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Novel Suspension-Phase Enzyme Reaction System Using Insoluble Extrusion Starch as Glycosyl Donor for Intermolecular Transglycosylation of L-Ascorbic Acid

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Abstract A novel suspension-phase enzyme reaction system for the intermolecular transglycosylation of L-ascorbic acid into 2-*O*-α-D-glucopyranosyl L-ascorbic acid supplementing extrusion starch as the glycosyl donor was developed using cyclodextrin glucanotransferase from *Thermoanaerobacter* sp. A high conversion yield compared to the conventional soluble-phase enzyme reaction system using cyclodextrins and soluble starch was achieved. The optimal reaction conditions were 2,000 units of cyclodextrin glucanotransferase, 20 g/l of L-ascorbic acid, and 50 g/l of extrusion starch at 50°C for 24 h. The new suspension-phase enzyme reaction system also exhibited several distinct advantages other than a high conversion yield, including a lower accumulation of oligosaccharides and easily separable residual extrusion starch by centrifugation or filtration in the reaction mixture, which will facilitate the purification of 2-*O*-α-D-glucopyranosyl L-ascorbic acid. The new suspension-phase enzyme reaction system seems to be potentially applicable as the industrial process for the production of thermally and oxidatively stable 2-*O*-α-D-glucopyranosyl L-ascorbic acid.

Key words: L-Ascorbic acid, 2-*O*-α-D-glucopyranosyl L-ascorbic acid, CGTase, new suspension-phase enzyme reaction system, intermolecular transglycosylation

L-Ascorbic acid (AA) is a well-known nutritional ingredient that is widely used to maintain general health. L-Ascorbic acid is also a cofactor of hydroxylases and monooxygenase in the synthesis of collagen, carnitine, and neurotransmitters [11, 17]. However, it is unable to sustain such physiological activity over a long time period because of thermal and oxidative degradation.

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To overcome the stability problem of L-ascorbic acid, the thermally and oxidatively stable derivative 2-*O*-α-D-glucopyranosyl L-ascorbic acid (AA2G) has been developed, in which one glucose unit is linked to the C2 hydroxyl group in the L-ascorbic acid molecule by an α-1,2-linkage through the intermolecular transglycosylation action of cyclodextrin glucanotransferase (CGTase) [1, 3, 11]. The thermal and oxidative stability of 2-*O*-α-D-glucopyranosyl L-ascorbic acid increased significantly compared with that of L-ascorbic acid, and therefore, it is being preferentially used in the food, pharmaceutical, and cosmetic industries as a stabilizer, quality-improving agent, antioxidant, bioactive agent, and UV absorbent [5, 13, 15, 19–21].

The intermolecular transglycosylation reaction of cyclodextrin glucanotransferase requires the glycosyl donors supplementing the glucose unit to the glycosyl accepter L-ascorbic acid. So far, only the soluble form of carbohydrate, such as α-, β-, and γ-cyclodextrins and soluble starch, has been used as the glycosyl donors. However, the conventional soluble-phase enzyme reaction system using the above soluble forms of carbohydrates raises several disadvantages associated with the homogeneous nature of the reaction system, where all biocatalyst, glycosyl donor, L-ascorbic acid, and transglycosylated 2-*O*-α-D-glucopyranosyl L-ascorbic acid are composing the soluble phase.

In a series of previous studies, the current authors developed several enzyme reaction systems using insoluble extrusion starch either as the substrate for production of α-, β-, and γ-cyclodextrins through intramolecular transglycosylation [9, 10], or as the glycosyl donor to obtain various transglycosylated forms of glucosides, such as stevioside, hesperidin, and salicin, through the intermolecular transglycosylation action of cyclodextrin glucanotransferase [2, 6, 15]. The structural feature of starch was changed into a swollen micelle form after an extrusion step, thereby transforming into a more favorable state suitable for access and the surface reactions

of various carbohydrase. A distinctly high transglycosylation yield and reaction rate was achieved without the accumulation of the recognizable amount of oligosaccharides in suspension-phase reaction systems compared to the conventional reaction system using soluble carbohydrates.

