Enzyme Wave

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AMANO

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From the Editor,

Since its establishment, Amano Enzyme Inc. has wanted to contribute to the regional community, and to preserve and nurture "symbiosis with nature, and Japanese culture and traditions which treasure objects." In order to put this goal into practice, Amano Enzyme Inc. opened the Amano Ceramic Art Club more than 20 years ago in Nishiharu-cho, Nishikasugai-gun, Aichi Prefecture where the company originated, by providing a building equipped with a kiln, potter's wheels and other instruments for the use of local residents. Today, many people participate in the club. The Amano Ceramic Art Club aims to improve local culture and heighten the level of club members' cultivation through ceramics. Every spring, the club holds the "Amano Ceramic Festival" in the garden of the Amano Enzyme chairman's residence, adjacent to the club building. At the festival, artworks made by the club members with their whole heart and soul are also sold. In the fall, the "Amano Ceramic Art Club Exhibit" is held in the Citizens Gallery in Nagoya City, Aichi Prefecture, where the head office of Amano Enzyme Inc. is located.

Aichi Prefecture is known for its ceramic ware, and ceramic ware produced in Seto City and Tokoname City is particularly famous. Incidentally, several nationally important large scale construction projects are underway near the two cities. One of the projects is the "EXPO 2005 AICHI," which will be held from March 25, 2005 to September 25, 2005 near Seto City with the theme, "Nature's Wisdom." The other project is the "Chubu International Airport," which is under construction at full pitch at an offshore site near Tokoname City, with the aim of opening in March 2005. Amano Enzyme Inc. is supporting both projects.

artwork made by one of the Amano Ceramic Art Club members

Tofu : Traditional Japanese Soybean Food

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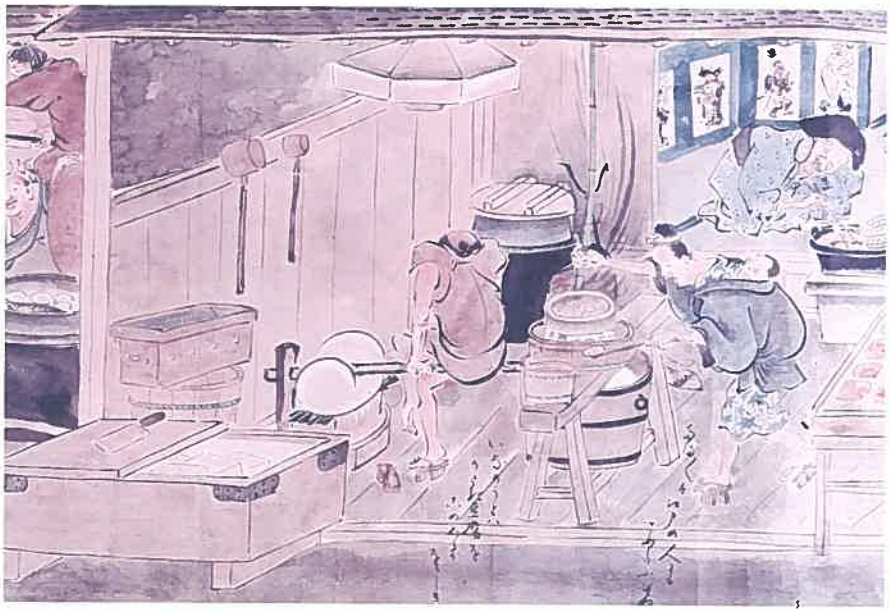
History of tofu

Tofu, as well as soy sauce and miso, originated in China. In China tofu was made more than 2000 years ago, and first introduced to Japan 1000 years ago by Buddhist priests during the Nara era when Buddhism flourished in Japan. At that time tofu was used exclusively in the imperial court, nobles and at Buddhist temples. Tofu became more popular during the Kamakura and Muromachi era (12th-16th century), but still was not known among common people. During the Edo era (17th-19th century) tofu gradually became a common daily food and was marketed by many tofu makers who controlled its unit price and weight. The publication of "Tofu : the best 100 dishes" and its sequels were issued and greatly promoted interest in tofu. Demand for tofu (including deep-fried tofu) increased throughout the 20th century. Regional tofu maker associations were formed and developed into a national union. While the production of tofu during World War II temporarily decreased because of a shortage of soybeans, production soon recovered to prewar levels. Recently various physiologically important substances have been found in tofu and the health benefits from tofu have resulted in making tofu quite popular internationally during the last decade. Today there are over 15,000 tofu makers and tofu is an indispensable daily dish and source of nutrients in the Japanese diet.

