

Transglycosylation of Rebaudioside A by β -Fructofuranosidase

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Rebaudioside A (RebA) was subjected to β -2.6 transglycosylation with β -fructofuranosidase from *Arthrobacter sp.* K-1 and sucrose as a source of fructose units. The yield of transglycosylation depends significantly on the concentration of the acceptor, the donor and the enzyme, as well as the reaction time. At the weight ratio of RebA to sucrose of 1: 1, the degree of transfructosylation in 24 hours was only 5.4%, while at a ratio of 1 : 5, it reaches to more than 23%. It was revealed that transfructosylation proceeds more efficiently in the concentrated solutions, the higher the total concentration of sucrose and RebA, the greater the yield of fructosylated RebA. To determine the effect of pH on transfructosylation, β -fructofuranosidase was incubated with a solution of 1% RebA and 10% sucrose at 40°C for 15 hours at various pH values. It was also revealed that with an increase in the amount of the enzyme, the reaction accelerates. The most optimal were quantities of 50-100 units per 1 g of sucrose. The reaction of transfructosylation of stevioside is examined, and an organoleptic evaluation of fructosylated derivatives of fructosyl-RebA, fructosyl-stevioside and fructosyl-rubuzoside is also shown. Isolation and purification of fructosylated RebA was carried out by ethanol precipitation and purification on the columns filled with macroporous Diaion HP-20 resin. The resulting product is possessing improved sensory characteristics and can be used as low-calorie sweetener.

Keywords: transfructosylation, β -fructofuranosidase, cultivation of *Arthrobacter sp.* K-1, β -Fructofuranosidase (FFase) activity, reaction conditions for transfructosylation of RebA, isolation and purification of fructosyl-RebA, taste profile of fructosylated derivatives

Stevia rebaudiana Bertoni is a plant species native to the South America and is now cultivated in many parts of the world. *Stevia* leaves are naturally sweet and extracts of *Stevia rebaudiana* have been used commercially to sweeten foods and beverages (Kennelly, 2002; Kennelly, 1985).

Extract of *Stevia rebaudiana* contains mixture of different ent-kaurene-type diterpene glycosides, which have a common base – steviol – and differ from each other by carbohydrates residues at C-13 and C-19 positions (Kinghorn et al., 1985; Kennelly, 2005). Some of the steviol glycosides were isolated and identified such as stevioside, rebaudiosides A, B, D, E, F, G, I, H, L, K, J, M, N, O and some others, and as well dulcoside A and B, rubusoside, steviolmonoside and steviolbioside. Rebaudioside A (RebA) and stevioside are the main components of the leaf, they have been studied and characterized as a high intensity sweeteners (Geuns, 2003)¹. Each of these steviol glycosides has its own unique taste profile (DuBois, 2011, ; DuBois, 1985) and sweetness intensity which can vary from 30 to

450 times sweeter than sugar (Abelyan et al., 2012; Kinghorn et al., 1985)².

In addition to the sweet taste, they have different pharmacological properties. With their regular consumption, the content of sugar, radionuclides and cholesterol in the body decreases, cell regeneration and blood coagulation improves, the growth of tumors is inhibited, blood vessels are strengthened. It also shows choleric, anti-inflammatory and diuretic properties, prevents the formation of ulcers in the gastrointestinal tract (Abelyan et al., 2012; Kinghorn, 1985; Toskulkao, 1994).

Glycosides of stevia possess residual bitterness and taste profile, which affects its quality characteristics (Chaturvedula, 2011; DuBois, 1985). They can be corrected by modifying glycosides with the help of intermolecular transglycosylation reactions under the action of various enzymes, during which the addition of other carbohydrates in the abovementioned positions C13 and C19 occurs. The exact amount of

¹ Morita, T., Morita, K., Kanzaki, S. Novel stevia variety and method of producing sweetener // US Patent Appl. 201110023192.-2011.

² Morita, T., Fujita, I., Matsuura, F., Ota, M. (2010). New steviol glycoside // WO2010/038911. - 2010.

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carbohydrate units in these positions determines the degree of sweetness of glycosides in stevia (Abelyan et al., 2012).

The enzymes used for transglycosylation are pullulanase, isomaltase (Lobov, 1991), β -galactosidase (Kitahata et al., 1989) and dextrin dextranase (Yamamoto et al., 1994), and donors are – pullulan, maltose, lactose and partially hydrolyzed starch respectively. However, they allow eliminate bitterness only partially due to the low yield of derivatives with the required characteristics. Best results were obtained with transglycosylation using microbial cyclodextrin glucanotransferase (CGTase) in the presence of starch as a donor of glucose units (Abelyan et al., 2012).

