System Development from Organic Solvents to Ionic Liquids for Synthesizing Ascorbyl Esters with Conjugated Linoleic Acids

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Abstract: The aim of this paper is to screen suitable reaction systems for the modification of antioxidants through enzymatic synthesis. Enzymatic esterification of ascorbic acid with conjugated linoleic acid (CLA) was investigated as a model. Four organic solvents and five different enzymes were evaluated. Results show that only Novozym® 435 turned out to be a useful enzymatic preparation for the production of ascorbyl-CLA ester. The optimum reaction conditions in the organic solvent system were 4 h at 55°C and at a molar ratio of 5 (CLA/ascorbic acid). The esterification reaction was transferred to an ionic liquid system for the purpose of improving solubility of the polar substrate and avoiding the application of organic solvents. From screening experiments, it was evident that only methyltrioctylammonium triflouroacetate (*t*OMA·TFA) could provide a proper reaction environment for production of ascorbyl-CLA ester when using Novozym® 435 as biocatalyst. It was possible to significantly increase the productivity (150 g/l) through the increase of ascorbic acid solubility in ionic liquids by super saturation together with the increase of reaction temperature to 70°C, far beyond than that in organic solvents (35 g/l) after preliminary optimizations for both systems.

Keywords: Ascorbic acid, CLA, Organic solvents, Ionic liquids, Esterification.

INTRODUCTION

An increasing amount of evidence compiled over the last 30 years, supports the nutritional benefits of dietary ω-3 polyunsaturated fatty acids (PUFAs), which present in high amounts in marine oil [1]. However, ω-3 PUFAs are highly susceptible to oxidative deterioration because of a high degree of unsaturated nature, and it will lead to the formation of unpleasant fishy off-flavors, reactive free radicals, and aldehydes. Oxidative deterioration seems to be particularly prominent in emulsions and complex food systems, and the particular mechanisms of oxidation can differ significantly between different food emulsion systems [2]. Moreover, the efficacy of antioxidants seems to be influenced by their localization in the food systems, which is dependent on the polarity of the antioxidant and on the emulsifier used. Therefore, it is necessary to develop new antioxidants based on natural sources with improved physical properties. These antioxidants are designed to be placed where they are needed and to have the right anti-oxidative properties required in the particular food system (e.g. free radical scavenging or metal chelating properties).

Ascorbic acid (Vitamin C) is an antioxidant which can be widely found in nature, however it is a hydrophilic compound and difficult to apply in cosmetics or in the presence of fats and oils. A simple method is to esterify ascorbic acid with a fatty acid, which will result in an amphiphilic molecule which will not only improve solubility of ascorbic acid in hydrophobic media but will also enhance its radical scavenging performance [3]. The majority of the previous investigations concerns the esterification of ascorbic acid with palmitic, oleic or linoleic acids in organic solvent systems and resulted in relatively lower productivities [3-7]. This paper investigates the enzymatic esterification of ascorbic acid with conjugated linoleic acid (CLA). In recent years, the nutritional benefits of CLA have been studied extensively, and especially the weight reducing and cancer suppressing effects have received significant attention [8, 9]. Additionally, antioxidant properties of CLA have also been proposed. Even though the radical scavenging activity of CLA was lower than those of the commercial antioxidants such as α-tocopherol, ascorbic acid and BHT, a concentration dependant reduction of free radicals in model assays were observed [10]. Likewise, it has been shown that the oxidative stability of eicosapentaenoic acid (EPA) was increased by esterification with ascorbic acid [11]. The present investigations concern the optimization of the enzymatic esterification in a solvent system with respect to enzymes, solvents, reaction temperatures, and molar ratios for the purpose of increase of volumetric productivity.

