International Journal of
Medicinal Chemistry & Analysis

www.ijmca.com

e ISSN 2249 - 7587
Print ISSN 2249 - 7595

Research article

ENZYMATIC SYNTHESIS OF ASCORBYL-CONJUGATED LINOLEIC ACID ESTER USING IONIC LIQUIDS AND NOVOZYM® 435: OPTIMIZATION AND BIOCATALYTIC POTENTIAL IN ANTIOXIDANT MODIFICATION

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ABSTRACT

This paper aims to explore the utilization of enzyme synthesis for modifying antioxidants. Specifically, enzymatic esterification with conjugated linoleic acid (CLA) is investigated as a model for studying the esterification of ascorbic acid. Five different enzymes and four organic solvents were assessed in this study. Among them, Novozym® 435 emerged as the most effective catalyst for producing ascorbyl-CLA ester. The optimal reaction conditions were determined to be at 55°C, with an ascorbic acid to CLA molar ratio of 5. Ionic liquids were employed to facilitate the esterification of polar substrates and eliminate the need for organic solvents. The use of Novozym® 435 as a biocatalyst in the most favorable reaction environment led to the successful production of ascorbyl-CLA ester. Additionally, preliminary optimizations revealed that the solubility of ascorbic acid could be substantially increased (up to 150 g/l) by supersaturating it in ionic liquids, surpassing the solubility achieved in organic solvents (35 g/l). Moreover, elevating the reaction temperature by 70°C further enhanced ascorbic acid solubility in both systems.

Keywords: Ionic Liquids, Organic Solvents, Esterification, Vitamin C, Cla, and Organic Solvents.

INTRODUCTION

Marine oil contains a high amount of polyunsaturated fatty acids (PUFAs), which may offer nutritional benefits. Over the past 30 years, research has broadened to support this hypothesis [1]. Aldehydes, reactive free radicals, and unpleasant fishy flavors will result, due to the high degree of unsaturation, which makes these fats very susceptible to oxidative deterioration. In food emulsions and complex food systems, oxidative deterioration is a particularly prevalent problem, and the mechanism of oxidation differs significantly from one emulsion system to another. As well as this, the efficacy of antioxidants in foods appears to depend on the way they are localized, which depend on both their polarity and the emulsifier they are bound with. As a result, new antioxidants with improved physical properties need to be developed from natural sources. A food system needs antioxidants that have the proper antioxidant properties and are placed where they are needed (e.g. free radical scavenging, metal chelating, etc.)

Despite its widespread availability in nature, ascorbic acid (Vitamin C) has the disadvantage of being a hydrophilic compound, making it difficult to apply to cosmetics or in oily areas. To make ascorbic acid more soluble in hydrophobic media, one can esterify it with a fatty acid, resulting in an amphiphilic molecule that also enhances its radical-scavenging capabilities.

A relatively low productivity level has been observed in previous studies investigating Palmitic, oleic, and linoleic acids are esterified with ascorbic acid in organic solvents [3-7].

CLA and ascorbic acid are esterified enzymatically in this paper. Studies have been conducted extensively over the past few years on CLA's nutritional benefits, particularly its ability to reduce weight and suppress cancer.

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In addition, CLA has been shown to possess antioxidant properties. In model assays, CLA reduced free radicals by concentration dependent on concentration, even though it was not as effective as commercial antioxidants tocopherol, ascorbic acid, or BHT. Esterification of eicosapentaenoic acid (EPA) with ascorbic acid has also been shown to increase the oxidative stability [11]. For increasing volumetric productivity, the present investigations aim to optimize An enzymatic esterification system with solvents and enzymes.

A growing number of enzyme synthesis reactions use ionic liquids as reaction mediums. Ionic liquids have been used to esterify ascorbic acid, but few studies have been published [12]. Adamczak M and Bornscheuer UT recently reported that in ionic solvent systems ascorbyl oleate can be synthesized with high yields using ascorbyl oleate. Our recent study demonstrated much higher volumetric productivity using Ionic liquid and organic solvent mixtures contradicted these results. In order to avoid using organic solvents, ionic liquids are used to esterify ascorbic acid and CLA. In order to increase product yield, the ionic liquid reaction conditions were optimized concentration by selecting five ionic liquids and screening them for their ability to provide a useful environment for enzymatic reactions.

A review of the state of the art for organic solvent systems is presented, followed by a focus on pure Volumetric productivity is emphasized in ionic liquid systems. Ascorbyl esters can be synthesized enzymatically, a comparison between the two systems can be made to highlight the pros and cons.

METHODS AND MATERIALS

Chemistries and reagents

We obtained various chemicals and reagents (>96%), from Sigma-Aldrich Company (St Louis, USA), free fatty acids (97% - 99%) are available. In addition, it contains 60% polyunsaturated fatty acids.