On the other hand, the inclusion of fructose via transfructosylation may lead to a derivatives of glycosides with improved taste characteristics (Chaturvedula, 2011; Darise et al., 1984; Ishikawa, 1990; Ishikawa, 1991).

β -transfructosylation schematically can be represented as follows:

Hydrolysis

Aldosyl-F \rightarrow Aldose + Fructose

Self-transferase reaction

Aldosyl-F + Aldosyl-F \rightarrow Aldosyl-F-F + Aldose

Transferase reaction to acceptor

Aldosyl-A + F \leftrightarrow F-A + Aldose

Note: F – is the residue of fructose, A - acceptor.

The purpose of this work is – the study of β -2,6-transfructosylation of RebA using β -fructofuranosidase (FFase) from *Arthrobacter* sp. K-1 and sucrose as a source of fructose units in order to improve its taste characteristics and potentially use it as sweetener itself.

Methods

Microbial cultures *Arthrobacter* sp. K-1 (FERM P-3192) (Japan), *Arthrobacter* sp. 10137 (Collection Of Industrial cultures, China), *Microbacterium saccharophilum* NBRC 108778 (Japan), *Aspergillus niger* IMI303386, (UK) *Schwanniomyces occidentalis* ATCC 26077 and *Aureobasidium pullulans* DSM2404 (Germany) used as producer of β -fructofuranosidase (FFase) (Fujita, 1990; Akimaru, 1991; Ishikawa, 1991).

Cultures were maintained on “Nissui” nutrient agar containing 0.2% of yeast extract.

Subsurface cultivation was carried out in a conical flasks at 30°C for 48 hours on a liquid nutrient medium, containing 1.2% yeast extract, 0.8% polypeptone, 4.0% lactose, 0.4% $(\text{NH}_4)_2\text{HPO}_4$, and 0.1% $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ (pH 7.0).

420ml of culture fluid was transferred to 10L fermenter, containing 8L of nutrient medium (pH 7.0), consisting of 5.0% of corn extract, 3.0% of sucrose, 0.4% $(\text{NH}_4)_2\text{HPO}_4$ and 0.4% $\text{MgSO}_4 \times 7\text{H}_2\text{O}$.

Cultivation was carried out at 37°C for 24 hours, with constant aeration and pH controlled in the range of 6.8-7.2 using 2N NaOH.

The culture fluid was centrifuged at 12,000 rpm (20 min, 4°C), filtered through a 0.45 mm cellulose-acetate filter and concentrated on ultrafilters. The extracellular enzyme concentrate was stored at 4°C.

β -Fructofuranosidase (FFase) activity was determined by the amount of reducing sugars released as a result of sucrose hydrolysis. One unit of FFase activity was determined as the amount of enzyme needed to release 1 μmol of reducing sugar per minute. Mixture of the crude enzyme (0.5 ml) and 0.5 ml of 40% (w/v) sucrose solution (in 50 mM phosphate buffer, pH = 6.5) was incubated at 30°C with constant stirring for 10 minutes. The reaction was stopped by boiling. The amount of reducing sugars was determined using 3,5-dinitrosalicylic acid (DNS) method

The glycoside content was determined by HPLC using ZORBAX NH_2 column (4.6 mm x 250 mm), at column temperature of 40°C and using an acetonitrile-water mixture in a ratio of 80:20 (v / v) as the mobile phase, flow rate of 1 ml / min, DAD detector, UV (210 nm).

Also, the reagents produced by “Shandong Huaxian Stevian Co., LTD” (PRC), «Wako Pure Chemical Industries», LTD (Japan) and Sigma-Aldrich (USA) were used.

Obtaining transfructosylated RebA

Forty grams (40 g) of sucrose was added into 54 ml of reverse osmosis purified water (pH 6.5) and stirred at 50°C for 30 minutes until fully dissolved. 6 g of high purity RebA (> 98%) was added to the solution and stirred at 50°C until complete dissolution. 400 units of β -fructofuranosidase were added to the resulting solution and the reaction was carried out at 40 ° C for 48 hours with constant stirring. The reaction was stopped by heating to 95°C for 10 minutes, then treated with activated carbon (2% of solids) at 70°C for 30 minutes, deionized on Amberlite FCP22 (H +) and

Amberlite FPA53 (OH⁺) ion exchange resins using the conventional scheme. The combined filtrate was passed through a column filled with DIAION HP-20 resin (1.6 x 50 cm) in a ratio of glycosides to gel of 10% (weight / volume). After the adsorption, the column was washed with 300 ml of distilled water and 10% methanol solution. Elution of the transglycosylated products was performed sequentially using 5 "column volumes" of 70% methanol in total.

