离子液体对 β -糖苷酶催化合成红景天苷的影响

毕艳红, 王朝宇*, 茅燕勇, 郑尚永, 张海江, 时 号

淮阴工学院生命科学与化学工程学院, 江苏淮安 223003

摘要: 研究了混合溶剂体系中阴离子分别为 BF_4^- , PF_6^- , Cl^- , Br^- 和 I^- 的 14 种离子液体对来源于黑布林糖苷酶催化合成红景天苷反应的影响。结果表明, 在最佳反应条件下, β -糖苷酶的反应初速度和红景天苷的产率分别为 $3.3 \text{ mmol}/(\text{L}\cdot\text{h})$ 和 24.5% 。离子液体咪唑阳离子的烷基链长 ($\text{C}_2\sim\text{C}_{10}$) 对 β -糖苷酶的活性影响较大, 当烷基链长为 C_6 时, 糖苷酶表现出较高的催化活性。

关键词: 离子液体; 红景天苷; 糖苷化反应; 黑布林籽; 混合溶剂

中图分类号: O643 **文献标识码:** A

收稿日期: 2012-02-23. 接受日期: 2012-04-13.

*通讯联系人. 电话/传真: (0517)83591165; 电子邮箱: biowzy@126.com

基金来源: 江苏省高校自然科学研究项目 (10KJB530001); 苏北科技发展计划项目 (BC2010441); 淮安市科技支撑计划 (工业) 项目 (HAG2011005); 淮安市科技支撑计划 (农业) 项目 (SN1143, SN1052).

本文的英文电子版(国际版)由Elsevier出版社在ScienceDirect上出版(<http://www.sciencedirect.com/science/journal/18722067>).

Ionic Liquid Effects on the Activity of β -Glycosidase for the Synthesis of Salidroside in Co-solvent Systems

BI Yanhong, WANG Zhaoyu*, MAO Yanyong, ZHENG Shangyong, ZHANG Haijiang, SHI Hao

School of Life Science and Chemical Engineering, Huaiyin Institute of Technology, Huai'an 223003, Jiangsu, China

Abstract: The preparation of salidroside was successfully carried out in fourteen ionic liquids (ILs)-containing systems using β -glycosidase from black plum seeds for the first time. The optimum conditions were determined for $\text{C}_6\text{MIm}\cdot\text{BF}_4$, pH, phosphate buffer content, and molar ratio of tyrosol to D-glucose to be 1% (v/v), 5.9, 20% (v/v), and 8:1, under which the initial reaction rate and yield were $3.3 \text{ mmol}/(\text{L}\cdot\text{h})$ and 24.5% , respectively. Moreover, the effects of 1-alkylimidazolium-based ILs possessing different alkyl chain lengths from C_2 to C_{10} and a variety of anions including BF_4^- , PF_6^- , Cl^- , Br^- , and I^- on enzyme activity in co-solvent systems were investigated. The results indicate that the optimal chain length of the alkyl substituent on the imidazolium ring of the cation was C_6 .

Key words: ionic liquid; salidroside; glucosylation; black plum seed meal; co-solvent

Received 23 February 2012. Accepted 13 April 2012.

*Corresponding author: Tel/Fax: +86-517-83591165; E-mail: biowzy@126.com

This work was supported by the Natural Science Research Project of Higher Education of Jiangsu (10KJB530001), the Subei Technology and Development Program of Jiangsu (BC2010441), the Science and Technology Supporting Program (Industry) of Huai'an City (HAG2011005), and the Agriculture Supporting Program of Huai'an City (SN1143, SN1052).

English edition available online at Elsevier ScienceDirect (<http://www.sciencedirect.com/science/journal/18722067>).

