – allopurinol, benzbromarone, probenecid and so on, which are based on inhibition of uric acid synthesis or enhancement of urinary excretion of uric acid, have been clinically used; however, occasional side effects of these medicines are a stumbling block for chronic use. In spite of their low frequency, the side effects of allopurinol could be life-threatening and occur more often in patients with renal insufficiency (Fam; 1998, Harris et al.; 1999, Lipshultz et al.; 2003, Schlesinger et al.; 2004).

Many factors, including genetics, insulin resistance, hypertention, renal insufficiency, obesity, diet, and use of diuretics, contribute to hyperuricemia. Gout is, therefore, a life-style related disease. Dietary factors have been estimated to account for about 12% of causes of gout (Chen et al.; 2008). With the change in eating habits, gout has become prevalent worldwide. Onset of gout has a strong association with the consumption of alcohol, fructose-sweetened drinks, meat, and seafood (Terkeltaub; 2010, Weaver; 2008). A ketogenic diet impairs the ability of the kidney to excrete uric acid, due to competition for transport between uric acid and ketones (Forster; 1979). The gene SLC2A9 encodes a protein that helps to transport uric acid into renal cytomembrane. Several single nucleotide polymorphisms of this gene are known to have a significant correlation with blood uric acid (Brandstatter et al.; 2008). In addition, it has been demonstrated that hyperuricemia is closely associated with other lifestyle-related diseases (Hosoya; 2008) .

Uric acid is the final metabolite of endogenous and food-derived purine bases in humans. To control the blood uric acid level, patients suffering from hyperuricemia are encouraged to consume foods with a low-purine content. For this purpose, low-purine alcoholic beverages are commercially available in Japan (Shibano et al.;1996, Tofler et

al.; 1981). However, it is difficult for individuals to control their blood uric acid level only using low-purine foods.

The preliminary experiment using normal rat suggested that oral administration of protease digest of shark cartilage has potential to moderate hyperuricemia. The present study was carried out to confirm the anti-hyperuricemic effect of the protease digest of shark cartilage using oxonate-induced hyperuricemic rat model. In addition, the present study aimed to identify the active component.

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Chapter 2.

Uric acid lowering effect by ingestion of proteolytic digest of shark cartilage and its basic fraction

ABSTRACT

Supplementing with proteolytic digest of shark cartilage to give 1.5% and 2.5% in the diet significantly moderated hyperuricemia in rats induced by potassium oxonate in the diet. On the other hand, no significant effect was observed by a diet containing 2.5% proteolytic digest of cow cartilage. Hyperuricemia induced by a single intraperitoneal injection of potassium oxonate was also moderated by single ingestion of the proteolytic digest of shark cartilage in a dose-dependent manner. The proteolytic digest of shark cartilage was fractionated on the basis of the isoelectric point into acidic, weak acidic and basic fractions. Significant anti-hyperuricemic activity was observed only in the basic fraction, and ingestion of chondroitin sulfate free from protein, one of the major constituents of the digest, had no significant anti-hyperuricemic activity. Basic components, possibly peptide, might be responsible for anti-hyperuricemic activity in this rat model.

INTRODUCTION

As described chapter 1., patients suffering from gout are encouraged to eat foods with low purine content to control the blood uric acid level by food, as uric acid is also a metabolite of food-derived purine. For the patient suffering from gout, low purine beverages have been developed (Fukui; 2011, Fukuuchi et al.; 2013). Recently, it has been reported that oral ingestion of chitosan can moderate hyperuricemia by suppressing absorption of purine into blood (Shirogane et al.; 2004). To the best of our knowledge, there are few peer-reviewed papers have demonstrated that ingestion of food components display anti-hyperuricemic activity based on xanthine oxidase inhibition.

Shark cartilage consists of proteoglycan, collagen and various proteins with biological activities (Lee et al.; 1984). Highly purified chondroitin sulfate C, polysaccharide moiety of proteoglycan, has been used for medical purposes (Ameya et al.; 2006) . In addition to medical products, various forms of shark cartilage products have been prepared as food ingredients. It has been suggested that these products might have beneficial effects on patients suffering from cancer, osteoarthritis, rheumatoid arthritis and so on, although their efficacy is controversial (Miller et al.; 1998, Hyodo et al.; 2005). Recently, a food-grade proteolytic digest of shark cartilage has been prepared on an industrial scale (Murota et al.; 2003). As mentioned chapter 1., in the preliminary

experiment, it was observed that ingestion of the proteolytic digest of shark cartilage tended to decrease the serum uric acid level of normal rat, suggesting that the digest has the potential to moderate hyperuricemia.

Rats have uricase which metabolizes uric acid to allantoin. Then, rats has low serum uric acid. Therefore, normal rats are not suitable for anti-hyperuricemic research. For this purpose, potassium oxonate-induced hyperuricemic rat model has been established. Potassium oxonate is an uricase inhibitor. It was checked in advance that the digest did not affect uricase activity.

The objectives of chapter 2. were to confirm the anti-hyperuricemic activity of the proteolytic digest of shark cartilage prepared in an industrial scale, using the potassium oxonate-induced hyperuricemic rat model and to estimate the component responsible for anti-hyperuricemic activity.

MATERIALS AND METHODS

Materials and Reagents

A crude proteolytic digest of shark cartilage was prepared on an industrial scale according to the method described by Murota et al (2006). Briefly, shark cartilage was suspended in water and heated in an autoclave at 12°C, before being digested with a food-grade Bacillus licheniformisprotease. The digest was clarified by filtration, passed through activated carbon, and then spray-dried. This product, referred to SCP, can be obtained commercially from Maruha Nichiro. (Tokyo, Japan). Analytical grade chondroitin sulfate and the Uric Acid Test Wako Kit were purchased from Wako Pure Chemicals (Osaka, Japan). Agarose powder was purchased from Nacalai Tesque (Kyoto, Japan). Potassium oxonate was purchased from Across Organics (Geel, Belgium).

Fractionation of Components in digests of shark cartilage.

The fractionation of components in the digest was performed by ampholyte-free preparative isoelectric focusing (autofocusing) according to the method of Hashimoto et al. (2005) using autofocusing apparatus with 10 sample compartments (75 mm in length x 80 mm in width x 85 mm in height). The digest was dissolved in deionized water to give 10% (w/v) and loaded into sample compartments No. 5 and 6. The other sample

compartments were filled with deionized water. Autofocusing was carried out 500 V for 12 h.

Animal Experiments

Female Sprague-Dawley strain rats and male Wistar rats were purchased from Charles River Laboratories Japan (Yokohama, Japan). Male Sprague-Dawley strain rats were purchased from Japan SLC (Hamamatsu, Japan). All animals were caged in the same building and allowed free access to diet pellets and tap water. They were allowed to adapt to the environment for a week before being used. This study was conducted in accordance with the standards established by the guide for the care and use of laboratory animals of Maruha Nichiro Holdings Central Research Institute (Tsukuba City, Japan).

Experiment 1. Hyperuricemia was induced by the method of Dan et al (1994). Nine-week-old female Sprague-Dawley strain rats (average body weight, 221.1 g) were fed a diet containing 2.5% (w/w) potassium oxonate (hyperuricemic; HU diet). Shark cartilage digest was supplemented to the HU diet to give 2.0% (SCP diet). The compositions of the HU and SCP diets are shown Table 1. Eighteen rats were fed the HU or SCP diet for 41 days. In addition, another 12 rats were fed the HU diet for 29 days and then divided into two groups; one group (n=6) continued to ingest the HU diet and

another group (n=6) was switched to the SCP diet for an additional 12 days. Rats were fasted for more than 12 h before blood drawing. The diets used in all experiments were prepared by Oriental Yeast (Tokyo, Japan).

Experiment 2. Shark cartilage digest was suspended in distilled water at a test material concentration of 0.02–0.2 g/mL (w/v) and administered at a constant volume of 5 mL/kg body weight to 8-week-old male Sprague-Dawley rats (average body weight, 285.4 g) via a stomach sonde to give a dosage of 0.1, 0.3 and 1.0 g/kg body weight. One hour later, the rats were i.p. injected with potassium oxonate solution (250 mg/2 mL of 3% gum arabic saline/kg body weight) to elevate the blood uric acid level (Osada et al.; 1993). Whole blood samples were collected 1 h after oxonate injection.

Experiment 3. Four-week-old male Sprague-Dawley rats (average body weight, 140.1 g) were fed diets containing 3% potassium oxonate supplemented with Shark cartilage digest, cow cartilage digest or purified chondroitin sulfate (CS) for 28 days. Rats were fasted for more than 12 h before blood drawing.

Experiment 4. Acidic, weak acidic and basic fractions of shark cartilage digest prepared by autofocusing were evaluated for their anti-hyperuricemic activity. The fractions were freeze-dried and re-suspended in distilled water and administered daily at a constant volume of 5 mL/kg body weight to 7-week-old female Sprague-Dawley rats

(average body weight, 201.3 g) to give a dosage 380, 250, 370 mg/kg body weight for acidic, weak acidic and basic fractions, respectively, via a stomach sonde. The dosage of each fraction was determined on the basis of the recovery of each fraction by autofocusing. The rats were fed a purified diet (Oriental Yeast) and administered with the test components for 22 days. Rats were fasted for more than 12 h before blood drawing.

Preparation of Sera

Blood was collected from the caudal artery, or the abdominal aorta under anesthesia and left to stand for 0.5–1 h at room temperature to clot. Serum was collected by centrifugation and stored at -30°C until used.

Analytical Procedures

Protein, mucopolysaccharide, lipid, moisture and heavy metals were determined according to the Association of Official Analytical Chemists method (1990). Amino acid analysis was performed according to the method of Bidlingmeyer et al. (1984) with a slight modification (Sato K et al.; 1992). Chondroitin sulfate was determined by the method of Bowness (1957). Serum uric acid was measured using an Uric Acid Test Wako Kit based on the phosphotungstic acid method.

Statistical Analyses

Data are presented as the means±standard deviation. Differences between the means were evaluated by Student's t-test with Holm adjustment (P=0.05) or analysis of variance followed by Dunnett, Bonferroni/Dunn or Fisher's protected least significant difference method (P=0.05). Statistical studies were performed with StatView Version 5.0 (SAS Institute, Cary, NC).

RESULTS

Chemical Characterization of Crude and Fractionated Proteolytic Digest of Shark Cartilage

The crude proteolytic digest of shark cartilage digest was characterized for its proximate composition and amino acid profile, as shown in Tables 2 and 3. The digest consisted of mainly peptide/protein and acidic polysaccharides containing uronic acid, namely mucopolysaccharide. Cellulose acetate electrophoresis and compositional analyses revealed that chondroitin sulfate C is a major constituent of the mucopolysaccharide fraction of the digest (data not shown). The digest contained hydroxyproline, a modified amino acid specifically distributed in collagen, at 3%. In collagen, hydroxyproline accounts for approximately 8% of the total amino acids. On the basis of these data, (the proteins in the digest) consisted of peptides derived from collagen (30%) and other proteins.

The shark cartilage digest was fractionated by autofocusing. As shown in Fig. 1, pH gradient from 3 to 11 was formed. Fractions 1–6 showed approximately pH 3. On the basis of pH value after autofocusing, fractions 1–6, 7 and 8, and 9 and 10 were combined and used as the acidic, weak acidic and basic fractions in the following experiments, respectively. The acidic fraction was rich in chondroitin sulfate C. The weak acidic

fraction was rich in peptide. As shown in Fig. 2, collagen-specific amino acids (hydroxyproline and hydroxylysine) were distributed in all fractions in approximately the same ratio. The peptide in the acidic and weak acidic fractions showed comparable amino acid composition, while the basic fraction was characterized with smaller acidic amino acids and higher basic amino acids than other fractions.

Experiment 1. The addition of potassium oxonate to the diet significantly increased the serum uric acid level to approximately 3.5mg/dl in the control group, as shown in Fig. 3A. On the other hand, supplementation of shark cartilage digest to the control diet suppressed potassium oxonate-induced hyperuricemia. Twenty-eight days after the administration of oxonate, the shark cartilage digest (SCP) group showed a significantly lower serum uric acid level than its counterpart. Rats fed the HU diet containing oxonate for 28 days showed significantly lower serum uric acid. The serum uric acid level continued to increase in the HU diet group, while it stopped increasing in the group switched to the SCP diet. Twelve days after the supplementation of SCP, the serum uric acid level was significantly lower in the SCP group (Fig. 3B). There was no difference in body weight gain among groups (data not shown).

Experiment 2. Hyperuricemia was also induced by a single i.p. injection of potassium oxonate. As shown in Fig. 4, oral administration of Shark cartilage digest decreased serum uric acid in a dose-dependent manner.

Experiment 3. To compare the uric acid-lowering effect with shark cartilage digest, enzymatic hydrolysate of cow cartilage digest and purified shark CS were used. After supplementation with 2.5% cow cartilage digest and CS for 28 days, no significant uric acid-lowering effect was observed, while a significant effect was again observed in the SCP group (Fig. 5). There was no difference in body weight gain among groups (data not shown).

Experiment 4. The components in shark cartilage digest were fractionated on the basis of the isoelectric point. After administration of test components for 22 days, only the basic fraction showed a significant uric acid-lowering effect even in smaller dosage than that of shark cartilage digest (Fig. 6). There was no difference in body weight gain among groups (data not shown).

DISCUSSION

In the hyperuricemic rat model based on long-term ingestion of oxonate in the diet, supplementation of shark cartilage digest from the start and 29 days after oxonate administration suppressed the development of hyperuricemia. A similar effect was observed in both male and female rats of two strains. On the other hand, the proteolytic digest of cow cartilage digest does not have this potential activity. This suggests that shark cartilage digest has the potential to moderate hyperuricemia, which must be proven by clinical study.

