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Enzymatic Synthesis of Resveratrol Oligosaccharides (Gluco-oligosaccharides) and their Enhanced Application as Antidementia Drugs that Cross the Blood-brain Barrier (BBB)

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A B S T R A C T

This study synthesized resveratrol oligosaccharides (*gluco oligosaccharides*) by two-step enzymatic glycosylation, using glucosyltransferase from *Phytolacca americana* and cyclodextrin glucanotransferase. Although resveratrol hardly crosses the blood–brain barrier (BBB) in the mouse brain, the synthesized resveratrol oligosaccharides incorporated into the mouse brain tissue crossed the BBB. Our study indicated that resveratrol modified with oligosaccharides might have gained a BBB-crossing ability. Furthermore, during the Y-maze test using senescence-accelerated mouse prone 8, our investigations revealed that the time spent in the novel Y-maze arm by the resveratrol-oligosaccharides-treated mice was longer than that spent in the novel arm by the resveratrol-treated-mice. Therefore, this study established that since resveratrol oligosaccharides could penetrate the BBB of mouse brain samples and be incorporated into the mouse's brain tissue, they could also enhance spatial learning.

Introduction

Alzheimer's disease is one of the most intractable neurodegenerative disorders. Notably, the β-amyloid peptides in the brain's learning and memory regions, such as the cortex and hippocampus, are typical of Alzheimer's (Berardi *et al.*, 2009; Quadros Gomes *et al.*, 2018). Therefore, preventing β-amyloid

peptide aggregation is the primary therapeutic strategy for treating Alzheimer's (Berardi *et al.,* 2009). Previously, resveratrol has been reported as a natural antioxidant in grapevine species, currently attracting clinical attention due to its versatile biological properties, especially its antiinflammatory and neuroprotective activities (Jubilee *et al.,* 2003). For example, while it decreases the aggregation of β-amyloid peptides in the hippocampus of patients with Alzheimer's, resveratrol also prevents hippocampal damage; protects neurons against Aβ1–42-induced disruption of spatial learning, memory, and synaptic plasticity; and rescues the reduction of SIRT1 expression in hippocampal rats (Jubilee *et al.,* 2003).

In the brain, the blood–brain barrier (BBB) exists as a selective semipermeable border that prevents solutes in the circulating blood from nonselectively crossing the extracellular fluid of the central nervous system where neurons exist (Daneman *et al.,* 2015). It comprises endothelial cells of the capillary wall, astrocyte end-feet ensheathing the capillary, and pericytes fixed firmly in the capillary basement membrane (Ballabh *et al.,* 2004). Importantly, while the BBB system allows the passage of some small molecules by passive diffusion, it also permits the selective transport of various nutrients, ions, organic anions, and macromolecules, such as glucose and amino acids, crucial to neuronal functioning (Ballabh *et al.,* 2004).

So far, we have developed a drug delivery system using drugs' chemical and biological glycosylation. As a result, curcumin was identified as one of the most useful neuroprotective agents. However, when curcumin was intraperitoneally injected into a mouse, while only a small amount could be incorporated into the mouse brain tissue because it hardly crossed the BBB in the mouse brain, the glycosylated curcumin, i.e., curcumin oligosaccharides (gluco oligosaccharides), successfully penetrated the BBB and were incorporated into the mouse brain tissue (Hamada *et al.,* 2020). Therefore, using our brain–drug-delivery technique and the glycosylation of neuroprotective chemicals, we studied the crossing ability of resveratrol oligosaccharides (gluco oligosaccharides) through the BBB in mouse brain. We also investigated whether the glucooligosaccharide modification of resveratrol enhances its crossing ability through the mouse brain's BBB, after which the resveratrol oligosaccharides' effects on spatial learning of senescence-accelerated mouse prone 8 (SAMP8) were examined.

Materials and Methods

General

Resveratrol was purchased from FujifilmWako Pure Chemical Co. However, C57BL mice (eight weeks) and senescence-accelerated mouse prone 8 (SAMP8) (eight weeks) were purchased from Japan SLC Inc. (Shizuoka, Japan).

