EnzymeWave

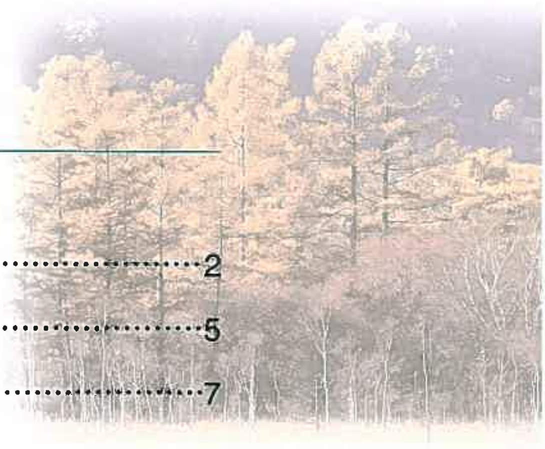
Volume 4

Soy Sauce (Shoyu) and Soy Paste (Miso)
~Typical Japanese fermented food~

Wine and Tea : Applications for β-glycosidase

NBRC : NITE Biological Resource Center

AMANO



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From the Editor,

The corporate policy of Amano Enzyme Inc. is to "contribute to society by developing enzymes that are widely used in everyday products". Since the establishment of Amano over one hundred years ago, and especially since Amano's entry into the enzyme business during the last half century, Amano has realized the goals of its corporate policy through the recognition and acceptance of Amano enzymes by customers worldwide.

As a way to fulfill the Amano corporate policy, Amano has established a series of symposiums on enzyme applications initiated in May 2000. The goal of the symposiums is to further the promotion of enzymes through a study of enzyme applications. To that end, Amano grants an award in recognition of significant advancement to the enzyme industry through the study of enzyme applications.

At each symposium, the recipients of awards are invited to present lectures on up to five carefully selected topics. In addition, leading scientists in Japan are invited to present keynote lectures. The following keynote lectures have been presented in the past:

"Exploration of Microbiological Catalysts and Industrial Use", Professor Emeritus Hideaki Yamada, Kyoto University (2000)

"Development of Biotechnology from Fermentation Technology", Professor Teruhiko Beppu, Nihon University (2001)

"Acetobacter Enzymes and Their Uses", Professor Osao Adachi, Yamaguchi University (2002)

Amano Enzyme Inc. is looking forward to welcoming you to the fourth Symposium which will be held in June, 2003, in Nagoya, Aichi Prefecture, Japan.

Soy Sauce (Shoyu) and Soy Paste (Miso)

~ Typical Japanese fermented food ~

For more than 1000years, people in Asia have been consuming soybeans in a variety of traditional soy foods. Today many popular soy foods are available worldwide including tofu, soy milk, soy sauce, soy paste and tempeh. Soy foods can be divided into two categories: fermented and non-fermented. Traditional fermented soy foods in Japan include soy paste (*miso*), soy sauce (*shoyu*) and *natto*. Soy sauce and soy paste are flavoring agents having similar aroma and flavor. Both are made by a two-step fermentation process from cereals and soybeans with a mixture of molds, yeast and bacteria. The first step involves fermentation with mold to produce proteolytic and amylolytic enzymes in the starter culture (*koji*); in a second fermentation with yeast and lactic acid bacteria in the presence of high salt concentration the characteristic aroma and flavor is produced.

