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Ultrasound-promoted enzymatic synthesis of troxerutin esters in nonaqueous solvents

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ABSTRACT

Comparative studies of enzymatic acylation of troxerutin by the alkaline protease from *Bacillus subtilis* under ultrasound and shaking were carried out in nonaqueous media. Using divinyl dicarboxylates (CH_2=CH–OOC–$(CH_2)_n$–COO–CH=CH_2, n = 2, 3, 4, 7, 8, 11) featuring different chain length as acyl donors, troxerutin was regioselectively acylated at B ethoxyl group, whether under ultrasound or shaking. Ultrasonic treatment increased the reaction rate and led to high conversion. Several factors, such as pre-irradiation on the enzyme, the power and frequency of the ultrasound, operation manner, as well as the length of the acyl donors were investigated. Using enzyme pre-irradiated for 8 h, the conversion of troxerutin was increased to 87.3% compared with 56.3% obtained from the untreated enzyme. Experimental results also showed that continual ultrasound caused greater rate acceleration than interval ultrasound. Powers of 100, 150 and 200 W, frequencies of 40, 80 and 100 kHz all showed significant improvement on the transesterification, with the greatest effect observed at 150 W, 80 kHz. The acceleration effect increased as the chain length of the acyl donors decreased from C_{13} to C_4.

1. Introduction

As an environmentally benign and convenient method, ultrasound has been widely used in the field of chemical synthesis, including materials science, food chemistry and pharmaceutical chemistry [1–6]. Based on the phenomenon of cavitation, ultrasound can accelerate chemical transformation, affect product distribution, improve yields and produce novel chemical reactions compared with traditional methods. However, its application in enzymatic reactions has been less extensively explored [7,8]. Ribeiro et al. reported that ultrasound accelerated the enzymatic resolutions of ethyl 3-hydroxy-3-phenylpropanoate without altering the reaction enantioselectivity and the enzyme activity [9]. Using ultrasonication in the lipase Amano PS-catalyzed acylation of 1,2-azido alcohols with vinyl acetate, the rates of acyl transfer increased to 3.5–10 times compared with magnetic stirring [10]. Other successful examples include ultrasound accelerated hydrolysis of ester catalyzed by Porcine pancreatic lipase [11], enzymatic synthesis of glucose esters [12] and acylation of KGM by Novozym 435 [13].

Flavonoids are widely used in food, cosmetics, medicines and various commodity preparations [14] due to their antioxidant, antimicrobial, anti-inflammatory, anti-tumor and anti-angiogenic activities [15,16], while their application is still limited by their low stability and solubility in lipophilic or hydrophilic media [17]. Due to the high specificity and selectivity of biocatalysts, many groups have studied the derivatization of flavonoids by enzymatic methods to improve their stability or modify the solubility [18–22]. Examples include enzymatic esterification of rutin and naringin with unsaturated fatty acids [23], regioselective acylation of aglycone flavonoids (quercetin, naringenin, hesperetin, and chrysin) and flavonoid glycosides (naringin and isoquercetin) catalyzed by Pseudomonas cepacea lipase (PSL-C) [24] and Candida antarctica lipase B [19], respectively.

To improve the reaction of enzymatic flavonoids derivatization, there have been many reports on the modification of the reaction media. Katsoura et al. performed the lipase-catalyzed selective acylation of flavonoids in ionic liquids, finding that the results were significantly better than those obtained from conventional organic media [25]. However, to the best of our knowledge, the manner used in most enzymatic acylation of flavonoids is traditional stirring, and no investigation of the ultrasonic acceleration on the reaction has been reported. In this work, we evaluated the influence of ultrasound on the transesterification of troxerutin and divinyl dicarboxylates in organic solvents catalyzed by alkaline protease from *Bacillus subtilis*, and synthesized a series of troxerutin esters. To evaluate the effect of ultrasound in the reaction, a comparative study was made between ultrasound and shaking. Meanwhile, we investigated the acceleration effect of other factors,

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including the operational manner, the power (100, 150 and 200 W), the frequency (40, 80, and 100 kHz) of the ultrasound irradiation and the chain length of the acyl donors.

2. Materials and methods

2.1. Materials

Alkaline protease from *B. subtilis* was obtained from Wuxi Xuemei Enzyme Co. Ltd (Wuxi, P.R. China). Troxerutin was provided by Sichuan Yabao Guangtai Pharmaceutical Co. Ltd. (Sichuan P.R. China). All other chemicals were of the highest purity commercially available. Divinyl carboxylates were prepared and purified as described by the patent [26].

2.2. Analytical methods

The acylation position of the product was established by ^{1}H NMR and ^{13}C NMR (Bruker Advance DMX 500). DMSO-d_6 was used as the solvent and the chemical shifts were expressed in ppm with reference to Me_4Si, coupling constants (J) in Hz. Mass spectra were obtained on a Bruker Esquire-LC instrument for electro-spray (ESI-MS) measurements (solvent: methanol). Qualitative analysis of troxerutin ester was performed by TLC on silica using ethyl acetate:methanol:H_2O (15:3.6:1, by vol.) as eluent.

Quantitative analysis of troxerutin and troxerutin esters was determined by HPLC. The troxerutin esters for HPLC external standard were prepared and purified by column chromatography (purity > 98%). The analysis of the transesterification was performed by HPLC using a Lichrospher RP 18 column and an absorbance UV detector (λ_{max} = 350 nm). The flow rate was 1 mL/min. Linear gradient for 15 min from 50% to 80% methanol in water was employed (2.60 min (troxerutin), 7.78 min (2a), 7.70 min (2b), 7.16 min (2c), 8.52 min (2d), 7.32 min (2e), 7.93 min (2f)). All experiments were carried out in duplicate.

2.3. Ultrasonic treatment

Sonication was performed using a Jiangsu Kunshan KQ-200VDE ultrasonic cleaner with three frequencies of 40/80/100 kHz and 200 W output power which can be adjusted. The 250 mL reaction flask was located in the ultrasonic bath, with the surface of the reactants slightly lower than the level of the water. The temperature of the water in the ultrasonic cleaner can be adjusted within a range from 20 to 80 °C and maintained within ±1 °C.

