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Lipase-catalyzed regioselective synthesis of monoester of pyridoxine (vitamin B_6) in acetonitrile

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Abstract

Candida antarctica lipase B (Novozyme 435)-catalyzed esterification of pyridoxine (PN) was studied. In order to improve the solubility of PN and to elevate the production of 5-*O*-acetylpyridoxine (5-AcPN), two media, acetonitrile and 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF_6), were used in the reaction. The site-selectivity in [bmim]PF_6 was different from that of acetonitrile, i.e., in [bmim]PF_6, the reactions provided 4-*O*-acetylpyridoxine (4-AcPN) as the major product by employing acetic anhydride as acyl donor; however, the main product was 5-AcPN in acetonitrile. By employing different acyl donor in the transesterification reaction, it was found that the acyl donor affected not only the degree of conversion but also the regioselectivity. In addition, the influence of several parameters such as water activity, reaction temperature, substrate mole ratio and enzyme loading on the esterification of PN were analyzed systematically. As a result, *C. antarctica* lipase B (Novozyme 435)-catalyzed esterification of PN gave a maximum conversion of 99% and the best regioselectivity of 93% in acetonitrile when vinyl acetate was used as the acyl donor.

Keywords: Pyridoxine; Novozyme 435 lipase; Regioselective esterification; Site-selectivity; [bmim]PF_6

1. Introduction

Pyridoxine (PN) is one of the three members (pyridoxine, PN; pyridoxal, PL; and pyridoxamine, PM; see Scheme 1) of the vitamin B_6 group, and it is the most important form of commercial vitamin B_6. In vivo, vitamin B_6 is an essential cofactor for a large number of enzymes involved in the metabolism of amino acids (Baldessari, Mangone, & Gros, 1998). Also, PN and its ester derivatives have broad applications in food industry, cosmetics and medical supplies (Baldessari et al., 1998). Unfortunately, PN molecule has three hydroxyl groups and leads to a high solubility in water, which brings about a great deal of mass loss when it is used. In order to increase the lipid solubility of PN, which would be expected to increase their skin and cellular absorption, esterification methodology provides an excellent route to obtain more apolar derivatives. In addition, there is a growing demand for regioselective esterification of PN that could provide precursors to synthesize other vitamin B_6 derivatives (Brown, Johnston, Suckling, & Halling, 1993; Yang, Shih, & Liu, 1991).

Regioselective esterification of PN is an arduous task since the pyridine-ring possesses three hydroxyl groups (including one primary -OH and two secondary –OH) of similar reactivity; as a result, it is very difficult to discern among these three groups from a chemical point of view. Moreover, the chemical methods leading to such regioselective analogues involve multi-step protection and deprotection procedures owing to its multiple hydroxyl groups. Enzymatic reactions in nonaqueous media by hydrolytic enzymes such as lipases provide an increasingly valuable tool to achieve regioisomer of PN ester. By employing enzymatic technology, the reactive processes can be carried out under mild conditions with a broad range of substrates and which is also environmentally friendly. The high degree of regioselectivity exhibited by the enzymes in

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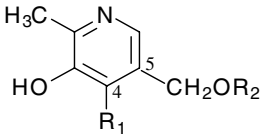


1. PN: R_1=CH_2OH R_2=H; 2. PM: R_1=CH_2NH_2 R_2=H
3. PL : R_1=CHO R_2=H; 4. PNP: R_1=CH_2OH R_2=RO_4^{2-}
5. PMP: R_1=CH_2NH_2 R_2=RO_4^{2-} 6. PLP: R_1=CHO R_2=RO_4^{2-}

Scheme 1. The structural formula of vitamin B6.

certain sugar molecules (Griswold & Miller, 2003; Kurahashi, Mizutani, & Yoshida, 2002; Mastihubová & Biely, 2004; Plou et al., 2002) and other compounds (Altreuter, Dordick, & Clark, 2002; Bonrath, Karge, & Netscher, 2002; Diaz, Ferrero, Fernandez, & Gotor, 2002) are especially interesting. Furthermore, the chemical instability and multifunction of PN provide an excellent opportunity to take advantage of biocatalysis.

To improve the solubility of PN, which has a strong polarity, we choose acetonitrile (MeCN) and 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF_6) as solvent. Besides, ionic liquids as a new type of alternative media are becoming a growing focus, and which is a benign solvent of polyhydroxyl groups compounds. Recently, ionic liquids employed as reaction media have been reported to have positive influences not only on the enzyme activity (Nara, Harjani, & Salunkhe, 2002), but also on the enantioselectivity and regioselectivity (Kim, Song, Choi, & Kim, 2001; Kim, Choi, Lee, & Ahn, 2003; Nara, Mohile, Harjani, Naik, & Salunkhe, 2004). Although enzymes have been successfully applied to the regioselective transformations of mono- and oligosaccharides (Riva, Nonini, Ottolina, & Danieli, 1998), there is little information pertaining to the enzymes on esterification of PN previously (Baldessari & Mangone, 2002), especially a systematic analysis of the regioselective biotransformation of PN in acetonitrile and even in ionic liquid.

