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The Enzyme-Catalysed Stereoselective Transesterification of Phenylalanine Derivatives in Supercritical Carbon Dioxide

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The *subtilisin Carlsberg* catalysed transesterification of *N*-acetyl phenylalanine methyl ester (1), *N*-acetyl phenylalanine ethyl ester (2), *N*-trifluoroacetyl phenylalanine methyl ester (3) and *N*-trifluoroacetyl phenylalanine ethyl ester (4) was studied in supercritical carbon dioxide. The water content of the reaction affects the reactivity of the system; for the transesterification of the methyl esters with ethanol the optimum concentration of water was determined to be about 0.74 M, while for the transesterification of the ethyl esters with methanol the optimum concentration of water was about 1.3 M. The conversion is also dependent upon the concentration of alcohol; for ethanol, 2% v/v gives the maximum conversion, whilst for methanol, only 0.8–1.2% v/v is required. This is probably due to a difference in the solubility of the substrates in the two alcohol/supercritical carbon dioxide mixtures. The reaction is highly stereoselective, in all cases no evidence for reaction of the D-isomer could be detected by chiral gas chromatography.

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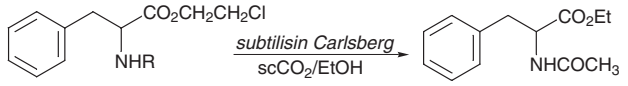
Introduction

Supercritical fluids and, in particular, supercritical carbon dioxide ($scCO_2$), are rapidly becoming 'green' alternatives to organic solvents in chemical processes. The use of supercritical fluids in place of organic solvents for extraction purposes is now an established industrial procedure although the use of supercritical fluids as a solvent for chemical reactions dates back to the 19th century.[1] More recently, supercritical fluids have been used for reactions such as asymmetric hydrogenation,[2] Diels–Alder addition[3] and the Fischer–Tropsch synthesis of wax.[4] The use of supercritical fluids for biocatalytic processes is a relatively new development and was first reported in 1985.[5–7] The solvent properties of supercritical fluids can be readily changed by altering either the temperature or pressure of the system. This ability to tune the solvent makes supercritical fluids attractive media in which to conduct biocatalytic reactions and this is now a rapidly expanding area of research.[8,9] The enzyme-catalysed transesterification reaction is probably the most widely studied enzymatic reaction in supercritical fluids.[1] The selective transesterification of triglycerides in $scCO_2$ is particularly appealing as it can provide a commercially viable pathway for the modification of fats and oils.

Pasta et al. reported the *subtilisin Carlsberg* catalysed transesterification reaction between *N*-acetyl-L-phenylalanine chloroethyl ester and ethanol in supercritical carbon dioxide (Scheme 1).[10] The report clearly demonstrated that



Scheme 1

the supercritical system was superior to similar systems utilizing organic solvents. The effect of temperature and the carbon dioxide/ethanol ratio upon the reaction was investigated, but no comment appeared concerning the stereoselectivity of the reaction in $scCO_2$ or the importance of water to the reaction. The stereospecificity of the *subtilisin Carlsberg* catalysed transesterification of *N*-acetyl-D/L-phenylalanine ethyl ester with methanol has been investigated in supercritical fluoroform[11] but no reports have appeared concerning the stereoselectivity of the reaction in $scCO_2$.

Results and Discussion

In order to examine the stereoselectivity of the *subtilisin Carlsberg* catalysed transesterification of phenylalanine esters in $scCO_2$, four phenylalanine derivatives were prepared: *N*-acetyl phenylalanine methyl ester (1), *N*-acetyl phenylalanine ethyl ester (2), *N*-trifluoroacetyl phenylalanine methyl ester (3) and *N*-trifluoroacetyl phenylalanine ethyl ester (4). The *N*-acetyl and *N*-trifluoroacetyl derivatives were prepared to determine whether the *N*-protecting group affected either the reactivity

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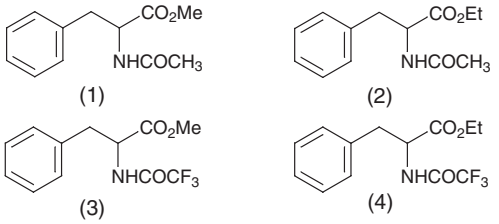
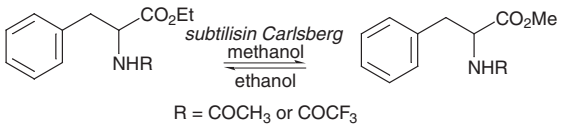


or the stereoselectivity of the reaction. The D, L and D/L derivatives of all four compounds were prepared in moderate to good yields with no loss of chirality as evidenced by chiral gas chromatography.

