Biosynthesis of (S)-naproxen starch ester by *Carica papaya* lipase in intermittent opening reaction mode

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Abstract: Inorder to brought S-naproxen into small intestine, an optically pure (S)-naproxen starch ester was produced by lipase through enantio-selective trans-esterification of racemic naproxen methyl ester with pretreatment starch in solvent system. With carefully selection of the reaction medium (isooctane), lipase (Carica Papaya Lipase, CPL) and the reaction mode (intermittent opening), a high conversion rate (48.6%) and enantiomeric excess of product (99.6%) was obtained. The slow release macromolecular (S)-Naproxen had been synthesized to improve the efficacy of racemic naproxen and overcome its side effects. The enanitomeric ratio of CPL (E=52.5) was higher than CRL (E=22) and greatly influenced by the byproduct methyl alcohol. The intermittent opening reaction mode was the effective way to remove the inhibition of methyl alcohol and to improve the enantio-selectivity of CPL. S-naproxen starch was confirmed by HPLC and ^{1}H NMR. This method may also apply to preparation the other optically pure 2-phenylpropionic acid derivatives. S-naproxen starch was a new optically pure derivatives possessing emulsifying and slow release properties would be widely applied to the food, pharmaceutical and biomedical industries.

Keywords: Intermittent opening reaction mode; Inhibition of methyl alcohol: Lipase-catalyzed in solvent system; (S)-naproxen starch ester; Trans-esterification synergy resolution

INTRODUCTION

Naproxen are 2-phenylpropionic acid derivative that could be widely applied to treatment the inflammatory diseases, such as rheumatoid arthritis and osteoarthritis. Not only the anti-inflammatory property of naproxen resides primarily in the S-enantiomer, but also the R-enantiomer has side effects (Morrone *et al*., 2010; Zuo *et al*.. 2011; Elif *et al.*, 2011). The strategies to obtain the S-naproxen have been reported, such as asymmetric synthesis(Smith et al., 2009; Sonawane *et al*., 1992), bio-catalytic kinetic resolution (Serpil *et al*., 2007; Panke *et al*., 2002), preferential crystallization (Rajesh *et al.*, 2012), and chiral chromatography separations of race-mates (William *et al.*, 1992). Some potential function of naproxen has not been embodied because of the pharmacological and physicochemical property. Such as naproxen is advocated for use in colorectal but it could not go into intestine because of the pharmacological (Tang *et al.*, 2013). Consequently developing their bio-reversible derivatives such as pro-drug to decrease the toxicity induced by NSAIDS and improve its new function is necessary.

In this work, S-naproxen starch ester has been prepared using lipase as catalyst in solvent system. And the S-naproxen could be brought in to small intestine because of the synergy of starch. This new S-naproxen derivative, maybe could be used to treat colon.

MATERIALS AND METHODS

Chemicals and enzyme

Starch was corn starch and purchased from Harbin Sheng Da Reagent Company, China and pretreatment in our laboratory. Naproxen of analytically grade was purchased from Shanghai Chemical Co., China. Novozym 435 (N435; Lipase B from *Candida Antarctica* immobilized on macro-porous acrylic resin; 10 U/mg) was purchased from Novozymes, Denmark, *Carica Papaya Lipase* (CPL; 120U/mg), *Candida Rugosa Lipase* (CRL; 890U/mg), *Wheat Germ Lipase* (WGL; 7.6U/mg) and Porcine *Pancreatic Lipase* (PPL; 10U/mg) was purchased from Sigma, Germany. All the other chemicals are of analytically grade.

Starch pretreatment

According to the method of Yan (Yan *et al.*, 2014), the 9% aqueous solution of sodium hydroxide/urea (2:1, w:w) was pre-cooled to below -10°C. Then the 5% dry native starch was added within 5 min by stirring at 3000 r/min until the solution was transparent. The transparent starch solution was neutralized to PH=7 with HCl (15%). After that the starch was precipitated out from the neutral starch solution by adding 50mL of ethanol drop-wise. And then, the precipitates were washed by successive centrifugations in 95% of ethanol until no HCl remained. Thereafter, they were washed with 100% of ethanol to remove water. The resulting precipitates were vacuum dried at 50°C for 24h.