The study of the lipase regioselective esterification of PN is this research line, with the aim of a convenient synthetic method to obtain high 'asymmetric' derivatives and high overall conversion. In the present work, a highly regioselective esterification of PN by Novozyme 435 lipase, which provides not only an effective protection for one of the three hydroxyl groups but also a method for the synthesis of regioselective monoester is described. Firstly, the results of our studies on different site-selectivity by using ionic liquid, [bmim]PF_6, as reaction media to esterize PN for the first time are reported. Secondly, several acyl donors were tested on the reaction and the right acyl donor was found. In addition, the influence of several parameters on the transesterification was analyzed systematically.

2. Experimental

2.1. Lipases and chemicals

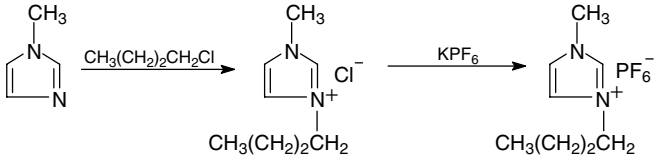
Pyridoxine (PN) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Novozyme 435 (immobilized on acrylic resin, 7400 PLU/g) was from Novo. Industrie. (Bagsvaerd, Denmark). Acetonitrile was of chromatographic grade and was obtained from Merck & Co. (Darmstadt, Germany). While all other reagents were purchased from local sources. The ionic liquid [bmim]PF_6 was prepared according to the procedure described by Park and Kazlauskas (2001) (see Scheme 2).

2.2. Enzymatic reactions

All enzymatic reactions were carried out in a temperature-controlled incubator shaker at 210 rpm. The procedure for the enzymatic esterification of 1 (see Scheme 1) in acetonitrile is described as a representative. Reactions were performed by adding Novozyme 435 lipase (35 mg) and acyl donor (0.18 mmol) to a solution of the PN (10.2 mg, 0.06 mmol) in acetonitrile (3 ml) and shaking the reaction mixtures at 35 °C during the times indicated in the text. Samples of the biotransformation were withdrawn at different times, then diluted with deionized water and analyzed by HPLC. Besides, in order to study the effect of one point such as a_w on the conversion and regioselectivity of the reaction, we have followed the strategy of keeping all the other experimental conditions constant while only a_w was changed.

2.3. HPLC analysis

PN transformational resultants were analyzed using high-performance liquid chromatography (HPLC, Agilent (Waldbronn, Germany) 1100 (quaternary pump, degasser)) equipped with an UV detector. HPLC analysis was conducted by employing a C-18 column (ZORBAX 300SB-C18 4.6 mm ID × 150 mm (5 μm), Agilent Technologies, Palo Alto, CA) with detection at 292 nm. The mobile phase was solution A (water containing 0.1% acetic acid) and solution B (acetonitrile containing 1% acetic acid) in all cases at a 1 ml min^{-1} flow of A/B gradient (0% B at 0 min, linear gradient to 100% at 7 min, maintained for 1 min and finally to 0% at 9 min). The percentages of several resultants were computed from the respective peak areas.



Scheme 2. Synthesis of ionic liquid [bmim]PF_6 by two steps method.

The conversion of the reaction was quantified in terms of the mole percentage transesterification, i.e., the ratio of PN consumed to the total amount of PN before reaction. In addition, the regioselectivity (Rs) is defined as:

$$\mathrm{Rs} = \frac{M_1}{M_2} \times 100\%$$

where M_1 is the mole percentage of 5-AcPN (5-*O*-acetylpyridoxine) formed, and M_2 is the mole percentage of PN consumed.

2.4. Setting initial a_w in closed system

Due to the distribution of water in organic reaction system (e.g., bound to the enzyme or dissolve in the organic solvent), the thermodynamic water activity (a_w) is a better parameter than the water content to determine the amount of water associated with the enzyme and thereby to truly correlate with the enzyme catalytic activity (Alston & Freedman, 2002; Lee & Parkin, 2001). Among the available methods for controlling a_w, the simple and convenient way is preequilibration of the reaction components in the presence of saturated salt solutions, and the method is particularly suitable for the reaction, which does not generate water.

The a_w of the acetonitrile, enzyme, and substrate were adjusted before starting the reaction by the following method: acetonitrile, PN and Novozyme 435 lipase were incubated in a chamber containing a desired saturated salt solution (Goderis et al., 1987), and the system was allowed to reach equilibrium for 4 days at 20 °C for a desired a_w. Molecular sieves were used to generate the nearly anhydrous condition ($a_w = 0.054$). The following salts were used in this work: LiCl, KAc, $MgCl_2$, $CuCl_2$, NaCl.

