Asian Journal of Chemistry; Vol. 23, No. 8 (2011), 3413-3416

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Selective Esterification and Antioxidant Activity of Rutin

QING HUO[1], XUEMEI GU[2,*], QIANG LIN[1], YUJIAO SUN[1], CHENXIA XU[1] and LEI XIAO[1]

[1]Biochemical Engineering College of Beijing Union University, Beijing 100023, P.R. China
[2]Beijing Military General Hospital, Beijing 100700, P.R. China

*Corresponding author: Tel: +86 10 52072020; E-mail: huo_q2002@yahoo.com.cn

(*Received*: 26 August 2010; *Accepted*: 15 April 2011) AJC-9814

Rutin is a natural medicine which is widely presented in plants. Modified by the esterification of rutin in the introduction of long hydrocarbon chain molecules, in order to increase its solubility and stability in the lipophilic, thus increasing the antioxidant activity. This paper reports the reaction solvents, acyl donor chain length, molecular sieve and enzyme. Experimental results show that *tert*-amyl alcohol is the best solvent for the reaction; *Rhizopus arrhizus* whole-cell lipase or Lipase Novozym435 is used as biological catalyst enzyme; the molar ratio of lauric acid:rutin is 5:1, the reaction temperature is 60 ºC, the reaction time is *ca.* 96 h, within the esterification, we should add molecular sieve in order to increase the esterification conversion. Antioxidant effect is tested by the ultraviolet spectrophotometer. The ester has stronger scavenging effect for $^{\bullet}OH$ and $^{\bullet}O_2^-$ than rutin. So the ester has more antioxidant capacity than rutin and mannitol.

Key Words: Rutin, Esterification modification, Thin layer separation, Oxidation resistance.

INTRODUCTION

Nowadays, the biological activity of rutin is paid more attention. It is approved that rutin has many physiological function and medicinal value. It can prevent a free radical oxidation with antiaging, antiinflammatory, antitumor, immunomodulatory and so on. Therefore, rutin has positive significance for preventing disease and human health[1,2].

Flavanoid is in the form of glucoside in nature. Glucoside is polarity, most of them exhibit a low solubility and stability in non-polar media like oil. A solution to improve the hydrophobic nature of flavonoids consists in the esterification of the hydroxyl functions by fatty acids. Therefore, flavanoid through esterification get the long hydrocarbons chain can increase the level of solubility and increase its resistance oxidation[3].

The antioxidant activity of aglycone is modified by the esterification is better than glucoside. This is due to the better lipophilic of aglycone which plays a role core in biomembrane hydrophobic layer. Flavanoid though esterification modify increase the level of solubility and more antioxidant. However, usual chemistry modification has a bad selectivity. Many hydroxyls of flavanoid may participate in esterification that usually combined with or shielding the main active group. Although the stability of the product increased, but decreased antioxidant effect. In addition, the chemistry of esterification involves three steps: (i) group protection, (ii) esterification and (iii) out of group protection. The process is complex, so that is difficult to handle[4-7].

To overcome this problem, the enzymatic catalysis can be envisaged because it is generally thought to be regioselective. The process can be conducted under mild conditions of temperature and pressure. This method operation is simple and with high efficiency[8-11].

EXPERIMENTAL

Palmitic acid, stearic acid, lauric acid, alcohol, formic acid, acetic ether, all of them are CP grade, methanol and other reagents are of analytical grade. (Beijing Chemistry Factory); *tert*-amyl alcohol, butanol, tertiary butyl alcohol and *n*-hexane are CP grade (Beijing Chemistry Reagent Company); rutin, content ≥ 95 % (Beijing Chemistry Reagent Company); *Rhizopus arrhizus* whole-cell lipase, *Rhizopus arrhizus* extracellular lipase, *Rhizopus arrhizus* solid-state fermentation mycelia lipase, polyurethane immobilized *Rhizopus arrhizus* mycelial lipase (Beijing University of Chemical Technology); Lipase Novozym435 (Novzymes (China) Biology Technology Limited Company); molecular sieve (China-America Shanghai Global Molecular sieve Limited Company); chromatography silica gel, 300-400 order (Qingdao Ocean Chemistry Factory); deionized water.