2.4. Ultrasonic pre-irradiation on alkaline protease from B. subtilis

0.5 g alkaline protease from *B. subtilis* was dispersed in 100 mL pyridine and the suspension was put in an ultrasound bath of 100 W at 50 °C for a fixed time (1, 2, 4, 8 and 12 h, respectively) before 0.7 g troxerutin and 0.66 g divinyl succinate were added. Then the mixture was kept shaking for 12 h. Aliquots (0.2 mL) were transferred at timed intervals and stored at −20 °C for later HPLC analysis.

2.5. Transesterification reactions

To initiate the reaction, 2.5 g alkaline protease from *Bacillus subtilis* was added to a mixture of troxerutin (2.8 g, 3.77 mmol) and divinyl dicarboxylate (15 mmol) in pyridine (100 mL). The reaction was carried out with shaking at 250 rpm and in an ultrasound bath at 50 °C, respectively. Aliquots (0.2 mL) were transferred at timed intervals and stored at −20 °C for later HPLC analysis. The reaction was terminated by filtering off the enzymes. The filtrates were concentrated under reduced pressure and the products were obtained by column chromatography purification using ethyl acetate:methanol:H_2O (15:3.6:0.5 by vol.) as eluent.

2.6. Vinylsuccinyl-troxerutin (2a)

Yellow power (yield: 24.8%), R_f 0.31; ^{1}H NMR (DMSO-d_6), δ (ppm): 12.49 (s, 1 H, OH_5), 7.84 (s, 1 H, $H_{2'}$), 7.77 (d, 1 H, J = 7.2 Hz, $H_{6'}$), 7.21 (dd, 1 H, J = 6.27 Hz, J = 14.05 Hz, –OCH=), 7.14 (d, 1 H, J = 7.7 Hz, $H_{5'}$), 6.73 (s, 1 H, H_8), 6.38 (s, 1 H, H_6), 5.34 (d, 1 H, J = 7.3 Hz, $H_{1''}$), 4.88 (m, 1 H, OCH=CH_2), 4.65 (m, 1 H, OCH=CH_2), 4.40 (m, 3 H, 2 H of A acylated, 1 H of B acylated), 4.30 (m, 1 H, $H_{1'''}$), 4.26 (m, 1 H, H of B acylated), 4.12–4.06 (m, 4 H, H of A), 3.75 (m, 4 H, H of B), 3.7–3.1 (10 H, H of rhamnoglucosyl), 2.68 (m, 4 H, -CH_2-CH_2-), 0.96 (d, 3 H, J = 6.2 Hz, CH_3 of rhamnosyl); ^{13}C NMR (DMSO-d_6): 177.9 (C-4), 165.1 (C-7), 161.3 (C-9), 156.9 (C-5), 156.9 (C-2), 151.5 (C-4′), 148.2 (C-3′), 134.2 (C-3), 123.8 (C-1′), 123.0 (C-6′), 113.9 (C-5′), 113.6 (C-2′), 105.5 (C-10), 101.8 (C-1″), 101.4 (C-1‴), 98.9 (C-6), 93.4 (C-8), 76.8 (C-3″), 76.4 (C-5″), 74.6 (C-2″), 72.2 (C-4‴), 71.1 (C-3‴), 71.1 (C-2‴), 70.9 (C-4″), 70.8 (C-A), 70.6 (C-A), 68.7 (C-5‴), 67.6 (C-6″), 67.24 (C-A), 63.2 (C-B), 60.0 (C-B), 59.8 (C-B), 18.1 (C-6‴), 173.3 (C=O), 170.0 (C=O), 141.6 (OCH=CH_2), 98.7 (OCH=CH_2), 28.8 ($(CH_2)n$), 28.7 ($(CH_2)n$); IR (KBr): 3411 cm^{-1} (OH), 1745 cm^{-1} (C=O), 1646 cm^{-1} (C=C); ESI–MS (m/z): 891.2 $(M + Na)^+$.

2.6.1. Vinylglutaryl -troxerutin (2b)

Yellow power (yield: 33.1%), R_f 0.32; ^{1}H NMR (DMSO-d_6), δ (ppm): 12.49 (s, 1 H, OH_5), 7.85 (s, 1 H, $H_{2'}$), 7.74 (d, 1 H, J = 7.2 Hz, $H_{6'}$), 7.22 (dd, 1 H, J = 6.4 Hz, J = 14.08 Hz, -OCH=), 7.17 (d, 1 H, J = 6.6 Hz, $H_{5'}$), 6.75 (s, 1 H, H_8), 6.38 (s, 1 H, H_6), 5.35(d, 1 H, J = 7.6 Hz, $H_{1''}$), 4.89 (m, 1 H, OCH=CH_2), 4.65 (m, 1 H, OCH=CH_2), 4.47 (m, 3 H, 2 H of A acylated, 1 H of B acylated), 4.32 (m, 1 H, $H_{1'''}$), 4.26 (m, 1 H, H of B acylated), 4.12–4.05 (m, 4 H, H of A), 3.75 (m, 4 H, H of B), 3.71–3.04 (10 H, H of rhamnoglucosyl), 2.47 (m, 2 H, –CH_2–COOCH=CH_2), 2.26 (m, 2 H, –CH_2–COO-troxerutin), 1.80 (m, 2 H, other CH_2 of glutaridioyl part), 0.97 (d, 3 H, J = 6.2 Hz, CH_3 of rhamnosyl); ^{13}C NMR (DMSO-d_6): 177.9 (C-4), 165.1 (C-7), 161.3 (C-9), 156.9 (C-5), 156.1 (C-2), 150.7 (C-4′), 148.1 (C-3′), 134.2 (C-3), 123.4 (C-1′), 123.0 (C-6′), 114.9 (C-5′), 113.5 (C-2′), 105.5 (C-10), 101.7 (C-1″), 101.4 (C-1‴), 98.9 (C-6), 93.3 (C-8), 76.8 (C-3″), 76.4 (C-5″), 74.6 (C-2″), 72.1 (C-4‴), 71.0 (C-3‴), 71.0 (C-2‴), 70.9 (C-4″), 70.8 (C-A), 70.6 (C-A), 68.4 (C-5‴), 67.4 (C-6″), 67.2 (C-A), 62.9 (C-B), 60.0 (C-B), 59.7 (C-B), 18.15 (C-6‴), 172.9 (C=O), 170.4 (C=O), 141.6 (OCH=CH_2), 98.6 (OCH=CH_2), 33.4 ((CH_2)n), 32.8 ($(CH_2)n$), 32.5 ($(CH_2)n$); IR(KBr): 3385 cm^{-1} (OH), 1732 cm^{-1} (C=O), 1645 cm^{-1} (C=C); ESI–MS (m/z): 905.1 $(M + Na)^+$.

