COMBI-CLEA FOR REACTIONS IN CONTINUOUS FLOW SUPERCRITICAL CO_2

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Abstract

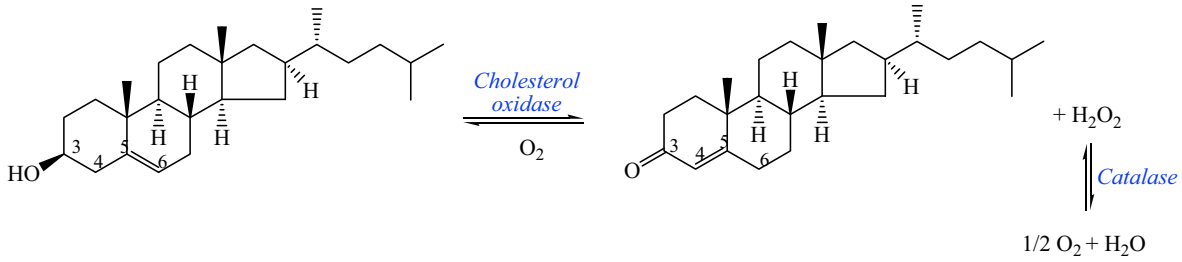
The production of pharmaceuticals in a 'green' way is a concern in the recent years. In this work, we have examined an oxidation reaction in a continuous flow supercritical CO_2 system using individual CLEA (Cross Linked Enzyme Aggregate) of cholesterol oxidase and combi-CLEA of cholesterol oxidase and catalase. Besides the activity studies, the research was aimed to be extended to kinetic studies and modelling of the system to optimise the flow of H_2O_2 between the two enzymes and the production of cholestenone. The reaction examined is the oxidation of cholesterol (Scheme 1). Cholesterol oxidase (chox) catalyses the oxidation of cholesterol using O_2 to 4-cholesen-3-one, a precursor of interest in the pharmaceutical industry. H_2O_2 is produced as by-product which can be reduced to water and oxygen using the enzyme catalase. The elimination of H_2O_2 is important as it can inhibit the first reaction.

Introduction

Enzymes are catalysts with a great potential due to the fact that they usually operate at mild conditions with high chemo-, regio- and enantioselectivity. Biocatalytic reactions conducted in alternative solvents, such as supercritical CO_2 can provide chemical transformations with a lower environmental impact (1-5).

The reaction of interest in the current study is the enzymatic oxidation of cholesterol. The reaction was investigated via a combi-enzymatic reaction of cholesterol oxidase and catalase. The total output of the coupled reaction is the oxidation product (4-cholesten-3-one), water and oxygen (Scheme 1). The main product, 4-cholesten-3-one, has a fat accummulation-inhibitory effect, it is used as an active component for the prevention of obesity and precursor to contraceptive pill steroid.

The oxidation of cholesterol produces H_2O_2 as a by-product which can inhibit the oxidase. In order to prepare a one-pot enzymatic catalyst, catalase is combined with cholesterol oxidase to decompose H_2O_2 increasing the yield of cholestenone.



Scheme 1. Reaction pathway for the enzymatic oxidation of cholesterol.

Cholesterol oxidase immobilised on glass beads has previously been shown to be active in a continuous flow $scCO_2$ system for the oxidation of cholesterol and also for quantifying cholesterol in food samples (7-10). Both cholesterol oxidase and catalase have been previously immobilised on solid supports, however, their carrier-free immobilisation has not been previously reported. Using the CLEA technique, carrier-free biocatalyst can be prepared and therefore there is only a very small amount of inactive mass carried by the catalyst. In addition, a combi-chox-catalase CLEA has not been reported in the literature. The individual and combi-CLEAs have significant potential to be active in a continuous flow $scCO_2$ according to the success of previously reported investigations.

Carbon dioxide is a well-known green solvent (11). The main specific benefits of using supercritical CO_2 to conduct the oxidation of cholesterol are the high miscibility of $scCO_2$ with O_2 and the solubility of cholesterol in $scCO_2$. This is 40-fold greater than its solubility in water (12-14).