The manufacture of tofu

In the past, tofu was made at a small scale early in the morning by family labor and sold within the same day in the neighborhood. This was natural, because tofu contains a large proportion of water and can easy deteriorate, and is also very fragile. Today, because of the advent of new equipment and the rising cost of labor, large scale production of tofu predominates (Photo 1).

The production of soybean milk is the first step in the manufacture of tofu. Soybeans are soaked in water over night and then ground with running water using a stone-mill or grinder. The resulting mash is heated to boiling, and then filtered to separate soybean milk and spent residue (*Okara*). A coagulant is then added to the soybean milk and mixed. After discarding the supernatant (whey) the white coagulated mass is transferred to a perforated wooden or metal box laid with cloth inside and pressed to remove excess whey. The resulting white molded square shaped mass is taken out of the box while in water, cooled in water and then cut for sale. Nowadays tofu is packed in a plastic case, sealed, cooled and marketed.



とうふや 文化二年（一八〇五年）北尾 政美 画

国会図書館蔵

A scene of tofu manufacture at Edo era

Photo 1

This type of tofu is called *momen* tofu ("cotton tofu") (Fig 1). Calcium sulfate, *nigari* (made from sea water) or magnesium chloride is used as coagulant. The hardness and texture of the tofu can vary depending on the variety of soybeans, the concentration and temperature of soybean milk, the amount of coagulant added and the way for adding coagulant. The weight of tofu for sale is usually 200-300g. The yield of tofu from 10kg soybeans is 40-50kg. The residue (*Okara*) has some use as a food, but mainly is used in feed and fertilizer, and sometimes thrown away as waste.

Kinugoshi tofu ("silken tofu") is made regionally and seasonally. Soybean milk of higher concentration (less water added to soybeans than for *momen* tofu) is used. The soybean milk is totally coagulated to a homogeneous gel without separating whey using calcium sulfate and magnesium chloride as coagulant and sometimes with glucono-δ-lactone.

"Soft tofu" and "filled tofu" are also on the market. Soft tofu is made from soybean milk of intermediate concentration between *momen* and *kinugoshi*. After coagulating to a soft gel, it is transferred to a box for *momen* tofu, then pressed with a light weight and molded.

It can be characterized as *momen* tofu but having a *kinugoshi* like texture. Filled tofu is made from soybean milk of higher concentration, like *kinugoshi* tofu. After cooling the soybean milk, coagulant is added and then the mixture is placed in a plastic container, sealed, heated to induce coagulation and then cooled. Since the tofu is not directly touched by human hands during production and transportation, the tofu is more hygienic, not easily broken and has a longer shelf-life than other tofu. Filled tofu, therefore, is popular for large scale factory production.

Deep-fried tofu is also very popular. There are several kinds of deep-fried tofu : ordinary *aburaage*, *ganmodoki* and *atsuage*. Ordinary *aburaage* is made from thin partially drained rectangular tofu. It is deep-fried with vegetable oil at 110-120℃ during the first stage of production to form a sponge-like texture due to expansion. Then it is fried at 180-200℃ during the second stage of production to make the surface of the tofu hard in order to prevent shrinking. The color of the surface is yellow or pale yellow. To form this sponge like texture the heating conditions for tofu making have to be mild, perhaps to optimize the denaturation of tofu proteins. *Ganmodoki* is made by mashing tofu with *yamaimo* (a kind of yam), cut vegetables and some varieties of seeds, followed by casting and finally two steps deep frying. *Ganmodoki* means wild goose- like. In the past it was made for Buddhist priests who were vegetarians. *Namaage* is made by deep-frying tofu directly at 180 -200℃ resulting in no change of texture.