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Preparation of racemic naproxen methyl ester

According to the method of Brook (Brook *et al.*, 1983), the 5.5g racemic naproxen, 40mL methanol and 40mL anhydrous benzene was added to a round bottom flask equipped with a water reflux condenser heating reflux for 4-5h. After reflux, the mixture solution was washed with 5% $NaHCO_3$ and distilled water. The organic phase was dried with anhydrous $NaSO_4$ for 12h. The solvent was evaporated by pressure dryness. The solid was re-crystallized using petroleum ether and methanol. The white crystal is the methyl ester of racemic naproxen. The conversion of naproxen was 100%.

General procedure for lipase trans-esterification

All the substrates must be pre-equilibrated up to the fixed water activities (a_w< 0.01). Firstly, the racemic naproxen methyl ester was dissolved using amount of isooctane. Secondly, the pretreated starch was added and mixed evenly with racemic naproxen methyl ester. The third, the lipase was added into the reaction system to initiated the trans-esterification. To conduct the reaction in 15mL solvent, a 3:1 mol ratio of racemic naproxen methyl ester to pretreated starch, reaction temperature at 55-75°C and 200r/min stir. The removal of residual racemic naproxen methyl ester from S-naproxen starch ester was accomplished by washed again and again with 10mL of isooctane and then dried at 75°C. The residual racemic naproxen methyl ester was recycled from 10mL of isooctane by vacuum evaporation.

Calculation of the conversion of racemic naproxen methyl ester, ee_p and E

The enantiomric excess of residual racemic naproxen methyl ester (ee_s) and conversion of racemic naproxen methyl ester (C) were determined by high performance liquid chromatography (HPLC)(Chen *et al.*, 1982).

The enantiomric excess of product (ee_p) was calculated as formula (1):

$$ee_p = ee_s \times \frac{1-C}{C} \qquad (1)$$

The enanitomeric ratio of lipase (E) was calculated as formula (2):

$$E = \frac{\ln(1-C)(1-ee_s)}{\ln(1-C)(1+ee_s)} \qquad (2)$$

The enantiomric excess (ee) was calculated as formula (3):

$$ee = \frac{R-S}{R+S} \qquad (3)$$

R is the content of R-naproxen; S is the content of S-naproxen.

^{1}H NMR spectra analysis

^{1}H NMR spectra were recorded at room temperature in DMSO-d_6 by using tetramethylsilane (TMS) as an internal reference on a Brucker DRX-400 NMR spectrometer (Germany) at 100 MHz. The determination conditions as followed: capillary voltage was 3KV; Scan range was 200-900 (m/z); Ionizing voltage (cone) was ES + 10v, 30 v; ES - 10 v, 30v. Ion source temperature: 130°C. Removal solvent temperature was 180°C; Chromatographic column: XTerraR MS C18 (2.1 x 150 mm), 5μm; The amount of sample was 2mg (Tang *et al.*, 2013).

HPLC analysis

HPLC analysis of naproxen, chromatographic column was Chiralcel OD-H (5μm, 4.6 mm × 250mm). Samples were filtered with 0.45μm Milli-pore filters and eluted with n-hexane and isopropyl alcohol (95:5, v/v) at 0.5 mL•min^{-1}. The eluent was monitored at 254nm and the column oven was set at 30°C. The sample size was 7.5 μL (Lucia *et al.*, 2008).

RESULTS

Screening of lipases

In order to select a suitable lipase possessing S-stereo preference, racemic naproxen methyl ester was subjected to trans-esterification with pretreatment starch in presence of immobilized *Candida antarctica* (Novozym435), *Porcine Pancreatic Lipase* (PPL), *Wheat Germ Lipase* (WGL), *Carica Papaya Lipase* (CPL) and *Candida Rugosa Lipase* (CRL) using isooctane as solvent. The lipase of CRL and CPL could catalyze the trans-esterification of (S)-naproxen methyl ester but Novozym 435 showed the desired R stereo preference. The PPL and WGL could not catalyze trans-esterification of the racemic naproxen methyl ester with pretreatment starch, as reported in table 1. These results showed that CRL and CPL not only could tolerate isooctane, but also the conformation match with racemic naproxen methyl ester. All the next reactions were carried out in presence of CRL or CPL as catalyst.

