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Fermented Tofu, Tofuyo

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1. Introduction

Fermented tofu (soybean curd) is widely distributed in East and Southeast Asia. It is known in mainland China and Taiwan as *toufu-ru*, *toufu-ju*, *furu*, *rufu*, *sufu*, *funan*, *fuyu*, *tou-yu*, *tau-zu*, in the Philippines as *tafuri*, in Malaysia as *tau ju*, in Thailand as *tau-fu yee*, and in Vietnam as *chao* or *dau-phu-nyu*. In Japan, fermented tofu, known as *tofuyo*, is found only in Okinawa Prefecture (Shurtleff & Aoyagi, 1975; Su, 1986; Yasuda, 1983b, 1994). Surprisingly, fermented tofu is not found in mainland Japan and Korea, which are located in East Asia. In most Western scientific literature, Chinese fermented tofu has been introduced as *sufu*. However, the term *sufu* is not familiar to most Chinese people, while *furu* or *toufu-ru* seems to be more commonly known. In Japan, fermented tofu has been introduced as “nyufu” (*rufu*) or “funyu” (*furu*) (Ohta, 1965). According to Su (1986), *sufu* is prepared by overgrowing soybean curd with a mold of the genus *Actinomucor*, *Mucor*, or *Rhizopus* and further fermented in a salt-brine/rice-wine mixture. During fermentation, the mold and rice wine mixture imparts additional flavor to the product. *Sufu* is usually comprised of red, pale yellow or white colored blocks (2 to 4 cm² and 1 to 2 cm thick). Commercial products of both white *sufu* (*furu*) and red *sufu* (*rufu*) made in Taiwan are shown in Fig. 1. Pale yellow or white *sufu* are untreated, whereas red *sufu* is colored with red koji which is prepared by growing the mold *Monascus anka* or *Monascus purpureus* on cooked rice. Other additives are frequently incorporated into the brine solution, imparting either additional color or flavor to the various types of *sufu*. *Sufu* is consumed directly as a condiment (for example, as a seasoning for hot breakfast rice: gruel or Chinese bread) or is cooked with vegetable or meats. *Sufu* adds zest to the bland taste of a rice-vegetable diet. These descriptions are very interesting in comparison with the following description of *tofuyo* from Okinawa.

Tofuyo is a fermented tofu indigenous to Okinawa Prefecture, Japan. It is a low-salt vegetable cheese product prepared from tofu by the action of microorganisms. It is nutritionally rich, with high quality of protein, fat, and other nutrients. *Tofuyo* is a creamy cheese-like product with a mild flavor, fine texture, and good taste. Traditionally, *tofuyo* is directly consumed as a dish eaten with *awamori*, a traditional distilled liquor in Okinawa, or as cakes served at teatime for ladies (Fig. 2).

Red *furu* was brought to Okinawa from Fujian, China, and was introduced during the period of the Ryukyu dynasty, around the 18th century. Because the product was brined and had a strong taste, it was initially unpalatable to the Okinawan people in its original form. Therefore, it was re-created using *awamori* instead of salt by cooks in the dynasty for a more palatable taste that was milder in flavor. It was named *tofuyo*. Its other beneficial



Fig. 1. Commercial products of sufu made in Taiwan Left hand side; white sufu (furu), Right hand side; red sufu (rufu)



Fig. 2. Tofuyo and awamori

properties, such as the smooth cheese-like texture, being a valuable source of protein, and improving blood circulation have made tofuyo treasured, not only as a nutritious side dish but also as a health food that has been eaten after illness for centuries. Improvement in

blood circulation is considered to be caused by the ethyl alcohol contained in awamori, which is used as one of the raw materials. During the Ryukyu dynasty, the royal family and high society relished tofuyo as a health food and high-level gourmet food; however, tofuyo was hardly known to commoners (Yasuda, 1983b).

As the secret techniques of tofuyo production have been passed on for generations only in select homes, few people have knowledge of this food. In order to develop tofuyo manufacturing, we have investigated the features of its production and scientifically analyzed its characteristics. The outcomes were transferred to the local industry and the market for tofuyo is now developing.

The scientific aspects of tofuyo have yet to be fully clarified. In this chapter, the history of tofuyo, its production, chemical characteristics and physiologically functional properties are described, as well as the role of the fungus *Monascus* in its production.

2. History of tofuyo

There have been few reports on the history of tofuyo since the one written in Japanese by Yasuda in 1983 (Yasuda, 1983b; 1994b). Shurtleff & Aoyagi introduced the tofuyo that was described in 1983 in their special report on the history of traditional fermented soy foods, written in English (Shurtleff & Aoyagi, 2007). In this section, the history of tofuyo from these literatures is outlined.

2.1 History of fermented tofu

Fermented tofu is considered to have originated in China; however, the exact time and place of its origin remain uncertain. Hong (1985) reported that the first description concerning fermented tofu was found in "*Peng Long Ye Hua*" written by Li Ri-Hua in the Ming dynasty (1368-1644), where it was prepared with molded tofu. Reference to furu appeared in the famous book on Chinese medicinal and herbal materials, "*Pen Ts'ao Kang Mu*" (*Honzo Kou Moku*, in Japanese) published by Li Shih-Chen in 1596. In this book, furu was prepared without the molding procedure. From these old records, it appears that there were two techniques involved in furu production, treatment with and without mold. Although the name rufu appears in the section of animals of this book, it is not fermented tofu, but refers to milk-curd, which is produced from the coagulation of protein in milk under acidic conditions.

During the Ch'ing dynasty (1644-1912), many records on fermented tofu production appeared, indicating that the molding procedure had become mainstream. The making of fermented tofu with the molding procedure is described in the book, "*Shi Xian Hong Mi*" written by Wang Zi-Zhen, in the middle of the Kang-Xi period (1681-1706). Interestingly, the author found that the fermented tofu was made with the "red koji" in this record. From this description, it is strongly suggested that the origin of tofuyo is the fermented tofu described herein. References to rufu often appear in documents of that time. Rufu seemed to be one of the finest products in the district of Jiang Nan, China, and was introduced in a kind of guidebook on local production, "*Jiang Nan Tong Zhi*" (1736). In this record, rufu was not milk-curd, but fermented tofu. It was revealed that the methods of making fermented tofu recorded in "*Xing Yuan Lu*" written by Li Shi-Ting in the middle of the Quianlong period (1757-1776) are similar to present-day methods. Fermented tofu, rufu, was also described in "*Sui Yuan Shi Dan*" written by Yuan Mei (1782), and the white type of rufu (containing

shrimp eggs) or famous production sites, and other aspects, were introduced in this document. From a variety of literature, it can be concluded that the development and spread of fermented tofu occurred in the Ch'ing dynasty, and became popular in the diets of people in mainland China and Taiwan, and continues to be enjoyed even today. As the Chinese fermented tofu spread to other countries in East and Southeast Asia, it was given its own name in each country, as described in the Introduction. From that time to the present, the fermented tofu of each country has been traditionally made in the home or small cottage industries.

2.2 History of tofuyo

Although fermented tofu has never been widely known nor consumed in mainland Japan, it has a long and interesting history in Okinawa Prefecture, where a mellow, delicious product named tofuyo has been enjoyed for nearly 200 years. Okinawa is one of Japan's southern prefectures, and consists of hundreds of the Ryukyu Islands in a chain over 1,000 km long, extending southwest from Kyusyu to Taiwan. The Ryukyu Kingdom existed in this area before the Japanese Meiji Period (1868). Since the islands are located in the center of the East China Sea and are relatively close to Japan, China and Southeast Asia, the Ryukyu Kingdom became a prosperous trading nation. However, four years after the beginning of the Meiji period (1872), the kingdom was officially annexed by Japan.