Results and discussions

It was previously shown that the reaction of transfructosylation of stevioside with the help of FFase in the presence of sucrose followed the Ping-Pong Bi-Bi mechanism (Figure 1) (Lobov, 1991).

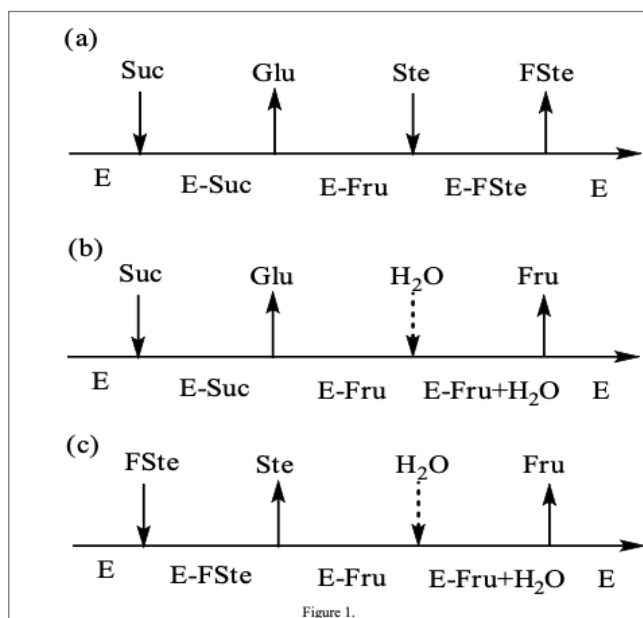


Figure 1. Schematic diagram of the Ping-Pong Bi-Bi mechanism for each reaction

Note: (a) Synthesis of fructosyl-stevioside; (b) Hydrolysis of sucrose; (c) Hydrolysis of fructosyl-stevioside (Suzuki et al., 2002, pp. 1033-1048)

The free enzyme (E) reacts with sucrose (Suc) to form the first complex (E-Suc). Then glucose (Glu) is released from the E-Suc complex, with the formation of the second E-Fru complex. This complex interacts with stevioside, which leads to the formation of the third complex E-FSt and subsequent release of FSt. In this system, along with the reaction of transfructosylation, the hydrolysis of sucrose and fructosil-stevioside occurs. The synthesis of fructosil-stevioside is inhibited by glucose and fructose. A conceptual diagram of the overall reaction mechanism is shown in Figure

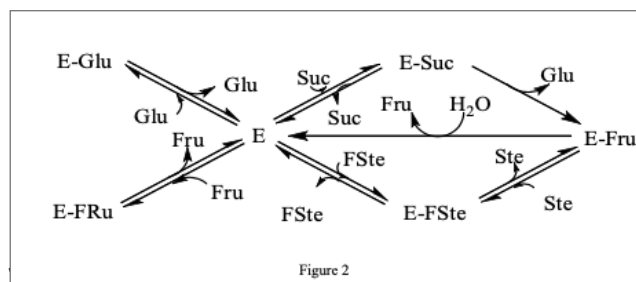


Figure 2. Conceptual diagram of the general reaction mechanism of fructosil-stevioside synthesis (Suzuki et al., 2002)

For transfructosylation, the enzyme (50 units) was incubated with sucrose (2.0 M) in the presence of stevioside or rubusoside (0.05 M) in 50 mM phosphate buffer (pH 6.5; 31 ml) at 40°C for 16 hours. The reaction was stopped by heating to 100°C for 10 minutes, then centrifuged, and the reaction mixture was purified on adsorption resin Diaion HP-20 and silica gel. Derivatives obtained were identified as β -D-fructofuranosyl-2,6- β -D-glucopyranosyl ester of steviolbioside and β -D-fructofuranosyl-2,6- β -D-glucopyranosyl ester of steviolmonoside for stevioside and rubusoside respectively (Figure 3). At the same time, the sweetness of the components did not increase, however, the taste characteristics of both derivatives significantly improved over the original glycosides. This was especially pronounced for fructosylated stevioside where the quality of taste, bitterness and aftertaste were comparable to the aspartame (Table 1) (Abelyan et al., 2012; Fujita, 1990).