The reactions were initiated by mixing the preequilibrated phases and Novozyme 435 lipase in closed 10 centrifuge tube. The resultant mixture was assayed by HPLC in regular time.

3. Results and discussion

3.1. Selection of suitable reaction media

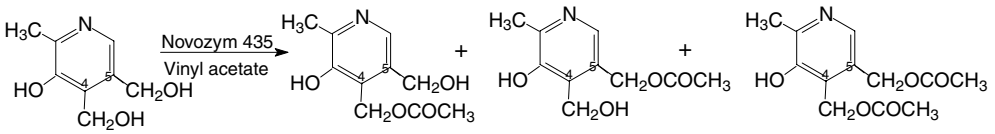
Biocatalyst performance in organic solvent is known to be highly sensitive to the solvent selection. In enzymatic reaction, the possibility of manipulating not only the reactivity, but also the regioselectivity, by the proper choice of the media, is of great interest. The versatility of ionic liquid as solvent for such diverse applications is due to the favorable attribute of an 'ideal solvent' possessed by them.

With Novozyme 435 lipase, we employed acetonitrile and [bmim]PF_6 as media for regioselective esterification of PN. The reaction was carried out at 35 and 50 °C in the presence of vinyl acetate, acetic anhydride and ethyl acetate, respectively. The detailed results are reported in Table 1. As can be seen, the reaction afforded monoester as a major product and also a little di-acetylated product (4,5-di-*O*-acetylpyridoxine) from PN (see Scheme 3). It must be pointed out that when using ethyl acetate as acyl donor, the result of reaction in acetonitrile is in agreement with that of Baldessari et al. (1998), i.e., the main product is 5-AcPN. The relative monoester ratios shown in Table 1 prove the high reactivity of both C-4 and C-5 hydroxyl groups of PN in enzymatic transesterifications (see Scheme 3). In [bmim]PF_6, when acetic anhydride was used, the reaction provided 4-AcPN (4-*O*-acetylpyridoxine) as the major product together with 5-AcPN as the minor product. Compared with the result, it has shown different site-selectivity in the presence of either vinyl acetate or ethyl acetate as acyl donor. Thus, the 5-AcPN becomes the predominant product. This allows controlled selective production of monoester or diester by simply changing the acyl donor.

Table 1
The esterification of PN by lipase under various conditions

Lipase	Solvent	Acyl donor (Acyl/PN)	Reaction time (h)	Conversion[a] (%)	A:B:C[b]
Novo435	[Bmim]PF_6 ($a_w = 0.105$)	Acetic anhydride (3:1)	2 h (50 °C)	63.5	82:4:14
Novo435	[Bmim]PF_6 ($a_w = 0.105$)	Vinyl acetate (3:1)	6 h (50 °C)	36.4	4:83:13
Novo435	[Bmim]PF_6 ($a_w = 0.105$)	Ethyl acetate (3:1)	6 h (50 °C)	27.5	14:74:12
Novo435	MeCN ($a_w = 0.160$)	Acetic anhydride (3:1)	2 h (35 °C)	98.9	0:64:36
Novo435	MeCN ($a_w = 0.160$)	Vinyl acetate (3:1)	2 h (35 °C)	99.1	0:96:4
Novo435	MeCN ($a_w = 0.160$)	Ethyl acetate (3:1)	6 h (35 °C)	26.9	0:96:4

Reaction conditions: initial mole ratio of acyl donor to PN, 3; amount of solvent, 3 ml; enzyme loading, 35 mg Novozyme 435 or 24 mg MIE.
[a] Conversion were determined by HPLC analysis.
[b] A, 4-AcPN; B, 5-AcPN; C, diester of PN.



Scheme 3. Esterification of PN.

However, whichever acyl donor was used in acetonitrile, the main product in all Novozyme 435 lipase-catalyzed transesterifications was always the 5-AcPN. Thus, reaction media and acyl donor both have effects on the degree of conversion and regioselectivity. The different regioselectivity exhibited by two media makes it feasible to synthesize different regioisomers, whose properties may vary considerably. In these experiments, through exploiting organic solvent and ionic liquid to examine the esterification of PN, acetonitrile gives the best result of transesterification by Novozyme 435 lipase. So we propose the use of acetonitrile as a general strategy to acylate hydrophilic substrates PN.

3.2. Selection of acyl donors

With the aim of obtaining the object derivative, 5-AcPN, from acylating the C-5 primary hydroxyl, we chose three acyl donors to detect the feasibility. It is known that in lipase-catalyzed transesterifications, reactions take place via the formation of an acyl-enzyme intermediate (Kawase, Sonomoto, & Tanaka, 1992). As a consequence, the nature of the acyl donor has a notable effect on reactivity.