Application of ionic liquids as reaction medium for enzymatic synthesis is becoming increasingly widespread. However, very few studies on the esterification of ascorbic acid in ionic liquids have been published [12]. A very recent study on the synthesis of ascorbyl oleate in ionic liquids has been conducted by *Adamczak M* and *Bornscheuer UT* [13], where they showed that a high production of ascoybyl oleate can be achieved in ionic solvent systems. In contrast to these previous studies, we have studied the use of mixture systems between ionic liquids and organic solvents, where much

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higher volumetric productivity has been demonstrated [14]. In order to create the possibility of avoiding the application of organic solvents, this paper investigates and compares the change of esterification of ascorbic acid with CLA, when transferred to an ionic liquid system from organic solvent systems. Five ionic liquids were selected and screened for their ability to provide a useful environment for the enzymatic reaction and the reaction conditions in the selected ionic liquid systems were optimized to increase product concentration.

The present study evaluates the organic solvent system first based on the state of the art, and then looks further into the pure ionic liquid system with a focus particularly on volumetric productivity. A comparison can then be made to demonstrate the possible pros and cons of the two systems for the enzymatic synthesis of ascorbyl esters.

MATERIALS AND METHODS

Chemical and Reagents

L-Ascorbic acid (99%), molecular sieves (3 Å, 4-8 mesh), free fatty acids (97% - 99%) and organic solvents (>96%) were purchased from Sigma-Aldrich Co. (St Louis, USA). Conjugated linoleic acid (CLA) with >80% (*9Z,11E*)- and (*10E, 12Z*)-isomer content, was obtained from Congnis Deutschland GmbH (Manheim, Germany). Mixture of DHA/EPA (5:1) was purchased from Promega A/S, Denmark, and it contains 60% of polyunsaturated fatty acid. All ionic liquids with minimum 98% of purity were purchased from Solvent Innovation GmbH (Köln, Germany). Methyltrioctylammonium trifluoroacetate (*t*OMA·TFA) with 99% of purity was from Merck KGaA (Darmstadt, Germany). Novozym® 435, Lipozyme® RM IM, and Lipozyme® TL IM were provided by Novozymes A/S (Bagsvaerd, Denmark). Lipase AK 20 and Lipase PS 30 were provided by Amano (Nagoya, Japan). All other chemicals and solvents were analytical grade.

Typical Experimental Procedure

A traditional solvent system was composed of ascorbic acid (2.1 mmol), CLA (8.5 mmol), molecular sieves (0.75 g) and 15 ml of organic solvent. Reactions were initiated at 55 ^{o}C and 400 rpm by adding 410 mg enzyme. Screening of enzymes was performed in tert-butanol under the same conditions demonstrated above. The conditions used in the traditional solvent system were also applied for examination of initial concentration of ascorbic acid and molar ratio between CLA and ascorbic acid. Reaction mixtures were continuously stirred at atmospheric pressure and the temperature was controlled by a thermostatic water bath.

In an ionic liquid system, reactions were conducted at 55 ^{o}C in the presence of 282 mg Novozym® 435 instead. Systems was composed of ascorbic acid (1.22 mmol), CLA (6.09 mmol) and ionic liquids (0.5 ml). Reaction mixtures were stirred at 300 rpm under 3 mbar vacuums, while pressure was controlled by a vacuum-pump (VWR PC301). Effects of temperature (from 55 ^{o}C to 70 ^{o}C) and initial concentration of ascorbic acid (from 0.81 M to 2.44 M) were studied base on the same condition as above.

