Preparation, characterisation and applications of immobilised lipase

Abstract of the Ph.D. Thesis

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Introduction

Lipases are enzymes that catalyse the hydrolysis of the ester bonds of lipids. As important digestive enzymes of oils and fats, lipases are to be found in all living organisms. In terms of application, lipases are highly significant; for example, they have long been used in the dairy industry, and they now also play a leading role in many areas of modern biotechnology. To date, mostly the microbial lipases have been employed with success, and fewer examples are to be found of the application of lipases from the mammalian pancreas. For this reason, I set out to examine porcine pancreas lipase (PPL).

Immobilised enzymes (e. g. attached to a solid support, or locked in membranes) possess numerous advantageous properties relative to the soluble forms. For instance, they can be operated, separated or controlled more easily; and the characteristics or stability of the enzymes are often favourable in consequence of the immobilisation. Enzymes can be attached to a large number of different support materials, and by many different methods. Microbial lipases are employed mainly in immobilised form. The immobilisation method is in most cases adsorption, or deposition, or rarely covalent binding. The characteristics of the support materials (e. g. the size and porosity of the granules, or the hydrophobicity) can vary widely. There are few precedents concerning the examination or application of immobilised mammalian pancreas lipases.

For the choice of the support most suitable for the immobilisation of an enzyme, it is worth trying different materials and coupling methods, and examining their effects on the activity and stability of the enzyme. The reaction conditions of the immobilisation (e. g. the pH, enzyme loading, and so on) can influence both the quantity and activity of the attached lipase. The catalytic properties, specificity and stability of the enzyme can also change because of the immobilisation.

The natural reaction medium for enzyme catalysis is usually aqueous, but enzymes have proved to be catalytically active in alternative media. Organic solvents (also containing a little water) are employed most frequently as alternative reaction media. Recently, enzymatic conversions are frequently performed in these non-conventional

media, in view of their advantages. For example, the solubility of hydrophobic substrates increases, catalysis carried out in an organic medium can be integrated more easily into the steps of a chemical synthesis, the enzyme loss from the reactor or support decreases, and there is less risk of microbial infections. Organic media can also open up new catalytic reaction routes. For example, at a low water content, the equilibrium of the catalysis can change; in this way, hydrolytic reactions can become reversible, which makes the synthesis of different important molecules possible through the use of hydrolytic enzymes.

Carbohydrates esterified with different (long-chain) fatty acids are currently prepared and applied in large quantities, in order to utilise their excellent surfactant properties. The interest in these natural, biodegradable, non-ionic detergents has increased by virtue of their potential industrial applications. Fructose fatty acid esters also belong in this group of detergents, but they can hardly be produced in the conventional chemical way. Any hydroxy group in fructose can react with fatty acids, resulting in a mixture of different esters, which decreases the surface activity of the product, among other disadvantages. The regiospecifity of enzymatic syntheses solves this problem, in addition to the advantage of the mild reaction conditions of enzyme catalysis.

Fructose esters can be produced by lipases (or other esterases) in two different ways: direct esterification, with the use of free fatty acids as acyl donors, or transesterification, when the acyl donor is usually the ester of a fatty acid and an alcohol. Triglycerides are rarely applied for the transesterification of fructose. These promising substrates have the advantage of being available in large quantities in nature, and in addition, triglycerides are applicable directly, without the costly preparation of a substrate (e. g. in the case of fatty acids or their esters).

Prior to the application of PPL in organic media, it is necessary to examine its stability in the solvents. Choice of the organic solvent most advantageous for the enzymatic catalysis must be based on the scanning of different types of solvents, and examination of the effects of the solvent hydrophobicity on the catalysis. The operation and stability of the enzyme can also be influenced decisively by the water content of the reaction medium, just as other reaction conditions (pH, temperature, and the quantity and proportions of the substrates). Immobilised enzymes can also be employed with success in organic media, but since the microenvironment of the enzymes differs basically from that in aqueous media, the effects of the support and the linking methods on the conformational stability and catalytic activity of the enzyme in the non-conventional media need to be reconsidered.

Objectives

1. One of my aims was to produce several forms of immobilised PPL, with the application of different supports and connecting methods. I planned to examine the effects of the immobilisation on the catalytic properties and stability of PPL in an aqueous system, and to compare the applicability of the produced enzyme forms for the hydrolysis of olive oil.

2. I set out to study the influence of different organic solvents on the stability of PPL.

3. Fructose ester synthesis catalysed by PPL in organic solvents was considered as a model reaction. It was also necessary to compare the applicability of PPL forms immobilised on different supports for fructose ester synthesis.

