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Production of novel bioactive compounds by enzymes, and their application to food*

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Abstract: Recently, people have been paying greater attention to their health and, as a result, the need to use physiologically functional foods was found on the market. For these reasons, the market size of "foods for specified health use" (FOSHU) in Japan has grown and was approximately ¥700 billion in 2008.

Many enzymes such as amylases and proteases have been used in food manufacturing because of their diversity, specificity, and mild condition in reaction.

The aim of this investigation was the production of novel bioactive compounds by three kinds of transglycosylation reaction of the amylolytic enzymes, and the research and development of physiologically functional foods using these compounds.

Phosphoryl oligosaccharides of calcium (POs-Ca) are a complex with Ca and phosphoryl oligosaccharides prepared from potato starch by a hydrolysis (transglycosylation to H_2O) of amylolytic enzymes. The chewing gum included POs-Ca prevented dental caries in humans.

Highly branched cyclic dextrin (HBCD) was produced from amylopectin by branching enzyme (intramolecular transglycosylation), which had a relatively narrow molecular-weight distribution compared with commercially available dextrins. The sports drink containing HBCD enhanced swimming endurance in mice and humans.

α-Glycosylhesperidin (G-Hsp) was produced from starch and hesperidin, a flavonoid found abundantly in citrus fruits, by the intermolecular transglycosylation using cyclodextrin glucanotransferase. Oral administration of G-Hsp improved rheumatoid arthritis in mice and humans, and poor blood circulation in women.

In this study, we looked to prove that this enzymatic modification technique was useful in creating unique and effective physiologically functional foods. These functional foods are expected to improve the health and quality of life of many people.

Keywords: branching enzyme; cyclodextrin glucanotransferase; dental caries; functional foods; glycosylhesperidin; highly branched cyclic dextrin; phosphoryl oligosaccharides; rheumatoid arthritis; transglycosylation; sports drinks.

INTRODUCTION

Since ancient times, there has been the concept that food and medicine have a common origin; this concept has been especially prominent in China, India, Europe, and Japan. In Japan, studies of the physiological function in foods began in 1984 as a national project under the sponsorship of the Ministry of

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Education, Science, and Culture; in 1991 the Ministry of Health and Welfare initiated a world-first policy by legally approving physiologically functional foods, terming them "foods for specified health use" (FOSHU) [1,2]. The first FOSHU product was hypoallergenic rice, made widely available after multisite clinical intervention trials [3] were reported in the journal *Nature*, under the title, "Japan explores the boundary between food and medicine" [4]. Japanese food companies have sought to produce physiologically functional foods to improve people's quality of life, and under the FOSHU registration system, the market size and number of FOSHU in 2008 were approximately ¥700 billion and more than 850 products, respectively; the market size increased five-fold in these 10 years.

Among the compounds used in physiologically functional foods, some of them were produced using enzymes, e.g., peptides produced with protease (which brings about opioid [5], vasorelaxing [6], antihypertensive [7], or immunostimulating effects [8], etc.) and oligosaccharides [9] produced by amylolytic enzymes (which promote the proliferation of bifidobacteria, the absorption of minerals, and immunopotentiating activities, among others).

The aim of this study was to contribute to the maintenance and improvement of human health through the provision of physiologically functional foods; we therefore prepared novel bioactive compounds originating from food materials with enzymes and applied them to these foods. The material and enzymes that we used were starch and amylolytic enzymes, such as α-amylase, glucoamylase, pullulanase, branching enzyme, and cyclodextrin glucanotransferase. As shown in Fig. 1, their reactions tended to follow one of three patterns: (1) hydrolysis (i.e., transglycosylation to H_2O, in which glucose moiety in starch or dextrin is transferred to H_2O, to produce smaller molecules), (2) intramolecular transglycosylation (i.e., in which glucose moiety is transferred to another part of the same molecule, to produce cyclic compounds such as cyclodextrins), and (3) intermolecular transglycosylation (i.e., in which glucose moiety in starch is transferred to another molecule, to produce new glycosides). Transglycosylation is defined as the transfer of a glycosidically bound saccharide to another hydroxyl group. In this study, we produced: (1) phosphoryl oligosaccharides (POs) from starch, by hydrolysis; (2) highly branched cyclic dextrin (HBCD) from starch, by intramolecular transglycosylation; and (3) α-glycosylhesperidin (G-Hsp) from starch and hesperidin, by intermolecular transglycosylation. We then applied these products to chewing gum, to prevent dental caries; sports drinks, to increase athletic endurance; and food, to alleviate rheumatoid arthritis symptoms and improve blood circulation.

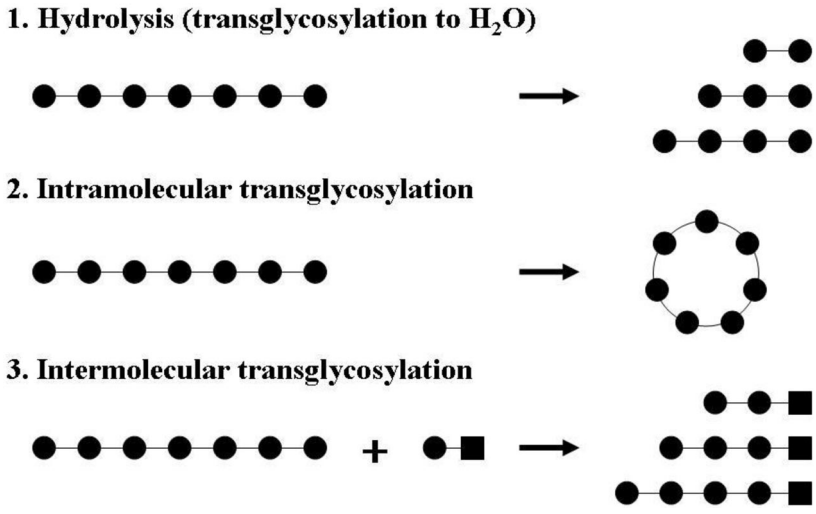

Fig. 1 Three types of transglycosylation reaction in amylolytic enzymes. ●: glucose; ■: acceptor compound; –: α-1,4 glycosidic linkage.

PHOSPHORYL OLIGOSACCHARIDES OF CALCIUM AND THEIR APPLICATION (TRANSGLYCOSYLATION TO H_2O)

Starches from various sources have been used widely—not only in dairy-based diets, but also in food manufacturing, as a nutrient, texture modifier, and a component of such sweeteners as glucose and maltodextrin. Starch consists of two components, amylose and amylopectin; the former is an essentially linear glucan linked with α-1,4 glycosidic linkages, while the latter is highly branched with α-1,6 glycosidic linkages.

It was reported that the phosphate (P) groups are located mostly in amylopectin, with very little presence in amylose [10]; ester phosphorus is widely distributed in starch from various plants. Among its components, potato starch is well known to contain esterified phosphoryl groups [10,11], especially, potato amylopectin contains 100–1,000 ppm of ester phosphorus, and about 70 % of the P groups are linked to C-6 of the glucosyl residues, with the rest being linked to C-3 [12].

In the industrial production of glucose from starch, amylolytic enzymes such as α-amylase, glucoamylase, isoamylase, and pullulanase are hindered by P groups linked to glucosyl residues. Hence, glucose and POs are produced; whereas the former is widely used as a food material and in intravenous fluids, the latter is discarded as an unused material.