As shown in Table 2, SCP predominantly consists of mucopolysaccharides (chondroichin sulfate C) and peptides. The ingestion of purified shark chondroitin sulfate C did not show anti-hyperuricemic activity. This result is consistent with the acidic fraction, which contains a higher amount of chondroitin sulfate C, having no anti-hyperuricemic activity. On the other hand, the basic fraction showed higher anti-hyperuricemic activity. In addition, the water extract of shark cartilage without proteolytic digestion did not show a significant uric acid lowering effect (data not shown). These facts suggest that basic peptide fraction might be responsible for anti-hyperuricemic activity.

Amino acid analysis revealed that the protein/peptide fraction consists of collagen- and other protein-derived peptide; however, the active peptide has not been identified. The preliminary experiment revealed that the shark cartilage digest has no in vitro inhibitory activity against xanthinoxidase, which is a key enzyme for uric acid synthesis. Therefore, the in vitro assay system based on the inhibition of xanthinoxidase cannot be used for identification of the active peptide. In addition, the peptides in the microbial protease digest might be further degraded by peptidases in digestive tracts and blood of rat and change their biological activity. It is, therefore, the in vitro activity of peptide in the diet could not be directly linked to the in vivo activity of peptide. Interestingly, a single administration of shark cartilage digest can suppress transient elevation of serum uric acid by i.p. injection of oxonate in a dose-dependent manner, as shown in Fig. 4. Based on this result, in vivo activity-guided fractionation of the peptide is performed as mentioned in chapter 4., in order to identify the active component, which would allow us to elucidate the mechanism for uric acid-lowering effect.

TABLES AND GRAPHS

Table 1. Composition of hyperuricemic and shark cartilage digest diets in experiment 1

(%)

	Hyperuricemic diet (HU diet)	Shark cartilage digest diet (SCP diet)
Corn starch	38.0	38.0
Casein	25.0	25.0
α-corn starch	10.0	10.0
Cellulose	8.0	8.0
Soybean oil	6.0	6.0
Mineral mix (Oriental)	6.0	6.0
Vitamin mix (Oriental)	2.0	2.0
sucrose	2.5	1.0
Potassium oxonate	2.5	2.5
Shark cartilage digest	-	1.5

Table 2. Proximate composition of crude proteolytic digest of shark cartilage.

Component	
Protein	53 %
Mucopolysaccharide	43 %
Lipid	0.01 %
Moisture	4.1 %
Heavy Metal	less than 10 ppm

Table 3. Amino acid profiles of crude proteolytic digest of shark cartilage.

Amino Acids (g/100g)	Shark cartilage digest
Hyp	3.6
Asp	4.1
Thr	2.2
Ser	2.2
Glu	5.8
Pro	5.1
Gly	10.7
Ala	6.2
Cys	2.0
Val	1.7
Met	3.9
Ile	4.2
Tyr	0.4
Phe	1.3
Lys	2.3
His	0.7

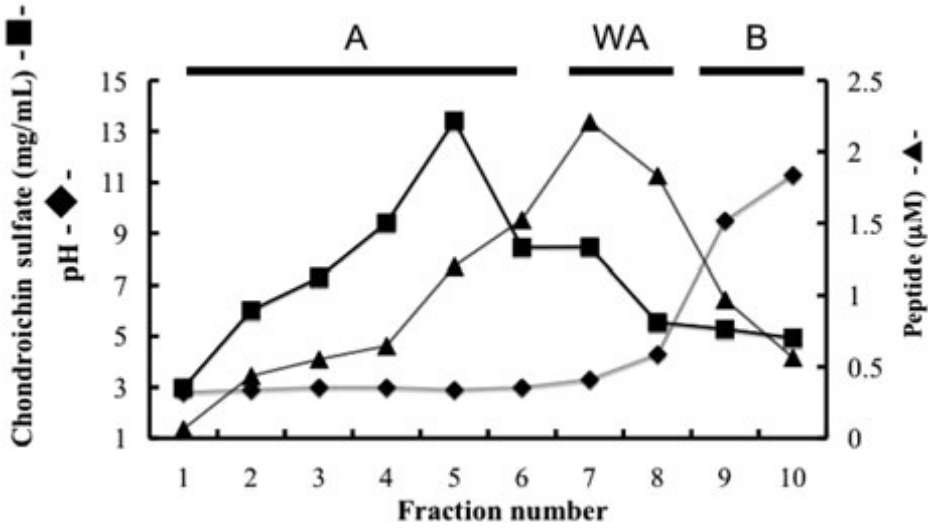

Figure 1. PH gradient and contents of chondroitin sulfate C and peptide in the autofocusing fractions.

Peptide content is expressed as sum of constituent amino acids. Acidic (A; Fr. 1–6), weak acidic (WA; Fr. 7 and 8), and basic (B; Fr. 9 and 10) were collected for the following experiments.

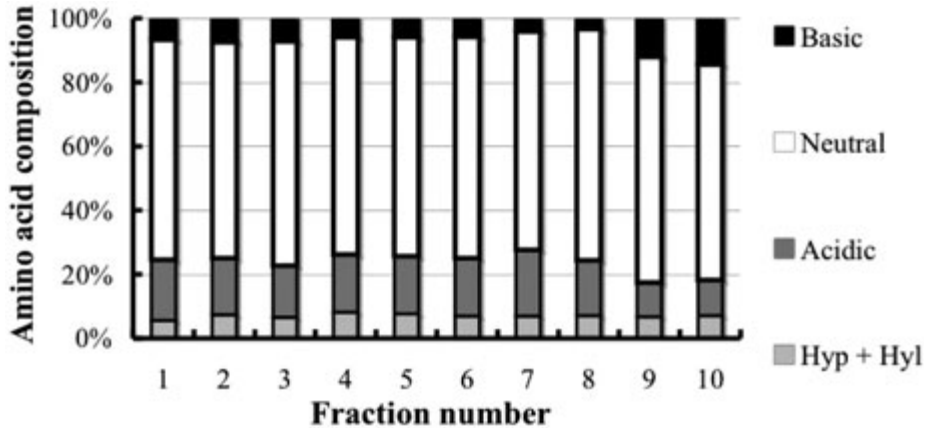

Figure 2. Amino acid composition of autofocusing fractions.

Acidic; Asp and Glu after hydrolysis, neutral; sum of Ser, Gly, Thr, Ala, Pro, Tyr, Val, Met, Ile, Leu and Phe, basic; sum of His, Arg and Lys. Hyp and Hyl represent hydroxyproline and hydroxylysine, respectively.

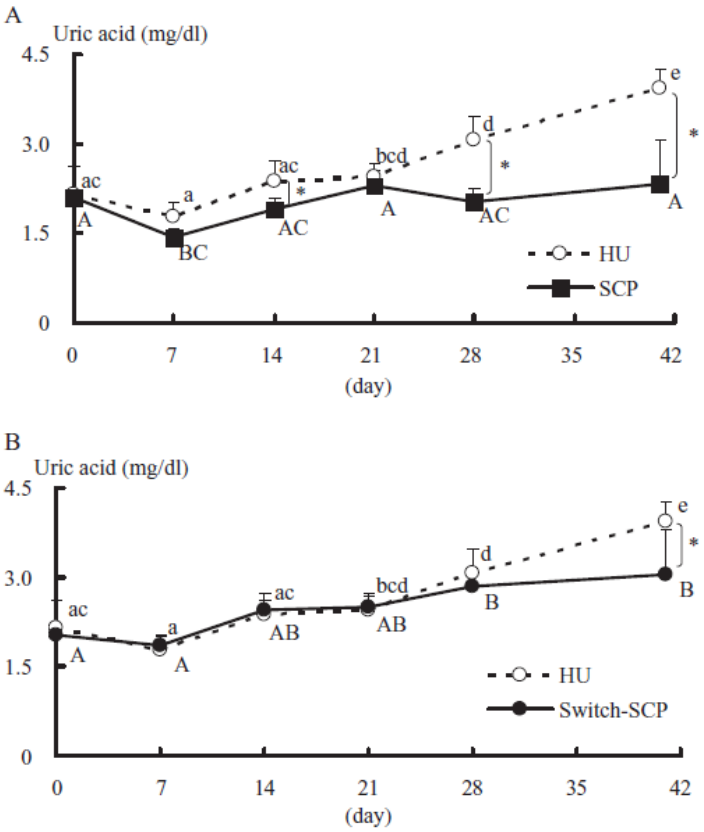

Figure 3. Effects of supplementation of shark cartilage digest in hyperuricemic diet on development of hyperuricemia.

(A) Rats received hyperuricemic (HU) or Shark cartilage digest (SCP) diet from the start of the experiment; (B) Rats received HU diet for 28 days and then received HU or SCP diet. The composition of HU and SCP diets is shown in Table 1. Data represent the means ± standard deviation for 6 animals. * indicates significant difference between HU and SCP groups $p < 0.05$; Dunnett). Different letters (capital; SCP or switch-SCP, small; HU) indicate significant difference within a group ($P < 0.05$; Student's t test with Holm adjustment).

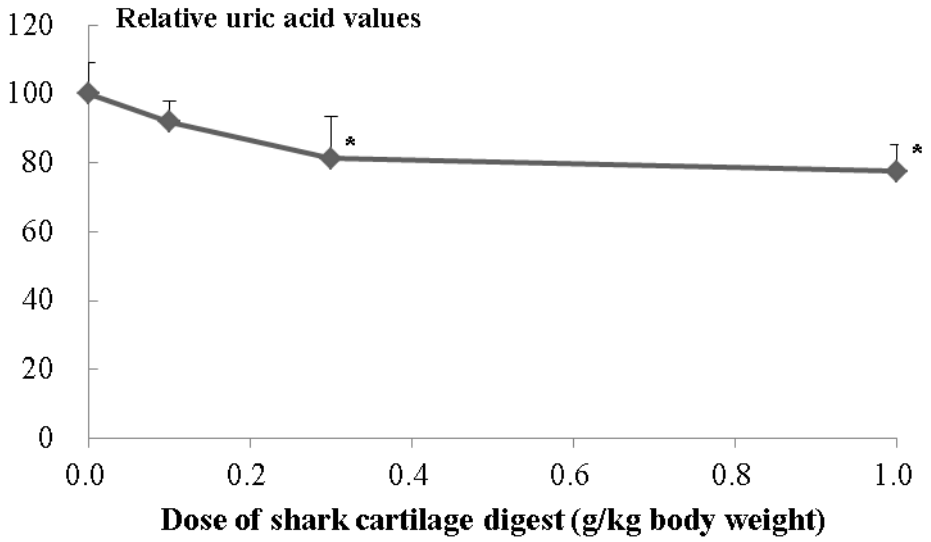

Figure 4. Dose – dependent uric acid lowering effect of shark cartilage digest

Rats received vehicle and shark cartilage digest in different dosages via stomach sonde and i.p. injected with potassium oxonate. Uric acid values were standardized against that of the vehicle group as 100. Data represent the means ± standard deviation for 6 animals. * indicates significant difference between vehicle and shark cartilage digest medication groups ($P < 0.05$; Dunnett).

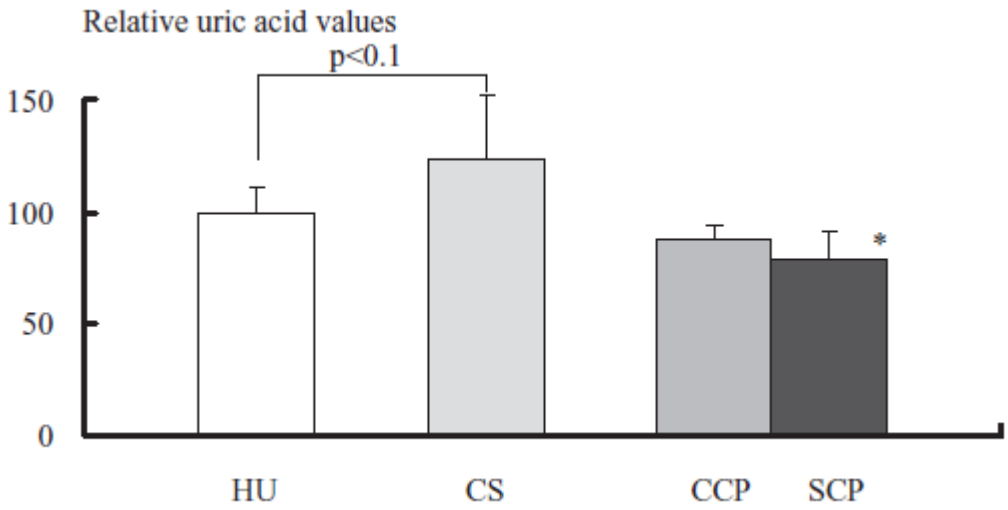

Figure 5. Comparison of serum uric acid lowering effect with cow cartilage digest and purified chondroitin sulfate C.

Rats were fed the HU diet containing 3% oxonate and supplemented diets with 2.5% shark cartilage digest (SCP), cow cartilage digest (CCP), or purified chondroitin sulfate C (CS). Uric acid values were standardized against that of the HU diet group as 100. Data represent the means ± standard deviation for 6 animals. Statistical significance between HU and CS was evaluated by Student's t test. *indicates significant difference between HU and shark cartilage digest groups ($P < 0.05$; Dunnett).