Synthesis of resveratrol β-D-glucoside

Resveratrol β-D-glucoside was synthesized through the enzymatic glucosylation of resveratrol. First, the cDNA of glucosyltransferase from *Phytolacca americana* (*Pa*GT) was cloned into pQE30, after which the resulting plasmids were transformed into *E.coli* M15 cells. Subsequently, expression and purification of *Pa*GT were performed as described previously (Hamada *et al.,* 2020). The purified enzyme solution was first dialyzed with 50 mM Tris-HCl (pH 7.2) containing 5 mM dithiothreitol, then stored at–80°C.

After that, glucosylation reactions were performed at 35°C for 24 h in 5 mL of 50 mM potassium phosphate buffer (pH 7.2) supplemented with a substrate, UDP-glucose, and enzyme. The reaction was finally stopped by adding 1.5% trifluoroacetic acid, and high-performance liquid chromatography (HPLC) was used to analyze the reaction mixture. Notably, the reaction mixture was extracted with *n*-butanol (*n*-BuOH). The *n*-BuOH fraction was initially concentrated by evaporation, after which the residue was dissolved in water. Then, while the water fraction was applied to Diaion HP20, washed

with water, and eluted with methanol, the methanol solution was subjected to preparative HPLC.

Synthesis of resveratrol oligosaccharides

Resveratrol oligosaccharides were prepared using enzymatic procedures: First, the reaction mixture containing resveratrol β-D-glucoside, soluble starch, and cyclodextrin glucanotransferase (CGTase) from *Bacillus macerans* (purchased from Amano Pharmaceutical Co. Ltd.), was incubated in sodium phosphate buffer (pH 7.0) at 40°C for 24 h. Then, the mixture was centrifuged at 3000 ×gfor 10 min. Subsequent investigations revealed that the supernatant contained glycosides, i.e., resveratrol maltoside, resveratrol maltotrioside, resveratrol maltotetraoside, and resveratrol maltopentaoside.

BBB penetrating ability test of resveratrol oligosaccharides

The mice were intraperitoneally injected once with resveratrol oligosaccharides or resveratrol (control) to test their BBB penetration abilities. One hour later, they were sacrificed by cervical dislocation, after which their brain tissue samples were quickly processed by rinsing with cold sodium phosphate buffer, then frozen and stored at −20°C. Subsequently, resveratrol was extracted, after which its concentration in the brain sample was determined using HPLC as previously described, with some modifications (Hamada *et al.*, 2020). Tissue samples were first homogenized in sodium acetate buffer (0.1 M, pH 6.0), and tissue homogenates were ultrasonicated in 0.1% Triton X-100 for 10 min. Then, in a flask containing the homogenate mixture with Triton X-100, 10 mg/ml α-glucosidase (Amano enzyme Co. Ltd., Aichi, Japan), 10 mg β-glucosidase (Amano enzyme Co. Ltd., Aichi, Japan), and 10 mg β-glucuronidase (NacalaiTesque, Inc.) were added and incubated at 36°C for one hour. Organic compounds were finally extracted with ethyl acetate. After three extraction steps, ethyl acetate was evaporated. Samples were dissolved in methanol to analyze them. Finally, the extracted resveratrol was quantified by HPLC using a reverse-phase column. The mobile phase comprised 31% acetonitrile and 69% water (flow rate: 1.0 ml/min, detector temperature: 40°C).

Y-maze test

A Y-maze with three arms was constructed with gray plastic, then it was equipped with a partition that isolates an arm. The experiment involved a 5-min trial 1, separated by a 40-min interval, followed by a 5-min trial 2. During the familiarization phase (trial 1), one arm (arm C: novel arm) of the Y-maze was closed with a partition. Then, while we placed one SAMP8 in one arm (arm A) of the two remaining arms (arms A and B) and the mouse allowed to explore the maze for five minutes, the partition was removed after a 40-min interval. Afterward, for five minutes, the mouse had free access to all three arms during the retrieval phase (trial 2). Notably, although the mouse was video recorded during trial 2, the time of the novel arm (arm C) exploration was only recorded when the mouse put his hind feet in that arm. Then, the percentage of time spent in the novel arm C was compared to the random exploration (33%) of the three maze arms. Finally, resveratrol was intraperitoneally injected once into three mice (the control group), whereas resveratrol oligosaccharides were intraperitoneally injected once into the three mice (the resveratrol-oligosaccharides-treated group).