Micro structure of tofu (*kinugoshi*) by scanning electron micro scope
(protein network and oil droplets : ×20,000) (by Rieko Hirose)

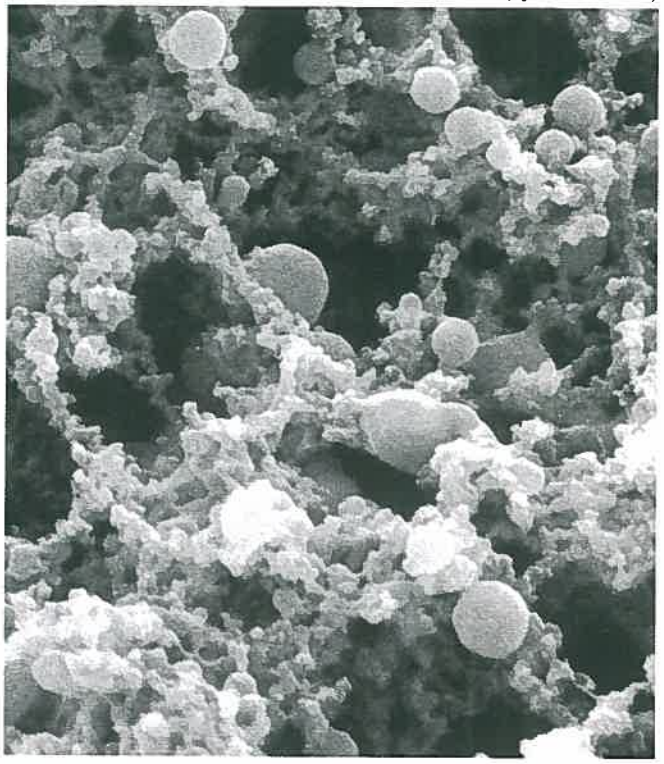
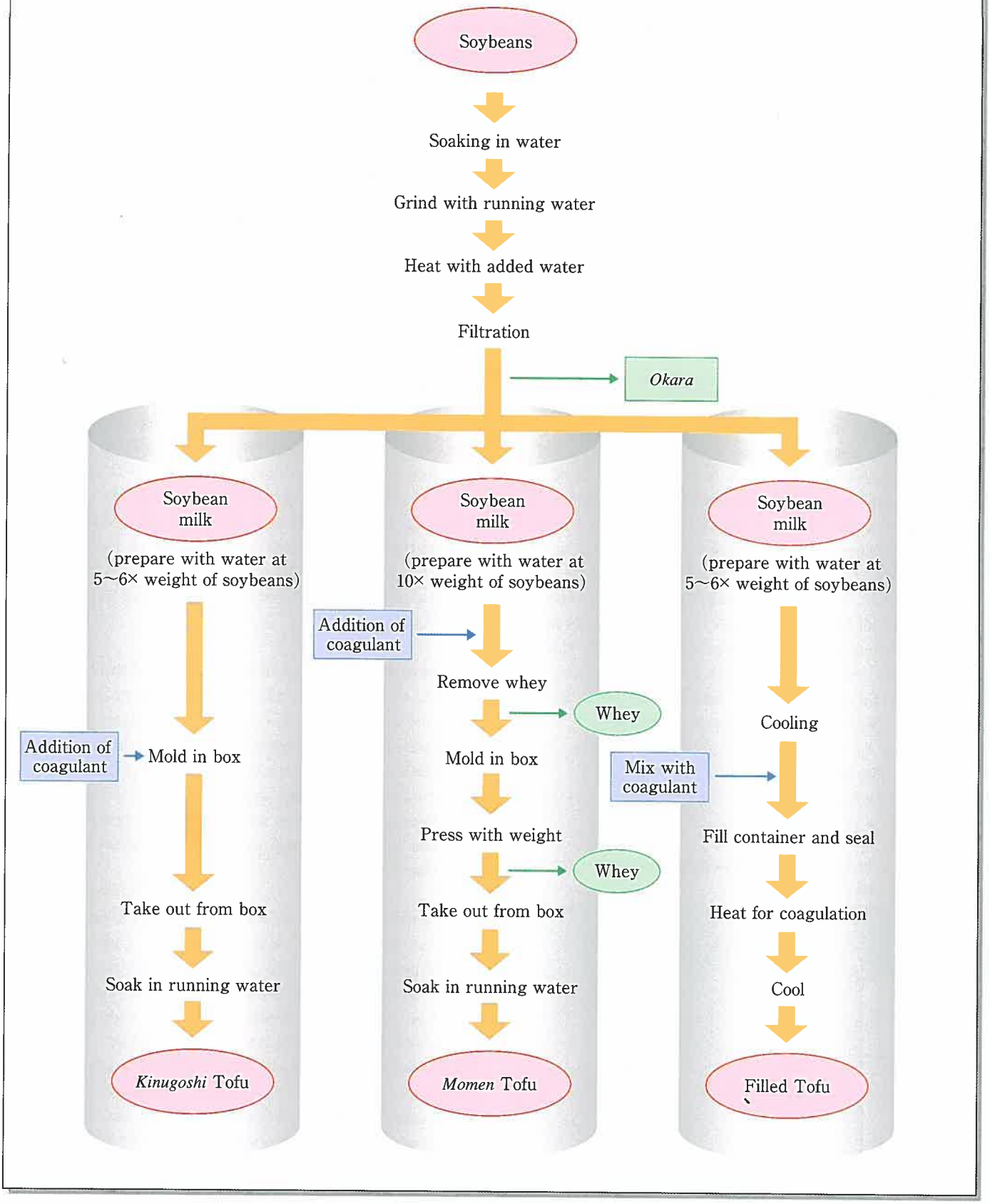


