Journal of Molecular Catalysis B: Enzymatic 82 (2012) 12–17

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Enzymatic preparation of a natural sweetener rubusoside from specific hydrolysis of stevioside with β-galactosidase from *Aspergillus* sp.

Hui-da Wan[a], Guan-jun Tao[a], Doman Kim[b], Yong-mei Xia[a,*]

[a] State Key Laboratory of Food Science and Technology, School of Chemical and Materials Engineering, Jiangnan University, Wuxi, Jiangsu 214122, China
[b] Department of Biotechnology and Bioengineering, Chonnam National University, Gwangju 500-757, Republic of Korea

ARTICLE INFO

Article history:
Received 5 January 2012
Received in revised form 17 May 2012
Accepted 17 May 2012
Available online 29 May 2012

Keywords:
Stevioside
Rubusoside
β-Galactosidase
Hydrolysis

ABSTRACT

Rubusoside is a precious bioactive sweetener which mainly exists in Chinese sweet tea plant (*Rubus suavissimus* S. Lee), while stevioside is an abundant natural sweetener with bitter aftertaste. In this work, a β-galactosidase from *Aspergillus* sp. presented specific hydrolytic activity on β-1,2 glucosidic linkage of stevioside, and converting stevioside to rubusoside. The hydrolytic activity was weak on the natural stevioside analogs, which makes the process a promising approach to produce rubusoside and utilize stevioside. The highest steviosides conversion was 98.3%, accompanying with a rubusoside yield of 91.4%.

1. Introduction

Rubusoside (Ru, 13-O-β-glucosyl-19-O-β-D-glucosyl-steviol) is a rare natural sweetener mainly existed in extract of Chinese sweet tea plant [1,2] (*Rubus suavissimus* S. Lee; known in Japan as Tenryocha or Tencha [3]). The tea plant grows only in southern China with variable yearly yield depending on local climate. Rubusoside is also a minor component in extract of *Stevia rebaudiana* leaf. However, there are few reports focused on synthetic rubusoside. For example, rubusoside was found as an intermediate in a chemo-enzymatic preparation of rebaudioside A, another sweetener that also exists in *Stevia rebaudiana* leaf [4]. Jiang et al. [5] reported that a bacterium (*Chryseobacterium* sp.) fermentation liquid or the β-glucosidase produced from the fermentation stimulated the hydrolysis of stevioside (St, 13-O-β-sophorosyl-19-O-β-D-glucosyl-steviol) into rubusoside, but the results lack enough structure characterization to exclude the isomers.

As an analog of rubusoside, stevioside exists in *Stevia rebaudiana* leaf as an abundant component, which is much less valuable than rubusoside because of its bitter aftertaste [6–8]. Selective cleavage of β-1,2-glucosidic linkage of sophorosyl moiety at C13 of stevioside can produce rubusoside. Whereas, the hydrolysis of stevioside may produce steviol, isosteviol, steviolmonoside, steviolbioside or their mixtures depending on the catalyst and reaction conditions (Scheme 1), because that stevioside possesses three glycosidic bonds (β-linked sophorose, β-1,2-D-glucopyranosyl on C13 and an ester β-glucosidic linkage on the C19 carboxyl group) [9–13].

In fact, most of the hydrolysis products of stevioside exist naturally and exhibit potential pharmaceutical properties, such as anti-inflammatory and anti-tumor activity [12,14–16]. However, few researchers applied hydrolysis of stevioside to improve its taste. A possible reason is that people always focus on the fact that the mono- and di-glycosylation at the 13-O-β-sophorosyl moiety can effectively improve the sweetness character [17].

Among the hydrolysis enzymes, β-galactosidases (β-D-galactoside galactohydrolase, EC 3.2.1.23) are known to catalyze both hydrolysis and transgalactosylation [18,19]. For example, Danieli et al. [20] demonstrated that bovine β-galactosidase (1,4-galactosyltransferase) could catalyze the galactosylation of stevioside and steviolbioside with UDP-galactose, affording corresponding galactosyl derivatives with improved sweetness.

An interesting and valuable hydrolysis using β-galactosidase from *Aspergillus* sp. was noticed when the galactosylation of stevioside was expected, in which rubusoside was obtained as the main product. Therefore, in this work, a β-galactosidase from *Aspergillus* sp. was employed to catalyze the hydrolysis of stevioside, its substrate specificity and regioselectivity as well as the hydrolysis conditions were investigated.