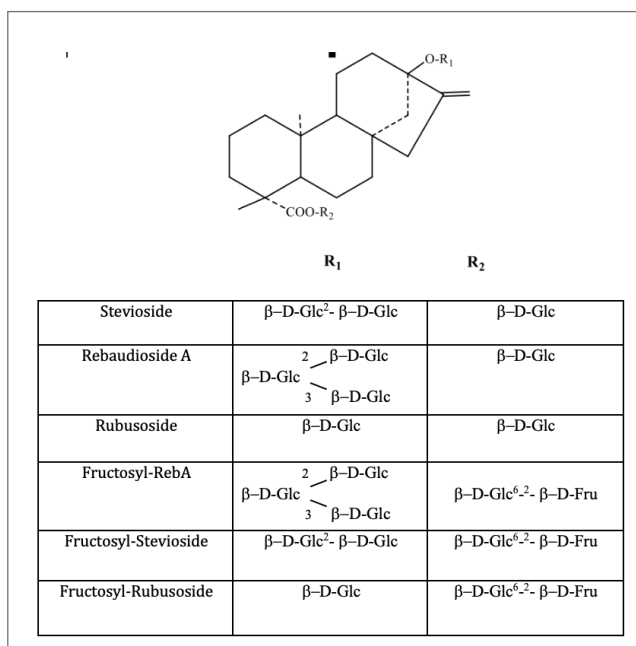


Figure 3. Fructosylated glycosides of Stevia

In our experiments, preliminary results of the transfructosylation of RebA showed that β -fructofuranosidase produced by *Arthrobacter* sp. K-1 is the most effective. At the same time, the enzyme catalyzed the formation of a mono- β -2,6-fructosylated at the 19-O-glucosyl residue of the RebA with a rather high yield (frucosyl-RebA), i.e. this β -fructofuranosidase is strictly specific for position C19 (Figure 4).

Table 1
Organoleptic evaluation of fructosylated derivatives (Abelyan et al., 2012)

Component	Bitterness	Aftertaste	Overall taste quality
Fructosyl-stevioside	4.6	4.2	2.9
Fructosyl-rubusoside	2.6	2.7	1.5
Stevioside	3.3	3.5	1.5
RebA	3.7	3.5	1.9
Aspartam	4.7	4.3	3.0

Note: The evaluation was carried out in sample solutions with concentrations corresponding to the sweetness of a 5% sucrose solution. 5, Best; 4, Significantly better; 3, better; 2, slightly better; 1, worse

All further experiments were performed using this enzyme.

Obtaining transfructosylated RebA

The aqueous and 10% methanol fractions contained only sucrose, glucose and fructose, while products of transglycosylation were in the 70% methanol fraction. The 70% methanol fraction was concentrated under the vacuum until full evaporation of methanol and the glycoside content was analysed by HPLC.

The yield of transfructosylation reaction depends largely on the concentration and ratio of the acceptor, donor and enzyme, as well as on reaction time. Transfructosylation is more effective with an excess of sucrose. The yield of the mono-fructosylated

derivative is 70%, provided that the reaction mixture contains 0.5% RebA, 5% sucrose, and the reaction time is 17 hours.

The ratio of the substrates had a definite effect on the degree of transfructosylation. The highest yield of fructosyl-RebA was achieved with excessive amounts of sucrose. Thus, at a weight ratio of RebA to sucrose of 1: 1, the degree of transfructosylation in 24 hours was only 5.4%, while at a ratio of 1 to 5, it reaches to more than 23%. The degree of transfructosylation increases significantly with the duration of the reaction, as shown for 24 hours, 48 hours and 72 hours (Table 2 and Figure 5).

Table 2
The effect of concentration of the acceptor and reaction time on the formation

Ratio (w/w)		Reaction time, hour	Transfructosylation, % Fru-RebA
RebA	Sucrose		
1	2	24	5.4
		48	20.1
		72	32.0
1	3	24	6.9
		48	24.6
		72	36.5
1	4	24	9.3
		48	29.2
		72	38.5
1	5	24	23.3
		48	32.7
		72	41.2
1	6	24	24.7
		48	35.4
		72	44.3
1	7	24	33.6
		48	38.7
		72	48.8

Note: Fru-RebA (total solids level at 35%; 40°C; pH 6.5)

