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Improving the Hydrophobic Properties of Starch-Protein Raw Material by Enzymatic Modification

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Abstract

*In this study, starch-protein raw material was esterified with oleic acid, vinyl laurate as well as rape oil as an acyl donor. Chloroform was used as a reaction medium and immobilized lipase from *Candida antarctica* as a catalyst. The degree of substitution (DS) of the products was determined by the volumetric method. The effect of the reaction parameters such as the kind of acyl donor or scale of the process on the DS, was studied. The product with the highest DS (in small scale - 0.11) was found in the reaction carried out at 50 °C for 6 h with oleic acid. Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) analyses confirmed the esterification of the starch-protein material. The hydrophobicity of non-esterified material and the final product was evaluated using the sessile drop method according to European Pharmacopeia 8.0. The possibility of processing the product of esterification into pellets to be used for film and fittings production was examined.*

Key words: lipase, esterification, starch-protein raw material, long-chain fatty acid, organic solvents.

Introduction

At present petroleum is crucial for the global economy, with industry totally dependent on petroleum and its products, such as raw material for the chemical industry or for the production of polymers. However, its decreasing resources and the increasing ecological awareness of societies prompt the industry to seek alternative raw materials. Among the many polymers used to develop biodegradable films, starch has been the focus of investigations because it is a natural polymer from renewable resources, abundant and low cost [1 - 3]. Starch is a natural biopolymer obtained from potato as well as cereal grains, mainly rice, corn and wheat. Unfortunately its strong hydro-

philic character limits possibilities to obtain new materials based on starch. In general, starchy materials are very sensitive to environmental conditions such as moisture or water. Among the possible means to overcome this limitation is the modification of starch, which can make it possible to achieve a product with improved water resistance [4]. A few kinds of modification have been described, basically chemical, physical, enzymatic and genetic. Esterification by chemical means often employs harsh chemicals like solvents, alkali, pyridine as well as catalysts at high temperatures, and also requires sophisticated instrumentation. These conditions restrict their use in industries like food and pharmaceutical as they involve environmental, economic and safety risks. Processes with enzymes, especially lipases and proteases as a catalyst seem to be environmentally friendly alternative approaches. Enzymes catalyse more selective processes than chemical ones, and reactions may be performed under mild conditions of temperature and pressure, avoiding polymer degradation. Lipases and proteases belong to the class of hydrolases, and for ester formation low water content media are thermodynamically favoured. Therefore alternative enzymatic procedures should be in, for example, organic solvents, or organic solvent mixtures as a reaction medium.

Enzymatic acylation of poly- and oligo-saccharides in conventional conditions is often carried out in DMF or DMSO as a reaction medium [5 - 8]. DMSO

is the only conventional solvent that is known to dissolve starch. But DMSO is also an extremely polar and hygroscopic organic solvent, and it is difficult to remove water from this reaction system. And removing water – an esterification by-product - is essential for the reaction catalysed by hydrolases. If water is not expelled from the reaction medium, it shifts the equilibrium of the reaction towards hydrolysis [9]. However, as in the case of many water-miscible solvents, the addition of DMSO to the reaction medium poses an even greater problem for enzymatic catalysis - the loss of catalytic activity of an enzyme [10]. DMF and DMSO also create problems during down-stream processing because it is quite difficult to wash them out of the esterification product, and because of their high boiling temperature (189 °C and 153 °C respectively), the solvent residue is difficult to remove by drying. A similar problem is encountered for another category of solvents of increased popularity in recent years - ionic liquids. The isolation of the product can be extremely difficult mainly due to the low volatility of this kind of solvent [11].

Therefore, the purpose of this paper is to present some results of research an organic solvent favorable due to much easier the product separation and better stability of the enzyme. For the obtaining new biopolymer was used not starch but functional flour obtained by the controlled hydrothermal treatment of classics flour-cereal products. Main component of this functional flour is starch, but

it contains also substances like proteins, minerals, oligosaccharides and fats.