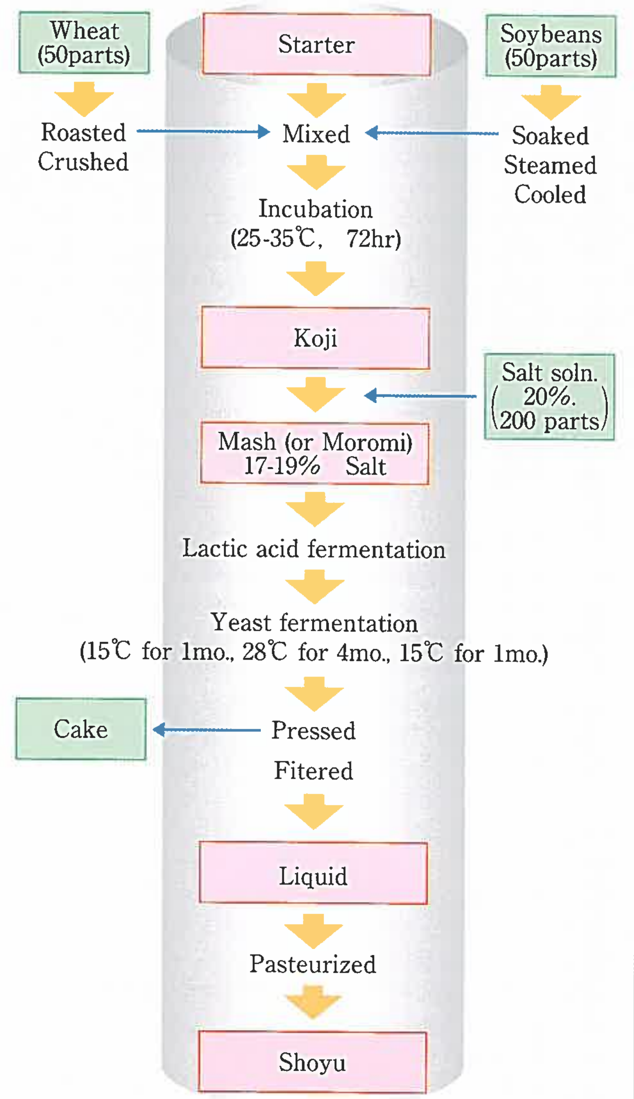
Soy Sauce (*Shoyu*)

Shoyu is a seasoning agent with a salty taste and a distinct aroma and is widely consumed in Asian countries as a condiment and coloring agent in the preparation of food and for table use. It is a dark brown liquid, stable at ambient temperature, which does not require refrigeration during storage due to its low water activity and high salt content.

Shoyu is produced by fermentation of a mixture of soybeans, wheat grain, water and salt. The production process consists of three steps: *koji* production, brine fermentation and refining. (A flowsheet for the process is shown in Figure 1.)

A scene of shoyu manufacture at old days

Figure 1: Flowsheet for manufacture of Shoyu



Koji is a mixture of proteolytic enzymes for converting soybean proteins into peptides and amino acids, and amylases for hydrolyzing starch into sugars. In *koji* production, defatted soybean flakes or whole soybeans are soaked in water and then cooked at a pressure of 6-7kgcm^{-2} for 20-30s in a continuous cooker; separately, wheat is heated in a continuous roaster with hot air at 150℃ for 30-40s at atmospheric pressure. The wheat is then cracked into 4 or 5 pieces per kernel. In making traditional soy sauce, the cooked soybeans are mixed with an equal amount of roasted wheat and then inoculated with 0.1-0.2% of starter mold (*Aspergillus oryzae* or *Aspergillus sojae spores*). After incubation at 25℃ for 72h, the *koji* becomes a greenish yellow mass as a result of mold growth and sporulation.

In the second step, the harvested *koji* is mixed with 20% salt brine and transferred by a pump into fermentation tanks. The selected lactic acid bacteria (*Pediococcus halophilus*) is cultured and added to the mash. To control growth the mash is kept at 15℃ for the first month while the pH decreases from 6.5 to 5.0 with the formation of lactic acid. Then cultures of *Saccharomyces rouxii* (osmophilic yeast) and *Candida sp*, are added as a starter culture for alcohol formation. The temperature of the mash is allowed to rise to 28℃ until alcohol fermentation starts. In the manufacturing process, 80-90% of the constituent proteins and starch are broken down into amino acids and sugars. Glutamic and aspartic acid are the major amino acids present in *shoyu*. Glutamic acid, produced from glutamine by glutaminase activity found in *koji*, is the most important component required for *shoyu* taste. The major sugars in *shoyu* are arabinose, glucose and galactose, with total sugar content about 4.45% as glucose.

The final process is refining which includes pressing, filtration, pasteurization at 70-80℃ for a few minutes and then packaging.