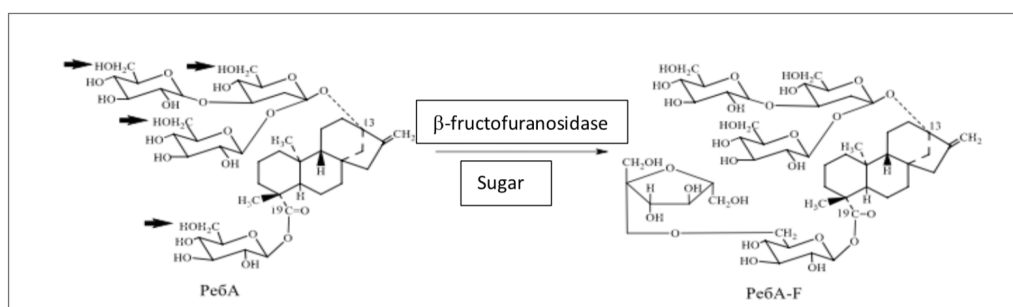


Figure 4. Transfructosylation of RebA

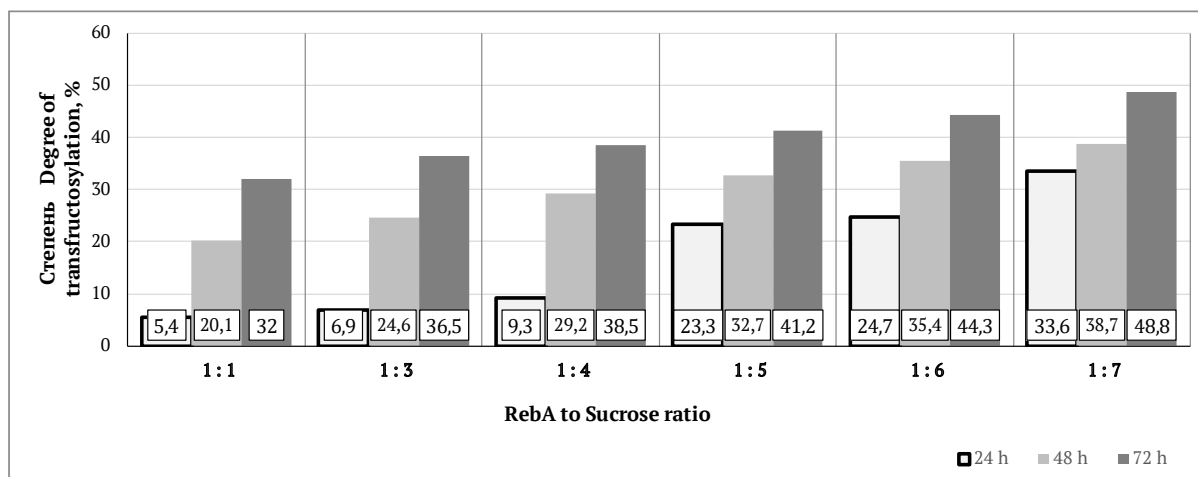


Figure 5. The effect of the ratio of sucrose and RebA, as well as reaction time on the degree of transfructosylation

The effect of concentration in the reaction mixture. The efficiency of transglycosilation would increase proportionately with increase concentration of the substrates, i.e reaction was more efficient in the concentrated solutions and higher combined concentration of RebA and sucrose resulted in higher yield of fructosylated RebA (Figure 6).

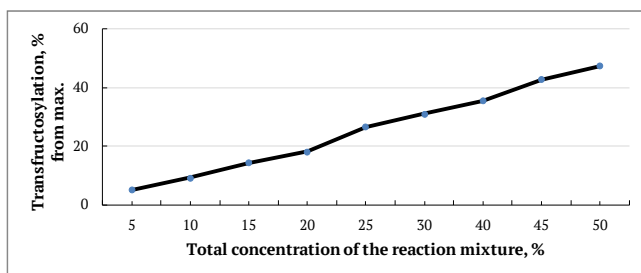


Figure 6. The effect of the concentration of the reaction mixture on the degree of transfructosylation (ratio of sucrose to RebA = 10:1; 48hours; 40°C).

The effect of pH, temperature and amount of enzyme. In order to determine the effect of pH on transfructosylation, the FFase was incubated with a solution of 1% RebA and 10% sucrose at 40°C for 15 hours at various pH values. It was revealed that the transfructosylation reaction proceeds most effectively in the pH range 6.5-8.0 (Figure 7).

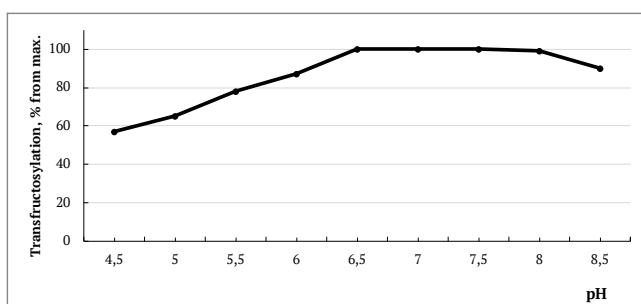


Figure 7. The effect of pH on transfructosylation of RebA with β -fructofuranosidase from Arthrobacter K-1

To determine the most suitable temperature, the enzyme was incubated at different temperatures with a solution of 1% RebA and 10% sucrose at pH 6.7 for 15 hours with constant stirring. The reaction has a distinct temperature optimum at 40°C. Deviation from this leads to the sharp decline in the efficiency of the reaction (Figure 8).