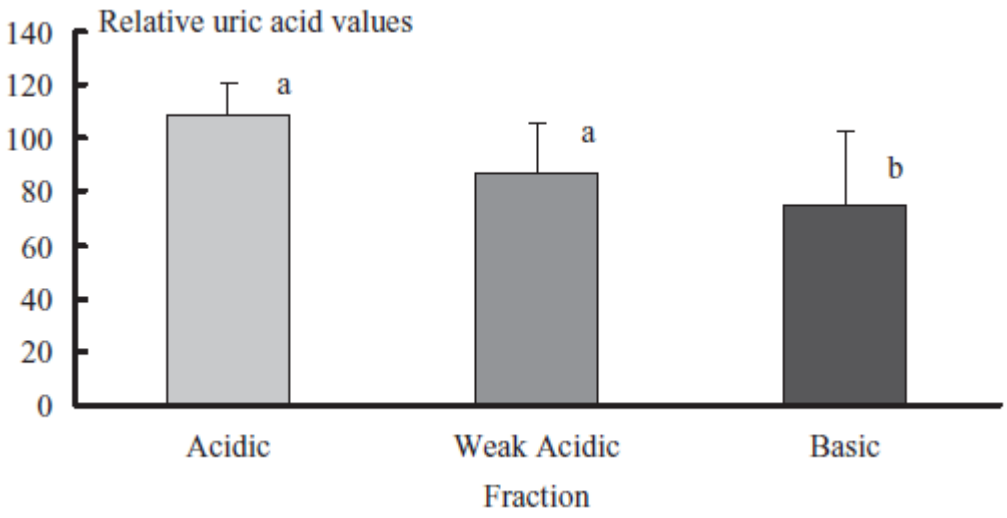

Figure 6. Comparison of uric acid lowering effect of autofocusing fraction of shark cartilage digest.

Uric acid values were standardized against that of the HU diet group as 100. Data represent the means ± standard deviation for 6 animals. Different letters indicate significant difference within groups ($P < 0.05$; Bonferroni/Dunn).

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Chapter 3.

Moderation of potassium oxonate-induced hyperuricemia in rats via the ingestion of an ethanol-soluble fraction of a shark cartilage proteolytic digest

ABSTRACT

A shark cartilage proteolytic digest was fractionated by selective extraction with 75% ethanol. Peptides in the digest were recovered in the 75% ethanol-soluble fraction. The ethanol-soluble fraction showed lower viscosity and higher solubility in water than the crude digest. Oxonate-induced hyperuricemia in rat was suppressed by the oral administration of the ethanol-soluble fraction of the digest (1 g/kg body weight/day) for 28 days. Single administration of the ethanol-soluble fraction also reduced blood uric acid level in a dose-dependent manner. A significant decrease in the serum uric acid level was observed at a dosage of 300 mg/kg. The 50% inhibitory dose value (ID50) of the ethanol-soluble fraction was estimated to be 262 mg/kg. Administration of the ethanol-soluble fraction of the digest (1 g/kg body weight/day) significantly increased serum inhibitory activity against xanthine oxidase, whereas the ethanol-soluble fraction had no in vitro xanthine oxidase inhibitory activity. These facts suggest that peptides in

the ethanol-soluble fraction might be converted into smaller peptides with xanthine oxidase inhibitory activity during digestion and absorption process.

INTRODUCTION

In chapter 2., it is reported that ingestion of a shark cartilage proteolytic digest moderated hyperuricemia in a potassium oxonate induced rat model and suggested that peptide fraction might be responsible for the anti-hyperuricemic activity (Murota et al.;2010). However, the underlying mechanism for the anti-hyperuricemic activity of the digest has remained to be solved. In addition, the crude digest of shark cartilage showed high viscosity in water due to presence of chondroitin sulfate, which makes difficult to sallow it down by human subject. The objective of the present study was to prepare a peptide fraction with lower viscosity and higher anti-hyperuricemic activity from the crude digest, which is suitable for human trial.

MATERIALS AND METHODS

Materials and reagents

A proteolytic digest of shark cartilage was prepared in the same method as described in chapter 2. The digest was powdered and suspended in 75% ethanol and stirred for 60 min. After centrifugation at 3000g for 10 min, the supernatant was collected. Then, the extract was dried under vacuum and then resolved in a minimum amount of water, before being powdered by spray drying or freeze drying. A Wako Uric Acid Test Kit, bovine milk xanthine oxidase, xanthine and allopurinol was purchased from Wako Pure Chemical (Osaka, Japan). Potassium oxonate was purchased from Acros Organics (Geel, Belgium).

Animals experiment

Female Sprague–Dawley rats and male Wistar rats were purchased from Charles River Laboratories Japan (Yokohama, Japan). All diet pellets were purchased from Oriental Yeast (Tokyo, Japan). All animals were housed (temperature: 22–24°C, humidity: 40–60%) under a 12 h light–dark cycle in the same building and given free access to their diet pellets and filtered water. The rats were allowed to adapt to their environment for a week before being used. This study was conducted in accordance with

the standards established by the Guide for the Care and Use of Laboratory Animals of Maruha Nichiro Holdings Central Research Institute (Tsukuba City, Japan).

Experiment 1: Hyperuricemia was induced according to the method of Dan et al (1994). Seven week-old female Sprague–Dawley rats (mean body weight of 199.8 g) were divided into three groups (n=7 for each group). Two of the groups were fed on a diet containing 2.5% (w/w) potassium oxonate (HU diet) for 28 days. The other group was fed on a normal diet. The compositions of the HU and normal diets are shown in Table 1. The ethanol-soluble fraction was suspended in distilled water at a concentration of 0.2 g/ml (w/v) and orally administered to the one of the HU groups once daily at a constant volume of 5 ml/kg body weight via a stomach sonde for 28 days. The other groups were administered with same volume of distilled water. The rats were fasted for 12 h before the blood collection from the abdominal aorta under anesthesia.

Experiment 2: Seven-week-old female Sprague–Dawley rats (average body weight of 201.6 g) were divided into two groups (n= 6 for each group) and fed on a normal diet for 28 days. The ethanol-soluble fraction was suspended in distilled water at a concentration of 0.2 g/ml (w/v) and administered orally to one of the groups once daily in the same manner as in experiment 1. Another group was administered the same volume of distilled water. The rats were fasted for 12 h before their blood was collected.

Experiment 3: Hyperuricemia was induced according to the method of Osada et al. (1993) with slight modifications. Six-week-old male Wistar rats (mean body weight of 161.2 g) were fed on a normal diet. The rats were divided into five groups (n= 6 for each group). Each test component was administered orally 2 h before the blood collection. The crude digest and the ethanol-soluble fraction were suspended in distilled water at a concentration of 0.2 g/ml (w/v) and administered orally at a constant volume of 5 ml/kg body weight to one group of rats each via a stomach sonde. Allopurinol was dissolved in water to give a concentration of 0.01 g/ml (w/v) and administered orally in the same manner as the crude digest and the ethanol-soluble fraction to another group of rats. One hour later, the rats were intraperitoneally injected with potassium oxonate solution (250 mg/2 ml of 3% gum Arabic saline/kg body weight) in order to elevate their blood uric acid level. Whole blood samples were collected 1 h after the oxonate injection.

Experiment 4: Hyperuricemia was induced in the same manner as in experiment 3. Six-week-old male Wistar rats (mean body weight of 172.1 g) were divided into four groups (n= 6 for each group). The rats were administered 0 (vehicle), 30, 100, or 300 mg/kg body weight of the peptide fraction; i.e., the ethanol-soluble fraction was suspended in distilled water at a concentration of 0, 6, 20, or 60 mg/ml (w/v) and administered orally at a constant volume of 5 ml/kg body weight via a stomach sonde.

One hour later, the rats were intraperitoneally injected with potassium oxonate solution (250 mg/2 ml of 3% gum arabic saline/kg body weight) in order to elevate their blood uric acid level. Whole blood samples were collected 1 h after the oxonate injection. To estimate the 50% inhibitory dose value (ID50), serum uric acid values were standardized against those of the normal and vehicle groups, which were taken as 0% and 100%, respectively.

Experiment 5. Six-week-old male Wistar rats (mean body weight of 150.9 g) were divided into two groups (n=6 for each group). The rats were administered orally 0 (vehicle) or 1 g/kg body weight of the ethanol-soluble fraction and intraperitoneally injected with potassium oxonate solution in the same manner in experiment 3. Whole blood samples were collected 1 h after the oxonate injection. The blood samples were stood for 0.5–1 h at room temperature to clot. The serum was then collected by centrifugation and stored at -30 °C until use.

Xanthine oxidase inhibitory activity assay

The xanthine oxidase assay was performed according to the method of Osada et al. (1993) with slight modifications. Xanthine was dissolved in 50 mM Tris–HCl buffer, pH 7.4, to give 15 mM. Xanthine oxidase was resolved in the same buffer to give 0.1

mU/μl. The ethanol-soluble fraction was dissolved in the same buffer to give a suitable concentration. The substrate (40 μl), enzyme (10 μl), and sample solution (1950 μl) were mixed and incubated for 10 min at 37ºC. The reaction was terminated by addition of 50μl of 3.2% (v/v) perchloric acid dis-solved in the same buffer. Absorbance at 292 nm was measured. Allopurinole and the buffer were used as positive and negative controls, respectively. Inhibitory activity is expressed as remaining activity against the negative control (%). For the evaluation of effect of ingestion of the ethanol-soluble fraction on the serum xanthine oxidase inhibitory activity, 200 μ l of serum from the rats in experiment 5 were mixed with 3 vol. (v/v) of ethanol. The precipitate was removed by centrifugation at 16,300g for 3 min. The supernatant was dried under vacuum and dissolved in the same buffer (1950 μ l) and used as sample.

Other analytical procedures

The protein, mucopolysaccharide (glycosaminoglycan), lipid, moisture, and ash contents in the crude extract and ethanol-soluble fraction were determined according to the method of the Association of Official Analytical Chemists (1990). Amino acid analysis was performed according to the method of Bidlingmeyer et al. (1984) with a slight modification (Sato et al.; 1992). The determination of chondroitin sulfate was

performed according to the method of Bowness (1957). Serum uric acid was measured using a Wako Uric Acid Test Kit (Wako Pure Chemical, Osaka, Japan) or the HPLC-UV method of the Committee on Biochemical Constituents and their Analytical Reagents (1993). The viscosity of a 20% sample water solution was measured using an RC-500 (Toki Sangyo, Tokyo, Japan) at 100 rpm and 25 °C. To estimate water solubility, the quantity of water required to completely dissolve 1 g sample at 25 °C was determined. The molecular weights of the peptides were estimated by gel filtration chromatography using a TSKgel G3000PW× l column (7.8×300 mm; Tosoh, Tokyo, Japan) equilibrated with 45% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and equipped with a Waters alliance 2965 HPLC system (Waters, Milford, MA, USA). The column was calibrated with glutathione (0.3 kDa), bradykinin (1.1 kDa),a-melanocyte stimulating hormone (MSH, 1.7 kDa), insulin chain B oxidized (3.5 kDa), aprotinin (6.5 kDa), cytochrome C (12.4 kDa), carbonic anhydrase (29.0 kDa), albumin (66.0 kDa), and alcohol dehydrogenase (150.0 kDa) as molecular weight markers, and a plot of log molecular weight versus Ve/Vt was used to estimate molecular weight, where Ve is the elution volume of each protein, and Vt is the retention volume of the total permeation peak.

Statistical analyses

Data are presented as the mean ± standard deviation. Differences between means were evaluated using the Student's t-test or an analysis of variance followed by Dunnett's test or the Bonferroni/Dunn method ($p<0.05$). Statistical analyses were performed with StatView Version 5.0 (Abacus Concept, Berkeley, CA, USA).

RESULTS

Characterization of the ethanol-soluble fraction

The 75% ethanol-soluble fraction had a yellow-white powdery appearance. The crude digest and ethanol-soluble fraction had their compositions and physical properties characterized as shown in Table 2. Acidic polysaccharides containing uronic acid, namely glycosaminoglycans, accounted for nearly half of the crude digest. On the other hand, most of the glycosaminoglycans had been removed from the ethanol-soluble fraction. Protein accounted for 99% of the ethanol-soluble fraction. Removing the glycosaminoglycans decreased the viscosity of the fraction to 1/27th of that of the crude digest and improved its solubility in water at 25°C by 3-fold compared to that of the crude digest. As shown in Table 3, the ethanol-soluble fraction had an amino acid composition similar to type II collagen. In shark type II collagen, hydroxyproline accounts for approximately 8% (mol/mol) of all amino acids. On the basis of its hydroxyproline content, approximately 75% of the peptides in the ethanol-soluble fraction were derived from type II collagen. As shown in Fig. 1, approximately 90% of the peptides in this fraction were distributed in the 0.3–7 kDa molecular mass range, and their mean molecular mass was estimated to be 1.5 kDa.

Animal experiments

Experiment 1: When the vehicle was administered, the serum uric acid levels of the rats that consumed the HU diet for 28 days were increased compared with those of the rats that ate the normal diet. The administration of the ethanol-soluble fraction (1 g/kg body weight) suppressed the development of hyperuricemia (Fig. 2A). There was no difference in body weight gain among any of the groups (data not shown).

Experiment 2: In the normal rats, there was no significant difference in the serum uric acid level between the rats received the ethanol-soluble fraction (1 g/kg body weight) and vehicle for 28 days (Fig. 2B). There was no difference in body weight gain among any of the groups (data not shown).

Experiment 3: In this experiment, hyperuricemia was induced in rats via a single intraperitoneal injection of oxonate. One hour before the oxonate injection, the test samples were administered orally. As shown in Fig. 3, a single dose of allopurinol decreased the serum uric acid level of the rats to below the normal level. A single administration of the ethanol-soluble fraction (1 g/kg body weight) also significantly decreased the serum uric acid level of the hyperuricemic rats in comparison to the administration of the vehicle and same dose of the crude digest.