The earliest known reference to fermented tofu in Japan comes from Osaka. In 1883, Ka Hitsu Jun published the famous book, "*Tofu Hyaku Chin Zokuhen*" (The Sequel to One Hundred Favorite Tofu Recipes). In this book, "red tofu" and the other fermented tofu, "tofu-ji", were introduced. According to the book, how to make the red tofu was a family-secret, and few details on its production were provided. The other description was clearly of Chinese style red furu, because the materials not only included red koji from China, shiro zake (white sake, Chinese distilled liquor named "Bai-Jiu"), *sansho* (Japanese spice, this spice seems to be used instead of chili), but also refer to it as tofu-ji and use the same method of preparation. However, this fermented food subsequently disappeared from and can no longer be found in mainland Japan. On the other hand, because relations between the Kingdom of Ryukyu and China were close at that time, it strongly suggests that furu production methods were brought to Ryukyu from China (probably from Fujang) in cultural exchanges between the two countries at that time. However, there is very little information available on fermented tofu in Ryukyu. Red tofu, fermented tofu, furu or tofuru were not described in the Ryukyu's Old Language Dictionary "*Kongo Kensyu*" (1711), or in the book on the History of Ryukyu "*Ryukyu Koku Yurai Ki*" (1713) edited by the Government of Ryukyu Ohfu.

Red furu was likely introduced to Ryukyu during the late 1700s. Since the product was brined and had a strong taste and smell, it was not immediately accepted in its original form by the people. Therefore, it has been re-created using awamori. Namely, the processes of preparing molded tofu and fermenting the mold-overgrown curd in salt-brine were eliminated by cooks in the dynasty, resulting in a more palatable taste that was milder in flavor. Thereafter, they were able to decrease the amount of salt in the original recipe, and increase the shelf life without salt. Thus, an elegant, mellow and delicious fermented food, dubbed *tofuyo*, was newly created.

The earliest known indirect reference was in the book "*Account of a Voyage of Discovery to the West Corea and the Great Loo-Choo Island*" written by Basil Hall in 1818. In this book, "Corea"

refers to “Korea”. Interestingly, Ryukyu was called the great Loo-Choo islands on the old map at that time. The Englishman Hall and his party visited Naha harbor in 1816, on route from China, where they were entertained by the Government of Ryukyu Kingdom and served the local cuisine. They were served “hard boiled eggs, cut into slices, the outside of the white being colored red...sackee (the liquor; awamori)...and something like cheese.” The red color is thought to be the red koji-pigments produced by the genus *Monascus*, and the cheese-like food may be the fermented tofu, tofuyo, which is often made with red koji.

The earliest known direct reference to tofuyo and red koji was in “*Gozen Hon Sou*” written by the physician Tokashiki Tsukan Peichin in 1832. Peichin is a term for a high-ranking officer. In this book, foods, and medicinal and herbal materials in Okinawa were described in the context of medicine and pharmacy. He describes that “Tofuyo has a delicious flavor and is good for the stomach. It makes eating a pleasure and is good for various types of sickness”. From these descriptions, it was considered that tofuyo was consumed by the royal family and the upper classes as a medicinal food and as a side dish. This group established the methods of making tofuyo, but the secret of its preparation has been a stubbornly guarded family-secret. In fact, there were no references to it from 1832 until 1938. It was not mentioned in the accounts of visits to Ryukyu by the English captain Frederick W. Beechey (1831) and the famous American Commander Mathew C. Perry (1857), nor in any documents from the Meiji period (1868-1912). This may explain why its use did not spread and why so few references have been made to it. During the early Showa period (1926-1989), home-made tofuyo was popular only among the upper classes in the cities of Shuri (the former capital) and Naha (the new capital) in Okinawa. However, its production was not clarified at all. Ladies ate it with tea, while men took it with awamori. Typically, a small cube was placed on a small dish and eaten with chopsticks or a tooth-pick (Fig. 2). The product was never widely known by regular people because it was only rarely made at home. In 1938, Shojuun Danshaku, who was a descendant of the Ryukyu Royal Family and a well-known connoisseur, wrote an article entitled “In Praise of Tofu” in the magazine, *Monthly Ryukyu*. He noted that tofuyo is one of the best rare and tasty foods in the world, if not the best.

After World War II, the special family-secret of making tofuyo techniques was, for many years, inherited only at select homes; few people are aware of the food. As it is a unique low-salt soybean cheese, its industrialization has been greatly expected. However, in order to develop its production, it is necessary to clarify features of the food making that depend upon intuition and experience, and to establish the manufacturing technology in proportion to mass production. Thus, we revealed its features, and scientifically established the basic technology involved (Yasuda, 1990). The outcome was transferred to the local industry during the mid 1980s. Subsequently, tofuyo that reflects the gastronomic culture in the age of the Ryukyu dynasty has been marketed. Furthermore, the red type of tofuyo is popular, and its production been greatly developing. Nowadays, attention is being paid to this food as a health food that is cholesterol free, and is a low-salt fermented soybean cheese. Therefore, it would also be suitable for western people because it could be utilized in almost the same way as cheese.

3. Production of tofuyo

Three steps are normally involved in making tofuyo: preparing tofu and dehydrated tofucubes (Yasuda & Hokama, 1984a; Yasuda et al., 1992), making koji (Yasuda et al., 1981;

1983a), and soaking and ripening (Yasuda et al., 1993; 1994a, 1995). A flowchart for tofuyo preparation is shown in Fig. 3.



Fig. 3. Process for making tofuyo

3.1 Processes for making tofu and dehydrated tofu-cubes

To make tofu, soybeans were first washed, soaked in water, and then ground with water. The ground mixture was heated and then strained through a coarse cloth to separate the soybean milk from the insoluble residues. The soybean milk was then heated, and a coagulant was added to coagulate the protein. The coagulated milk was then transferred to a cloth lined wooden box, and pressed with a weight on top so as to remove the whey. In this way, tofu was formed. In general, tofu has a bland taste and high water content. The water content of regular tofu in Japan is 86%. It is suggested that the texture of tofu affects the quality of tofuyo. It is preferable that the water content of tofu used for making tofuyo be lower than that of regular tofu. Hardness of the tofu was from 10 to 11×10^3 Pa and the moisture was approximately 76%. The product has a suitable elasticity and fine texture (Yasuda & Hokama, 1984a).

To prepare dehydrated tofu, the tofu was cut into 2-cm cubes, and the cubes were dehydrated at room temperature for between 24 to 48 h. The dehydrated cubes were washed with awamori, and subsequently used as the material for tofuyo. The physical properties of dehydrated tofu-cubes are also important factors in determining the quality of tofuyo. If the dehydrated tofu-cubes are too soft, they will fall apart during maturation. On

the other hand, if they are too hard, it will be difficult to impart a good texture and taste to the tofuyo.

In order to establish a suitable texture of the cubes for making tofuyo, they were prepared using tofu with different values of hardness. It was found that the product of the best quality showed a value of 17.8×10^3 Pa and was obtained using tofu-cubes dehydrated to 28.9×10^3 Pa. This product was bland, smooth, and had a fine texture, like soft cheese. Thus, tofuyo with a value between 14 to 18×10^3 Pa was preferred. The preferred hardness of the dehydrated cubes was between 20 to 30×10^3 Pa (Yasuda & Hokama, 1984a). The relationship between tofuyo hardness and dehydration method is shown in Table 1. Tofuyo was made with dehydrated tofu-cubes prepared following methods and having the same value of hardness. The hardness of the product prepared at room temperature for between 24 to 48 h had a value of 17.5×10^3 Pa, agreeing with the preferred value as mentioned above. This product had a bland taste. However, the tofuyo prepared with tofu-cubes dehydrated by mechanical methods, such as by warm-wind, in the oven, or under vacuum, were not palatable and did not progress in maturation. Therefore, the best quality of tofuyo was obtained with tofu-cubes (hardness: from 20 to 30×10^3 Pa) that were gradually dehydrated at room temperature, and had a hardness of 14 to 18×10^3 Pa.

