Influence of the Ionic Liquid Cosolvent on Glucosylation Reactions Catalyzed by β-Glycosidase for Production of Salidroside

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The enzymatic synthesis of salidroside catalyzed by β-glycosidase from *Malus pumila* seed meal has been successfully carried out in ionic liquids-containing systems for the first time. The optimum conditions were screened out and the best yield of 24.9 % was received in the $C_4MIm·PF_6$-containing cosolvent system. Moreover, the influence of ionic liquids on the performance of the enzyme has been discussed, using both hydrophobic and hydrophilic ionic liquids possessing various anions and different alkyl chain lengths (C2-C10) in imidazolium cations.

Key Words: Ionic liquids, Salidroside, Glucosylation, *Malus pumila* seed meal.

INTRODUCTION

Over the last couple of years, ionic liquids (ILs) have received an ever-increasing attention as biocatalytic reaction media for a wide range of chemical processes, owing to their unique physicochemical properties, such as the environmentally benign nature, recyclability and ability to dissolve both polar and non-polar compounds[1]. For instance, many excellent reviews by Rantwijk *et al.*[2], Sowmiah *et al.*[3] and Gorke *et al.*[4] have manifested the advantages of the ionic liquids-containing enzymatic systems for the purpose of preparing the fine chemicals with markedly high product yield, shorter reaction time, enantio- or region-selectivity and better enzyme stability as well.

Glycosides and their analogs, an important group of natural compounds, show multiple biological and pharmacological activities and have been used extensively in food, cosmetics and medicine preparations[5]. Among these compounds, salidroside, mainly obtained from the plant *Rhodiola sachalinensis* A. Bor., has been reported to have convincing activities in the therapy of inflammation, fatigue, cardiovascular disease and cancer[5,6]. Recently, enzymatic preparation of salidroside has emerged as a promising one-step procedure instead of the tedious isolation and purification steps from the medicinal plant[7-9], which is on the edge of extinction. However, these biotransformations in synthetic media containing pure organic solvent and buffer solution have traditionally suffered from low product yields[10,11].

In our previous study, we had successfully performed the β-glycosidase-catalyzed synthesis of salidroside using unpro-tected and unactivated donors in ionic liquid-containing systems, with a wide range of yields ranging from 4.9-24.5 %[12], depending on the nature of the ionic liquids. As a further extension of our research on solvent engineering strategies, the performance of β-glycosidase from *Malus pumila* seed meal in ionic liquid-containing systems for producing salidroside was investigated. Additionally, the influence of ionic liquids with various anions and different alkyl chain lengths in cations and was also examined.

EXPERIMENTAL

1-Butyl-3-methylimidazolium nitrate ($C_4MIm·NO_3$), N-hexylpyridinium *bis*(trifluoromethyl sulfonyl) imide ($C_6Py·Tf_2N$), 1-butyl-3-methyl imidazolium octylsulfate ($C_4MIm·C_8SO_4$), 1-ethyl-3-methylimidazolium tetrafluoroborate ($C_2MIm·BF_4$), 1-butyl-3-methylimidazolium tetrafluoroborate ($C_4MIm·BF_4$), 1-hexyl-3-methylimidazolium tetrafluoroborate ($C_6MIm·BF_4$), 1-ethyl-3-methylimidazolium hexafluorophosphate ($C_2MIm·PF_6$), 1-butyl-3-methylimidazolium hexafluorophosphate ($C_4MIm·PF_6$), 1-hexyl-3-methylimidazolium hexafluorophosphate ($C_6MIm·PF_6$), 1-octyl-3-methylimidazolium hexafluorophosphate ($C_8MIm·PF_6$), 1-butyl-3-methylimidazolium chloride ($C_4MIm·Cl$), 1-butyl-3-methylimidazolium bromide ($C_4MIm·Br$), 1-ethyl-3-methylimidazolium iodide ($C_2MIm·I$), 1-propyl-3-methyl-imidazolium iodide ($C_3MIm·I$), 1-butyl-3-methylimidazolium iodide ($C_4MIm·I$), 1-hexyl-3-methylimidazolium iodide ($C_6MIm·I$), 1-decyl-3-methylimidazolium iodide ($C_{10}MIm·I$) and standard salidroside were purchased from Sigma-Aldrich. *Malus pumila* seeds were obtained from food processing

company. Tyrosol was from Aladdin. All other chemicals were also from commercial sources and of the highest purity available.

Determination of β-glycosidase activity: The glucosylation activity of the *Malus pumila* seed meal was determined as described by our previous report with a specific activity of 23.4 U/g[12].