2.6.2. Vinyladipoyl-troxerutin (**2c**)

Yellow power (yield: 18.5%), R_f 0.33; ^{1}H NMR (DMSO-d_6), δ (ppm): 12.49 (s, 1 H, OH_5), 7.84 (s, 1 H, $H_{2'}$), 7.73 (d, 1 H, J = 7.2 Hz, $H_{6'}$), 7.20 (dd, 1 H, J = 6.24 Hz, J = 14.08 Hz, -OCH=), 7.14 (d, 1 H, J = 7.6 Hz, $H_{5'}$), 6.73 (s, 1 H, H_8), 6.38 (s, 1 H, H_6), 5.34 (d, 1 H, J = 7.3 Hz, $H_{1''}$), 4.89 (m, 1 H, OCH = CH_2), 4.64 (m, 1 H, OCH = CH_2), 4.40 (m, 3 H, 2 H of A acylated, 1 H of B acylated), 4.31 (m, 1 H, $H_{1'''}$), 4.26 (m, 1 H, H of B acylated), 4.12–4.06 (m, 4 H, H of A), 3.74 (m, 4 H, H of B), 3.7–3.1 (10 H, H of rhamnoglucosyl), 2.44 (m, 2 H, -CH_2-COOCH=CH_2), 2.38 (m, 2 H, -CH_2-COO-troxerutin), 1.58 (m, 4 H, other CH_2 of hexanedioyl part), 0.99 (d, 3 H, J = 6.2 Hz, CH_3 of rhamnosyl); ^{13}C NMR (DMSO-d_6): 178.1 (C-4), 165.2 (C-7), 161.4 (C-9), 157.0 (C-5), 157.0 (C-2), 151.7 (C-4′), 148.3 (C-3′), 134.4 (C-3), 123.8 (C-1′), 123.0 (C-6′), 115.6 (C-5′), 113.7 (C-2′), 105.7 (C-10), 101.9 (C-1″), 101.4 (C-1‴), 99.0 (C-6), 93.5 (C-8), 76.9 (C-3″), 76.5 (C-5″), 74.7(C-2″), 72.3 (C-4‴), 71.3 (C-3‴), 71.1 (C-2‴), 71.0 (C-4″), 70.9 (C-A), 70.7 (C-A), 68.8

(C-5‴), 67.6 (C-6″), 67.4 (C-A), 63.0 (C-B), 60.1 (C-B), 59.9 (C-B), 18.3 (C-6‴), 173.3 (C=O), 170.8 (C=O), 141.8 (OCH=CH_2), 98.6 (OCH=CH_2), 33.6 ((CH_2)n), 33.2 ((CH_2)*n*), 24.3 ((CH_2)*n*); IR (KBr): 3412 cm^{-1} (OH), 1726 cm^{-1} (C=O), 1648 cm^{-1} (C=C); ESI–MS (*m*/*z*): 919.1 $(M + Na)^+$.

2.6.3. Vinylnonoanedioyl-troxerutin (2d)

Yellow power (yield: 15.8%), R_f 0.34; ^1H NMR (DMSO-d_6), δ (ppm): 12.50 (s, 1 H, OH_5), 7.85 (s, 1 H, $H_{2'}$), 7.75 (d, 1 H, *J* = 8.4 Hz, $H_{6'}$), 7.21 (dd, 1 H, *J* = 6.0 Hz, *J* = 13.8 Hz, -OCH=), 7.15 (d, 1 H, *J* = 8.4 Hz, $H_{5'}$), 6.75 (s, 1 H, H_8), 6.38 (s, 1 H, H_6), 5.41 (d, 1 H, *J* = 10.0 Hz, $H_{1''}$), 4.91 (m, 1 H, OCH=CH_2), 4.61 (m, 1 H, OCH=CH_2), 4.41 (m, 3 H, 2 H of A acylated, 1 H of B acylated), 4.32 (m, 1 H, $H_{1'''}$), 4.26 (m, 1 H, H of B acylated), 4.12–4.06 (m, 4 H, H of A), 3.75 (m, 4 H, H of B), 3.71–3.00 (10 H, H of rhamnoglucosyl), 2.39 (t, 2 H, *J* = 7.2 Hz, -CH_2-COOCH=CH_2), 2.34 (t, 2 H, *J* = 7.2 Hz, -CH_2-COO-troxerutin), 1.52, 1.25 (m, 10 H, other CH_2 of nonanedioyl part), 0.99 (d, 3 H, *J* = 6.2 Hz, CH_3 of rhamnosyl); ^{13}C NMR (DMSO-d_6): 177.9 (C-4), 165.1 (C-7), 161.3 (C-9), 156.9 (C-5), 156.9 (C-2), 151.4 (C-4′), 148.0 (C-3′), 134.2 (C-3), 122.8 (C-1′), 122.7 (C-6′), 115.3 (C-5′), 115.1 (C-2′), 105.5 (C-10), 101.8 (C-1″), 101.3 (C-1‴), 98.8 (C-6), 93.3 (C-8), 76.8 (C-3″), 76.4 (C-5″), 74.6 (C-2″), 72.1 (C-4‴), 71.0 (C-3‴), 71.0 (C-2‴), 70.9 (C-4″), 70.8 (C-A), 70.6 (C-A), 68.7 (C-5‴), 67.4 (C-6″), 67.2 (C-A), 62.8 (C-B), 60.0 (C-B), 59.7 (C-B), 18.1 (C-6‴), 173.4 (C=O), 170.8 (C=O), 141.6 (OCH=CH_2), 98.4 (OCH=CH_2), 33.8 ((CH_2)n), 33.4 ((CH_2)n), 24.7 ((CH_2)n); IR (KBr): 3377 cm^{-1} (OH), 1735 cm^{-1} (C=O), 1647 cm^{-1} (C=C); ESI–MS (*m*/*z*): 961.3 $(M + Na)^+$.