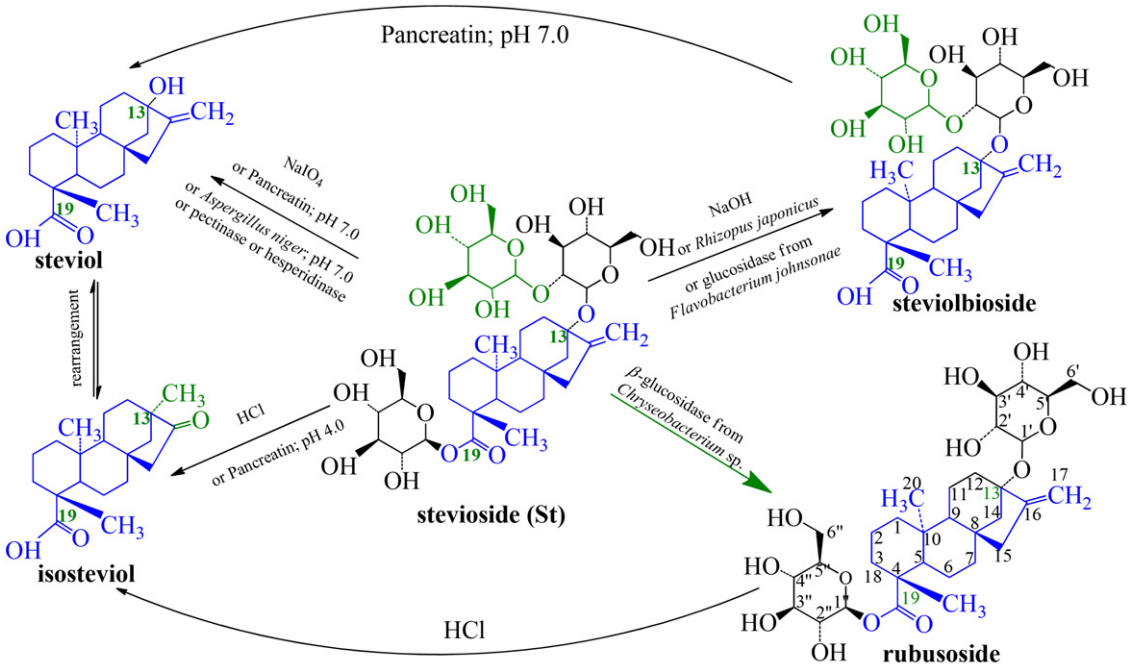
2. Materials and methods

2.1. Enzyme and chemicals

β-Galactosidases from *Aspergillus* sp. (CICIM F0620, from CCTCC) and *Sulfolobus* sp. (ATCC No35092) were provided by Dr Wu

* Corresponding author. Tel.: +86 510 85327973; fax: +86 510 85919625.
E-mail addresses: ymxia@jiangnan.edu.cn, ymxia@126.com (Y.-m. Xia).

1381-1177/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.molcatb.2012.05.018



Scheme 1. Hydrolysis of stevioside using different catalysts.

Jing from the State Key Laboratory of Food Science and Technology at Jiangnan University. β-Galactosidase from *Kluyveromyces lactis* (Maxilact® LG 2000, 2000 NLU/g) was provided by Royal DSM N.V. β-Galactosidase from *Kluyveromyces lactis* (Lactozym 3000L HP-G, 3000LAU/mL) was presented by Novozymes (China). α-Galactosidase from *Asperillus niger* was provided by Amano Pharmaceutical Co., Ltd. (China). Stevioside (≥97%, HPLC) was purchased from GLG Life Tech. Co., Ltd. (China). Natural rubusoside (98.6%, HPLC) was provided by Professor Chen Quan-bin at Guangxi Normal University China. Steviol glycosides extracted from *Stevia rebaudiana* leaf (30.0% stevioside, 35.9% rebuside A, 11.2% rebaudioside C, 22.9% others, HPLC) and rebaudioside A (≥99%, HPLC) were provided by Changzhou Niutang Chemical Plant Co., Ltd. *o*-Nitrophenyl β-D-galactopyranoside was purchased from Sigma Chemical Co. Na_2CO_3, CH_3COONa, CH_3COOH, $Na_2HPO_4{\cdot}12H_2O$ and $NaH_2PO_4{\cdot}2H_2O$ were purchased from Sinopharm Chemical Reagent Co., Ltd., China. All other reagents were of analytical grade unless otherwise stated.