Experimental

Materials

All materials were purchased from common commercial sources. CO_2 (>99.95 %) and O_2 (ultrapure, >99.9 %) were purchased from Carburos Metalicos. Cholesterol (96 %) was purchased from Alfa Aesar, 4-cholesten-3-one (98 + %) was purchased from Acros and 5 cholestene-3-one (98 %) was purchased from Aldrich. Sodium azide (99+ %), sodium borohydride (>96 %) and glutaraldehyde (25 wt %) were bought from Fluka. Dextran polyaldehyde was prepared by us. Dextran from *Leuconostoc mesenteroides* (average mol wt 5M-40M, Sigma) was oxidised using sodium iodine (Montplet and Esteban SL. Barcelona). Cholesterol oxidase from *Nocardia sp.* was purchased for Merck and catalase from Bovine liver was purchased from Sigma. Isopropanol (>99 %) was purchased from Sigma.

Reaction apparatus

A schematic diagram of the reactor and its components can be seen in Figure 1. The data reported here has been obtained from a steady state condition of a continuous experiment.

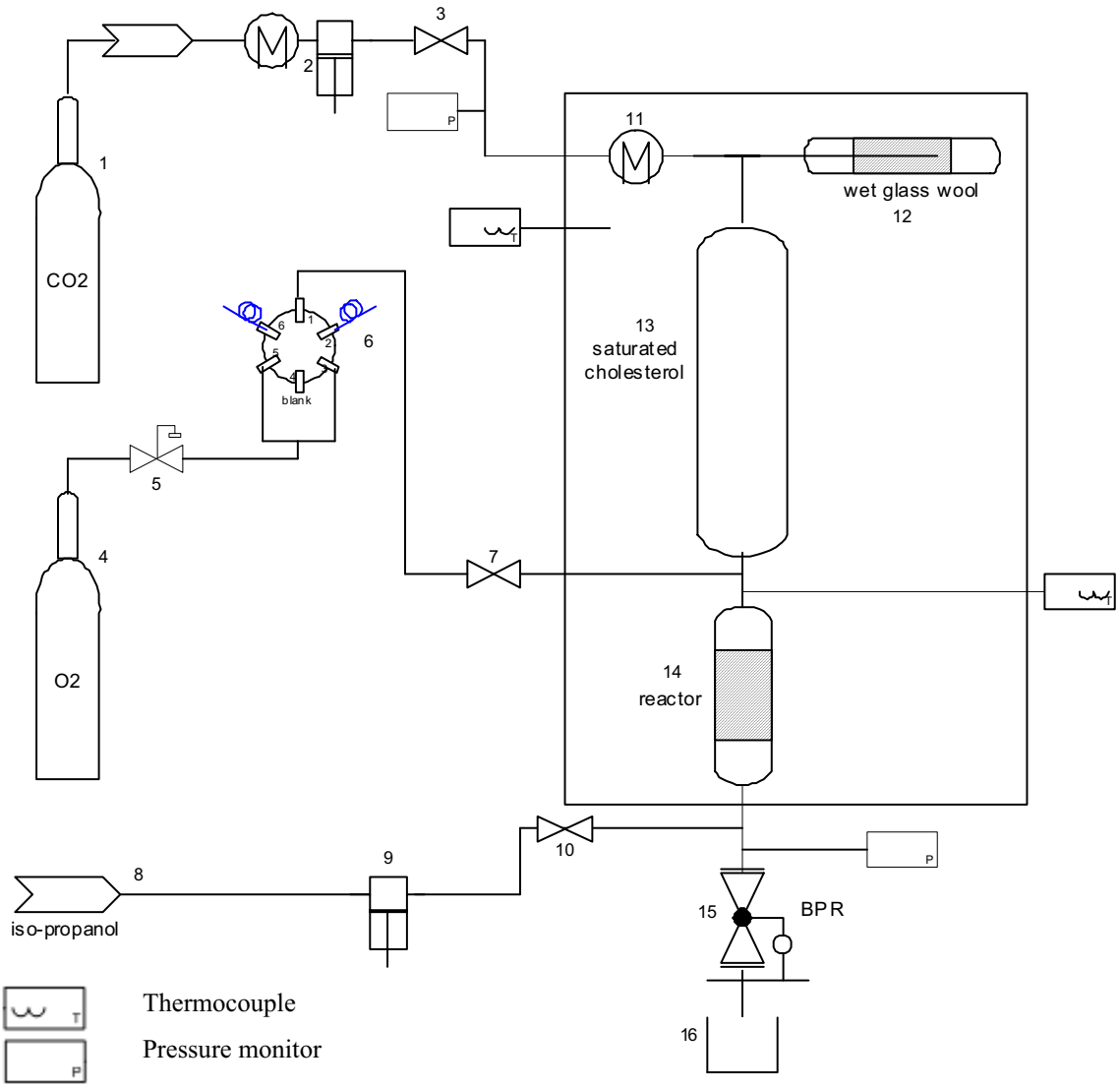
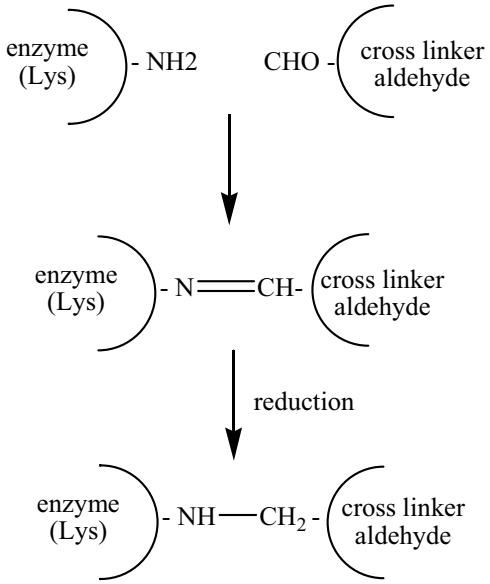