In this work, a suspension-phase enzyme reaction system using extrusion starch was applied for the intermolecular transglycosylation of L-ascorbic acid to 2-*O*-α-D-glucopyranosyl L-ascorbic acid. The reaction conditions were optimized, and the reaction characteristics of the suspension-phase enzyme reaction system were elucidated in terms of the profiles of the intermediate compounds, cyclodextrin, maltooligosaccharide, and residual extrusion starch. The efficacy of the new suspension-phase enzyme reaction system was evaluated for its potential application in the production of thermally and oxidatively stable 2-*O*-α-D-glucopyranosyl L-ascorbic acid.

MATERIALS AND METHODS

Cyclodextrin Glucanotransferase

Cyclodextrin glucanotransferase from *Thermoanaerobacter* sp. (EC 2.4.1.19, Novo Nordics Co., Ltd., Denmark) was used as the transglycosylating enzyme for intermolecular transglycosylation of L-ascorbic acid. The specific activity of cyclodextrin glucanotransferase was 1,200 units/mg proteins.

L-Ascorbic Acid and Glycosyl Donor

L-Ascorbic acid (Sigma Chemical Co., U.S.A.) and 2-*O*-α-D-glucopyranosyl L-ascorbic acid (Hayashibara Co. Japan) were used as the standard materials. Maltooligosaccharides (G_2–G_7, Sigma Chemical Co., U.S.A.), α-, β-, and γ-cyclodextrins (CycloLab, Ltd., Hungary), dextrin, soluble starch (Sigma Chemical Co., U.S.A.), and extrusion starch were used as the glycosyl donors. The extrusion starch was prepared from corn starch, with an equilibrated moisture content of 18%, using a single screw extruder at a feed rate of 300 g/min and a screw speed of 200 rpm, and the degree of gelatinization of the extrusion starch was 63.5% [8].

Determination of CGTase Activity

The CGTase activity was determined using the methods of Kitahata and Okada [7], where the starch hydrolyzing activity was measured using 1% (w/v) soluble starch in a 0.02 M sodium acetate buffer (pH 5.5) at 37°C for 30 min. One unit of activity was defined as the amount of CGTase corresponding to a 1% increase in transmittance at 660 nm per min. The concentration of protein was determined by the Lowry method [14].

Intermolecular Transglycosylation Reaction

The intermolecular transglycosylation reaction of L-ascorbic acid into 2-*O*-α-D-glucopyranosyl L-ascorbic acid was carried out using 2,000 units of cyclodextrin glucanotransferase per gram of L-ascorbic acid, 20 g/l of L-ascorbic acid, and 50 g/l of carbohydrate as the glucosyl donor in a 0.02 M sodium acetate buffer (pH 5.5) in a dark bottle at 50°C and 200 rpm for 24 h. The amounts of cyclodextrin glucanotransferase, L-ascorbic acid, and carbohydrate as the glycosyl donor were varied to optimize the reaction conditions.

Determination of L-Ascorbic Acid and 2-*O*-α-D-Glucopyranosyl L-Ascorbic Acid

The L-ascorbic acid and 2-*O*-α-D-glucopyranosyl L-ascorbic acid concentrations were measured using an HPLC (Gilson Co., France) equipped with a μBondapak C18 column (Waters Co., U.S.A.). The mobile phase was 0.1 M KH_2PO_4-0.1 M H_3PO_4 (pH 2.0), at a flow rate of 0.5 ml/min; the UV detector was at 240 nm.

Analysis of Cyclodextrins and Maltooligosaccharides

The residual α-, β-, and γ-cyclodextrins and maltooligosaccharides (G_2–G_7) in the reaction mixture were analyzed using HPLC: Inertsil NH_2 column (GL Sciences Inc., Japan), acetonitrile/water (65/35) as the mobile phase, at a flow rate of 1 ml/min, with an RI detector. The concentration of reducing sugar was determined by the DNS method [18].