2.2. Galactosidase hydrolytic activity assay

The hydrolytic activity was determined using *o*-nitrophenyl β-D-galactopyranoside as substrate [21]. The increase in the absorbance at 420 nm (T6 New-Century, Beijing Purkinje General Instrument Co., Ltd.) caused by the release of *o*-nitrophenol was measured to calculate the hydrolytic activity. The reaction mixture containing 1.8 mL acetate buffer (50 mM, pH 4.5), 100 μL enzyme solution (dissolved and diluted using the aforementioned buffer) and 100 μL *o*-nitrophenyl β-D-galactopyranoside (20 mM) was shaken at 60 °C for 10 min, and then quenched by 1 mL Na_2CO_3 (1 M). One unit (U) of hydrolytic activity is defined as the amount of enzyme required to release 1 μmol *o*-nitrophenol per min under the above reaction conditions.

2.3. Enzymatic hydrolysis of stevioside by β-galactosidase

In a typical reaction, 20 mL stevioside solution (10 g/L) and β-galactosidase (0.8 kU/g stevioside) were mixed in a 50 mL Erlenmeyer flask, shaken at 60 °C for 24 h. The reaction mixture was then boiled for 3 min to deactivate the enzyme and the precipitated enzyme was removed by centrifugation. The supernatant was purified with column chromatography over silica gel (200–300 mesh) to obtain the products eluted by CH_2Cl_2:CH_3CH_2OH:H_2O (5:4:1, v/v/v).

The product was analyzed with the HPLC system (Waters 2996, United States) equipped a NH_2 column (APS-2 HYPERSIL, Thermo, United States) and a photodiode array detector. A mixture of acetonitrile and water was used as eluent, gradient from 75:25 (v/v) to 50:50 (v/v) at 1 mL/min. LC–MS-MS profile was taken from Waters Acquity UPLC system (BEH HILIC column, acetonitrile and water (80:20, v/v), 0.3 mL/min, column temperature: 30 °C, collision energy: 20–55 eV, polarity: ES^-). NMR spectra were recorded at AVANCE III 400 MHz Digital NMR Spectrometer (Bruker, USA). The conversion of stevioside was calculated as following:

$$\text{coversion} = \frac{C_0 - C_t}{C_0} \times 100\%$$

Here C_0 is the initial stevioside concentration (g/L), C_t is the real time stevioside concentration in the reaction mixture (g/L). The stevioside concentration was determined with a standard calibration curve. Product yields were calculated according to the percentages of chromatographic areas and calibrated with that of stevioside.

The concentration of glucose in the reaction mixture was determined with SBA-50 Glucose Biosensor (Biology Institute of Shandong Academy of Sciences, Shangdong, China). All tests were performed in triplicate.

3. Results and discussion

3.1. Enzyme screen

Initially, five galactosidases were assayed for their hydrolytic activity on stevioside (Fig. 1, a: 40 °C; b: 70 °C).

