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MINI-REVIEW

Biochemical aspects of red koji and tofuyo prepared using *Monascus* fungi

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Abstract Red koji or red mold rice is prepared by growing a genus *Monascus* on steamed rice. For centuries, it has been used in Asia for the production of fermented foods including red rice wine and fermented tofu. Although red koji is an important source of various hydrolytic enzymes critical for food fermentation, information on the enzymatic properties in red koji has been limited. Hydrolytic enzymes produced by *Monascus* fungi may play important roles in ripening of tofuyo (Japanese fermented tofu) regarding the chemical and physical properties of the product. This review provides an introduction of red koji, its properties, and the application of hydrolytic enzymes, especially aspartic proteinases and carboxypeptidases from *Monascus* fungi. We also describe tofuyo and a novel fermented soybean protein food using a microbial action originating from red koji.

Keywords *Monascus* · Koji · Aspartic proteinases · Carboxypeptidases · Fermented tofu · Tofuyo

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Introduction

Monascus is a genus of filamentous fungi that has traditionally been used in the microbial fermentation industry in Asia. These ascomycetes are considered to belong to the family Monascaceae, order Eurotiales. *Monascus purpureus*, *Monascus pilosus*, and *Monascus ruber* are industrially useful microorganisms (Kuba-Miyara and Yasuda 2012). Red koji has been used for the production of natural colorants and fermented foods such as red rice wine, vinegar, and fermented tofu (tofuyo in Japan and furu in China) and as an additive for preserving fish, meat, and vegetables (Su 1975; Yasuda 1983a; Yasuda 1983b; Lin et al. 2008). It has also been used as a Chinese herbal medicine, promoting digestive absorption and blood circulation (Wang and Lin 2007).

Recent studies have confirmed that red koji has medicinal characteristics including antihypertensive, antihypercholesterolemic, antimicrobial, antioxidative, and anticarcinogenic properties (Aniya et al. 2000; Lee et al. 2006; Wang and Lin 2007; Kuba et al. 2009; Tsukahara et al. 2009). Great attention has been given to the bioactive substances produced by *Monascus* fungi in terms of health foods and medicine, and some reviews have been published (Endo 1981; Yasuda 1983a; Yasuda 1983b; Wang and Lin 2007; Lin et al. 2008; Kuba-Miyara and Yasuda 2012).

Although *Monascus* fungi are important microorganisms for the production of traditional fermented foods in Asia, information about their enzymatic properties has been limited. In order to understand the fermentation of tofuyo or other fermented foods, it is important to know the enzymatic aspects of red koji. In this review, we focused on some hydrolytic enzymes, especially proteinases, peptidases, and amylases, produced by *Monascus* fungi. Based on our research, we furthermore introduce the application of aspartic proteinases and carboxypeptidases produced by *Monascus*

fungi. We also provide a Japanese fermented tofu called tofuyo and a novel fermented soybean protein food made with unique soybean protein gel and microbial fermentation.

A short history of red koji utilization in Asia

Utilization of red koji is thought to have originated in China; however, the exact time and place remain uncertain. The first records concerning red koji making and utilization in China has been considered to be in about the ninth century based on old literature search (Hong 1981). At that time, red koji had been used for the production of alcoholic beverages and preparation of moromi-mash for soaking meat according to his literature research. It is moreover recorded that red koji might improve blood circulation and spleen and stomach health as the physiological effect cited in a famous book on Chinese medicine and herbs, *Pen Ts'ao Kang Mu* (*Honzo Komoku*, in Japanese), written by Li Shih-Chen (1596). Details on how to make red koji are described in *Tien Kung K'ai Wu* (*Ten Ko Kai Butsu*, in Japanese) by Song Yin-Xing (1637). These old literatures have contributed to the modern manufacturing technique of fermentation industry using *Monascus* fungi in mainland China and Taiwan.

In Okinawa Prefecture, Japan, red koji has also been traditionally used for tofuyo making and as a colorant for celebratory foods such as cooked red rice, red boiled eggs, red fish-cake (kamaboko in Japanese), red cuttlefish meat, and so on since the eighteenth century (Yasuda 1983a; Yasuda 1983b). The earliest record concerning utilization and physiological effect of red koji in Okinawa, *Gozen Honso*, written by the physician Tokashiki Tsukan, appeared in 1832. However, *Monascus* fungi have never been widely known or utilized in mainland Japan at that time. Attention to these fungi for the production of natural food pigments or bioactive substances is comparatively recent. Nowadays, *Monascus* fungi are utilized in the food industries for the production of natural food colorants, health supplements, and medicines in Japan.

Production of red koji

Red koji is prepared from polished rice as it is essential that the koji mold quickly penetrates the rice kernel. Polished rice is soaked in water for around 18 h at room temperature and then excess water is drained off. The swollen rice is cooked with steam at atmospheric pressure for 60 min, cooled to 35 °C, and inoculated with a *Monascus* starter. The rice is placed in wooden or bamboo trays for incubation. Temperature, moisture, and aeration are important factors that must be controlled. After incubation at 32 °C for around 7 days, red koji is harvested (Yasuda et al. 1983; Yasuda 2011). Pigments, hydrolytic enzymes, and other useful metabolites are produced during cultivation.

Fungal hydrolytic enzymes for food fermentation

Hydrolytic enzymes play critical roles during fermentation of such products as miso paste, soy sauce, Japanese sake, and distilled liquor known as "awamori" or "shochu" in Japan. The original proteins are converted into peptides and free amino acids by hydrolysis of proteinases and/or peptidases produced by the fungi. Starches are changed to glucose and oligosaccharides by hydrolytic reactions of α-amylases and glucoamylases. Lipids are degraded to fatty acids and glycerol by lipases. Those of *Aspergillus* species have been well investigated in relation to the production of fermented foods and their details have been in print as in some books such as *Koji-Gaku* (*Koji Science*) edited by Murakami (1986), *Koji* by Ichishima (2007), and *Aspergillus* as edited by Machida and Gomi (2010). Although fungi in *Monascus* are also important industrial microorganisms in Asia, little attention has been given to the enzymological properties of these microorganisms. In this review, we introduce aspects of some hydrolytic enzymes produced by *Monascus* fungi.