Figure 1. The small scale continuous flow reactor. Parts are labelled as follows: 1: CO_2 cylinder, 2: CO_2 cooling unit and HPLC pump, 3: Parker ball valve, 4: O_2 cylinder, 5: Manual pressure regulator, 6: Waters switching valve for O_2 dosage (two-position six-port switching valve), 7: Parker ball valve, 8: substrate/solvent reservoir, 9: HPLC pump, 10: Parker ball valve, 11: Water bath heater, 12: Vessel for CO_2 saturation with H_2O, 13: Cholesterol vessel, 14: Reactor, 15: GO valve - manual back pressure regulator (BPR), 16: Collection vial. All components are connected by 1/16" 316 stainless steel tubing. The feed streams are mixed in a heated T-piece mixer before being passed through the cholesterol vessel (14.92 cm^3 in volume) and the reactor (0.7 cm^3 in volume). The cholesterol extraction was performed up flow. Products are collected by continuous depressurisation of the reaction mixture using a manual backpressure regulator, and analysed by reverse phase HPLC.

Methods

Preparation of the cross-linked enzyme aggregates (CLEA®s)

Individual CLEAs of cholesterol oxidase (from *Nocardia sp.*) and catalase (from Bovine liver) as well as combi-CLEAs of the two enzymes were prepared. The 'CLEAtion' requires two steps, physical aggregation and cross-linking (Scheme 2). The enzymes needed no special handling. All procedures were performed at 4 °C using cold solvents.



Scheme 2. Reaction of the enzyme's amino group and the cross linker's aldehyde group.

The first step is the precipitation of the enzyme that is commonly performed with organic solvents. The most common precipitants are ethanol, iso-propanol, acetone, tert-butanol, dimethoxyethane (ethyleneglycol dimethyl ether), acetonitrile and ammonium sulfate can be also used as precipitant. The precipitants (except tert-butanol) were all screened for the aggregate formation of cholesterol oxidase and catalase using 1 to 5 ratio (vol) of enzyme aliquot to precipitant. The precipitation was performed using 2.08 mg/mL cholesterol oxidase solution and 50 mg/ml catalase solution in a 5 mM sodium phosphate solution (pH 7). Cholesterol oxidase was used in low concentration due to its high price. 1800 μL cold precipitant was measured in a 2 mL centrifuge tube and 300 μL enzyme solution was added drop wise while an intense stirring. The solution was stirred for 30 minutes and centrifuged afterwards at 3000 rpm for 10 min. The supernatant was decanted and the pellet was redissolved in 1800 μL 5 mM phosphate buffer (pH 7). The enzyme activity was measured in both supernatant and the enzyme aliquot of the redissolved pellet.