As POs have a negative charge based on the P groups within the molecule, cations such as Ca and iron will attach to the molecule. By mixing Ca with POs, phosphoryl oligosaccharides of calcium (POs linked with calcium; POs-Ca) are produced; Ca is then solubilized. Therefore, this study focused on the use of POs-Ca as solubilized Ca.

Production of POs from potato starch

To produce glucose from potato starch, starch was hydrolyzed into glucose with a combination of α-amylase (which hydrolyzes an α-1,4 glycosidic linkage at random with endo-wise), glucoamylase (which hydrolyzes an α-1,4 glycosidic linkage from the reducing end of starch), and pullulanase (which hydrolyzes an α-1,6 glycosidic linkage). The reaction mixture containing glucose and residual oligosaccharides (POs) was separated with an ion-exchange column; the POs were then fractionated into two fractions, PO-1 and PO-2. The components of the POs were analyzed via high-performance anion-exchange chromatography and a pulsed amperometric detector system [13]. Fraction PO-1 was the major component of POs and comprised maltotriose, maltotetraose, and maltopentaose to which one P group was attached [14]. Fraction PO-2 predominantly comprised maltopentaose and maltohexaose to which at least two phosphoryl groups were attached [15]. The structure of fraction PO-1 is shown in Fig. 2.

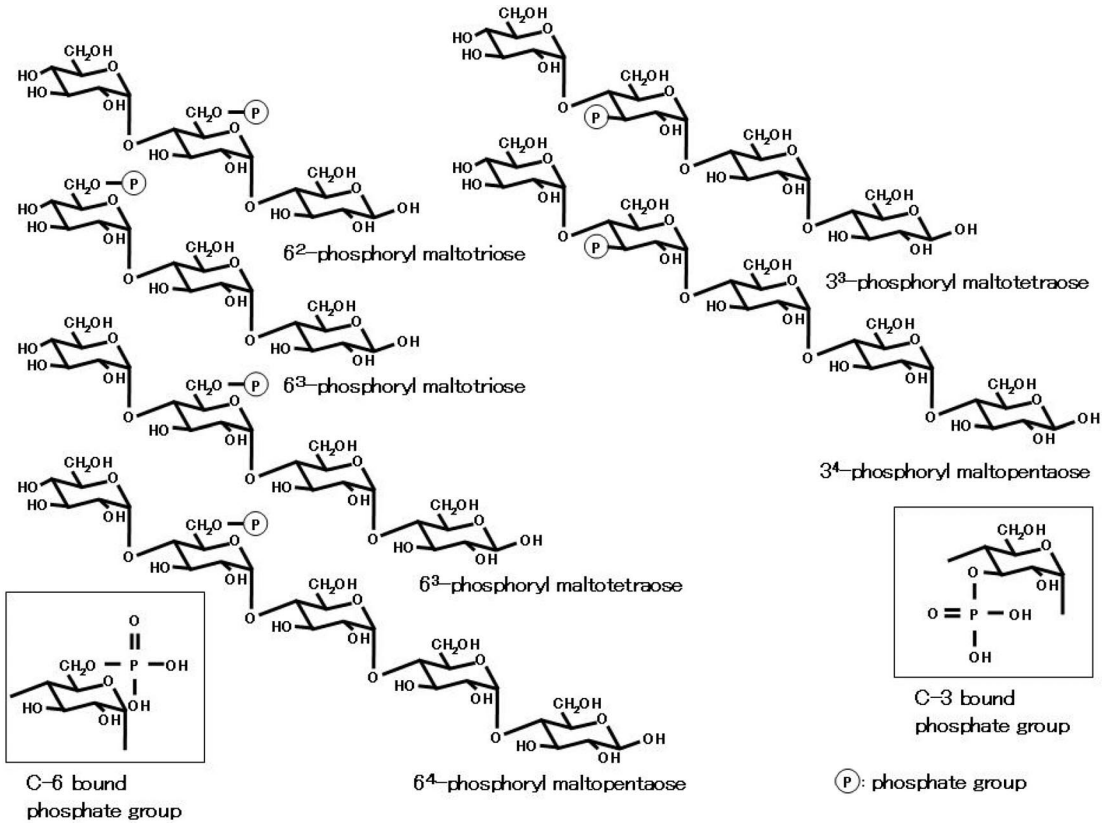

Fig. 2 Structures of PO-1 fraction of POs.

Prevention of dental caries by POs-Ca

Figure 3 illustrates the cycle of demineralization (i.e., the process of mineral loss from tooth enamel during the early stages of dental decay) and remineralization (i.e., the reversal of the demineralization of tooth enamel) that occurs in an oral cavity. In general, after every meal, plaque is always produced at the surface of the tooth enamel by cariogenic bacteria such as *Streptococcus mutans*. In that plaque, organic acid is produced by bacteria, and the pH of the oral cavity then decreases. This promotes demineralization in the enamel region of the teeth. When oral hygiene and the secretion of saliva are sufficient, remineralization occurs through the buffer action of saliva and by the supply of Ca and P ions from saliva. However, if dental cleaning or the secretion of saliva in the oral cavity is insufficient, the tooth will proceed to decay, creating dental caries. Therefore, it is expected that research into compounds that prevent dental caries will be highly advantageous.

The effects of the daily use of a chewing gum containing 2.5 % POs-Ca on the remineralization of enamel were examined. First of all, an in vitro human saliva immersing (HSI) test was conducted using POs-Ca [16]. Two types of chewing gum were prepared; one contained 2.5 % POs-Ca and 46 % xylitol as a sweetener (POs-Ca gum), and the other contained 48.5 % xylitol without POs-Ca (xylitol gum). Both gums were sugar-free. While 12 volunteers (6 males and 6 females; mean age, 29.9 year-old) chewed each gum (weighing 3 g) for 20 min, all saliva stimulated was collected for the first 10 min (Fs) and last 10 min (Ls) separately. The volume and mineral contents in the collected saliva were analyzed; the results thereof are shown in Table 1. The salivary volume of Fs and Ls secreted on account of the chewing of gum with or without POs-Ca were nearly equal, and not significantly different. The

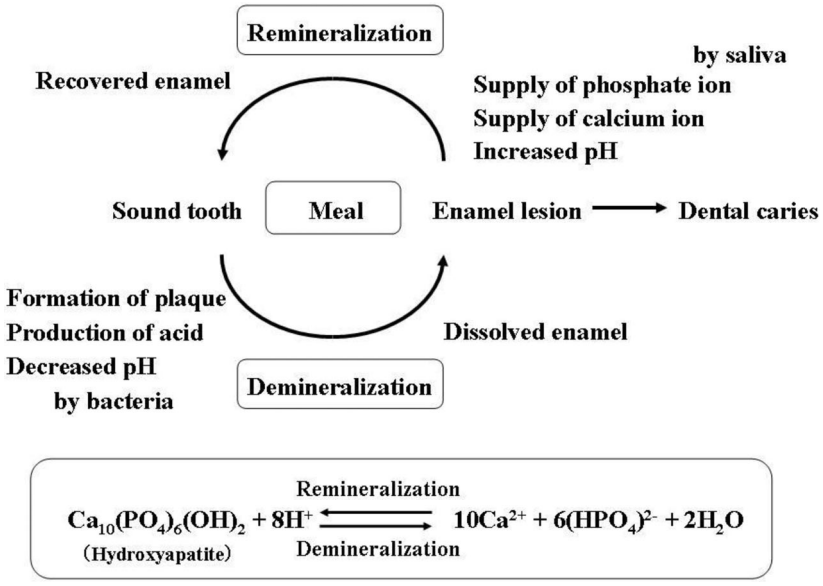

Fig. 3 Cycle of demineralization and remineralization of tooth enamel in an oral cavity.

concentrations of inorganic P in both groups' saliva were not significantly different, either. However, the concentration of Ca in Fs when chewing POs-Ca gum was much higher than that in Fs when chewing xylitol gum; the difference therein was statistically significant. Therefore, the Ca/P ratio values in Fs from the POs-Ca gum were significantly higher than those from xylitol gum. Although the Ca/P ratio value was found to decrease gradually during gum-chewing, the value at the beginning of chewing POs-Ca gum was 1.12—nearly the same value of hydroxyapatite in enamel (i.e., 1.67). POs-Ca was thus proven to provide Ca in the oral cavity. This result seemed suitable for remineralization.