2.6.4. Vinylsebacoyl-troxerutin (**2e**)

Yellow power (yield: 16.5%), R_f 0.35; ^1H NMR (DMSO-d_6), δ (ppm): 12.49 (s, 1 H, OH_5), 7.84 (s, 1 H, $H_{2'}$), 7.72 (d, 1 H, *J* = 7.0 Hz, $H_{6'}$), 7.20 (dd, 1 H, *J* = 6.21 Hz, *J* = 14.04 Hz, –OCH=), 7.14 (d, 1 H, *J* = 7.4 Hz, $H_{5'}$), 6.73 (s, 1 H, H_8), 6.38 (s, 1 H, H_6), 5.34 (d, 1 H, *J* = 7.3 Hz, $H_{1''}$), 4.89 (m, 1 H, OCH=CH_2), 4.63 (m, 1 H, OCH=CH_2), 4.39 (m, 3 H, 2 H of A acylated, 1 H of B acylated), 4.31 (m, 1 H, $H_{1'''}$), 4.26 (m, 1 H, H of B acylated), 4.12–4.06 (m, 4 H, H of A), 3.74 (m, 4 H, H of B), 3.7–3.1 (10 H, H of rhamnoglucosyl), 2.40 (t, 2 H, *J* = 7.2 Hz, –CH_2–COOCH=CH_2), 2.33 (t, 2 H, *J* = 6.9 Hz, –CH_2–COO–troxerutin), 1.58, 1.22 (m, 12 H, other CH_2 of sebacoyl part), 0.99 (d, 3 H, *J* = 6.2 Hz, CH_3 of rhamnosyl); ^{13}C NMR (DMSO-d_6): 177.9 (C-4), 165.1 (C-7), 161.3 (C-9), 156.9 (C-5), 156.9 (C-2) 151.5 (C-4′), 148.1 (C-3′), 134.3 (C-3) 123.7 (C-1′), 123.1 (C-6′), 115.6 (C-5′) 113.8 (C-2′), 105.5 (C-10), 101.8 (C-1″), 101.4 (C-1‴), 98.8 (C-6), 93.3 (C-8), 76.8 (C-3″), 76.4 (C-5″), 74.6 (C-2″), 72.2 (C-4‴), 71.1 (C-3‴), 71.1 (C-2‴), 70.9 (C-4″), 70.8 (C-A), 70.6 (C-A), 68.7 (C-5‴), 67.6 (C-6″), 67.2 (C-A), 62.8 (C-B), 60.0 (C-B), 59.8 (C-B), 18.1 (C-6‴), 173.4 (C=O), 170.8 (C=O), 141.7 (OCH=CH_2), 98.4 (OCH=CH_2), 33.8 ((CH_2)n), 28.9 ((CH_2)n), 24.8 ((CH_2)n); IR (KBr): 3410 cm^{-1} (OH), 1728 cm^{-1} (C=O), 1649 cm^{-1} (C=C); ESI–MS (*m*/*z*): 975.2 $(M + Na)^+$.

2.6.5. Vinyltridecandioyl-troxerutin (**2f**)

Yellow power (yield: 10.6%), R_f 0.36; ^1H NMR (DMSO-d_6), δ (ppm): 12.50 (s, 1 H, OH_5), 7.85 (s, 1 H, $H_{2'}$), 7.74 (d, 1 H, *J* = 8.7 Hz, $H_{6'}$), 7.22 (dd, 1 H, *J* = 6.2 Hz, *J* = 14.0 Hz, –OCH=), 7.15 (d, 1 H, *J* = 8.8 Hz, $H_{5'}$), 6.75 (s, 1 H, H_8), 6.38 (s, 1 H, H_6), 5.38 (d, 1 H, *J* = 6.0 Hz, $H_{1''}$), 4.92 (m, 1 H, OCH=CH_2), 4.60 (m, 1 H, OCH=CH_2), 4.42 (m, 3 H, 2 H of A acylated, 1 H of B acylated), 4.32 (m, 1 H, $H_{1'''}$), 4.26 (m, 1 H, H of B acylated), 4.13–4.06 (m, 4 H, H of A), 3.75 (m, 4 H, H of B), 3.70–3.04 (10 H, H of rhamnoglucosyl), 2.41 (m, 2 H, -CH_2-COOCH=CH_2), 2.33 (m, 2 H, -CH_2-COO-troxerutin), 1.52, 1.21 (m, 18 H, other CH_2 of tridecanoyl part), 0.96 (d, 3 H, *J* = 6.2 Hz, CH_3 of rhamnosyl); ^{13}C NMR (DMSO-d_6): 177.9 (C-4), 165.1 (C-7), 161.2 (C-9), 156.9 (C-5), 156.9 (C-2), 150.7 (C-4′), 148.0 (C-3′), 134.2 (C-3), 123.3 (C-1′), 123.0 (C-6′), 115.4 (C-5′), 113.7 (C-2′), 105.4 (C-10), 101.8 (C-1″), 101.4 (C-1‴), 98.8 (C-6), 93.3 (C-8), 76.8 (C-3″), 76.3 (C-5″), 74.6 (C-2″), 72.1 (C-4‴), 71.0 (C-3‴), 71.0 (C-2‴), 70.9 (C-4″), 70.8 (C-A), 70.6 (C-A), 68.7 (C-5‴), 67.6 (C-6″), 67.4 (C-A), 62.9 (C-B), 59.9 (C-B), 59.7 (C-B), 18.1 (C-6‴), 173.4 (C=O), 170.8 (C=O), 141.6 (OCH=CH_2), 98.4 (OCH=CH_2), 33.8 ((CH_2)n), 29.2 ((CH_2)n), 24.8 ((CH_2)n); IR (KBr): 3392 cm^{-1} (OH), 1735 cm^{-1} (C=O),1647 cm^{-1} (C=C); ESI–MS-(*m*/*z*): 1017.3 $(M + Na)^+$.