Selection of solvent

With the intention of improving the enantio-selectivity of CRL and CPL in the trans-esterification of naproxen ester, in order to consider it for an enantiomeric preparative resolution, there action was repeated in different solvents as reported in table 2.

Although the trans-esterification could carry out in all the considered solvents, except acetone, the enantio-selectivity of lipase and the conversion of substrate decreased with the decrease of log P of solvent. That may be due to the toxicity of solvent on lipase increase with the decrease of its logP. Not only CPL but also CRL presented the best enantio-selectivity (E=52.13 of CPL and 22.25 of CRL) when using isooctane as the reaction medium. So, isooctane had been selected as the reaction medium. That not only could dissolve naproxen methyl ester to reduce the viscosity of the reaction system, but also could maintain the activity of lipase.

Table 1: Trans-esterification of racemic naproxen methyl ester with pretreatment starch by different Lipase

Lipase from	Time (d)	Temperature (°C)	ee_p (%)	ee_s (%)	Conversion (%)	Stereo-preference	E
All the reactions were repeated for 3 times. Reacrion condition: the amount of enzyme is 10%, the ratio of starch to racemic naproxen methyl ester is 1:3, rotation speed is 200 r/min, 50°C to 60°C.							
Carica Papaya (CPL)	4	60	95.9±0.58	44.9±0.56	31.9±0.78	S	52±1.36
Candida Rugosa (CRL)	6	60	82.8±0.98	65.0±0.63	45.1±0.69	S	21±1.21
Wheat Germ (WGL)	6	50	-	-	3.6±0.32	S	-
Porcine Pancreatic (PPL)	6	50	-	-	-	-	-
Candida Antarctica (N435)	4	65	86.3±0.52	34.1±0.24	28.3±0.21	R	19±0.86

Table 2: Effect of organic solvent on the trans-esterification of naproxen methyl with starch

Organic solvent	$\log^P$	Conversion (%)		ee_p (%)		ee_S (%)		E	
		CPL	CRL	CPL	CRL	CPL	CRL	CPL	CRL
isooctane	4.7	31.9 ±0.38	45.1 ±0.17	95.9 ±0.45	82.8 ±0.46	44.9 ±0.26	68.0 ±0.46	52.1 ±1.23	21.5 ±0.98
n-hexane	3.5	19.82 ±025	15.78 ±0.36	72.53 ±0.25	36.58 ±0.41	17.89 ±0.52	13.59 ±0.65	7.42 ±069	7.14 ±0.25
Methyl benzene	2.5	30.15 ±0.45	12.19 ±0.36	53.84 ±0.35	26.64 ±0.36	23.24 ±0.36	3.77 ±0.72	4.16 ±0.36	1.73 ±0.32
tertiary amyl alcohol	1.5	59.46 ±0.36	29.82 ±0.59	42.82 ±0.49	6.05 ±0.29	62.80 ±0.47	2.57 ±0.85	8.34 ±0.25	5.52 ±0.12
tertiary butanol	0.8	57.27 ±0.25	28.35 ±0.25	15.10 ±0.25	2.54 ±0.32	16.54 ±0.49	1.01 ±0.23	1.57 ±0.36	0.93 ±0.23
acetone	-0.23	-		-		-		-	

All the reactions were repeated for 3 times. Reacrion condition: the amount of enzyme is 10 % (W:W), the ratio of starch to racemic naproxen methyl ester is 1:3 (mM: mM), rotation speed is 200 r/min, 65°C (CRL) or 60°C (CPL); CPL is Carica Papaya Lipase, CRL is Candida Rugosa Lipase; ee_p is the enantiomric excess of product; ee_s is the enantiomric excess of residual racemic naproxen methyl ester; E is the enanitomeric ratio of lipase.