Method	Hardness (10^3 Pa)	
	Dehydrated tofu-cube	Tofuyo
A	28.6	27.8
B	25.9	25.0
C	24.9	22.4
D	26.5	17.5

Tofu-cubes were dehydrated in warm wind-drying box (Method A), oven-drying box (Method B) or vacuum-drying box (Method C) at 50°C for 4-6 h. Tofu-cubes were also dehydrated at room temperature (Method D) for 24-48 h. Tofuyo was prepared by these dehydrated tofu-cubes.

Table 1. Relationship between hardness of tofuyo and dehydration methods

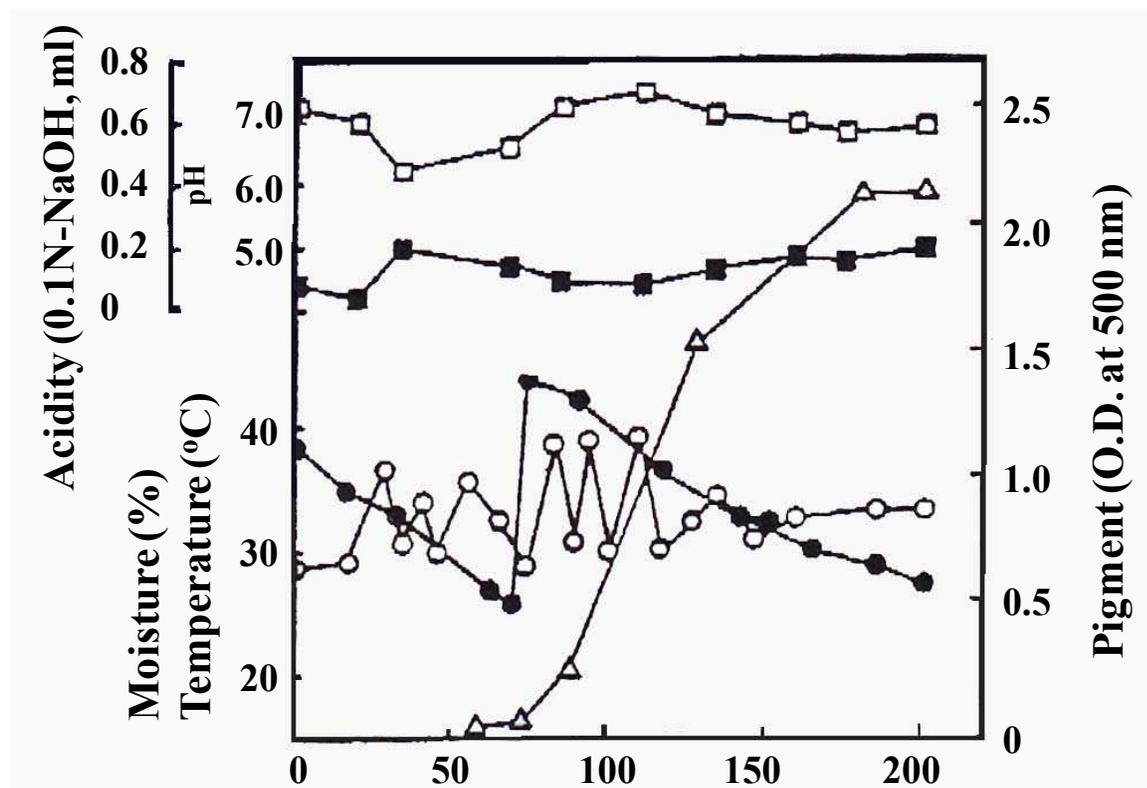
During dehydration, microorganisms such as bacteria grew on the surface of the tofu-cubes. The microorganisms may develop enzyme systems having higher proteolytic and lipolytic activities, because the bacteria grew on tofu which was protein and lipid rich. Furthermore, the microorganisms may play an important role in "pre-fermentation", which affects the quality of tofuyo by partially degrading the soybean protein of tofu. We selected *Bacillus* sp. TYO-67, which has high proteinase activity, for examining the above hypothesis by the use of a pure culture (Yasuda et al., 1992). The protein content of the cubes inoculated with the bacterium during dehydration was lower than that of those un-inoculated. Thus, it was confirmed that the former was more degraded than the latter. The ripening time of tofuyo prepared using the dehydrated tofu-cubes that were inoculated with the isolated bacterium

was shorter than that prepared with un-inoculated cubes. Therefore, the tofu-cube dehydration process at room temperature was characterized as being “pre-fermented” by bacteria (Yasuda et al., 1992). Later, the microorganism was identified and named as *Bacillus pumilus* TYO-67. An alkaline serine proteinase produced by this strain was purified and characterized (Yasuda et al., 1999; Aoyama et al., 2000a; 2000b).

3.2 Processes for making koji

Rice koji was prepared from polished rice, since it was essential that the mold quickly penetrate the rice kernel. Polished rice was soaked in water over night and the excess water was drained off. The swollen rice was cooked with steam at atmospheric pressure for 60 min, cooled to 35°C, and inoculated with a starter of the genus *Monascus* or *Aspergillus oryzae*. The incubated rice was placed in wooden trays. During the development of the koji, temperature, moisture, and aeration are extremely important factors and must be rigidly controlled. After incubation at 32°C for 7 days, the red koji, prepared using the genus *Monascus*, was harvested (Yasuda et al., 1983a). After incubation at 32°C for nearly 48 h, the yellow koji prepared using *A. oryzae* was harvested (Yasuda et al., 1981).

The data concerning the changes in temperature, moisture, pigment, pH, and acidity of the koji during cultivation are shown in Fig. 4. After 40 h of inoculation, the temperature of the



Cultivation was carried out at 32°C for the time indicated. Steamed rice was inoculated with seed containing *Monascus purpureus*, and cultured in an incubation box. Koji samples were taken for analysis at the indicated time.

-○-; Temperature, -●-; Moisture, -△-; Pigment, -□-; pH, -■-; Acidity

Fig. 4. Changes in temperature, moisture, pigment, pH, and acidity of red koji during cultivation

material increased to around 40°C. The temperature was controlled by hand mixing. The level of moisture decreased during incubation. After 50 h of incubation, the level of moisture was reduced to 30% or less, and the koji materials were moistened by water spray. These treatments resulted in marked enhancement of microbial growth and koji-pigment formation. The pH and acidity of the koji were not greatly affected during incubation. Although it is not shown in this figure, proteinase, α -amylase and glucoamylase activities were maximal at 150 h (Yasuda et al., 1983a). Thus, the optimum incubation period of the koji production was 150 h after the inoculation of the seed culture.