■ Materials and methods

Materials

The materials used in the study: pure oleic acid, acetone and chloroform purchased from Avantor Performance Materials Poland S.A. (Gliwice, Poland); vinyl laurate – Aldrich Chemistry (Sigma-Aldrich, Germany); pure rape oil, Rapso, VOG Polska Sp. z o.o. (Poland); lipase B from *C. antarctica* (Novozym 435 - lipase immobilized on macroporous acrylic resin; specific activity: 10,000 U/g was purchased from Novozymes (Denmark). Functional flour Q-Farin C 1000 obtained by controlled hydrothermal treatment of classic wheat flour (Lubella, LCC, LP; Poland); this material contained c.a. 55% starch, 14% protein and 6% moisture; described in detail in [12].

Process of enzymatic modification

Initially wheat flour was dried at 105 °C. The dried starch-protein material was then added to the chloroform at a concentration of 10% (m/v). Tests were performed in two variations – on a small and on a bigger scale. On the small scale (variant 1) suspensions containing 0.2 g of Q-Farin C 1000 were heated in 25 ml glass bottles for 1 h in a shaking water bath. Then the acyl donor (oleic acid, vinyl laurate or rape oil) and biocatalyst were added. A constant molar ratio of the starch part of the functional flour (anhydroglucose unit, AGU) to the acyl donor (1:1) was maintained, as well as a constant weight ratio of the immobilized lipase (0.5 g per 1 g of the functional flour). A molecular sieve in the amount of 0,15 g/bottle was used to remove water. The synthesis of esters was carried out on a thermostated shaker at a temperature 40 and 50 °C for 4, 6 or 24 h, and the mixture was shaken at 100 r.p.m. After the completion of the reaction, the mixture was cooled to room temperature and the products were precipitated using acetone. Then the precipitates were centrifuged and washed with anhydrous alcohol to separate out the main product of the esterification. The solid residues were washed again thrice to eliminate the chloroform and untreated reagents. Finally the products were dried at room temperature for 48 h and then subjected to physicochemical analysis.

On a bigger scale (Variant 2), suspensions containing 50 g of Q-Farin C1000 were heated in 1 l a chemical reactor (Radleys Reactor Ready) for 1 h at 50 °C. Then the acyl donor (oleic acid, vinyl laurate or rape oil) and biocatalyst were added. A constant molar ratio of the starch part of the functional flour (anhydroglucose unit, AGU) to the acyl donor (1:1) was maintained, as well as a constant weight ratio of the immobilized lipase (0.25 g per 1 g of the functional flour). The synthesis of esters was carried out at temperature 50 °C for 4, 6 or 24 h, and the mixture was stirred at 140 r.p.m. After the completion of the reaction, the mixture was cooled to room temperature and the products separated from the mixture in a separatory funnel and precipitated using acetone. The next steps were as described above.

Determination of the degree of substitution (DS)

The DS of fatty acid derivatives of starch-protein material were determined according to the volumetric method [7]. Half a gram of dried-to-constant weight modified flour was dispersed in 20 ml of acetone and 10 ml of 0.5 mol/l NaOH solution. The mixture was stirred at room temperature for 1 h. After this time, a few drops of phenolphthalein were added as an indicator and the mixture was titrated using 0.5 mol/l HCl solution. A blank sample was simultaneously titrated using functional flour instead of its ester.

Proton nuclear magnetic resonance spectroscopy (¹H NMR)

¹H NMR spectra of the samples were recorded using a Bruker Avance III spectrometer (Billerica, MA, US) at 500 MHz. The samples were dissolved in DMSO-d₆ (99.8% D, Armar Chemicals, Döttingen, Switzerland) and the solution was then heated at 70 °C in a water bath for 12 h. Next 0.70 ml of the solution was placed in a 5 mm NMR tube. All of the NMR measurements were carried out at 22 °C with a 5 mm BBI probe head and z-gradient coil. Prior to Fourier transformation of the FIDs, the Lorentzian apodization function (0.3 Hz) was applied and the spectra were zero-filled twice. These measurements were carried out at the Centre of Molecular and Macromolecular Studies (Polish Academy of Sciences, Lodz, Poland).

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of the samples were recorded using a Genesis Series FTIR™

spectrophotometer (Unicam, ARI Mattson, USA). The powdered samples were mixed with an analytical grade KBr and then pressed into discs. Their spectra were recorded in the range of 4000 – 400 cm⁻¹, with 16 scans in total. The accuracy of the reading position of characteristic bands of the FTIR curves was ±1 cm⁻¹.