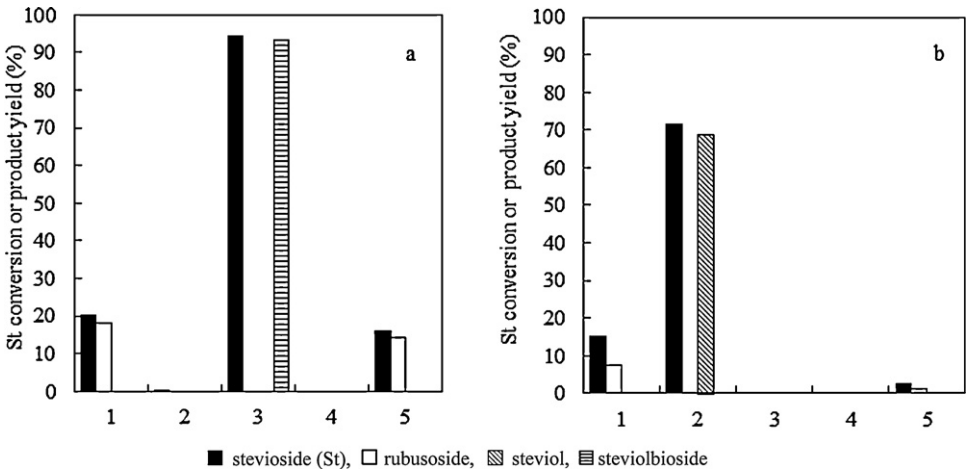


Fig. 1. Hydrolysis of stevioside in the presence of β-galactosidases. The enzymes used were: 1. *Aspergillus* sp., 14 kU/g stevioside; 2. *Sulfolobus* sp., 1 kU/g stevioside; 3. *Kluyveromyces lactis* (Maxilact® LG 2000), 20 KNLU/g stevioside; 4. *Kluyveromyces lactis* (Lactozym 3000L HP-G), 3KLAU/g stevioside; 5. α-Galactosidase from *Asperillus niger*, 10 kU/g stevioside. All experiments were carried out for 3 h with 10 g stevioside/L.

Among the assayed galactosidases, β-galactosidase from *Kluyveromyces lactis* (Lactozym 3000L HP-G) did not show obvious hydrolytic activity on stevioside. β-Galactosidases from *Sulfolobus* sp. or from *Kluyveromyces lactis* (Maxilact® LG 2000) stimulated the hydrolysis of stevioside, but produced steviol and steviolbioside, respectively. While β-galactosidase from *Aspergillus* sp. and α-galactosidase from *Asperillus niger* induced a specific production of rubusoside, but the latter possessed poor thermal stability (Fig. 1b).

Therefore, the β-galactosidase from *Aspergillus* sp. was chosen for the subsequent experiments, in which rubusoside and minor transglycosylation products were found in an extra long reaction (Fig. 2); the products were then identified with LC–MS-MS (see also supplementary material Fig. S1) and NMR profiles (Table 1 and supplementary material Figs. S2 and S3).

As reported in literature, β-galactosidases are usually used to cleave β-1,4 or β-1,6 galactosidic linkage of glycosides, and/or accelerate the transglycosylation [21–23]. Apparently, in this experiment, the β-galactosidase possessed β-glucosidase activity and hydrolyzed β-1,2 glucosidic linkage of stevioside selectively. Actually, as the member of glycoside hydrolase family, β-galactosidase or β-glucosidase could play dual roles under certain circumstances [24–27].

3.2. Transglycosylation of stevioside

Some β-galactosidases including β-galactosidase from *Aspergillus* sp. have good transglycosylation activity in the synthesis of galactooligosaccharides from lactose [21,22,28]. Fig. 2 indicates that there were some transglycosylated steviosides (LC–MS-MS profile, supplementary material Fig. S4) except the majority of hydrolysis product. To investigate the competition between the hydrolysis and transglycosylation, extra glucose or lactose was employed to verify the transglycosylation of stevioside in the presence of the β-galactosidase in water, respectively. However, the β-galactosidase presented very weak transglycosylation activity just as it did in the hydrolysis of stevioside in the absence of glucose (Fig. 3).

In addition, in the hydrolysis of stevioside, the glucose level in the reaction mixture was 2.13 g/L when stevioside conversion reached 95.0%. This is equivalent to the calculated glucose level (2.12 g/L) if 95.0% of β-1,2 glucosidic linkages of stevioside were cleaved from stevioside.

3.3. The substrate specificity of the hydrolytic activity of the β-galactosidase

To investigate the substrate specificity of the hydrolytic activity of the β-galactosidase, a commercial crude *Stevia rebaudiana* leaf extract containing a family of steviol glycosides (supplementary material Fig. S5) was examined using the β-galactosidase on their hydrolytic activity as shown in Table 2 and Fig. 4.

Fig. 4 discloses a very interesting result that none of the analogs of stevioside, i.e. rebaudioside A, rebaudioside C, and other steviol glycosides including rubusoside itself, performed remarkable hydrolysis in the presence of the β-galactosidase. This hydrolysis is specific for stevioside, which truly offers an efficient process to produce rubusoside specifically, the bioactive sweetener obtained mainly from nature before.

