Enzymatic Transesterification between Sugar Alcohol and Oils

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Summary: Pancreatic lipases from bovine and porcine in crude form were immobilized on phenolic resin and utilized for transesterification reactions between sugar alcohol and various oils in nearly dry pyridine. The enzymatically generated esters of sugar alcohol were purified by column chromatography and identified as primary monoesters by TLC.

Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are ubiquitous biocatalysts whose biological function is to catalyze the hydrolysis of triacylglycerol to yield fatty acids, reformation of fats, synthesis of various classes of glycerides and the optical resolution of chemically synthesized racemic acids or alcohols [1,2]. These catalysts are capable of catalyzing a variety of alternative enzymatic reactions with considerable commercial biotechnological potential including the transesterification reactions in anhydrous non-aqueous systems [3,4], the interesterification of oils and fats to modify the composition of triglyceride mixture [5], the synthesis of esters with application in the food, cosmetic, drug, detergent, paint and other industries or as surfactants [3,6,7] and the synthesis and selective acylation of peptides [8].

Enzyme immobilization often provides a means for easy separation of the catalyst from the reaction mixture and allows its continuous or intermittent use over extended periods of time.

Lipases in immobilized condition from various microbial and animal sources have been performed by different methods [9-11] for various applications. In the present study the authors have prepared phenolic resin in the laboratory, utilized the resins for immobilization of lipases from bovine and porcine pancreas powder by crosslinking with glutaraldehyde. These immobilized enzymes were used for the production of biosurfactants in dry pyridine between sugar alcohol and various plant oils.

Results and Discussion

Yield of the cross-linking procedure

The recovery of porcine and bovine pancreatic lipases incubated with the glutaraldehyde-treated phenolic resins were 43 and 55% respectively. The immobilized enzymes were utilized for about three times without any appreciable change in their activity. The enzymatic activity was completely preserved after three months storage at 4°C.

Transesterification reactions of sugar alcohol with triolein, olive and soybean oils were carried out in anhydrous pyridine using immobilized pancreatic lipases. TLC analysis of the enzymatic reaction products giving a single spot indicated the formation of monoesters of equimolar mixture of sorbitol to oleic acids. ^{13}C-NMR studies [12] of these monoesters formed by porcine pancreatic lipase in soluble form revealed that sorbitol monooleates are acylated at C-1 and C-6 position indicating the selective transesterification reaction acylating only on primary hydroxyl groups. Table-1 shows the acylation of sorbitol by triolein, olive and soybean oils catalyzed by immobilized pancreatic lipases in pyridine.

Transesterification of carboxylic acid with an alcohol in aqueous medium, catalyzed by lipases has a considerable influence, both on the product and substrate. In non-aqueous solvent the situation is completely change [3]. But sugars are insoluble in many of non-aqueous solvents except solvent like pyridine which can dissolve sugar but the reaction rate is slow when used as a solvent with porcine pancreatic lipase catalyzed reaction [13]. Sugar alcohols used nowadays for production of surfactants chemically [14], provides a good alternative and are therefore, used in the present investigation.

Table-1: Sorbitol acylation by triolein, olive and soybean oils catalyzed by immobilized pancreatic lipases in pyridine*.

Oils	Triolein	Olive oil	Soybean oil
Amount of sorbitol (mg)	325	325	325
Yield obtained of sorbitol monoester (mg) by IPPL	32	25	29
Yield obtained of sorbitol monoester (mg) by IBPL.	74	80	123

*Conditions: 325 mg sorbitol + 1.8 ml of triolein, olive and soybean oils + 25 ml of dry pyridine and 250 mg of immobilized bovine and procine pancreatic lipases (IBPL and IPPL) added. Suspension shaken (150 rev min^{-1}) at 40°C for 48 hrs and the product recovered and purified as described in procedure for transesterification.

Oils are triglycerides of saturated and unsaturated fatty acids [15]. In oilve oil the fraction of oleic acid is approximately 83.5% and soybean oil contains oleic acid to about 26%. Porcine pancreatic lipase transfer fatty acid moieties from an oil to a primary hydroxyl group of sorbitol without a striking preference for the nature of the fatty acid and its position in the glycerides but in the case of bovine pancreatic lipase the specificity of transesterification reaction has not yet been reported. Although chemical methods for the production of monoesters of sorbitol and fatty acids exist [16], they are not economically feasible, and the corresponding esters are not commercially available. The industrially utilized alternative method [17] is very harsh and involves high temperatures and concentrated H_2SO_4 as a catalyst; consequently, sorbitol undergoes dehydration prior to acylation and the resultant products are sorbitan monoesters. Enzymatically formed sugar alcohol monoesters possess superior surface-active properties compared to chemically produced sorbitan monoesters. This is most likely due to higher hydrophilicity of sorbitol vs. sorbitan and consequently, a more optimal hydrophilic lipophilic balance value [18].

Experimental

Lipase from procine pancreas (type II crude), triolein, olive and soybean oils, sugar alcohol (D-sorbitol), chemically prepared esters of sorbitols and fatty acids like sorbitan monolaurate, monopalmitate, monostearate and monooleate were

obtained from Sigma Co. St. Louis USA. Bovine pancreatic lipase was isolated in the author's laboratory. These enzymes were kept at 40°C for 60 min and desiccated under vacuum for 30 min. Phenolic resin used was prepared in the laboratory. Pyridine (Sigma) was used without further purification except from drying with potassium hydroxide. TLC was carried out on precoated silica gel plates obtained from Aldrich. Solvent system consisted of chloroform-methanol-water (64:10:1) was used. Silica gel for column chromatography (60 um particle size) was obtained from Merck. All other compounds and solvents used in the present study were of reagent grade.