Two reactions were studied: the transesterification of a phenylalanine methyl ester to the corresponding ethyl ester using ethanol, and the transesterification of a phenylalanine ethyl ester to the corresponding methyl ester using methanol (Scheme 2). The transesterification reactions were carried out using *subtilisin Carlsberg* that had been buffered at pH 7.8 using a phosphate buffer. Reactions involving enzyme preparations, which had been buffered at pH values above or below this, resulted in substantially lower conversions. The enzyme reactions were performed using a batch process in a 15 mL stainless steel reaction vessel containing a magnetic stirring bar. The vessel was pressurized to 1650 psi with dry carbon dioxide and immersed in a water bath at 47°C for 1 h. Longer reaction times had little effect upon the conversion. Gas chromatography was used to measure the extent of reaction and the conversions quoted are an average of at least two repetitions.



Scheme 2

Water Concentration

It has been shown that for enzymatic reactions carried out in non-aqueous media a small amount of water is required for enzyme activity.[12] In the earlier paper by Pasta et al. no comment was made regarding the addition of water to the reaction mixture[10] and we were interested to determine whether water played a role in the activity of this $scCO_2$ system. In order to control the water content of the reaction, the carbon dioxide was passed through a drying tube (4 Å molecular sieves) and the alcohols were dried using established procedures.[13] The optimal water content for the transesterification of the L-phenylalanine derivatives was determined by conducting reactions with varying amounts of added water (Fig. 1). These reactions all contained 2% v/v of the appropriate alcohol.

As expected, little transesterification occurred in the absence of water. As the water content was increased, the conversion increased, reached a maximum and in some cases decreased. A similar relationship between enzyme activity

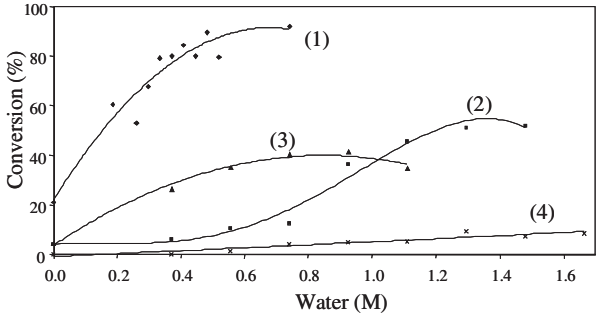


Fig. 1. The effect of water concentration upon the *subtilisin Carlsberg* catalysed transesterification of phenylalanine derivatives in $scCO_2$.

and water content was evident in the same reaction carried out in supercritical fluoroform.[14] The optimal amount of water is dependent upon the alcohol present in the reaction. For the transesterification of the methyl esters with ethanol the optimum concentration of water is about 0.74 M, while for the transesterification of the ethyl esters with methanol the optimum concentration of water is about 1.3 M. This difference is probably due to differences in the solubility of water in $scCO_2$/ethanol and $scCO_2$/methanol mixtures. Hydrophilic solvents tend to strip the essential water layer from the enzyme, consequently more water is required to maintain the appropriate amount of water around the enzyme in a hydrophilic solvent. The maximum water solubility in $scCO_2$ containing 450 mM ethanol at 40°C and 13 MPa is 2.9 g/L.[15] Whilst the maximum water solubility in $scCO_2$/methanol mixtures has not been reported, it would be expected to be higher than for the corresponding ethanol/$scCO_2$ mix, due to the increased hydrophilicity of methanol compared with ethanol. Hence, the optimal water content is higher for the reactions involving the methanol/$scCO_2$ system.