UV spectrophotometer: UV analysis is applied to determine the content of rutin as reported by Zheng-Xiang[4]. Rutin concentration is calculated using the standard sample as the calibration standard. Rutin dissolved in methanol, A good linear relationship is obtained over the range of 0.0116 mg/mL < C < 0.0696 mg/mL and the regression is y = 2.28448x + 0.1701 (R = 0.9996), where y is the absorbance at 256.5 nm, x is the concentration of rutin (mg/mL) and R is the regression coefficient.

In the reaction process, measure the reaction liquid at 256.5 nm, calculate the content of rutin samples, the esterification were determined.

HPLC: HPLC analysis is performed using a Waters liquid chromatographic systems (Waters company, USA) HPLC system, A diamonsil C18 column (250 mm × 4.6 mm i.d., 10 μm) is employed for the separation of samples. The detector is set to 254 nm and injection volume is 20 μL. methanol + water = 58:42 is adopted as mobile phase. Flow rate is 1.0 mL/min. All HPLC are performed at 25 ± 1 ºC. All solutions are prepared in 58 % aqueous methanol and filtered through 0.45 μm membranes (Chromatography Science and Technology Co., Tianjin, China) before HPLC analysis, the retention time of rutin was 33.5 min. The chromatographic peak of the rutin was confirmed by comparing its retention time and UV spectrum with that of the reference standard. The working calibration curve based on rutin standard solutions showed good linearity over the range of 1.875-4.375 μg. The regression line was $y = 2 \times 10^6 x + 475288$ ($R^2 = 0.9992$, n = 8), where Y is the peak area of rutin and X is the concentration of rutin (μg).

Experimental methods

Water content in organic medium has a great influence on the catalytic esterification reactions. With high water content, usually leads to low rate of conversion yield, therefore before the reaction, water treatment is necessary. Rutin, palmitic acid, stearic acid and lauric acid dry in the dryer for 1 week. *tert*-Amyl alcohol, butanol, tertiary butyl alcohol and *n*-hexane use 4A molecular sieve for 5 days, prior to the activation 4A (150 ºC, 24 h).

Synthesis: Rutin and acyl donor (such as palmitic acid, stearic acid and lauric acid), with a fixed ratio in solvent (such as *tert*-amyl alcohol, butanol, tertiary butyl alcohol and *n*-hexane). In 60 ºC add Novozym435 lipase (4 mg/mL), shaking the bottle. After 24 h, according to 1 L reaction liquid by adding 100 g molecular sieves 4A to remove the water produced in esterification. The reaction time is 96 h, filter the enzyme and molecular sieve[12,13].

The products are isolated by TLC, using ethyl acetate, formic acid and ethanol as developing solvent component. Adjusting the component ratio, lution and ester can separation. Different colours are taken down and analyzed.

Oxidation resistance test

Hydroxyl radicals: $^{\bullet}OH$ group is produced by EDTA-Na-Fe(II)-H_2O_2 (Fenton) system. $^{\bullet}OH$ can make *Crocus sativus* red T fade, according to the degree of faded with colorimetric method to measure the content $^{\bullet}OH$ group. In pH 7.4 reaction system for the solution of the phosphoric acid buffer, *Crocus sativus* red T (520 μg/mL) 0.2 mL, 2 mmol EDTA-2Na-Fe(II) 0.7 mL, different concentrations of selected liquid 1.0 mL, 6 % of H_2O_2 0.4 mL, mixing 0.5 h in 37 ºC water heat. Then measuring absorbance in wavelength 520 nm with spectrophotometer, experimental results with clearance E %, ED_{50} means that 50 % clearance drug concentration in system.

