Esterification of Fructose-oleic Acid by tert-Butanol/Dimethyl Sulfoxide and by 2-Methyl-2-butanol/Dimethyl Sulfoxide

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Abstract: In this study two different strategy were followed to obtain a D-fructose-oleic acid ester. One of the strategies has been well established enzymatic synthesis of an ester bond. The other strategy excluded the biocatalyst and only used a mixture of two organic solvents as the reaction media, 2-methyl-2-butanol/dimethyl sulfoxide or tert-butanol/dimethyl sulfoxide for the production of D-fructose-oleic acid ester. Ester products obtained were characterised by using FT-IR, NMR, by MS. Product yield was also assessed by HPLC. Results of structural analyses and yield measurement indicated that two approaches produced almost identical ester products.

Key words: fructose, oleic acid, sugar-fatty acid esterification, tert-butanol, 2-methyl-2-butanol, dimethyl sulfoxide

1 Introduction

Monosaccharides have important roles in the world of living entities. Hexoses are generally energy units and storage materials in plants and animals. Hexose oligomers are known as surface antigens, especially in Gram (-) bacteria. Fructose is a common fruit sugar in plants. D-ribose is one of the most common and unique molecule of the biosphere. It is the basic subunit of nucleotides where it binds the phosphate and the base. It is also found in the structure of other nucleotides which participate in the electron transfer. In summary, in the evolution of macromolecules, the ribose has been selected as the structural unit. In this context, the prebiotic synthesis of essential carbohydrates, monosaccharides, is as important as those of the amino and nucleic acids. The existence of a "glyco-world" can even be envisioned before the "world of RNA". It is surprising, however, that carbohydrate evolution appears to have been paid almost no attention.

Sugar esters are non-ionic biosurfactant molecules consisting of a carbohydrate unit as the hydrophilic head, and one or more fatty acids as the lipophilic tail. It has been well established that sugar esters could be synthesized by chemical or enzymatic processes. Enzymatic esterification has seen much more importance, due to its relatively mild reaction conditions, and better stereo- or region-selectivity. By controlling the degree of esterification and the type of fatty acid and sugar, it is possible to synthesize ester products with differing properties. They have some advantages over chemically synthesized surfactants because they can be prepared from renewable sources. They are tasteless, odourless, stable, and non-irritating in a wide pH range.

Sugar esters have mainly been used as the emulsifying agent in cosmetics, healthcare, pharmaceutics. In food industry, fructose esters can be added in mayonnaise and sauces, can be used in the ripening of cheeses, in bakery, and in the fermented meat products. Sugar esters can exert some antibiotic, anti-tumour, and insecticidal activities. Hence, sugar esters are biocompatible, biodegradable, and have no detectable cytotoxicity.

The restrictive parameter in the synthesis of sugar-fatty acid esters is substrate solubility in organic reaction media. Sugars dissolve poorly in most of the organic solvents, and some other organic media which dissolve sugar are detrimental for enzyme activity. In addition, most of those organic solvents, which meet these two basic conditions,
are immiscible, that is, they form distinct phases. In many of the esterification studies, two pairs of organic solvents have been chosen and utilized as the reaction media: dimethyl sulfoxide / tert-butanol and dimethyl sulfoxide / 2-methyl-2-butanol. Unfortunately, however, control reactions, to be performed in the absence of a catalyst or bio-catalyst, have not been included in these studies.

In this study, it has clearly been demonstrated that, besides the enzymatic esterification with Candida antarctica lipase B, the above-mentioned two organic reaction media, dimethyl sulfoxide / tert-butanol and dimethyl sulfoxide / 2-methyl-2-butanol, could by themselves be perfectly used in the esterification of fructose with oleic acid. This esterifying capacity of the organic reaction media has previously been proven for two other monosaccharides, glucose and ribose (in press).

2 Methods

2.1 Reaction procedures

Esterification reactions conditions with immobilized lipase B of Candida antarctica (S) and control reactions (C), in the absence of a catalyst, were summarised below (Table 1). The reaction time AND temperature were 24 h and 55°C, respectively. Esterification reactions with immobilized lipase B of Candida antarctica (S) and control reactions (C), in the absence of a catalyst, were performed under the same conditions (Fig. 1). The esterification procedure described by Deng and Zimmermann was followed with minor modifications: final reaction volume (25 mL) included 20% DMSO (v/v), 80% tert-butanol (v/v), or 20% DMSO (v/v), 80% 2-methyl-2-butanol (v/v), 0.2 M D-fructose (0.72 g), and 0.2 M oleic acid (1.128 g). The enzymatic reactions included 140 mg immobilised Candida antarctica lipase B. Water produced during esterification was removed by using 2 g molecular sieve into the reaction mixture. Esterification was performed for 24 h in a shaker incubator at 200 rpm and 55°C. Samples were then centrifuged at 5,000 rpm and supernatants were transferred into fresh tubes and stored at –20°C.