3. Results and discussion

3.1. Enzymatic acylation of troxerutin

Transesterification of troexrutin with divinyl dicarboxylates occurred in the presence of alkaline protease from *Bacillus subtilis*, affording mono-substituted troxerutin derivatives 2 (Scheme 1). The products obtained were purified by silica gel column chromatography and characterized by ESI–MS, FT-IR, ^1H NMR and ^{13}C NMR. The data were shown in the experimental section.

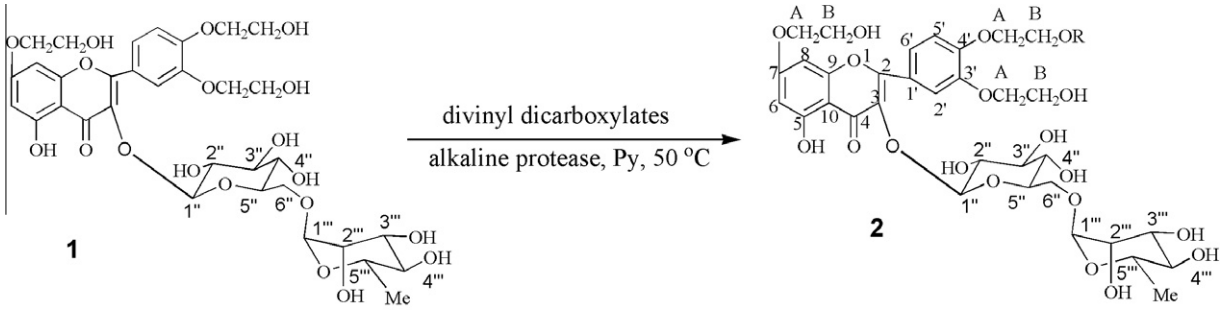
For troxerutin, there are three kinds of hydroxyl groups: phenol hydroxyl, secondary hydroxyl groups in sugar moiety, and primary hydroxyl groups in –OCH_2CH_2OH. The DS (Degree of Substitution) of the products was determined by ^1H NMR and MS. In the ^1H NMR spectrum of 2c, the ratio of proton integration for $(CH_2)_n$ groups and troxerutin showed the product to be mono-substituted ester. The ESI-MS analyses were in accordance with the ^1H NMR results. Similar results were observed in 2a–f.

The position of acylation was determined by ^{13}C NMR. In the ^{13}C NMR spectrum of troxerutin, the ^{13}C NMR signals of C-B appeared at around 60.0 ppm, while C-A appeared at about 70.8 ppm. In the ^{13}C NMR spectrum of transesterification product 2c, one of the signals of C-A was shifted upfield from 70.8 to 67.4 ppm, and one of the signals of C-B was shifted downfield from 60.0 to 63.0 ppm. The chemical shifts of the other resonances did not obviously change. This indicates that the structure of the esterification product should be two [27]. Acyl donors featuring different chain lengths were used for the transesterification of troexrutin and ^{13}C NMR spectra analysis indicated that the transesterification all selectively occurred at the B primary hydroxyl position. The results also showed that the regioselectivity of the enzyme under ultrasound or shaking was the same.

3.2. The effect of ultrasonic pre-irradiation on the activity of alkaline protease from Bacillus subtilis

Figs. 1 and 2 show the influence of ultrasound pre-irradiation on the catalytic activity of alkaline protease from *B. subtilis*. From Fig. 1, it can be seen that ultrasonic pre-irradiation of the enzyme suspension led to an obvious increase of the catalytic activity. The conversion of troxerutin increased with the time prolongation of ultrasonic pre-irradiation. For enzyme pre-irradiated for 1, 2, 4, 6, or 8, conversion of 70%, 74%, 80%, 83% or 87.3% were obtained over 4 h, while untreated enzyme gave 56.3% conversion under the *same conditions.* Consistent with the report of Shah et al. [28], pre-irradiation of the enzyme increased the conversion rate. Meanwhile, Shah et al. found that *oversonication of the sample* gave low conversion, while in our case prolonged enzyme pre-irradiation (10 h, 12 h, or 16 h) did not substantially affect the conversion.

In accordance with Fig. 1, Fig. 2 shows that ultrasonic pre-irradiation of the enzyme suspension increased the yield of transesterification. For enzyme pre-irradiated for 8 h at 100 W, a yield of 44% was obtained over 2 h, while untreated enzyme gave only 17% yield after the same reaction time. Pre-irradiation of 1, 2, 4, 6, or 8 gave the highest yield of mono-substituted troxerutin esters over 4 h reaction, while untreated enzyme afforded the highest



divinyl dicarboxylates: $H_2C{=}HCOOC{-}(CH_2)n{-}COOCH{=}CH_2$ (n=2,3,4,7,8,11)

R: $CO(CH_2)_2COOCH{=}CH_2$ (**2a**); $CO(CH_2)_3COOCH{=}CH_2$ (**2b**); $CO(CH_2)_4COOCH{=}CH_2$ (**2c**); $CO(CH_2)_7COOCH{=}CH_2$ (**2d**); $CO(CH_2)_8COOCH{=}CH_2$ (**2e**); $CO(CH_2)_{11}COOCH{=}CH_2$ (**2f**).