$$E\ \% = (A_{sample} - A_{blank}) / A_{sample} \times 100\ \%$$

Super oxide anion radical: Pyrogallol in alkaline conditions can release an oxygen free radicals. Super oxide anion radical and NBT form purple compounds which can be measured in the wavelength of 530 nm. The absorption value (A) reflects the content of $^{\bullet}O_2^-$. Take 0.1 mol/L (pH 8.2) of *Tris*-HCl buffer solution 2.5 mL in tube, warm-water bath at 25 ºC 20 min preheat samples, add different concentrations sample 0.2 mL, 0.98 mmol/L NBT 0.6 mL, 10 mmol/L of pyrogallol 0.3 mL, after mixed reaction at 25 ºC water bath 4 min, immediately with 8 mol/L of 0.1 mL HCl to terminate the reaction and the wavelength of 530 nm absorbance was determined at A values. Useful distilled water instead of a blank sample. Determination results in clearance rate $E\ \% = (A_{blank}-A_{sample}) / A_{blank} \times 100\ \%$[14].

RESULTS AND DISCUSSION

Effect of the acyl donors: 1 g Rutin, 0.605 g enzymes, 150 mL butanol and the acyl donor (such as palmitic acid, stearic acid or lauric acid) were mixed in the 250 mL bottle. The molar ratio of acyl donor:rutin is 5:1, three flasks are in shaking bed, temperature control in 55-60 °C, reaction 24 h later, add the molecular sieve, continue the reaction. After 96 h, the reaction was stopped. The experimental data in different solvents is shown in Fig. 1.

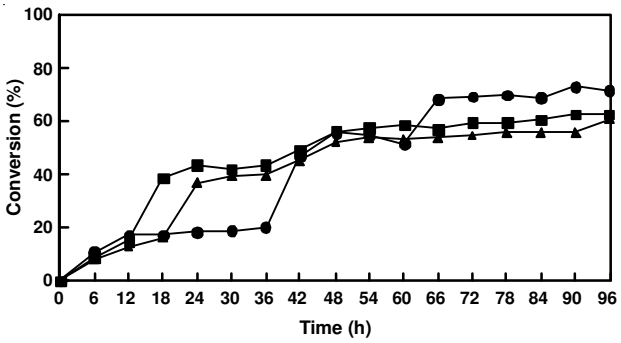

Fig. 1. Effect of acyl donor on the conversion ■ palmitic acid, ▲ stearic acid, ● lauric acid

Using lauric acid as acyl donor, the ester formed slowly at beginning. The shorter carbon chain, the lower melting point. Due to the catalytic temperature of 60 °C is beneficial to lauric acid solvent, the reaction rate increased rapidly in 36 h. Since then, the conversion of lauric acid as acyl donor is significantly higher than palmitic acid and stearic acid as acyl donor.

Effect of solvent on the reaction: Take 1 g rutin, 1.64 g lauric acid (the molar ratio of lauric acid:rutin is 5:1), 0.605 g enzymes, 150 mL solvent (*tert*-amyl alcohol, butanol, tertiary butyl alcohol or *n*-hexane) in the 250 mL bottle, four flasks are in shaking bed, temperature control at 55-60 °C, after 24 h of the reaction, add the molecular sieve and continue the reaction. After 96 h, stop reaction. Experimental data in different solvents is shown in Fig. 2-5.