As shown in Fig. 1, when using ethyl acetate, a lowering of the degree of conversion was observed, though the 5-OH position of PN was much more reactive than others. It is well known that acetic anhydride and vinyl acetate are both activated esters and far superior acetylating reagents to ethyl acetate. In Fig. 1, when the reaction was carried out with acetic anhydride as acyl donor, the degree of conversion was considerably high but, unexpectedly, the regioselectivity was only about 65%. In contrast, for esterification of PN with vinyl acetate, the result showed the most effective, not only the degree of conversion, but also the regioselectivity. Wang, Lalonde, Momongan, Bergbreiter, and Wong (1988) demonstrated that the rate of transesterification of hydroxyl-containing compounds with vinyl esters was about 20–100 times faster than that with alkyl esters (the vinyl alcohol formed during the process tautomerises to the low-boiling-point acetaldehyde, shifting the equilibrium towards the ester formation). Thus in general, vinyl acetate was used in the following studies. However, one must be cautious since it has recently been reported that several lipases (e.g. from *Candida rugosa* and *Geotrichum candidum*) lose most of their activity when exposed to acetaldehyde (Weber, Stecher, & Faber, 1995).

3.3. Effect of water activity

Apart from the fact that vinyl acetate was employed as acyl donor, we investigated how to optimize the process by analyzing the effect of several parameters such as water activity, reaction temperature, substrate mole ratio, enzyme loading as a way to improve it.

Water has multiple roles in regulating lipase activity while also functioning as the hydrolysis of products (not only monoester but also diester of PN) in the reaction. The amount of water presented in the reaction systems has long proven to be one of the most important factors, because enzymes require a minimum amount of water, or the so-called essential layer of water, to maintain their structure and flexibility and thereby activity (Alston & Freedman, 2002; Klibanov, 2001). Especially, acetonitrile possesses strong polarity and is capable of competing with lipase for its essential water effectively. It is therefore particularly important to address the optimization of water conditions for improving the lipase-catalyzed esterification of PN in the acetonitrile system.

The conversion and regioselectivity for the esterification of PN in acetonitrile at various water activities are reported in Fig. 2. As can be seen, the substrate conversion proved

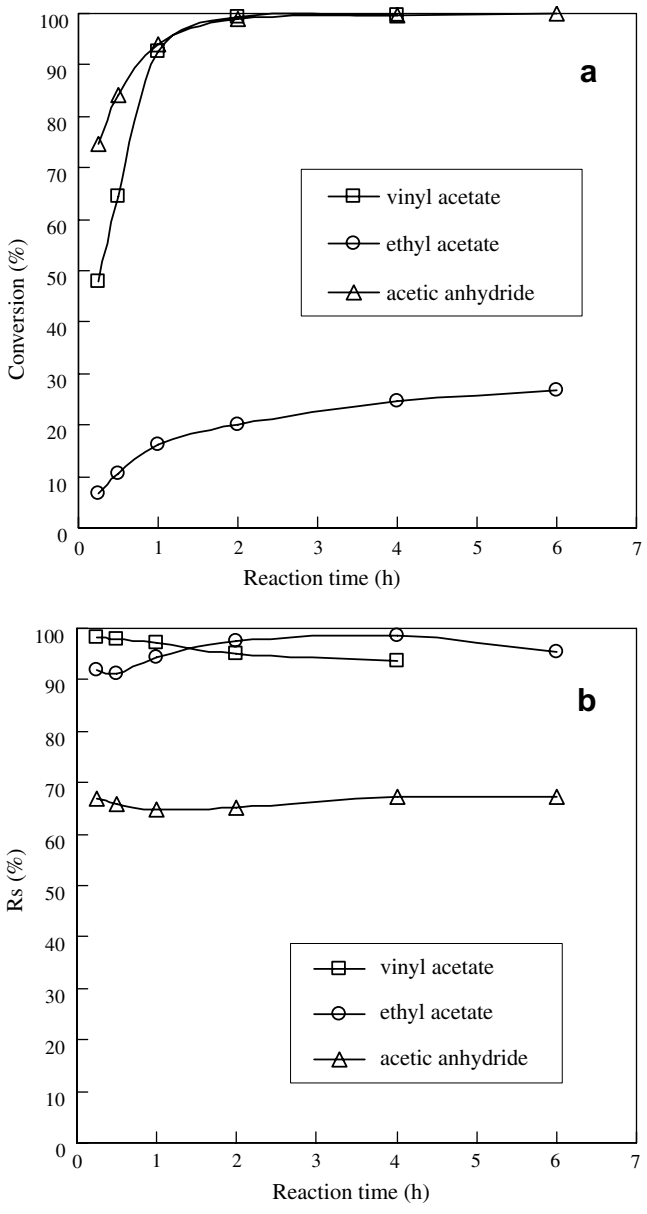


Fig. 1. Selection of acyl donors in the lipase-catalyzed PN esterification in acetonitrile: (a) conversion of PN and (b) regioselectivity (Rs) (reaction conditions: acetonitrile 3 ml, temperature 35 °C, Acyl/PN mole ratio 3:1, Novozyme 435 35 mg, a_w 0.160).