Table 1 Analysis of volumes and mineral contents in saliva.

		Fs		Ls		
	POs-Ca	Means±SD	P^a	Means±SD	P^a	P^b
Salivary volume (mL)	+	20.34±4.13	ns	9.35±3.24	ns	**
	-	20.74±4.43		9.65±3.35		**
Ca (mM)	+	6.29±2.44	**	1.72±0.27	*	**
	-	1.69±0.41		1.39±0.37		ns
P (mM)	+	5.62±1.41	ns	6.22±1.31	ns	ns
	-	6.15±1.35		6.49±1.15		ns
Ca/P	+	1.12±0.31	**	0.27±0.05	*	**
	-	0.28±0.08		0.22±0.05		ns

P^a; for POs-Ca vs. Xylitol, P^b; for Fs vs. Ls, ns; not significant, *; $p<0.05$, **; $p<0.001$

Fs; collected whole saliva for the first 10 min, Ls; collected whole saliva for the last 10 min.

To evaluate the effect of POs-Ca on remineralization, we proceeded with an in vitro experiment. Bovine enamel slabs were demineralized with lactate. They were immersed in the mineral solution with or without POs-Ca for 1 week at 37 °C. After this treatment, the level of remineralization in the enamel slabs were analyzed by a previously described method [17,18] and a scanning electron microscopy. Mineral loss in the enamel slabs with POs-Ca was recovered more than that without POs-Ca, as shown in Fig. 4. From these results, it was determined that POs-Ca promoted a greater amount of remineralization activity.

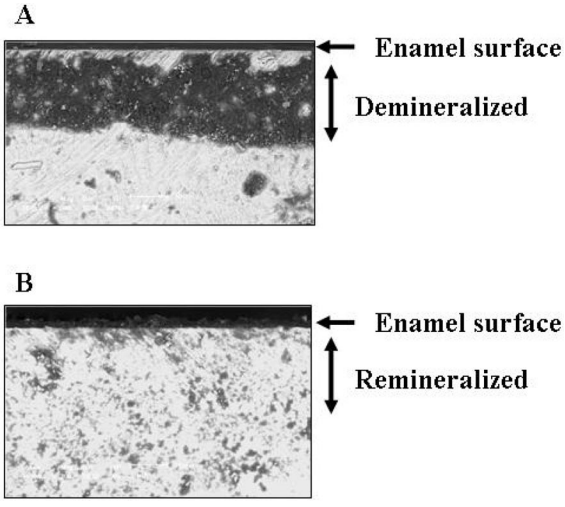

Fig. 4 Effects of POs-Ca on remineralization of tooth enamel. Bovine tooth slabs were demineralized in a 0.1 M lactate gel (pH 5.0) at 37 °C for 3 weeks; they were then immersed in a mineral solution containing 20 mM Hepes buffer (pH 7.0), 1.5 mM $CaCl_2$, and 0.9 mM KH_2PO_4, with (B) or without 0.07 % POs-Ca (A). The dental caries-like lesions were scanned with an electron microscope.

Based on the aforementioned results, the effects of chewing gum containing POs-Ca on tooth-enamel remineralization were investigated in situ. Twelve healthy adult volunteers (6 males and 6 females; mean age, 21.0 year-old) were randomly divided into 3 groups, each of which used, in a double-blind manner, chewing gums with or without POs-Ca (based on xylitol as a sweetener), or sucrose-based placebo chewing gum. Each volunteer wore a palatal appliance to which a demineralized bovine enamel disk was attached, as described above for the in vitro experiment; each volunteer chewed one of the gums 4 times a day (after each meal and before bed time) for 4 weeks. The chewing time was 20 min, and the palatal appliance was kept in the oral cavity for an additional 20 min. Remineralization of each enamel disk attached to the palatal appliance was evaluated by lesion depth, which was the depth of the demineralized part in the enamel disk from its surface. The lesion depths of the enamel disks were shown in Fig. 5 [19]. The remineralization rates (i.e., the percentage of lesion depth with respect to that after initial demineralization) in the group chewing POs-Ca gum were 67, 54, and 76 % at the first, second, and fourth week, respectively. The corresponding values in the group that chewed the gum without POs-Ca were 12–23 %—obviously much lower than those in the POs-Ca gum group. The values in the placebo gum group were much lower than those of these two groups.

Based on these results, a chewing gum containing POs-Ca obtained FOSHU accreditation from Japan's Ministry of Health and Welfare in 2003.

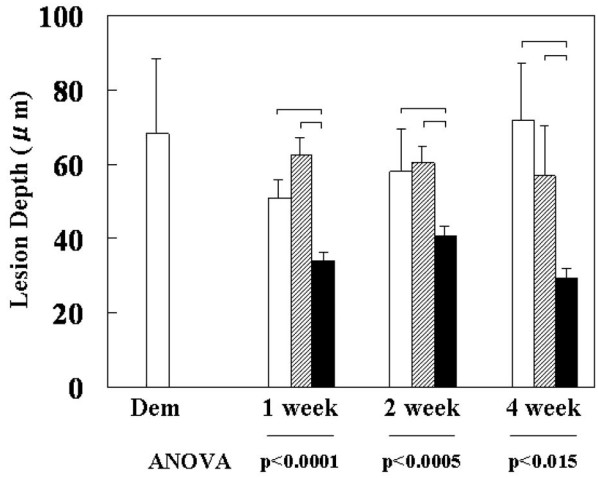

Fig. 5 Comparison of lesion depth by durations of intraoral experiments. □: placebo chewing gum (based on sucrose as a sweetener); ▨: chewing gum without POs-Ca (based on xylitol); ■: chewing gum with POs-Ca (based on xylitol). Dem: initial lesion depth of demineralized enamel disk. Horizontal bars indicate statistically significant difference at $p < 0.05$ (Turkey multiple comparison).

HIGHLY BRANCHED CYCLIC DEXTRIN AND ITS APPLICATION (INTRAMOLECULAR TRANSGLYCOSYLATION)

Starch and its derivatives—such as oligosaccharides—have been widely used in the food industry, as described above, because various types of amylolytic enzymes that react to starch have been found in various sources (microorganisms, plants, insects, and animals) and applied in an industrial scale [20].

Whereas branching enzyme (1,4-α-D-glucan: 1,4-α-D-glucan 6-α-D-(1,4-α-D-glucano)-transferase, EC 2.4.1.18; BE) is well known to catalyze the α-1,6 glycosidic linkage of amylopectin and glycogen, it has been reported that the enzyme synthesizes a new dextrin called HBCD. Its structure has been analyzed in great detail [21,22].