The second step of CLEAtion is the cross-linking of the solid enzyme particles. The most commonly used compound is glutaraldehyde, however, other materials are also used e.g. dextran polyaldehyde, polymeric dialdehydes, paraformaldehyde. In this study, glutaraldehyde and dextran polyaldehyde were used as cross-linkers. Glutaraldehyde was used in 10 mM and 50 mM concentrations. Dextran

polyaldehyde (20 % oxidised) was used in 100 μL/mL solution and 200 μl/mL solution concentrations. The cross-linking was performed in the same solution as the precipitation. The cross-linker was added to the suspension in an appropriate concentration while an intense stirring. After 18 hours stirring, the CLEAs were centrifuged at 3000 rpm for 10 min and washed two times with 5 mM phosphate buffer pH 7 (the volume of the phosphate buffer used for the washing was half of the total volume of the suspension) to remove the unreacted cross-linker. The supernatants were decanted and kept for further analysis. The washed CLEAs were resuspended in 5 mM phosphate buffer pH 7 and stored at 4 °C.

In some cases, a reduction step was also included using $NaBH_3CN$ or $NaBH_4$ as reducing agent in 0.5 mg/mL concentration. The cold borohydride solution was added to the suspension of the CLEAs (the phosphate buffer was removed prior to the addition of the solution of the borohydride solution) and stirred for 30 minutes. The CLEAs were centrifuged at 3000 rpm for 10 min and washed twice with 5 mM phosphate buffer pH 7 (the volume of the phosphate buffer used for the washing was half of the total volume). The supernatants were decanted and kept for further analysis. The CLEAs were resuspended in 5 mM phosphate buffer pH 7 and stored at 4 °C.

The combi-CLEAs were prepared using the procedure described above. The enzymes were co-precipitated.

Oxidation of cholesterol in aqueous media

The cholesterol oxidation was performed in 20 mM sodium phosphate buffer (pH 7) containing Triton X-100 surfactant in 0.5 mL/L concentration. The cholesterol solution was prepared in iso-propanol (IPA). Cholesterol oxidase from *Nocardia sp*. was prepared using 100 U (16 U/mg solid) enzyme in 3 mL 5 mM phosphate buffer (pH 7).

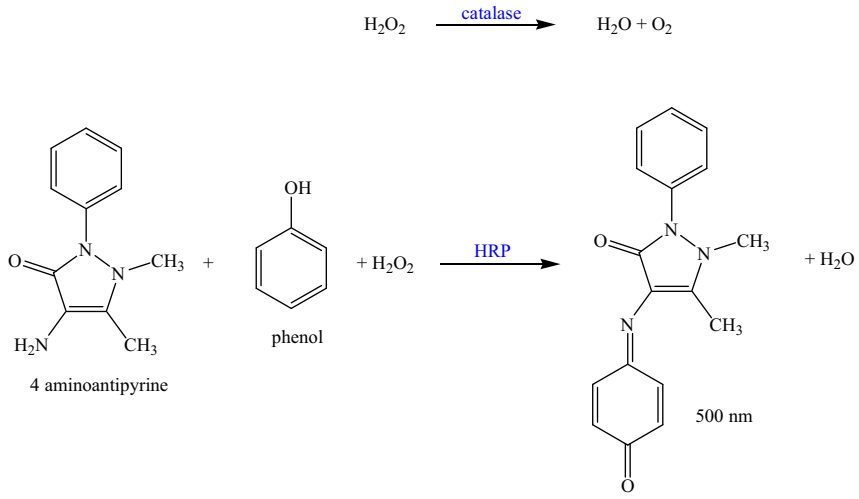
The reaction was carried out in a 100 mL round bottom flask at room temperature using O_2 as the oxidant. The reaction mixture consisted of 27.3 mL buffer solution, 2.5 mL cholesterol solution in IPA and 0.2 mL cholesterol oxidase. The flask was sealed with suba-seals that allowed charging the flask with O_2 using a syringe. The reaction mixture was stirred with a magnetic stirrer bar. The reaction was run up to 5 hours. The samples for the HPLC analysis (described below) were prepared by mixing 750 μl sample and 250 μl 0.2 M NaOH. The NaOH solution effectively quenched the reaction and therefore real time analysis could be performed.