Results and Discussion

Enzymatic synthesis of resveratrol β-D-glucoside

Plasmids prepared with pQE30, including the cDNA of plant glucosyltransferase from *Pa*GT, were transformed into *E.coli* M15 cells. Then, the expressed glucosyltransferase *Pa*GT was used for glucosylation. After stopping this reaction using trifluoroacetic acid, the reaction mixture was analyzed by HPLC, indicating compounds' formation. Subsequently, the reaction mixture was extracted with *n*-BuOH, after which the purification of the *n*-BuOH fraction by preparative HPLC gave

the product, which was finally identified as resveratrol β-D-glucoside (Figure 1).

Enzymatic synthesis of resveratrol oligosaccharides

Subsequently, resveratrol β-D-glucoside was subjected to glycosylation, using CGTase as a biocatalyst (Figure 2). Then, it was incubated with soluble starch and CGTase from *Bacillus macerans* for 24 h. After centrifuging the reaction mixture, we observed that the supernatant contained resveratrol oligosaccharides (Figures 3).

BBB penetration by resveratrol oligosaccharides

Brain tissue samples were processed as described in the Materials and Methods. After homogenizing tissue samples in sodium acetate buffer, the homogenates were ultrasonicated and treated by hydrolysis with glycosidases. Afterward, the products were extracted with ethyl acetate to prepare a brain sample.

The obtained resveratrol was subsequently quantified by HPLC analysis of the brain sample using a reverse-phase column. Thus, resveratrol was detected at 228 ng per 1 g of mouse brain tissue.

Subsequently, resveratrol oligosaccharides were intraperitoneally injected. The HPLC analysis results of the brain sample indicated that resveratrol oligosaccharides were incorporated into the mouse brain tissue.

Investigations also revealed that the brain sample of the mouse treated with resveratrol (control) contained little amount of resveratrol, indicating that it hardly migrated to the mouse brain tissue. These results suggest that resveratrol oligosaccharides, which were intraperitoneally injected into mice, could smoothly penetrate the BBB in the mouse brain.

Y-maze test findings for resveratrol oligosaccharides-injected-SAMP8

In the Y-maze test using SAMP8, the time spent in the novel arm of the Y-maze by the mice (n = 3) intraperitoneally injected with resveratrol-oligosaccharides (resveratrol-oligosaccharides-treated mice), was longer than that spent by the control mice (n = 3), into which resveratrol alone was intraperitoneally injected (Table 1).Investigations also revealed that the percentage of time spent in the novel arm by resveratrol-oligosaccharides-treated mice was higher than that of the time spent by the control mice. These results suggest that resveratrol oligosaccharides penetrated the BBB and were incorporated into the brain tissue of SAMP8, enhancing spatial learning of the mouse by 25% (approximately 1.3-fold) compared to the control.

Resveratrol oligosaccharides were synthesized following a biocatalytic synthetic procedure, using *Pa*GT and CGTase as biocatalysts. Enzymatic hydrolysis using three glycosidases of the brain tissue homogenates of mice, to which resveratrol oligosaccharides were intraperitoneally injected, yielded resveratrol, suggesting that resveratrol oligosaccharides migrated into the brain tissue through the BBB in mouse brain. Alternatively, previous studies have reported that the glucosides of ketoprofen and indomethacin could significantly inhibit the glucose transporter (GluT1)-mediated uptake of glucose, indicating its affinity to the transporter (Gynther *et al.*, 2009; Berardi *et al.*, 2009). Furthermore, these glucoconjugates could temperature-dependently penetrate the BBB, indicating that the glucosylation of drugs enhances their BBB-crossing ability and that the brain uptake of the conjugates is carrier-mediated (Gynther *et al.*, 2009). Consistent with these studies, we also observed that resveratrol oligosaccharides can cross the BBB in the mouse brain and be incorporated into brain tissue.

Table.1 Spatial-learning of SAMP8 in the Y-maze test.

SAMP8 group	Time spent in the novel arm[1] (s)	Percentage total time (%)
Resveratrol-treated mice (control) (*n* = 3)	104	34.6
Resveratrol-oligosaccharides-treated mice (*n* = 3)	130	43.3

[1]The exploration periods of the novel arm (arm C) recorded only when the mouse puts his hind feet in that arm.