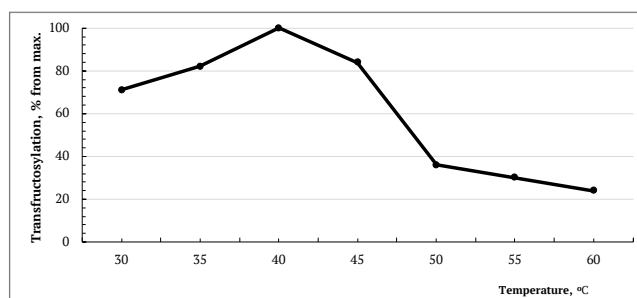


Figure 8. The effect of temperature on transfructosylation of RebA with β -fructofuranosidase from Arthrobacter K-1.

It was also revealed that the reaction is accelerated with an increase in the amount of enzyme. The optimal amounts were 50-100 units per 1 g of sucrose (Figure 9).

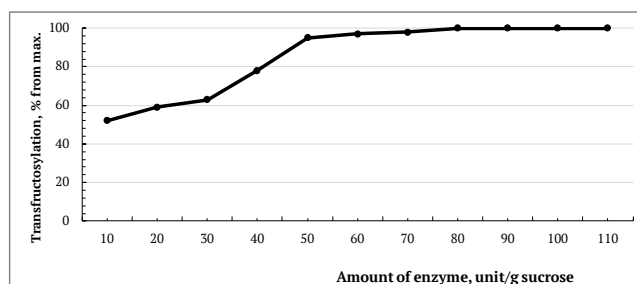


Figure 9. The effect of amount of enzyme β -fructofuranosidase on transfructosylation of RebA

Isolation and purification of fructosylated RebA. Forty nine grams (49 g) of RebA and 340 g

of sucrose was dissolved in 1L of deionized water, β -fructofuranosidase was added in the amount of 80 units per gram of sucrose and the reaction was carried out at 40°C for 48 hours with constant stirring.

The reaction was stopped by boiling, then the reaction mixture was cooled, an equal volume of absolute ethanol was added and the precipitate was filtered off. The filtrate was decolorized with activated carbon (1%) at 50-60°C for 20 minutes. The ethanol was evaporated and the residue was purified on two columns filled with Diaion HP-20 macroporous resin. The columns were successively washed with 10 volumes of water and 10% ethanol and the adsorbed glycosides were eluted with 5 volumes of 70% methanol. The eluate from the first

column was a mixture of the large part of unmodified RebA and fructosyl-RebA, while the second column contained more than 80% of fructosylated RebA (Figure 10a, b).

Further purification of fructosyl-RebA was carried out by crystallization and recrystallization from the minimal amount of absolute methanol, in which it is practically insoluble. The purity of the obtained white fructosyl-RebA crystals reaches up to 95% (Figure 11).

The fructosyl-RebA identification was performed by mass spectrometry (MS) analysis using an Agilent 6110 Series Quadrupole LC / MS with column C₁₈ silica gel.