Scanning electron microscopy (SEM)

Morphological changes in the samples were observed using a scanning electron microscope – ESEM type Quanta 200 of FEI Co (USA).

Physical-mechanical properties

Physical-mechanical testing of pellets was carried out according to appropriate Polish standards using a testing machine for static tests (Instron). The max. stretching force, tensile strength, elongation at max. strain and Young's modulus were measured according to the PN-EN ISO 527-3:1998 standard and compressive strength - PN-EN ISO 604:2006 standard.

Wettability of powders - sessile drop method

According to the European Pharmacopoeia 8.0. method, samples of powder were compacted as a disc in an attempt to make the surface smoother. For this purpose, the powder was moulded and pressed at a pressure of 7.85 MPa. A drop of water (volume 0.004 ml) was placed on the disc, allowing direct measurement of the contact angle by geometric construction on a photomicrograph using Lorentzen-Wette AB equipment.

Preparation of pellets

After modification, Q-Farin was mixed with glycerol 20 wt.% at 75 °C for 30 min. The mixture was conditioned for 24 hours in a sealed container and then extruded using a co-rotating twin screw extruder having eleven heating zones with a simultaneous degassing of volatile components in the sixth zone of the plasticizing unit. The extrusion process was carried out using the following temperature profiles 40/100/120/120/120/120/120/120/105/120/120 °C, in which the screw rotation was 50 r.p.m. Finally – granulation. The preparation of pellets of unmodified functional flour was similar, but the addition of 25 wt.% of glycerol was needed.

Table 1. Enzymatic esterification of starch-protein material using various acyl donors on a small scale (Variant 1) and bigger one (Variant 2): **Process conditions:** Variant 1: vol. of chloroform: 2 ml, conc. of starch-protein material 10% (w/w), molar ratio of the starch part of the functional flour (anhydroglucose unit, AGU) to the acyl donor (1:1), amount of enzyme 0.5 g per 1 g of the functional flour; time of reaction 6 h; Variant 2: vol. of chloroform: 500 ml, conc. of starch-protein material 10% (m/v), molar ratio of the starch part of the functional flour (anhydroglucose unit, AGU) to the acyl donor (1:1), amount of enzyme 0.25 g per 1 g of the functional flour; time of reaction 24 h.

Acyl donor	Degree of substitution	
	Variant 1	Variant 2
oleic acid	0.11	0.04
rape oil	0.06	0.01
vinyl laurate	0.05	0.01

Results and discussion

Reaction on a small scale (Variant 1)

At the first stage of the research an organic solvent had to be chosen on a small

scale which would be an alternative solvent for DMSO and DMF for this kind of reaction. In this step oleic acid as an acyl donor acid was used. Examined was the possibility of using as a reaction medium

a number of solvents more hydrophobic than DMSO and DMF, including petroleum ether, hexane, acetonitrile, acetone and chloroform. In all trials a molecular sieve was introduced to remove water produced in the reaction from the reaction medium. The best results were obtained in chloroform. In the next step esters (vinyl ester of lauric acid and rape oil – ester of long-chain fatty acids and glycerol) as co-substrates in chloroform were used. Results are presented in **Table 1** (Variant 1) as a degree of substitution (determined by the volumetric method). The degree of substitution of a starch ester (DS) can be defined as the number of hydroxyl groups that are substituted by acyl groups in each anhydroglucose unit. The theoretical maximum value of the DS is 3, because each glucose unit contains three reactive hydroxyl groups. In the material tested the starch content was 55 w/w % in the initial material containing 6% moisture, and after drying at 105 °C (first stage of the process described in ‘Process of enzymatic modification’) the content of starch in the dry mass of the material was 58.5 w/w%. In this case the maximum value of the DS (calculated from the dry weight of the total product) is 1.75.