Fig.1 Synthesis of resveratrol β-D-glucoside by biocatalytic glucosylation of resveratrol with *Pa*GT.

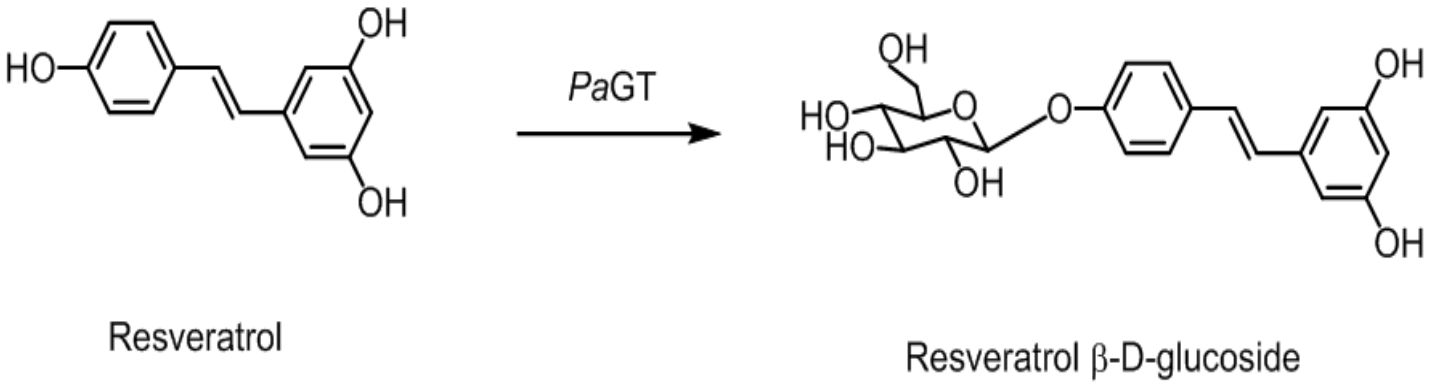


Fig.2 Synthesis of resveratrol oligosaccharides (glucooligosaccharides) by biocatalytic glycosylation of resveratrol β-D-glucoside with CGTase.

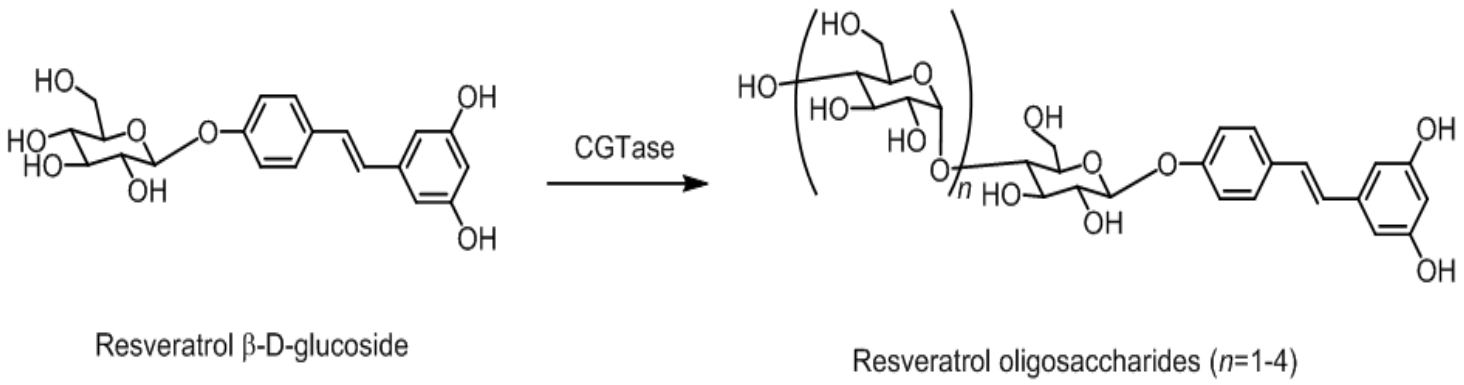
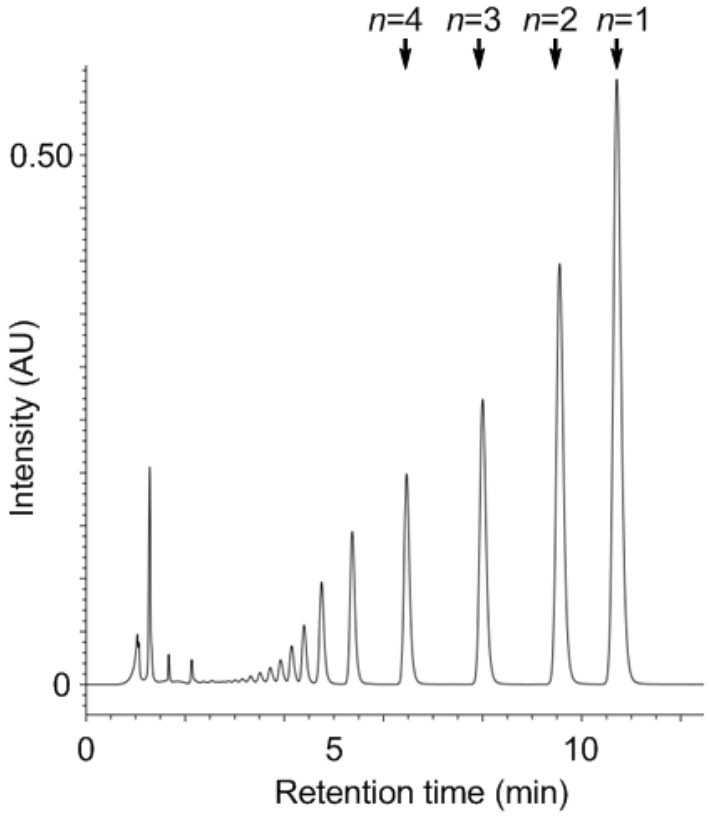