Aspartic proteinases

Proteinases cleave peptide bonds at internal positions within proteins/peptides to produce peptide fragments. They are classified into four groups based on their catalytic mechanisms: (1) aspartic proteinases, (2) serine proteinases, (3) cysteine proteinases, and (4) metallo proteinases (Hartley 1960). Aspartic proteinases (acid proteinases) have been widely employed in the food industry. Among the reported fungal aspartic proteinases, penicillopepsin, aspergillopepsin, rhizopuspepsin, and mucorpepsin have been extensively studied (Ichishima 2000; Yegin et al. 2011). However, the enzymes from *Monascus* fungi have not been clarified sufficiently.

Tsai et al. (1978) and Liang et al. (2006) purified aspartic proteinases from *Monascus kaoliang* and *M. purpureus*, respectively. We also purified and characterized aspartic proteinases from *M.* sp. no. 3403 (*M. purpureus*) (Yasuda et al. 1984; Lakshman et al. 2011b) and *M. pilosus* (Lakshman et al. 2010). The molecular mass of aspartic proteinases was estimated to be 42 kDa from *M. purpureus* (MpuAP) (Yasuda et al. 1984; Lakshman et al. 2011b) and 43 kDa (MpiAP1) and 58 kDa (MpiAP2) from *M. pilosus* (Lakshman et al. 2010), showing monomeric protein, respectively. MpuAP and MpiAP1, among them, were an acidic non-glycoprotein, whereas MpiAP2 was an acidic

glycoprotein with 27 % carbohydrate content. All enzyme activities showed a maximum at a pH of around 3.0, their stabilities being in the range of pH 2.5–6.0, and exhibited maximum at 55 °C in the range of pH 2.5–3.0 against casein and human hemoglobin as the substrate commonly used. These enzyme activities were strongly inhibited by addition of pepstatin A and showed a competitive inhibition (i.e., K_i value of 26 nM for MpuAP activity), that is, these enzymes were categorized into the typical aspartic proteinase. The lyophilized form of MpuAP was well preserved at 30–40 °C for 7 days without any stabilizer. Substrate specificity tests of these enzymes on various natural proteins indicated that these enzymes are capable of acting on a broad range of natural substrates. Interestingly, these fungal aspartic proteinases purified from the genus *Monascus* hydrolyzed animal proteins such as cytochrome *c*, human hemoglobin, Hammarsten milk casein, and whey protein concentrate (WPC) more favorably than vegetable proteins such as soybean protein (SP) and wheat gluten.

To investigate the cleavage mechanism against the peptide bonds, the oxidized insulin B-chain was digested by each enzyme. A comparison of the cleavage sites of the oxidized insulin B-chain with other aspartic proteinases is summarized in Fig. 1 (Lakshman et al. 2011b). MpuAP hydrolyzed seven peptide bonds and preferentially hydrolyzed five sites of their cleaving sites in the oxidized insulin B-chain: Gln^4–His^5, His^{10}–Leu^{11}, Ala^{14}–Leu^{15}, Gly^{23}–Phe^{24}, and Phe^{24}–Phe^{25}. Of these preferred cleaving sites, His^{10} –Leu^{11} and Gly^{23} –Phe^{24} were characteristic sites of MpuAP among fungal aspartic proteinases including other *Monascus* enzymes. The peptide bonds of Gln^4–His^5, Gly^{23}–Phe^{24}, and Phe^{24}–Phe^{25} were specific to all aspartic proteinases of the genus *Monascus* (Hwang and Hseu 1980; Lakshman et al. 2011b).

On the other hand, MpiAP1 and MpiAP2 enzymes hydrolyzed 11 peptide bonds and seven peptide bonds in the oxidized insulin B-chain, respectively (Lakshman et al. 2010). Of these cleavage sites, four peptide bonds (Leu^{11}–Val^{12}, Leu^{15}–Tyr^{16}, Tyr^{16}–Leu^{17}, and Phe^{24}–Phe^{25}) and three peptide bonds (Leu^{15}–Tyr^{16}, Tyr^{16}–Leu^{17}, and Phe^{24}–Phe^{25}) were preferentially hydrolyzed by MpiAP1 and MpiAP2, respectively. Only Leu^6–Cys^7 bond was a specific site of MpiAP1 compared with other fungal aspartic proteinases including *Monascus* enzymes. The cleavage sites of Leu^{15}–Tyr^{16} and Tyr^{16}–Leu^{17} appear to be common to all the fungal aspartic proteinases except for MpuAP (Ichishima et al. 1995; Mains et al. 1971; Panneerselyam and Dhar 1981; Tanaka et al. 1977). In this regard, the substrate specificity of MpuAP was demonstrably different from other fungal aspartic proteinases, including those from *Monascus* fungi, and MpiAP1 and MpiAP2 appear to be different enzymes which share some similar properties (Lakshman et al. 2010; Lakshman et al. 2011b).

Amino acid sequence analyses of MpiAP1 and MpiAP2 from *M. pilosus* showed that the N-terminal amino acid sequence of MpiAP1 was EKGSVNTYPEPQDAEYLFAV