Calculation of Conversion Yield

The 2-*O*-α-D-oligoglucopyranosyl L-ascorbic acid family (AA-2G_n) in the reaction mixture was digested by amyloglucosidase (E.C. 3.2.1.3., Sigma Chemical Co., U.S.A.) from *Rhizopus* at 55°C for 24 h to obtain a pure form of 2-*O*-α-D-glucopyranosyl L-ascorbic acid. The conversion yield of L-ascorbic acid (AA) into 2-*O*-α-D-glucopyranosyl L-ascorbic acid (AA2G) was then calculated as follows:

$$\text{Conversion yield (\%)} = \frac{[\text{Pure AA2G produced}]}{[\text{Initially added AA}]} \times 100$$

RESULTS AND DISCUSSION

Evaluation of Glycosyl Donors for Intermolecular Transglycosylation of L-Ascorbic Acid

Table 1 compares the glycosyl donor specificities of insoluble extrusion starch and other forms of soluble carbohydrates including maltooligosaccharides (G_2–G_7), α-, β-, γ-cyclodextrins, dextrin, and soluble starch for the intermolecular transglycosylation of L-ascorbic acid to 2-*O*-α-D-glucopyranosyl L-ascorbic acid. The long-chain polymeric saccharides, such as soluble starch and insoluble extrusion starch, showed better donor specificity compared with the low molecular weight carbohydrates, such as maltooligosaccharides

Table 1. Glycosyl donor specificities for intermolecular transglycosylation of L-ascorbic acid using CGTase from *Thermoanaerobacter* sp.

Glycosyl donors	Phase	Conversion yield (%)
α-Cyclodextrin	Soluble	19.7±1.2
β-Cyclodextrin	Soluble	19.9±1.5
γ-Cyclodextrin	Soluble	18.5±0.9
Maltose (G_2)	Soluble	1.8±0.2
Maltotriose (G_3)	Soluble	2.0±0.1
Maltotetraose (G_4)	Soluble	2.5±0.2
Maltopentaose (G_5)	Soluble	3.4±0.3
Maltohexaose (G_6)	Soluble	5.4±0.2
Maltoheptaose (G_7)	Soluble	6.6±0.5
Dextrin	Soluble	18.7±1.8
Soluble starch	Soluble	19.4±1.5
Extrusion starch	Suspended	26.1±1.4

Reaction conditions: 20 g/l of ascorbic acid, 50 g/l of each donor, and 2,000 units of CGTase from *Thermoanaerobacter* sp./g of L-ascorbic acid at 50°C, 200 rpm for 24 h.

(G_2–G_7), α-, β-, γ-cyclodextrins, and dextrin. The highest conversion yield of 26.1% was obtained when the extrusion starch was used as the intermolecular glycosyl donor compared with other carbohydrates, which ranged from 1.8 to 6.6% for maltooligosaccharides, 18.5 to 19.9% for cyclodextrins, 18.7% for dextrins, and 19.4% for soluble starch.

Optimal Conditions for Intermolecular Transglycosylation of L-Ascorbic Acid into 2-*O*-α-D-Glucopyranosyl L-Ascorbic Acid

The optimal transglycosylation condition was determined by changing the amounts of cyclodextrin glucanotransferase, L-ascorbic acid used as the glycosyl acceptor, and extrusion starch used as the glycosyl donor. As shown in Fig. 1, the conversion yield increased proportionally as the amount of cyclodextrin glucanotransferase increased up to 2,000 units per gram of L-ascorbic acid, and then thereafter remained at a constant level. An excess amount of cyclodextrin glucanotransferase seems to lead to several undesirable side reactions, such as the formation of an excess amount of intermediate compound cyclodextrins through the intramolecular tranglycosylation and the decomposition of the converted 2-*O*-α-D-oligoglucopyranosyl L-ascorbic acid family (AA-2G_n) by the disproportionation reaction of cyclodextrin glucanotransferase.

Fig. 2 illustrates the effect of the L-ascorbic acid and extrusion starch concentrations on the conversion yields, changing L-ascorbic acid from 10 to 100 g/l (A) and extrusion starch from 10 to 100 g/l (B). The maximum conversion yield of 26.1% was achieved when 20 g/l of L-ascorbic acid and 50 g/l of extrusion starch, corresponding to the mixing ratio between the glycosyl donor and acceptor of 2.5. The conversion yield even decreased at the high