Figure 1 : Flow sheet for making Tofu



Note : weight of waters is 5-10 times weight of soybeans used and includes water absorbed by soaking and added during and after grinding

Deep frying apparatus that continuously changing oil temperature from low to high were developed and there are many large scale *aburaage* factories today. The longer shelf-life of *aburaage* also promotes mass production.

Current tofu production and market situation

The amount of soybeans consumed for tofu production is about 500,000t (1/3 is for *aburaage*) per year (Table 1). Over half of these soybeans are imported from USA and other foreign countries. Table 1 shows the trend in the number of tofu makers during the last decade, which indicates a gradual decrease in number. However the average amount of soybeans per year utilized by each tofu maker is increasing, as the total yearly amount of soybeans used for tofu remains essentially unchanged. The mass production of tofu has been promoted by labor-saving methods and mechanization of processing as well as technical developments in storage and transportation of the product. In addition, the establishment of a cold chain system and development of plastic film containers should be pointed out. Besides filled tofu, a hot-pack system of *momen* and *kinugoshi* tofu is now noticed. In this system *momen* or *kinugoshi* tofu, still hot (50℃), is transferred to a plastic case, sealed, heated in hot water (80℃) and then cooled in water at 3℃. Since this tofu contains less microorganisms, its shelf-life is longer than ordinary tofu. Large scale tofu factories consume over 10t soybeans per day and produce 200,000 units of tofu (200 g each). The product from these factories is sent to mass-sale stores, restaurants and dining rooms of various institutions. In contrast, small-scale factories consuming 60-90kg of soybeans per day, often sell their product directly to consumers. The fresh product, sold face to face, is welcomed by consumers.

Table 1: Yearly trend of total amounts of soybeans for tofu and number of tofu makers

Year	Amounts (1,000t)	Numbers
1990	494	21,819
1992	494	20,140
1994	493	18,780
1996	492	17,599
1998	495	16,804
2000	492	15,994
2001	492	15,600

The annual expenditure for tofu including *aburaage* is about 9,000 yen per household, the largest among soybean foods. Tofu and *aburaage* are ready to serve at the table after a short time for preparation. It is a characteristic of Japanese tofu dishes to minimize a change of texture, flavor and color of the tofu (Photo 2, 3, 4). This is in contrast to Chinese dishes, which modify the texture, flavor and color of tofu. In the future the Chinese style of tofu cooking may be more popular in Japan.

Since food regulations are becoming strict, labeling is recently an important issue. In Japan the name of the product, list of materials, weight of contents, time limit for best quality, method of storage and name of producer must appear on the label. In the case of tofu, except filled tofu which is more preservable, the time limit for consumption (eating) is used instead of time limit of the best quality. Although the date of production is not required, tofu makers usually print it on the label. The use of GMO (Genetically Modified Organisms) soybeans must be indicated on the label. Although no-use of GMO soybeans does not need to be mentioned, tofu makers print it on the label. The Ministry of Health and Labor prescribes a storage temperature below 10℃ and as guide the number of microorganisms in 1 g tofu should be below 10^5 and in the case of filled tofu should be below 10^3 and negative for colon bacillus.