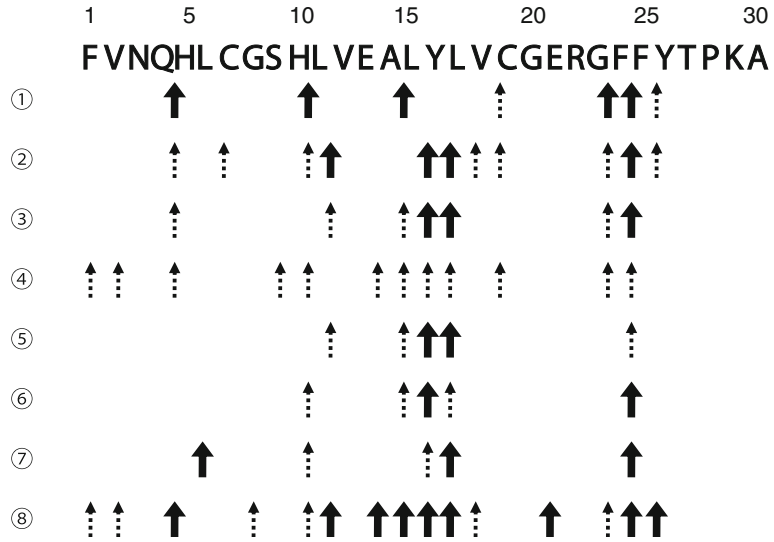

Fig. 1 Comparison of cleavage sites of various fungal aspartic proteinases on the oxidized insulin B-chain. *1* aspartic proteinase from *M. purpureus* (Lakshman et al. 2011b). *2* MpiAP1 from *M. pilosus* (Lakshman et al. 2010), *3* MpiAP2 from *M. pilosus* (Lakshman et al. 2010), *4* aspartic proteinase from *M. kaoliang* (Hwang and Hseu 1980), *5* aspartic proteinase from *Rhizopus hangchow* (Ichishima et al. 1995), *6* aspartic proteinase from *Aspergillus saitoi* (Tanaka et al. 1977), *7* aspartic proteinase from *A. fumigatus* (Panneerselvam and Dhar 1981), *8* aspartic proteinase from *Penicillium janthiellum* (Mains et al. 1971). Cited from Fig. 2 in Lakshman et al. (2011b), with permission from the Society for Industrial Microbiology and Biotechnology

and that of MpiAP2 was AKGSVNTYPE-QDA-YLTAV (Lakshman et al. 2010). Although both enzymes showed over 90 % similarity among 20 amino acid residues, their N-termini were different form each other. Their similarity of alignment with other fungal aspartic proteinases revealed an approximately 72 % similarity between *Monascus* aspartic proteinase [EMBI accession number Q76KL4] and other aspergillopepsins [EMBI Q0CSP0 and Q06902]. These results suggest that there are some common ancestral proteinases in Ascomycota.

The pattern of degradation of soybean protein by *Monascus* aspartic proteinase was investigated by SDS-PAGE in order to reveal the role of the enzyme in tofuyo ripening (Yasuda and Sakaguchi 1998). It was found that digestion of soybean protein progressed as follows: initially the α′-, α-, and β-subunits in β-conglycinin and then the acidic subunit in glycinin were degraded. However, the basic subunit of glycinin still remained, and some polypeptide bands (around 10 kDa) were formed. The degradation rate of soybean protein was affected by the ethyl alcohol concentration in the enzyme reaction mixtures. These results were similar to tofuyo ripening as described by Yasuda et al. (1993) and are included in the following discussion of tofuyo. Thus, it can be concluded that *Monascus* aspartic proteinases are key enzymes in tofuyo ripening.

Enzymes are important in food biotechnology because they are key catalysts for the production of useful compounds and also for the elimination of undesirable substances from raw materials. Much attention has recently been paid to the role of health-promoting foods in disease prevention (Arai et al. 2001). Hypertension is a serious risk factor in cardiovascular disease, leading to high mortality. Controversy surrounds the effect of the angiotensin I-converting enzyme (ACE, EC 3.4.15.1) on hypertension. This enzyme converts angiotensin I (DRVYIHPFHL) to the potent pressor peptide, angiotensin II (DRVYIHPF), and also degrades the depressor peptide bradykinin (Yang et al. 1971).

ACE inhibitors from various foods have been studied, and some peptides such as tripeptide (Takano 1998) and dipeptide (Kawasaki et al. 2000) have been used as supplementary foods to improve hypertension. Proteinases are frequently used in protein hydrolysis to obtain ACE-inhibitory peptides. Microbial alkaline serine proteinases are utilized in the production of ACE inhibitors from food proteins (Matsufuji et al. 1994); however, there have been few reports of the use of microbial aspartic proteinases for the production of ACE inhibitors. We investigated the production of ACE-inhibitory peptides from soybean protein, using *Monascus* aspartic proteinase (Kuba et al. 2005). The major components of soybean protein, β-conglycinin and glycinin, were hydrolyzed by this enzyme, resulting in the isolation of four ACE-inhibitory peptides. Those from β-conglycinin hydrolysis were identified as LAIPVNKP and LPHF and those from glycinin hydrolysis as SPYP and WL. The compound WL was identical to one isolated from tofuyo (Kuba et al. 2003), as described in the next section. The inhibitory activity of SPYP markedly increased after successive digestion by pepsin, chymotrypsin, and trypsin in vitro. However, that of LAIPVNKP markedly decreased after successive digestion by these gastrointestinal proteinases. This is important for bioactive peptide design, which must take gastrointestinal digestion into account. This enzyme contributes to the formation of ACE-inhibitory peptides and also has the potential to produce various bioactive peptides from food proteins.

Furthermore, proteinases can be expected to remove undesirable elements from food proteins, as will be described later. It is well known that milk proteins provide good nutrition because of their excellent amino acid profiles. Unfortunately, the majority of protein components in whey are eventually discarded as by-products in the cheese industry. Whey protein has potential use in value-added foodstuffs such as dietary supplements, dairy products, and animal feed. However, cow's milk allergy is one of the major causes of food hypersensitivity (Monaci et al. 2006). The antigenicity of cow's milk proteins in humans are well known, and it has been suggested that the main allergenic components are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin, and α-casein, main protein components in milk whey and almost absent or present in much lower quantities in human milk (Malik et al. 1988; Svenning et al. 2000). In order to reduce the antigenicity of whey proteins using gastric proteinases (Schmidt et al. 1995), plant proteinases (papain and bromelain), bacterial proteinases (alcalase and neutrase), and combinations of extracellular proteinases with gastric proteinases have been tested in the manufacture of low-antigenic whey protein hydrolyzates (Nakamura et al. 1993a; 1993b). A very limited number of reports can be found on the application of fungal proteinases for the production of hypoallergenic whey protein hydrolyzates.