The products of esterification of the functional flour with the highest degree of substitution were observed in reactions carried out at 50 °C for 6 h. It was not necessary to extend the reaction period. The best result - DS 0.11 – was observed for oleic acid as the esterifying component (**Table 1**). To confirm the esterification reaction, a sample of the best product was subjected to Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) analyses. FTIR spectra of the functional flour (Q-Farin C1000) and Q-Farin oleate are presented in **Figure 1**. In the spectrum for the initial material, a strong broad band appeared with three peaks between 900 and 1200 cm⁻¹. These can be attributed to C O stretching vibrations, which are the most characteristic for polysaccharides (starch fingerprint) [13]. Peaks at 1300 – 1720 nm are assigned to specific protein components amide I (1580 – 1720 cm⁻¹) and amide II (1480 – 1580 cm⁻¹) (protein fingerprint) [14]. Other characteristic bands between 3000 and 3500 cm⁻¹ can be assigned to stretching vibrations from hydrogen-bonded hydroxyl groups both in the proteins and starch. In the FTIR spectrum of the Q-Farin oleate a new band

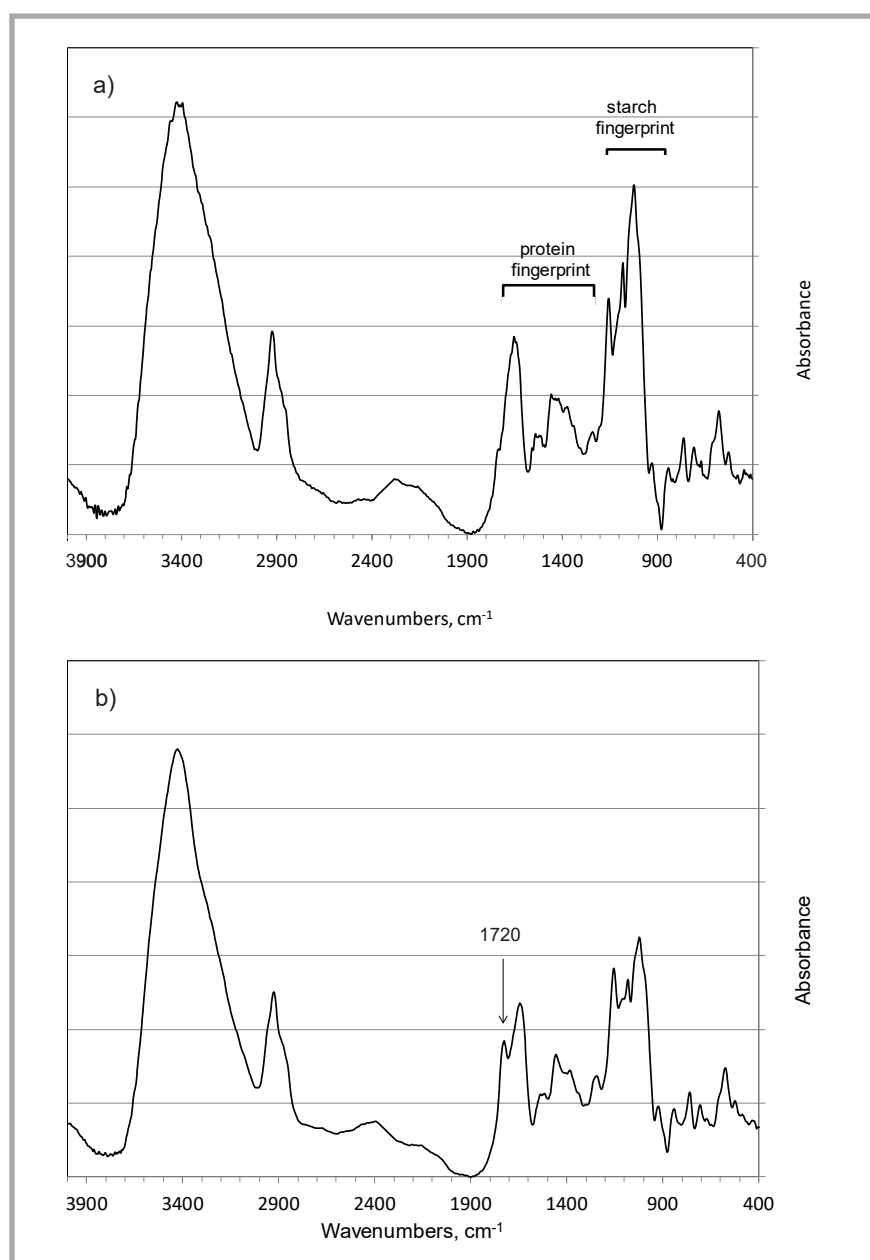


Figure 1. FTIR spectrum of initial Q-Farin C1000 (A) and Q-Farin oleate (B).