Experiment 4: As shown in Fig. 4, a single administration of the ethanol-soluble fraction moderated hyperuricemia in a dose-dependent manner. A significant decrease in the serum uric acid level was observed at a dosage of 300 mg/kg. The 50% inhibitory dose value (ID50) of the ethanol-soluble fraction was estimated to be 262 mg/kg.

Xanthine oxidase inhibitory activity

As shown in Fig. 5A, the ethanol-soluble fraction did not show significant in vitro xanthine oxidase inhibitory activity even at relatively high dose. However, ingestion of the ethanol-soluble fraction significantly increases xanthine oxidase inhibitory activity in 75% ethanol-soluble fraction of the serum of rat (Fig. 5B).

DISCUSSION

In the present study, a peptide fraction of nearly 99% purity was prepared from the shark cartilage microbial protease digest using simple selective 75% ethanol-extraction method. This fraction shows anti-hyperuricemic activity in two animal models by using different strains of rat. The efficacy at 1 g/kg body weight was significantly higher than the crude digest. On the other hand, purified chondroitin sulfate, one of the major glycosaminoglycans present in the crude shark cartilage digest, had no anti-hyperuricemic effect (chapter 2.). Taking these findings together, it can be concluded that the peptides in the digest are responsible for its anti-hyperuricemic activity in this rat model. In addition to increase of anti-hyperuricemic activity, the present ethanol-soluble fraction shows significantly lower viscosity and higher solubility than the crude digest by removal of glycosaminoglycans. Then, human subject can easily take it without difficulty in comparison to the crude digest. Low viscosity and high solubility in water are also advantageous with respect to manufacturing efficiency, which makes easy in filtration, etc. This ethanol soluble-fraction equivalent to the present peptide fraction can be also produced as a byproduct in the process of medical grade chondroitin sulfate.

The present ethanol-soluble fraction predominantly consisted of peptides with molecular weights of less than 7 kDa and displayed a similar amino acid composition to

collagen. However, on the basis of their hydroxyproline content, approximately 25% of the peptides in the present fraction are derived from non-collagenous extracellular matrix proteins. To the best of our knowledge, there have been no reports about peptides with anti-hyperuricemic activity, except for imidazole dipeptides. They mildly moderate fatigue and consequently fatigue induced hyperuricemia in humans (Nishitani et al.; 2009, Quinlan et al.; 1997). However, only negligible amounts of β-alanine, a constituent of imidazole dipeptides, were detected in the HCl hydrolysate of the present ethanol-soluble fraction. Therefore, non-imidazole peptides are responsible for the anti-hyperuricemic activity of the present ethanol-soluble fraction. Xanthine oxidase is a key enzyme for uric acid synthesis. Therefore, food components that are able to inhibit xanthine oxidase have been tested (Yoshizumi et al.; 2005). However, few peer-reviewed papers have demonstrated that ingestion of food components display anti-hyperuricemic activity based on xanthine oxidase inhibition. The present study in this chapter clearly indicates that inhibition of xanthine oxidase is involved in the anti-hyperuricemic activity by ingestion of the ethanol-soluble fraction (Fig. 5B). However, the ethanol-soluble fraction had no significant in vitro xanthine oxidase inhibitory activity (Fig. 5A). Then, conventional in vitro activity-guided fractionation based on xanthine oxidase inhibition assay cannot be used for identification of the active peptide in the ethanol-soluble fraction.

The apparent inconsistency between in vitro and in vivo results could be explained as follow. After ingestion of the ethanol-soluble fraction, smaller peptides or their metabolites with xanthine oxidase inhibitory activity might be produced from parent peptides in the ethanol-soluble fraction during digestion, absorption, metabolism processes. A further study based on in vivo activity-guided fractionation for identification of the anti-hyperuricemic peptides in the digest is described in chapter 4.

Peptides in a shark cartilage microbial protease digest are responsible for the anti-hyperuricemic activity by oral administration. The peptide fraction of the shark cartilage digest with higher anti-hyperuricemic activity and lower viscosity than crude digest can be prepared using by simple 75% ethanol extraction, which can be used for the preparation of food ingredients. Therefore, this preparation has potential as a functional food ingredient with anti-hyperuricemic activity and consumer-friendly textural properties. Inhibition of uric acid synthesis is demonstrated to be involved in the anti-hyperuricemic activity of the present preparation.

TABLES AND GRAPHS

Table 1. Compositions of normal and oxonate diets used in experiment 1.

(%)

	Normal diet	Hyperuricemic (HU) diet
Corn starch	38.0	38.0
Casein	25.0	25.0
α-corn starch	10.0	10.0
Cellulose	8.0	8.0
Soybean oil	6.0	6.0
Mineral mix (Oriental)	6.0	6.0
Vitamin mix (Oriental)	2.0	2.0
Sucrose	5.0	2.5
Potassium oxonate	-	2.5

Table 2 Approximate composition and physical properties of a shark cartilage digest and its ethanol-soluble fraction.

Component	Crude shark cartilage digest	Peptide fraction
Protein	53%	99%
Mucopolysaccharides	43%	0.6%
Lipids	< 0.1%	0.1%
Apparent viscosity of 20% solution at 25 °C	35.1 mPa·s	1.3 mPa·s
Water solubility (1g)	6.0 ml	2.0 ml

Data are shown as the mean values of duplicate determinations.

Table 3. Amino acid (g/100 g of protein) profile of the ethanol-soluble fraction.

	Peptide fraction extracted from the crude digest	Type II collagen
Hyp	6.4	7.6
Asp	5.8	5.2
Thr	2.7	2.3
Ser	3.2	2.5
Glu	8.6	10.0
Pro	8.8	8.7
Gly	16.8	16.5
Ala	7.5	6.8
Val	1.5	1.9
Met	1.8	1.2
Ile	4.1	1.4
Tyr	1.4	0.9
Phe	2.5	2.1
Lys	2.8	2.9
His	1.2	0.9
Arg	6.1	5.9

His	1.2	0.9
Arg	6.1	5.9

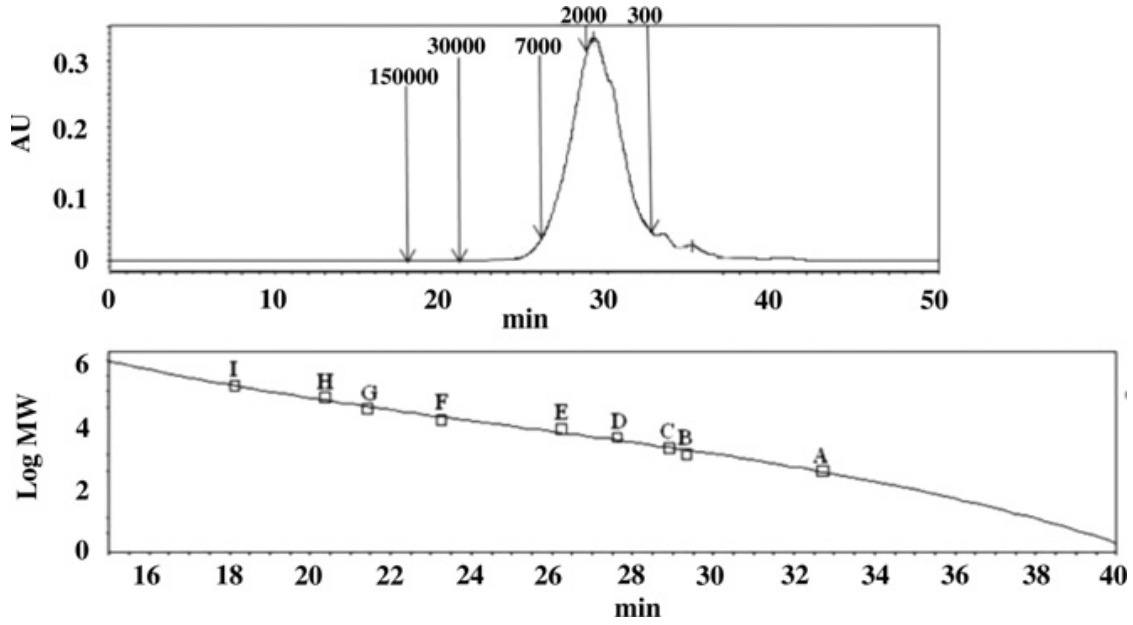

Figure 1 Elution profile of the ethanol-soluble fraction on gel filtration chromatography and a profile of standard proteins .

The column was calibrated with A: glutathione (0.3 kDa), B: bradykinin (1.1 kDa), C: alpha-MSH (1.7 kDa), D: insulin chain B oxidized (3.5 kDa), E: aprotinin (6.5 kDa), F: cytochrome C (12.4 kDa), G: carbonic anhydrase (29.0 kDa), H: albumin (66.0 kDa), and I: alcohol dehydrogenase (150.0 kDa) as molecular weight markers, and a plot of log molecular weight versus (Ve/V0) was used to estimate molecular weight (lower graph).

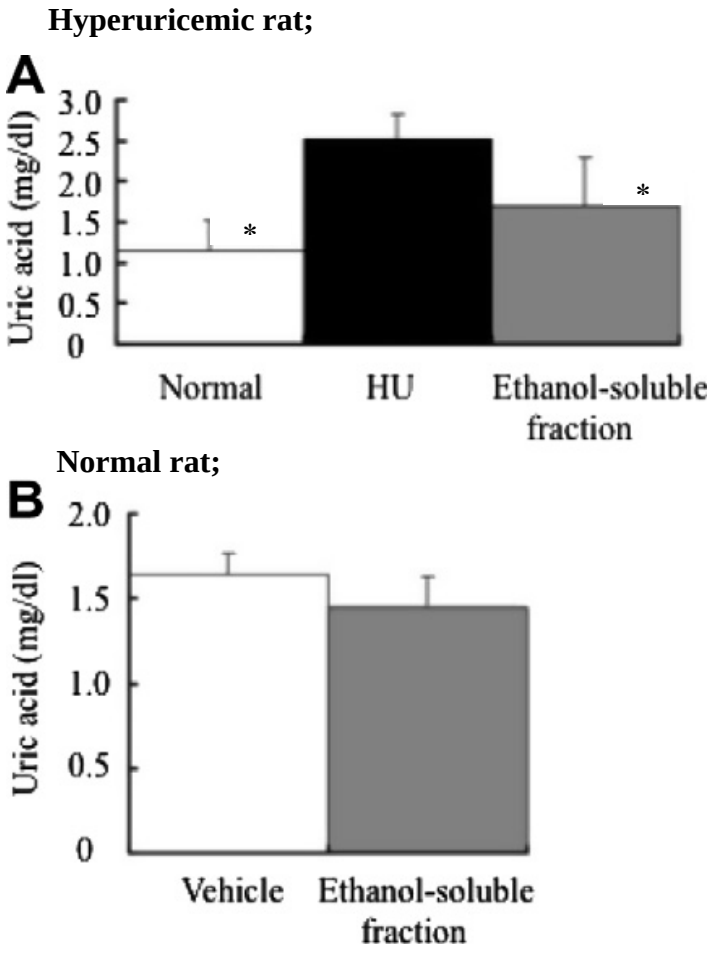

Figure 2. Effect of the orally administered ethanol-soluble fraction on the serum uric acid level of rat models.

Graphs are presented for the serum uric acid level of a rat model. A: Rats were fed the HU diet containing 2.5% oxonate or the normal diet and then received vehicle (normal and HU) or the ethanol-soluble fraction for 28 days (experiment 1). * Indicates a significant difference to the HU group ($p < 0.05$; Dunnett). B: Rats were fed the normal diet and then received vehicle or the ethanol-soluble fraction for 28 days (experiment 2).

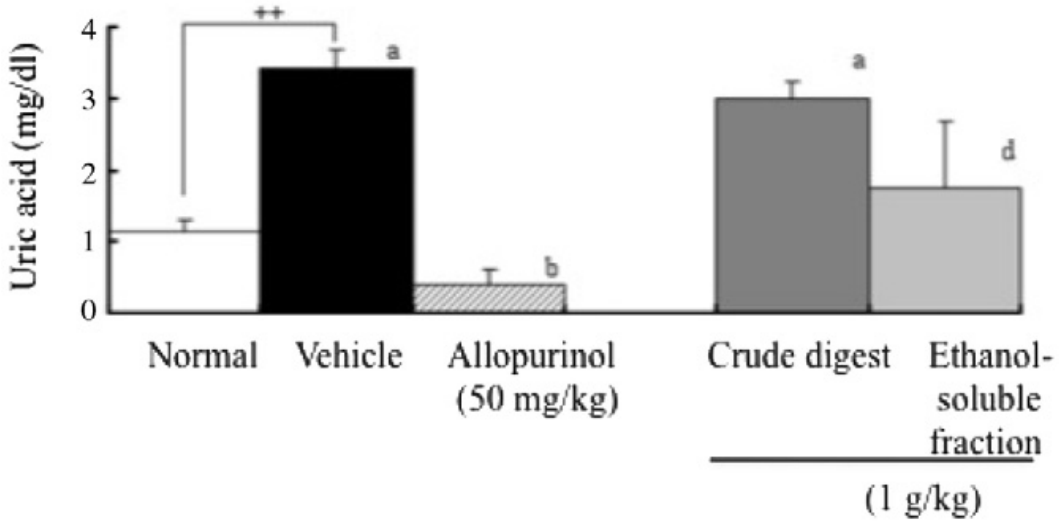

Figure 3. The serum uric acid lowering effects by single administration of the ethanol-soluble fraction, crude digest, and allopurinol.