To investigate the possibility of reducing the antigenicity of milk whey proteins, enzymatic hydrolyzates of the whey protein were evaluated by competitive inhibition ELISA in our laboratory (Lakshman et al. 2011a, 2011b). Out of the three main components of whey protein, casein and α-LA were efficiently degraded by MpuAP from *M. purpureus* (Lakshman et al. 2011b). The sequential reaction of MpuAP and trypsin against the whey protein successfully degraded casein, α-LA, and β-LG with the highest degree of hydrolysis. The hydrolyzates obtained using the MpuAP-trypsin combination showed the lowest antigenicity compared with single applications of pepsin and trypsin or a pepsin–trypsin combination. We suggest that the storage-stable MpuAP and trypsin combination will be productive in making

hypoallergenic bovine whey protein hydrolyzates. We also examined the potential application of both MpiAP1 and MpiAP2 in the production of low-antigenic whey protein (Lakshman et al. 2011a). Hydrolyzates of WPC were prepared enzymatically using MpiAP1, MpiAP2, pepsin, and trypsin, singly and in combination. Electrophoretic analyses of WPC component degradation showed that casein is the component most favored by all enzymes. α-LA was completely digested by the fungal aspartic proteinases, MpiAP1 and MpiAP2, but not by the gastrointestinal proteinases (pepsin and trypsin), and β-LG was effectively digested only by trypsin. The complete digestion of all WPC components was accomplished by using combinations of *Monascus* aspartic proteinases and trypsin. The lowest antigenicity was observed by competitive inhibition ELISA in the WPC hydrolyzates treated with MpiAP1/MpiAP2 and trypsin. From these results, it is suggested that *Monascus* aspartic proteinases can be effectively used to make hypoallergenic bovine milk whey protein hydrolyzates.

Alkaline serine proteinases

Although some extracellular aspartic proteinases from *Monascus* fungi have been reported as described above, only one report of serine proteinases has been published. Aso et al. (1989) reported some properties of alkaline serine proteinases produced by *Monascus* species grown on wheat bran media. They found alkaline proteinase activities in the crude extracts prepared from six strains of five species and one variety of *Monascus* using a printing technique on a casein-containing slab gel. The enzymes, which showed the highest activities at around pH 9, were stable in the pH range of 5 to 10 after incubation at 4 °C for 24 h. Thermal treatment over 40 °C for 20 min decreased enzyme activities. Enzyme activities were completely inhibited by phenylmethylsulfonyl-fluoride (PMSF) and were insensitive to *o*-chelating and SH reagents. These enzymes were therefore characterized as serine proteinases, distinct from chymotrypsin- or trypsin-type enzymes because of their insensitivity to affinity-labeling reagents *N*-α-tosyl-L-lysyl chloromethyl ketone and *N*-tosyl-L-phenylalanyl chloromethyl ketone. However, further enzymatic properties of alkaline serine proteinases from genus *Monascus* have not yet been clarified.

Carboxypeptidases

Carboxypeptidases are exopeptidases that cleave peptide bonds at points within proteins, liberating amino acids sequentially from the C-terminus. We purified serine carboxypeptidases from *M. purpureus* (Liu et al. 2004a) and *M. pilosus* (Liu et al. 2004b) as homogenous preparations using SDS-PAGE.

The purified enzyme, MpuCP, from *M. purpureus* was a heterodimer with a molecular mass of 132 kDa, consisting of two subunits of 64 and 67 kDa (Liu et al. 2004a). It was characterized as an acidic glycoprotein with an isoelectric point of 3.67 and 17 % carbohydrate content. The activity of this enzyme was strongly inhibited by piperastatin A, diisopropylfluoride, PMSF, and chymostatin, suggesting that it is a chymotrypsin-like serine carboxypeptidase. Benzyloxycarbo-tyrosyl-L-glutamic acid (Z-Tyr-Glu) was the best substrate for the enzyme, followed by Z-Glu-Phe, Z-Glu-Tyr, Z-Phe-Ala, and Z-Ala-Glu. The enzyme prefers peptides containing an aromatic amino acid or glutamic acid in the C-terminal position (PI') or the penultimate position (PI) of the C-terminus, suggesting that it also possesses chymotrypsin-like activity.

We also purified two serine carboxypeptidases, MpiCP-1 and MpiCP-2, from *M. pilosus* (Liu et al. 2004b). MpiCP-1 was a homodimer with a native molecular mass of 125 kDa, consisting of two identical subunits of 65 kDa, while MpiCP-2 was a high molecular mass homo-oligomer with a native molecular mass of 2,263 kDa, consisting of 38 identical subunits of 59 kDa. This is unique among the already known carboxypeptidases and distinguishes MpiCP-2 as the largest known carboxypeptidase. Both purified enzymes were acidic glycoproteins. MpiCP-1 had an isoelectric point of 3.7 and a carbohydrate content of 11 %; in MpiCP-2, these values were 4.0 and 33 %, respectively. The optimum pH and temperature were 4.0 and 50 °C for MpiCP-1 and 3.5 and 50 °C for MpiCP-2, respectively. PMSF strongly inhibited MpiCP-1 and completely inhibited MpiCP-2, suggesting that they are both serine carboxypeptidases. Of the substrates tested, Z-Tyr-Glu was the best for both enzymes. The substrate specificities of the carboxypeptidases from *M. purpureus* and *M. pilosus* as described above are consistent with the finding that glutamic acid is one of the most abundant free amino acids found in matured tofuyo (Yasuda et al. 1993; Yasuda 2010). Thus, carboxypeptidases from *Monascus* fungi are concluded to play an important role in the formation of taste-associated compounds (amino acids) from soybean protein during the maturation of tofuyo.