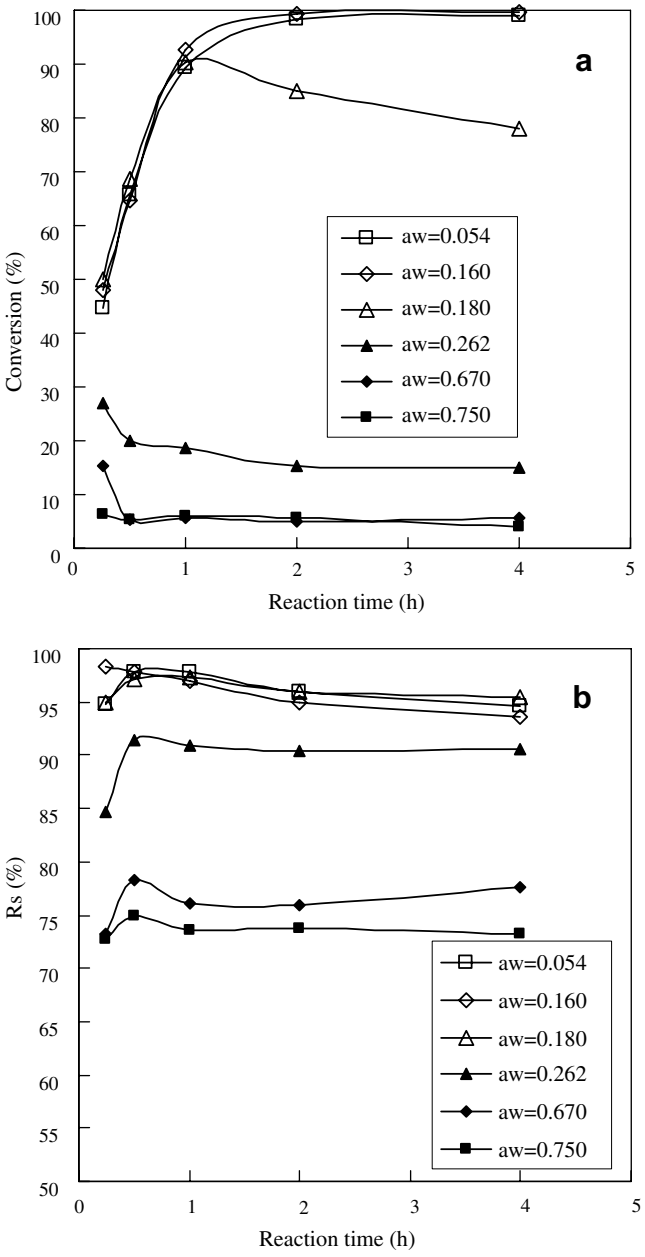
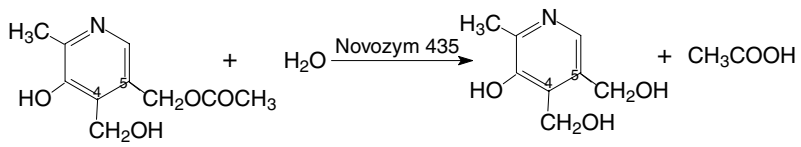


Fig. 2. Effect of a_w on lipase-catalyzed PN esterification in acetonitrile: (a) conversion of PN and (b) regioselectivity (Rs) (reaction conditions: acetonitrile 3 ml, temperature 35 °C, Acyl(vinyl acetate)/PN mole ratio 3:1, Novozyme 435 35 mg).

to be highly dependent on water activity. The moderate optimal water activity ($a_w < 0.16$) could be rationalized by the controversial effects of water on the enzyme-catalyzed synthetic reactions in organic media. The results from HPLC analysis showed that, at any time point, products with higher degrees of regioselectivity and yield were obtained at $a_w < 0.16$ as compared with that of $a_w > 0.18$, as seen in Fig. 2. Especially, when $a_w = 0.18$, there was also a fast reaction rate at initial stage and the conversion of PN attained 90.45% at 1 h, but unfortunately, the conversion started to go down thereafter. This phenomenon is similar to the report of Chang, Chou, and Shaw (2001). It is well known that hydrolytic enzymes such as lipases could catalyze not only esterification/transesterification, but also hydrolysis of ester (Yang, Wang, & Kuo, 2004). One possible explanation is that hydrolysis would be favored over ester bond formation with more and more ester of PN generated in the presence of abundant amount of water in the reaction system (see Scheme 4). The strong competing hydrolysis led to much lower esterification extent at higher water activities. This could be caused by the fact that excessive water shifted the reaction thermodynamic equilibrium in favor of the side hydrolysis reaction, although trace water in the system satisfied the requirement of the enzyme for holding essential water layer to perform its catalytic functions properly. In Fig. 2a, the reaction rate becomes much lower by changing a_w from 0.18 to 0.26, at the same time, the regioselectivity also drops deeply. The higher water activities imply excessive water and thereby increase competition of water for the acyl-enzyme intermediate. Moreover, enzymes with more free water molecules are less thermally stable (Volkin, Staubli, Langer, & Klibanov, 1991). Thus, it can be concluded that an optimal water activity is very important for lipase-catalyzed PN monoester synthesis.