Graph is presented for the serum uric acid level of a rat model (experiment 3) administered orally the ethanol-soluble fraction, crude digest, or allopurinol followed by oxonate treatment. Normal; rat without oxonate treatment. Different letters indicate a significant difference within groups ($p < 0.05$; Bonferroni/Dunn). $^{++}$ Indicates a significant difference between the vehicle group and the normal or crude digest group ($p < 0.01$; Student's t-test).

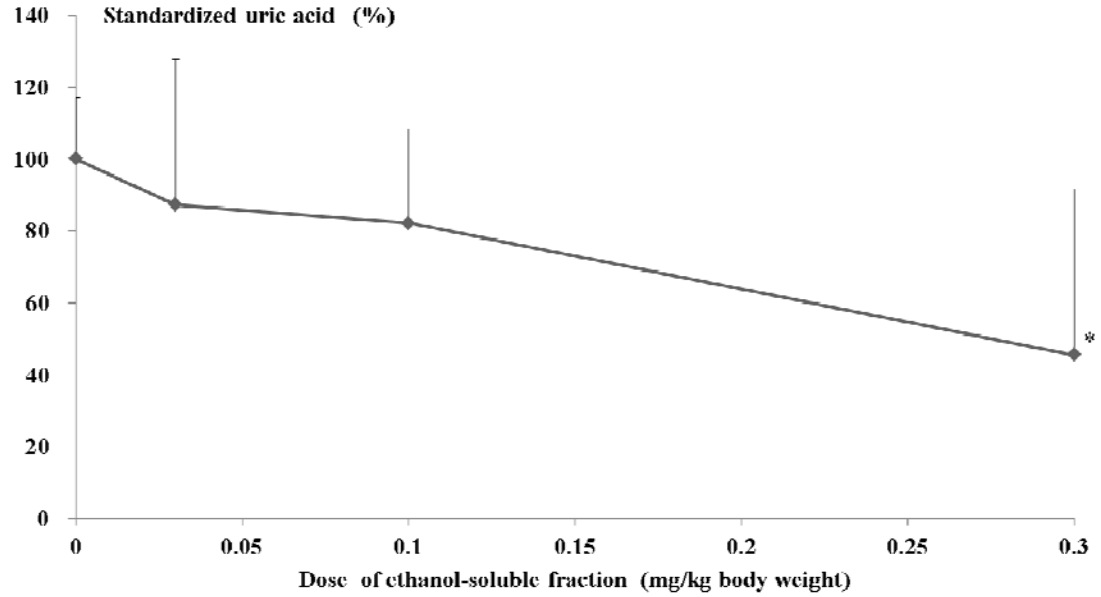

Figure 4. Dose-dependent serum uric acid lowering effect of the ethanol-soluble fraction.

Graph is presented for the serum uric acid level of a rat model administered the ethanol-soluble fraction orally in the dose-finding study (experiment 4). To estimate ID50, serum uric acid values were standardized against those of the normal and vehicle groups, which were taken as 0% and 100%, respectively. * indicates a significant difference between the vehicle group ($p < 0.05$; Dunnett).

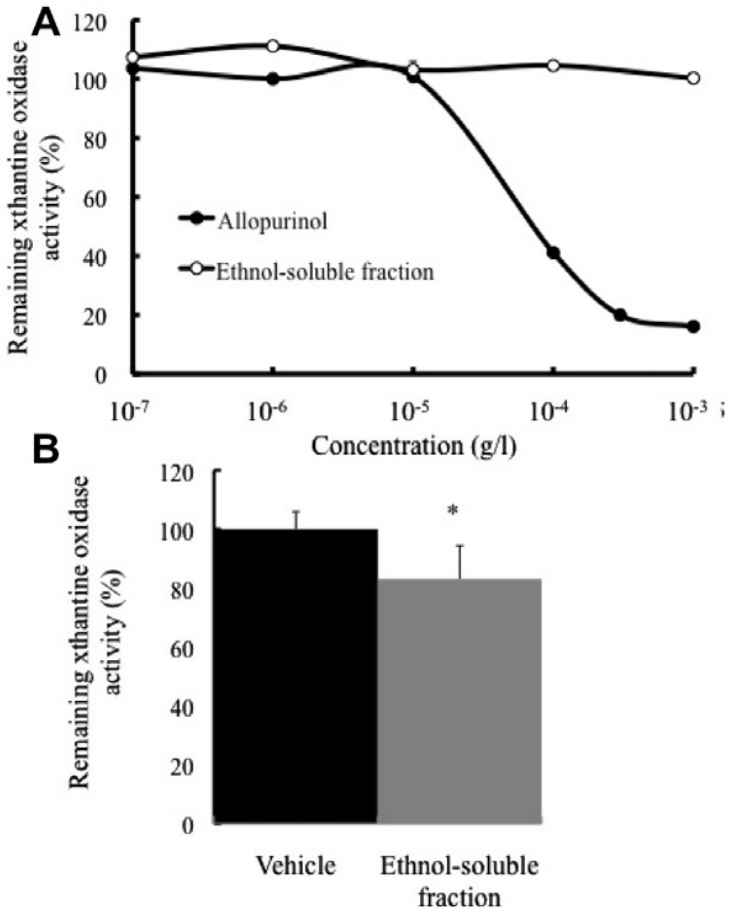

Figure 5. In vitro and in vivo xanthine oxidase inhibition by the ethanol-soluble fraction of shark cartilage digest.

A:Remaining xanthine oxidase activity after addition of the ethanol-soluble fraction. Allopurinol was used as positive control.

B: Remaining xanthine oxidase activity after addition of the ethanol-soluble fraction of the serum of Wister strain rat after ingestion of vehicle and ethanol-soluble fraction (experiment 5). The remaining activity was standardized against that of the vehicle groups, which were taken as 100%.

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Chapter 4.

Identification of anti-hyperuricemic peptides in the proteolytic digest of shark cartilage water extract by using in vivo activity-guided fractionation

ABSTRACT

A peptide that exerts anti-hyperuricemic activity after oral administration was identified from a microbial protease (alcalase) digest of the water extract of shark cartilage by in vivo activity-guided fractionation, using oxonate-induced hyperuricemic rats. Water extract of shark cartilage was first fractionated by preparative ampholine-free isoelectric focusing and followed by preparative reversed-phase liquid chromatography. The anti-hyperuricemic activity of the alcalse digests of the obtained fractions was evaluated using an animal model. Alcalase digests of the basic and hydrophobic fractions exerted anti-hyperuricemic activity. Eighteen peptides were identified in the alcalase digest of the final active fraction. These peptides were chemically synthesized and evaluated for anti-hyperuricemic activity. Tyr-Leu-Asp-Asn-Tyr and Ser-Pro-Pro-Tyr-Trp-Pro-Tyr lowered serum uric acid level via intravenous injection at 5 mg/kg body weight. Furthermore, orally administered Tyr-Leu-Asp-Asn-Tyr showed

anti-hyperuricemic activity. Therefore, these peptides are at least partially responsible for the anti-hyperuricemic activity of the alcalase digest of shark cartilage.

INTRODUCTION

As mentioned in chapter 2. and 3., it is demonstrated that oral administration of a crude proteolytic digest of shark cartilage lowers serum uric acid levels in the potassium oxonate-induced hyperuricemic rat (Murota et al.; 2010). The crude digest contained a mixture of chondroitin sulfate and peptides. The previous study as described in chapter 2. demonstrated that chondroitin sulfate has no significant anti-hyperuricemic activity. These results imply that peptides may be responsible for the anti-hyperuricemic activity (Murota et al.; 2012). However, the anti-hyperuricemic peptide in the digest has not yet been identified.

Administration of the ethanol-soluble fraction of the digest (1 g/kg body weight/day) significantly increased serum inhibitory activity against xanthine oxidase, whereas the ethanol-soluble fraction had no in vitro xanthine oxidase inhibitory activity. These facts suggest that peptides in the ethanol-soluble fraction might be converted into smaller peptides with xanthine oxidase inhibitory activity during digestion and absorption process.

The objective of the present study in this chapter is to identify the active peptide, which shows anti-hyperuricemic activity by oral ingestion in the animal model.

MATERIALS AND METHODS

Regents. Synthetic peptides were purchased from ILS (Ibaraki, Japan). These peptides were synthesized using the 9-fluorenylmethyloxycarbonyl (Fmoc) strategy. The purity of the peptides was checked by electron spray ionization mass spectrometer using a LCQ Advantage (Thermo Fisher Scientific, Waltham, MA). The Uric Acid Test Wako Kit, trifluoroacetic acid (TFA), acetonitrile (HPLC grade) were purchased from Wako Pure Chemical (Osaka, Japan). Potassium oxonate was purchased from Across Organics (Geel, Belgium). Shark cartilage chondroitin sulfate C was purchased from Sigma (St. Louise, MO). Alcalase (5U/g) was obtained from Novozymes (Bagsvaerd, Denmark). Other reagents were of analytical grade or better.

Preparation of Shark Cartilage Water Extract. The water extract of cartilage from blue shark (Prionace glauca) fin was prepared using the previously reported method (Kitahashi et al; 2012). Blue sharks were caught by longline fishing and was stored at Kesen-Numa Port in Miyagi Prefecture, Japan. The fin was collected and solar-dried. The dried fin was rehydrated in 50-60°C water for approximately 30 min. The fin was skinned and collagenous transparent fibers, which are used in gourmet Chinese cuisine, were removed. The remaining cartilage was solar-dried. The dried fin cartilage was crushed

with liquid nitrogen in a Lindex mill (Hosokawa Micron, Osaka, Japan). Three hundred grams of the fine powder (60 μm in average diameter) was mixed with 1.5 L of cold water. The suspension was allowed to stand for 30 min. with occasional stirring. The supernatant was harvested by centrifugation at 1000 × g using a No. 9 rotor (Tomy Seiko, Tokyo, Japan). The pellet was further extracted with 500 mL of cold water. The supernatants were combined and used for the following experiments

Fractionation of water-soluble components for animal experiment. Compounds in the water-extract were fractionated by ampholyte-free preparative isoelectric focusing, which is referred to as autofocusing, using the method described by Hashimoto et al. (2005) using an autofocusing apparatus with 10 sample compartments (75 mm in length × 80 mm in width × 85 mm in height for each compartment). The water-extract was placed in sample compartments No. 5 and 6. The other compartments were filled with deionized water. Autofocusing was carried out at 500 V for 18 h. The autofocusing fractions were freeze-dried.

The autofocusing fraction that exerted anti-hyperuricemic activity after alcalase digestion was subjected to preparative reversed-phase liquid chromatography. YFLC gel C18 (particle size 40 μm in diameter; Yamazen, Osaka, Japan) was packed into a column

(250 mm × 15 mm i.d.) and equilibrated with 10% (v/v) acetonitrile in the presence of 0.1% TFA. Two grams of the dried active autofocusing fraction without alcalase digestion was dissolved into 100 mL of the equilibrium solvent and loaded into the column using an MPLC system (Yamazen) at 10 mL/min. After loading of the sample, the column was washed with 100 mL of the equilibrium solvent. After elution of the non-absorbed components, the absorbed components were eluted with a linear gradient of acetonitrile from 10 to 80% in the presence of 0.1% TFA over 20 min. Fractions were collected every 1 min. Elution was monitored by absorbance at 280 nm.

Alcalase Digestion. Prior to conducting the animal experiment, an aliquot of each fraction was digested with alcalase. The sample was dissolved in water to yield 8% (w/v) and adjusted to pH 8.0 by addition of NaOH or HCl. Alcalase (liquid form) was added at a ratio of 0.04% (v/v) to the reaction mixture and reacted at 60°C for 4 h. The reaction was terminated by heating to 85°C for 10 min. The reaction product was freeze-dried and stored at −20°C until use.

Animal Experiment. Male Wistar rats were purchased from Charles River Laboratories Japan (Yokohama, Japan). All diet pellets were purchased from Oriental

Yeast (Tokyo, Japan). All animals were housed (temperature: 22-24°C, humidity: 40-60%) in the same building under a 12-hours light-dark cycle and given free access to diet pellets and filtered water. The rats were allowed to adapt to the environment for a week before the start of the experiment. This study was conducted in accordance with the standards established by the Guide for the Care and Use of Laboratory Animals of Maruha Nichiro Holdings Central Research Institute (Tsukuba-City, Japan).

The test components were suspended in distilled water at an appropriate concentration and administered at a constant volume of 5 mL/kg body weight to the rats via a stomach sonde. The doses of fractions (mg/kg body weight) are shown in Figures. The rats in the control group were administered the same volume of distilled water (vehicle). In some cases, test components were dissolved in saline and injected into the tail vein. Before the injection, the sample was sterilized by passing through a filter (0.45 μm pore size, GL Sciences, Tokyo, Japan). One hour after the administration of test components, rats received a potassium oxonate solution (250 mg/2 mL of 3% gum arabic saline/kg body weight) by intraperitoneal injection to elevate their blood uric acid level (Murota et al.; 2010, Murota et al.; 2012, Osada et al.; 1993). One hour after the oxonate injection, blood samples were collected from the caudal artery under pentobarbital anesthesia. Rats were starved for more than 12 hours before their blood was drawn.