Salidroside, a phenylpropanoid glycoside isolated from the root of the Chinese medicinal plant *Rhodiola sachalinensis* A. Bor., shows many pharmacological properties for the treatment of fatigue, inflammation, anoxia, cardiovascular disease, and cancer [1,2]. However, *R. sachalinensis* is on the edge of extinction due to the over-exploitation. In this sense, several strategies for preparing salidroside were

explored [3–5]. Fortunately, enzymatic synthesis of salidroside has been proven to a favorable and practicable option for this purpose owing to its simplicity, high selectivity, simple product isolation, and environmental friendliness compared with the use of cell/tissue culture and organic synthesis [6,7]. Recently, Xu and co-workers [8–11] synthesized salidroside using β -glycosidases from several

fruit seeds in co-solvent mixtures with good yields of 13.0%–15.8%.

Ionic liquids (ILs), as a potential replacement for organic solvents, have gained increasing attention for various enzymatic synthetic processes over the past decade as they are known to improve the specificity of the enzyme [12]. Since the first biocatalysis in ILs was reported in 2000, there are numerous examples of biocatalytic reactions that have been successfully carried out in such neoteric media [13]. The great enthusiasm for this methodology may be attributed to the “green” and “designable” properties of the ILs [14].

In many cases, enzymes showed a comparable or even higher activity, stability, and selectivity in IL-containing systems compared with conventional organic solvents [14]. However, very little work has been done on β -glycosidase-mediated preparation of β -D-glucopyranosides in such IL-containing systems. Encouraged by this, the enzymatic synthesis of salidroside was selected as a model reaction to elucidate the characteristics of the β -glycosidase from black plum seeds in a $C_6MIm \cdot BF_4$ -containing system for the first time. Moreover, the influence of the cations and anions in combination with ILs has been examined (Scheme 1).

1 Experimental

1.1 General procedure for the enzymatic synthesis of salidroside

The different seed meals were prepared as described by Yu et al. [10]. The reaction was performed in a 10 ml Erlenmeyer shaking flask capped with a septum containing 1.6 ml dioxane, 0.4 ml phosphate buffer (pH 6.8, 100 mmol/L), 0.5 mmol D-glucose, 5 mmol tyrosol, and different fruit seed meal at 50 °C and 200 r/min. Aliquots were withdrawn at specified time intervals from the reaction mixture, and then diluted 50 times with the corresponding mobile phase prior to HPLC analysis. One unit of β -glycosidase activity (U) was defined as the amount of enzyme required to produce 1 μ mol salidroside in the first hour under the above conditions. The specific activities of the black plum seed meal, apple seed meal, peach seed meal, bitter almond seed meal, and prune seed meal were 27.6, 23.4, 25.9, 13.5, and 19.7 U/g. 1-Ethyl-3-methylimidazolium tetrafluoroborate ($C_2MIm \cdot BF_4$), 1-butyl-3-methylimidazolium tetrafluoroborate ($C_4MIm \cdot BF_4$), 1-hexyl-3-methylimidazolium tetrafluoroborate ($C_6MIm \cdot BF_4$), 1-ethyl-3-methylimidazolium hexafluorophosphate ($C_2MIm \cdot PF_6$), 1-butyl-3-methylimi-

dazolium hexafluorophosphate ($C_4MIm \cdot PF_6$), 1-hexyl-3-methylimidazolium hexafluorophosphate ($C_6MIm \cdot PF_6$), 1-octyl-3-methylimidazolium hexafluorophosphate ($C_8MIm \cdot PF_6$), 1-butyl-3-methylimidazolium chloride ($C_4MIm \cdot Cl$), 1-butyl-3-methylimidazolium bromide ($C_4MIm \cdot Br$), 1-ethyl-3-methylimidazolium iodide ($C_2MIm \cdot I$), 1-propyl-3-methylimidazolium iodide ($C_3MIm \cdot I$), 1-butyl-3-methylimidazolium iodide ($C_4MIm \cdot I$), 1-hexyl-3-methylimidazolium iodide ($C_6MIm \cdot I$), 1-decyl-3-methylimidazolium iodide ($C_{10}MIm \cdot I$), and standard salidroside were purchased from Sigma-Aldrich. Black plum seeds were obtained from a food processing company. Tyrosol was from Aladdin. All other chemicals were from commercial sources and of the highest purity available.