3.4. Influence of reaction temperature

The effect of temperature on the reaction is shown in Fig. 3. HPLC analysis showed that the initial reaction took place slightly rapidly along with elevated temperature, which suggested an influence of temperature on the reaction rate. One explanation for the observation could be that the mobility of substrates increased with temperature and that they therefore became more accessible for the enzyme. However, the PN conversion was almost constant above 35 °C and nearly all PN were converted after about 2 h. Therefore, it also might indicate that temperature was not very crucial for such a reaction system. On the other hand, the degree of conversion was also considerably high at 2 h at lower temperature, but subsequently, the rate of hydrolysis was faster and faster due to high a_w (0.16), which led to the fall of the conversion. The phenomenon



Scheme 4. Hydrolysis of 5-AcPN.

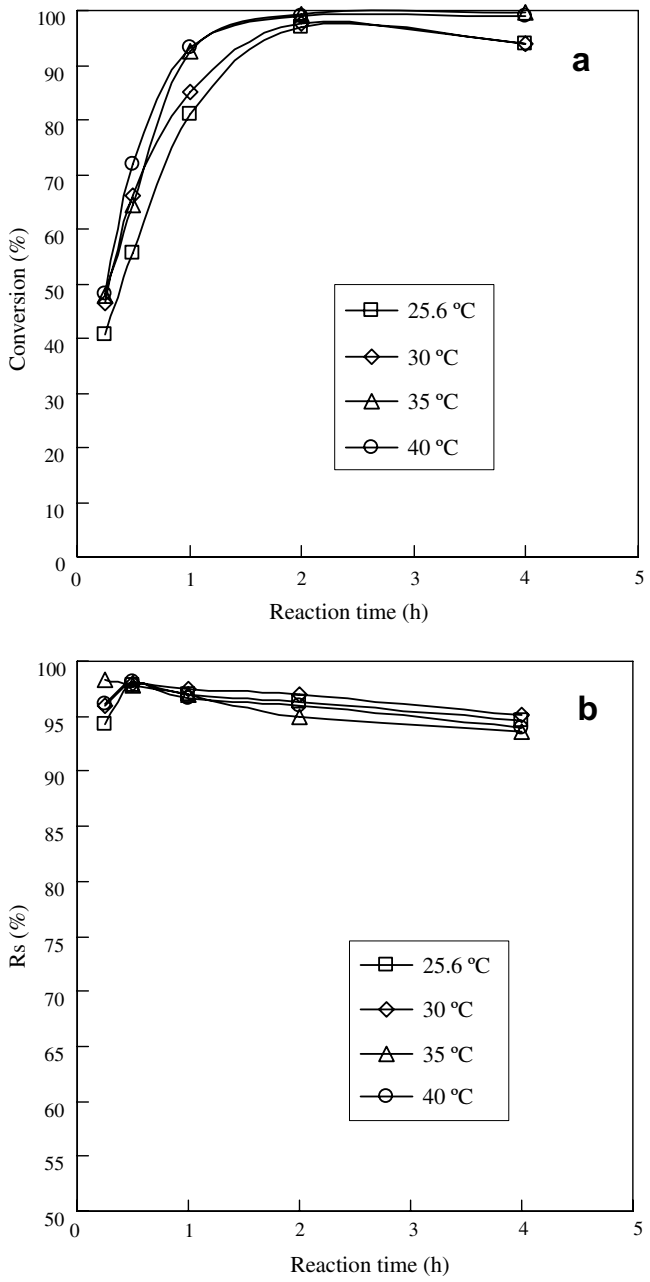


Fig. 3. Effect of reaction temperature on lipase-catalyzed PN esterification in acetonitrile: (a) conversion of PN and (b) regioselectivity (Rs) (reaction conditions: acetonitrile 3 ml, Acyl(vinyl acetate)/PN mole ratio 3:1, Novozyme 435 35 mg, a_w 0.160).

is similar to that of Kim, Lee, Lee, Kim, and Yoon (2000). Another interesting phenomenon is that the regioselectivity is not decreased (about 95%) despite the decline of conversion. It is important to point out that, during the esterification of PN, the regioselectivity goes up initially, and then follows a decreasing trend. All these observations can be explained as follows: (1) Owing to the existence of hydrolysis side reaction in the presence of excessive water, there is an 'over-equilibrium phenomenon' at low temperature. This can be understood with the aid of the concept 'inertia', so we name it 'reactive equilibrium-inertia'. (2) Increasing temperature is able to shift the equilibrium towards the ester formation. That is, high temperature is in favor of the formation of ester. (3) Although high temperature is capable of avoiding hydrolysis of ester (the experimental results in Fig. 3 show that high temperature is in favor of esterification of PN and therefore, represses hydrolysis of PN ester slightly), but too high temperatures can bring about the loss of enzyme activity.