Isolation of peptides in the alcalase digest of active fraction. Peptides were obtained from the alcalase digest of the active fraction by preparative reversed-phase liquid chromatography, and were resolved by a size-exclusion chromatography (SEC) and reversed-phase high performance liquid chromatography (RP-HPLC). One milligram of the alcalase digest of the active fraction was dissolved in 200 μL of 30% acetonitrile in the presence of 0.1% TFA and loaded into a Superdex peptide 10/30 HR (GE Healthcare, Buckinghamshire, UK), which had been equilibrated with 30% acetonitrile in the presence of 0.1% TFA at a flow rate 0.5 mL/min. Fractions were collected every 1 min. The SEC fractions were dried under vacuum and dissolved in 200 μL of 10% acetonitrile in the presence of 0.1% TFA and further fractionated using a Cosmosil 5C18-MS-II (250 × 4.6 mm i.d., Nacalai Tesque, Kyoto, Japan). Elution was performed using a linear gradient of acetonitrile from 10 to 80% in the presence of 0.1% TFA for 30 min at a flow rate of 1 mL/min. Absorbance at 214 nm was monitored. The column was maintained at 40°C.

Other Analytical Methods. Protein content was evaluated by amino acid analysis after HCl hydrolysis. Amino acid analysis was performed according to the method described by Bidlingmeyer et al. (1984) with slight modifications (Higaki et al.; 2003). The peptide

sequence was analyzed using the Edman degradation procedure using a PPSQ-21 (Shimadzu, Kyoto, Japan). Chondroitin sulfate was determined by the carbazole sulfate method using shark cartilage chondroitin sulfate C as a standard (Bowness; 1957).

The xanthine oxidase assay was performed according to the method of Osada et al. (1993) with slight modifications (Murota et al.; 2012) Xanthine was dissolved in 50 mM Tris–HCl buffer, pH 7.4, to give 15 mM. Xanthine oxidase was resolved in the same buffer to give 0.1 mU/mL. Sample was dissolved in the same buffer to give a suitable concentration. The substrate (40 μL), enzyme (10 μL), and sample solution (1950 μL) were mixed and incubated for 10 min at 37ºC. The reaction was terminated by addition of 50 μL of 3.2% (v/v) perchloric acid. Absorbance at 292 nm was measured. Allopurinole and the buffer were used as positive and negative controls, respectively. Inhibitory activity is expressed as remaining activity against the negative control (%). For the evaluation of effect of ingestion of Tyr-Leu-Asp-Asn-Tyr on the serum xanthine oxidase inhibitory activity, 100 μL of serum from the rats received the peptide, vehicle, and allopurinol were mixed with the buffer (1850 μL) and used as sample solution. Doses of peptide are shown in Figure legend.

Statistical Analysis. Data are presented as the mean ± standard deviation. Differences in serum uric acid levels were analyzed by one-way analysis of variance and Scheffe's post hoc test using StatView 4.11 (Abacus Concepts Inc., Berkeley, CA, USA).

RESULTS

As shown in Figure 1, the intact water-extract did not show significant anti-hyperuricemic activity. However, the alcalase digest showed significant uric-acid lowering activity to the oxonate-induced hyperuricemic rat by oral administration at 1 g/kg of body weight, indicating that peptides produced from the parent proteins in the water extract by alcalase digestion are responsible for the anti-hyperuricemic activity. The intact water-soluble components of the shark cartilage were fractionated on the basis of their isoelectric points by autofocusing. As shown in Figure 2, acidic (fraction number 1–5), weakly acidic (6–7), and basic fractions (8–10) were collected. Solid components were recovered from the acidic, weakly acidic, and basic fractions at 66, 24, and 10%, respectively. The acidic and weakly acidic fractions consisted of protein and chondroitin sulfate. In contrast, the basic fraction predominantly consisted of protein. The three fractions were digested with alcalase and the digests were orally administered to the oxonate-induced hyperuricemic rats. The dose of each fraction was determined on the basis of the recovery percentage. Only the alcalase digest of the basic fraction significantly reduced serum uric acid level at 0.1 g/kg of body weight, which is less than that of the water-extract (1 g/kg) or the other fractions.

The proteins in the basic fraction were further fractionated by preparative

reversed-phase liquid chromatography without alcalase digestion. As shown in Figure 3, three peaks (Fractions 2-4) were eluted by gradient elution after elution of the unabsorbed components (Fraction 1). These fractions were also digested with alcalase and evaluated for their anti-hyperuricemic activity. The dose of each fraction was determined on the basis of recovery percentage. Only the alcalase digest of Fraction 4 showed significant anti-hyperuricemic activity by oral administration at 50 mg/kg body weight.

The peptides in the alcalase digest of the Fraction 4 were first resolved by SEC. Fractions were collected every 1 min. The SEC fractions eluted between 29 and 35 min were subjected to analytical RP-HPLC. As shown in Figure 4, the two-dimensional HPLC provided excellent resolution of the constituent peptides. The peptides marked with large characters were collected and subjected to sequence analysis. The obtained sequences are summarized in Table 1. All peptides contained aromatic amino acids: tyrosine, phenylalanine, and tryptophan. On the basis of the sequence data, all peptides were chemically synthesized and evaluated for anti-hyperuricemic activity. As shown in Figure 5, Tyr-Leu-Asp-Asn-Tyr and Ser-Pro-Pro-Tyr-Trp-Pro-Tyr showed significant anti-hyperuricemic activity by intravenous injection at 5 mg/kg ($P<0.01$ and <0.05, respectively). However, Asp-Phe-Trp-Arg-Tyr (DFWRY) and other peptides did not show significant anti-hyperuricemic activity (data not shown). Oral administration of the

Tyr-Leu-Asp-Asn-Tyr also significantly reduced serum uric acid at 50 mg/kg body weight (Figure 5), while Ser-Pro-Pro-Tyr-Trp-Pro-Tyr did not show significant activity by oral ingestion. Oral administration of Tyr-Leu-Asp-Asn-Tyr also significantly increased serum inhibitory activity against xanthine oxidase at same dose (Figure 6). The xanthine oxidase inhibitory activity of Tyr-Leu-Asp-Asn-Tyr and possible fragment peptides is shown in Table 2. Asp-Asn, Leu-Asp-Asn, Asp-Asn-Tyr, and Leu-Asp-Asn-Tyr showed inhibitory activity, while Tyr-Leu-Asp-Asn-Tyr and constituting amino acids did not show the inhibitory activity. The IC_{50} of these fragment peptides were 13-23 times higher than that of allopurinol.

DISCUSSION

Over the last few decades, numerous studies have demonstrated that ingestion of food protein hydrolysates has significant biological effects, such as moderation of hypertension, hypertriglyceridemia, hypercholesteremia, etc. The active peptides have been identified using activity-guided fractionation based on in vitro assay systems using enzyme reactions and cell culture systems. Using this approach, many peptides have been apparently identified as active compounds. However, unlike other functional ingredients, proteins and peptides are further degraded into smaller peptides and constituent amino acids during digestion and absorption. As a result, the peptides in food may lose the potential biological activity detected by in vitro assays. Therefore, the in vitro activity of the peptides in the food cannot be directly linked to their biological activity after ingestion. To resolve these questions, the biological activity of potentially active peptides should be evaluated by feeding experiments using animal models. However, this approach requires relatively large amounts of peptide in comparison to the in vitro assays. For this approach, we have developed large-scale ampholyte-free preparative isoelectric focusing, referred to as autofocusing, which can process peptide samples up to 50-500 g (Murota et al.; 2012). Coupled with preparative reversed-phase chromatography, the parent protein, which produced anti-hyperuricemic peptides after alcalase digestion, was

recovered from the basic and hydrophobic fractions (Figures 3 and 4). The alcalase digest of the final fraction exerted significant uric acid-lowering activity at a 1/20 dose in comparison to the crude digest. All peptides identified in the digest consisted of aromatic amino acids. Similarity research analyses using FAST and BLAST protocols revealed that these peptide sequences are similar to mammalian hypothetical proteins, of which the function is unknown. Using peptides synthesized on the basis of the sequence data, two peptides were identified, which exerted anti-hyperuricemic activity by intravenous injection at 5 mg/kg of body weight. Among these peptides, oral administration of Tyr-Leu-Asp-Asn-Tyr at 50 mg/kg of body weight exerted anti-hyperuricemic activity, which indicates that this peptide plays a significant role in the anti-hyperuricemic activity of shark cartilage extract. However, the dose of Tyr-Leu-Asp-Asn-Tyr necessary for the significant anti-hyperuricemic activity by oral ingestion was comparable to the required dose of the alcalase digest of the final fraction (Fraction 4 in Figure 3). The presence of other peptides in the digest may enhance the anti-hyperuricemic activity of Tyr-Leu-Asp-Asn-Tyr by oral ingestion.

Our previous study demonstrated that ingestion of a protease digest of shark cartilage increases serum inhibitory activity against xanthine oxidase, a key enzyme for uric acid synthesis, whereas the digest has no in vitro xanthine oxidase inhibitory activity.

Tyr-Leu-Asp-Asn-Tyr also had no in vitro xanthine oxidase inhibitory activity (Table 2), whereas it increased serum xanthine oxidase inhibitory activity by ingestion (Figure 6). It has been demonstrated that most of the food-derived peptides in blood are tri- and di-peptides even after ingestion of larger peptides.17-19 Then Tyr-Leu-Asp-Asn-Tyr may be further degraded into smaller peptides with xanthine oxidase inhibitory activity and absorbed into the blood system. Indeed, some di-, tri-, and tetra-peptides, which could be potentially derived from Tyr-Leu-Asp-Asn-Tyr, show xanthine oxidase inhibitory activity. Asp-Asn motif is contained in all xanthine oxidase inhibitory peptides. These facts suggest that these fragment peptides might be absorbed into blood and exert anti-hyperuricemic activity by inhibition of xanthine oxidase. Alternatively, the degradation peptides may induce endogenous xanthine oxidase inhibitors. Further studies on the metabolic fate of Tyr-Leu-Asp-Asn-Tyr and other peptides in the active fraction and on the identification of increased endogenous or food-derived xanthine oxidase inhibitors in serum after ingestion of these peptides are currently in progress to elucidate the mechanism of the anti-hyperuricemic activity.

The active peptide Tyr-Leu-Asp-Asn-Tyr in the alcalase digest of crude shark cartilage can be separated from chondroitin sulfate and other high molecular weight components by simple selective precipitation technique using 75% ethanol, which can

decrease dose to exert anti-hyperurecemic activity by ingestion (Iawi et al.;2005). Therefore, the peptide concentrate has potential as a functional food ingredient with anti-hyperuricemic activity.

TABLES AND GRAPHS

Table 1. Summary of sequences of peptides from the alcalase digest of the Fraction 4

Peak	Sequence
A	Leu-Pro-Tyr
B	Tyr-Leu-Asp-Asn-Tyr
C	Asp-Phe-Trp-Arg-Tyr
D	Ser-Pro-Pro-Tyr-Trp-Pro-Tyr
E	Ser-Leu-Pro-Tyr-Trp-Pro-Tyr
F	Ile-Asn-Tyr
G	Val-Tyr-Gln
H	Tyr-Asn-Leu
I	Leu-Tyr
J	Ser-Ile-Tyr-Asp
K	Phe-Tyr
L	Tyr-Leu
M	Arg-Tyr-Leu
N	Gly-Tyr-Leu
O	Tyr-Phe
P	Tyr-Tyr
Q	Ser-Asn-Trp-Gln
R	Phe-Tyr

Refer to Figure 4 for peak names.

Table 2. Xanthine oxidase Inhibitory activity of possible fragment peptides from Tyr-Leu-Asp-Asn-Tyr

Sample	Remaining activity (%) Dose (M) 1×10^{-5}	1×10^{-4}	3×10^{-4}	1×10^{-3}	IC_{50} (M)
Allopurinol	73.1±29.5	25.8±7.2*	11.5±10.6*	12.3±6.7*	3.8×10^{-5}
Tyr-Leu	102.2± 2.1	106.4±3.1	98.7±10.2	106.9±2.7	-
Leu-Asp	104.7±1.0	107.7±5.9	88.9±32.8	59.7±19.7	-
Asp-Asn	103.5±2.0	107.8±7.1	83.2±17.9	16.2±11.1*	5.0×10^{-4}
Asn-Tyr	105.2±4.1	108.3±6.0	101.8±9.1	86.0±13.1	-
Try-Leu-Asp	103.5±2.0	108.6±5.5	103.1±9.9	79.6±24.1	-
Leu-Asp-Asn	95.0±16.9	84.4±16.7	98.6±12.1	35.8±11.5*	7.6×10^{-4}
Asp-Asn-Tyr	106.6±7.4	87.7±28.5	86.6±18.2	45.7±12.7*	8.8×10^{-4}
Tyr-Leu-Asp-Asn	109.0±10.7	108.1±6.9	102.3±10.9	57.1±25.2*	-
Leu-Asp-Asn-Tyr	104.2±2.7	108.4±6.3	99.6±6.1	34.4±18.9*	7.5×10^{-4}
Try-Leu-Asp-Asn-Tyr	100.2±7.7	101.5±1.3	-	101.7±0.2	-

Allopurino and distilled water were used as positive and negative controls, respectively. Remaining activity of xanthine oxidase is expressed as percent of the negative control (mean ± standard deviation, n=3). *indicates a significant difference vs the negative control $P< 0.05$.