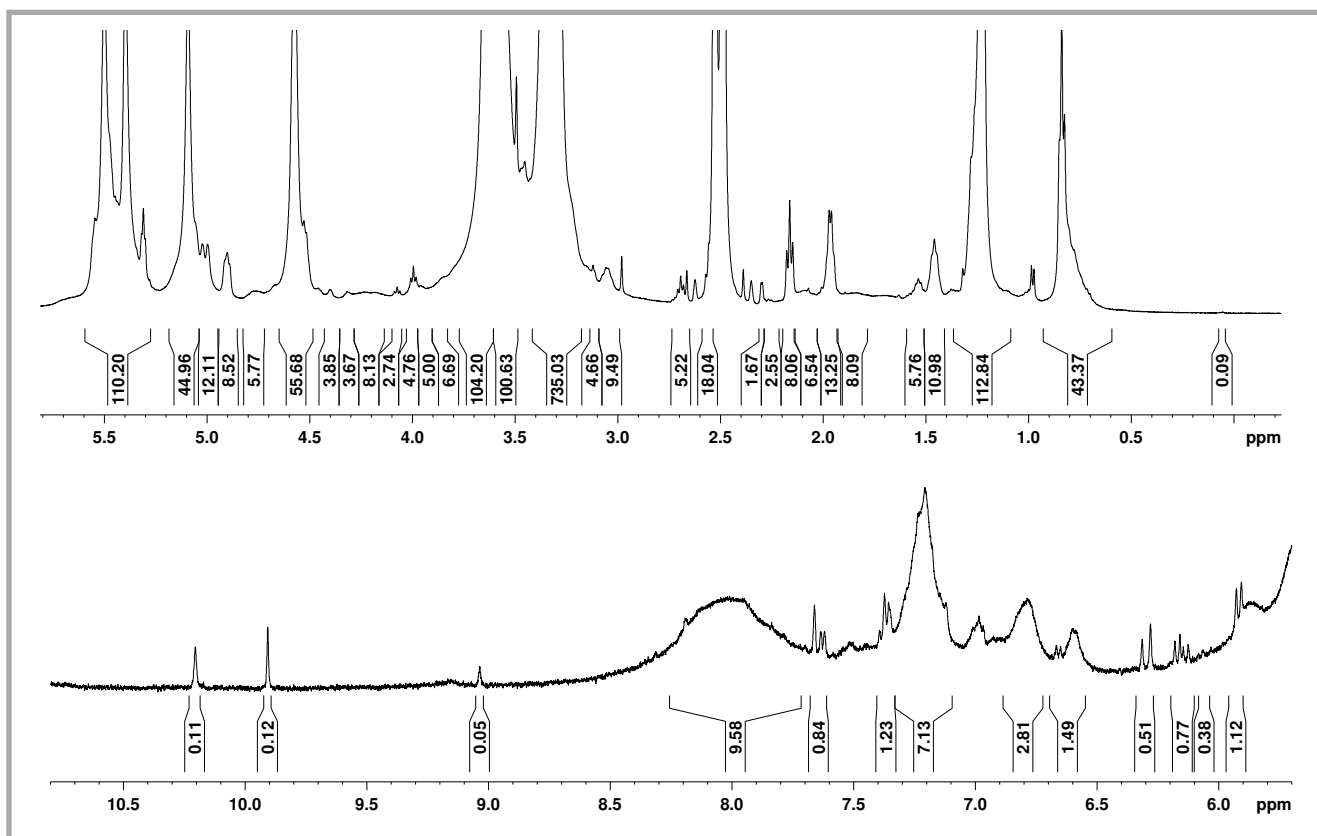


Figure 2. ^1H NMR spectra of Q-Farin oleate (with the DS 0.11).

appeared at 1720 cm^{-1} . The occurrence of this band is attributed to the carbonyl group in the ester, which is confirmation of the esterification of the starch part of the Q-Farin with oleic acid [15].