The observation that in some cases the conversion begins to decrease as the water concentration increases is consistent with hydrolysis of the ester to the corresponding carboxylic acid. Miller et al.[16] reported that the rates of inter-esterification of trilaurin and myristic acid, catalysed by a 1,3-specific lipase in $scCO_2$, declined with increasing water content due to hydrolysis of the product to the corresponding acid. For the transesterification of the phenylalanine derivatives in $scCO_2$, no trace of free acid could be detected by gas chromatography after the reaction, indicating that at the level of water used in this study, hydrolysis was not occurring. Once the optimal water level has been reached, increasing amounts of water may precipitate from the solvent leading to a build up of water droplets in the enzyme particles, which form a mass-transfer barrier, and a consequent decrease in reaction rate.[17,18] The precipitation of water from $scCO_2$ is the most likely cause of the drop in reaction rates in the present study.

Alcohol Concentration

Although $scCO_2$ is a non-polar fluid, the solubility of polar substrates in $scCO_2$ can be increased by the addition of a polar organic co-solvent.[19,20] The addition of a co-solvent can

lead to increases in reaction rate, although not always due to increased substrate solubility.[21] In the present study, the alcohol can be considered to be not only the reactant but also a co-solvent to aid in the solubilization of the phenylalanine derivatives in the $scCO_2$. The effect of alcohol content upon the transesterification of the L-phenylalanine derivatives was studied by varying the alcohol concentration from 0.3–2.7% v/v (Fig. 2). The optimum concentration of water as determined in the previous section was used in each of the reactions; i.e. 0.74 or 1.3 M as appropriate. The conversion appears to be dependent upon the concentration of the particular alcohol; for ethanol 2% v/v gives the maximum conversion, whilst for methanol only 0.8–1.2% v/v is required. This suggests that the different degrees of conversion may be due to differing solubilities of the phenylalanine derivatives in the two solvent mixtures.

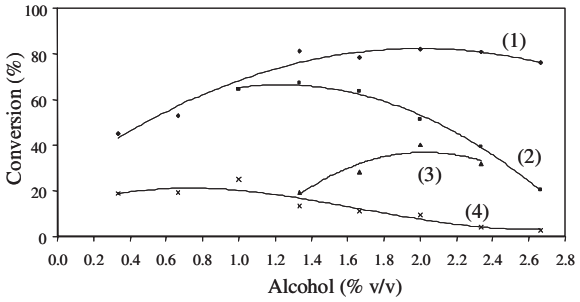


Fig. 2. The effect of alcohol concentration (% v/v) upon the *subtilisin Carlsberg* catalysed transesterification of phenylalanine derivatives in $scCO_2$.

It is also clear that each derivative shows different reactivity in this system, with *N*-acetyl-L-phenylalanine methyl ester (1) giving the best conversion (82%) with 0.74 M water. For the transesterification of the corresponding ethyl ester (2) the conversion is lower (52%) and more water is required (1.3 M). The nature of the protecting group on the nitrogen also influences the rate of transesterification. The *N*-acetyl derivatives are significantly more reactive than the corresponding *N*-trifluoroacetyl compounds.

Stereoselectivity

Enzymes are generally highly stereoselective and they have been shown to retain this property in $scCO_2$.[4] The stereoselectivity of *subtilisin Carlsberg* transesterification reactions has not been studied in $scCO_2$ although it has been shown to be stereoselective in supercritical fluoroform. In order to study the stereoselectivity of the transesterification of phenylalanine derivatives catalysed by *subtilisin Carlsberg* in $scCO_2$, a series of reactions, utilizing the optimum conditions previously determined for each derivative, were carried out using the L, D/L and D phenylalanine derivatives (Table 1). The conversion was measured using conventional gas chromatography whilst the enantiomeric composition of the product was determined using chiral gas chromatography.

The *subtilisin Carlsberg* catalysed transesterification of the phenylalanine derivatives in $scCO_2$ is a highly stereoselective process. The D-isomer does not react at all. When racemic material is added to the reaction only the L-isomer reacts and the extent of conversion is about half of that when pure L-isomer is added.

Table 1. Stereoselectivity of the *subtilisin Carlsberg* catalysed transesterification of phenylalanine derivatives in $scCO_2$

R^1, R^2, R^3	Configuration of starting material	Conversion (%)	Configuration of product (> 99% ee)
R^1 = Me, R^2 = $COCH_3$, R^3 = Et	L	92	L
	D/L	36	L
	D	0	—
R^1 = Me, R^2 = $COCF_3$, R^3 = Et	L	40	L
	D/L	15	L
	D	0	—
R^1 = Et, R^2 = $COCH_3$, R^3 = Me	L	67	L
	D/L	24	L
	D	0	—
R^1 = Et, R^2 = $COCF_3$, R^3 = Me	L	25	L
	D/L	13	L
	D	0	—

Conclusion

The *subtilisin Carlsberg* catalysed transesterification of a number of phenylalanine derivatives has been shown to proceed in a highly stereoselective manner in $scCO_2$; no evidence for reaction of the D-isomer could be detected. The concentration of both water and alcohol in the system is important in determining the optimum reaction conditions. This transesterification reaction in $scCO_2$ complements the previously reported reaction in supercritical fluoroform and highlights the high reactivity and selectivity which can be obtained in supercritical fluids.