General procedure for enzymatic synthesis of salidroside: In a typical experiment, the mixture (2.0 mL) of ionic liquid, phosphate buffer (100 mM, pH 6.0) and dioxane containing 0.5 mmol D-glucose, 3.0 mmol tyrosol and 6.0 U *Malus pumila* seed meal were incubated in a 10 mL Erlenmeyer shaking flask capped with a septum under predetermined reaction conditions (50 ℃, 200 rpm). Aliquots were withdrawn at specified time intervals from the reaction mixture and then diluted 50 times with corresponding mobile phase prior to HPLC analysis.

HPLC analysis: The reaction mixture was analyzed by RP-HPLC on a 4.6 mm × 250 mm (5 μm) Zorbax SB-C$_{18}$ 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.

RESULTS AND DISCUSSION

Screening the reaction solvent: The nature of the solvent such as polarity, hydrophobicity, dielectric constant and concentration will govern the hydration water of the enzyme molecular, protein conformation change and solubility of the substrate and product, thus affecting the activity, stability and specificity of the enzyme[1,13].

As shown in Table-1, among the several pure organic solvents tested, the β-glycosidase from *Malus pumila* seed meal displayed the highest yield in dioxane (12.0 %), moderate yields in THF (5.0 %) and acetone (6.8 %) and lower yields in other solvents (1.0-3.3 %). However, the synthetic activity of β-glycosidase was changed remarkably in co-solvent systems with different ionic liquids as additives than that observed in neat dioxane, suggesting that the nature of the anion in ionic liquid exerts an unexpected effect on the performance of the enzyme. With respect to the initial reaction rates and yields (4.3-10.2 %), β-glycosidase showed low or moderate activities in dioxane-ionic liquid cosolvents containing Tf$_2$N⁻, Cl⁻, Br⁻, NO$_3$⁻ and C$_8$SO$_4$⁻ anions, which seem to have negative effect on the enzyme. For ionic liquid based on I⁻, BF$_4$⁻ and PF$_6$⁻ anions, the higher synthetic activities were obtained with excellent yields (12.4-13.9 %).

The difference in the effects that the various ionic liquids had on the β-glycosidase may be attributable to several reasons, such as the hydrogen-bond basicity, hydrophilic or hydrophobic property, nucleophilicity of the anion and the viscosity of the ionic liquid[2,3]. In this case, the C$_4$MIm·BF$_4$ was considered as the most suitable ionic liquid for the salidroside preparation in cosolvent systems.

Optimization of enzymatic synthesis of salidroside: For a better understanding of the characteristics of the β-glycosidase in the C$_4$MIm·BF$_4$-containing system, several crucial factors influencing the reaction were also studied. Firstly, the impact

TABLE-1 EFFECT OF SOLVENTS ON ENZYMATIC SYNTHESIS OF SALIDROSIDE			
Solvents	V$_0$ (mM/h)	Time (h)	Yield (%)
Acetonitrile	1.3	60	3.3
Acetone	1.2	60	6.8
THF	0.4	72	5.0
tert-Butanol	0.7	72	9.3
DMSO	0.1	48	2.1
DMF	0.1	48	1.0
Dioxane	1.4	72	12.0
Dioxane-C$_4$MIm·Br	1.0	56	7.6
Dioxane-C$_4$MIm·Cl	1.0	56	6.1
Dioxane-C$_4$MIm·I	1.2	72	12.4
Dioxane-C$_4$MIm·NO$_3$	0.9	72	8.2
Dioxane-C$_6$Py·Tf$_2$N	0.7	48	4.3
Dioxane-C$_4$MIm·BF$_4$	1.4	72	14.8
Dioxane-C$_6$MIm·PF$_6$	1.4	72	13.9
Dioxane-C$_4$MIm·C$_8$SO$_4$	0.7	72	10.2

Reaction conditions: 6.0 U Malus pumila seed meal, 0.5 mmol D-glucose, 2.0 mmol tyrosol, 2.0 mL organic solvent or dioxane-IL (1 %, v/v) containing 0.2 mL phosphate buffer (100 mM, pH 6.2), 50 ℃, 200 rpm

of ionic liquid content on both of the initial reaction rate and yield was investigated. As shown in Fig. 1, the enzyme activity was enhanced as the ionic liquid content increased from 0.5-5.0 %. The maximal yield of 16.2 % was observed at 5.0 % of the ionic liquid content and was not further improved by increasing the ionic liquid content (5.0-11.0 %). High ionic strength and the severe dehydration of the enzyme caused by high concentration of hydrophilic C$_4$MIm·BF$_4$ might inactivate the enzyme[3,4], thus leading to poor results. Besides, high viscosity of the reaction mixture resulting from the high ionic liquid concentration can also be proposed as another reason for this phenomenon[14].
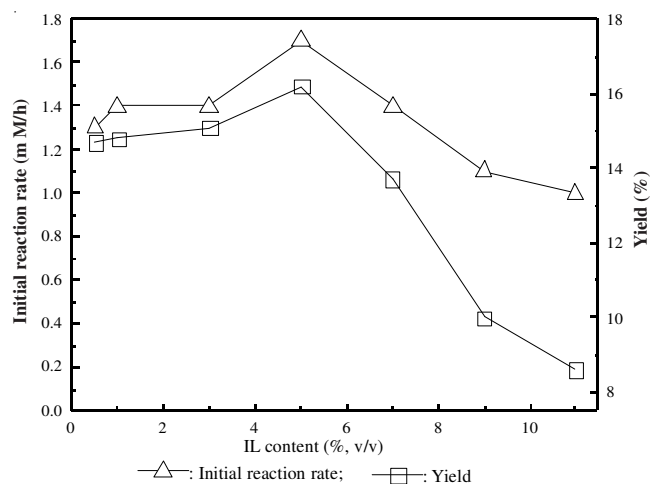