^1H NMR spectra for the Q-Farin C1000 oleate with a DS of 0.11 are presented in **Figure 2**. Chemical shifts are reported in parts per million. During esterification the starch part of Q-Farin was modified, and consequently the signals characteristic for starch oleate were observed. The NMR showed signals from three protons (a triplet peak) at around 0.8 ppm, which can be linked to the protons in the terminal methyl group of the acyl chain. The proton signal at 2.2 ppm may be referred to the methylene group, which occurs before the carbonyl group, and another proton peak at around 1.5 ppm is associated with the methylene group directly before it. Oleic acid is monounsaturated fatty acid, hence the signal at 5.3 ppm in the spectrum may be related to the protons associated with carbon atoms which are connected by a double bond, and at around 2.0 ppm signals of the methylene groups close to this double bond can also be observed. All the other groups included in the acyl

chain are observed as a signal around 1.25 ppm. [13]

In **Figure 3** the morphologies of the initial functional flour and Q-Farin oleate (DS 0.11) are presented. It can be seen in the SEM images that due to the hydrothermal treatment of flour there is no regular rounded starch granules in Q-Farin C1000. SEM measurements of Q-Farin indicate particle diameters in a wide range of about 5 to $100\text{ }\mu\text{m}$ and the shape is irregular. **Figure 3.B** illustrates that the modification of Q-Farin C1000 by enzymatic treatment in chloroform as a reaction medium altered the structure of the particles. The granules are

conglomerated, and the size observed is between approx. 20 and $200\text{ }\mu\text{m}$. The surface of the new particles is smoother than the initial one.

In order to evaluate the hydrophobic properties of the esterified products, tests were conducted by means of the sessile drop method, according European Pharmacopeia 8.0, is designed to assess the wettability of powders, where the smaller the wettability, the higher the hydrophobicity of the product. Therefore the method may be used to characterise the hydrophobicity of compacted formulations. The test involves measurement of the contact angle. The contact

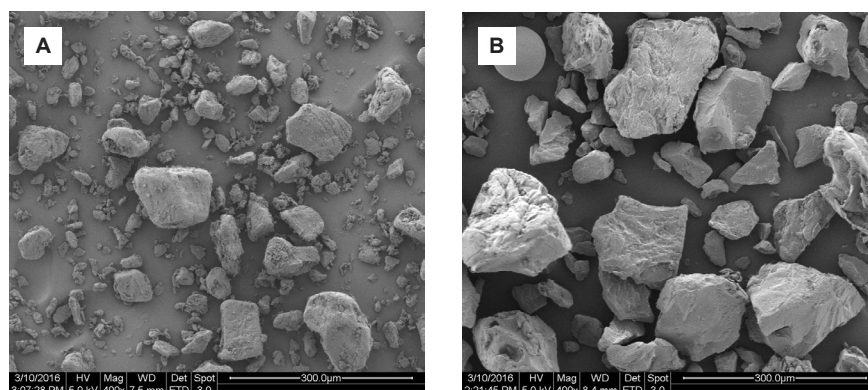


Figure 3. SEM images of initial Q-Farin C1000 (A) and Q-Farin oleate (B).