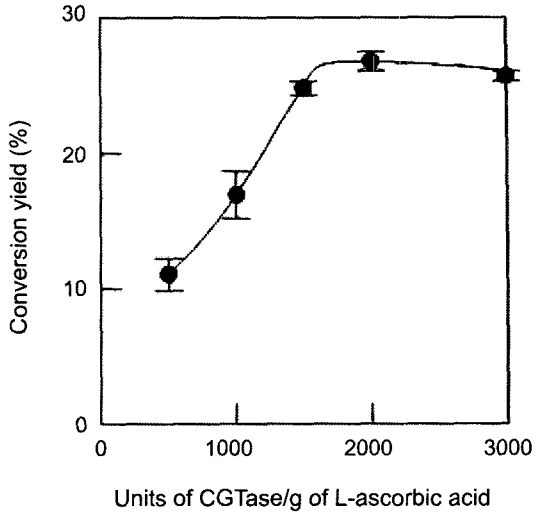

Fig. 1. Effect of CGTase concentration on intermolecular transglycosylation of L-ascorbic acid in a suspension-phase enzyme reaction system using extrusion starch.
The reaction was carried out at 500–3,000 units CGTase/g of L-ascorbic acid, 20 g/l of L-ascorbic acid, and 50 g/l of extrusion starch at 50°C, 200 rpm for 24 h.

extrusion starch concentration of over 50 g/l, due to the loss of the fluidity of the reaction mixtures interfering with the penetration of water molecules into the suspended insoluble extrusion starch.

Intermolecular Transglycosylation of L-Ascorbic Acid to 2-*O*-α-D-Glucopyranosyl L-Ascorbic Acid in a Suspension-Phase Enzyme Reaction System

Fig. 3A compares the progression of the intermolecular transglycosylation reaction of L-ascorbic acid in the new suspension-phase enzyme reaction system using extrusion

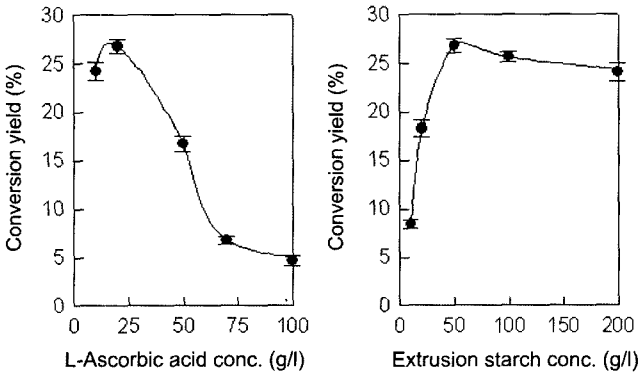

Fig. 2. Effect of L-ascorbic acid (**A**) and extrusion starch (**B**) concentrations on intermolecular transglycosylation of L-ascorbic acid in a suspension-phase enzyme reaction system using extrusion starch.
The enzyme reaction was performed with 10–100 g/l of L-ascorbic acid, 10–100 g/l of extrusion starch, and 2,000 units of CGTase/g of L-ascorbic acid at 50°C, 200 rpm for 24 h.