The quality of soy source is monitored by measurement of the pH, acidity, amino nitrogen, salt content, color, microbial contamination, and sensory attributes: color, aroma and flavor of the product. The most important aromatic compounds are HEMF (4-hydroxy-2-ethyl-5-methyl-3-furanon) and volatile compounds including esters, ketones and furans. These volatiles are formed by microorganisms during *koji* and brine fermentation.

There are several types of *shoyu* in Japan characterized by different fermentation processes that differ in the rate of mixture of wheat and soybean. The type of *shoyu* is classified by JAS (Japanese Agricultural Standard) and the properties of some Japanese *shoyu* are shown in Table I. Koikuchi is an example of fermented soy sauce that is very popular in Japan because of its strong aroma and attractive flavor.
Good *shoyu* stimulates the secretion of gastric juices and has the same effect as the caffeine in a cup of coffee. In other words, *shoyu* is an excellent appetizer and a digestive aid. A little *shoyu* gives taste to a dish and enhances its flavor.

Figure 2: Flowsheet for manufacture of Miso

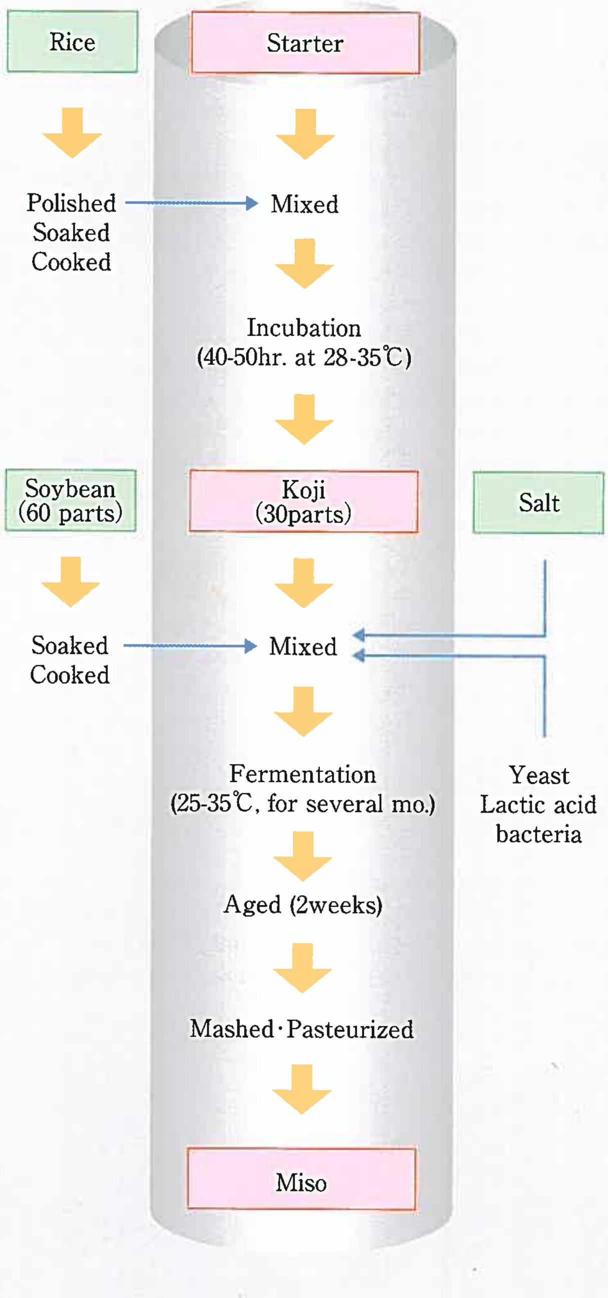


Table 1: Typical compositions of three varieties of shoyu

	Baume	NaCI (%,w/vol.)	Total - N (%,w/vol.)	Formol - N (%,w/vol.)	R -suger (%,w/vol.)	Alcohol (%,vol/vol.)	pH
Koikuchi	22.0	16.9	1.57	0.94	3.0	2.3	4.7
Usukuchi	22.2	18.9	1.19	0.80	4.2	2.1	4.8
Tamari	29.9	19.0	2.55	1.05	5.5	0.1	4.8

Soy Paste (*Miso*)

Miso or soybean paste is made from fermented soybeans, and occasionally in combination with wheat, barley or rice. Similar products are produced in China, Korea and other parts of Asia under a variety of names such as *Jang*, *Chiang* and *Tao-si*.