Catalase assay

The catalase activity test - the reduction of H_2O_2 - was performed in 20 mM sodium phosphate buffer (pH 7) containing Triton X-100 in 0.5 mL/L concentration. H_2O_2 was used as received 30 % w/w in water. Catalase aliquot from Bovine liver was prepared in 8.33 mg/mL concentration (2950 U/mg solid) in 5 mM phosphate buffer.

The catalase assay consisted of two steps (Scheme 3). The first reaction was carried out in a 100 mL round bottom flask at room temperature using 0.2 mL enzyme suspension/aliquot. The enzyme was added to a 1 M H_2O_2 solution and incubated for exactly 1 min at room temperature while stirring. Catalase activity was quenched by inhibitor addition (sodium azide). To determine the remaining H_2O_2, a coupling reaction was performed. A dye could be formed by a reaction occurring between 4-aminoantipyrine and phenol catalysed by horseradish peroxidise (HRP). The reaction was performed in a 5 mL vial at 37 °C. The reaction mixture consisted of 0.9 mL 20 mM sodium phosphate buffer

containing Triton X-100, 1 mL HRP reagent, 0.1 mL HRP solution and 3 mL sample. The HRP reagent consisted of a 6.25 mM 4-aminoantipyrine and a 100 mM phenol solutions in 1 : 1 ratio (vol). The activity of the enzyme could be calculated from the absorbance data (measured by UV-VIS spectroscopy at 500 nm) obtained for the second reaction (dye formation) of the assay. The dye formation reaction was used to determine the remaining H_2O_2 in the combi-enzymatic reaction.



Scheme 3. Dye formation. The Dye is formed by a reaction between 4-aminoantipyrine, phenol when H_2O_2 is reduced by horseradish peroxidise (HRP).

High performance liquid chromatography (HPLC)

The oxidation of cholesterol was monitored by HPLC. The difference in polarity of cholesterol, 4-cholesten-3-one and 5-cholesten-3-one allowed the separation. A Waters HPLC instrument equipped with a Waters 717 auto sampler was used. A reverse phase C18 column was used to analyse the samples using 100 % acetonitrile as mobile phase. The column was heated to 60 ºC isotherm. The flow rate of acetonitrile was 0.8 mL/min and a UV detector was used at 210 nm.

Results and Discussion

Enzyme and CLEAs activity tests

The enzyme and the CLEAs were tested before use exhibiting high activity. These results are not included in this paper but some of them will be included in the oral presentation.

Extraction of the cholesterol

Several different reactor setups were tested for the extraction of cholesterol. The reproducibility of cholesterol solubility has not been achieved in most of the cases. Presumably, the reason was the residence time. Anyhow, the objective of this study was to dissolve a quantity of cholesterol in order to perform the reaction subsequently. As the real amount of cholesterol can be know by HPLC analysis the total amount is not a critical variable in this initial stage of the reaction. This needs to be solved for performing a kinetic study.

Oxidation of the cholesterol

The reaction has been performed using an immobilised cholesterol oxidase in a carrier-free form (CLEAs) in a continuous flow of $scCO_2$ system. The oxidation reaction was examined using cholesterol oxidase (as received), cholesterol oxidase CLEAs and combi-chox-catalase CLEAs. The experiments were performed using the reactor setup shown in Figure 1.

The main results for the oxidation are presented in the tables below (Table 1-3). Cholesterol oxidase (300 U chox), cholesterol oxidase CLEA (300 U chox) and combi-chox-catalase-CLEA (200 U chox) were used as catalysts. The yield in the table was calculated as the ratio of the actual yield divided by the maximum theoretical yield in mass.