Scheme 1. Enzymatic synthesis of vinyl-troxerutin esters.

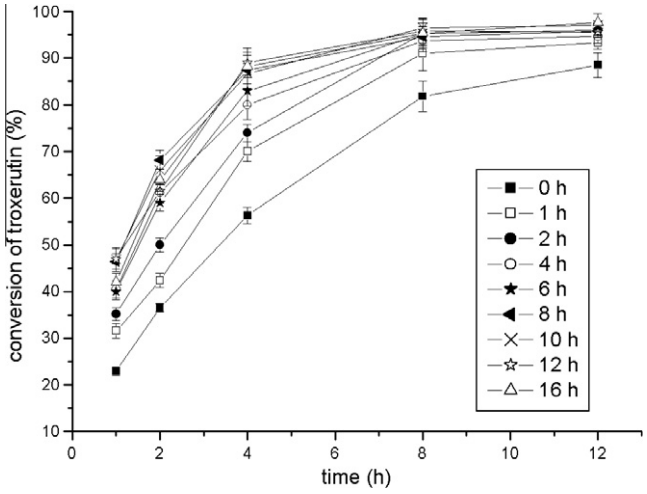


Fig. 1. The effect of ultrasonic pre-irradiation on the enzymatic transesterification of troxerutin with divinylsuccinate.

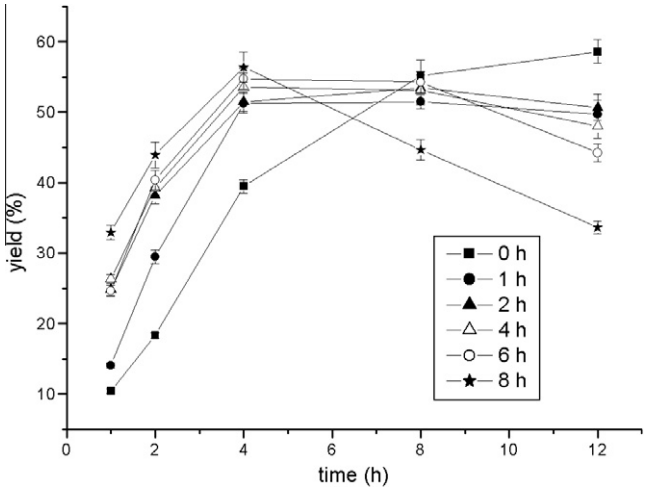


Fig. 2. The effect of ultrasonic pre-irradiation on the enzymatic transesterification of troxerutin with divinylsuccinate.

yield over 8 h. Due to the conversion of mono-substituted troxerutin to multi-substituted troxerutin with the further increase of the reactive time, there is a drop off in the yield of mono-substituted troxerutin ester for the pre-treated sample. The ultrasonic pre-treatment enhanced the activity of enzyme and increased the rate of reaction.

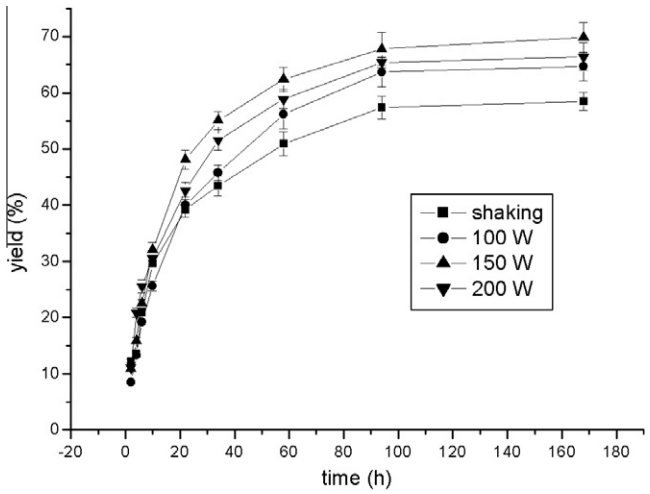


Fig. 3. Influence of continual ultrasound on the enzymatic acylation of troxerutin with divinyl glutarate in pyridine: (-●-) 10/0 min, 100 W continual ultrasound; (-▲-) 10/0 min, 150 W continual ultrasound; (-▼-) 10/0 min, 200 W continual ultrasound; (-■-) shaking.

3.3. Influence of power and intermittence of ultrasound

For reactions under ultrasound irradiation, the operational procedure and ultrasound power were important influencing factors. In this study, we selected three different powers of 40 kHz ultrasound, namely 100, 150, and 200 W, and investigated the effect of the manner in which the untrasound was generated.

Fig. 3 shows the effect of continual ultrasound irradiation on the yield after 168 h. From Fig. 3, it can be seen that the reaction was accelerated markedly with the increase in power output when it was below 150 W, while further increase in power output up to 200 W did not significantly affect the reaction rate. The intensity of the ultrasound irradiation affected the activity of the enzyme [28] and Chen et al. reported Novozym 435 reached its highest activity with an ultrasonic irradiation of 100 W [13]. Enhancement of the enzymatic reaction at low ultrasound intensities was probably due to a decrease in substrate inhibition and aggregation based on hydrogen bonding of molecules, in addition to the activation of enzyme. Sakakibara et al. found that high ultrasound intensities caused partial inactivation of the enzyme [29]. Our research

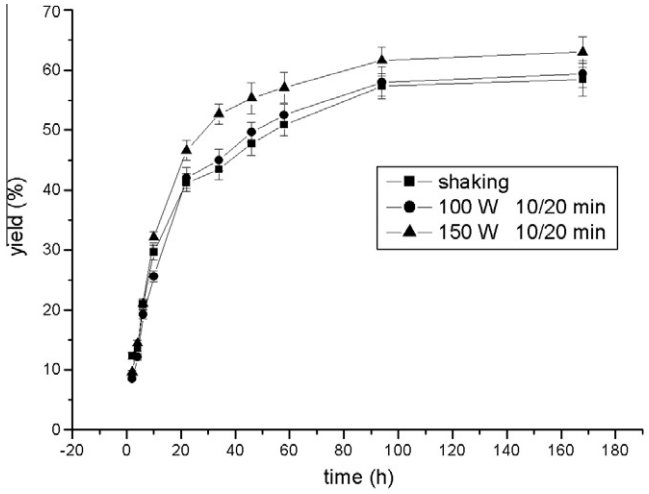


Fig. 4. Influence of intermittent ultrasound on the enzymatic acylation of troxerutin with divinyl glutarate in pyridine: (-●-) 10/20 min, 100 W intermittent ultrasound; (-▲-) 10/20 min, 150 W intermittent ultrasound; (-■-) shaking.