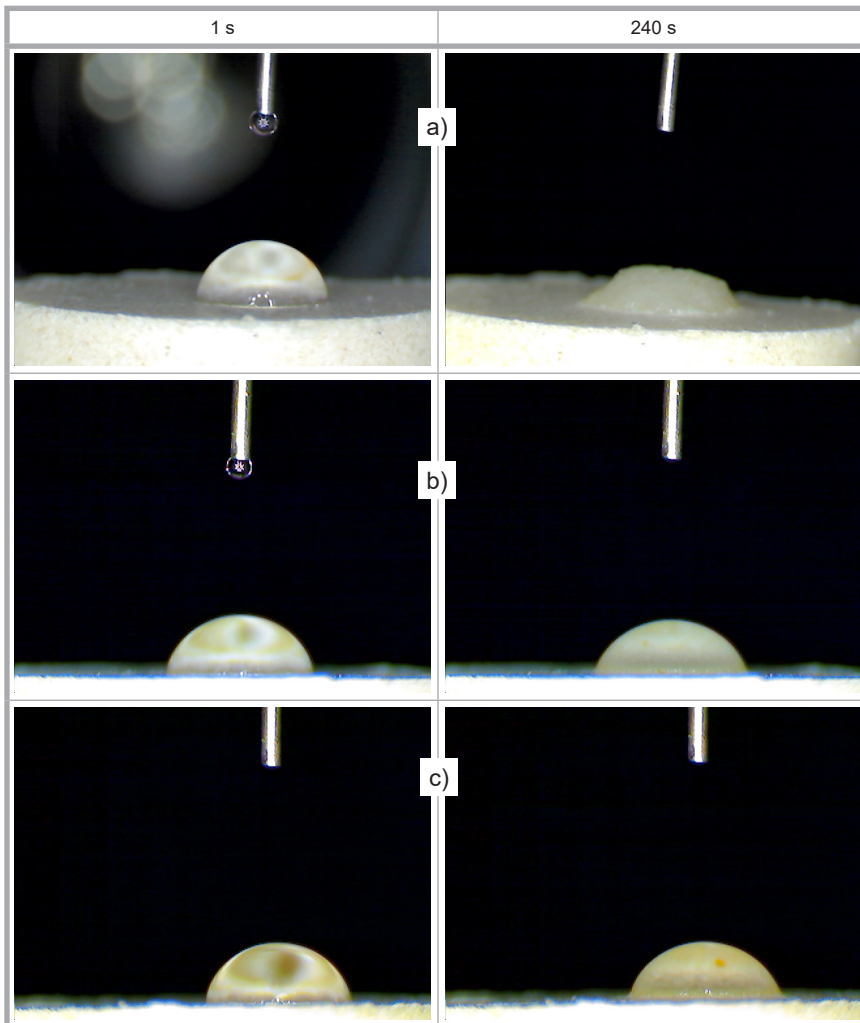


Figure 4. Drop of water on biopolymer discs made of Q-Farin C1000 and its derivatives; a) Initial Q-Farin C1000, b) Q-Farin oleate DS = 0.11, c) Q-Farin oleate DS = 0.04.

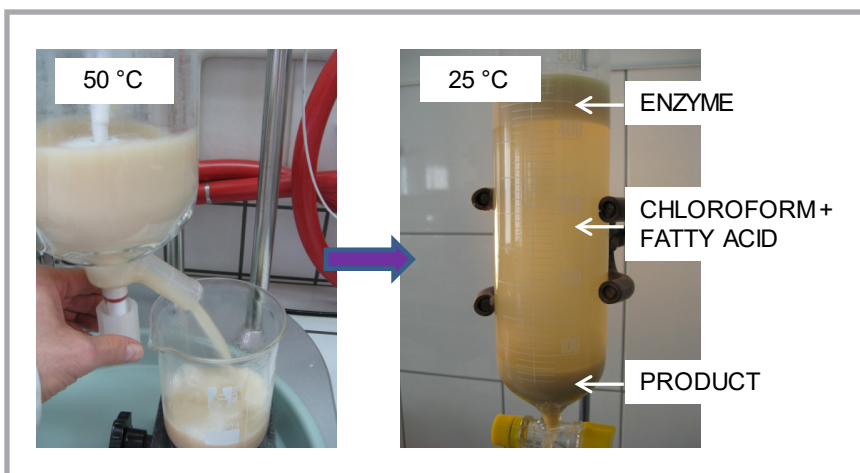


Figure 5. First step of downstream process using chloroform as a reaction medium.

angle (θ), occurring between a liquid and solid, is formed freely when a drop of liquid is placed on a solid surface. A small contact angle ($0 - 45^\circ$) proves very good wettability, and the bigger the contact angle using water as the wetting liquid means higher hydrophobicity of the ma-

terial. Moreover it is possible to use a sessile drop instrument in the dynamic measurement of porous solid/liquid systems where the contact angle decreases. By taking several contact angle measurements as a function of time, the rate of spreading accompanied by the penetra-

tion of a liquid droplet into a slightly porous solid may be studied.

The surface wettability of the initial functional flour and its acetylated derivative (DS 0.11) samples compacted as discs were determined (*Table 2*). The contact angle of this product is 11 degrees higher than for the initial material, indicating a higher hydrophobicity of the Q-Farin derivative. The lower change in the contact angle for modified flour compared to the initial one also shows a lower penetration of water into the disc. This result demonstrates the lower wettability of oleate than that of Q-Farin C1000. *Figure 4* shows drops of water on the initial Q-Farin and its derivative in 1 s and after 240 s of application. After 240 s on the disc of oleate the drop is still visible. After the same time on the disc of the initial material there is not a water drop visible, only the swelled flour.