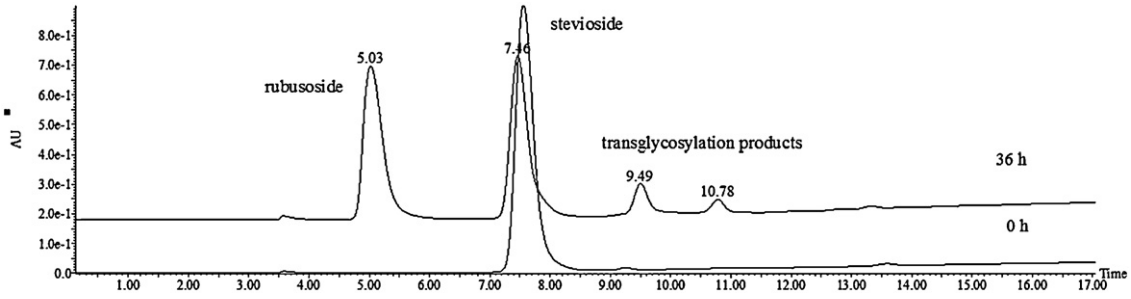


Fig. 2. HPLC chromatogram of the hydrolysis products of stevioside using β-galactosidase from *Aspergillus* sp. 60 °C, 0.4 kU/g steviosides, 80 g stevioside/L.

Table 1
^{13}C NMR profile of the main hydrolysis product of St.

	No.	Ru[a]	Ru[b]	Product[c]
Aglycone	1	40.8	41.0	41.1
	2	19.5	19.7	19.8
	3	38.4	38.6	38.7
	4	44.1	44.3	44.4
	5	57.4	57.6	57.7
	6	22.2	22.4	22.5
	7	41.7	41.9	42.1
	8	42.4	42.7	42.8
	9	54.0	54.2	54.3
	10	39.8	40.1	40.2
	11	20.7	20.9	21.0
	12	37.3	37.5	37.7
	13	85.9	86.2	86.3
	14	44.6	44.8	44.9
	15	47.8	48.0	48.1
	16	154.6	154.8	154.9
	17	104.4	104.6	104.8
	18	28.4	28.6	28.7
	19	177	177.2	177.3
	20	15.6	15.8	16.0
C13-O-Glu	1	95.9	96.1	96.3
	2	74.0	74.2	74.4
	3	79.1	79.3	79.5
	4	71.1	71.3	71.5
	5	79.4	79.5	79.7
	6	62.1	62.3	62.5
C19-O-Glu	1	99.7	99.9	100.1
	2	75.6	75.7	75.9
	3	78.8	79.0	79.2
	4	72.4	72.6	72.8
	5	78.1	78.3	78.4
	6	63.1	63.3	63.5

^{13}C NMR profile (δ: ppm).
[a] Natural Ru reported in [3].
[b] Natural Ru from Prof. Chen Quan-bin, referring to Section 2.
[c] The enzymatic hydrolysis product

3.4. Optimization of the hydrolysis conditions

Subsequently, Fig. 5 provides the optimization of the hydrolysis conditions including the reaction temperature, the initial concentration of stevioside, the enzyme loading, pH and the time course.

As shown in Fig. 5, the highest stevioside conversion of 98.3% and a rubusoside yield of 91.4% were obtained under the optimum

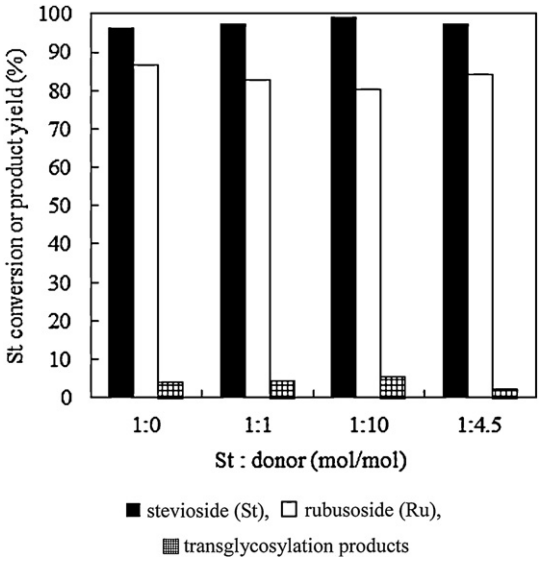


Fig. 3. Effect of glycosyl donors on the hydrolysis catalyzed by the β-galactosidase. 60 °C, 3 h, 14 kU/g stevioside, 10 g stevioside/L, stevioside:glucose = 1:1 or 1:10 (mol/mol), stevioside:lactose = 1:4.5 (mol/mol).

Table 2
HPLC–MS features of the steviol glycosides and transglycosylation products.