Experiment's procedures

Various solvents, including organic solvents, molecular sieves, and 15 ml of ascorbic acid (2.1 mmol), are commonly utilized in this process. An enzyme dose of 410 mg was employed, and the reaction was carried out at 55°C with a rotation rate of 400. The same conditions were applied to screen enzymes in tert-butanol. Additionally, the initial concentration of ascorbic acid and the molar ratio of ascorbic acid to CLA (1.6) were examined using the traditional solvent system. The temperature of the reaction mixtures was controlled by a thermostatic water bath while continuously stirred at atmospheric pressure. In a deviation from the traditional method, 282 mg of Novozym® 435 was utilized in ionic liquid at 55°C, with 0.5 ml of ionic liquids added to the system. The molar quantities of ascorbic acid (1.22 moles) and CLA (6.09 moles) were specified. An Air-Pump (VWR PC301) was employed to regulate pressure while stirring the reaction mixtures at 300 rpm. Furthermore, the effects of temperature (55°C to 70°C) and initial ascorbic acid concentration (0.81 M to 2.44 M) were investigated under the same conditions as above.

Analyses by HPLC

The mixture was periodically sampled, and dimethyl sulfoxide (DMSO) was added to dissolve the sample. The sample was then filtered through a PFTE membrane with a pore size of 0.45 μm. High-performance liquid chromatography (HPLC) with UV detection was performed at regular intervals using Hypersil C18 columns (250 mm x 4.6 mm, 5 μm) on Agilent HPLC systems. These systems included UV diode array detectors (UV-DADs), auto-samplers, online degassers, and column heaters. The eluents used were a mixture of methanol and acetonitrile (50/50 v/v) and water with phosphoric acid. A flow rate of 1 ml/min was used to inject and separate two liters of the diluted reaction mixture at 15°C. After 15 minutes, 80% of Solvent A was added to the solution, and the gradient was gradually increased to 100%. It took three minutes to return to initial conditions after maintaining this condition for two minutes. The products were detected under UV light at 240 nm. The amount of conjugated linoleic acid (CLA) ascorbate equivalents formed was calculated for each batch of reaction using the ascorbic acid per equivalent (APE) measure. APE values were determined for all analyses conducted in triplicate, with the APE percentage versus USD being below 5.3%.

A COSMO-RS-assisted calculation of Sodium ascorbate solubility

For measuring the solubility of ascorbic acid in ionic liquids and other solvents, COSMO-RS was utilized. Molecular COSMO files were generated using Turbomole 5.8. The solubility of ascorbic acid in ionic liquids was calculated using infinite dilution activity coefficients with CosmothermX_2.2 in non-iterative mode. Additionally, the solubility of ascorbic acid in organic solvents was calculated for comparison purposes. The predictions indicate that 100g of ascorbic acid can be dissolved in both ionic liquids by more than 15g each, whereas only 15g of ascorbic acid can be dissolved in 100g of other ionic liquids and organic solvents. According to our findings, BMIm PF6 and BMIm BF4 exhibit minimal solubility, dissolving approximately 6 grams of ascorbic acid per 100 grams of ionic liquid. In contrast, ECOENG 21 M appears to have superior solubility, with the ability to dissolve six grams of ascorbic acid per 100 grams of ionic liquid. As depicted in table 1, hexane shows minimal solubility for ascorbic acid, while tert-butanol exhibits higher solubility compared to other organic solvents.

RESULTS & DISCUSSION

Organic solvents for enzyme screening

In the past, various solvent systems have been used to perform this type of esterification [3, 15]. Our study suggested making a volumetric productivity evaluation based on these findings. Previous research had not addressed this issue. Immobilized lipases were first evaluated, followed by solvents.

Table 1: Depending on the ionic liquid used at 25°C, ascorbic acid and CLA should be soluble

The solvent	Solubility in Ionic Liquids or Solvents (g/100g)	
	AICD for ascorbic acid	CLA
tOMA·TFAb	>16[c]	>16[c]
Alectronic Engineering 218/EMIm OSb	>16[c]	>16[c]
A 21Mb version of ECOENG	7.0	>16[c]
A BMI of PF6b	0.7	0.3
BF4b BMIm	0.5	2.1
Acetate	<11[c]	Easily soluble
Butanol tert-butyl	5.98	Easily soluble
Butanol-2-methyl	4.1	Easily soluble
The hexane solvent	0	Easily soluble

[a]Based on COSMO-RS calculations. Information on methods can be found in the Methods Section.
[B]Sulfonyl-trioctyl-ammonium(OMA-TFA): ethyl-3-methyl-imidazoliumn-octylsulfate; ECOM 21M:. In this case, it is 1-butyl-3-methyl-imidazolium hexafluorophosphate: tetrafluoroborate;. PF6 is the general name for A 1-butyl-3-methylimidazolium hexafluorophosphate is not a positive charge; however, it has one-butyl-3-methylimidazolium fluoride is the chemical name for the substance; 1.
[c]The COSMO-RS predictions were inadequate.

