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RESEARCH NOTE

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Cryo- and Thermo-protective Effects of Enzymatically Synthesized β-Galactosyl Trehalose Trisaccharide

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Abstract The effects of β-(1,6)-galactosyl trehalose trisaccharide (β-GT) that was preferentially produced by *Escherichia coli* β-galactosidase on cryo- and thermo-protections of protein were investigated with those of other sugars at the level of 8% (w/v). As compared to a control without sugar additive, β-GT effectively enhanced 32-54% of the cryoprotection of fish actomyosin against repeated freeze-thawing and frozen storage, and also 49% of the protection against thermal inactivation of pyrophosphatase, respectively. As a result, it was significantly more effective than sorbitol and trehalose in both cryoprotection and thermoprotection. Thus, β-GT would be possibly applied as a sugar substitute for cryo- and thermo-protective applications of food protein.

Keywords: β-galactosyl trehalose, fish actomyosin, cryoprotection, thermoprotection

Introduction

Trehalose is a non-reducing disaccharide in which 2 glucose molecules are bonded in an α, α-(1↔1)-glucosidic linkage that makes it essentially non-reducing and highly resistant to hydrolysis (1). It is widely distributed in various organisms such as bacteria, yeast, fungi, insects, and plants, where it may serve as a source of energy and a protectant of proteins and cellular membranes from such environmental stress conditions as dehydration, heat, and freezing (2). Compared to other sugars like glucose and sucrose, trehalose is known to show greater function in protecting proteins and viable cells from freeze-thawing and frozen storage *in vitro* (3). It has been also reported in mycobacteria that trehalose-based oligosaccharides, being composed of trehalose and additional glucose residue in the α-(1→4)- and β-(1→6)-linkages, or galactose one in the α-(1→6)-linkage, were supposed to play a protective role (4).

Carbohydrates such as mono- and di-saccharides, low molecular weight polyols, and maltodextrins were found to be cryoprotective, which could be added as cryoprotectants to improve protein stability of leached fish muscle during frozen storage (5). Various oligosaccharides including branched-, fructo-, isomalto-, and galacto-oligosaccharides have been also recommended as effective and non-sweet cryoprotectants of fish and beef proteins (6,7). Generally, oligosaccharides have been widely used as a food ingredient due to their favorable properties, such as high water holding, low sweetness, low calories, growth factors for Bifidus, and no dental caries. We have recently prepared trehalose-based trisaccharide that is composed of trehalose and additional galactose preferentially attached in the β-(1→6)-linkage (8). We have also reported that the trisaccharide had markedly enhanced hygroscopicity and better physiological properties with trehalase-indigestibility (9). In this study, we aimed to evaluate the galactosyl trehalose trisaccharide as a cryoprotectant in fish protein and also a stabilizer against thermal inactivation of model protein, with comparing its effectiveness to those of other sugars.

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Materials and Methods

Materials Trehalose dihydrate (98% purity) was purchased from Hayashibara Biochemical Laboratories (Okayama, Japan). Sorbitol, sucrose, maltose, and baker's yeast pyrophosphatase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Commercial galactooligosaccharide (mainly composed of β-D-Gal*p*-(1→4)-α-D-Gal*p*, β-D-Gal*p*-(1→3)-α-D-Gal*p*, β-D-Gal*p*-(1→4)-α-D-Glc*p*, and β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*, 60% purity) was obtained from Samyang Genex Co. (Seoul, Korea). All other chemicals used were of reagent grade.

Enzymatic preparation of β-galactosyl trehalose trisaccharide β-(1→6)-Galactosyl trehalose trisaccharide (β-GT) was preferentially prepared by the transgalactosylation reaction of *E. coli* β-galactosidase with 30%(w/v) trehalose and 10%(w/v) lactose in 50 mM Tris-HCl buffer (pH 7.5) and purified by BioGel P2 gel filtration and recycling preparative HPLC according to the previous report (8).

Actomyosin preparation Actomyosin (AM) was prepared as previously described (6,10). It was extracted from freshly sacrificed fish, Alaska pollock, purchased from market by blending 10 g flesh in 100 mL of chilled 0.6 M KCl (pH 7.0) with a homogenizer. The extract was centrifuged at 5,000×g for 30 min (4°C). AM was precipitated by diluting with 3 times volume of chilled distilled water and collected by centrifuging at 5,000×g for 30 min (4°C). The precipitated AM was then dissolved with an equal volume of chilled 1.2 M KCl (pH 7.0) and used for further experiment (11).