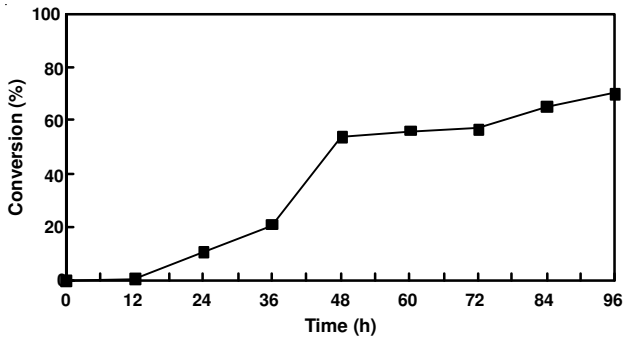

Fig. 2. Effect of tertiary butyl alcohol as solvent on the conversion

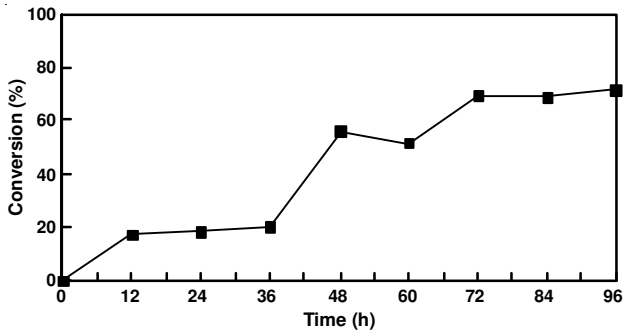

Fig. 3. Effect of butanol as solvent on the conversion

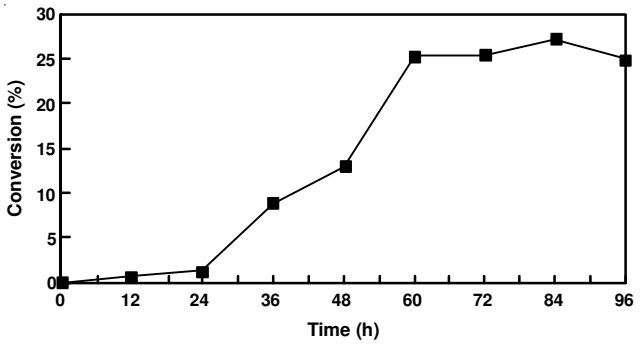

Fig. 4. Effect of *n*-hexane as solvent on the conversion

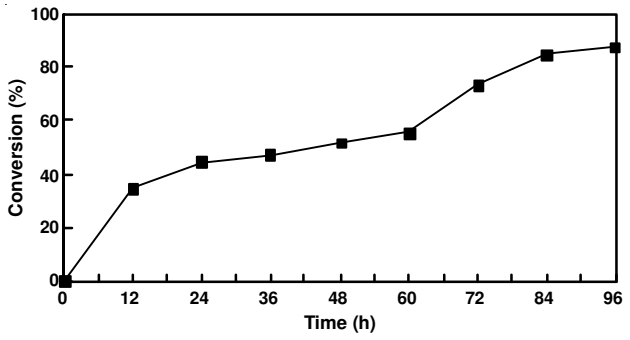

Fig. 5. Effect of *tert*-amyl alcohol as solvent on the conversion

From Figs. 2-5, it is observed that the initial reaction conversion rate is very low. This may be due to the various components of the reaction takes time to dissolve. About 50 h, the conversion speed up more obvious. About 96 h, the reaction reaches the maximum conversion rate. Each solvent comparison is given in Table-1.

TABLE-1
COMPARISON OF FOUR SOLVENTS

Solvent	Conversion rate (%)	Remark
t-Butyl alcohol	70.6	Solid in room temperature, analysis difficulties
Butanol	71.5	Easy to operate
n-Hexane	25.0	Low solubility of rutin
t-Amyl alcohol	87.5	Easy operation and high conversion

According to Table-1, it is clear that the best reaction solvent is *tert*-amyl alcohol. The highest conversion rate is up to 87.5 % and easy to operate.

Influence of reaction sieve: Take 1 g rutin, 1.64 g lauric acid (the molar ratio of lauric acid:rutin is 5:1), 0.605 g Lipase Novozym 435, 150 mL *tert*-amyl alcohol in the 250 mL bottle, two flasks are in shaking bed, temperature control in 55-60 ºC, reaction 24 h later, add the molecular sieve in one flask, according to 1 L reaction liquid by adding 100 g molecular sieves 4A, continue the reaction. After 96 h, stop reaction. Experimental data is shown in Fig. 6.