HPLC Analysis

Samples (20 µl) were taken from the reaction mixture at regular intervals, dissolved in 1 ml dimethyl sulphoxide (DMSO), filtered (PFTE membrane, 0.45 µm, Subware, Hillerød, Denmark) and analyzed by HPLC with UV-detection. HPLC analysis was conducted by using a Hypersil C18 column (250 × 4.6 mm, 5 µm, Supelcosil Inc., Bellefonte, PA) in an Agilent HPLC system. The system is equipped with an ultraviolet diode-array detector (UV-DAD), an autosampler, an online degasser and a column heater. The eluents were composed of solvent A (methanol and acetonitrile, 50/50 (v/v)), and solvent B (water with 1 wt% of phosphoric acid). A 2 µl volume of the diluted reaction mixture was injected and separated at 15 ^{o}C with a flow rate of 1 ml/min. The elution gradient was as follows: start with 80% of solvent A, and then increased to 100% over 15 minutes. This condition was maintained for 2 min and then brought back to initial conditions over 3 mins. Products were detected under 240 nm UV light. The formation of esterified product was determined as g CLA ascorbate equivalents/l (APE) in each reaction batch. All analyses were conducted in triplicate and the averages ($\overline{APE}$) were used for evaluation. The relative standard deviations ($|\pm SD|*100/\overline{APE}$) were below 5.3%.

Solubility Calculation of Ascorbic Acid Assisted by COSMO-RS

Based on an earlier study [15], a commercial program, COSMO-RS (COSMOlogic GmbH & Co KG, Leverkusen, Germany), was employed to calculate the solubility of ascorbic acid in ionic liquids or other solvents based on a quantum chemistry model. In general, generation of molecular COSMO files was implemented on Turbomole 5.8. Infinite dilution activity coefficients were used for solubility calculations of ascorbic acid in ionic liquids with a non-iterative mode on CosmothermX_2.2. In order for the comparison, we also calculated the solubility of ascorbic acid in the organic solvents used in above.

According to the prediction by the software, it shows solubility of ascorbic acid in tOMA·TFA and ECOENG 218 / EMIm OS is much higher than other ionic liquids and organic solvents – 100 g of each these two ionic liquids can dissolve more than 15 g of ascorbic acid. ECOENG 21 M is seemed to have some solubility to ascorbic acid (about 6 g in 100 g ionic liquid), but BMIm PF6 and BMIm BF4 are only seemed to have minor solubility. In organic solvents, it was predicted that solubility of ascorbic acid in acetone > tert-butanol > 2-methyl-2-butanol, and ascorbic acid is hardly dissolved in hexane (Table **1**).

RESULTS AND DISCUSSIONS

Enzyme Screening in Organic Solvents

There have been a number of studies to apply solvent systems for such esterifications previously [3, 15]. Based on these studies, we proposed to make a practical evaluation in terms of volumetric productivity. This aspect was not clear in previous researches. We first made an evaluation of available immobilized lipases, followed by an evaluation of sol-

Fig. (7). Optimization of reaction systems. Reaction systems were firstly optimized at 55 °C under vacuum (3 mbar) by varying concentration of ascorbic acid: 0.41 mmol/ml (▲), 0.82 mmol/ml ( ), 1.62 mmol/ml (△), 2.11 mmol/ml (◆). Afterwards, temperature was increased to 70 °C to evaluated the reaction under vacuum (3 mbar) (□) or without vacuum (■), while the concentration of ascorbic acid was kept as 2.11 mmol/ml. In each case of reaction, 15% of Novozym® 435 and 2 mmol/ml of CLA were applied.

g/l productivity in the selected ionic liquid system. This could be six times of that in the traditional solvents. It certainly demonstrates more potentiality of the system for further development.

CONCLUSIONS

These studies show that ascorbic acid can be esterified with the nutritionally important CLA in an organic solvent system as shown in literature for other esters such as ascorbyl palmitate. More interestingly, this enzymatic esterification was successfully transferred to an ionic liquid system consisting of *t*OMA·TFA. The enzymatic reaction in the ionic liquid system was preliminarily optimized. Overall high production up to 200 g ascorbyl ester/l reaction batch was obtained in comparison with maximum 35 g/l for the organic solvent system. This is a significant increasement considering the possibility of the volumetric productivity of the process. On the other hand, the reaction time will be longer for the ionic liquid system. The separation of product could be more difficult also for the ionic liquid system. Therefore, the decision of which one is better should be further evaluated. Obviously the study provides a preliminary evaluation in terms of volumetric productivity for the purpose to inspire more research.

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