Miso is produced by a process similar to soy sauce, except that cooked whole beans are mixed with steamed rice and fermentation takes place after the addition of salt and a limited amount of water. The production method for rice *miso* is shown in Figure 2.

Rice soaked in water overnight is steamed for 40-60min and seeded with spores of *Aspergillus oryzae* at a rate of 1g per kg of rice. The rice is then incubated at 30-35℃ for 40-50hr. The resulting *koji*, a source of enzymes to hydrolyze soybeans and rice components, is mixed with sodium chloride (13%), cooked soybeans steamed at 115℃ for 20min, pure cultured yeast (*Sacchaomyces rouzii*), lactic acid bacteria (*Pediococcus halophilus*) and a small amount of water compared to *shoyu* fermentation. The resulting *miso* is a solid paste and therefore does not require filtration. The fungus and yeast used in *miso* production are similar and sometimes the same as in soy sauce production. After the second fermentation for an appropriate period (1 week to 1 year) at 25-30℃, the resulting aged mixture is mashed and packaged as *miso*. You need not worry about using *miso* quickly since it can be refrigerated for up to a year.

There are many varieties of *miso* in Japan. Rice *miso* is made from rice, soybeans and salt. Barley *miso* is made of barley instead of rice and soybean *miso* is made of soybeans only. Rice *miso* is currently most popular in Japan. The color of *miso* varies from a creamy, yellowish white to a very dark brown; in general, the darker the color of *miso* the stronger the flavor. The nutritional components of *miso* are 11-19% protein, 4-9% fat, 13-36% carbohydrate, 1-15% ash and 42-47% moisture.

Miso is very important to the Japanese diet and is consumed by nearly all Japanese in the form of *miso* soup which is served for breakfast, but also for lunch and dinner. It may be thinned and used as a dressing for vegetables and as a picking medium.

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Wine and Tea: Applications for β-glycosidase

Wine Aroma

The aroma of wine, especially Muscatel, Riesling and Gewüztraminer, is defined by the presence of specific aromatic compounds, including monoterpene alcohol (e.g., α-terpineol, geraniol and citronellol), green alcohol ((Z)-3-hexenol) and phenolic alcohol (benzyl alcohol and 2-phenyl ethanol) (1). These aromatic compounds impart the fresh, floral and flowery sweet aromas characteristic of the wine; likewise, the characteristic aroma of Chardonnay is imparted by the presence of C13 norisoplenoids (e.g., vitispirane and damascenone).

The aromatic compounds in grape, grape juice and wine are present as diglycosidic precursors. The precursor compounds (6-O-α-L-rhamnopyranosyl β-D-glucopyranoside or 6-O-α-L-arabinofuranosyl β-D-glucopyranoside) are hydrolyzed during the process of wine making releasing the aromatic compounds that characterize the wine (2). The hydrolysis of the precursor compounds is carried out by a combination of glycosidases, including β-D-glucosidase, α-L-rhammosidase and α-L-arabinofuranosidase, originating from the yeast utilized in wine making (3) (figure 1). However, the hydrolysis of the precursors during wine making is not optimal; the activity of α-L-rhammosidase and α-L-arabinofuranosidase is relatively low and the crucial enzyme β-D-glucosidase is severely inhibited by free glucose.