There are many reports of improvements to the functional properties of soybean protein by enzymatic features. The enzymatic hydrolysis of soybean protein frequently leads to the production of a bitter taste arising from the natural degradation products of the proteolytic reaction. Arai et al. (1970) reported that the combination of aspergillopeptidase A and *Aspergillus* aspartic carboxypeptidase was capable of both a deodorization and a debittering effect to produce a bland soybean protein hydrolyzate. We examined the actions of pepsin and the admixture of pepsin and

carboxypeptidase from *Monascus* fungi on the hydrolysis of soybean protein (Liu and Yasuda 2005). The pepsin hydrolyzate of soybean protein was much more bitter and contained relatively smaller amounts of total free amino acids than the hydrolyzates obtained using the admixture of pepsin and MpiCP-1. In addition, hydrophilic and hydrophobic amino acids were present in almost equal proportions in the pepsin hydrolyzate, while mainly hydrophobic amino acids made up the hydrolyzate obtained with the admixture of pepsin and MpiCP-1. This suggested that MpiCP-1 suppresses and reverses the bitter taste that results from the pepsin hydrolysis of soybean protein by releasing mainly hydrophobic amino acids from the C-termini of the bitter components.

We thus concluded that this enzyme plays an important role in the formation of taste-associated amino acids and in the elimination of bitterness during the ripening of soybean fermented foods such as tofuyo. This information may provide clues to the applicability of *Monascus* carboxypeptidases in the modification of soybean protein and the elimination of bitterness.

α-Amylases

α-Amylase is an endohydrolase that cleaves α-1, 4-glucoside linkages at random sites in starch. α-Amylases are important enzymes in the production of fermented foods such as alcoholic beverages, miso pastes, and so on. The α-amylase activity in red koji is lower than in the yellow koji (prepared using *A. oryzae*) or white koji (prepared using *A. kawachii*), and thus little attention has been paid to enzyme characterization. Recently, Yoshizaki et al. (2010) reported that low-temperature cultivation of *Monascus* fungi dramatically enhanced the production of this enzyme. They found an interesting phenomenon—that incubation at 37 °C promoted maximum cell growth, while incubation at 25 °C resulted in enhanced α-amylase production. They succeeded in producing red koji with high α-amylase activity, and they also purified the enzyme homogeneously. The molecular mass of the purified enzyme was estimated to be 47 kDa. They observed that α-amylase was unstable at acidic pH and less resistant to heat (at <40 °C) than glucoamylase. Further characterization of this enzyme is needed.

Glucoamylases

Glucoamylase is an exo-hydrolyzing enzyme liberating D-glucose units from the non-reducing ends of amylose, amylopectin, and glycogen by hydrolysis of α-1,4-linkages in consecutive monomers, producing D-glucose as the sole product. The enzyme is industrially important, and many fungal glucoamylases, such those from *Aspergillus*, *Mucor*, and *Rhizopus*, have been purified and characterized (Ono et al. 1988; Selvakumar et al. 2008; Yu et al. 1999; Yamasaki et al. 1977; Yu and Hang 1991). However, information concerning *Monascus* glucoamylase is very limited. Iizuka and Mineki (1977, 1978), Yasuda et al. (1989), and Yoshizaki et al. (2010) purified glucoamylases from some *Monascus* strains. Two forms of an extracellular glucoamylase, MpuGA-I and MpuGA-II, were purified to homogeneity from *M. purpureus* in our laboratory (Tachibana and Yasuda 2007). The properties of these enzymes may be summarized as in the following discussion.

The molecular masses of these enzymes were estimated to be 60 kDa (MpuGA-I) and 89 kDa (MpuGA-II). These enzymes were characterized as glycoproteins with a carbohydrate content of 15.0 % in MpuGA-I and 16.2 % in MpuGA-II. Optimal pH was 5.0 for both enzymes, and the optimal temperatures were 50 °C (MpuGA-I) and 65 °C (MpuGA-II). We examined the relative rates of hydrolysis of various substrates by the purified enzymes. Both enzymes showed the highest hydrolysis activity toward short polymerized amylose Ex-I, having only the α-1,4-linkage, and they showed only moderate hydrolysis activity toward amylopectin. The K_m values for soluble starch were calculated to be 4.0 mg/ml (MpuGA-I) and 1.1 mg/ml (MpuGA-II). Interestingly, glucoamylases from *A. niger* (Ono et al. 1988) and *A. awamori* (Fierobe et al. 1998) hydrolyze various malt-oligosaccharides as well as soluble starch, whereas MpuGA-I and MpuGA-II from *M. purpureus* RY3410 clearly exhibited low hydrolyzing activity toward malt-oligosaccharides. The primary structure of the N-terminal amino acid sequence of MpuGA-I showed 72.7 % homology with that of MpuGA-II, and the N-terminal amino acid sequence of MpuGA-I showed high homology and similarity to other fungal glucoamylases belonging to the glucoside hydrolase family 15. *Monascus* glucoamylases may thus play an important role in the formation of glucose during the ripening of tofuyo (Yasuda et al. 1993).

Other enzymes

β-Glucosidase catalyzes the hydrolysis of β-glycosidic linkages in a variety of glucosides and is useful in the food industry for the enzymatic saccharification of cellulose and the release of phenolic compounds with antioxidant activity from fruit and vegetable residues. The enzyme also plays an important role in increasing the antioxidant ability of fermented soybean foods. Although β-glucosidases are widely produced by fungi including species of *Aspergillus*, *Trichoderma*, and *Penicillium* (Yan et al. 1998; Chen et al. 1992; Bhiri et al. 2008), the role of this *Monascus* enzyme has been

unclear. Daroit et al. (2008) reported the purification and characterization of extracellular β-glucosidase from *M. purpureus*. The maximum activity of the enzyme was observed over a wide range of temperature and pH, with optimal conditions at 50 °C and pH 5.5. Hydrolysis of *p*-nitrophenyl-β-D-glucoside, cellobiose, salicin, *n*-octyl-β-D-glucopyranoside, and maltose indicated that the enzyme had broad substrate specificity. It is therefore thought that the enzymes from *Monascus* fungi might greatly contribute to the antioxidative activity in tofuyo (Yasuda 2010) and sufu (Yin et al. 2005).