Koji is an important material for making tofuyo. It is a source of enzymes for converting starch into fermentable sugars and proteins into peptides and amino acids. The characteristics of suitable koji for making tofuyo are as follows. One is that it has high enzymatic activity, such as for proteinase, α -amylase and glucoamylase. Another is the provision of a good flavor and taste to the product. In order to enhance red koji enzyme activity, we examined the effect of steaming methods and rice varieties on the enzyme and pigment production (Table 2). Enzyme activities of the varieties of koji were indicated against relative activity of the koji prepared using non-glutinous rice by steaming under atmospheric pressure. *Monascus*-pigment was also indicated. In the system of steaming under atmospheric pressure, enzyme and pigment production of the koji prepared with glutinous rice was more abundant than that of non-glutinous rice. Traditionally, glutinous rice is used as a raw material for koji. α -Amylase and proteinase of the koji prepared with broken rice was the same as non-glutinous rice, but glucoamylase and pigment showed low production levels. However, with the steaming under high-pressure system, enzyme and pigment production of the koji prepared with non-glutinous rice was, in general, more abundant than that of broken rice from Thailand or glutinous rice. Thus, it was found that the highest production of enzymes and pigment in koji was obtained by employing autoclaved non-glutinous rice.

	Relative activity (%)					
	Steaming under atmospheric pressure			Steaming under pressure		
	A	B	C	A	B	C
α-Amylase	100	94	106	124	108	84
Glucoamylase	100	74	124	140	139	96
Proteinase	100	102	123	167	179	94
Pigment	100	68	138	417	183	61

A non-glutinous rice. B broken rice. C glutinous rice.

Table 2. Effect of steaming methods and varieties of rice on the production of enzyme and pigment in red koji

High enzyme activities in yellow koji by *A. oryzae* were also obtained in the same manner as in the case of red koji described above (Yasuda et al., 1981).

3.3 Soaking and ripening

The last step in making tofuyo was soaking and ripening. To make the moromi (soaking mixture; fermentation broth), red koji and/or yellow koji, awamori (43% of ethyl alcohol concentration) and a small amount of salt were mixed, and kept at 4°C for 24-48 h. The mixture was then ground and the result is known as moromi. The dehydrated cubes were immersed in the moromi and allowed to ripen at 25 to 30°C for 5 months. The ethyl alcohol concentration in the moromi was around 20% and the pH was from 5.6 to 6.0. It is unique and characteristic that the ripening was carried out under the presence of a high alcohol concentration. The quality of the product was affected by the variety of koji and the liquor in the moromi. According to our investigations, the product with red koji or the mixed koji with red koji and yellow koji was superior to that of yellow koji with respect to color and flavor (Yasuda et al., 1995). Awamori was the most important liquor for making tofuyo. Other liquor, such as whisky and shochu, which is distilled liquor from sweet potato or grains in Japan, were not suitable for the flavor of the product.

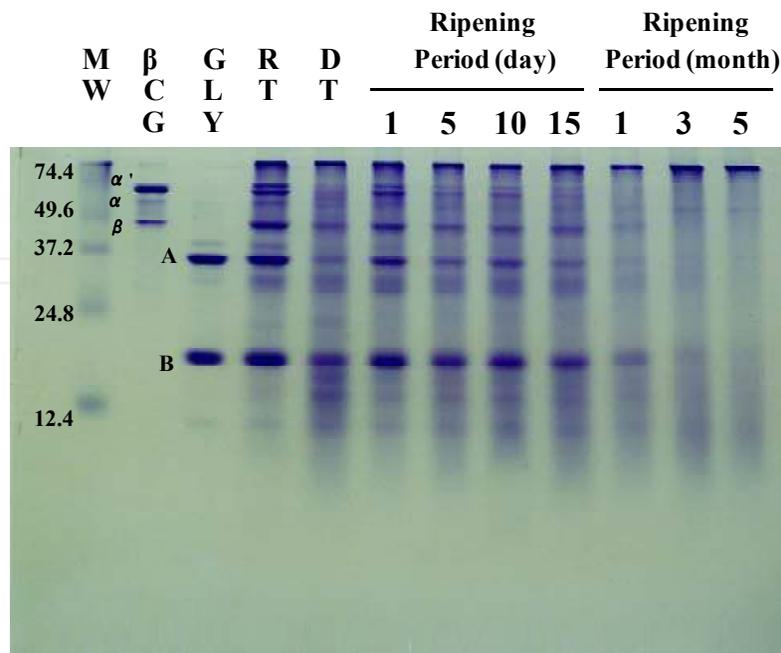
4. Characterization of tofuyo

4.1 Chemical changes in tofuyo during the ripening period

Although we have compared the characteristics of tofuyo made with red koji, yellow koji and mixed koji (Yasuda et al., 1993; 1994a; 1995; 1996), here the chemical characteristics of tofuyo prepared with the red koji is mainly described (Yasuda et al., 1993a). Chemical changes in tofuyo during the ripening period were examined. Crude protein and crude fat contents of tofuyo decreased, but reducing sugar content increased during the ripening period. Crude fiber was not detected. Sodium chloride content of the product was considerably constant (around 3%) throughout the ripening period. The sodium chloride content of tofuyo was lower than that of Chinese sufu (Wang & Hesselstine, 1970). Therefore, tofuyo is a low-salt fermented tofu.

Proteinase is an enzyme that plays a pivotal role in the maturation of tofuyo. Changes in proteinase activity in the moromi due to the koji during the ripening period were examined. Proteinase activity decreased during the ripening period due to the effect of the high alcohol concentration (20%; v/v) in moromi. Thus, it was suggested that the proteinase activity was controlled by the presence of ethyl alcohol originating in awamori, and the ripening might be gradually carried out. Therefore, soybean proteins may be degraded to a limited extent, and may also result in the formation of good physical properties, such as a smooth texture and optimal viscoelasticity.

In order to confirm its biochemical degradation, digestion of soybean protein during fermentation was examined by slab polyacrylamide gel electrophoresis (Fig. 5). Some bands originating from polypeptides of soybean globulin (such as the α' -, α -, and β -subunits of β -conglycinin, and the acidic subunit in glycinin) in the water-insoluble fraction of tofuyo disappeared after the 3-month ripening in the presence of ethyl alcohol from awamori, whereas the basic subunit in glycinin was recognized as a thin band. Furthermore, those of the other polypeptides (*Mr.* 30-32 kDa, 10-15 kDa) were also recognized. These polypeptides and the remaining glycinin, which has strong gel-forming ability, are closely related with the body of tofuyo (Yasuda et al., 1993; 1994a; 1995). Therefore, it is considered



MW : Standard proteins (MW-marker, kDa)
 β CG : β -Conglycinin, GLY : Glycinin
RT : Raw Tofu, DT : Dehydrated Tofu
 α' , α , β : α' , α and β -subunits in β -Conglycinin
A, B : Acidic and Basic subunits in Glycinin

Fig. 5. SDS-PAGE pattern of the water-insoluble fraction of tofuyo prepared by red koji at various ripening stages

that the glycinin greatly contributes to preserving the desired texture of tofuyo (Yasuda et al., 1996), and is partially converted to peptides and amino acids. On the other hand, some polypeptides in the water-soluble fraction of tofuyo, with molecular masses of 31, 25, 23, 21, and 10-12 kDa, were leveled off by proteinases during the fermentation (0-3 months). The nitrogen components of tofuyo during the ripening period were examined. The ratio of water-soluble nitrogen to the total nitrogen (called the protein solubility ratio or ripening ratio) reached 36.3% after the 3-month ripening. The ratio of 4%-trichloroacetic-acid soluble nitrogen to the total nitrogen reached 34.0% after the 3-month ripening. Thus, 94% of non-protein-nitrogen compounds, such as amino acids and peptides, in the water-soluble fraction of the product were converted from soybean protein by proteinases and peptidases during the fermentation. Free amino acids, such as glutamic acid and aspartic acid, may contribute to the formation of flavor, and released peptides may contribute to the physiological function of tofuyo. These phenomena were also observed in the products prepared using yellow koji and mixed koji (Yasuda et al., 1994a; 1995; Katsura, 1996).