Trans-esterification procedure

Fig. 1 and fig.2 showed the reaction process of biosynthesis of (S)-naproxen starch ester with *Carica Papaya Lipase* (CPL) and *Candida Rugosa Lipase* (CRL) in isooctane system. In the case of CRL as biocatalyst, the amount of racemic naproxen ester solution was 10 mM and the amount of lipase was 5%. The reaction could last for 6d. Specific reaction rate was 0.41mmol/hg-lipase at the initial stage of reaction, but the lipase enantio-selectivity decreased after 4d. When using *Carica Papaya Lipase* as catalyst, the reaction process continues for 4d and the initial reaction rate was 0.04mmol/hg-lipase, but the lipase enantio-selectivity could last till the end of the reaction. In isooctane system, the activity of CRL was

Thigher than CPL, but the enantio-selectivity of CPL (E=52.1) was better than CRL (E=21.5). In order to biosynthesis the optical purity (S)-naproxen starch ester, the CPL was selected as the catalyst. That may be because of CPL is plant protein, its active center structure of CPL conforms with naproxen more than other lipase.

Effect of reaction mode

An enzymatic trans-esterification synthesis process has been developed to directly prepare starch ester of (S)-naproxen from racemic naproxen methyl ester using CPL as the bio-catalysis in the isooctane. When the trans-esterification was conducted by the sealing reaction mode, the conversion of substrate and enantiomric excess of product was lower than by the intermittent opening.

Reaction mode, as showed in figs. 3 and 4. These results demonstrated that the byproduct methyl alcohol not only effected the enzyme activity but also effected the enantio-selectivity of CPL. When using the intermittent opening reaction mode, the methyl alcohol could be eliminated by intermittent opening the reaction system every 1d for 2h. But, that will let the volatilization of isooctane at the same time. So that must replenishing 15mL isooctane into reaction system after opening 2h and then sealing again. The ee_p was high to 99.6% and the conversion of substrate was improved to 48.6% by using intermittent opening reaction mode. If the trans-esterification proceeded in opening reaction mode completely, the

reaction could last for 2d only because of the volatilization of isooctane. So, the reaction mode of intermittent opening is the desired way to trans-esterification synthesis of (S)-Naproxen starch ester in isooctane by CPL. These results demonstrated that the byproduct methyl alcohol is the inhibitor of lipase catalysis the trans-esterification. So, how to remove it effectively form the reaction system is very important.

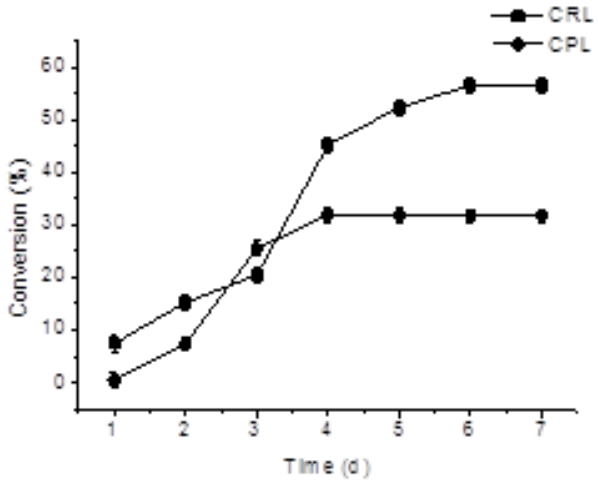

Fig. 1: The conversion of racemic naproxen methyl ester using CRL (■) and CPL (●) (Reacrion condition: the amount of enzyme was 10% (W:W), the ratio of starch to racemic naproxen methyl ester was 1:3 (mM: mM), speed of stir bar was 200 r/min, 65°C (CRL) or 60°C (CPL); CPL is Carica Papaya Lipase, CRL is Candida Rugosa Lipase)