Product	Retention time (min)	$(M-H)^-$
Steviolmonoside (Sm)	0.75	479.2
Rubusoside (Ru)	2.03	641.2
Steviolbioside (Sbio)	0.93	641.2
Dulcoside A (DA)	3.01	787.2
Stevioside (St)	4.75	803.2
Rebaudioside F (RF)	6.79	935.2
Rebaudioside C (RC)	9.08	949.2
Rebaudioside A (RA)	9.57	965.1
St-Glu1	12.14	965.1
Rebaudioside D (RD)	14.83	1127.2
St-Glu2	14.45	1127.2

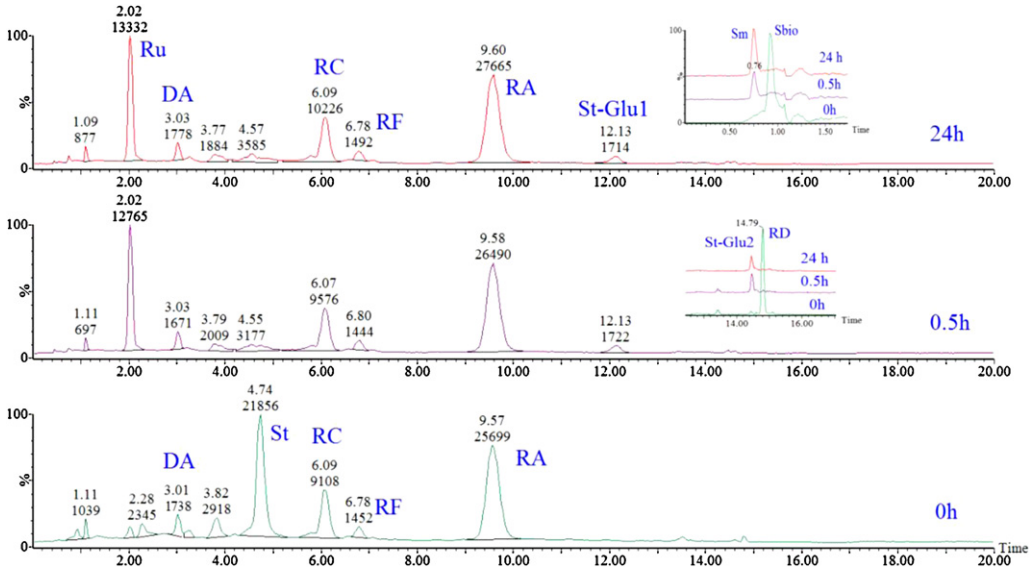


Fig. 4. Total ion chromatography of the reaction mixture of steviol glycosides. 60 °C, 10 g steviol glycosides/L, 5 kU/g steviol glycosides.