Both glucoamylase and α -amylase had high activities throughout the ripening period. Starches in koji were mostly converted to glucose by these amylases, and this can provide a desirable taste to the tofuyo. These phenomena were also observed in the products prepared using yellow koji and the mixed koji (Yasuda et al., 1994a; 1995).

Amino acids, peptides, and other taste-associated compounds, including saccharides, in tofuyo are discussed in more depth in other sections (4.3 and 4.4). Moreover, soybean lipids were digested to some extent into fatty acids by lipases in the moromi. The added awamori

can react with these fatty acids chemically or enzymatically to form esters, which may provide the pleasant aroma of tofuyo (Yasuda, 2010).

4.2 Physical changes in tofuyo during the ripening period

Tofuyo is a unique fermented soybean protein food that possesses a cream-cheese-like texture. Its texture is very different from that of the tofu that is the raw material for tofuyo. Therefore, textural changes in tofuyo during fermentation were investigated (Yasuda et al., 1996). 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 during the ripening period. Interestingly, creep analysis of tofuyo, ripened for 3 months, showed that each viscoelasticity coefficient was similar to that of commercial cream cheese or soft-type processed cheese. Thus, it was scientifically confirmed that tofuyo has a cheese-like texture.

In order to understand the ripening of tofuyo, changes in the microstructure of the product were also examined. Electron microscope observation revealed that fat globules seemed to bind with proteins. Moreover, the results of electron microscopy showed that the structure of the soybean protein forming the body of tofuyo changed from fibriform to small particles, which connected to each other during the ripening period (Fig. 6). These phenomena were in good agreement with the results of slab polyacrylamide gel electrophoresis, as shown in Fig. 5. It was thus considered that these phenomena contributed to maintaining the shape and unique texture of this product.

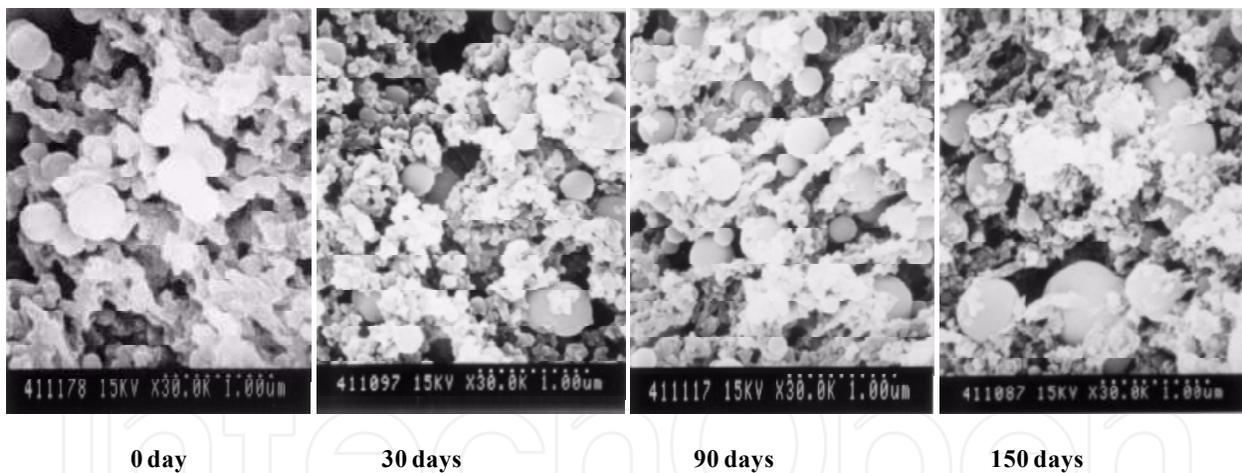


Fig. 6. Changes in form of soybean protein particles of tofuyo prepared by red koji during fermentation observed by a scanning electron microscopy

4.3 The flavor compounds in tofuyo

The chemical composition of tofuyo, after 3-month ripening, was as follows: 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). Thus, tofuyo is a fermented low-salt soybean food that is rich in proteins, lipids, and carbohydrates.

Amino acids greatly contribute to flavor formation. Changes in the free amino acids of tofuyo, prepared using red koji, during the ripening period are shown in Table 3. The data clearly indicate an increase in and the involvement of free amino acids after fermentation,

especially glutamic acid, alanine, aspartic acid, glycine and serine, which, after a 3-month ripening, may contribute to the pleasant taste. It is well known that glutamic acid and aspartic acid contribute to the pleasant umami taste or savory enhancement in foods. Phenylalanine is also rich in tofuyo. Recently, Lioe & Yasuda et al. (2004; 2007) carried out chemical and sensory studies on savory fractions obtained from soy sauces, and the presence of phenylalanine, as well as NaCl with glutamic acid, were found in the fractions. A potential synergistic effect of umami among free glutamic acid, salt and phenylalanine (the so called bitter amino acid) were observed (Lioe et al., 2010). Furthermore, this phenomenon of umami or savory enhancement by subthreshold aromatic amino acids in the soy sauce system has been established by the model experiment system (Lioe et al., 2005). Recently, we confirmed that l-phenylalanine also plays an important role in umami taste enhancement of glutamic acid in the presence of NaCl, both in tofuyo and soy sauce.

Amino acids (mg/100 g)	Raw tofu	Dehydrated tofu	Ripening period (month)			
			0.5	1	3	5
Aspartic acid	15	29	317	511	673	773
Threonine	23	26	33	78	143	148
Serine	10	53	78	209	410	481
Glutamic acid	20	56	283	609	864	881
Proline	9	49	85	83	86	147
Glycine	14	67	73	302	422	543
Alanine	24	90	219	414	676	702
Cysteine	0	0	3	5	6	9
Valine	15	19	38	88	145	124
Methionine	0	4	23	60	51	45
Isoleucine	3	15	40	85	126	137
Leucine	11	65	117	246	403	477
Tyrosine	0	10	51	114	153	150
Phenylalanine	14	50	65	128	200	173
Histidine	7	5	17	29	34	39
Lysine	8	4	13	78	152	216
Arginine	1	2	44	137	176	109
Total amino acids	174	544	1499	3176	4720	5154

Table 3. Changes in free amino acids of tofuyo prepared by red koji during the ripening period

Glucose is one of the major compounds, besides amino acids, involved in the taste component of tofuyo, with the other compounds being organic acids (e.g., malic, citric, lactic, and acetic acids), nucleotides and NaCl. It is strongly suggested that the desirable taste of tofuyo is formed by interactions between these components.

4.4 Physiologically functional properties of tofuyo

4.4.1 Angiotensin I-converting enzyme inhibitory peptides in tofuyo

Lifestyle-related diseases are a serious social problem, along with the current changes in peoples' dietary habits. Recently, much attention has been paid to the role of health-promoting foods in disease prevention. Hypertension is a serious risk factor for cardiovascular disease, and it is known to have high morbidity. It is well known that angiotensin I-converting enzyme (ACE) inhibitors have an excellent effect on hypertension. ACE is a dipeptidyl carboxypeptidase associated with the regulation of blood pressure. It converts angiotensin I to the potent pressor peptide, angiotensin II, and also degrades the depressor peptide bradykinin (Yang et al., 1971). ACE inhibitors from various foods have been recently studied in terms of their ability to prevent and alleviate hypertension. Physiologically functional foods enriched with ACE inhibitors, such as tripeptide (Val-Pro-Pro, Ile-Pro-Pro) from sour milk (Takano, 1998), dipeptide (Val-Tyr) from sardine muscle hydrolysate (Kawasaki et al., 2000) and dodecapeptide (Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys) from casein hydrolysate (Maruyama et al., 1987), are used as supplements to improve hypertension.