Production of HBCD from waxy starch

The actions of branching enzyme and α-amylase with respect to amylopectin are compared in Fig. 6: While the former split the inner chains connecting the cluster units (multi-branched glucan) of amylopectin (arrow heads in Fig. 6) and transferred the cluster unit to another site on the same substrate molecule (intramolecular transglycosylation), the latter hydrolyzed the α-1,4 glycosidic linkage in amylopectin at random. Therefore, branching enzyme synthesized dextrin with a relatively narrow molecular-weight distribution, while α-amylase synthesized various dextrins with a wide molecular-weight distribution, according to the reaction condition of the enzyme.

The structure and molecular weight of HBCD have been elucidated to be between those of amylopectin and oligosaccharides, and HBCD bears the properties of both amylopectin (i.e., little sweet taste, low osmolality, difficult to brown, etc.) and oligosaccharides (i.e., high water solubility, high stability in water, low viscosity, few specific odors that starches have, etc.).

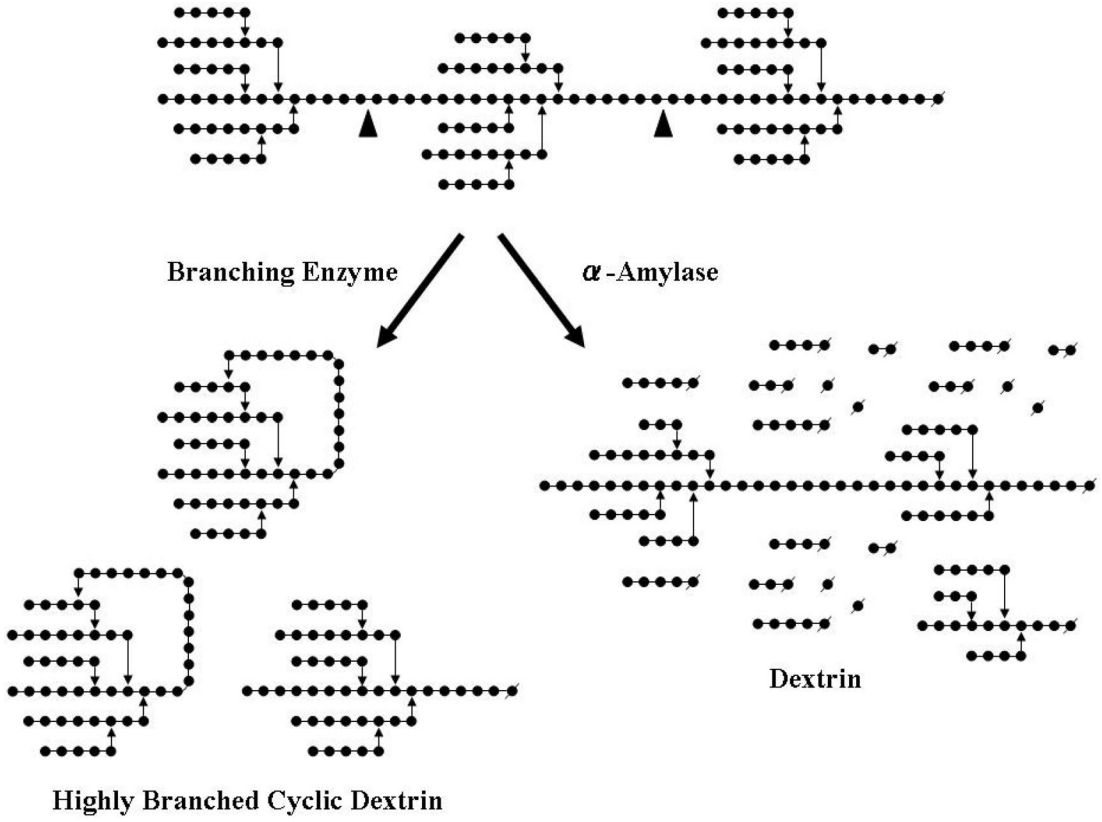

Fig. 6 Reaction pattern of branching enzyme to amylopectin, compared with α-amylase and structure of HBCD. ●: glucose; –: α-1,4 glycosidic linkage; →: α-1,6 glucosidic linkage; ◖: reducing end glucose; ▲: point of branching enzyme reaction.

Enhancement of endurance performance by HBCD

The main impetuses for ingesting sports drinks are the replacement of body fluids lost to sweat and the provision of an exogenous form of energy, typically carbohydrates. As the development of fatigue during prolonged exercise has been associated with the depletion of endogenous carbohydrate stores [23], and given that dehydration results in the loss of more than 2 % of body weight [24], the intake of water and exogenous energy is very important during exercise.

HBCD was found to bear many unique characteristics not found in most commercially available dextrins, as described above. Considering these properties, HBCD seemed an appropriate ingredient in sports drinks—as an energy source [25].

For this purpose, laboratory mice were exercised and administered HBCD solution. After being acclimated to the act of swimming, swimming time to exhaustion was measured for all mice; the average time was 64 ± 5 min (means ± SEM) in the adjustable-current pool, with the intensity of the exercise estimated at about 50 % VO_{2max} [26]. First, HBCD exhibited a dose-dependent effect on swimming endurance: The mean swimming time for the mice that had ingested 500 mg HBCD/kg of body weight was 75 ± 4 min, which is significantly different from that for the mice that had ingested the same volume of water and 166 mg HBCD/kg (65 ± 3 min), when orally administered 30 min after the starting of swimming. Therefore, 500 mg HBCD/kg of body weight was orally administered in this study.

To elucidate the effects of HBCD on swimming endurance, 300 µl of a 5 % HBCD solution, a 5 % glucose solution (as a control, because glucose is a structural unit of HBCD), or water (as a con-

trol) was administered to individual mice 10 min before, 10 min after, or 30 min after the starting of swimming. As shown in Fig. 7 (A-1, A-2), the mice that had ingested HBCD 10 min before swimming showed almost the same level of endurance as those that had ingested water, whereas those that had ingested glucose showed a 40 % poorer endurance performance than those given HBCD. The mice administered HBCD 10 min after the starting of swimming were able to swim significantly longer than those administered water or glucose (Fig. 7; B-1, B-2). The mice administered HBCD 30 min after the starting of swimming swam 20 % longer than those administered water, although the endurance performance of the former was similar to that of those given glucose (Fig. 7; C-1, C-2). These results indicated that, for our purposes, 10 min after the starting of swimming was the best time to administer HBCD to mice. To confirm reproducibility, three cross-experiments were conducted; the swimming