Increasing the scale of the process (Variant 2)

To enable the practical use of esterified Q-Farin derivatives, it was necessary to scale up the process. Therefore tests were carried out in a 1 l chemical reactor (Radleys Reactor Ready). Trials were carried out using all three acyl donors applied in the first step. Due to the high price of the catalyst, it was decided to reduce the addition from about 0.5 g per 1 g of Q-Farin to 0.25 g per 1 g. In order to obtain a product with maximum efficiency, it was necessary to extend the process to 24 hours. Unfortunately for all co-substrates the efficiency of the process decreased dramatically (*Table 1*). The best result - DS 0.04 was obtained for oleic acid.

However, there were a number of other advantages. The post-reaction mixture in the modification process in DMSO comprising esterified Q-Farin, enzyme, DMSO and oleic acid has the consistency of dough. The separation of the product from the other components of the mixture is time consuming and requires large amounts of ethanol. The enzyme separated is no longer active (own study, unpublished). However, in the case of using chloroform as the reaction medium, the separation of layers takes place during the cooling of the reaction mixture (*Figure 5*). Components of the reaction mixture can be separated in an easy way and the enzyme is still active. The enzyme recovered was then used in six subsequent

Table 2. Contact angle and change in the contact angle for Q-Farin C 1000 and its derivatives.

Material	Contact angle (θ), degrees	Change in contact angle, degrees/s
Q-Farin C1000	79	0.083
Q-Farin oleate DS 0.11	90	0.050
Q-Farin oleate DS 0.04	84	0.075

Table 3. Physical-mechanical properties of pellets from plasticized starch-protein raw materials.

Polymer pellet	Max. stretching force, N	Tensile strength, MPa	Elongation at max. strain, %	Young's modulus, MPa	Compressive strength, MPa
Initial Q-Farin C1000/ glycerol (25%)	4.2	0.8	104	1.4	1.4
Q-Farin oleate DS = 0.04/ glycerol (20%)	27.2	4.4	73	31	11.2

reactions and the product was obtained with the same yield.

The Q-Farin oleate of DS 0.04 obtained is characterised by improved hydrophobic properties compared to the initial flour (Table 2, Figure 4). Even such a low degree of substitution is sufficient to decrease the water wettability of the product. This Q-Farin derivative plasticized with glycerol was extruded and cut in the form of pellets which can be used in further processing operations for the preparation of films and mouldings. In the same way the pellets were prepared from unmodified Q-Farin, as described in 'Preparation of pellets'. The preparation of pellets from both materials was possible, but in the case of modified Q-Farin it required the use of a smaller amount of glycerol. Strength parameters of the two kinds of pellets are shown in Table 3. The pellets obtained from the esterified starch-protein raw material have superior physico-mechanical properties.

Conclusion

Chloroform was selected as an alternative solvent DMSO as the reaction medium for enzymatic esterification of starch products. Acylated Q-Farin derivatives were successfully synthesised by immobilised lipase *C. antarctica* using different types of acyl donors as co-substrates. The advantages of the solution developed over other solutions that use DMSO or DMF as the reaction medium are as follows: much easier separation of the product from the reaction medium during the downstream process, the ability to separate the immobilised enzyme from the product after the process while the separated enzyme still maintains its activity and it is possible to use it in the next step with similar efficiency, and the ability to accurately remove residual chloroform

from the product due to the much lower boiling temperature.

The Q-Farin derivative modified with long-chain fatty acid obtained has better hydrophobic properties compared to unmodified starch-protein material. And the pellets obtained from the esterified material are characterized by better physical-mechanical properties. These materials based on modified flour could be used to produce new biodegradable packaging materials. According to the opinion issued by COBRO - Packaging Research Institute, there are no contraindications to using this hydrophobic solvent in the process of polymer modification and the product modified to obtain foil packages.

Editorial note

The problems discussed in this article were presented on the 17th European Congress of Biotechnology ECO 2016 Cracow, Poland, by D. Ciechańska, L. Kaszuba, J. Kapuśniak ('Modification of Starch-protein Raw Materials for New Usable Products').

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