As it was of interest whether ACE inhibitors were present in tofuyo or not, ACE inhibitory activities were examined in our laboratory (Kuba et al., 2003). As a result, inhibitory activity was found in tofuyo extracts, with an IC_{50} value of 1.77 mg/ml. The IC_{50} value shows the concentration of inhibitor needed to inhibit the ACE reactions to 50%. That is to say, the ACE inhibitory activity is higher as the IC_{50} value is lower. Two ACE inhibitors were isolated to homogeneity from the extract by absorption and gel filtration column chromatographies, and by reverse-phase high-performance liquid chromatography (HPLC). Since both substances reacted with 2,4,6-trinitrobenzenesulfonic acid sodium salts, which bind only to primary amines and the sulfhydryl group, they both appeared to be peptide-like substances. The amino acid sequences of the substances, determined by Edman degradation, were Ile-Phe-Leu (IFL; IC_{50} value, 44.8 μ M) and Trp-Leu (WL; IC_{50} value, 29.9 μ M). A computer search of the SWISS-PROT protein sequence data base showed that the amino acid sequence of IFL was present in the primary structures of the α - and β -subunits of β -conglycinin, whereas WL was present in the primary structures of glycinin. Since the α' -, α -, and β -subunits in β -conglycinin and the acidic subunit in glycinin were degraded to low-molecular-weight elements during 3-month tofuyo fermentation, it is likely that IFL was liberated from β -conglycinin by proteinases produced by *M. purpureus* and/or *A. oryzae* used in the fermentation process. Although the basic subunit in glycinin cannot be easily degraded by these enzymes, as compared with each subunit in β -conglycinin or the acidic subunit in glycinin, WL might have been liberated from the basic subunit during long-term fermentation (3 months).

In order to examine the resistance of both IFL and WL peptides in tofuyo to digestion *in vivo*, changes in their IC_{50} values before and after treatment with gastrointestinal proteinases *in vitro* were examined. As shown in Table 4, the ACE inhibitory activities of both peptides were completely preserved after pepsin treatment. Although the ACE-inhibitory activity of WL was also completely preserved after pepsin, chymotrypsin, or trypsin treatment, that of IFL decreased to 62% and 75% of the original value following chymotrypsin and trypsin treatments, respectively. In spite of the successive digestion with pepsin, chymotrypsin and trypsin, the inhibitory activity of IFL was found to be 38%, and that of WL was 29%, of the original value. It has been reported that the IC_{50} values of "Ile-Phe" (Cheung et al., 1980,

Seki et al., 1995) and “Phe-Leu” (Eto et al., 1998), which are parts of Ile-Phe-Leu, were 930 and 16 μM , respectively. Thus, IFL obtained from tofuyo is likely to preserve its activity until it is degraded to its individual amino acids.

Digestion	IC ₅₀ ($\mu\text{g/ml}$)	
	IFL	WL
None	18	10
Pepsin	18	10
Chymotrypsin	29	11
Trypsin	24	10
Pepsin → chymotrypsin and trypsin	47	35

Table 4. Digestive stability of IFL and WL toward ACE inhibition

It is well known that di- and tripeptides are more rapidly absorbed and reach a higher concentration in the blood than individual amino acids (Claff et al., 1968; Chun et al., 1996). Short peptides (mean residue length of 3.2) in a soybean hydrolysate were more rapidly absorbed than the long ones (mean residue length of 5.2) when using a rat intestinal everted sac (Chun et al., 1996). Although it has not been demonstrated in the case of tofuyo, IFL and WL isolated from tofuyo are expected to be easily absorbed and to contribute to the antihypertensive effect via a transport system *in vivo*.

4.4.2 Antihypertensive and hypocholesterolemic effects of tofuyo in spontaneously hypertensive rats

Although ACE inhibitory activity was confirmed in tofuyo *in vitro*, the antihypertensive activity and other physiological functions of tofuyo *in vivo* remain to be clarified. We investigated the antihypertensive effects of tofuyo *in vivo* (Kuba et al., 2004). Four-week-old male spontaneously hypertensive rats (SHR) were fed a commercial diet for 4 weeks. The rats were housed individually at 25°C and 70% humidity under a 12-h light-dark cycle. At 8 weeks of age, the rats were divided into a control group and tofuyo group. The chemical composition, such as protein, carbohydrate, lipid, sodium chloride, and vitamins, of each diet was adjusted. Each group was fed a different diet for 6 weeks. The tofuyo group showed a similar growth rate to that of the control group. Diet intake during the experimental period did not differ greatly between two groups. The systolic blood pressure (SBP) of rats gradually increased with body weight. At 11 and 12 weeks of age, the SBP of rats in the tofuyo group tended to be lower than that in the control group, and at 13 weeks of age, the SBP of the tofuyo group (199.2 ± 4.4 mmHg) was significantly lower than that of the control (207.6 ± 4.4 mmHg). Several studies have examined the antihypertensive effect of ACE inhibitors in SHR by long-term feeding. The SBP of SHR fed sour milk (Nakamura et

al., 1996) and chicken essence hydrolyzate (Chen et al., 2002) had decreased 19 mmHg at 23 weeks of age and 26 mmHg at 24 weeks of age, respectively. The experimental period of our study seems to be shorter than those reports, and with a smaller decrease in SBP.

After feeding the experimental diets (for 14 weeks), ACE activities of various tissues were examined. The ACE activity of kidney was significantly lower in the tofuyo group (2.6 ± 0.8 mU/mg of protein) than that in the control group (4.9 ± 1.8 mU/mg of protein). It is known that persistent ACE inhibition in peripheral tissues, especially in the vascular wall and kidney, might be important for the prolonged antihypertensive effects of ACE inhibitors (Unger et al., 1985). As the elevation in SBP had been reversed by 14 weeks age, the ACE inhibition in kidney of the tofuyo group might be related to this antihypertensive effect. The authors also confirmed the presence of γ -amino butyric acid in tofuyo extracts. Thus, tofuyo is expected to have antihypertensive effects. Further investigations are required to clarify the antihypertensive mechanism of tofuyo and the dominant substances involved in its effect.

Serum lipids and total cholesterol in the tofuyo group were significantly lower than those in the control group. High density lipoprotein (HDL) cholesterol was significantly decreased in the tofuyo group. However, the ratio of HDL to total cholesterol in the tofuyo group tended to be higher than that in the control group. Triglycerol and phospholipid in the tofuyo group tended to be lower than that in the control group. These results indicate that tofuyo had hypocholesterolemic activity in serum. In addition, there were no differences in body weight and tissue weights between the groups, and no macroscopic liver lesions, such as fatty liver, were observed in either group. Thus, the hypocholesterolemic effect of tofuyo was probably not due to accumulation of lipids in the liver (Kuba et al., 2004). Inoue et al. (2006) also observed the influence of tofuyo on serum composition in high-fat-fed mice. Triglycerol values of the tofuyo group fed a high-fat diet plus tofuyo decreased by 1/2, while those of the HDL-cholesterol group increased over 2.6-fold as compared to the control group (fed a high-fat diet without tofuyo). From these results, it is expected that tofuyo contributed to the improved lipid metabolism in the mice.