α-Galactosidase catalyzes the disruption of the α-D-galactosidic linkages of both simple and complex oligo- and polysaccharides. Soybeans and other legumes contain the raffinose family of oligosaccharides. Because the enzyme cannot be secreted in the human gastrointestinal tract, presence of these saccharides causes flatulence. It has thus been expected that the enzyme will be useful in upgrading the nutrition of bean foods. Wong et al. (1986) purified and characterized α-galactosidase from *M. pilosus*. The molecular mass of this enzyme was estimated to be 150 kDa by gel filtration. The optimum conditions for enzyme reaction were pH 4.5–5.0 and 55 °C. Melibiose was hydrolyzed to galactose and glucose by the action of this enzyme. Raffinose was hydrolyzed to galactose and sucrose, while stachyose was hydrolyzed to galactose and sucrose, with raffinose as the intermediate compound. They consider therefore that α-galactosidase may play an important role in reducing the nondigestible oligosaccharides, raffinose, stachyose, and so on present in soybean during the ripening of fermented soybean foods such as tofuyo, sufu, natto, and miso paste.

Chitinase from *M. purpureus* was purified and characterized by Wang et al. (2002). The enzyme had a molecular mass of ~81 kDa and a p*I* of 5.4. This *Monascus* chitinase showed antimicrobial activities and proteinase activity. The enzyme was most effective on *Fusarium oxysporum* and *Fusarium solani* with inhibitory ratios of >70 % (85 and 70 %, respectively). The enzyme also inhibited the growth of various bacteria (*Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*). The enzyme or the microorganism may thus be useful in food preservation.

Ribonuclease was purified and characterized from *M.* sp. no. 3403 (*M. purpureus*) by Yasuda et al. (1995). The molecular mass of this enzyme was estimated to be 26 kDa by SDS-PAGE. The purified enzyme was active toward RNA but inactive toward untreated DNA, heat-denatured DNA, bis(*p*-nitrophenyl)phosphate, *p*-nitrophenyl phosphate, 3′- or 5′-adenylic acid, 3′- or 5′-guanylic acid, and 3′- or 5′-cytidylic acid. Homopolyadenylic acid was a good substrate for the enzyme. The optimum pH of the enzyme was 4.2 and its optimum temperature was 55 °C. Further characterization of this enzyme is needed.

Tofuyo—Japanese fermented tofu

Tofuyo is a vegetable protein food made from tofu in Okinawa Prefecture, Japan, by the action of microorganisms (Yasuda 2011). Tofuyo was found to be 29.2 % crude protein, 21.2 % crude fat, 7.4 % crude ash, 0 % crude fiber, 24.2 % reducing sugars, and 3.2 % salt (on a dry weight basis), that is, tofuyo may be a fermented low-salt soybean food. It is a creamy cheese-type product with a mild flavor, fine texture, and pleasant taste. Its other beneficial properties, such as being a valuable source of protein and improving blood circulation, have made tofuyo treasured for centuries not only as a nutritious side dish but also as a healthy food for ingestion after an illness. Some physiological functional properties of tofuyo have been clarified in our laboratory (Kuba et al. 2003; Yasuda 2010), and it attracts attention as a health food. Thus, we are certain that tofuyo will be accepted by Western people in the same way as cheese.

We have established in our laboratory how to make the best-quality tofuyo (Yasuda 1990; Yasuda 2011). Tofuyo is produced as follows: dehydrated tofu cubes are soaked into a mixture consisting of red koji prepared from *Monascus* fungi and/or yellow koji from *Aspergillus oryzae*, a small amount of salt, and awamori (Fig. 2). During the ripening period of tofuyo, crude protein and crude fat levels of tofuyo decreased, but reducing sugar levels increased. The sodium chloride content of tofuyo was relatively constant (around 3 %) throughout the ripening period and it is lower than that of Chinese sufu (Wang and Hesseltine 1970).

Proteinases play a pivotal role in the maturation of tofuyo. Proteinase activities in the moromi mash decreased during the ripening period due to ethyl alcohol concentration (20 %, *v*/*v*) originating in awamori (Yasuda et al. 1993). It was suggested that the enzyme activity could be controlled by the presence of ethyl alcohol originating in awamori and that the ripening of tofuyo might be gradually carried out. Therefore, we consider that the soybean proteins in tofu may be degraded to a limited extent and may also confer good physical properties such as smooth texture and optimal viscoelasticity in the product.

In order to confirm its biochemical degradation, the digestion pattern of soybean proteins during the ripening period was examined by using slab SDS-PAGE method (Yasuda et al. 1993). Some major bands originating from soybean globulin polypeptides (such as the α′-, α-, and

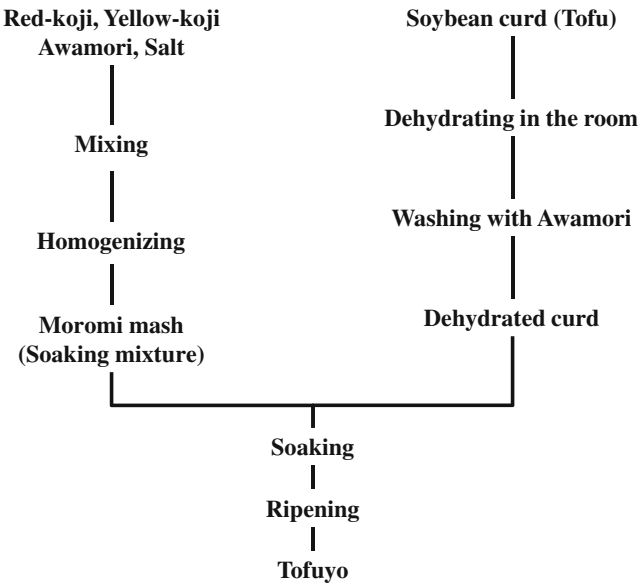

Fig. 2 The tofuyo-making process. Cited from Fig. 1 in Yasuda (2010), with permission from the Japanese Society for Food Science and Technology

β-subunits in β-conglycinin and the acidic subunit in glycinin) in the water-insoluble fraction of tofuyo disappeared after 3 months of ripening in the presence of ethyl alcohol from awamori, whereas the basic subunit in glycinin was observed as a thin band (Fig. 3). Furthermore, bands from