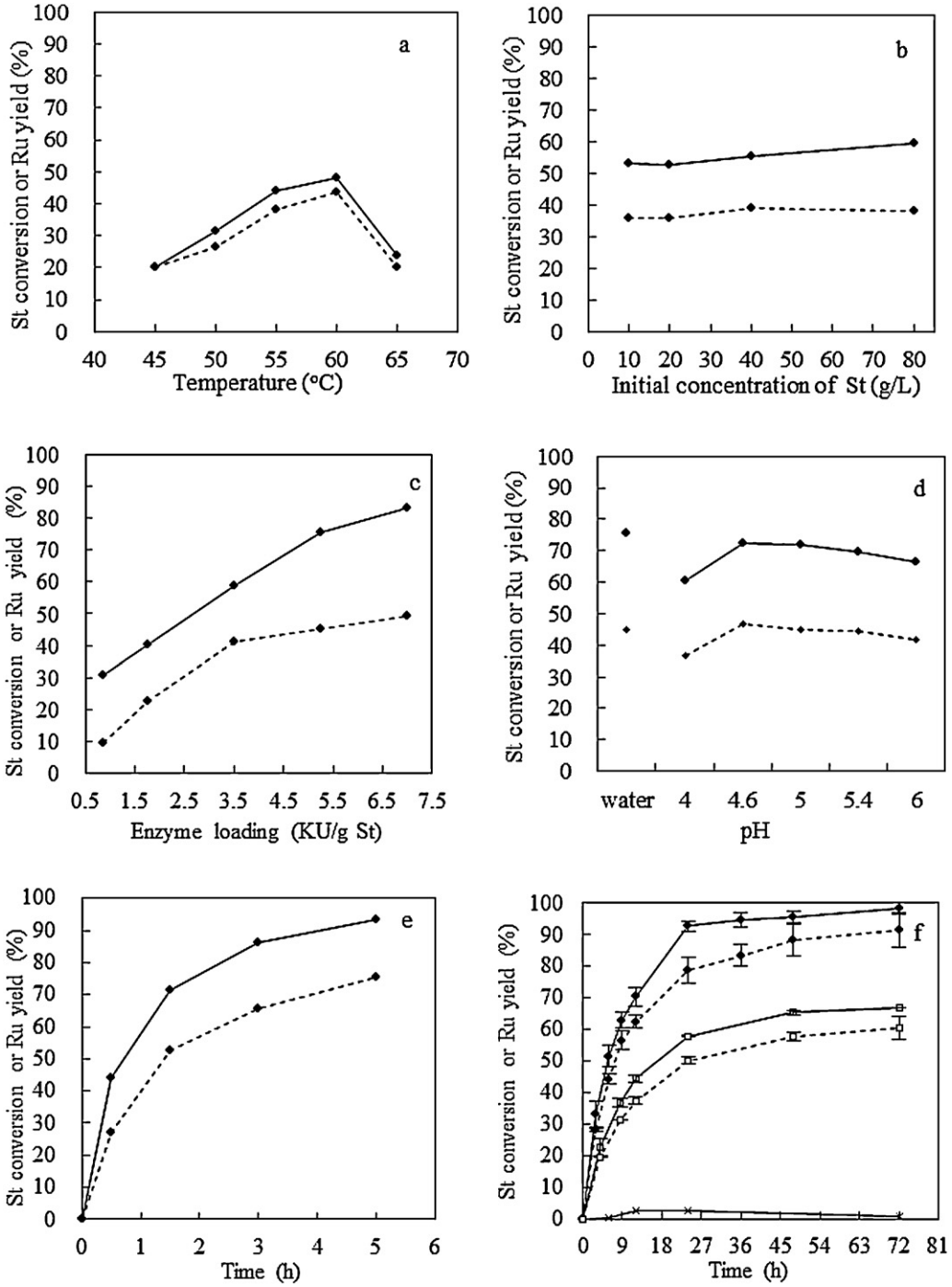


Fig. 5. Optimization of the hydrolysis of stevioside. a: 10 g stevioside/L, 3.5 kU/g stevioside, 1.5 h, DI water; b: 3.5 kU/g stevioside, 60 °C, 1.5 h, DI water; c: 80 g stevioside/L, 60 °C, 1.5 h, DI water; d: 80 g stevioside/L, 5.25 kU/g stevioside, 5 mL buffer, 60 °C, 1.5 h, acetate buffer (pH 4.0–5.4, 50 mM), phosphate buffer (pH 6.0, 50 mM); e: 80 g stevioside/L, 5.25 kU/g stevioside, DI water, 60 °C; f: 80 g stevioside/L, 60 °C, DI water.

conditions. The sharp turning in Fig. 5a indicated the β-galactosidase is thermally unstable, which can be improved by enzyme immobilization. The β-galactosidase presents highest hydrolytic activity in DI water, which might be because that the ionic strength effect on the enzyme activity.

4. Conclusions

A β-galactosidase from *Aspergillus* sp. was found to be able to catalyze the hydrolysis of stevioside selectively to produce rubusoside, a natural sweetener that is mainly obtained from Chinese sweet tea. The β-galactosidase presents weak transglycosylation activity in the transition of glucose or lactose onto stevioside as well as weak hydrolytic activity on the analogs of stevioside. The highest rubusoside yield was obtained in 72 h at 60 °C with 80 g stevioside/L and an enzyme loading of 0.8 kU/g stevioside in water.

Acknowledgments

Financial support from National Natural Science Foundation of China (31171752), Innovation Fund for the Industry-University Cooperative Research in Jiangsu Province (BY2010115), and the

Research Program of State Key Laboratory of Food Science and Technology (SKLF-ZZB-201207) are gratefully appreciated.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2012.05.018.