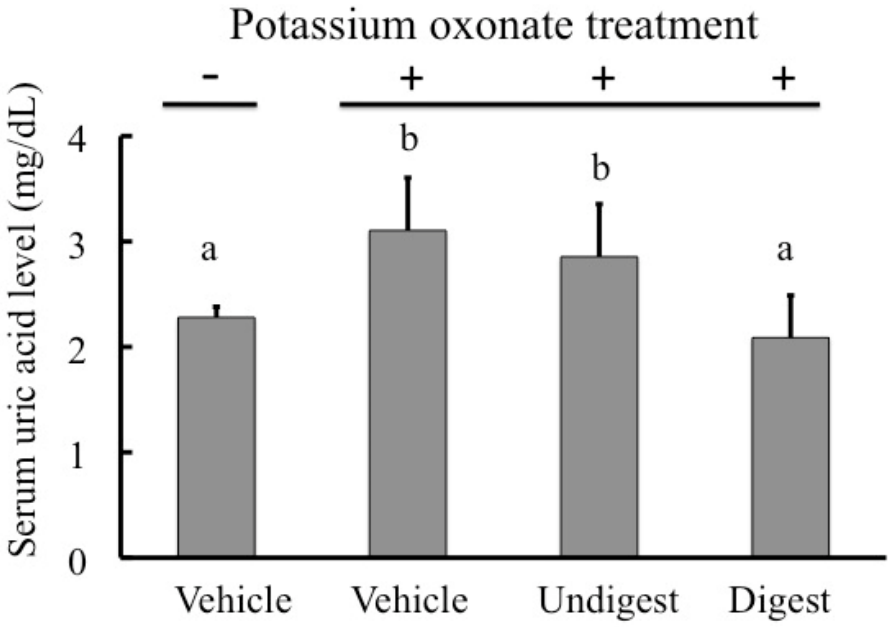


Figure 1. Effect of supplementation of the water extract of shark cartilage (undigest) and its alcalase digest (digest) on serum uric acid levels of rats treated with potassium oxonate.

Data present the mean ± standard deviation (n=6). Data points marked with different letters indicate significant differences ($P<0.01$).

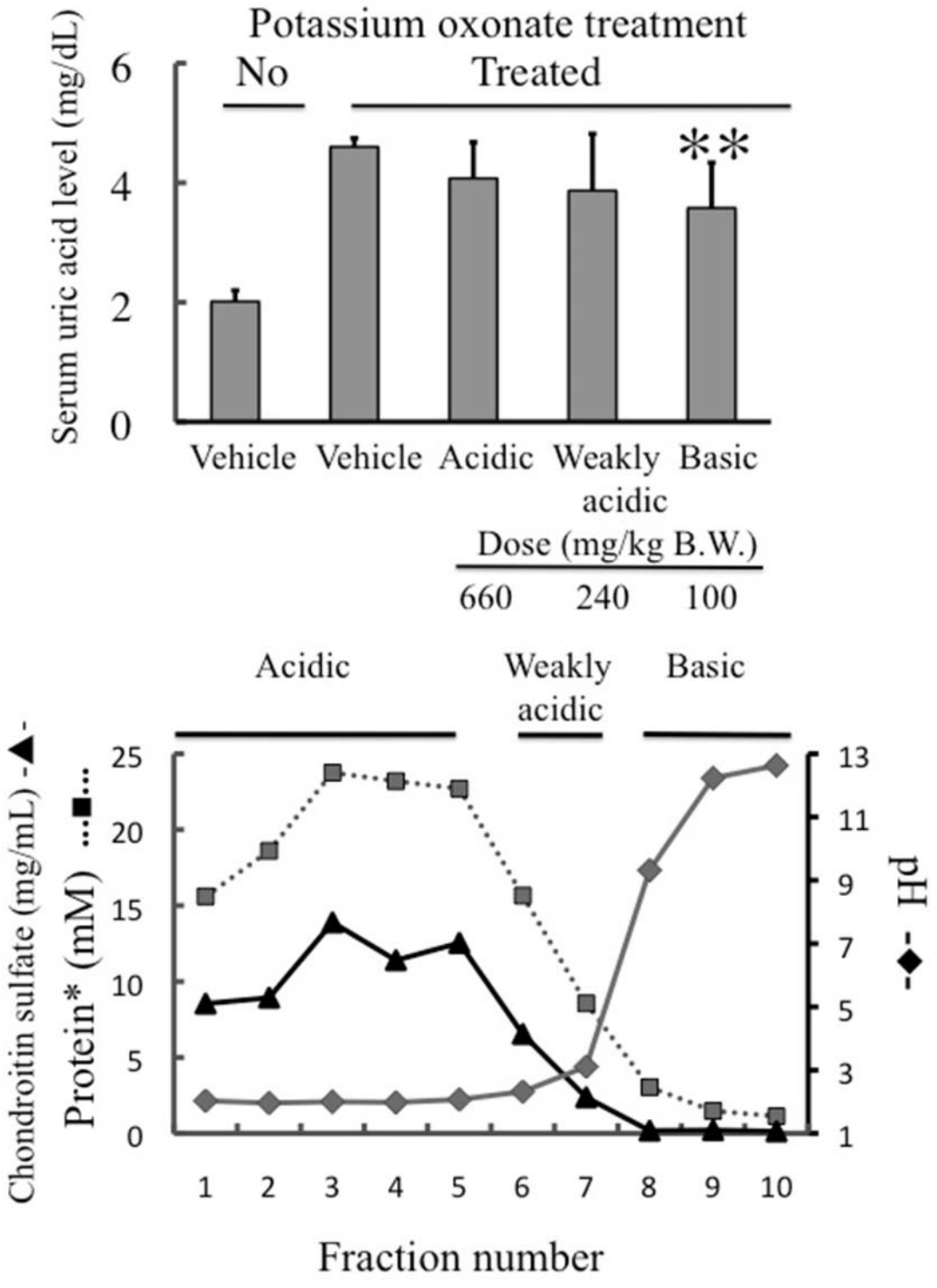


Figure 2. First purification of the protein that produces peptides with

anti-hyperuricemic activity after alcalase digestion using ampholyte-free preparative isoelectric focusing (autofocusing).

The water extract of shark cartilage without alcalase digestion was fractionated by autofocusing (lower). Acidic, weakly acidic, and basic fractions were collected as indicated by bars. *Protein content is expressed as the sum of the constituting amino acids in the HCl hydrolysate. An aliquot of each fraction was evaluated for anti-hyperuricemic activity after alcalase digestion (upper). Dosages were determined by the following equation: (ratio of each fraction to start material) × 1g/kg body weight. Data present the mean ± standard deviation (n=6). Data marked with ** are significantly different from the cntrol group treated with potassium oxonate at $P<0.01$.

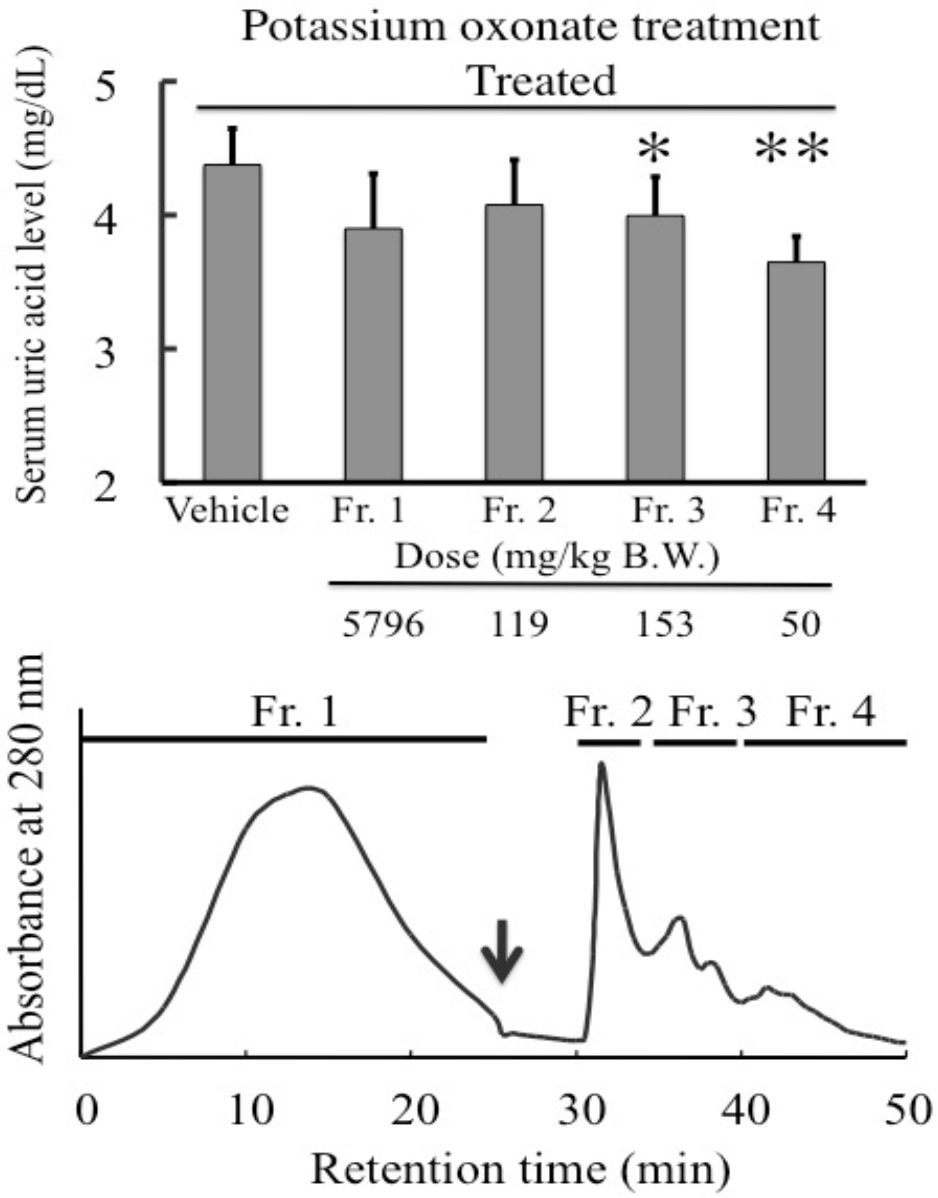


Figure 3. Second purification of the protein that produces peptides with anti-hyperuricemic activity after alcalase digestion by preparative reversed-phase chromatography.

The basic fraction shown in Figure 2, without alcalase digestion, was used (lower). The arrow represents the start of gradient elution. Effluents were collected every minute and gathered to Fraction (Fr.) 1-4, as shown. An aliquot of each fraction was evaluated for anti-hyperuricemic activity after alcalase digestion (upper). Dosages (mg/kg body weight) are presented in parentheses. Data present the mean ± standard deviation (n=6). * and ** indicate significant difference from the control group (Vehicle) treated with potassium oxonate at $P<0.05$ and 0.01, respectively

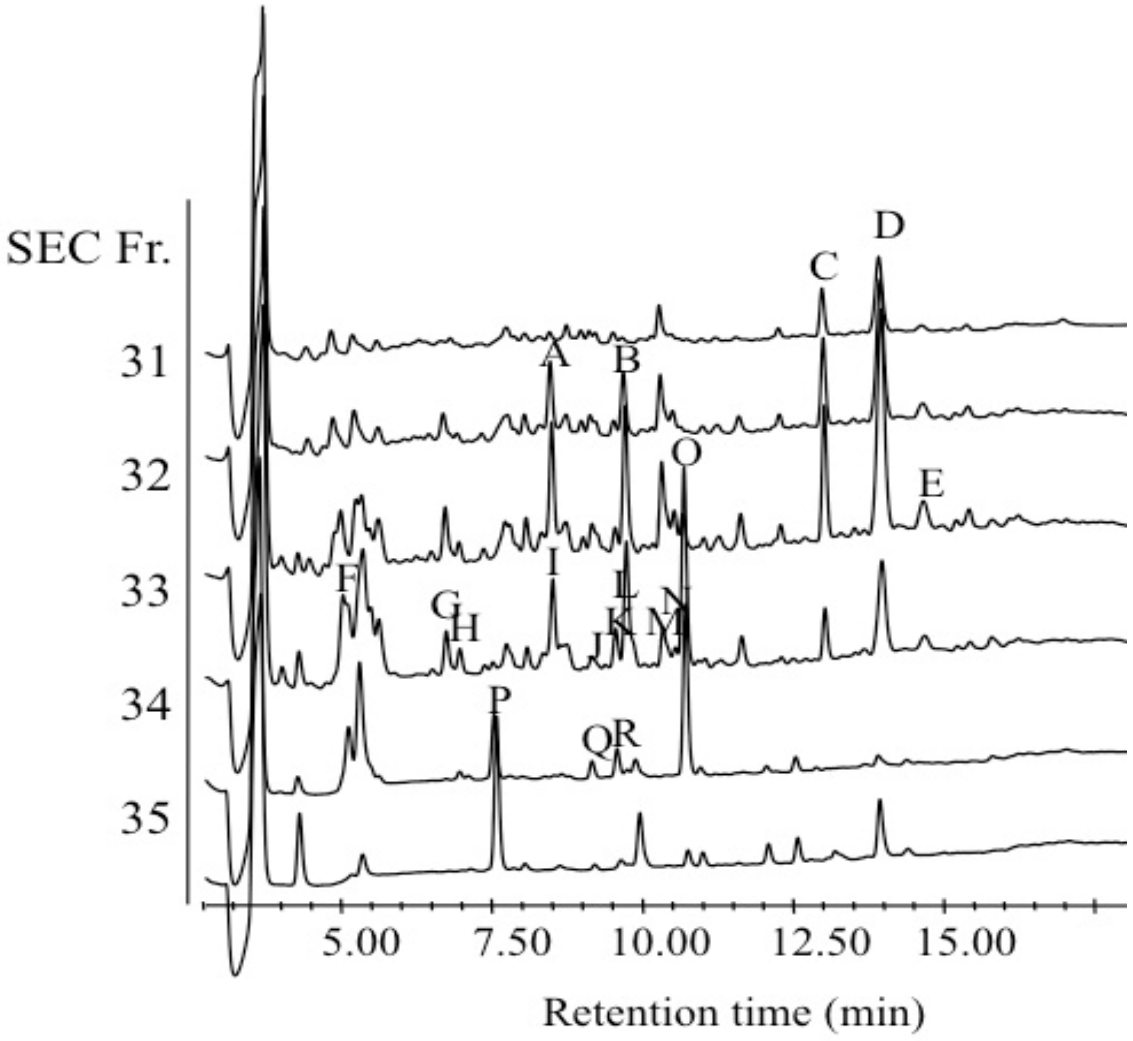


Figure 4. Isolation of peptides from the alcalase digest of the hydrophobic fraction (Fraction 4 in Figure 3).