Experimental

General

Gas chromatography was performed on a Shimadzu GC-17A with FID; the column was a BP-10 (15 m by 0.25 mm) with a phase thickness of 0.25 μm. For chiral gas chromatography a Chiraldex G-TA column (30 m × 0.25 mm) with a phase thickness of 0.125 μm was used. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded at 300 MHz on a Bruker DPX300 and refer to solutions with tetramethylsilane as the internal reference (δ 0.0 ppm).

Enzyme Preparation

Crude protease (*subtilisin Carlsberg* Type VIII: Bacterial with 14 units/mg solid, purchased from SIGMA–Aldrich) (215.1 mg) was dissolved in pH 7.8 phosphate buffer (2 mL) with gentle agitation. The pH of the solution was readjusted to pH 7.8 by titration with 0.1 M disodium hydrogen phosphate under slow agitation. The solution was frozen in liquid nitrogen and freeze-dried over 4 days. The modified protease (345.0 mg) was stored below 4°C in a sealed vial.

Preparation of Phenylalanine Derivatives

Using a procedure similar to that described by Yamada et al.,[22] borontrifluoride/diethyl ether complex (15 mL) was added to a solution of L-phenylalanine (1.47 g, 8.91 mmol) in methanol (30 mL) and the mixture refluxed for 24 h with an anhydrous calcium carbonate drying tube to exclude moisture. The solvent was then removed under reduced pressure. Dichloromethane (300 mL) was added and saturated potassium carbonate solution was added dropwise under strong agitation until the solution was approximately pH 8 (at that time the colour of the solution changed from dark pink to light yellow). The resultant solid was filtered off and discarded and the dichloromethane layer was separated and dried with magnesium sulfate. Removal of the dichloromethane gave crude L-phenylalanine methyl ester.

The crude L-phenylalanine methyl ester was immediately dissolved in dichloromethane (30 mL). Acetic anhydride (15 mL) was added dropwise under strong agitation and the mixture stirred at room temperature for 24 h. Removal of the solvent under reduced pressure followed by bulb-to-bulb distillation (220–230°C/1 mm) gave *N*-acetyl-L-phenylalanine methyl ester as colourless crystals (1.38 g, 70%) (m.p. 60–61°C, lit.[23] 60–61°C). ^{1}H NMR (300 MHz): δ 2.03, s, 3H, CH_3; 3.11, dd, *J* 6, 14 Hz, 1H, H3; 3.18, dd, *J* 6, 14 Hz, 1H, H3; 3.75, s, 3H, CH_3; 4.91, dt, *J* 6, 7.8 Hz, 1H, H2; 5.95, d, *J* 6 Hz, 1H, NH; 7.1, dd, *J* 1.5, 7.5 Hz, 2H, H2′, H6′; 7.3–7.4, m, 3H, H3′, H4′, H5′. Chiral gas chromatography showed only one enantiomer (> 99% ee).

A similar procedure was used for the preparation of the other derivatives; ethanol or trifluoroacetic anhydride was used in place of methanol or acetic anhydride as appropriate.

N-*Acetyl*-D/L-*phenylalanine methyl ester*. Yield 54%. Chiral gas chromatography showed two peaks in a 1 : 1 ratio indicating that the product was racemic.

N-*Acetyl*-D-*phenylalanine methyl ester*. Yield 60%. Chiral gas chromatography indicated a single enantiomer (> 99% ee).