1.2 Reusability

To assess the stability of the enzyme for repeated use, batch synthesis of the salidroside was conducted by the addition of 7.0 U black plum seed meal to 2 ml of a co-solvent system containing 0.02 ml $C_6MIm \cdot BF_4$, 0.4 ml phosphate buffer (pH 5.9, 100 mmol/L), and 1.58 ml dioxane at 50 °C and 200 r/min. The enzyme was recovered by filtration after reaching the maximum product yield, washed with fresh reaction mixture, and then dried. The residual activity was determined for the next batch reaction under the same conditions.

1.3 HPLC analysis

The reaction mixture was analyzed by RP-HPLC on a 4.6 mm $\times$ 250 mm (5 μ m) Zorbax SB-C18 column (Agilent Technologies Industries Co., Ltd., USA) using an Agilent G1311A pump and a UV detector at 275 nm. The mobile phase is a mixture of water and methanol at a flow rate of 1.0 ml/min. The volumetric ratio of water to methanol and the retention time for salidroside were 40/60 and 2.47 min.

2 Results and discussion

2.1 Effect of reaction medium

Several typical organic solvents and $C_6MIm \cdot BF_4$ were examined as alternative media for the synthesis of salidroside by using β -glycosidase from black plum seeds. Table 1 showed that *N,N*-dimethylformamide and dimethyl

Scheme 1. Enzymatic synthesis of salidroside.

sulfoxide had an inhibitory effect on the activity of the enzyme due to their hydrophilicity in nature. Of the six organic solvents tested, dioxane proved to be the most favorable one with respect to the product yield (14.9%). Interestingly, the enzyme performances were significantly affected in $C_6MIm \cdot BF_4$ -containing systems as compared with the other media. For instance, the product yield was substantially enhanced with an increasing concentration of $C_6MIm \cdot BF_4$ up to 1.0%, and a further increase in $C_6MIm \cdot BF_4$ content resulted in a poor outcome. A similar observation was found for the preparation of *N*-acetylglucosamine by β -galactosidase from *Bacillus circulans* [15]. This may be explained by the fact that the high concentration of hydrophilic $C_6MIm \cdot BF_4$ tends to strip more water molecules from the reaction system to hydrate itself, thus leading to a dehydrated enzyme and lower enzyme flexibility [16]. The high ionic strength, substrate ground-state stabilization and poorer mass transfer caused by a high concentration of $C_6MIm \cdot BF_4$ could also partly account for this [17,18].

Table 1 Effect of solvents on the enzymatic synthesis of salidroside

Solvent	V_0 (mmol/(L·h))	Time (h)	Yield (%)
Dimethyl sulfoxide	0.1	48	0.6
Dioxane	1.3	72	14.9
Acetone	1.0	64	4.2
<i>N,N</i> -Dimethylformamide	0.1	48	0.4
<i>tert</i> -Butanol	0.8	86	12.3
Tetrahydrofuran	0.3	72	4.2
Dioxane/ $C_6MIm \cdot BF_4$ (0.5%, v/v)	1.3	72	16.3
Dioxane/ $C_6MIm \cdot BF_4$ (1.0%, v/v)	1.5	72	20.2
Dioxane/ $C_6MIm \cdot BF_4$ (1.5%, v/v)	1.4	72	18.9
Dioxane/ $C_6MIm \cdot BF_4$ (2.0%, v/v)	1.4	72	18.2
Dioxane/ $C_6MIm \cdot BF_4$ (2.5%, v/v)	0.9	80	16.5
Dioxane/ $C_6MIm \cdot BF_4$ (3.0%, v/v)	0.6	86	14.2

Reaction conditions: 0.5 mmol D-glucose, 4.0 mmol tyrosol, 7.0 U black plum seed meal, 2.0 ml solvent containing 0.2 ml phosphate buffer (pH 6.8, 100 mmol/L) and various amounts of $C_6MIm \cdot BF_4$, 50 °C, 200 r/min.