References

[1] G.Y. Koh, G. Chou, Z. Liu, J. Agric. Food Chem. 57 (2009) 5000–5006.
[2] G.Y. Koh, K. McCutcheon, F. Zhang, D. Liu, C.A. Cartwright, R. Martin, P. Yang, Z. Liu, J. Agric. Food Chem. 59 (2011) 98–104.
[3] N. Sugimoto, K. Sato, H.M. Liu, H. Kikuchi, T. Yamazaki, T. Maitani, Shokuhin Eiseigaku Zasshi 43 (2002) 250–253.
[4] N. Kaneda, R. Kasai, K. Yamasaki, O. Tanaka, Chem. Pharm. Bull. 25 (1977) 2466–2467.
[5] Z.Y. Jiang, Y.R. Chen, H. Liu, Acta Microbiol. Sin. 51 (2011) 43–49.
[6] G. Brahmachari, L.C. Mandal, R. Roy, S. Mondal, A.K. Brahmachari, Arch. Pharm. 344 (2011) 5–19.
[7] P.K. Mishra, R. Singh, U. Kumar, V. Prakash, Global J. Biotechnol. Biochem. 5 (2010) 62–74.
[8] V. Chatsudthipong, C. Muanprasat, Pharmacol. Therapeut. 121 (2009) 41–54.
[9] H. Mizukami, K. Shiiba, H. Ohashi, Phytochemistry 21 (1982) 1927–1930.
[10] A.G. Avent, J.R. Hanson, B.H. De Oliveira, Phytochemistry 29 (1990) 2712–2715.
[11] K. Okamoto, H. Nakano, T. Yatake, T. Kiso, S. Kitahata, Biosci. Biotechnol. Biochem. 64 (2000) 333–340.
[12] H.M.S. Milagre, L.R. Martins, J.A. Takahashi, Braz. J. Microbiol. 40 (2009) 367–372.
[13] C. Wehrli, Application: WO 2011089031, DSM IP Assets B.V., Neth. (2011).
[14] J. Li, D. Zhang, X. Wu, Bioorg. Med. Chem. Lett. 21 (2011) 130–132.
[15] R.R. Sharipova, I.Y. Strobykina, V.E. Kataev, O.A. Lodochnikova, A.T. Gubaidullin, A.A. Stomakhin, Russ. J. Gen. Chem. 79 (2009) 2668–2672.
[16] H.P. Li, C.M. Dong, Y.M. Hou, H.M. Liu, Chem. Res. Chin. Univ. 25 (2009) 116–117.
[17] Y. Fukunaga, T. Miyata, N. Nakayasu, K. Mizutani, R. Kasai, O. Tanaka, Agric. Biol. Chem. 53 (1989) 1603–1607.
[18] Q. Husain, Crit. Rev. Biotechnol. 30 (2010) 41–62.
[19] P.L.H. McSweeney, P.F. Fox, M.J. Playne, R.G. Crittenden, Galacto-oligosaccharides and other products derived from lactose, in: P. McSweeney, P.F. Fox (Eds.), Advanced Dairy Chemistry, Springer, New York, 2009, pp. 121–201.
[20] B. Danieli, M. Luisetti, M. Schubert-Zsilavecz, W. Likussar, S. Steurer, S. Riva, D. Monti, J. Reiner, Helv. Chim. Acta 80 (1997) 1153–1160.
[21] H.-Y. Park, H.-J. Kim, J.-K. Lee, D. Kim, D.-K. Oh, World J. Microbiol. Biotechnol. 24 (2008) 1553–1558.
[22] K. Iwasaki, M. Nakajima, S.-i. Nakao, Process Phytochem. 31 (1996) 69–76.
[23] I. Mladenoska, E. Winkelhausen, S. Kuzmanova, Food Technol. Biotechnol. 46 (2008) 311–316.
[24] E. Ishikawa, T. Sakai, H. Ikemura, K. Matsumoto, H. Abe, J. Biosci. Bioeng. 99 (2005) 331–339.
[25] P. Nakkharat, D. Haltrich, J. Biotechnol. 123 (2006) 304–313.
[26] T. Hansson, P. Adlercreutz, Biotechnol. Lett. 24 (2002) 1465–1471.
[27] H.X. Fan, L.L. Miao, Y. Liu, H.C. Liu, Z.P. Liu, Enzyme Microb. Technol. 49 (2011) 94–99.
[28] P. Czermak, M. Ebrahimi, K. Grau, S. Netz, G. Sawatzki, P.H. Pfromm, J. Membr. Sci. 232 (2004) 85–91.