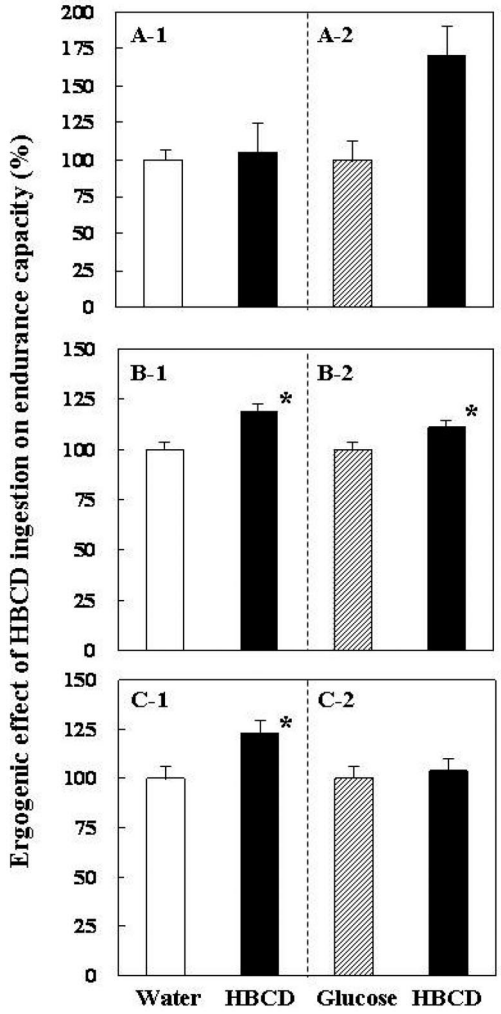

Fig. 7 Relationship between swimming endurance and carbohydrate ingestion. A: 10 min before (n = 9); B: 10 min after (n = 18); and C: 30 min after the onset of the swimming exercise (n = 14). Values in A-1, B-1, and C-1 represent the percentage (means ± SEM) of the swimming time for the mice that had ingested HBCD to that of the mice that had ingested water (as 100 %). Values in A-2, B-2, and C-2 represent the percentage (means ± SEM) of the swimming time for the mice that had ingested HBCD to that of the mice that had ingested glucose (as 100 %). Comparison vs. water (A-1, B-1, and C-1) and glucose (A-2, B-2, and C-2): $*p < 0.05$ (Student's t-test).

time to exhaustion of the mice administered HBCD, glucose, or water 10 min after the starting of swimming were 86 ± 6, 68 ± 5, and 65 ± 5 min, respectively. The mice that had ingested HBCD swam more than 20 % longer than the mice that had ingested glucose or water.

To elucidate the mechanism behind HBCD-based improvements in endurance performance, we examined the postprandial blood glucose and insulin levels after HBCD ingestion, during exercise. The mice were made to swim in the current pool, as described above, and HBCD, glucose, or water was administered to the mice 10 min after the starting of swimming. Another 10 min later—that is, 20 min after the starting of swimming—blood was taken from the neck vein; plasma glucose, insulin, and lactate were analyzed; and the results thereof are shown in Table 2. Plasma glucose in the mice administered HBCD and glucose had increased; apparently, these two substances provided energy to the mice. Additionally, plasma insulin levels had increased according to the concentration of plasma glucose.

Table 2 Changes to metabolic parameters in the serum.

	Treatment		
	Water	CCD	Glucose
Glucose (mg /dl)	74.1 ± 10.9 [a]	109.2 ± 12.5 [b]	147.7 ± 10.8 [c]
Insulin (pg / ml)	77.5 ± 41.3 [a]	250.9 ± 55.3 [a]	437.5 ± 67.5 [b]
Lactate (mM)	2.1 ± 0.5	1.2 ± 0.3	1.6 ± 0.4

1; Each solution was administered to the mice 10 min after the onset of swimming
2; Each value represents the mean ± SEM for 5 - 8 mice.
a-c; Means in the same row with no common superscripts are statistically significant

Due to its high molecular weight, HBCD requires more time than glucose for digestion in the small intestine during exercise. Therefore, the postprandial glucose and insulin responses in mice that had ingested HBCD were lower than those that had ingested glucose. On the other hand, as ingested glucose led to rapidly elevated plasma insulin levels, it is thought to cause hypoglycemia afterward [27]. The result, as shown in Fig. 7 (A-1, A-2), is that hypoglycemia was thought to have occurred in mice that had ingested glucose. As a result, the plasma lactate levels of the mice that had ingested glucose were also higher than those of the mice that had ingested HBCD.

From these results, HBCD is thought not to cause hypoglycemia, and to supply energy longer than glucose; consequently, it enhances swimming endurance in mice. This finding agrees with that of a study of human subjects, in which 7 swimmers (20.0 ± 0.3 year-old) who participated in the Japan Championship in 2003 ingested each 1.5 g/kg HBCD, glucose, or water (as a control), just before swimming. After the interval training (at VO_{2max} 75 %; swimming for 5 min and rest for 3 min), swimming time to exhaustion (at VO_{2max} 90 %) was measured in a current-flow pool. The percentage of swimming time to exhaustion was 163.1 % for HBCD, 91.6 % for glucose comparing with that for water (as 100 %), respectively, in which HBCD showed significant difference from water (Student's *t*-test, $p < 0.05$).

Gastric emptying time of HBCD-based sports drink

Since the nutrients and water are absorbed mainly from the intestine, not from the stomach, the balance between the concentration of carbohydrates and its osmotic pressure to accelerate gastric emptying has been under discussion. A high concentration of carbohydrates in a drink, which is intended to increase the amount of carbohydrates delivered as an energy source, delays gastric emptying due to high osmotic pressure, and thus decreases the amount of fluid available for absorption. Conversely, a low concentration of carbohydrates accelerates gastric emptying and thus increases the amount of fluid available, but

decreases the level of carbohydrates delivered. Therefore, the relationship between carbohydrate content and osmotic pressure in the formulation of sports drinks has been under great scrutiny.

First of all, we measured the rate of gastric emptying of HBCD and other carbohydrate solutions, using ultrasonographic techniques with 7 healthy men and 3 healthy women (26.2 ± 6.7 and 24.3 ± 2.3 year-old, respectively) [28]. As the relaxed cross-sectional area of the pylorus antrum has been reported to be well correlated with gastric volume—which was measured by an extracorporeal ultrasonic echo-image analyzer, after ingesting a drink [29]—the gastric emptying characteristics of the solutions are shown as gastric emptying times derived from gross half-gastric volumes. The gastric emptying times of the solutions increased as a function of increasing concentrations of HBCD, glucose, and other commercially available dextrins based on glucose unit [maltose, dextrin (DE = 40), dextrin (DE = 20), in which the dextrose equivalent (DE) is defined as the degree of hydrolysis of starch into glucose], as shown in Fig. 8. A strong correlation was observed between gastric emptying time and the osmotic pressure of these solutions, except with water, physiological saline, and HBCD solutions ($y = 0.047x + 9.856$; $R^2 = 0.875$; y: gastric emptying time; x: osmotic pressure). As HBCD has a relatively large molecular weight and contains only a small amount of low-molecular-weight dextrin, solutions thereof have very low osmotic pressure. For example, a 10 % HBCD solution has an osmotic pressure of 9 mOsm/kg; when sports drinks based on 10 % HBCD or 10 % dextrin (DE = 16) contain the same concentrations of minerals, vitamins, and organic acids, their osmotic pressures are 150 mOsm/kg and 269 mOsm/kg, respectively. HBCD-based sports drinks are transferred from the stomach to the small intestine significantly faster than the dextrin (DE = 16)-based one.

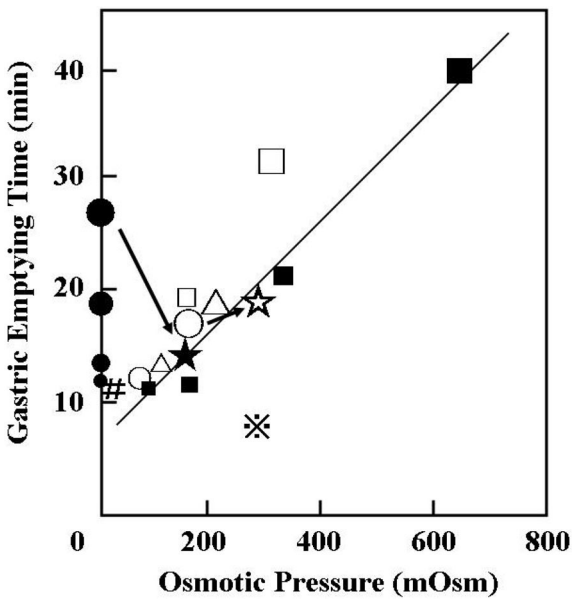

Fig. 8 Relationship between gastric emptying time and osmotic pressure of test solutions. ●: HBCD; ■: glucose; □: maltose; △: dextrin (DE = 40); ○: dextrin (DE = 20). Concentrations (%) are represented by the size of the symbols, from the smallest to the largest: 1.25, 2.5, 5.0, and 10.0 %. ★: HBCD-based sports drink; ☆: dextrin (DE = 16)-based sports drink; #: water; ※: physiological saline.