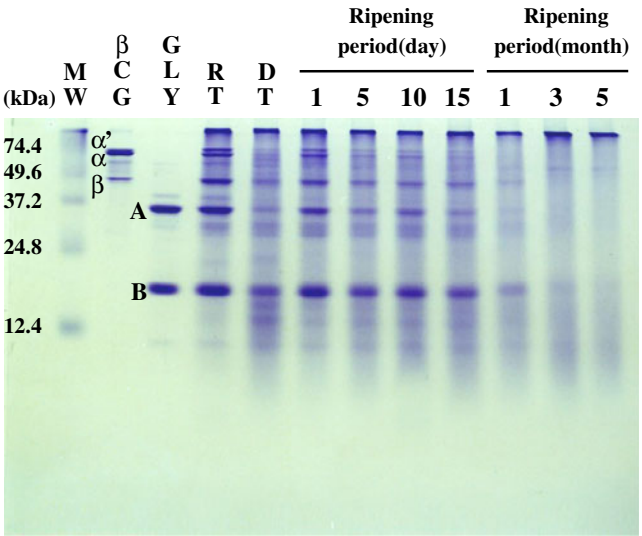

Fig. 3 Slab SDS-PAGE pattern of the water-insoluble fraction of tofuyo at various stages. Molecular masses were estimated by the mobilities of cytochrome *c* from horse heart and cross-linked cytochrome *c* (cytochrome *c* from monomer to hexamer). *MW* standard proteins described above, *βCG* β-conglycinin, *GLY* glycinin, *RT* raw tofu, *DT* dehydrated tofu. *Numerical values* in MW represent each molecular mass. *α′*, *α*, and *β* show the α′-, α-, and β-subunits in β-conglycinin; *A* and *B* show the acidic and basic subunits in glycinin. Cited from Fig. 2 in Yasuda (2010), with permission from the Japanese Society for Food Science and Technology

the other polypeptides (30–32 and 10–15 kDa) were also observed (Yasuda et al. 1993; Yasuda 2011). Similar phenomena described earlier were also confirmed in the degradation of soybean protein isolates by the enzymatic hydrolysis of *Monascus* aspartic proteinase (Yasuda and Sakaguchi 1998).

Because these polypeptides may greatly affect the texture of the tofuyo product, we investigated changes in the physical properties of the product during the ripening period (Yasuda et al. 1996; Yasuda 2011). The texture of tofuyo was dramatically different from that of the tofu which was the raw material of the product. Breaking stress and breaking energy values of the product decreased during fermentation. Creep analysis of tofuyo revealed that the values of each viscoelasticity coefficient also decreased. Interestingly, creep analysis of tofuyo ripened for 3 months showed that the viscoelasticity coefficients were similar to those of commercial cream cheese or soft processed cheese (Yasuda 2011). Internal microstructures of tofuyo showed that the structure of the soybean protein forming the tofuyo changed from fibrous to finely particulate, with the particles connected to each other. These physical and structural changes in soybean protein were considered to greatly affect the special texture of tofuyo, such as its smoothness in the mouth.

Some polypeptide bands in the water-soluble fraction of tofuyo disappeared during fermentation (0–3 months) (Yasuda et al. 1993). Ninety-four percent of the low molecular nitrogen compounds, such as amino acids and peptides, in the water-soluble fraction of the tofuyo product, were converted from soybean protein by the *Monascus* hydrolytic enzymes during fermentation. Glutamic acid, alanine, aspartic acid, glycine, and serine were major amino acids in tofuyo, and glutamic acid and aspartic acid may contribute to the pleasant taste of tofuyo. Meanwhile, unpleasant tastes derived from bitter peptides may be desirable to remove from the product by the reaction of these enzymes. Other liberated peptides may also contribute to the physiological property of tofuyo as will be described later.

Both glucoamylase and α-amylase were kept with high activities throughout the ripening period, although the activity of aspartic acid proteinases from *Monascus* fungi was suppressed by the presence of ethyl alcohol originating from awamori. Starches in koji were mostly converted to glucose by these amylases from *Monascus* fungi, giving a desirable taste to the tofuyo product. Glucose is one of the major compounds, in addition to amino acids, that are involved in the taste of tofuyo (Yasuda 2011). These observations strongly suggest that the desirable taste of tofuyo is formed by interactions between the amino acids mentioned earlier, glucose, NaCl, and organic acids originating in the koji.

Lifestyle-related diseases have become a serious social problem in modern society, and much attention has been paid to the role of health-promoting foods in disease prevention. Hypertension, for example, is a serious risk factor in cardiovascular disease. ACE-inhibitory peptides in various fermented foods have been investigated in order to reduce this risk. It is thus of interest whether ACE inhibitors are present in tofuyo; we surveyed this in our laboratory (Kuba et al. 2003). ACE-inhibitory activity was found in tofuyo extracts, and two ACE-inhibitory peptides (IFL and WL) were isolated from the tofuyo product. The IC_{50} values of IFL and WL were determined to be 44.8 and 29.9 μM, respectively. The peptide, WL, was identical to one obtained from soybean protein digestion by *Monascus* aspartic proteinase (Kuba et al. 2005). In order to evaluate the resistance of the IFL and WL peptides against gastrointestinal digestion in vivo, changes in their IC_{50} values before and after treatment with gastrointestinal enzymes (pepsin, chymotrypsin, and trypsin) were investigated in vitro. Despite successive digestion with pepsin, chymotrypsin, and trypsin, the IC_{50} values of IFL and WL were shown to be 120.0 and 110.3 μM. It has been reported that the IC_{50} values of "IF" (Cheung et al. 1980; Seki et al. 1995) and "FL" (Eto et al. 1998), which are parts of IFL, were 930 and 16 μM, respectively. IFL obtained from tofuyo is likely to preserve its activity until it is degraded into individual amino acids. These peptides isolated from tofuyo are therefore expected to be easily absorbed and contribute to the antihypertensive effect via an in vivo transport system.