2.2 Structural and yield analyses

Fourier-transform infrared spectroscopy (FT-IR, Perkin Elmer 400), high-performance liquid chromatography, and carbon and proton nuclear magnetic resonance (13C-NMR Bruker Spectrometer, 125 MHz and 1H-NMR, Varian INOVA 500 MHz) analyses were performed at Research Centre Laboratories, Erciyes University, Kayseri, Turkey. Mass spectrometry (Thermo Finnigan LCQ LC-MS/MS Spectrometer) analyses were carried out at Research Centre Laboratories, Istanbul University, Istanbul, Turkey. The conversion percentage of sugar monomers into sugar-fatty acid esters was taken as the reaction yield. The conversion percentage of sugar was assessed by HPLC (Agilent ZORBAX) using a carbohydrate analysis column (Agilent ZORBAX).

2.3 Cytotoxicity

2.3.1 Preparation of tissue culture

Fibroblasts are the most abundant cells in mammalian organisms and divide once every 24 hours on average. L929 mouse fibroblast cell line (ATCC cell line, NCTC clone 929) was used for the cytotoxicity test. Tissue culture Dulbecco’s minimum Eagle medium (DMEM: 10% fetal calf serum (FCS) and 2 mM/mL L-glutamine; Sigma, St. Louis, MO, USA) was prepared under 5% CO₂ and at 37°C. No antibiotics were included. Passages were performed when the culture reached the confluent state, i.e. when the cells covered about 75% of the culture flask base. The culture

![Table 1](image)

**Table 1** Yield in fructose ester under different conditions (oleic acid 0.2 M, 1.128 g; *Candida antarctica* Lipase B (CALB) Novozyme 435, 140 mg; molecular sieves (3 Å), 2 g; 55°C, 24 h).

| Entry | Molar ratio | Fructose/oleic acid | D-Fructose (g) | Solvent | Enzyme | Sugar conv. % |
|-------|-------------|---------------------|----------------|---------|--------|--------------|
| 9S    | 1:1         | 0.72                | TBU/DMSO       | +       | 98.90  |
| 12K   | 1:1         | 0.72                | 2M2B/DMSO      | -       | 98.90  |

**Fig. 1** Esterification reaction of oleic acid with D-fructose.
was washed with PBS (Phosphate buffered saline) after the medium was discharged. Cells were mobilized by partial trypsin treatment (0.05 trypsin + 0.02 % EDTA, WISENT Inc St. Bruno, Quebec, Canada). Digestion was terminated by adding fresh DMEM to the medium. Approximately \( 10^4 \) cells in a volume of 100 \( \mu \)L were placed in each of the 96-wells of ELISA plate.

2.3.2 XTT

Cytotoxic potential was assessed by using XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carboxyl]-2H-tetrazolium hydroxide] method. Three different sugar solutions (10\(^{-2}\), 10\(^{-3}\), and 10\(^{-7}\) M) were prepared. DMEM containing FBS and L-glutamine were used as media. XTT is a commercially available cell proliferation reagent (Biological Industries, USA) and allows the number of cells to be determined due to the cleavage of tetrazolium salt by the cell’s mitochondrial dehydrogenase enzyme (Berridge, M.V, 2005). Ten microliters of XTT was added onto each of the 100 \( \mu \)L cell suspension in the wells of the ELISA plate. The cells were then incubated for 2 h. The sample wells were read against negative controls at OD 450 (Thermo Scientific Microplate Photometer, Multiskan FC, USA). The percentage of live cells was calculated using the formula: \( \% = \left( \frac{OD_{450 \text{ (example)}} - OD_{450 \text{ (negative control)}}}{OD_{450 \text{ (negative control)}}} \right) \times 100 \). Readings were analysed by one-way variance analysis (ANOVA) and by Student’s \( t \)-test at 95% and at 0.05 margin of error.