Table 1. Cholesterol oxidase 300 U chox – yield of 4-cholesten-3-one (%)

CO_2 flow (ml/min)	**40 °C 130 bar**	**40 °C 160 bar**	**50 °C 130 bar**	**50 °C 160 bar**
2 ml/min	< 0.02	< 0.5	0	0
4 ml/min	0	< 0.15	0	0
6 ml/min	< 0.2	< 0.5	0	0
8 ml/min	0	< 0.16	0	0

Table 2. Cholesterol oxidase CLEA 300 U chox – yield of 4-cholesten-3-one (%)

CO_2 flow (ml/min)	40 °C 130 bar	40 °C 160 bar	50 °C 130 bar	50 °C 160 bar
2 ml/min	< 0.2	< 1.16	0	< 0.14
4 ml/min	0	0	< 0.05	< 0.16
6 ml/min	< 0.05	< 0.05	< 0.06	< 0.11
8 ml/min	0	0	< 0.06	< 0.14

Table 3. Combi-CLEA 200 U chox - yield of 4-cholesten-3-one (%)

CO_2 flow (ml/min)	40 °C 130 bar	40 °C 160 bar	50 °C 130 bar	50 °C 160 bar
2 ml/min	< 0.04	< 0.04	na	0
4 ml/min	< 0.03	< 0.02	na	0
6 ml/min	< 0.03	< 0.02	na	0
8 ml/min	< 0.6	0	na	0

Unfortunately, as it can be seen from the results shown in the tables above that the oxidation of cholesterol in a continuous flow $scCO_2$ system exhibited a very low conversion. Although the activities of the enzyme and the CLEAs were high (measured before the use in $scCO_2$), the reaction systems could not convert cholesterol in more than ca.1%.

Cholesterol oxidase CLEA 300 U chox at 160 bar, 40 ºC and 2 mL/min of CO_2 showed the highest conversion (1.16%).

Higher flow rates enhance mass transfer, but a longer reactor is required at the same time in order to keep constant residence time. This positive effect in the enzyme activity was previously reported (8) but in this case has not been demonstrated.

The enzymes may be active in flow $scCO_2$ but it is possible that they require a larger amount of water than it was provided in the experiments reported here (water-saturated CO_2 was used). A different reactor configuration needs to be tested in a further investigation.

Deactivation of the enzymes due to $scCO_2$ could be possible, but high activity was reported previously using immobilised cholesterol oxidase in a continuous flow $scCO_2$ system (7, 9). The possibilities of conformational changes under supercritical conditions and therefore deactivation of the enzyme could be possible. The effect of $scCO_2$ on the conformation of cholesterol oxidase was studied using EPR spectroscopy. There was no significant effect shown using a homogeneous enzyme (15). Here, CLEAs were examined for the reaction that might have been influenced by CO_2. Structural changes might have been caused under high pressure that could deactivate the enzyme. It is also possible that some of the active sites of the enzyme got blocked under high pressure due to compression. Further investigations needs to be performed.

Another possible cause for the low reaction rate could be the residence time (in this system the residence time in the reactor is 3.54 min at 2 mL/min CO_2 flow rate if the whole volume of the reactor

is filled with catalyst). A higher conversion could be possibly obtained by providing a longer reaction time and/or providing a more active enzyme per unit. The reaction was examined using 0.5 and 1 mL/min CO_2 flow rate but the yield of 4-cholesten-3-one was still very low (the yields are not presented here but they were similar to the ones listed in the table above).

Conclusions

Individual CLEAs of cholesterol oxidase and combi-CLEAs of cholesterol oxidase and catalase were successfully prepared with high activity. Differences were seen in the activity of the cholesterol oxidase exhibited in the individual CLEAs and in the combi-CLEAs. It is more likely to be due to mass transport limitation. The large amount of catalase around the cholesterol oxidase may slow down the transport of cholesterol to the active site of the enzyme which can result in slower reaction rate. However, it is also possible that some active site of cholesterol have become blocked during the cross-linking.

The oxidation of cholesterol was performed in continuous flow $scCO_2$ using cholesterol oxidase (as received), cholesterol oxidase CLEAs and combi-chox-catalase CLEAs. Unfortunately, very low yield was obtained in all experiments performed. Further investigation needs to be performed to determine whether: 1) the enzyme needs more water, 2) CO_2 deactivates the enzyme under high pressures or 3) longer residence times are required.

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