Using five different immobilized lipases, CLA and ascorbic acid are converted to CLA L-ascorbate ester. Novozym® 435 was shown to be the most efficient catalyst for esterifying ascorbic acid with CLA in tert-butanol. Compared to Lipase AK20, Lipase PS30, or Lipozyme® TL IM, Novozym® 435 produces higher yields for esterification in organic solvents. Nevertheless, as we can see from this study, Novozym® 435 showed a significant difference (20 times) compared to the other lipases used. Previous reports have not mentioned this. Achieving equilibrium at 55°C took four hours or less according to the initial tests.

Ascorbic Acid Concentration and Organic Solvent Screening

In four different organic solvents, Novozym® 435 forms esterified products. Two-methyl-2-butanol and tert-butanol produced large quantities of product, but 2-methyl-2-butanol yielded a much smaller amount. Neither Novozym® 435 nor hexane were essential to the esterification reaction. Both acetone and tert-butanol showed good conversion in previous studies. Hexane, too, did not show any reaction when these ascorbyl-ester enzymatic synthesis studies were conducted [3, 16]. Based on COSMO's predictions, intermediate hydrophilic solvents performed better compared to hydrophobic solvents, probably because they solubilized ascorbic acid more readily. Water, however, must remain in its active form to keep an enzyme active. Water, which otherwise keeps the enzyme active, is dissolved by highly hydrophilic solvents. Further experiments supported this hypothesis by demonstrating that the tert-butanol system was significantly more productive when ascorbic acid concentrations were increased above saturation limits. 10 grams of ascorbic acid in a liter produced 8.3 grams, 25 grams in a liter and 50 grams in a liter produced 26.1 grams and 32.3 grams, respectively. Accordingly, the maximum productivity is hampered by excessive ascorbic acid use at 35g/l, causing problems when handling the solid existing system.

Screening for free fatty acids

Additional experiments were conducted using free fatty acids in various states of unsaturation or carbon length. Ascorbic acid's esterification rate with different fats was examined in this study. Novozym® 435 was used as a catalyst in serial reactions in tert-butanol. Study participants included EPA/DHA (C20:5/CC22:6) and caprylic/lauric/palmitic acids (C12,C16,C18:1) and linoleic acids (C18:2). The length of the carbon chain and the number of double bonds had a significant impact on the esterification rate with ascorbic acid. Shorter carbon chains seem to result in higher esterification rates for free fatty acids. It starts out at a rate of 10.9 mg/ml/h when ascorbic acid meets caprylic acid. Free fatty acids, however, saw a steady decrease in initial reaction rate as carbon chains lengthened. Lauric acid, palmitic acid, and oleic acid began to react at 4, 5, and 0.4 mg/ml/h, respectively. The initial reaction rate for oleic acid was 4.1 mg/ml/h, while linoleic acid was 5.9 mg/ml/h. It is possible that PUFAs favor the esterification reaction. The longer carbon chain in EPA/DHA and the higher number of double bonds compare to those in oleic acid provide

another benefit of using EPA/DHA as a reaction substrate. In comparison with other free fatty acids, CLA was significantly more abundant at the beginning (33.6 mg/ml/h). Because CLA has double bonds conjugated and is UV-absorbent, this is likely the case.

The effect of substrate molar ratios

Ascorbic acid molar ratios between acyl donors and ascorbic acids in the interval R (1-10) was first examined. Ratios of 1 to 5 exhibited a four-fold increase, while 5 to 10 exhibited only minor increases. Using response surface modeling (detail study omitted), the optimal ratio was determined as 5. A linear increase between R and equilibrium conversion has been observed using ascorbic acid esterification in acetone at 50 °C in previous studies [5].

A screening procedure for ionic liquids

Although certain super saturation did improve the volumetric productivity significantly, it was the low solubility of ascorbic acid that was the major drawback for organic solvents. Nevertheless, increasing solubility is recommended. The possibility of using several ionic liquids with greater solubility was investigated [14]. With COSMO-RS (Leverkusen, Germany) ascorbic acid and CLA solubility calculations were carried out for eleven different ionic liquids. Ascorbic acid and CLA were soluble in five of these ionic liquids (Table 1), so these substances were tested as reaction media for enzyme esterification.