The antihypertensive effects of tofuyo in vivo were investigated with spontaneously hypertensive male rats (Kuba et al. 2004). The tofuyo-fed group showed a similar body weight to that of the control group. Diet intake whole experimental period did not differ greatly between both groups. The systolic blood pressure (SBP) of rats gradually increased along with weight gain. At 11 and 12 weeks old, the SBP in the tofuyo group tended to be lower than in the control group, whereas at 13 weeks the SBP in the tofuyo group was significantly lower than in the control group. After feeding experimental diets for 14 weeks, the ACE activity of kidney was significantly lower in the tofuyo group than in the control group. Unger et al. (1985) proposed that kidney might be important for the prolonged antihypertensive effects of ACE inhibitors.

It is well known that γ-aminobutyric acid (4-aminobutanoic acid; GABA) is the chief inhibitory neurotransmitter in the mammalian central nervous system and contributes to decreased blood pressure (Stanton 1963; Hayakawa et al. 2002). Red koji also produce GABA and it may be eventually contained into the tofuyo extracts (Kuba et al. 2009). Thus, tofuyo is expected to have antihypertensive effects from its ACE-inhibitory peptides and also from GABA. Some physiological properties such as erythrocyte deformability in mice that were fed a high-fat diet (Inoue et al. 2006), antioxidative activity, and DPPH (1,1-diphenyl-2-picrylhydraxzyl) radical scavenging activity were also found in the water-soluble fraction of tofuyo. We confirmed also the presence of aglycone-type isoflavones that were released from isoflavone glycosides by β-glucosidase in the tofuyo extracts.

Monascus hydrolytic enzymes may not only contribute to formation of good taste, flavor, and desirable texture but are also involved in the production of bioactive substances such as ACE-inhibitory peptides and antioxidants during the ripening period of tofuyo.

Development of a novel fermented soybean protein food product

In addition to serving as a nutrient-rich food, soybean is known to be an excellent base material for a variety of traditional Asian foods such as tofu and tofu-related foods, miso paste, soy sauce, natto, and tempeh. Owing to its various physical properties including gelation, viscosity, emulsification, and foaming, SP has generally been used in fabricated food products such as frozen tofu, sausages, bacon, and many other meat-like products.

Recently, little attention has been paid to SP gel in fermented products. We describe an innovative calcium gel consisting of a SP product, SP-Ca-Gel, for use in novel SP foods (Yasuda and Nagamatsu 1997; Yasuda et al. 2009). The production method could be beneficial for novel fermented soybean foods including new types of tofuyo. We speculated that fermentation of SP-Ca-Gel with a *Monascus* fungus may provide novel flavors and textures and also better nutrition and physiology to the soybean product. A novel SP product, fermented soybean food which is a tofuyo-like product, was established by employing a combination of fabrication and fermentation procedures (Fig. 4). The product using

Fig. 4 Fermented soybean protein gel foods. A red koji product is on the *right*, a mixed koji product is in the *center*, and a yellow koji product is on the *left*. Cited from Fig. 4 in Yasuda (2010), with permission from the Japanese Society for Food Science and Technology

Monascus fungus had a deep red color, smooth texture, and flavor just like traditional tofuyo.

To evaluate the quality of the product, we investigated changes in protein and nitrogen composition in the fermented product generated from SP-Ca-Gel and fermentation with a *Monascus* fungus (Yasuda et al. 2009). The results of SDS-PAGE analysis and nitrogen levels of the product during fermentation showed that the major soy proteins except for the basic subunit in glycinin were preferentially degraded by the hydrolytic enzymes in the moromi mash prepared by *Monascus* fungi, and the protein band corresponding to the basic subunit on SDS-PAGE gel remained even at the end of the ripening period. These phenomena were identical to those in traditional tofuyo. The ratios of water-soluble nitrogen and TCA-soluble nitrogen to total nitrogen reached 52.4 and 46.6 % after 60 days of ripening, respectively. Moreover, involvement of a large number of free amino acids, especially aspartic and glutamic acids, was shown in this novel fermented SP-Ca-Gel food product. This method has great advantages for improving food nutrition, including the ratio and/or balance of the essential amino acids, and for added physiological effects. Based on these advantages, a novel health food named "Daichi-no-tsubu (the Grain of the Earth)", which is rich in essential amino acids, calcium, and isoflavones, was developed by collaborating with our laboratory and local industry in Okinawa, Japan.

Conclusions

Monascus fungi are important microorganisms in food fermentation in Asia. To clarify the fermentation of tofuyo scientifically, some hydrolytic enzymes from *Monascus* fungi, *M. purpureus* and *M. pilosus*, were purified and characterized in our laboratory. Aspartic proteinases play important roles as key enzymes for ripening, chemical and physical properties, and formation of bioactive peptides in the tofuyo product. Furthermore, they have the potential to produce various bioactive peptides from food proteins and can also be effectively used to create hypoallergenic bovine milk whey protein hydrolyzates. Carboxypeptidases play important roles in the release of free amino acids (especially glutamic acid) from soybean protein that gives a pleasing taste to tofuyo. The enzymes thus also contribute to the removal of bitterness from food proteins. *Monascus* proteinases and carboxypeptidases could be potentially applied in the field of protein processing in the food industry. Glucoamylases, β-glucosidases, or α-galactosidases may also play a role in creating desirable tastes, increasing antioxidant ability, or reducing nondigestible oligosaccharides during the fermentation of tofuyo.

Tofuyo is an indigenous fermented tofu prepared in Okinawa Prefecture, Japan, using *Monascus* fungi. Fermentation of tofuyo is unique with respect to its soybean proteins, which undergo limited hydrolysis by proteinases in the presence of ethyl alcohol originating from awamori. The unique physical properties of tofuyo are formed during the ripening period. Soybean proteins are digested into amino acids (glutamic and aspartic acids, etc.) and peptides (IFL and WL) by microbial action. These free amino acids contribute to the formation of pleasing tastes and the released peptides also contribute to the physiology (e.g., ACE inhibition) of tofuyo.

A novel fermented foodstuff using both SP-Ca-Gel fabrication and microbial fermentation by *Monascus* fungus was developed in our laboratory. This product has potential nutritional benefits, such as improving the composition of essential amino acids and giving better physiological properties to soybean foods. It is therefore expected that red koji, including *Monascus* hydrolyzing enzymes, has potential benefits for the development of new foods that provide good physiological functionality for health.

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