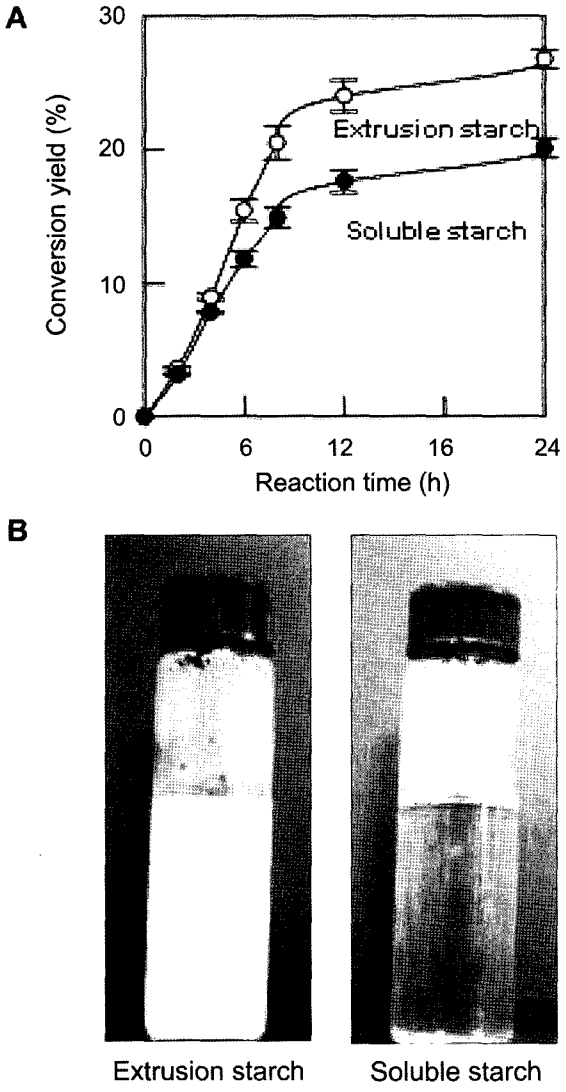

Fig. 3. Comparison of the progress of reaction in a suspension-phase enzyme reaction system with a conventional system using soluble starch.
(**A**) Progress of transglycosylation reaction using extrusion (●) and soluble starch (■) as glycosyl donors, and (**B**) phase features.

starch with the conventional soluble-phase enzyme reaction system, using soluble starch as the glycosyl donor. The intermolecular transglycosylation reaction proceeded more effectively in the new suspension-phase enzyme reaction system and the reaction was mostly completed within 12 h. The final conversion yield after 24 h was 26.1%, a significantly higher value compared with 19.4% in the conventional enzyme reaction system using soluble starch.

As shown in Fig. 3B, the extrusion starch formed a distinguishable suspension-phase enzyme reaction system, where the extrusion starch added as a glycosyl donor was completely suspended throughout the reaction mixture. Meanwhile, the soluble starch formed the usual homogeneous soluble-phase reaction system, thoroughly dissolved in the reaction mixture. A thoroughly swollen extrusion starch seemed to generate a sufficient surface area that can transfer the glycosyl residues from the nonreducing end of the micelle structure. Furthermore, the extrusion starch was further fragmented into a large number of small-sized particles as the enzyme reaction proceeded, thereby generating new surfaces for the intermolecular transglycosylation [10].

Characteristics of the Suspension-Phase Enzyme Reaction System for Intermolecular Transglycosylation of L-Ascorbic Acid

In Fig. 4, the profiles of intermediate compounds, cyclodextrins (A) and maltooligosaccharides (B), were monitored in order to characterize the transglycosylation in the suspension-phase enzyme reaction system. A large amount of cyclodextrins was accumulated equally in both reaction systems, especially during the initial 6 h, in which the transglycosylation reaction occurs most actively. The final cyclodextrin concentrations in both systems were 27.3 and 27.0 g/l, respectively.

The intermolecular transglycosylation of L-ascorbic acid seems to be carried out via two steps: the synthesis of intermediate compound cyclodextrins from the extrusion starch, and then the transfer of the glycosyl unit from cyclodextrin molecules to the glycosyl acceptors, as observed in our previous works [2, 15], in which the intermolecular transglycosylation of a glycosyl unit to stevioside was carried out in a similar suspension-phase reaction system supplementing the extrusion starch and raw starch as the glycosyl donors. A distinct difference between them can be found in the pattern of the accumulation of soluble maltooligosaccharides (G_1–G_7) after the completion of the transglycosylation reaction. As shown in Fig. 4B, only a negligible amount of maltooligosaccharides of 0.23 g/l was accumulated in the new suspension-phase reaction system; meanwhile, a large quantity of maltooligosaccharides of up to 7.38 g/l was generated in the conventional enzyme reaction system using soluble starch as the glycosyl donor.

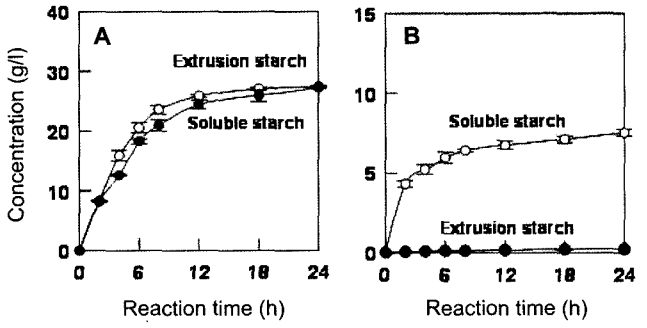

Fig. 4. Profiles of intermediate cyclodextrins (**A**) and maltooligosaccharides (**B**) in a suspension-phase enzyme reaction system using extrusion starch (○) and a conventional enzyme reaction system using soluble starch (●).