Nutrition and physiological functionality of tofu

Soybeans are rich in protein and fat, but soybeans are also hard and are not digestible by simple cooking or roasting. Furthermore soybeans contain substances which inhibit the action of digestive enzymes or have hemolytic action. However, in soybean milk and tofu the hard tissue of soybeans is destructed by grinding, the indigestible fibers are removed as *okara*, and undesirable substances are inactivated by heating.

The protein and oil in soybeans is extracted in the soybean milk and tofu at a high yield. As shown in Table 2 tofu is rich in protein and oil. The quality of the protein is high, and is especially rich in lysine. Recently tofu protein was found to reduce cholesterol in blood. Fatty acids of soybean oil are rich in linolic and linolenic acid which are indispensable to human nutrition and also are effective in preventing hardening of the arteries. Tofu also contains many other substances that originate in soybeans, such as saccharose, inorganic salt, vitamins B_1, B_2 and E. Recently several physiologically functional substances in tofu were identified: 1. Saponin has antioxidative activity which limits damage from the aging process, 2. Lecithin which promotes metabolic function, 3. Isoflavone which is effective in

Table 2 : Composition of tofu, *aburaage* and soybeans (in 100g) (From 5th edition of Standard Tables of Food Composition in Japan)

	Energy (Kcal)	Water (g)	Protein (g)	Lipid (g)	Carbo-hydrate (g)	Ash (g)	Minerals(mg)					Vitamins(mg)				Dietary Fibers (g)
							Calcium	Phospho-rus	Iron	Magne-sium	Potas-sium	E	B_1	B_2	Niacin	
Momen tofu	72	86.8	6.6	4.2	1.6	0.8	120	110	0.9	31	140	0.6	0.07	0.03	0.1	0.4
Kinugoshi tofu	56	89.4	4.9	3.0	2.0	0.7	43	81	0.8	44	150	0.3	0.10	0.04	0.2	0.3
Soft tofu	59	88.9	5.1	3.3	2.0	0.7	91	82	0.7	32	150	0.4	0.07	0.03	0.1	0.4
Filled tofu	59	88.6	5.0	3.1	2.5	0.8	28	83	0.8	62	200	0.6	0.15	0.05	0.3	0.3
Abura age	386	44.0	18.6	33.1	2.5	1.8	300	230	4.2	130	55	2.6	0.06	0.03	0.1	1.1
Ganmodoki	228	63.5	15.3	17.8	1.6	1.8	270	200	3.6	98	80	2.4	0.03	0.04	0.2	1.4
Namaage	150	75.9	10.7	11.3	0.9	1.2	240	150	2.6	55	120	1.4	0.07	0.03	0.1	0.7
Soybeans	417	12.5	35.3	19.0	28.2	5.0	240	580	9.4	220	1900	3.6	0.83	0.30	2.2	17.1

Note : Carbohydrate=100−(Water+Protein+Lipid+Ash)

preventing osteoporosis and some kinds of cancer and 4. Oligosaccharide which promote the growth of bifidobacterium ("beneficial bacteria") in the intestine. Compared to *momen* tofu, *kinugoshi* and filled tofu contain the above substances in higher concentration because the whey is not removed.

New technologies in tofu production

1. Continuous process of molding of *momen* tofu

In an effort to scale up tofu manufacturing, a process for the continuous production of soybean milk was completed in 1960. However, there were problems in the molding process. The development of a small perforated box for coagulation could eliminate the cutting process and make a more efficient total continuous system of tofu production.

2. Development of long-life tofu

Extending the shelf-life of tofu has been successful by the development of the filled tofu and hot-pack system already explained. However, to extend the shelf-life of tofu, especially for consumers in remote areas, ordinary soybean milk is pasteurized with a plate heater at high temperature for a short time period. The milk, after cooling, is mixed with microbial free coagulant, filled aseptically in a pasteurized plastic case, sealed and then heated for coagulation. The resulting product is stable for several months at ambient temperature. There is another type of long-life tofu. It is made by sterilizing filled tofu at over 100℃ for 20-30 min. It is stable for more than 6 months at ambient temperature, but is not on the market because of undesirable flavor caused by severe heating.