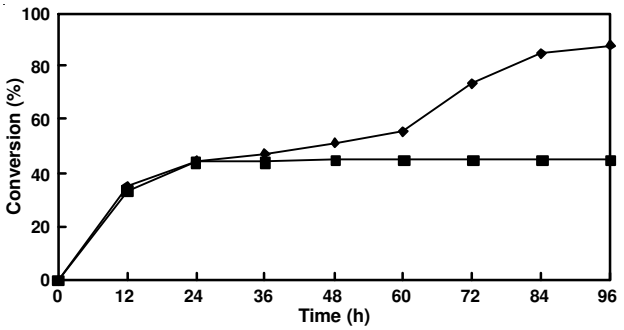

Fig. 6. Influence of reaction sieve ■ without molecular sieve ◆ add molecular sieve

With or without added molecular sieve has great influence on the reaction yield. Molecular sieve absorb water which is produced by esterification. Esterification reaction intermediate is "acyl donors -enzyme". Water can hydrolyse intermediate, the more intermediate, the higher yield, so to promote reaction the sieve is added.

Effect of enzyme: Take 1 g rutin, 150 mL *tert*-amyl alcohol, 1.64 g acyl donor lauric acid and enzyme (such as fungus ball of lipase, immobilization of lipase, bacteria, Lipase Novozym 435) in the 250 mL bottle, The amount of enzyme is 4 mg/mL, four flasks are in shaking bed, temperature control in 55-60 °C, reaction 24 h later, add the molecular sieve, continue the reaction. After 96 h, stop reaction. Experimental data in different enzymes is shown in Table-2.

Table-2 shows that due to stronger stability, the yield of the whole cells of *Rhizopus arrhizus* lipase is higher than the conversion rate of extracellular lipase. *Rhizopus arrhizus* solid-state fermentation mycelia lipase has the lowest conversion rate. It may be due to less exposure to oil during fermentation process, the enzyme activity is lower. Polyurethane immobilized *Rhizopus arrhizus* mycelial lipase has low yield is due to lower content of the enzyme. Summary, Novozym 435 Lipase and *Rhizopus arrhizus* whole-cell lipase are very good enzyme in esterification.

TABLE-2
EFFECT OF ENZYME IN THE ESTERIFICATION

Lipase	Conversion rate (%)
Rhizopus arrhizus whole-cell lipase	89.5
Rhizopus arrhizus extracellular lipase	50.3
Rhizopus arrhizus solid-state fermentation mycelia lipase	26.7
Polyurethane immobilized *Rhizopus arrhizus* mycelial lipase	44.3
Novozym 435 Lipase	87.5

Thin layer separation: Separate the molecular sieve and product by filter, then liquid mixtures are separated by TLC. the best developing solvent ratio is ethyl acetate:ethanol:formic acid = 8:1:1, this condition can effectively separate ester and rutin, expand agents 15.3 cm, rutin 12.6 cm, ester 4.8 cm, R_f value of rutin is 0.31.

Oxidation resistance experiment: Antioxidant effect is tested by the ultraviolet spectrophotometer. $^{\bullet}OH$ group is produced by EDTA-Na-Fe(II)-H_2O_2 (Fenton) system. Pyrogallol in alkaline conditions can release an oxygen free radicals $^{\bullet}O_2^-$. The scavenging $^{\bullet}OH$ and $^{\bullet}O_2^-$ are showed in Tables 3 and 4.

TABLE-3
MANNITOL, RUTIN AND ESTER SCAVENGING $^{\bullet}OH$

Sample	System concentration (mg/mL)	E %	EC_{50} (mg/mL)
Mannitol	1.62	14.28 ± 0.060	5.24
	4.05	37.85 ± 0.810	
	6.48	60.98 ± 0.870	
Rutin	0.63	18.47 ± 0.060	1.71
	1.52	46.17 ± 0.180	
	2.53	73.87 ± 0.060	
Ester	0.58	32.41 ± 0.050	0.89
	0.87	48.15 ± 0.546	
	1.56	87.26 ± 0.060	