Ascorbyl-CLA ester levels could only be obtained by enzymatic esterification in tOMA•TFA. When tOMA•TFA was used to react at 55 °C, about 11 g/l ascorbyl-CLA ester was obtained. The ascorbyl-CLA esters were only formed in small quantities (1-5 g/l) under the same conditions as in tOMA•TFA when reactions were performed with BMIm BF4, BMIm PF6, ECOENG 218/EMIm OS and ECOENG 21M. Following optimization of the reaction system, those four ionic liquids that produced less ester were excluded.

According to the calculated solubility values (Table 1), the highest production was obtained with the tOMA•TFA- system. When compared to other ionic liquids, tOMA• TFA exhibited the highest solubility of ascorbic acid. tOMA• TFA also dissolved well in CLA, so the esterification was performed using tOMA• TFA as a reaction medium. A significant amount of ascorbyl-CLA ester was not formed by applying ECOENG 218/EMIM.OS to ascorbic acid or CLA. Ionic liquid systems are not solely dependent on substrate solubility to influence enzyme efficiency. Catalytic activity of enzymes is also affected by ionic interactions between the ionic liquid and the enzyme, such as the ability of the ionic liquid to bind water. Moreover, since n-octyl sulphate anion has an amphiphilic nature, its potential surface activity may influence its behavior as a reaction medium. There may be a molecule-to-molecule interaction between the anion and CLA, which could limit how much CLA is available to the enzyme, thus reducing production.

BMIM BF4 was used in the present study to produce ascorbyl-CLA ester, which was considerably lower than that produced by palmitic acid in a previous study [12]. Despite higher amounts of ascorbic acids and fatty acids, while enzyme dosage was six times higher, there was still very little ascorbyl ester produced in the present study. Based on the results, fatty acid type affects the final production. However, BMIM BF4 has been shown to have very low solubilities of glucose despite being a hydrophilic ionic liquid. Ascorbic acid also had a low calculated solubility. Low production was probably caused by this. Last but not least, it seems that BMIM PF6 is considered an ionic liquid that is hydrophobic, in contrast to BMIM BF4. When Novozym® 435 is heated to 50°C after 48 hours, it retains at least 50% of its activity [20]. BMIM PF6 produced low production, which can also be explained by low solvent levels of both substrates. Ascorbic acid esterification using BMIM PF6 was similar to studies conducted in the past [8]. Hydrophobic or hydrophilic properties of ionic liquids do not appear to have a direct relationship with enzyme activity as far as we know [18]. There are other factors affecting enzyme activity beyond hydrophobicity and solubility.

Ascorbic acid concentration in ionic liquids is influenced by its initial concentration

Ascorbic acid concentrations above their saturation limits significantly increased product concentrations, similar to the esterification in tert-butanol systems. The productivity of the ester product increased almost six times when the ascorbic acid concentration was doubled from 0.41 to 0.82 mmol/ml. The amount of ascorbic acid was increased 6-fold from 0.47 mmol/ml to 2.44 mmol/ml and the amount of ascorbyl-CLA ester produced increased 8-fold after 32 hours.

Conversion in Ionic Liquids at Different Temperatures and Pressures

Low volatility enables higher reaction temperatures with ionic liquids. After 32 h, the volumetric productivity was increased by 60% by increasing the temperature from 55°C to 70°C and significantly increasing the reaction rate. There is no doubt that the increase in temperature has resulted in the reduction of viscosity. In contrast, Arrhenius law also predicted an increase in enzymatic activity at the temperature range. As a further observation, we observed that vacuum vacuums did not increase product formation further when removing the water formed during esterification. Compared to 3 mbars, atmospheric pressure resulted in a 40% higher volumetric productivity. The enzyme could be in an inactive state at 3 mbars because too much water is removed. Water may have been isolated from the reaction

equilibrium by ionic liquids as well. In order to gain a deeper understanding of the vacuum effect, further studies should be conducted.

Ionic liquids are capable of achieving productivity levels up to 200 g/l, which is a clear conclusion for the chosen system. A traditional solvent could cost six times as much. A further development of the system is definitely possible based on this demonstration.

CONCLUSIONS

CLA is nutritionally important and can be esterified with ascorbic acid in organic solvents like ascorbyl palmitate, which has been shown in literature. An ionic liquid system containing toMA•TFA was successfully converted to an enzymatic esterification process. Ionic liquid systems have been optimized for enzymatic reactions. A high level of production was achieved with ascorbyl ester up to 200 grams per reaction batch, compared with a maximum amount of 35 grams per reaction batch with organic solvents. The volumetric productivity of the process can increase significantly as a result of this increase. In contrast, the ionic liquid system will require longer reaction times. Ionic liquid systems may also have difficulty separating products. In order to make a better decision, further evaluation is necessary in order to determine which one is best. As a preliminary evaluation, the study will hopefully inspire further research on volumetric productivity.

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