3. Use of enzymes in tofu production

Recently, transglutaminase has been found to improve the texture of tofu. Transglutaminase is added to soybean milk with coagulant to promote the formation of intermolecular peptide linkage of soybean protein. The resulting tofu is more homogeneous and elastic. It may also be used for tofu from soybean milk prepared without removing *okara*, though the effect is not the same as tofu from ordinary soybean milk.

Tofu can be fortified with soluble dietary fiber prepared from *okara* by treating with cellulase, hemicellulase and pectinase. Soybean milk, after cooling, is mixed with those enzymes-digested solubles of *okara* and coagulant, filled, sealed and heated for coagulation. The product contributes fiber supplement to the Japanese diet. Dietary fiber reduces cholesterol in blood, improves intestinal microflora and promotes excretion of unnecessary metabolites. The application may also be helpful in extending the use of *okara*.

Photo 2

Photo 3

Photo 4

Enzymes for Determination of Glucose

The number of people with diabetes is rapidly increasing each year. Recent studies by WHO estimate up to 240 million people worldwide will suffer from diabetes by 2010. Treatment for diabetes today is centered on alleviating complications due to diabetes and careful monitoring of blood sugar levels to evaluate the effectiveness of diet and other treatments to slow the progress of the disease. In addition, urine and blood glucose levels are important markers for diagnosing diabetes.

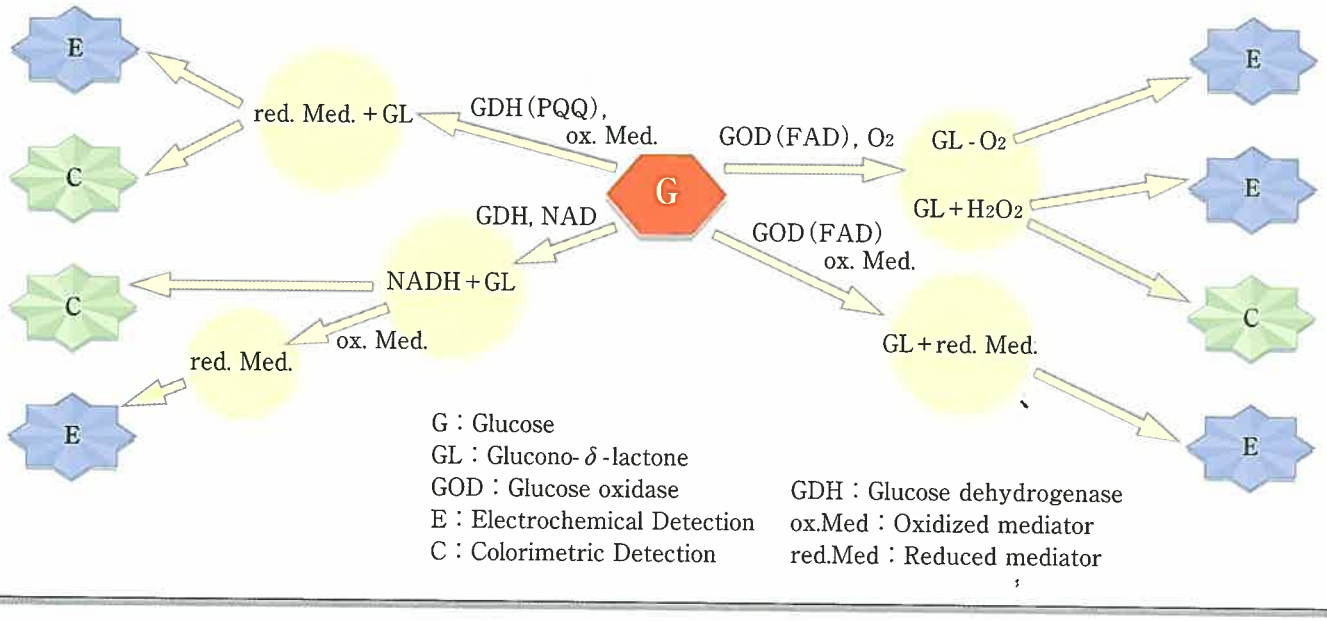
It is therefore essential that fast and simple methods to let patients determine their own glucose levels are widely available. Recently, the advent of self-monitoring of blood glucose (SMBG) devices has made this possible. These devices have became increasingly popular in the 1990's. Patients are now able to determine their own level of blood glucose by themselves as they go about their daily lives without complicated instruments and techniques. Only a small amount of blood from fingertip or forearm is drawn and placed onto a test strip of the devices, the level of glucose in the blood is determined by an enzymatic reaction which develops color or generates an electrochemical signal in proportion to the amount of glucose present.