Fig. 1. Effect of ionic liquid content on enzymatic synthesis of salidroside. Reaction conditions: 0.5 mmol D-glucose, 2.0 mmol tyrosol, 6.0 U *Malus pumila* seed meal, 2.0 mL solvent containing 0.2 mL phosphate buffer (100 mM, pH 6.2) and various amounts of C$_4$MIm·BF$_4$, 50 ℃, 200 rpm

The effect of the buffer pH on synthetic activity in the ionic liquid-containing cosolvent system is shown in Fig. 2a. The initial rate and yield were clearly boosted in the pH range of 5.6-6.0 and then decreased at pH 6.0-6.6. The highest yield was achieved at pH 6.0 (18.3%). Understandably, buffer
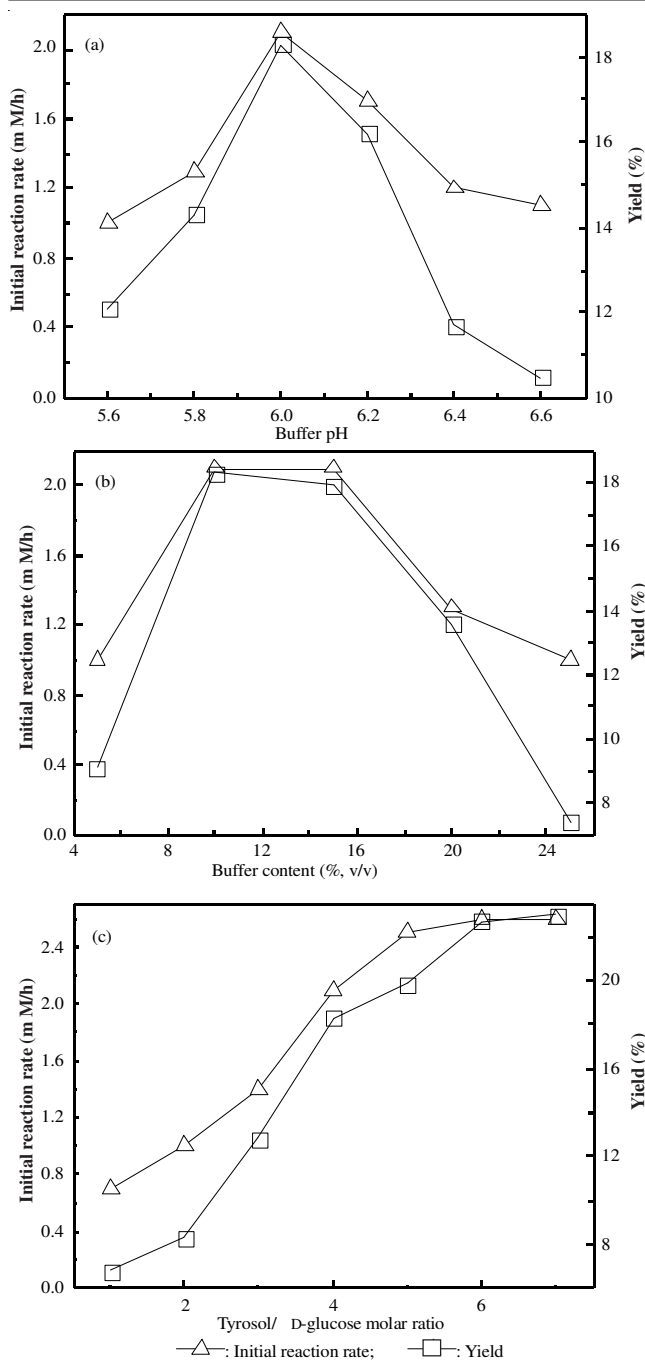
Fig. 2.   Effect of buffer pH (a), buffer content (b) and molar ratio of tyrosol to D-glucose (c) on the enzymatic synthesis of salidroside. Reaction conditions: 0.5 mmol D-glucose, 6 U *Malus pumila* seed meal, 0.1 mL C₄MIm·BF₄, 50 ºC, 200 rpm. (a) 2.0 mmol tyrosol, 0.2 mL phosphate buffer (100 mM), 1.7 mL dioxane; (b) 2.0 mmol tyrosol, 2 mL solvent containing various amounts of phosphate buffer (100 mM, pH 6) and dioxane; (c) various amounts of tyrosol, 0.2 mL phosphate buffer (100 mM, pH 6), 1.7 mL dioxane