As participants in endurance events frequently suffer from gastrointestinal disorders such as belching, flatulence, heartburn, nausea, abdominal pain, lateroabdominal stitches, regurgitation, an urge to defecate, and diarrhea, the effects of ingesting HBCD-based sports drinks on gastrointestinal disorders and fatigue during exercise were also investigated [30].

Seven untrained volunteers (34.3 ± 2.8 year-old) participated in an experiment consisting of a preliminary exercise for 9 min (3 min of cycling at each of 3 loads: 56, 71, and 85 W on a cycling ergometer), a 10-min rest, and 30 min of exercise (10 min of cycling at each of 3 loads: 71, 85, and 99 W), all at 25 °C. Volunteers were administered sports drinks containing 10 % HBCD, 10 % glucose, 10 % dextrin (DE = 16), or water (as a control) just after a 9-min exercise. The mean gastric emptying time after the ingestion of an HBCD-based sports drink was significantly faster than that of a glucose-based one. The number of gastrointestinal disorders—i.e., the degree of subjective flatulence and the number of belches—was small with HBCD-based drinks during exercise, compared with other drinks. Consequently, volunteers who ingested HBCD-based sports drinks were able to continue to exercise comfortably, with little fatigue.

These results mentioned above revealed the effects of HBCD on endurance performance, compared with other drinks based on glucose or dextrin with glucose units. When HBCD was ingested, it was transferred from stomach to intestine more quickly to be absorbed as energy, as shown by the ultrasonograph techniques. Furthermore, not only carbohydrate but also other nutrients such as minerals, amino acids, water, and so on would be utilized quickly during exercise. In addition, the ingestion of HBCD was found to utilize as a carbohydrate energy, which raised plasma glucose level and did not raise plasma insulin level so much compared with the ingestion of glucose. Therefore, it did not raise plasma lactate level and, consequently, the endurance performance in mice and humans would be improved.

α-GLYCOSYLHESPERIDIN AND ITS APPLICATION (INTERMOLECULAR TRANSGLYCOSYLATION)

Hesperidin, discovered in 1936 by Szent-Gyorgi as vitamin P [31], is a flavonoid that is abundant in citrus fruits such as Satsuma mandarin oranges (*Citrus unshiu* Marc.) and Valencia oranges (*Citrus sinensis* Valencia). It is also a major component of the traditional Chinese medicine Chen-pi. Hesperidin is well known to exert many biological functions; for example, antioxidative, antiinflammatory, antiviral, and anticarcinogenic [32]. However, since hesperidin is not adequately soluble in aqueous solutions and may not be absorbed well from the intestinal tract, it has not yet been used in the fields of food and medicine.

Production of G-Hsp from starch and hesperidin

To solubilize hesperidin, we planed to transglycosylate hesperidin into its glycosides with an enzyme in an alkaline pH, because it is almost insoluble in water or alcohol but is freely soluble in alkali. In screening alkalophilic bacteria from soil, we found cyclodextrin glucanotransferase [1,4-α-D-glucan 4-α-D-(1,4-α-D-glucano)-transferase (cyclizing), EC.2.4.1.19; CGTase] from an alkalophilic *Bacillus* sp. A2-5a; it was stable and exhibited relatively strong activity in an alkaline pH [33].

α-Glycosylhesperidin (4^G-α-D-glucopyranosylhesperidin (G-Hsp) and series of oligoglucosides; Fig. 9) were synthesized from hesperidin and starch by the enzyme at pH 10; G-Hsp was approximately 300 times more soluble than hesperidin in the aqueous phase [34]. G-Hsp was also found to be absorbed from the intestine about 3 times more readily than hesperidin [35]. Therefore, it would be interesting to determine whether G-Hsp exhibits any biological activity in vivo.

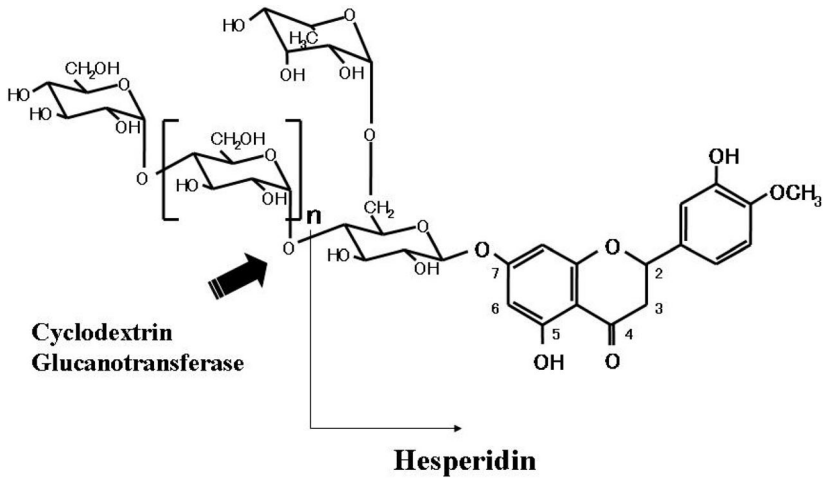

Fig. 9 Structure of G-Hsp. n = 0: monoglycosylhesperidin; n = 1: diglycosylhesperidin; n = 2: triglycosylhesperidin; n = 3: tetraglycosylhesperidin.

Improvement of rheumatoid arthritis by G-Hsp

Rheumatoid arthritis is a chronic autoimmune disease of unknown etiology; it causes long-term joint damage, chronic pain, and loss of function in affected joints [36]. Although a large number of disease-modifying antirheumatic drugs have been developed, such as methotrexate [37,38], biological therapies such as anti-TNF-α antibodies [39–41], and other treatments, they are often associated with adverse effects. Therefore, safer approaches for treating rheumatoid arthritis are required.

Recently, it was reported that the oral administration of hesperidin suppressed collagen-induced arthritis (CIA) in mice [42]. Therefore, the effects of G-Hsp on CIA mice were investigated [35].

To determine the therapeutic effects of G-Hsp on CIA progression, 3 mg G-Hsp was orally administered to CIA mice (body weight: 30 g) 3 times a week, from day 31 after priming with type II collagen emulsified with complete Freund's adjuvant (10 days after the booster dose). Clinical scores on days 52, 54, and 56 were significantly improved by treatments with 3 mg G-Hsp, compared with the control (Fig. 10). To assess the preventive effects of G-Hsp in CIA development, in a separate experiment, 3 mg of G-Hsp was orally administered to CIA mice 3 times a week, starting from the onset of disease (i.e., day 21 after priming). The administration of G-Hsp resulted in significant improvements in clinical scores at later stages, similar to those undergoing therapeutic treatment.

In histopathological observations, the extent of inflammation among knee joints (increases in synovial and inflammatory cells and pannus formation) in the G-Hsp group improved more than those in the control group. The synthesis of proteoglycan was reduced and the matrix metalloprotease activated in the CIA mice, and then each joint was damaged. In the case of G-Hsp group, the former was recovered and the latter was inhibited. Such was not the case in the control group. These results indicate that the administration of G-Hsp may improve the symptoms of rheumatoid arthritis.