Ca^{2+}-ATPase activity In order to assay the ATPase activity in fish muscle, 1 mL of AM solution (0.6 M KCl, pH 7.0) prepared was mixed with 0.5 mL of 0.5 M Tris-HCl buffer (pH 7.0), 0.5 mL of 0.1 M $CaCl_2$, 7.5 mL of deionized water, and 0.5 mL of 20 mM ATP solution (pH 7.0). Then, the mixture was incubated for 5 min at 25°C. The reaction was stopped by adding 5 mL of chilled 15% trichloroacetic acid. The concentration of inorganic phosphate (Pi) released during incubation was measured (12). The specific activity of Ca^{2+}-ATPase was defined as the amount (μM) of inorganic phosphate released per mg protein at 25°C for 1 min.

Effect of sugars on freeze-thaw and frozen storage stabilities of actomyosin To investigate the effect of sugars on AM stability during freezing and thawing, samples were subjected to 2 freeze-thaw cycles, which caused significant AM denaturation without sugar additives. AM solutions with 8%(w/v) various sugars were frozen in liquid nitrogen (−196°C) for 3 min. The frozen samples were held in a bath at −5°C for 60 min and then thawed at 25°C for 5 min (10). After the freeze-thaw cycles, the samples were stored in ice-water (4°C) and Ca^{2+}-ATPase activity was measured. Denaturation of actomyosin during frozen storage (−18°C) with/without sugar additives was also measured for about 2 weeks by using the Ca^{2+}-ATPase activity.

Effect of sugars on thermal inactivation of pyrophosphatase To investigate a protein stabilization of β-GT and other sugars against thermal inactivation, the activity of pyrophosphatase was determined by measuring the amount of total phosphate released at the end of incubation (13). The enzyme solution with 8%(w/v) sugar additives were incubated at 55°C for a certain period up to 30 min, and after aliquots of samples were taken, reactions were performed by adding each enzyme sample in 100 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$ and 2 mM tetrasodium pyrophosphate at 25°C for 5 min. For the control, the enzyme solution contained no sugar additive. The reaction was quenched after 5 min by adding 2 volume of 20%(w/v) trichloroacetic acid, and the concentration of inorganic phosphate released was measured (12). All analyses above in the cryo- and thermo-protections were performed in duplicate.

Results and Discussion

Denaturation and conformational changes of myofibrillar proteins have been known to cause a loss of functional properties and deteriorative changes of frozen stored fish muscle (14). The functional properties of actomyosin in fish muscle allow its use as a protein ingredient in a variety of fabricated products such as seafood analogs and sausages. After freeze-thawing cycles of AM extracted from Alaska pollock with/without 8%(w/v) carbohydrates, the remaining Ca^{2+}-ATPase activity was measured as index of cryoprotection for each sample, because the remaining Ca^{2+}-ATPase activity is shown to correlate well with the stability of fish protein (6,15). Some oligo-saccharides have been recommended as an effective cryoprotectant to substitute sucrose and/or sorbitol commercially used (16,17). Trehalose is also well known to protect proteins from the damage caused by freezing (1). Thus, β-GT would be expected to show a protective effect on the freeze-induced denaturation of fish protein, which was finally determined using a model of actomyosin system with treatments of sugar additions.

A gradual decrease of Ca^{2+}-ATPase activity by freeze-thawing was observed for the control and sugar-treated samples (Fig. 1). The activity of unfrozen sample was regarded as 100% to compare the AM stabilities in unfrozen to frozen-thawed samples. About 74% of the activity was lost in the control after 2 cycles of freeze-thawing. However, the reduction of the activity in sugar-treated groups was significantly retarded. The amounts of the activity loss were 55% for galactooligosaccharide, 51% for trehalose, 47% for sorbitol, and 42% for β-GT, respectively, after 2 cycles of freeze-thawing. It was apparent that sugar treatment reduced the extent of AM denaturation during freeze-thawing repeats. Based on molar concentrations of the sugars (8%) employed, β-GT (0.16 M) was much more effective in the cryoprotection than trehalose (0.23 M) and sorbitol (0.44 M) tested. When the samples were stored at −18°C for about 2 weeks, the Ca^{2+}-ATPase activities for the control and sugar-treated groups were also decreased gradually (Fig. 2). The decreases in the activity after the 2 week frozen storage were 69% for the control, 19% for galactooligosaccharide, 18% for trehalose, 23% for sorbitol, and 15% for β-GT, respectively. These results confirmed that the present sugar additives retarded AM denaturation rate during frozen storage and consequently indicated that β-GT was better in the effectiveness of the cryoprotections than trehalose and sorbitol used. Accordingly, it may be proposed that β-GT would be an effective substitute as cryoprotectant for frozen storage of fish protein. The higher ability of β-GT in protecting protein against freezing may be considered probably with relation to the increased power of water absorption/binding, as compared to trehalose. One of