3.5. Influence of substrate mole ratio

The effect of substrate mole ratio can be in two ways. On one hand, the increase of vinyl acetate amount will increase

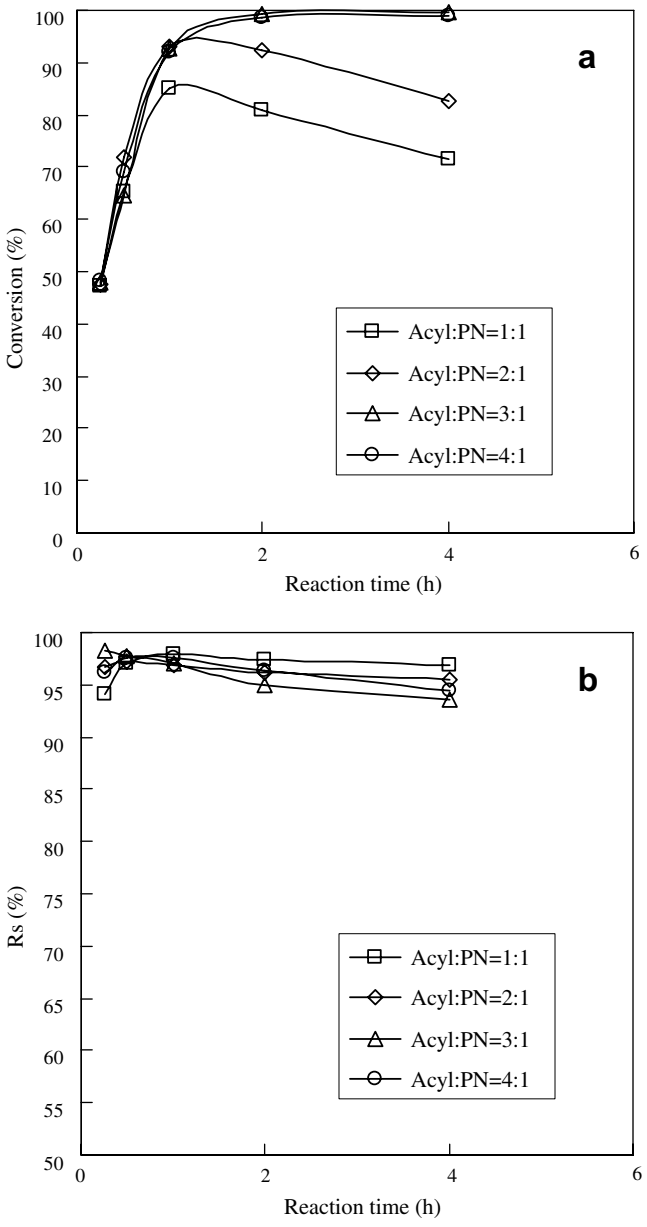


Fig. 4. Effect of substrate mole ratio on lipase-catalyzed PN esterification in acetonitrile: (a) conversion of PN and (b) regioselectivity (Rs) (reaction conditions: acetonitrile 3 ml, temperature 35 °C, Novozyme 435 35 mg, a_w 0.160).

the theoretical reaction equilibrium value. On the other hand, it can also raise the reaction rate of transesterification and reduce the rate of hydrolysis. To evaluate the influence of ratio of Acyl/PN on the reaction, various amounts of vinyl acetate were added to the reaction at water activity of 0.16 and $T = 35$ °C. Fig. 4 showed time course data of the PN ester synthesis with controlled ratio of vinyl acetate to PN. As can be seen, the initial rate was very rapid and the conversion reached 45% in 15 min. In addition, the process of transesterification has different behaviors at different vinyl acetate loadings. The conversion of PN shows a monotonic rise at Acyl/PN > 3, however, at Acyl/PN < 3, the conversion of PN emerged a peak with a maximum value around $t = 1$ h and then declined. A possible reason is that the hydrolysis rate increased with PN ester 1 h later at Acyl/PN < 3. This stresses the fact that variation in the conversion is not necessarily monotonic when acyl donor is relatively insufficient. We can draw a conclusion that the amount of vinyl acetate has a potential impact on the esterification of PN.