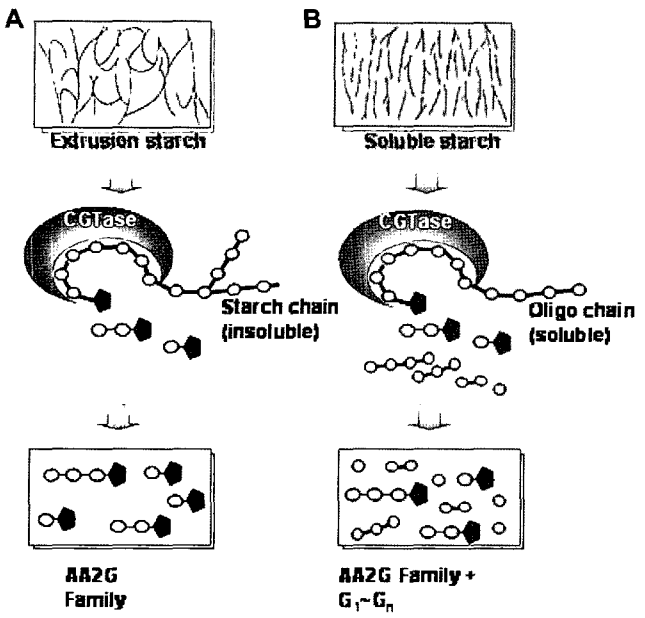

Fig. 5. Hypothetical model for intermolecular transglycosylation of L-ascorbic acid using the catalytic action of cyclodextrin glucanotransferase from micelle-chains in extrusion starch (**A**) and from oligo-chains in soluble starch (**B**).

A hypothetical schematic model comparing both reaction systems was constructed, as shown in Fig. 5: one from the micelle-chains in insoluble extrusion starch (A) and the other from the oligo-chains in soluble starch (B). The transglycosylation reaction from extrusion starch can proceed at the nonreducing end of the micelle in the surface of insoluble extrusion starch; therefore, the accumulation of maltooligosaccharides can be minimized. Meanwhile, the reaction from soluble starch proceeds exo-wisely from the end of the partially digested oligo-chains in soluble starch, and consequently, a significant amount of glucose and maltooligosaccharides will be left after the transglycosylation reaction [2, 9, 15]. The lower accumulation of residual maltooligosaccharides in the new suspension-phase enzyme reaction system will facilitate the purification of 2-*O*-α-D-glucopyranosyl L-ascorbic acid from the reaction mixture.

Comparison of the Performances of a Suspension-Phase Enzyme Reaction System and a Conventional Enzyme Reaction System

Table 2 compares the overall performance of both reaction systems, including a high conversion yield and lower residual maltooligosaccharides, as discussed in previous sections. The insoluble extrusion starch of 31.9 g/l that remained after the completion of the transglycosylation reaction was readily separable by simple centrifugation. It will also facilitate the separation and purification of 2-*O*-α-D-glucopyranosyl L-ascorbic acid, in addition to the lower accumulation of the residual maltooligosaccharides. The distinct advantages of the novel suspension-phase enzyme reaction system will facilitate the development of an industrial process for the production of a thermally and oxidatively stable 2-*O*-α-D-glucopyranosyl L-ascorbic acid.

Table 2. Comparative performances of a suspension-phase reaction system using extrusion starch and a conventional reaction system using soluble starch as the glycosyl donor.

Process criteria	Extrusion starch	Soluble starch
Initial L-ascorbic acid conc. (g/l)	20.0	20.0
AA2G conc. (g/l)	10.6±0.6	7.8±0.6
Conversion yield (%)	26.1±1.4	19.4±1.5
Half-reaction time (h)[a]	5.6	5.2
Residual cyclodextrin (g/l)	27.3±0.2	27.0±0.4
Residual maltooligosaccharides (g/l)	0.2±0.0	7.3±0.4
Separable residual starch (g/l)[b]	31.9±0.6	<0.05
Purification of AA2G	Easy	Difficult

[a]Time required for half level of maximum conversion yield.
[b]Centrifuged in preparative centrifuge at 8,000 rpm.

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