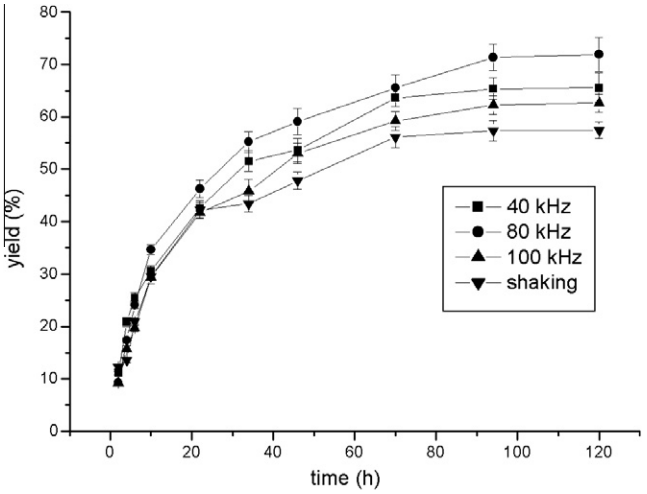


Fig. 5. Influence of the frequency of continual ultrasound on the enzymatic acylation of troxerutin with divinylglutarate in pyridine: (■) 40 kHz, 150 W continual ultrasound; (●) 80 kHz, 150 W continual ultrasound; (▲) 100 kHz, 150 W continual ultrasound; (▼) shaking.

showed no inactivation of the enzyme with 40 kHz ultrasound, and a suitable ultrasound power was necessary.

The effect of interval ultrasound on the yield after 168 h is shown in Fig. 4. Both figures show that ultrasound caused an acceleration of the transesterification, and the yields under continual or interval ultrasound were higher than those of shaking over the same reaction time. Furthermore, it can be observed that continual ultrasound caused greater rate acceleration than interval ultrasound. For example, the yields under continual ultrasound of 150 W were 55.1% after 34 h and 69.9% after 168 h, respectively, whereas the yields were only 52.7% and 63.0% under interval ultrasound (10 min ultrasound/20 min shaking). However, the equilibrium yields under continuous and interval ultrasounds were nearly equivalent, not significantly higher than that of shaking.

The same influence of ultrasound power and operational procedure on the conversion of troxerutin was observed (not shown in this paper). Conversions of troxerutin under three continual ultrasound powers (100, 150, and 200 W) or two interval ultrasound powers (100 and 150 W) were higher than that under shaking. An 86% conversion of troxerutin could be obtained after 168 h in an ultrasound bath (150 W), whereas only 69% was observed in shaking under the same conditions.

3.4. Influence of the frequency of ultrasound

The frequency is an important influencing factor for reactions under ultrasound irradiation, but research on this subject is still poor. Suchkova et al. investigated ultrasound acceleration of enzymatic fibrinolysis at different frequencies and found markedly greater enhancement at midkilohertz frequencies of 27, 40 and 100 kHz [30]. We investigated the enzymatic acylation of troxerutin with vinylglutarate at three different frequencies (40, 80, and 100 kHz) under an output power of 150 W. As shown in Fig. 5, the yields under ultrasound were higher than that of shaking over the same reaction time and the frequency of 80 kHz afforded the highest yield. For example, the yield under continual ultrasound of 80 kHz reached 71.9% after 120 h, whereas the yields were 65.1% at 40 kHz, 61.2% at 100 kHz, and 57.4% under shaking, respectively.

3.5. Influence of chain length of the acyl donors

To study the influence of chain length of the acyl donors on the acylation of troxerutin, we chose six divinyl dicarboxylates (CH_2═CH–OOC–$(CH_2)_n$–COO–CH═CH_2, n = 2, 3, 4, 7, 8, 11) as the acyl donors and studied the influence of continual ultrasound on the reaction.

The comparative results in Figs. 6–8 show that the chain length affected the reaction. Whether under ultrasonication or shaking, continual ultrasound increased the reaction rate and the yields of troxerutin etser significantly. Using divinyldecanedioate as acyl donor, the yields under continual ultrasound was 26.1% after 168 h, whereas the yield was 20% by shaking (Fig. 7). The reaction rate increased with the decrease in chain length of the acyl donor. The yield of the acylation with divinylbutanedioate reached 58.5% after 2 h under ultrasound, while it took 12 h to reach the same yield by shaking. Prolongation of reaction time led to the acylation of other primary hydroxyl groups and resulted in the decrease of the yield of mono-ester (Fig. 6). This is most probably due to the high reactivity of divinylbutanedioate and it might be easier to access the active pocket of the enzyme with decreasing chain length.