4.4.3 Effect of tofuyo on erythrocyte deformability in high-fat-fed mice and other bioactivities

Inoue et al. (2006) examined the influence of tofuyo on erythrocyte deformability in high-fat-fed mice. After ICR mice were fed a high-fat diet (HFF), tofuyo paste was given to the animal over a 3-week period. In this experiment, "HFF + physiological saline solution" or "normal diet + physiological saline solution" were used as control groups. After 3 weeks, erythrocyte deformability was measured. The group given "HFF + tofuyo" showed a higher erythrocyte deformability value (0.532 ± 0.04 at 30 Pa) than the control group given "HFF + physiological saline solution" (0.498 ± 0.01). On the other hand, this value was almost similar to that of the control group given "normal diet + physiological saline solution". Thus, tofuyo might contribute to the improvement in erythrocyte deformability.

Antioxidative activity and DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity were found in the water-soluble fraction of tofuyo, which we are now purifying for identification of the compounds. On the other hand, we confirmed the presence of isoflavones that have DPPH radical scavenging activity in 70% ethyl alcohol soluble fractions of tofuyo. Further studies are needed to identify the chemical compounds that showed antioxidative activity and to clarify the physiological effects *in vivo*.

It is suggested that other interesting physiologically bioactive compounds are present in tofuyo. Of note are the microbial metabolites formed during fermentation, such as bioactive peptides and oligosaccharides, as well as bioactive substances originating in soybean, such as isoflavones, saponins, soybean proteins, and essential fatty acids.

5. Microbial biochemistry in tofuyo production

Microorganisms commonly used to the manufacturing of tofuyo belong to the genera *Bacillus*, *Monascus*, and *Aspergillus*. The genus *Bacillus* grows on the surface of tofu-cubes during the dehydration process, and plays an important role in the pre-fermentation of tofuyo, as described in section 3.1. The microorganisms involved in the main fermentation are molds. Mold belonging to the genus *Monascus* produces a red color, and is known as the *Redkoji* mold.

The mold *Monascus* was inoculated onto steamed rice, resulting in the production of red molded rice called red koji or *Beni koji*. This red koji has been historically used for many years in the fermentative production of red sufu, red rice wine and for Chinese medicine. This koji has also been traditionally used for tofuyo production and as a colorant used in the cooking of celebration foods, such as red cooked rice, red colored boiled eggs, red squid, red kamaboko (fish cake), and so on, of Okinawa Prefecture, Japan (Yasuda, 1983b). *A. oryzae* is a very important industrial microorganism for the production of miso paste and Japanese sake, for example. The fungal proteinases, peptidases, amylases and lipases play important roles in tofuyo fermentation. Especially, proteinases are very important enzymes for the maturation of the product (Yasuda & Sakaguchi, 1998; Yasuda, 2004).

5.1 Characterization and application of aspartic proteinases from *Monascus* fungus

Proteinases cleave peptide bonds at internal points within proteins/peptides to produce peptide fragments. They are classified based on their catalytic mechanisms as (1) serine proteinases, (2) cysteine proteinases, (3) aspartic proteinases, and (4) metallo proteinases. Although proteinases produced by the genus *Monascus* are important enzymes for tofuyo production, its enzymatic properties have not yet been fully clarified. Tsai et al. (1978) purified acid proteinases from *M. kaoliang*, and we purified aspartic proteinases from *M. sp.* No. 3403 (*M. purpureus*) (Yasuda et al., 1984b) and *M. pilosus* (Lakshman et al., 2010) as homogenous preparations using SDS-polyacrylamide gel electrophoresis. The molecular mass of the enzyme from *M. purpureus* was estimated to be 40 kDa by gel-filtration and 43 kDa by sedimentation method; therefore, it was determined to be a monomeric structure. The enzyme was active in acidic regions, with the maximum at around pH 3.0. The inhibition of pepstatin A was competitive with Hammarsten milk casein (The K_i value was 26 nM). On the other hand, two acid proteinases, MpiAP1 and MpiAP2, were purified to homogeneity from *M. pilosus*. Both purified enzymes were monomeric structures, with molecular masses of around 43 and 58 kDa, respectively. The former was an acidic non-glycoprotein, whereas the later was an acidic glycoprotein with 27% carbohydrate content. Although amino acid sequence analysis of both enzymes (MpiAP1 and MpiAP2), 20 amino acids in length, showed over 90% similarity, their amino-terminal amino acids differed from each other. Both enzymes were optimally active at 55°C and pH 2.5-3.0 against casein or human hemoglobin. The $T_{1/2}$ values of MpiAP1 and MpiAP2 were 65 and 70°C, respectively. Both enzymes were completely inhibited by pepstatin A. Milk casein and

hemoglobin were good substrates for these enzymes. Eleven cleavages were detected using the oxidised insulin B-chain as a peptide for the proteolytic specificity test of MpiAP1, while seven cleavages were detected for MpiAP2.

Degradation of soybean protein by *Monascus*-proteinase was investigated in order to reveal the role of the enzyme in the process of tofuyo ripening (Yasuda & Sakaguchi, 1998). It was found that the digestion of soybean protein by this enzyme progressed as follows: initially the α' -, α - and β -subunits in β -conglycinin, and then the acidic subunits in glycinin, were degraded. However, the basic subunit of glycinin still remained, and some polypeptide bands (around 10 kDa) were formed during the enzyme reaction. It is considered that the difficulty in degrading the basic subunit depends upon the substrate specificity of the enzyme. The degradation rate of soybean protein by this enzyme was affected by the ethyl alcohol concentration in the reaction mixtures. Namely, *Monascus*-proteinase gradually degraded the tofu protein (especially each subunit in β -conglycinin and the acidic subunit in glycinin) in the presence of ethyl alcohol in the moromi and formed polypeptides (around 10 kDa), peptides, and amino acids during the ripening. However, the enzyme could hardly degrade the basic subunit in glycinin, as shown in Fig. 5, section 4.1. Thus, it was concluded that *Monascus*-proteinase is a key enzyme for tofuyo ripening.

Proteinases, such as pepsin, chymotrypsin and trypsin, are frequently used in protein hydrolysis to obtain ACE inhibitory peptides. Microbial alkaline proteinases are also utilized in the production of ACE inhibitors from food proteins (Matsufuji et al., 1994; Matsui, 2003); however, there are few reports on the use of microbial acid proteinases for the production of ACE inhibitors. We investigated the production of ACE inhibitory peptides from soybean protein using *Monascus*-acid proteinase, prepared in our laboratory. Soybean proteins, β -conglycinin and glycinin were hydrolysed by the acid proteinase (Kuba et al., 2005). The degree of hydrolysis and ACE inhibitory activities increased with increasing proteolysis time. After 10 h of incubation, the IC_{50} values of β -conglycinin and glycinin hydrolysates were determined using ACE from pig lung as 0.126 mg/ml and 0.148 mg/ml, respectively. Four ACE inhibitory peptides were isolated; those from β -conglycinin hydrolysis were identified as LAIPPVNK and LPHF, and those from glycinin hydrolysis as SPYP and WL. It should be noted that WL has been purified from tofuyo extract described in section 4.4.1. Therefore, the result from this study suggested that ACE inhibitory peptides in tofuyo might have been derived from soybean protein by the action of *M. purpureus* acid proteinase. The inhibitory activity of SPYP markedly increased after successive digestion by pepsin, chymotrypsin and trypsin *in vitro*. The activity of the split peptide SPY, with tyrosine at the C-terminus, was expected to increase after digestion. However, that of LAIPVNKP markedly decreased after successive digestion by these gastrointestinal proteinases. Susceptibility to absorption, as well as resistance to digestion by gastrointestinal proteinases, is essential for the anti-hypertensive effect of ACE inhibitory peptides *in vivo*. Matsui (2003) found that 18 di- and tri-peptides derived from β -conglycinin, including WL, were absorbed intact via small intestine membrane transport in rats. WL, isolated from glycinin hydrolysis and tofuyo, is expected to exhibit an antihypertensive effect *in vivo*. The effect of tofuyo ingestion on ACE inhibitory activity is due to the relationship between peptide chemical structure and tofuyo metabolism. This is especially important from the viewpoint of bioactive peptide design, which must take metabolism into account. This enzyme not only contributes to the formation of ACE inhibitory peptides, but also has the potential to produce various bioactive peptides from food proteins.