To confirm the effects of G-Hsp on rheumatoid arthritis in humans, 19 volunteers with rheumatoid arthritis were divided into 2 groups; one (n = 9) was administered 3 g of G-Hsp per day, while the other (n = 10) was administered 3 g of dextrin (DE = 16) as a placebo for 12 weeks, using a double-blind method.

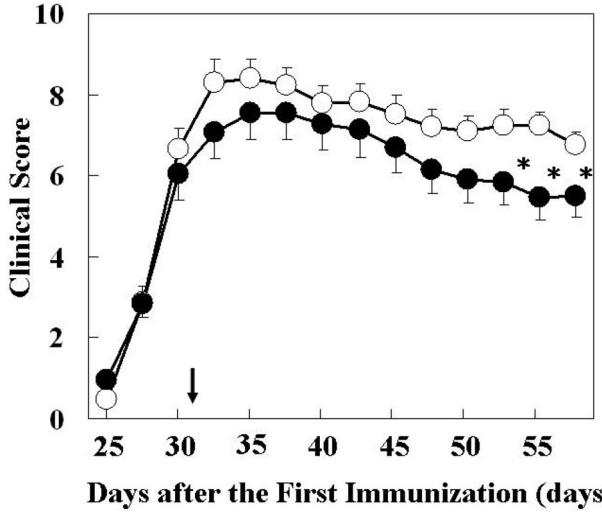

Fig. 10 Therapeutic effects of G-Hsp on CIA in mice. DBA/1J mice with CIA were orally administered water (○; control, $n = 18$) or G-Hsp (●; $n = 17$) after the onset of arthritis. Arrow shows the beginning of ingestion of G-Hsp in mice. Comparison vs. control: $*p < 0.05$ (Scheffe's post-hoc test).

As shown in Fig. 11A, the physician's global assessment of disease activity using a 100-mm visual analog scale (VAS) of the American College of Rheumatology's (ACR) 20 criteria decreased at weeks 4 and 8 in both the placebo and G-Hsp groups, compared with baseline (in the placebo group, significantly decreased at week 8; in the G-Hsp group, significantly decreased at weeks 4, 8, and 12). Although the VAS evaluation worsened in the placebo group at week 12, the G-Hsp group showed a tendency to improve. Although there was no significant difference in the VAS values between the

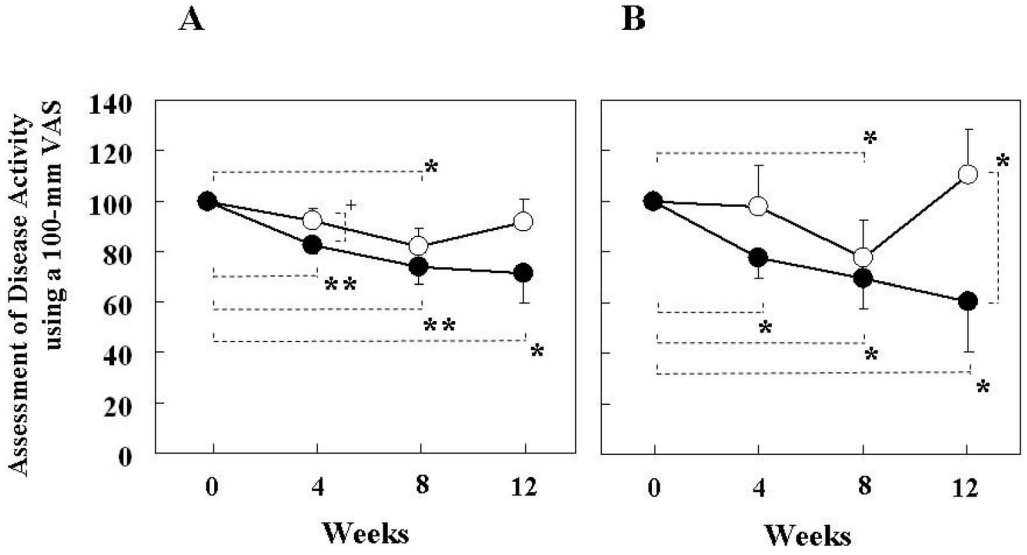

Fig. 11 Assessment of disease activity using a 100-mm VAS. A: Physician's global assessment of disease activity; B: Patient's global assessment of disease activity, using a 100-mm VAS in ACR20. Placebo: ○; G-Hsp: ●. Comparison vs. baseline. +: $p < 0.10$; *: $p < 0.05$ and $**p < 0.01$ (Student's t-test). Comparison vs. placebo: $+p < 0.10$ and $*p < 0.05$ (Mann–Whitney U-test).

placebo and G-Hsp groups, the G-Hsp group showed a tendency to improve at week 4 ($p = 0.078$). In addition, the global assessment of disease activity by patients themselves had the same tendency as that made by the physician; it was shown that the G-Hsp group significantly improved more than the placebo group at week 12 (Fig. 11B).

As VAS scoring is subjective, we evaluated the concentration of C-reactive protein (CRP) in plasma, as an objective indicator (Fig. 12). CRP in plasma is known to increase with inflammation. In the G-Hsp group, CRP decreased significantly compared with the placebo group during the study period; after the cessation of G-Hsp ingestion, CRP increased gradually. In the placebo group, the CRP value did not improve during the study period [35].

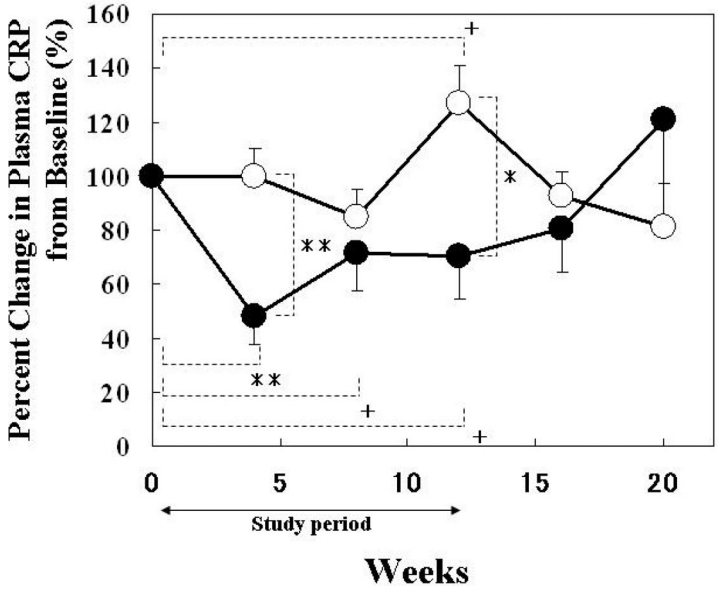

Fig. 12 Change in the plasma CRP concentration from baseline in the patients. Patients were administered Placebo (○) or G-Hsp (●), and the plasma CRP concentration was assayed every 4 weeks for 5 months. Comparison vs. baseline: **; $p < 0.01$, *; $p < 0.05$, +; $p < 0.10$ (by Student's *t*-test). Comparison vs. placebo group: **; $p < 0.01$, *; $p < 0.05$ (by Mann–Whitney *U*-test).

In general, therapeutic efficacy was evaluated by a physician using ACR20 criteria for the diagnosis of rheumatoid arthritis [43]. The administration of G-Hsp was found to be more effective than that of placebo, with respect to symptom alleviation. Therefore, G-Hsp will be useful as a complementary/alternative medicine in standard antirheumatoid therapy. However, a long-term study with a larger sample size (e.g., patients in various stages and classes of rheumatoid arthritis) is necessary.