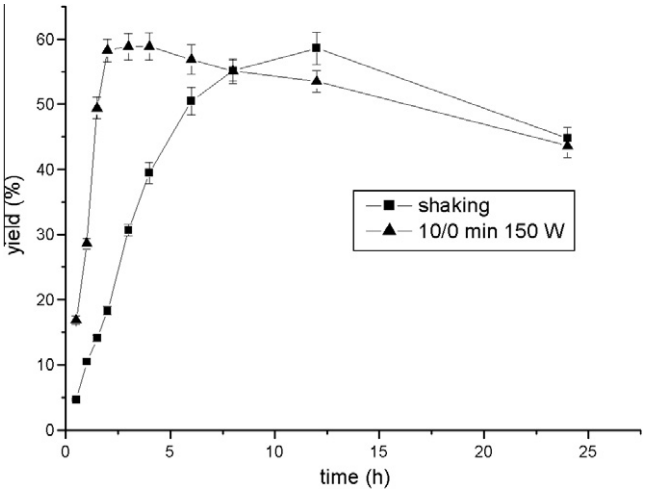


Fig. 6. Ultrasound acceleration of the enzymatic acylation of troxerutin with divinylbutanedioate (n = 2) in pyridine. (▲) 10/0 min, 80 kHz, 150 W continual ultrasound; (■) shaking. Conditions: 0.0037 M troxerutin, 0.015 M divinylbutanedioate, 20 mg/mL alkaline protease from *B. subtilis*, 50 °C, yields determined by HPLC.

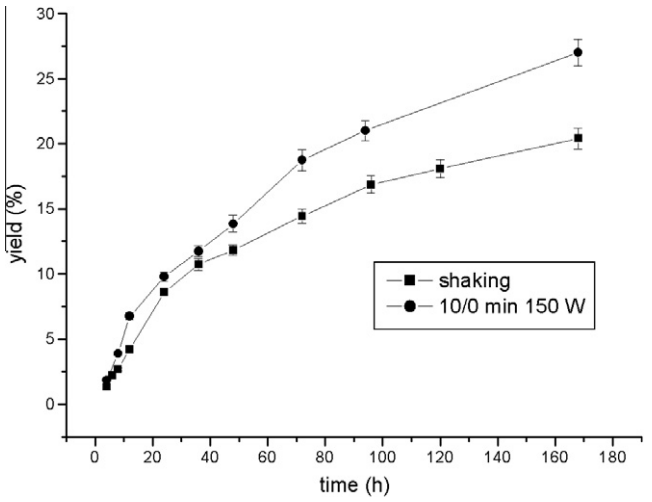


Fig. 7. Ultrasound acceleration of the enzymatic acylation of troxerutin with divinyldecanedioate ($n = 8$) in pyridine. (▲) 10/0 min, 80 kHz, 150 W continual ultrasound; (■) shaking. Conditions: 0.0037 M troxerutin, 0.015 M divinyldecanedioate, 20 mg/mL alkaline protease from *B. subtilis*, 50 °C, yields determined by HPLC.

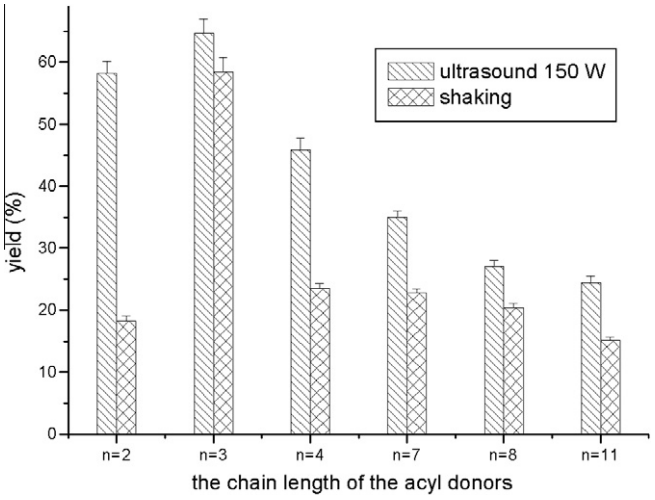


Fig. 8. Influence of chain length of the acyl donors (CH_2=CH–OOC–$(CH_2)_n$–COO–CH=CH_2, $n = 2, 3, 4, 7, 8, 11$) on the synthesis of troxerutin esters. Conditions: 0.0037 M troxerutin, 0.015 M acyl donors, 20 mg/mL alkaline protease from *B. subtilis*, pyridine, 2 h for $n = 2$, 168 h for $n = 3, 4, 7, 8$ and 11.

Some other groups also investigated the relationship between the chain length of the acyl donors and the activity of the enzyme. Ardhaoui et al. studied the effect of acyl donor chain length on the acylation of esculin and rutin catalyzed by Novozym 435 and observed that higher conversion yields were obtained with aliphatic acids having high carbon chain length (>C_{12}) [31]. Pedersen et al. reported that initial reaction rate increased with decreasing chain length of the acyl donor for disaccharides when fatty acids (chain length C_4–C_{12}) were used as donors. The highest initial reaction rate and yield were obtained with the shortest chain-length acyl donor [32]. Chen et al. studied the acceleration effect of ultrasound on Novozym 435-catalyzed acylation of konjac glucomannan, and found a decrease with an increase in the chain length of the acyl donors from C_2 to C_{18} [13]. In our research on the enzymatic synthesis of glucose esters, we observed the same trend [12]. In enzymatic reactions, factors as enzyme, solvent, substrate, acyl donor et al. could all affect the reaction results. This paper showed that application of ultrasound did not change the trend that the time of reaction was shortened with decreasing chain length of the acyl donor.

4. Conclusions

We investigated the regioselective acylation of troxerutin catalyzed by the alkaline protease from *Bacillus subtilis* under ultrasound and shaking, and obtained a series of mono-substituted troxerutin esters. Ultrasonic pre-irradiation or ultrasound irradiation showed a remarkable acceleration on the reaction rate without changing the regioselectivity of the enzymatic acylation. The acceleration effect increased as the chain length of the acyl donors decreased from C_{13} to C_4. The performance of the enzymatic acylation under ultrasound also depended on the power of the ultrasound irradiation, the operational procedure and the frequency of the ultrasound irradiation. From the experimental results presented in this work, it was evident that continual ultrasound caused greater rate acceleration than interval ultrasound, and the optimal power and frequency were 150 W and 80 kHz, respectively.

Acknowledgments

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