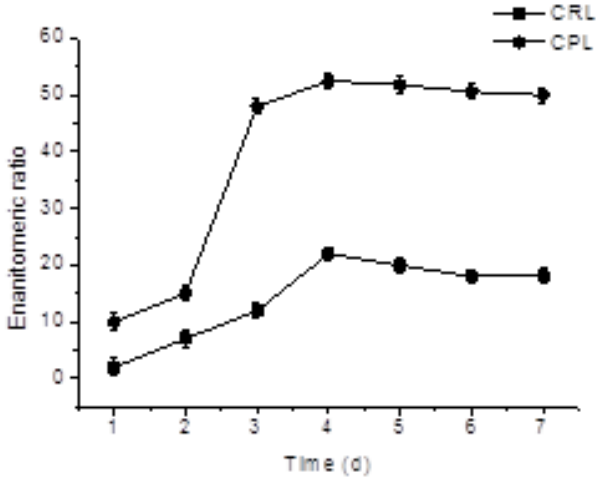

Fig. 2: The enanitomeric ratio of CRL (■) and CPL (●) (Reacrion condition: the amount of enzyme was 10% (W: W), the ratio of starch to racemic naproxen methyl ester was 1:3 (mM: mM), speed of stir bar was 200 r/min, 65°C (CRL) or 60°C (CPL); CPL is Carica Papaya Lipase, CRL is Candida Rugosa Lipase)

^{1}H NMR analyses

Naproxen Starch Ester was solubled in DMSO-d_6 with one drop of TFA-d_1. Fig. 5(a), (b) and (c) show the typical ^{1}HNMR spectra of pretreatment starch, naproxen starch ester product and naproxen methyl ester respectively. The overlapped peaks in the region δ7.15–7.81ppm and at δ3.86ppm, δ3.37ppm, δ2.51ppm, δ1.43ppm correspond to the aromatic nucleus atoms of the Naproxen (Fig. 5b) (Wang *et al.*, 1999). The overlapped peaks at δ5.5–2.5ppm are assigned to the starch protons (Elomaa *et al.*, 2004; Junistia *et al.*, 2008). In comparison with the spectra of the naproxen methyl ester and starch, the major change of naproxen starch ester is the presence of $COOCH_2$ proton absorption at δ 3.6ppm. The red shift of pretreatment starch protons at δ1.25-1.0ppm was attributed to the change of the chemical environment. These results could prove that the naproxen was molecular attached to starch.

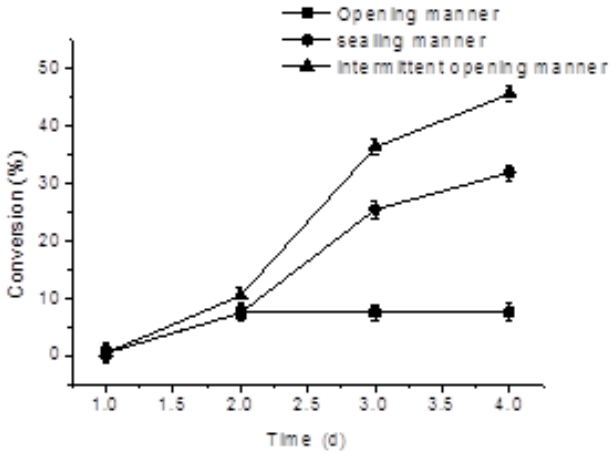

Fig. 3: The conversion of racemic naproxen methyl ester by opening reaction mode (■), sealing reaction mode (●) and intermittent opening reaction mode (▲) (Reacrion condition: the amount of CPL was 10% (W: W), the ratio of starch to racemic naproxen methyl ester was 1:3(mM: mM), speed of stir bar was 200 r/min, 60oC; CPL is Carica Papaya Lipase)

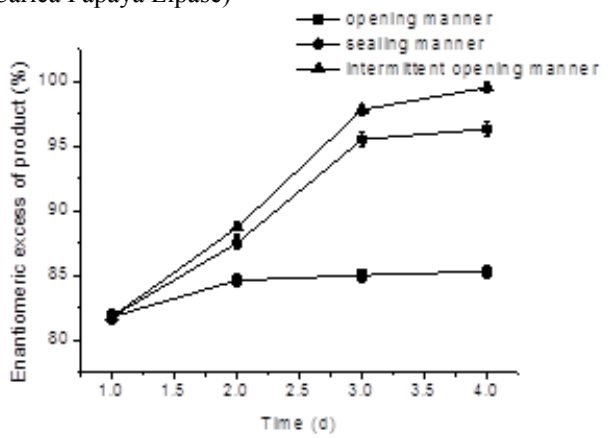

Fig. 4: The conversion of racemic naproxen methyl ester by opening reaction mode (■), sealing reaction mode (●) and intermittent opening reaction mode (▲) (Reacrion condition: The amount of CPL was 10% (W:W), the ratio of starch to racemic naproxen methyl ester was 1:3(mM: mM), speed of stir bar was 200 r/min, 60°C; CPL is Carica Papaya Lipase)