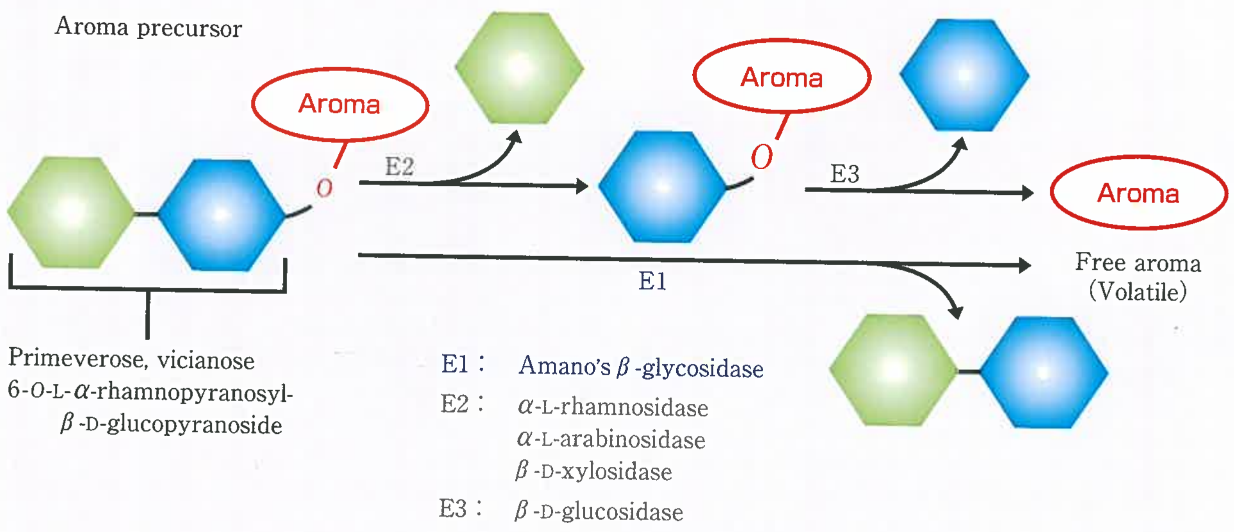
Tea Aroma

The aroma of tea is one of the most important characteristics used to rank the quality of tea. The presence of aromatic compounds including (Z)-3-hexenol, 1-hexanol, geraniol, linalool, benzyl alcohol, 2-phenyl ethanol and methyl salicylate imparts the fresh and flowery sweet aroma found in green tea, oolong tea and black tea (4). These aromatic compounds also exist as diglycoside precursors (6-O-D-xylopyranosyl β-D-glucopyranoside (β-primeveroside)) that are hydrolyzed during tea preparation by endogenous β-primeverosidase which is found in tea leaves. This enzyme is apparently responsible for the hydrolysis of all aromatic precursor compounds found in tea (5). It has been reported that a significant amount of aromatic precursors remains in tea leaf extract after tea preparation and thus it should be possible to increase the aroma of tea by adding exogenous enzyme (6).

Amano's β-glycosidase

In order to develop enzymes to enhance the aroma of wine and tea, Amano Enzyme Inc. initiated a search for an activity capable of hydrolyzing *p*-nitrophenyl β-D-primeveroside to *p*-nitrophenol and primeverose. Several β-primeverosidase - like enzymes were found from microbial sources; these enzymes are referred to

Figure 1: One step hydrolysis of aroma precursor by Amano's β-glycosidase

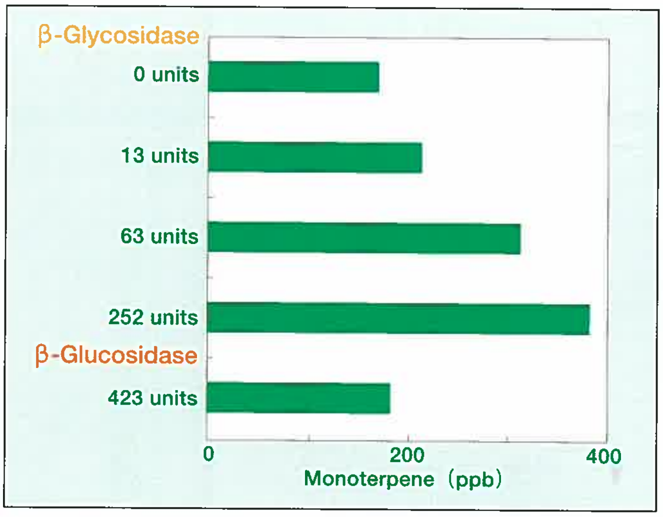


as β-glycosidase until they are more extensively characterized.