Lipase assay

A photometric assay using p-nitrophenyl laurate (pNPL) was used for quick analysis of lipase activity. An emulsion of pNPL was prepared by taking 1.0 ml of 20 mM pNPL in acetone (64 mg/10 ml acetone) and added to a mixture of 4.0 ml of 1% polyvinylalcohol (PVA) and 8.0 ml of acetate buffer (0.1 M, pH 3.8) solution and emulsified. The emulsion was diluted with deionised water to a total volume of 20 ml (1.0 mM, pNPL). The reaction mixture consisted of 2.3 ml Tris-Cl buffer (0.1 M, pH 8.6) and 0.6 ml of emulsified substrate were taken in the cuvette and mixed thoroughly. The cuvette was thermostated at 37°C for 5 min. At zero time 100 ul of the enzyme solution was added to the reaction mixture and mixed gently. The lipase reactions were monitored at 405 nm with a uv/vis spectrophotometer (Hitachi U-100), using an extinction coefficient (405 nm) of p-nitrophenol of 2.2×10^{6} $1\ mol^{-1}\ cm^{-1}$

One unit of lipase activity was defined as the amount of enzyme that liberated 1 umol of p-nitrophenol per min. from pNPL at optimum conditions.

Phenolic resins preparation

A 0.33 M solution of hydroquinone was prepared in 4.2 M boiling HCl and refluxed. In the refluxing solution 40 ml of aqueous formaldehyde solution (38%) was added dropwise. The resultant brown gelatinous suspension was further refluxed for 180 min. with constant stirring before the addition of cold water. Brown gelatinous mass settled, the upper layer of supernatant was removed by filteration, washed with cold water and then the gel was dried under vacuum. This method produced 34 g dried source of the brown coloured resin with active -OH groups on the surface.

Immobilization procedure

The enzymes used in the present study were immobilized on phenolic resins according to estab lished methods [19] by crosslinking with glutaraldehyde. Phenolic resins (4 g) was boiled in 5% HNO_3 for 30 min, washed with water and dried in an oven. The dried resin was derivatized with an aqueous solution of 3-aminopropyltriethoxysilane at pH 3.45 for 150 min. at 80°C with constant stirring. The alkylamino phenolic resin was filtered off, washed with water and dried. This silanization process was repeated to ensure maximum activation of the resin and treated with 30 ml of 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.0) for 60 min at room temperature. The resin was washed thoroughly with water and then with phosphate buffer twice.

Crude lipase preparations (1.0 g of porcine pancreas powder and 1.0 g of bovine pancrease powder (lab. isolated) were separately suspended in 40 ml of phosphate buffer (0.01 M, pH 7.0) with gradual stirring for 30 min at room temperature, centrifuged at 13000 x g for 20 min. The supernatants were dialyzed overnight against the same buffer at 4°C to remove low molecular weight substances and then centrifuged. The enzyme solutions were concentrated by burying the dialysis bag with solution in a jar containing sucrose and kept it in a refrigerator.

Porcine and bovine pancreatic lipases 37500 and 30600 units were separately immobilized on 2 g aliquots of the derivatized resins and the suspensions were stirred at 4°C for 180 min at 150 rev min^{-1} and left overnight in a refrigerator. The buffer phase was recovered and the resins were washed with cold water followed by phosphate buffer (0.1 M, pH 6.0), dried under vacuum, stored at 4°C and utilized whenever required. The protein contents of the residues were measured [20] to evaluate the yield of the immobilization procedure.

Procedure for transesterification

Transesterification between triolein, olive and soybean oils and sugar alcohol catalyzed by immobilzied pancreatic lipases were carried out in

100 ml glass flasks separately. Reactions were initiated by adding 250 mg of immobilized lipases into 25 ml pyridine containing 325 mg sugar alcohol (D-sorbitol, 71.3 mmol/l) and 1.8 ml triolein, olive soybean oils (81.3 mmol/l). The temperature of the reaction system was controlled at 40°C by immersion in a water bath (Kottermann Labortechni, Karl Kolb, Germany) with shaking (150 rev min^{-1}). The reactions were stopped after 48 hrs by filtering the enzyme. The solvent containing the products and un-reacted materials was removed by vacuum evaporation. The residues were first washed with toluene and then dissolved in methanol (10-15 ml). The resultant methanol solution was passed through a column (10 x 150 mm) packed with dry silica gel, eluted with chloroform-methanol-water (64:10:1, v/v/v) at a flow rate of 40 ml h^{-1}. The effluent was concentrated at low temperature (35-40°C) and the products formed were identified by thin layer chromatography using precoated silica gel plates. After developing in the mobile phase, chloroform-methanol-water (64:10:1, v/v/v), the plates were dried, sprayed with 2.5% H_2SO_4 in ethanol followed by heating at 60°C for 20 min.

Conclusion

Bovine and porcine pancreatic lipases in immobilized form are more economical from the practical stand point when dry pyridine is utilized as reaction medium for transesterification process. The yield of sorbitol monoester was higher with immobilized bovine pancreatic lipase as compared to the immobilized porcine pancreatic lipase.

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