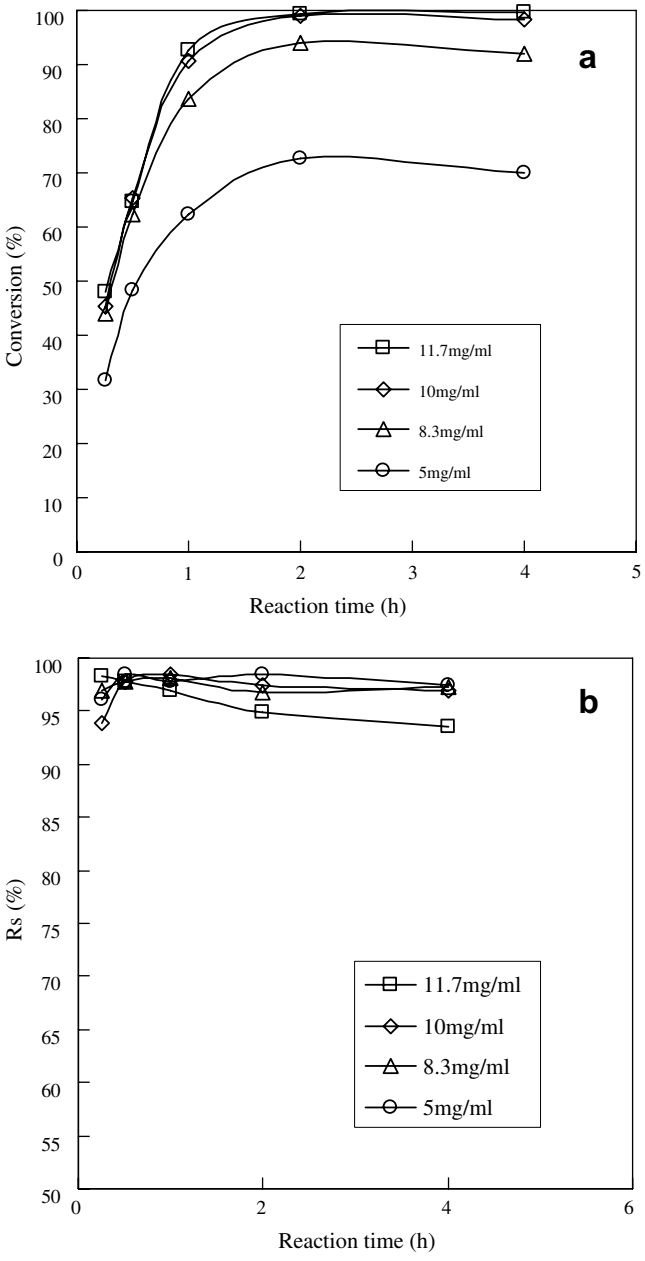


Fig. 5. Effect of enzyme loading on lipase-catalyzed PN esterification in acetonitrile: (a) conversion of PN and (b) regioselectivity (Rs) (reaction conditions: acetonitrile 3 ml, temperature 35 °C, Acyl(vinyl acetate)/PN mole ratio 3:1, a_w 0.160).

3.6. Influence of enzyme loading

To study the effect of enzyme loading, different amounts of Novozyme 435 were used in order to determine the minimum amount of enzyme required to obtain the maximum conversion in acetonitrile. In the enzyme loading study, esterification of PN was conducted at the Novozyme 435 loading of 11.7, 10, 8.3, 5 mg/ml acetonitrile, respectively. Fig. 5 depicts the effect of enzyme loading on the conversions. It was found that lower amount of enzyme loading led to lower conversion. As the enzyme loading increased, the conversion of PN also increased, however, when the enzyme loading was raised to 10 mg/ml acetonitrile, a further increase in the enzyme loading was not capable of enhancing the conversion. This observation is similar to that reported by Ryu, Kim, Kim, Baek, and Oh (2003). It must be emphasized that, when the reaction is carried out to a certain degree in the presence of a low enzyme loading, there is a slight decline in conversion due to hydrolysis. This can be explained in detail by that the transesterification rate is comparatively slow with a low enzyme loading which leads to slow equilibrium, at the same time, the hydrolysis rate is relatively faster, as a consequence, the conversion cannot reach a high level. After the addition of an extremely high amount of Novozyme 435, a 99.6% conversion and 93.6% regioselectivity was achieved after 4 h.

4. Conclusions

The results showed that, in acetonitrile, Novozyme 435 lipase displayed high activity towards the 5-OH of PN. However, it showed much more activity towards the 4-OH in ionic liquid. A plausible explanation of the different site-selectivity was that ionic liquid changed the conformation of lipase, and sequentially, the 4-OH became more active owing to sterically hindered effect. In addition, different acyl donors also showed significant effect on PN conversion and regioselectivity. By employing vinyl acetate in acetonitrile, the influences of water activity, temperature, substrate mole ratio and enzyme loading on the transesterification reaction have been investigated. As a result, enzymatic esterfication of PN exhibits a profound difference in response to the changes in a_w. Under the optimal operating conditions, a maximum conversion of 99% can be attained, and the regioselectivity can exceed 93%. The results are of general interest for developing industrial processes for the preparation of PN monoester useful for food additives

and cosmetic formulations and the synthesis of other PN derivatives.

Acknowledgments

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