Fig.3 HPLC analysis results of resveratrol oligosaccharides (glucooligosaccharides).



The hippocampus is a critical brain area for cognitive and memory functions, making it a sensitive area in Alzheimer's (Berardi *et al.*, 2009). In a previous study, a significant reduction in the neurodegeneration of the hippocampus was observed after intracerebroventricular injection of resveratrol in an animal model, which was associated with a decrease in SIRT1 acetylation (Rimando *et al.*, 2004; Evers *et al.*, 2004). Another study also showed that mice treated with resveratrol for 45 d had reduced β-amyloid toxicity (Shan *et al.*, 2014). This finding indicates that the onset of neurodegeneration may be delayed by chemopreventive agents, such as resveratrol, which protects against β-amyloid formation and oxidative stress (Berardi*et al.*, 2009). Accordingly, a recent report showed that resveratrol protected neurons against β-amyloid 1–42-induced disruption of spatial learning and memory, rescuing the reduction of SIRT1 expression in hippocampal rats (Berardi *et al.*, 2009). Similarly, resveratrol also effectively reduced central nervous system damage and decreased the ischemia and toxicity induced by β-amyloid peptide, indicating its potential therapeutic use in neurodegenerative diseases (Niles *et al.*, 2006). These previous findings are consistent with our study, which suggests that resveratrol oligosaccharides are chemopreventive agents that can protect neurons against the β-amyloid 1–42-induced disruption of spatial learning and memory in the hippocampus of SAMP8 and enhance spatial learning. Additionally, we recently reported that the glycosylated curcumin, i.e., curcumin oligosaccharides (gluco-oligosaccharides), successfully penetrated the BBB in the mouse brain and can be incorporated into their brain tissue (Hamada *et al.*, 2020). Therefore, based on these results, our findings suggest that the gluco oligosaccharide modification of neuroprotective chemicals, such as resveratrol and curcumin, enhances their crossing ability through the BBB in the brain, thus, proposing that the brain–drug-

delivery technique of neuroprotective chemicals by glycoside (gluco oligosaccharide) modification is useful for preparing new antidementia drugs.

Further studies on the transporter that recognizes oligosaccharide conjugates as substrates and the neuroprotective property of oligosaccharide conjugates are currently in progress in our laboratory.

Declaration of Conflicting Interests

The author(s) declare no potential conflicts of interest regarding the research, authorship, and/or publication of this article.

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