content is another most important parameter in non-aqueous enzymology apart from the appropriate buffer pH. As depicted in Fig. 2b, the satisfied result was in good accordance with the buffer content of 10 %, which was regarded as the optimal value. This suggested that proper buffer pH and content were beneficial to maintain the dimensional structure of the enzyme protein, thus keeping its catalytic activity. Furthermore, reasonable substrate ratio is necessary for this enzymatic reverse reaction and the optimum ratio of tyrosol to D-glucose

for salidroside synthesis conducted in the C₄MIm·BF₄-containing system was showed to be 6.0 from the data summarized in Fig. 2c.

Effect of cation chain length of the ionic liquid: In ionic liquid-containing reaction media, it is already well-known that enzyme synthetic activity not only depends on the anion of ionic liquid, but also on the cation[15,16]. To gain a deeper insight into the effect of ionic liquids, the catalytic specificity of the β-glycosidase on the glucosylation reaction was then examined in media containing methyl imidazolium ionic liquids (MIm) of different cation chain lengths (C$_n$MIm$^+$, n = 2-10) and in combination with three different anions of BF$_4^-$, PF$_6^-$ and I$^-$.

Table-2 showed that although the initial reaction rates were comparable with or lower in 13 ionic liquids-containing cosolvents than that observed in the pure dioxane, the improved yields (20.9-24.9 %) were gained except for those (17.5-17.7 %) in C₂MIm·I- and C₃MIm·I-containing systems (Table-2, entries 9-10). This can be partially explained by the higher viscosity stemming from the addition of ionic liquid into the reaction medium, thereby increasing mass transfer resistance[15].

TABLE-2
EFFECT OF THE ILS ON ENZYMATIC
SYNTHESIS OF SALIDROSIDE

Entry	Ionic liquids	V_0 (mM/h)	Time (h)	Yield (%)
1	–	2.6	80	19.6
2	C₂MIm·BF₄	2.4	84	21.9
3	C₄MIm·BF₄	2.6	84	22.7
4	C₆MIm·BF₄	2.4	86	21.2
5	C₂MIm·PF₆	2.4	84	21.5
6	C₄MIm·PF₆	2.7	84	24.9
7	C₆MIm·PF₆	2.2	90	20.3
8	C₈MIm·PF₆	2.2	90	20.2
9	C₂MIm·I	1.7	84	17.5
10	C₃MIm·I	1.7	84	17.7
11	C₄MIm·I	2.0	90	20.9
12	C₆MIm·I	2.4	90	22.6
13	C₁₀MIm·I	2.4	90	23.0

Reaction conditions: 0.5 mmol D-glucose, 3.0 mmol tyrosol, 6.0 U *Malus pumila* seed meal, 0.1 mL ionic liquids, 0.2 mL phosphate buffer (100 mM, pH 6.0), 1.7 mL dioxane, 50 ºC, 200 rpm.

In the case of two widely used ionic liquids based on BF$_4^-$ and PF$_6^-$ anions, an increment in hydrophobic alkyl chain length in imidazolium cation improved synthetic activity up to a maximum, after which declined slightly with the further elongation of the alkyl chain, implying that appropriate chain length of the cation had poor water-stripping ability and low viscosity[14,16], which were beneficial to maintain the enzyme catalytic activity. Interestingly, the β-glycosidase showed considerable activity with moderate or good yields (17.5-23.0 %) when the I$^-$-based ionic liquids were added into reaction systems. Zhao[15] has reported that the enzyme performance followed the Hofmeister series in systems involving ionic liquids. In that case, the β-glycosidase activities should be lower in I$^-$-based ionic liquids than those observed in Cl$^-$- and Br$^-$-based ionic liquids. Clearly, these results were not in accordance with the aforementioned regulation. To our best of knowledge, the unexpected results might be attributed to the hydrogen-bond networks formed from the interaction

among the ionic liquids, water and substrate, with the exception of the ionic impact of the ionic liquids on the active site of the enzyme.

Conclusion

The possibility of ionic liquids containing various anions and different alkyl chain lengths in cations as additives in reaction media for the enzymatic production of salidroside catalyzed by β-glycosidase from *Malus pumila* seed meal has been demonstrated. The results indicated that the use of ionic liquid as cosolvent brought about a clear change in the performance of the enzyme. Under the optimum conditions, the highest 24.9 % yield was observed when $C_4MIm \cdot PF_6$ was added into the cosolvent system.

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