5.2 Characterization and application of serine carboxypeptidases from genus *Monascus*

Carboxypeptidases are a kind of exopeptidases. The enzymes cleave peptide bonds at points within the proteins and remove 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-polyacrylamide gel electrophoresis. The purified enzyme from *M. purpureus* was a heterodimer with a molecular mass of 132 kDa, consisting of two subunits of 64 kDa and 67 kDa. It was characterized as an acidic glycoprotein, with an isoelectric point of 3.67 and 17% carbohydrate content. The enzyme was strongly inhibited by piperastatin A, diisopropylfluoride (DFP), phenylmethylsulfonylfluoride (PMSF), and chymostatin, suggesting that it is a chymotrypsin-like serine carboxypeptidase. Benzyloxycarbonyl-L-tyrosyl-L-glutamic acid (Z-Glu-Tyr) was the best substrate for the enzyme. On the other hand, two serine carboxypeptidases, MpiCP-1 and MpiCP-2, were purified to homogeneity from *M. pilosus*. MpiCP-1 is a homodimer with a native molecular mass of 125 kDa, composed of two identical subunits of 65 kDa, while MpiCP-2 is a high-mass homooligomer with a native molecular mass 2,263 kDa, composed of 38 identical subunits of 59 kDa. This is unique among carboxypeptidases and distinguishes MpiCP-2 as the largest known carboxypeptidase. The purified enzymes were both acidic glycoproteins. MpiCP-1 has an isoelectric point of 3.7 and a carbohydrate content of 11%, while for 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. Substrate specificities of carboxypeptidases from *M. purpureus* and *M. pilosus* described above are consistent with the findings that glutamic acid is the one of the most abundant free amino acids found in matured tofuyo (Yasuda et al., 1993; Yasuda, 2010). Thus, carboxypeptidases from the genus *Monascus* are concluded to play an important role in the formation of taste-associated compounds (amino acids) from soybean protein during the maturation of tofuyo.

The action of pepsin and the admixture of pepsin and carboxypeptidase from the genus *Monascus* on the hydrolysis of soybean protein were studied (Liu & Yasuda, 2005). The results showed that the pepsin hydrolysates of soybean protein were much more bitter and contained relatively smaller amounts of total free amino acids than the hydrolysates obtained with the admixture of pepsin and the enzyme. In addition, hydrophilic and hydrophobic amino acids were present in almost equal proportions in the pepsin hydrolysates, while mainly hydrophobic amino acids made up the hydrolysates obtained with the admixture of pepsin and the enzyme. Thus, this enzyme not only plays an important role in the formation of taste-associated compounds, but also in the elimination of bitterness during the ripening of tofuyo.

The information obtained from the investigation may provide clues to the applicability of *Monascus* carboxypeptidase in the modification of soybean protein, and how bitterness is eliminated.

5.3 Characterization of glucoamylases from genus *Monascus*

Glucoamylases are exo-hydrolyzing enzymes that liberate 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. In tofuyo production, glucoamylases play an important role in providing taste to the product. Two forms of an extracellular glucoamylase, MpuGA-I and MpuGA-II, were purified to homogeneity from *M. purpureus* in our laboratory (Tachibana & Yasuda, 2007). The properties of the enzyme were summarized as follows. 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. The optimal pH was 5.0 for both enzymes, and the optimal temperatures were 50°C (MpuGA-I) and 65°C (MpuGA-II). The relative hydrolysis rates of various substrates by the purified enzymes were examined. Each final preparation showed the highest hydrolysis activity toward short polymerized amylose Ex-I having only the α -1,4-linkage. Amylopectin was moderately hydrolyzed by both the enzymes. The K_m values for soluble starch were calculated to be 4.0 mg/ml (MpuGA-I) and 1.1 mg/ml (MpuGA-II). The primary structure of the N-terminal amino acid sequence of MpuGA-I shows 72.7% homology with that of MpuGA-II, and the N-terminal amino acid sequence of MpuGA-I showed high homology and similarity with other fungal glucoamylases that belong to the glucoside hydrolase family 15.

6. Conclusions

Tofuyo is a fermented tofu indigenous to Okinawa Prefecture, Japan. The origin of tofuyo is considered to be red furu, from China. It was created as a mellow and delicious food, as well as a low-salt health food. Various aspects of tofuyo production, including optimal conditions, tofu and koji preparation, as well as soaking and ripening, were clarified in our laboratory.

The main components comprising the body of tofuyo consist of basic subunit in glycinin and other polypeptides (M_r : 55 kDa, 11-15 kDa). The soybean proteins were digested into peptides and amino acids during maturation. Free glutamic acid and aspartic acid were strongly related to the desirable taste (*umami*-taste) of the product. The other hand, some of the peptides released from soybean protein in tofuyo have bioactivity. Angiotensin I-converting enzyme (ACE) inhibitors have an excellent effect on hypertension. Some of the peptides (IFL and WL) liberated from tofuyo inhibited the ACE activity that produces the vasopressor peptides. The systolic blood pressure of rats in the tofuyo group was significantly lower than that in the control group. Additionally, tofuyo might contribute to an improvement in erythrocyte deformability in mice.

Homogenous preparations of proteinase from *M. purpureus* and *M. pilosus* were characterized as aspartic enzymes. The enzyme plays an important role as a key enzyme for ripening of tofuyo. Furthermore, this enzyme not only contributes to the formation of ACE inhibitory peptides, but also has the potential to produce various bioactive peptides from food proteins. Homogenous preparations of carboxypeptidases from *M. purpureus* and *M. pilosus* were characterized as serine carboxypeptidases. These enzymes are involved to the release of amino acids from soybean protein and impart a pleasing taste to tofuyo. Furthermore, the enzymes also contribute to the removal of bitterness during the hydrolysis of soybean protein. Homogenous preparations of glucoamylase from *M. purpureus* were characterized. The enzymes contribute to the production of d-glucose from starch and play a role in the desirable taste of tofuyo.

Tofuyo is a health food that is cholesterol free, and is a low-salt fermented soybean cheese. The potential health benefits of tofuyo such as antihypertensive and hypocholesterolemic effects, erythrocyte deformability, antioxidative and DPPH radical scavenging activities are clarifying. Therefore, tofuyo is expected to widely spread as a health food. It would also be suitable for western people because it could be utilized in almost the same way as cheese. In order to spread tofuyo as health food, it is necessary to clarify the details of functional properties in this product. The author expects that tofuyo will develop into the health food of the world in the future.

7. References

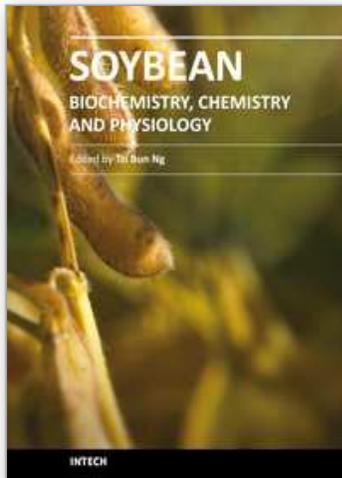
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