2.2 Catalytic performance of various enzymes in $C_6MIm \cdot BF_4$ -containing systems

It is well known that various β -glycosidases from several fruit seeds have been found to be capable of synthesizing β -D-glucopyranoside [8–10,19]. Therefore, four other potential seed meals, including apple seed meal, peach seed meal, bitter almond seed meal, and prune seed meal, were subsequently examined for the preparation of salidroside in $C_6MIm \cdot BF_4$ -containing systems. As shown in Table 2, all the tested seed meals showed good catalytic activity in the co-solvent systems. In terms of its initial reaction rate and product yield, the black plum seed meal gave the most

Table 2 Enzymatic synthesis of salidroside by β -glycosidases from various sources

Source	Specific activity (U/g)	V_0 / (mmol/(L·h))	Time (h)	Yield (%)
Black plum seed	27.6	1.5	72	20.2
Apple seed	23.4	1.2	72	16.0
Peach seed	25.9	1.4	72	18.6
Bitter almond seed	13.5	0.7	72	10.1
Prune seed	19.7	1.0	72	15.2

Reaction conditions: 0.5 mmol D-glucose, 4.0 mmol tyrosol, 7.0 U seed meal, 2.0 ml solvent containing 0.2 ml phosphate buffer (pH 6.8, 100 mmol/L), 0.02 ml $C_6MIm \cdot BF_4$, 1.78 ml dioxane, 50 °C, 200 r/min.

promising result.

2.3 Optimization of the reaction conditions and enzyme reusability

To better understanding the reaction, various influential variables on the reaction were studied. The effect of phosphate buffer pH from 5.6 to 7.1 was first examined. Figure 1(a) showed that both the highest initial reaction rate and maximum product yield were obtained at pH 5.9. It is well documented that different biological molecules require different water density equilibriums especially when the synthetic reaction is reversible [17]. Control experiments indicated that the reaction markedly accelerated with increasing phosphate buffer content from 5% to 20% (Fig. 1(b)). A further increase in buffer content led to a clear decline in the initial reaction rate and yield, mainly resulting from the high flexibility of the enzyme and the hydrolysis of the product. Meanwhile, the most suitable substrate ratio of tyrosol to D-glucose was 8.0, as shown by the favorable initial reaction rate (3.3 mmol/(L·h)) and excellent yield (24.5%, Fig. 1(c)).

Studies on plum seed meal reusability were also carried out by using the recovered enzyme over several cycles. The results (Fig. 2) showed that the enzyme sharply lost activity (62% loss in activity) after three cycles of the reaction, and then decreased gradually from the 4th to the 6th cycle. This indicates that the stability of the enzyme is not ideal with successive reuse, probably due to conformational changes of the enzyme.

2.4 Effect of the ILs

It is well recognized that the physicochemical properties of ILs can be fine-tuned by modifying the cation, anion, or attached substituent, thus improving the performance of the enzyme [13,20]. To compare the impact of the ionic composition of ILs during the enzymatic glucosylation, fourteen ILs based on the 1-alkyl-3-methylimidazolium cation

Fig. 1. Effect of buffer pH (a), buffer content (b), and molar ratio of tyrosol to D-glucose (c) on the enzymatic synthesis of salidoside. Reaction conditions: 0.5 mmol D-glucose, 7.0 U black plum seed meal, 0.02 ml $C_6\text{MIm}\cdot\text{BF}_4$, 50 °C, 200 r/min. (a) 4.0 mmol tyrosol, 0.2 ml phosphate buffer (100 mmol/L), 1.78 ml dioxane; (b) 4.0 mmol tyrosol, 2.0 ml solvent containing various amounts of phosphate buffer (pH 5.9, 100 mmol/L) and dioxane; (c) various amounts of tyrosol, 0.4 ml phosphate buffer (pH 5.9, 100 mmol/L), 1.58 ml dioxane.

Fig. 2. Reusability of black plum seed meal. Reaction conditions: 0.5 mmol D-glucose, 4.0 mmol tyrosol, 7.0 U black plum seed meal, 0.02 ml $C_6\text{MIm}\cdot\text{BF}_4$, 0.4 ml phosphate buffer (pH 5.9, 100 mmol/L), 1.58 ml dioxane, 50 °C, 200 r/min.