3 Results

3.1 Structural analysis

3.1.1 9S: 6-O-Fructose oleate

This ester product was synthesized in the DMSO: tert-butanol reaction media in the presence of biocatalyst Candida antarctica lipase B. The compound 9S (1.75 g, 98.90%) as a pale yellow liquid: IR \( \nu (\text{cm}^{-1}) \): 2876, 2969 (aliph. CH), 2923, 1722 (ester C=O), 1719 (ester C-O). \(^1\)H-NMR (500 Mhz, [D6]DMSO): -OH peak of oleic acid (11 ppm) (SDBS) no longer observed. \(^13\)C-NMR (125 Mhz, [D6] DMSO): \( \delta = 175.85 \) (CH\( \_\_COO \)).

3.1.2 9C: 6-O-Fructose oleate

This ester product was synthesized in the DMSO: tert-butanol reaction media in the presence of biocatalyst Candida antarctica lipase B. The compound 9C (1.75 g) as a pale yellow liquid: IR \( \nu (\text{cm}^{-1}) \): 2876, 2969 (aliph. CH), 1718 (ester C=O), 1187 (ester C-O).

3.1.3 12S: 6-O-Fructose oleate

This ester product was synthesized in the DMSO: 2-methyl-2-butanol reaction media in the presence of biocatalyst Candida antarctica lipase B. The compound 12S (1.75 g) as a pale yellow liquid: IR \( \nu (\text{cm}^{-1}) \): 2876, 2969 (aliph. CH), 1718 (ester C=O), 1187 (ester C-O).

3.1.4 12C: 6-O-Fructose oleate

This ester product was synthesized in the DMSO: 2-methyl-2-butanol reaction media in the absence of biocatalyst Candida antarctica lipase B. The compound 12C (1.75 g, 98.90%) as a pale yellow liquid: IR \( \nu (\text{cm}^{-1}) \): 2876, 2967 (aliph. CH), 1719 (ester C=O), 1186 (ester C-O). \(^1\)H-NMR (125 Mhz, [D6] DMSO): \( \delta = 174.90 \) (CH\( \_\_COO \)).

The band belonging to the functional ester group C=O is in the range of 1718-1722 cm\(^{-1}\). The IR spectra of the both 6-O-Fructose oleate, produced in the presence and in the absence of the enzyme, were found similar to those of both 6-O-Fructose oleate, produced in the presence and in the absence of the enzyme, were found similar to those of the literature, residing between 1725 and 1736 cm\(^{-1}\)\(^{15, 16}\).

It has been reported that the feruloyl esterase compound, synthesized in a hexane and tert-butanol mixture and had

![Fig. 2](image-url) \(^{13}\)C-NMR.

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a stress of C=O ester at 1725 cm$^{-1}$. The carbon atoms represented by the peaks seen in the NMR plot of the fructose oleate samples (9S and 9C) were assigned (Fig. 2). Ester bonds were presented between 174.90 and 175.85 ppm range. The presence of carbon peak belonging to the ester functional group in sugar esters has generally been taken as the sign that the esterification reaction took place.

The strongest non-shielding effect among carbon atoms is seen in carbonyl carbons. Any functional group that increases the electron density on the carbonyl group causes a shift to the right in the spectrum. The electron density in the ester is therefore higher than in that of the acid group. While the carbonyl group in the acid resonates, there should be no resonance in the ester group. Expectedly, COOH carbon in acetic acid was seen at 190.7 ppm and ester carbon at 175.7 ppm$^{17}$. To sum up, in line with the expectations, carbon NMR spectra indicated that acid and alcohol groups were successfully replaced by the functional ester groups during the esterification reactions.

The fructose oleate sample (9C, 6-O-fructose oleate) was chosen and used for the analysis by $^{13}$C-NMR. The proton distribution of 6-O-Fructose oleate 9C was similar to the proton shift values in the literature findings$^{18, 19}$. The most striking finding in this analysis was that the COOH proton, presented with a peak at 10-14 ppm, seemed to have disappeared in the spectra of 9C fructose oleate sample (Fig. 3)$^{20}$.

3.2 Cytotoxicity

XTT test and subsequent statistical analyses indicated that 6-O-fructose oleate did not have a significant cytotoxic effect on mouse fibroblasts.

4 Conclusion

This study clearly demonstrated that another common monosaccharide, D-fructose could form ester bonds with a fatty acid in organic media devoid of any catalyst or biocatalyst. The FT-IR spectra of the two samples (C, and S) appeared to be almost identical (Fig. 6 Supp). A mechanism for his esterification was suggested (Fig. 4). This is the SN2 mechanism where the oxygen of the primary alcohol attacked to that of the carbonyl group of the oleic acid.
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Supporting Information
This material is available free of charge via the Internet at http://dx.doi.org/jos.69.10.5650/jos.ess20142

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