The addition of β-glycosidase during fermentation of Muscatel wine results in an increase in the total amount of five monoterpenes (linalool, α-terpineol, geraniol, nerol and citronellol) (figure 2) as well as a clear increase in floral and sweet aroma as monitored by an olfactory panel test. As a control, α-D-glucosidase from *Aspergillus niger* was added to a separate batch of Muscatel wine. The effect of β-glycosidase was superior to that of β-D-glucosidase (figure 2) as expected since β-D-glucosidase is greatly inhibited by free glucose and the effectiveness of β-D-glucosidase is dependent on the presence of other enzymes, especially α-L-rhamnosidase and α-L-arabinosidase, in a two step reaction to liberate aromatic compounds. In contrast, β-glycosidase is not inhibited by free glucose and liberates aromatic compounds directly from disaccharide precursors in a one step reaction and would therefore be expected to be more effective in aroma production.

The aroma of tea was also significantly improved by the addition of Amano's β-glycosidase as measured by the concentration of aromatic compounds in tea after enzyme treatment (table 1) and also by an olfactory panel test that found an increase in freshness in green tea (and corresponding decrease in the typical aroma characteristic

Figure 2



Total amount of 5 monoterpenes (linalool, geraniol, nerol, citronellol, α-terpineol) in Muscatel wine. Amano's β-glycosidase was added in the process of fermentation. Commercial β-glucosidase was also tested for comparison.

Table 1: Effect of Amano's β-glycosidase on tea extract

Compound	Ratio		
	GT	OT	BT
Z-3-Hexenol	1.8	9.1	6.6
Z-Linalool oxide	1.6	7.4	
E-Linalool oxide		∞	1.7
Benzaldehyde	1.8	2.9	32.6
Linalool	1.2	6.1	4.4
Geranial	1.5	∞	∞
Methyl salicylate	2.9	∞	∞
Geraniol	6.6	7.0	2.4
Benzyl alcohol		∞	∞

GT; green tea, OT; oolong tea, BT; black tea.
Analyzed by GC/MS. Values on the table show the ratio of peak area to control (no enzyme addition).

of old or low grade green tea) and an increase in the floral and sweet aroma of black and oolong tea (characteristic of high grade tea). The results obtained with Amano's β-glycosidase were significantly better than results with β-D-glucosidase (data not shown).

The application work described above suggests that Amano's β-glycosidase can not only reinforce the characteristic aroma of wine and tea, but can also improve the aroma by releasing new aromatic compounds not typically found in wine or tea. It is also clear that the application of Amano's β-glycosidase may be extended to other materials as well as wine and tea.

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NBRC : NITE Biological Resource Center

The NITE Biological Resource Center (NBRC) was established on April 1 in 2002 under the National Institute of Technology and Evaluation (NITE). The location of NBRC is in Kisarazu city, Chiba Prefecture and their mission is the isolation, identification of microorganisms and preservation of biological resources (microorganisms, DNA clones, culture broths, etc.) in support of biotechnology R&D with the potential of industrialization. Microorganisms are a group of organisms endowed with an abundance of diversity that also have a strong link with human life.

NBRC Activities

Preservation and Distribution of Stock Culture Collection

- Offer microbial culture collection
- Explore and isolate microorganisms
- Improve the quality of cultures/study new methods of Taxonomy

Development and Offer of Biological Usefulness for Industry

- Offer microorganisms, culture broths and genes
- Preserve and offer of DNA clones

Education and Consultation

- Accept researchers and technicians from all over the world in order to facilitate technology exchange and education
- Offer services for the isolation and identification of microorganisms
- Resource for techniques in taxonomy and cultivation of microorganisms

Collaborative Relationships with Foreign Countries

- Make project and material transfer agreements according to the Convention on Biological Diversity
- Utilize the biological resources of foreign countries

You can reach the NBRC at the following homepage.

http://www.bio.nite.go.jp/nbrc/e-home/

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Nov.2002