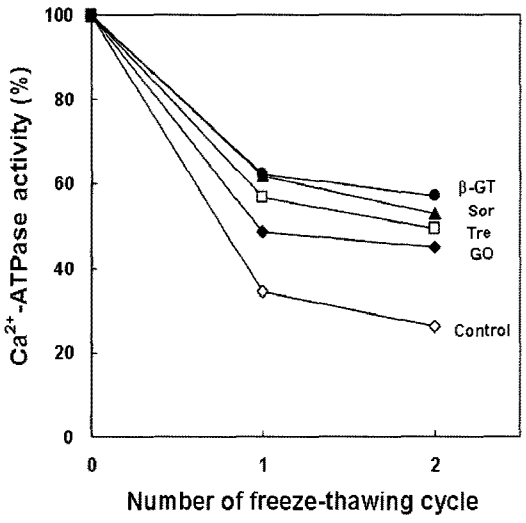

Fig. 1. Effect of β-GT on the Ca^{2+}-ATPase activity in fish actomyosin during repeated freeze-thawing in comparison with those of other carbohydrates. (●) β-GT, β-Galactosyl trehalose; (▲) Sor, sorbitol; (□) Tre, trehalose; (◆) GO, galactooligosaccharide; (◇) Control, no sugar added.

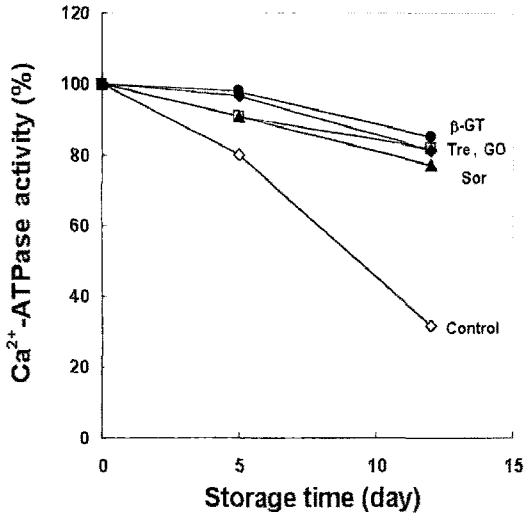

Fig. 2. Effect of β-GT on the Ca^{2+}-ATPase activity in fish actomyosin during frozen storage at -18°C in comparison with those of other carbohydrates. (●) β-GT, β-Galactosyl trehalose; (▲) Sor, sorbitol; (□) Tre, trehalose; (◆) GO, galactooligosaccharide; (◇) Control, no sugar added.

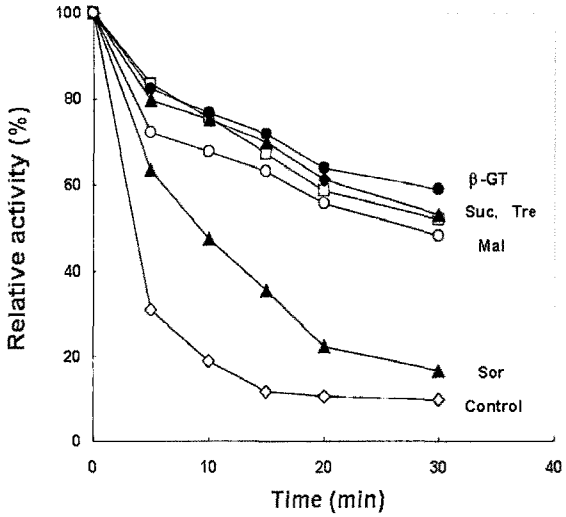

Fig. 3. Time course of thermal inactivation of pyrophosphatase at 55°C in the presence of several carbohydrates. (●) β-GT, β-Galactosyl-trehalose; (◆) Suc, sucrose; (□) Tre, trehalose; (○) Mal, maltose; (▲) Sor, sorbitol; (◇) Control, no sugar added.

critical mechanisms for the protective role of trehalose against freezing is thought to be a replacement of water molecules that are hydrogen bonded to the end groups of proteins (18). Actually, β-GT was shown to have higher water binding property than trehalose (9). Thus, it is likely that by more effectively substituting for water, β-GT might maintain AM proteins more undenatured and active.

In addition, trehalose has been described to act as one of best stabilizers in protecting enzymes against thermal inactivation due to its larger hydrated volume based on the water holding (13). That is, it can substitute and bind more water molecules from the proteins. It is suggested that this higher water binding character is largely contributed to protecting proteins from thermal denaturation. Interestingly, β-GT showed approximately 6-40% better protection against thermal inactivation than did other sugars used and the control on the basis of weight percent concentration (Fig. 3). As a result, β-GT was also thought to be an effective stabilizer for protein in solution against thermal denaturation, which was supposed to be attributed to the high water attracting property.

In conclusion, it was shown that β-GT had similar or better effectiveness alternatively in the cryoprotection of fish protein and in the thermoprotection of heat-labile protein, as compared to representative sugars commercially used. Therefore, it was positively suggested that β-GT might be applied as an effective sugar substitute for the cryoprotection and thermoprotection of food protein, as well as an alternative non-digestible oligosaccharide with the bifidogenic and non-cariogenic properties (9).

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