($C_n\text{MIm}^+$, $n = 2-10$) combined with the anions BF_4^- , PF_6^- , Cl^- , Br^- , and I^- were employed in co-solvent systems.

Table 3 illustrates that the enzyme synthetic activity not only depend on the anions of ILs, but also on the cations. Also, the results suggested that the reaction rates were comparable with or lower in IL-containing systems than that observed in a control reaction system. For the BF_4^- -based ILs, however, the initial reaction rate and maximum yield ascended with increasing alkyl chain length from C_2 to C_6 , showing a similar trend to those obtained with $C_n\text{MIm}\cdot\text{PF}_6$ and $C_n\text{MIm}\cdot\text{I}$ ($n = 2-6$). Among all the ILs tested, the best results were observed in a $C_6\text{MIm}\cdot\text{BF}_4$ -containing system with a yield of 24.5%. One explanation is that the longer hydrophobic alkyl chain in the cation has a lower tendency to “absorb” the essential water from the enzyme, thus maintaining its activity [16]. However, the initial reaction rate and yield dropped with further elongation of the alkyl chain of the ILs, such as $C_8\text{MIm}\cdot\text{PF}_6$ and $C_{10}\text{MIm}\cdot\text{I}$. The increment viscosity of the ILs caused by the longer alkyl chain

may lead to a decrease of substrate and product mass transfer rate, resulting in a lower yield [21]. Interestingly, in contrast, the addition of $C_4\text{MIm}\cdot\text{Cl}$ and $C_4\text{MIm}\cdot\text{Br}$, two ILs that have hydrogen bonding potential, reduced the activity of the enzyme with unsatisfactory results.

Recently, Zhao [18] reported that the kosmotropic anions and chaotropic cations of the ILs were capable of stabilizing the structure of proteins, while chaotropic anions and kosmotropic cations destabilize them. According to the literature, the relative orders of the kosmotropicity and chaotropicity of ions in stabilizing the enzyme are $\text{Cl}^- > \text{Br}^- > \text{I}^- > \text{BF}_4^- > \text{PF}_6^-$ and $C_2\text{MIm}^+ > C_3\text{MIm}^+ > C_4\text{MIm}^+ > C_6\text{MIm}^+ > C_8\text{MIm}^+ > C_{10}\text{MIm}^+$, respectively. Obviously, the results confirmed by our study showed that the enzyme activity was not consistent with this sequence of ions. We suspected

Table 3 Effect of the ILs on the enzymatic synthesis of salidoside

Ionic liquid	V_0 /(mmol/(L·h))	Time (h)	Yield (%)
—	3.2	72	18.8
$C_2\text{MIm}\cdot\text{BF}_4$	2.9	72	18.9
$C_4\text{MIm}\cdot\text{BF}_4$	2.9	68	22.6
$C_6\text{MIm}\cdot\text{BF}_4$	3.3	64	24.5
$C_2\text{MIm}\cdot\text{PF}_6$	2.3	72	17.2
$C_4\text{MIm}\cdot\text{PF}_6$	2.9	72	21.2
$C_6\text{MIm}\cdot\text{PF}_6$	3.0	72	24.3
$C_8\text{MIm}\cdot\text{PF}_6$	2.7	72	19.0
$C_4\text{MIm}\cdot\text{Cl}$	0.2	56	4.9
$C_4\text{MIm}\cdot\text{Br}$	0.2	56	9.5
$C_2\text{MIm}\cdot\text{I}$	0.7	56	11.6
$C_3\text{MIm}\cdot\text{I}$	0.9	56	13.0
$C_4\text{MIm}\cdot\text{I}$	1.0	72	16.2
$C_6\text{MIm}\cdot\text{I}$	2.9	72	21.9
$C_{10}\text{MIm}\cdot\text{I}$	2.3	72	17.4

Reaction conditions: 0.5 mmol D-glucose, 4.0 mmol tyrosol, 7.0 U black plum seed meal, 0.02 ml ILs, 0.4 ml phosphate buffer (pH 5.9, 100 mmol/L), 1.58 ml dioxane, 50 °C, 200 r/min.

that when water was involved in the enzymatic reaction, the interactions between the ions of the IL and the substrate or product may be also responsible for the above-mentioned phenomena in addition to the viscosity of the IL and the change in the native microenvironment of the enzyme.