The alcalase digest was first subjected to size-exclusion chromatography (SEC). Fractions were collected every minute. SEC fractions (Fr. 31–35) were further fractionated by RP-HPLC. Peaks marked with large characters were collected and subjected to sequence analysis. Sequences of the identified peptides are summarized in Table 1.

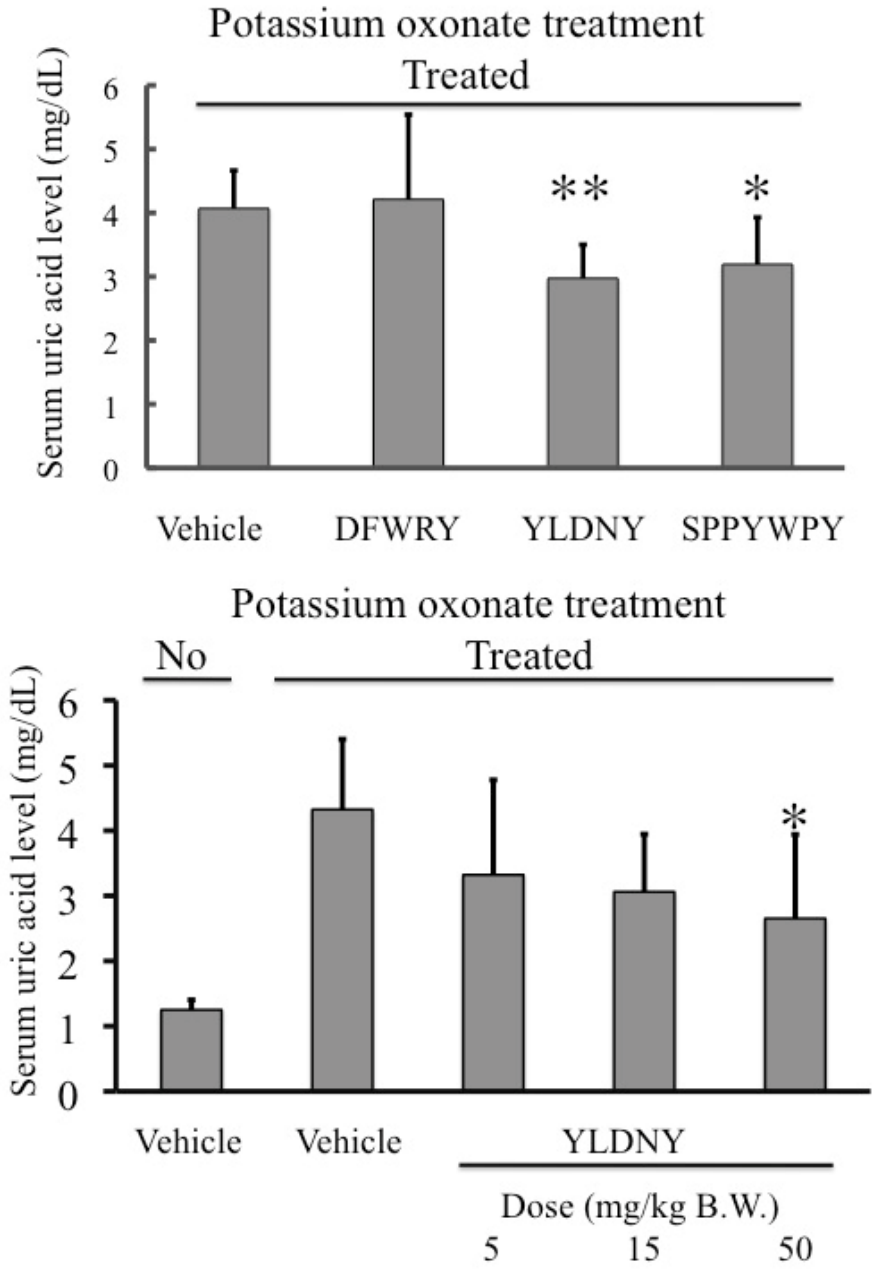


Figure 5. Anti-hyperuricemic activity of the synthetic peptides.

Asp-Phe-Trp-Arg-Tyr (DFWRY), Tyr-Leu-Asp-Asn-Tyr (YLDNY), and Ser-Pro-Pro-Tyr-Trp-Pro-Tyr (SPPYWPY) were injected into the tail vein (5 mg/kg body weight) (upper). YLDNY was also orally administered (lower).

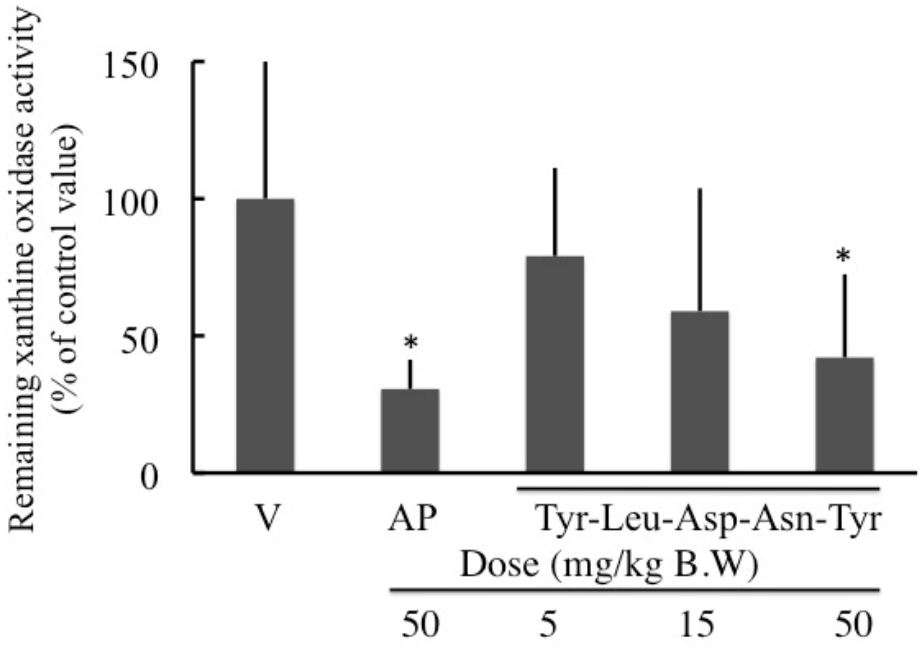
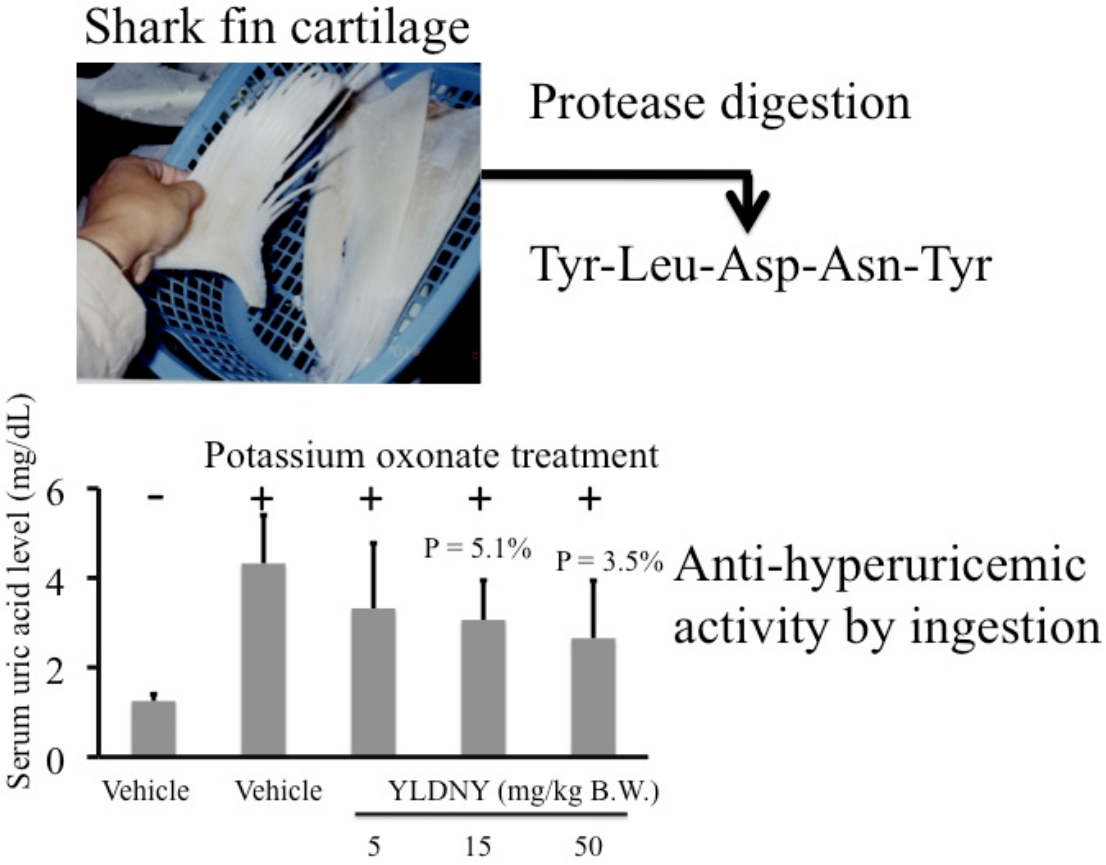


Figure 6. Inhibitory activity against xanthine oxidase by serum from the rat that had ingested vehicle (V), allopurinol (AP), and Tyr-Leu-Asp-Asn-Tyr 2 h before collection of blood.

Data presents the mean ± standard deviation (n=6). * indicates significant difference from the control group (V) at $P<0.05$.


Shark fin cartilage
Protease digestion
Tyr-Leu-Asp-Asn-Tyr
Potassium oxonate treatment
Serum uric acid level (mg/dL)
6
4
2
0
-
+
+
+
+
P = 5.1%
P = 3.5%
Vehicle
Vehicle
YLDNY (mg/kg B.W.)
5
15
50
Anti-hyperuricemic activity by ingestion

TOC

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Chapter 5. General discussion and conclusion.

Xanthine oxidase is a key enzyme for uric acid synthesis. Therefore, food components that are able to inhibit xanthine oxidase have been tested to obtain serum uric acid lowering food ingredient (Yoshizumi, et al.; 2005). However, few peer-reviewed papers have demonstrated that ingestion of food components display anti-hyperuricemic activity based on xanthine oxidase inhibition. For one reason, the components, which had been screened by in vitro inhibitory assay against xanthine oxidase, might be metabolized and lose the potential inhibitory activity during digestion and absorption process. To break this situation, uric acid lowering activity was evaluated by feeding experiment using oxonate-induced hyperuricemia rat model. After confirming the uric acid lowering activity of proteolytic digest of shark cartilage, in vivo activity-guided fractionation based on large-scale fractionation and animal experiment was used to identify the active peptide.

Tyr-Leu-Asp-Asn-Tyr was successfully identified by this approach as serum uric acid lowering peptide by ingestion. Tyr-Leu-Asp-Asn-Tyr intact does not show xanthine oxidase-inhibiting activity, while ingestion of Tyr-Leu-Asp-Asn-Tyr decreased serum xanthine oxidase activity. Therefore, Tyr-Leu-Asp-Asn-Tyr cannot be identified by using

the conventional in vitro xanthine oxidase inhibitory assay, which indicates advantage of the present approach to the conventional one.

Only Tyr-Leu-Asp-Asn-Tyr exerted serum uric acid lowering effect by oral administration in the active fraction that had been prepared by autofocusing and preparative reversed phase liquid chromatography. These facts indicate that Tyr-Leu-Asp-Asn-Tyr plays significant role in serum uric acid lowering effect in the proteolytic digest of shark cartilage. However, effective dose for serum uric acid lowering effect of Tyr-Leu-Asp-Asn-Tyr was comparable to the final active fraction, which indicates that other peptides in the active fraction might enhance the activity of Tyr-Leu-Asp-Asn-Tyr, while these peptides do not exert uric acid lowering activity alone. Effect of other peptides in the active fraction on uric acid lowering effect of Tyr-Leu-Asp-Asn-Tyr should be examined. Identification of Tyr-Leu-Asp-Asn-Tyr and other peptides in the active fraction enable examine it.

Mechanism for decrease of serum xanthine oxidase activity by ingestion of shark cartilage digest and Tyr-Leu-Asp-Asn-Tyr remains to be solved. As Tyr-Leu-Asp-Asn-Tyr has no inhibitory activity against xanthine oxidase, its fragment peptides might directly inhibit it. Alternatively, Tyr-Leu-Asp-Asn-Tyr or its fragment peptides might induce endogenous inhibitors. Further investigation is necessary to elucidate the mechanism for

the decrease in the serum uric acid level observed after the intake of crude shark cartilage extract and Tyr-Leu-Asp-Asn-Tyr.

The serum uric acid lowering activity in the crude shark cartilage extract can be concentrated by removal of chondroitin sulfate by ethanol precipitation, which can be used for food manufacture. By this simple treatment, dose for serum uric acid lowering activity can be decreased, which would facilitate human trial. If serum uric acid lowering effect of the ethanol extract is confirmed by well-designed human trial, it would be used as functional ingredient to moderate hyperuricemia.

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