Improvement in blood circulation by G-Hsp

In Japan, many women feel cold—not only in winter, but also in summer, in air-conditioned environments. One reason for this is poor blood circulation. Therefore, we looked to investigate blood-circulation improvements facilitated by the administration of G-Hsp.

Following the ingestion of 50–3600 mg/person G-Hsp and subsequent testing, 250 mg G-Hsp was found to be the minimum dose for improvements of blood circulation to be evident. So, G-Hsp (250 mg) or placebo (250 mg sucrose) was administered to 11 women (29.6 ± 3.9 year-old) with coldness of the extremities, using a double-blind, placebo-controlled, cross-over methodology [44]. After ingestion of

G-Hsp or placebo, the skin surface temperature after cold-loading at 15 °C for 1 min was recorded, with time, using thermography (Fig. 13). Whereas the skin surface temperature in the placebo group (<30 °C) was not improved 40 min after cold-loading, that in the G-Hsp group (33–34 °C) was improved to preloading levels, just 20 min after cold-loading. Both the width of blood vessels and blood flow in the finger were reduced after cold-loading, but recovered gradually in the G-Hsp group; recovery in the placebo group was slower than that in the G-Hsp group. From these results, it can be concluded that the administration of G-Hsp leads to an extension in the width of blood vessels and therefore an increase in blood flow—both of which consequently lead to a recovery of skin surface temperature. Furthermore, the continuous administration of G-Hsp (250 mg/day for 7 days) maintains this effect, and may alleviate poor blood circulation.

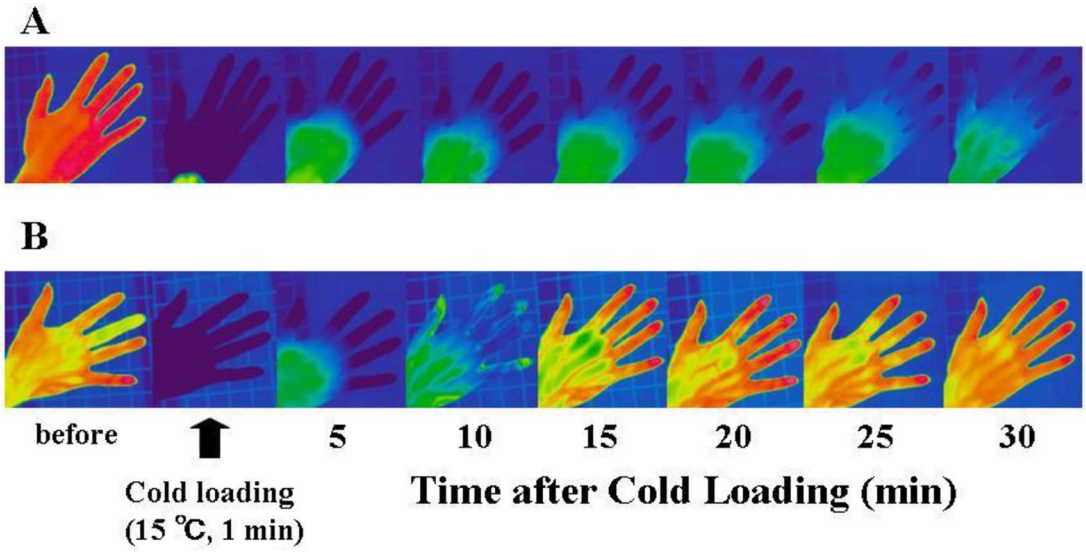

Fig. 13 Effect of G-Hsp on the improvement of poor blood circulation in women. A: Placebo (250 mg sucrose) or B: G-Hsp (250 mg) was administered to women with coldness of the extremities; skin surface temperature after cold-loading at 15 °C for 1 min was recorded, with time, using thermography.

Figure 14 offers a summary comparison of the bioactivities of G-Hsp and hesperidin. Hesperidin, bearing many bioactivities, was transglycosylated into G-Hsp; its solubility and absorption into the body increased approximately 300- and 3-fold, compared with those of hesperidin, respectively. G-Hsp still retained the bioactivities of hesperidin, e.g., anticarcinogenic characteristics [45] and improvements to bone mass density [46]. Furthermore, newly discovered benefits—namely, blood-circulation improvements and alleviation of rheumatoid arthritis symptoms—were found during our study.

Because most bioactive compounds exert their activities after being absorbed into the body, improvements in absorption are very important with regard to the compounds' use in food and medicine; transglycosylation by enzymes, in particular, will be a very useful tool, as described in this study.

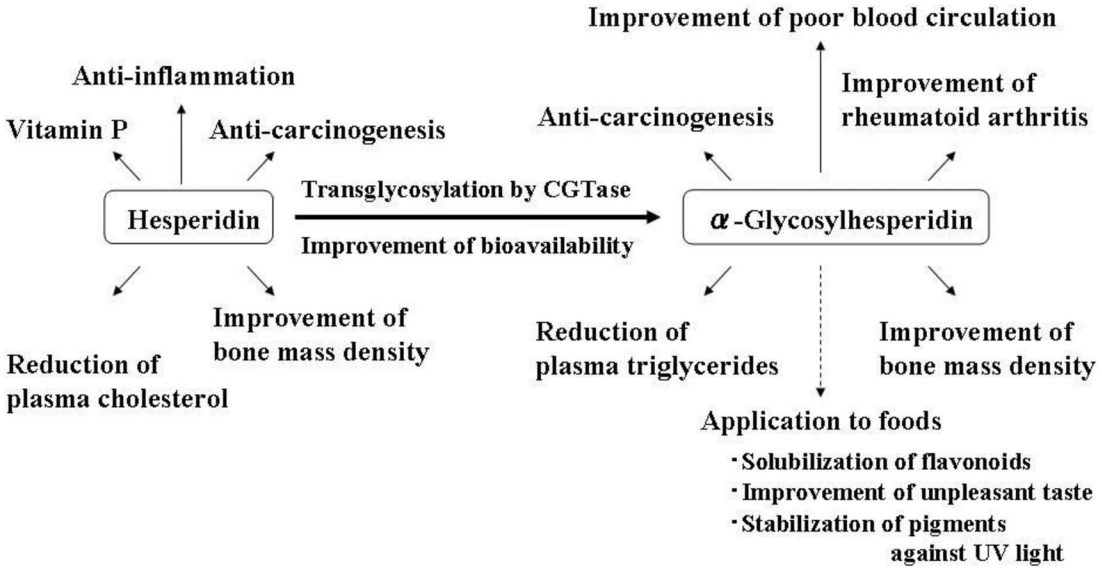

Fig. 14 Summary of bioactivities of G-Hsp.

CONCLUSIONS

Recently, consumers paid much greater attention to their health, and in order to respond to various needs, many kinds of physiologically functional foods have been needed.

As modifications of compounds can change the structure, characteristics, and bioavailability of them, it is possible to create novel functions. This study looked to prove that enzymatic modifications are useful in creating unique and effective physiologically functional foods.

There are a variety of environmental conditions on the earth—including low or high pressure or temperature, high salt concentrations, and low or high pH—and a wide variety of organisms live in each of them, and we can cull a variety of enzymes from them. By using such enzymes, it is possible to produce novel physiologically functional foods that are suitable for people's needs—which is to say, make them healthier and improve their quality of life.

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