Emulsion Capacity

Emulsion capacity of starch, S-naproxen starch and (R, S)-Nproxen were evaluated by visual inspection of the emulsions after different storage times at room temperature. The measurement results of emulsion capacity of the emulsions are shown in fig. 6. The S-naproxen starch not only obtained the emulsion capacity (53.2%), but also it was higher than satrch (5.3%). The emulsion capacity of (R,S)-Nproxen was not be measured. The better emulsion capacity of S-naproxen starch make it easy to digestive absorption by colon.

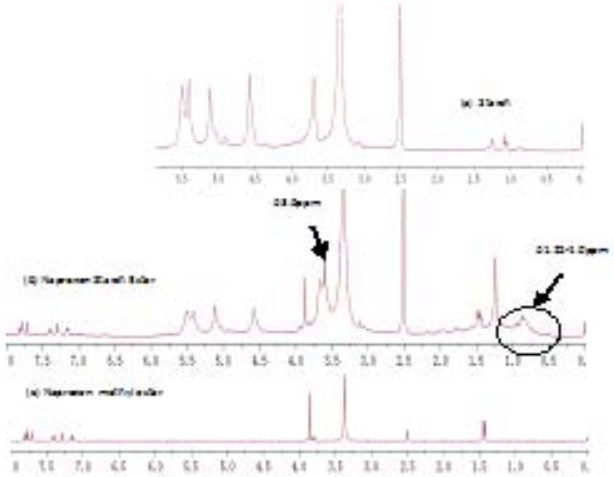

Fig. 5: ^{1}HNMR spectra of pretreatment starch (a), naproxen starch ester (b) and naproxen methyl ester (c).

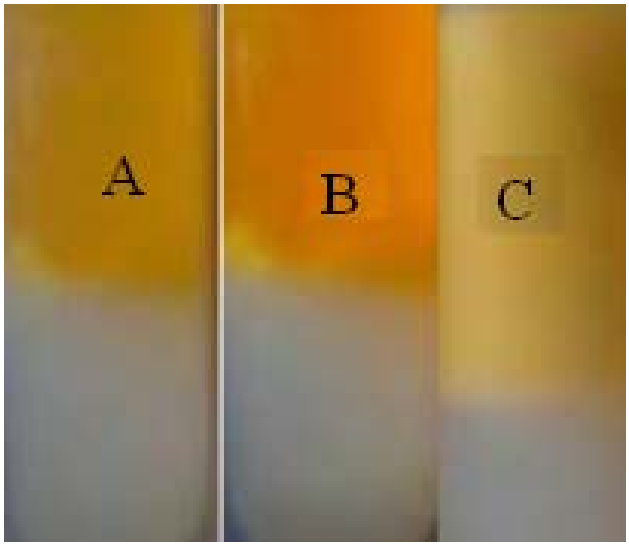

Fig. 6: The emulsion of Starch (A),(R,S-naproxen) (B) and S-naproxen starch (C)were centrifuged for 15 min.

CONCLUSION

A new macromolecular (S)-Naproxen has been enzymatic synthesized by trans-esterification racemic naproxen methyl ester with starch in organic solvent. The (S)-Naproxen starch ester has been synthesized by the enantio-selectivity of lipase to improve the efficacy of racemic naproxen and overcome its side effects. The CPL presented a higher enantio-selectivity than other enzymes in trans-esterification synthesis of S-naproxen starch eater in isooctane. But the byproduct (methyl alcohol) was the inhibitor of this trans-esterification. The intermittent opening reaction mode has been proposed to eliminate methyl alcohol that could improve the enantiomeric excess of product and the conversion of substrate obviously.

DISCUSSION

The method of one step esterification synergy resolution synthesis by an enzyme could be used in resolution and derivatization of other 2-phenylpropionic acid derivative. But the range of application and reaction mechanism was undefined. In the future, this work would not only focus on studying the molecular structure and the biological activity of S-naproxen starch ester, but also on the reaction mechanism of lipase.

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