Since Amano launched glucose oxidase in 1976, we have been making great efforts to develop and improve enzymes useful for the determination of glucose; because glucose detection principles vary, the specifications required for enzymes utilized for glucose detection also vary. Fig. 1 shows different methods of glucose determination. For example, one method utilizes the detection of hydrogen peroxide or dissolved oxygen, while another method is based on the detection of an electrochemical signal via a suitable mediator (biosensor). Early SMBG devices relied on detection of glucose colorimetrically, but the trend has now shifted to the use of biosensors.

Glucose oxidase

Glucose oxidase is produced from *Aspergillus niger* and coenzyme FAD (flavin adenine dinucleotide) is bound to the enzyme. Glucose oxidase is quite stable and has a high specificity for glucose (Table 1) and is therefore widely used for the determination of glucose. Amano has developed and launched various glucose oxidase products each with a specific application. For example, glucose oxidase for electrode and colorimetric methods that detect hydrogen peroxide must have all traces of catalase removed by purification because catalase decomposes hydrogen peroxide and would therefore compromise the detection method. On the other hand, glucose oxidase for use in the oxygen electrode method must have sufficient amounts of catalase present to eliminate hydrogen peroxide which would interfere with the determination of dissolved oxygen. In addition, glucose oxidase with a high specific activity is required for glucose determination methods relying on immobilized enzyme.

Figure 1: Principle of Glucose Determination



Glucose Dehydrogenase

As described previously, most currently used SMBG devices utilize biosensor technology, not only for personal use at home but also for diagnostic use in hospitals. Ideally, enzymes used for biosensor technology should not be affected by concentration of dissolved oxygen. Since the concentration of dissolved oxygen is greater in arterial blood then in capillary blood, glucose determination by glucose oxidase biosensors would erroneously underestimate glucose levels in arterial blood. Therefore, glucose dehydrogenase, which is not affected by dissolved oxygen, is becoming the enzyme of choice for biosensors.

a.NAD dependent Glucose Dehydrogenase

Amano has launched NAD (nicotineamide adenine dinucleotide) dependent glucose dehydrogenase produced from *Bacillus megaterium*. Site-directed mutagenesis studies have contributed to an improvement in the stability of this enzyme. The mutant that shows a drastic improvement in thermostability (Fig. 2) contains an amino acid substitution in the 252nd position from the amino terminal (glutamine to leucine) and has no alteration in substrate specificity (Table 1). However, it requires the addition of NAD to the reaction.

b.PQQ dependent Glucose Dehydrogenase

Amano has just developed a new type of glucose dehydrogenase from *Acinetobacter* sp.. Coenzyme PQQ (pyrroloquinoline quinone), which is called "the third coenzyme", is bound to this enzyme. The reaction rate is highest for known enzymes that catalyze glucose and it is also unaffected by dissolved oxygen. Therefore it is of great interest; yet a broad substrate specificity (Table 1) is a problem since the enzyme reacts with reducing saccharides other than glucose. Improvement in substrate specificity by genetic engineering is ongoing at Amano.

As diabetes reaches epidemic proportions it will be even more critical to develop new technology to monitor and control blood glucose levels. Amano will continue the challenge to develop new enzymes for the determination of glucose.