Materials and methods

Measurement of hydrolytic activity of PPL: The hydrolytic activity of PPL was determined by pH-stat titration with previously emulsified olive oil as substrate. The reaction mixture used for activity measurements contained 5 ml of substrate emulsion, 2.5 ml of distilled water and 125 µl of a 20% solution of sodium taurocholate; the pH of the

mixture was adjusted to 8.9 with the use of 0.2 M NaOH (about 300-350 μl of which was needed). With an automatic titrator, the formation of fatty acids was followed at pH 8.9. In the case of the other substrates (triolein, tristearin and tributyrin), the activity measurements were performed under analogous conditions.

Preparation of immobilised PPL: The PPL was covalently bound to polyacrylamide beads (Akrilex C-100) activated by carbodiimide, or to a Silochrome-aldehyde support. The PPL was attached to Sorsilene, Celite and Al_2O_3 by physical adsorption, and to Dowex resin by ionic bonds in addition to the adsorption.

Hydrolysis of olive oil: Hydrolysis was carried out at 37 ^{o}C, and pH 8.9, during continuous stirring (300 rpm). The substrate: 0.15 g of olive oil was mixed previously in 15 ml of distilled water for 1 minute, without the use of an emulsifier, and the reaction was started by the addition of the different PPL forms with an activity of 50 U. The quantity of fatty acids formed during the hydrolysis was determined with a pH-stat titrator.

Stability of PPL in organic solvents: The stability of the PPL was studied in 100 mM Tris/HCl buffer (pH 7) containing organic solvents in different concentrations (between 20 and 80%,v/v). The PPL was incubated at 25 ^{o}C for 120 minutes, and aliquots were then taken, in which the residual activity of the PPL was determined by a standard method.

Synthesis of fructose esters by immobilised and free PPL forms: The reaction mixture contained 150 μmol of fructose and 750 μmol of acyl donor (butyric acid or tributyrin) in 15 ml of acetonitrile. The reaction was initiated by addition of the PPL preparation (immobilised, or freely suspended PPL for comparison) with a hydrolytic activity of 300 U. Synthesis was carried out at 30 ^{o}C, in a thermostated, stirred batch reactor. The water content was 0.6%. 300 μl of reaction mixture was taken from time to time as sample, and analysed by TLC.

Thin-layer chromatography (TLC): The fructose esters formed in the synthesis were isolated on reactivated silica gel plates (Kieselgel-60, Merck) with the use of a mixture of chloroform : methanol : acetic acid : water (80:10:8:2). Plates were sprayed with diphenylamine-aniline-phosphoric acid developer reagent, and the fructose ester spots appeared after heating at 100-120 ^{o}C. Plates were analysed with use of the computer program Gelbase Pro&Gelbase/Gelblot (UVP Ultra Violet Products), which is suitable for the determination of optical density. Fructose esters were identified by means of ^{13}C NMR.

Results

1.

All of the PPL forms (immobilised on aluminium oxide, Celite or Sorsilene by adsorption; on Dowex resin by ionic bonds; and on Akrilex C-100 and Silochrome-aldehyde by covalent linking) were active, with the activity between 28.82 and 1114.84 U/g xerogel. PPL could be attached readily both to hydrophilic (e.g. Akrilex C-100) and hydrophobic (Sorsilen) supports, and consequently the hydrophobicity of the supports exerted only a slight effect on the activity of the immobilised enzyme. As regards the stability of the bound enzyme, the supports that linked PPL strongly proved to be the most appropriate for the immobilisation (e. g. Akrilex C-100 and Sorsilen). Covalently attached Akrilex-PPL provided the best activity and stability too, and for this reason we studied this PPL form in detail.

2.

The thermal stability of Akrilex-PPL was better than that of the soluble PPL: after 60 minutes of incubation at 50 ^{o}C, the soluble form retained 20% of its starting activity, while the residual activity of the immobilised form was 49%. pH 6 was most favourable for the stability of the immobilised PPL: it exhibited sensitivity to alkaline pH. The immobilised PPL was crosslinked by glutaraldehyde or difluoronitrobenzene to improve its resistance to alkaline pH. The crosslinked and immobilised forms exhibited better pH and thermal stability, and also stability against urea, as compared with the immobilised PPL.

3.