TABLE-4
MANNITOL, RUTIN AND ESTER SCAVENGING $^{\bullet}O_2^-$

Sample	System concentration (mg/mL)	E %	EC_{50} (mg/mL)
Mannitol	1.61	14.63 ± 0.960	5.13
	4.00	39.86 ± 0.870	
	6.43	61.77 ± 0.610	
Rutin	0.61	22.36 ± 1.280	1.42
	1.50	53.62 ± 0.920	
	2.41	82.98 ± 1.350	
Ester	0.21	6.47 ± 0.890	1.36
	0.71	24.69 ± 1.080	
	1.00	35.87 ± 0.949	

The ester has stronger scavenging effect for $^{\bullet}OH$ and $^{\bullet}O_2^-$ than rutin. The amounts scavenging of rutin 50 % (EC_{50}) $^{\bullet}OH$ and $^{\bullet}O_2^-$ are 1.71 and 1.42 mg/mL. The amount scavenging of ester 50 % (EC_{50}) $^{\bullet}OH$ and $^{\bullet}O_2^-$ are 0.89 and 1.36 mg/mL. So the ester has more antioxidant capacity than rutin and mannitol.

Conclusion

The purposes of esterification is to increase the rutin lipophilic character, in order to enhance its antioxidant activity. The feasibility of the enzymatic esterification of rutin is demonstrated. This reaction is catalyzed by the *Rhizopus arrhizus* whole-cell lipase or Lipase Novozym435. To improve the conversion yield, water produced during esterification is removed continuously by the addition of molecular sieves 4A. *tert*-Amyl alcohol is the best solvent for the reaction, the highest conversion yield obtained is 87.5 %. Enzymatic synthesis in organic media is controlled by numerous biochemical and physical-chemical parameters.

ACKNOWLEDGEMENTS

This work was supported by Biochemical Engineering College of Beijing Union University Subject Construction Projects.

REFERENCES

1. G. Di Carlo, N. Mascolo, A.A. Izzo and F. Capasso, *Life Sci.*, **65**, 337 (1999).
2. D. Zhang, J.X. Tai and Q. Fu, *Food Fermen. Ind.*, **6**, 52 (1999).
3. J.P.E. Spencer, G.G.C. Kuhnle, R.J. Williams and C. Rice-Evans, *Biochem. J.*, **372**, 173 (2003).
4. W. Li and Z.-X. Ning, *Food Sci.*, **26**, 505 (2005).
5. Q. Wang, A. Tang and B. Shi, *Nat. Prod. Res. Dev.*, **13**, 12 (2001).
6. H. Fujii, T. Nakagawa, H. Nishioka, E. Sato, A. Hirose, Y. Ueno, B. Sun and T. Yokozawa, *J. Agric. Food. Chem.*, **55**, 1525 (2007).
7. C. Wei and T.K. Lin, *Chin. J. Synth. Chem.*, **16**, 133 (2008).
8. S. Gayot, X. Santarelli and D. Coulon, *J. Biotechnol.*, **101**, 29 (2003).
9. A. Kontogianni, V. Skouridou, V. Sereti, H. Stamatis and F.N. Kolisis, *J. Mol. Catal. B: Enzym.*, **21**, 59 (2003).
10. N. Nakajima, K. Ishihara, H. Hamada, S. Kawabe and T. Furuya, *J. Biosci. Bioeng.*, **90**, 347 (2000).
11. M. Ardhaoui, A. Falcimaigne, S. Ognier, M. Engasser, P. Moussou, G. Pauly and M. Ghoul, *J. Biotechnol.*, **110**, 265 (2004).
12. E.W. Tan and B.Q. Chen, *CIESC J.*, **7**, 1685 (2010).
13. J.J. Zhao and D. Li, *Chem. Ind. Eng. Progress*, **9**, 1311 (2007).
14. R. Zheng and Z.Y. Deng, *J. Nucl. Agric. Sci.*, **6**, 602 (2007).