N-*Acetyl*-L-*phenylalanine ethyl ester*. Yield 41% (m.p. 91–92°C, lit.[24] 92–94°C). ^{1}H NMR (300 MHz): δ 1.25, t, *J* 7.2 Hz, 3H, CH_3; 2.17, s, 3H, CH_3; 3.09, dd, *J* 5.7, 14.1 Hz, 1H, H3; 3.16, dd, *J* 5.7, 14.1 Hz, 1H, H3; 4.17, q, *J* 7.2 Hz, 2H, CH_2; 4.86, dt, *J* 5.7, 7.8 Hz, 1H, H2; 5.89, d, *J* 6.3 Hz, 1H, NH; 7.1, dd, *J* 1.5, 7.2 Hz, 2H, H2′, H6′; 7.24–7.28, m, 3H, H3′, H4′, H5′. Chiral gas chromatography showed one peak only (> 99% ee).

N-*Acetyl*-D/L-*phenylalanine ethyl ester*. Yield 69%. Chiral gas chromatography showed two peaks in a 1 : 1 ratio indicating that the product was racemic.

N-*Acetyl*-D-*phenylalanine ethyl ester*. Yield 64%. Chiral gas chromatography indicated a single enantiomer (> 99% ee).

N-*Trifluoroacetyl*-L-*phenylalanine methyl ester*. Yield 43% (m.p. 51–52°C, lit.[25] 52–53°C). ^{1}H NMR (300 MHz): δ 3.17, dd, *J* 5.4, 14.1 Hz, 1H, H3; 3.24, dd, *J* 5.7, 14.1 Hz, 1H, H3; 3.78, s, 3H, CH_3; 4.88, dt, *J* 5.7, 7.5 Hz, 1H, H2; 6.62, s, 1H, NH; 7.06, dd, *J* 2, 7.5 Hz, 2H, H2′, H6′; 7.25–7.31, m, 3H, H3′, H4′, H5′. Chiral gas chromatography showed only one enantiomer (> 99% ee).

N-*Trifluoroacetyl*-D/L-*phenylalanine methyl ester*. Yield 57%. Chiral gas chromatography showed two peaks in a 1 : 1 ratio indicating that the product was racemic.

N-*Trifluoroacetyl*-D-*phenylalanine methyl ester*. Yield 60%. Chiral gas chromatography indicated a single enantiomer (> 99% ee).

N-*Trifluoroacetyl*-L-*phenylalanine ethyl ester*. Yield 43% (m.p. 56–57°C, lit.[26] 57–58°C). ^{1}H NMR (300 MHz): δ 1.30, t, *J* 7.2 Hz, 3H, CH_3; 3.19, dd, *J* 5.7, 14.1 Hz, 1H, H3; 3.26, dd, *J* 5.7, 14.1 Hz, 1H, H3; 4.25, q, *J* 7.2 Hz, 2H, CH_2; 4.87, dt, *J* 5.7, 7.8 Hz, 1H, H2; 6.78, br s, 1H, NH; 7.1, dd, *J* 2.1, 7.2 Hz, 2H, H2′, H6′; 7.2–7.4, m, 3H, H3′, H4′, H5′. Chiral gas chromatography showed one peak only (> 99% ee).

N-*Trifluoroacetyl*-D/L-*phenylalanine ethyl ester*. Yield 64%. Chiral gas chromatography showed two peaks in a 1 : 1 ratio indicating that the product was racemic.

N-*Trifluoroacetyl*-D-*phenylalanine ethyl ester*. Yield 83%. Chiral gas chromatography indicated a single enantiomer (> 99% ee).

Transesterification Reactions

The following is the general procedure that was used for all of the reactions using $scCO_2$.

N-Acetyl-L-phenylalanine methyl ester (15 mg) and modified protease (10 mg) were placed in a 15 mL stainless steel vessel, which contained a magnetic stirring bar, and dried ethanol (300 μL) and distilled water (200 μL) were added. Liquid carbon dioxide was pumped into the vessel to 1650 psi and the reaction stirred at 47°C for 1 h. The reaction vessel was then cooled in a water–ice mixture (to convert the supercritical carbon dioxide into liquid carbon dioxide), the pressure was carefully released and the gas bubbled through dichloromethane. Dichloromethane was used to wash all parts of the reaction vessel and then filtered. The dichloromethane solutions were combined and subjected to gas chromatography, which showed a mixture of *N*-Acetyl-L-phenylalanine methyl ester and *N*-Acetyl-L-phenylalanine ethyl ester in an 8 : 92 ratio; the two components were identified by comparison with authentic samples. Chiral gas chromatography showed no evidence of the D-enantiomer.

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