Comparisons were made as concerns the substrate specificity of the different PPL forms with the use of triolein, tributyrin and tristearin as substrates. All of the enzyme forms were able to hydrolyse these substrates, but while tributyrin was most favourable for the soluble PPL because of its flexible enzyme structure, the crosslinked and immobilised forms hydrolysed triolein at maximum rate. The dependence of the enzyme activity on the substrate concentration was very similar for the soluble and immobilised forms, and the active PPL molecules are therefore most probably situated on the surface of the granules. The soluble and the immobilised PPL had the same pH optimum, pH 8.9, because the immobilisation did not alter the structure of the active sites of the enzyme. The thermal optima of the enzyme activity were 32-33 ^{o}C, 45 ^{o}C and 47 ^{o}C for the soluble, immobilised and crosslinked-immobilised PPL, respectively. This indicates a more inflexible molecule structure, and at the same time a better thermal stability due to the immobilisation and crosslinking.

4.

The activities of the different PPL forms were compared in the hydrolysis of olive oil. It can be stated that the immobilised and immobilised-crosslinked lipases hydrolysed the olive oil much more quickly and led to greater productivity than for the soluble form.

5.

Prior to the application in organic media, we studied the stability of the PPL in mixtures containing organic solvents and water in different compositions. In dimethyl sulphoxide (DMSO), the PPL retained its activity up to 40% DMSO concentration, but lost its activity at 50-60% DMSO concentration. In 1,4-dioxane, acetonitrile or ethanol, the activity of the PPL changed according to a minimum curve as a function of the organic solvent amount. When the stability of PPL immobilised on Akrilex was examined in these solvents, considerable differences from the stability of soluble PPL were not observed. Water-immiscible organic solvents were also studied: while the activity of the PPL decreased in toluene, in ethyl acetate no decrease of activity was observed.

6.

Fructose butyrate synthesis served as a model reaction for study of the operation of PPL in organic media. Butyric acid (in esterification) or tributyrin (in transesterification) was used as acyl donor for the fructose ester synthesis. In both types of reaction, two products were identified: fructose-1 and 6-butyrate, in a ratio of 0.54:0.46.

7.

Several solvents were used as medium for ester synthesis (*n*-hexane, cyclohexane, toluene, 2-methyl-2-propanol, acetonitrile, DMSO and pyridine), and the more polar solvents, which possessed smaller log P values (e. g. 2-methyl-2-propanol, acetonitrile and pyridine), proved to be advantageous for the synthesis. Acetonitrile was the most suitable for the catalysis.

8.

The effect of the number of carbon atoms (between 1 and 19) in the fatty acids on the ester synthesis was also examined. It was found that the extent of ester production increased with increasing chain length of the fatty acids, up to C13-C17. In the examined fatty acids containing about 10 carbon atoms, unsaturation (the presence of carbon-carbon double bonds) had a positive effect on the PPL-catalysed ester synthesis. Among the substrates employed, the mono-unsaturated undecenic acid yielded the most fructose ester.

9.

Investigation of the influence of the reaction conditions on fructose butyrate synthesis revealed (among others) that PPL demands the presence of only a very small amount of water (<0.3%) during the synthesis in organic media. This feature of PPL can be

advantageous both in esterification and in transesterification. The thermal optimum of the enzyme activity was observed at 40-45 ^{o}C, in contrast with the optimum for the most microbial lipase, which is usually at about 60 ^{o}C. The optimum ratio of fructose to acyl donor (butyric acid or tributyrin) was 1:5; and the pH optimum of the esterification was at 7.4.

10.

For the establishment of practical applicability, fructose ester syntheses with different immobilised or freely suspended PPL forms were compared. For esterification, all of the immobilised forms produced more fructose ester than free lipase. In transesterification, the PPL forms immobilised on Celite and Sorsilene provided the best conversion. In contrast with the observations in aqueous media, the hydrophobicity of the support used for immobilisation had a noticeable influence on the reaction rate and conversion of the enzyme forms in organic media: in esterification the hydrophobic support Sorsilene and in transesterification the less hydrophobic Celite proved to be the most suitable support.

11.

The operational stability of the PPL forms was also investigated. The lipases immobilised covalently on Akrilex, or bound on Celite or Al_2O_3 by adsorption, were the most stable. In organic media (in contrast with the conventional media) binding effects weaker than covalent bonds seemed to be sufficient to retain the stability of the immobilised enzyme forms.

12.

The esterification of fructose (with butyric acid) was compared with the transesterification (with tributyrin) with the application of both immobilised and free PPL forms. It was established that transesterification provided better efficiency than esterification, the difference between the conversion of esterification to transesterification even attaining about one order of magnitude.

To summarise, the series of immobilised lipases that I produced and examined furnish a good selection for application for alternative technical purposes. In aqueous medium Akrilex-lipase was the most suitable enzyme form in hydrolysis, whereas in esterification in organic medium the lipase attached to Sorsilene, and in transesterification the PPL bound to Celite provided the best conversions.

Our results can contribute to a broadening of the technical application of lipases through exploration the characteristics of the examined lipase forms.

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