Reference

1) Method in Enzymology Vol Ⅸ p.82
2) J Biol Chem(1989) 264(11) 6381-6385
3) Biosensors (1986) 2(2) 71-87

Figure 2: Stability of GDH(NAD dependent) in Solution

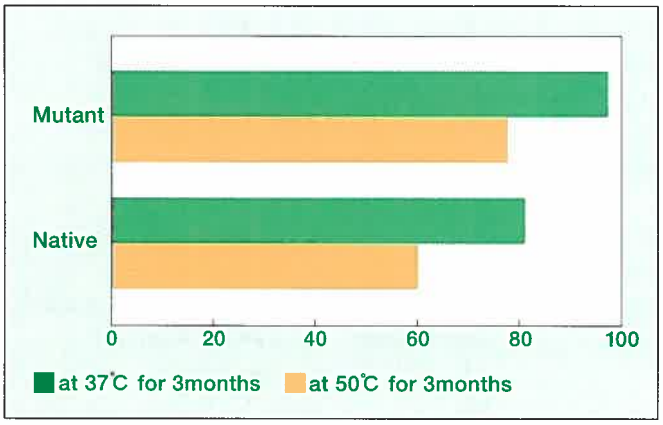


Table 1: Substrate Specificity

	Glucose oxidase	Glucose dehydrogenase (NAD dependent)	Glucose dehydrogenase (PQQ dependent)
D-Glucose	100	100	100
D-Galactose	0	0	12
D-Mannose	3	10	17
Maltose	0	0	71
Sucrose	0	0	0
Fructose	0	0	0
Lactose	0	1	56

Revision of Japanese Pharmaceutical Regulations

The Japanese Pharmaceutical Affairs Law (PAL) will be drastically revised in 2005. This article presents major points of the revision.

The current Pharmaceutical Affairs Law (PAL) was introduced in 1961 to implement regulations necessary for ensuring the quality, effectiveness, and safety of drugs and medical devices. The PAL has since been revised several times. However, with the advent of the 21st century, the following shortcomings have been identified with regard to the PAL in its current form:

- The PAL may not be keeping up with international standards and with changes in socioeconomic circumstances.
- Some of the current legal framework may not be relevant to the control of new drug classes that make use of bio/genomic technology.

Thus it was decided to make revisions to the PAL from the perspectives indicated in Table 1.

Table 1: Points of the revision

- Improvement of post-marketing safety measures and revision of approval/authorization system
- Clarification of corporate responsibility for safety measures
- Revision of manufacturing approval system based on international harmonization
- Legislative preparations to ensure the safety of biological products
- Major revision of safety measures for medical devices
- Respond to new medical devices that use varied technologies and materials

Revision of Approval System

The strictures of the PAL in its current guise are based entirely on the premise that each drug is developed by a manufacturer that makes the product at its own manufactory. The new PAL will be changed from a system that is focused on the act of manufacturing to that of an integrated sales and marketing system. Therefore instead of having an authorized drug producer with a manufactory as the necessary founding premise for drug approval, under the revised plan it will be necessary to have a manufacturer/marketing company with quality control and post-marketing safety management structures in operation.

Another important change under the revised plan is that any factory that manufactures drugs must have approved buildings and facilities and must have obtained authorization to manufacture drugs. Overseas factories must be certified rather than authorized, after going through a similar procedure.

Manufacturing approval will be granted after a review of quality, effectiveness, and safety has been conducted. In addition, manufacturing and marketing approval may be granted once it is verified that the factory has a requisite permit and that its product(s) conform(s) to the GMP. With the adoption of the new procedures, the present manufactured item-based authorization, which focused on the act of manufacturing as its basic premise, will be abolished. In another development, drug substances that were subject to production approval are no longer so under the new manufacturing and marketing approval system, because they are not distributed to medical institutions. As a consequence, the DMF system implemented in the USA and Europe was adopted in the form of a drug substance register. Other matters to be considered in the future are listed in Table 2.

Table 2: Other topics under review

Manufacturing and marketing company authorization

- Standards related to quality control methods
- Standards related to post-manufacturing and marketing safety management methods

Production industry authorization

- Standards related to plant building and facilities

Certification of foreign manufacturers

- Certification methods

Manufacturing and marketing approval

- Review methods
- Extent of minor changes that do not require application for partial change of approval

Drug Master File

- Items that should be registered

Biological products

- Labeling/record storage, etc.

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