3 Conclusions

It can be concluded that the synthesis of salidroside using β -glycosidase from black plum seeds can be successfully conducted in several IL-containing systems. Among the ILs examined, 1-alkylimidazolium ILs with linear C_6 were shown to be the most suitable for the reaction especially when the enzymatic reaction was performed in a $C_6MIm \cdot BF_4$ -containing system. Additionally, the results confirmed by our study indicate that there was no direct correlation between the catalytic activity of the enzyme and the sequence of the kosmotropic and chaotropic characteristics of the ions. Further investigation into the correlation of the ions based on ILs with low- or high-density water in enzymatic reactions will be reported in due course.

References

- 1 Hu X L, Lin S X, Yu D H, Qiu S F, Zhang X Q, Mei R H. *Cell Biol Toxicol*, 2010, **26**: 499
- 2 Qian E W, Ge D T, Kong S K. *J Ethnopharmacol*, 2011, **133**: 308
- 3 Akita H, Kurashima K, Nakamura T, Kato K. *Tetrahedron: Asymmetry*, 1999, **10**: 2429
- 4 Wu S X, Zu Y G, Wu M. *J Biotechnol*, 2003, **106**: 33
- 5 王梦亮, 郭春侠. 催化学报 (Wang M L, Guo C X. *Chin J Catal*), 2011, **32**: 1051
- 6 Akita H, Kawahara E, Kishida M, Kato K. *J Mol Catal B*, 2006, **40**: 8
- 7 Yang R L, Li N, Zong M H. *J Mol Catal B*, 2012, **74**: 24
- 8 Lu W Y, Lin G Q, Yu H L, Tong A M, Xu J H. *J Mol Catal B*, 2007, **44**: 72
- 9 Tong A M, Lu W Y, Xu J H, Lin G Q. *Bioorg Med Chem Lett*, 2004, **14**: 2095
- 10 Yu H L, Xu J H, Lu W Y, Lin G Q. *J Biotechnol*, 2008, **133**: 469
- 11 Tong A M, Xu J H, Lu W Y, Lin G Q. *J Mol Catal B*, 2005, **32**: 83
- 12 Lau R M, Van Rantwijk F, Seddon K R, Sheldon R A. *Org Lett*, 2000, **2**: 4189
- 13 Zhang Q H, Zhang S G, Deng Y Q. *Green Chem*, 2011, **13**: 2619
- 14 van Rantwijk F, Sheldon R A. *Chem Rev*, 2007, **107**: 2757
- 15 Kaftzik N, Wasserscheid P, Kragl U. *Org Process Res Dev*, 2002, **6**: 553
- 16 Zhao H, Jackson L, Song Z Y, Olubajo O. *Tetrahedron: Asymmetry*, 2006, **17**: 2491
- 17 Gorke J, Srienc F, Kazlauskas R. *Biotechnol Bioprocess Eng*, 2010, **15**: 40
- 18 Zhao H. *J Mol Catal B*, 2005, **37**: 16
- 19 王琴琴, 郁蕙蕾, 赵娜, 李春秀, 尚亚卓, 刘洪来, 许建和. 催化学报 (Wang Q Q, Yu H L, Zhao N, Li Ch X, Shang Y Z, Liu H L, Xu J H. *Chin J Catal*), 2012, **33**: 275
- 20 汪薇, 宗敏华, 娄文勇. 催化学报 (Wang W, Zong M H, Lou W Y. *Chin J Catal*), 2011, **32**: 1003
- 21 Lou W Y, Chen L, Zhang B B, Smith T, Zong M H. *BMC Biotechnology*, 2009, **9**: 90