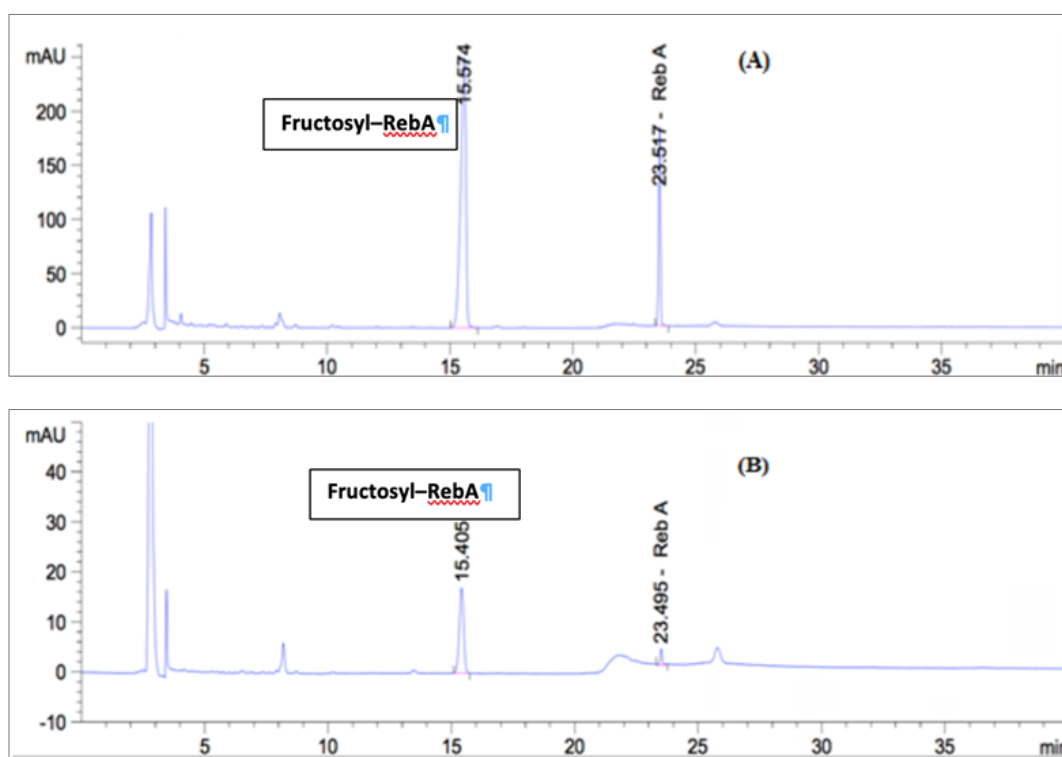


Figure 10. HPLC charts of eluates from the first (A) and the second columns (B)

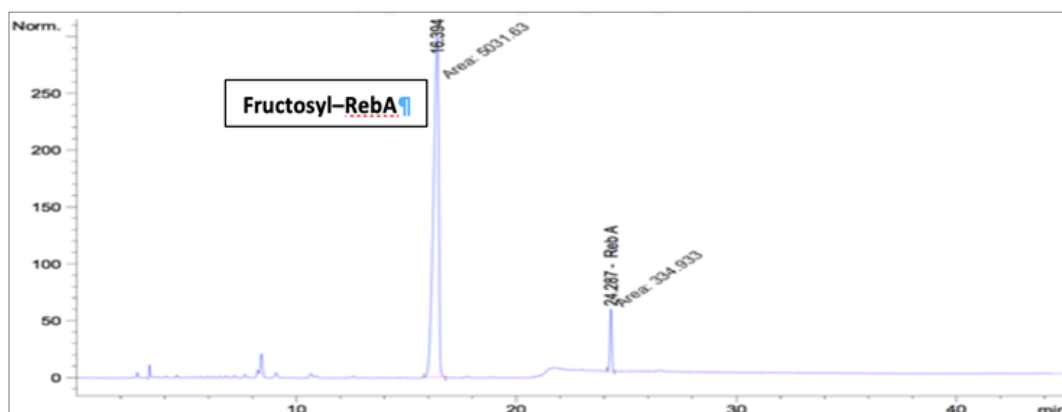


Figure 11. HPLC chart of fructosyl-RebA after recrystallization from absolute methanol

Fructosyl-RebA, $C_{44}H_{70}O_{25}$, is β -2,6-fructosylated at 19-O-glucosyl group and identified as β -D-fructofuranosyl-2,6- β -D-glucopyranosyl ester steviol-13-O- β -D-glucopyranosyl-1,2- β -D-glucopyranosyl-1,3- β -D-glucopyranoside with molecular weight of 1128 (Figure 12).

According to the results of sensory analysis, it was shown that transfructosylation leads to an improvement in the taste profile, aftertaste, bitterness, and palatability. The resulting flavor profile is comparable to the flavor characteristics of aspartame. There was a decrease in bitterness and an improvement in the taste profile relative to RebA. The taste characteristics of fructosylated RebA are quite comparable with those of aspartame (Table 3). However, in low pH drinks, the fructofuranose bond is not stable enough and can hydrolyse under conditions over time. Fructosyl-RebA can be excellent tasting and soluble sweetener in the production of low-calorie table top sweeteners (Chaturvedula et al., 2011, pp. 16-26; Fukunaga et al., 1989, pp. 1603-1607).

Thus, as a result of comparative studies, the possibility of efficient transfructosylation of RebA using β -fructosyltransferase in the presence of an excess amount of sucrose was shown. The product has excellent taste characteristics and good solubility and can be used in various foods and beverages as a low-calorie sweetener.

Table 3

Organoleptic evaluation of fructosylated derivatives (Ishikawa et al., 1990, pp. 3137-3143)

Component	Bitterness	Aftertaste	Overall taste quality
Fructosyl-rebA	4.9	4.5	2.7
Fructosyl-stevioside	4.6	4.2	2.9
Fructosyl-rubusoside	2.6	2.7	1.5
Stevioside	3.3	3.5	1.5
RebA	3.7	3.5	1.9
Aspartam	4.7	4.3	3.0

Note: The evaluation was carried out in sample solutions with concentrations corresponding to the sweetness of a 5% sucrose solution. 5, Best; 4, Significantly better; 3, better; 2, slightly better; 1, worse.

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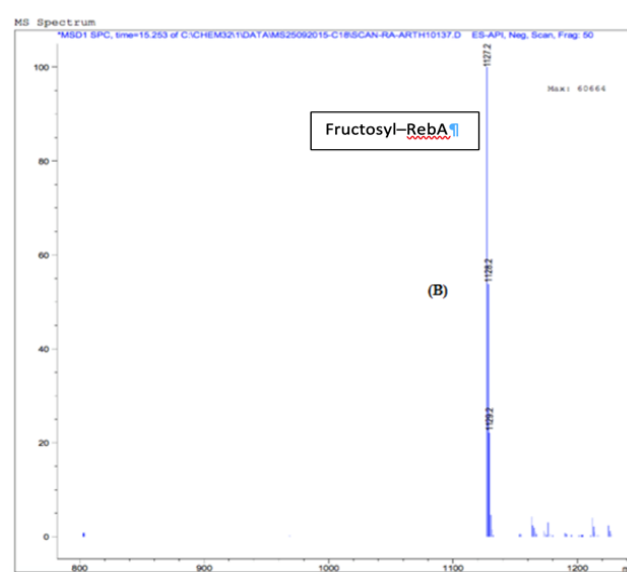
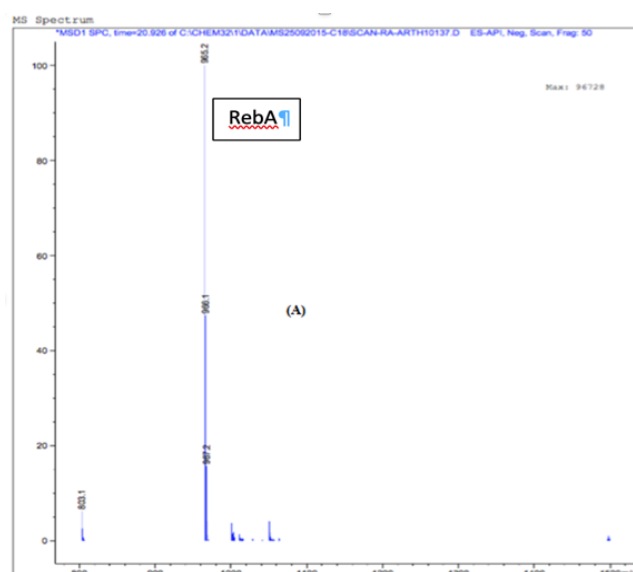


Figure 12. LC/MC analysis of RebA (A) and fructosyl-RebA (B)

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Трансгликозилирование Ребаудиозида А β-фруктофуранозидазой

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Ребаудиозид А (RebA) подвергали β-2,6-трансгликозилированию β-фруктофуранозидазой из *Arthrobacter* sp. K-1 и сахаразы как источник звеньев фруктозы. Выход трансгликозилирования существенно зависит от концентрации акцептора, донора и фермента, а также от времени реакции. При массовом соотношении RebA к сахарозе 1:1 степень трансфруктозилирования за 24 часа составила всего 5,4%, а при соотношении 1:5 она достигает более 23%. Выявлено, что трансфруктозилирование протекает эффективнее в концентрированных растворах, чем выше общая концентрация сахарозы и RebA, тем выше выход фруктозилированного RebA. Для определения влияния pH на трансфруктозилирование β-фруктофуранозидазу инкубировали с раствором 1% RebA и 10% сахарозы при 40 °С в течение 15 часов при различных значениях pH. Также было обнаружено, что с увеличением количества фермента реакция ускоряется. Наиболее оптимальными были количества 50-100 единиц на 1 г сахарозы. Исследована реакция трансфруктозилирования стевиозида, а также показана органолептическая оценка фруктозилированных производных фруктозил-RebA, фруктозил-стевиозида и фруктозил-рубузозида. Выделение и очистку фруктозилированного RebA осуществляли осаждением и очисткой этанолом на колонках, заполненных макропористой смолой Diaion HP-20. Полученный продукт обладает улучшенными сенсорными характеристиками и может быть использован в качестве низкокалорийного подсластителя.

Keywords: transfructosylation, β-fructofuranosidase, cultivation of *Arthrobacter* sp. K-1, β-Fructofuranosidase (FFase) activity, reaction conditions for transfructosylation of RebA, isolation and purification of fructosyl-RebA, taste profile of fructosylated derivatives

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Как цитировать

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