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THE OBTAINING OF STARCH – AND OLEIC ACID – BASED ESTER AND ITS PROPERTIES

S u m m a r y

Nowadays, esters of saccharides and fatty acids are obtained mainly using chemical methods. An alternative method of saccharide esterification with fatty acids is a process using a lipase biocatalyst. The objective of the study was to develop a method to enzymatically esterify starch with a lipase preparation isolated from the *Candida antarctica* yeast. The esterification reaction of starch was performed at a low temperature (60 ºC) using non-toxic reagents. The reaction medium was also non-toxic. The obtained product of this reaction was a starch oleate that could be a thickening agents' constituent owing to its hydrophilic and hydrophobic properties.

The starch ester obtained through the enzymatic esterification reaction was analysed using a HNMR (Nuclear Magnetic Resonance) technique. The degree of substituting starch with oleic acid was DS = 0.13. The DSC (Differential Scanning Calorimeter) analysis proved that the enzymatic modification of starch caused the thermal characteristics of the product obtained to change. The heat appearing specific for the starch oleate phase transition was three times lower than that of the native starch. The correlation was also determined between rheological properties of water suspensions of starch and starch oleate and temperature. The starch oleate was characterized by a rapid increase in its viscosity during the initial phase of heating and, as the temperature rose, by a slow decrease in its viscosity.

Key words: starch, esterification, lipase, oleic acid

Introduction

Owing to their properties, saccharide and fatty acid-based esters are produced on an industrial scale. Saccharose is used to esterify fatty acids including long-chain fatty acids. Saccharose esters are the food ingredients referred to as E 473 [11]. At present,

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research studies are carried out on the synthesis of such esters as: maltose [7], fructose [12], and glucose [4], as well as on the starch-based esters [2, 3, 8].

Saccharide-based esters have many free hydroxide groups; therefore, they exhibit high affinity to water molecules. A fatty acid esterified in sugar plays a role of the alkyl (hydrophobic) chain. Owing to this structure, the saccharide- and fatty acid-based esters are able to form thermostable emulsions. The esters of the compounds as mentioned above, which are the ideal non-ionic surfactants, exhibit emulsifying and stabilizing properties along with other physical and chemical properties, which make them suitable for industrial applications.

Today, the saccharide- and fatty acid-based esters are mainly produced with the use of chemical methods. The chemical synthesis of esters must be performed in the media that contain polar organic solvents, such as: pyridine, dimethylosulfoxide (DMSO), and NN-dimethylformamide (DMF). Those solvents are very toxic to humans, thus, it is impossible to apply them in the food industry. The esterification can also be successfully performed without a solvent, at a melting temperature of the saccharide (169 - 170 °C). However, at these temperatures, discoloration (darkening) of the product occurs. When chemical methods are used, the blends of mono- and polyesters are produced instead of one ester only.

The lipase-catalyzed esterification is a good alternative to the chemical methods. With the use of this enzyme, the esterification reaction is performed either in the medium containing apolar solvents or in a two-phase (water-solvent) system. The reaction temperatures are much lower (30 - 50 °C), when the esterification is performed using chemical methods. Specific actions of the enzymes affect the resulting structure of the products that are free of any discolorations [5, 6, 15, 17]. However, the enzyme substitution is expensive and therefore not used on a large scale.

The sources of lipases are bacteria, yeast, fungi, and, also, some plant and animal species [13]. The lipases are glycoproteins (containing 2 - 15 % of carbohydrates, mainly mannose) active within a wide range of pH and temperatures [14].

Up to date, the starch has been esterified with long-chain fatty acids, using, mainly, chemical methods. Thus, the esters obtained exhibit thermoplastic properties similar to those found in the low-substituted starch. Consequently, the starch esters are suitable to produce biodegradable plastic products [2].

The objective of the present study was to esterify starch with the use of a long-chain fatty acid and with yeast enzymes.

Material and methods

The study material comprised: *Candida antarctica* yeast obtained from the company '*Deutche Sammlung von Mikroorganismen und Zellkulturen GmbH'*; potato starch called "Superior" and manufactured in a potato plant '*Przedsiębiorstwo Prze-*

mysłu Ziemniaczanego S.A. in Niechlów; oleic acid (*Fluka Chemika* Co.); lauric acid (*Przedsiębiorstwo Odczynników Chemicznych S.A*, Gliwice); tert – butanol (Fluka Chemika Co.); a Sigma - Aldrich molecular sieve (4 Å, 8 - 12 mesh); analytically pure acetone (Przedsiębiorstwo Odczynników Chemicznych S.A., Gliwice); olive oil (Aceites Ybarra S.A., Seville, Spain), arabic gum, (Fluka Chemika); and yeast culture of *Candida antarctica.*

The yeast culture of *Candida antarctica* was developed on an YM medium, at 30 ºC, during a 5 day period.

Isolating the extracellular lipase from a culture medium of the yeast of Candida antarctica sp.

The culture medium, containing the *Candida antarctica* yeast and all the enzymes present in the yeast, was centrifuged in a K80 centrifuge (MLW Co.) and, next, the supernatant obtained was separated from the yeast sediment. Afterwards, the supernatant containing lipase (separated from the yeast) was densified, using a laboratory vacuum evaporator, and lyophilized for further densification and reduction of the moisture content. The resultant lipase called lyophylysate was refrigerated and stored in an airtight container. This procedure ensured the biological value and the structure of the enzyme.

Determining the lipolytic activity of the enzyme obtained during the research [1]

A mixture necessary to determine the lipase activity was prepared in 150 ml conical flasks. It contained 50 ml of olive oil, 10 ml of 5 % solution of arabic gum, and 40 ml of distilled water. A 20 ml portion of the mixture and 0.25 g of the enzyme preparation were placed in conical flasks. A control sample was one with no enzyme preparation added. The reaction was carried out in a shaker at 50 °C for 3 h. Afterwards, to each flask, 5 ml of 96 % ethanol and 75 ml of distilled water were added. The quantity of the released fatty acids was determined while titrating the samples in 0.05 M NaOH in the presence of phenolphthalein. The lipolytic activity was expressed in U (μM fatty acid produced in 1 hour by 1 g of enzyme).

Esterifying the starch enzyme with oleic acid [18]

The starch was dried in a vacuum drier until a stable dry matter was obtained [15]. Next, a blend containing 20.0 g of starch and 31.4 g of oleic acid (1 mol equivalent of glucose corresponds to 1 mol of fatty acid) was prepared and supplemented with 2.5 g of the isolated enzyme. The medium contained 102.8 g of tert-butanol (a double weight equivalent of saccharide and fatty acid). The resultant water (a by-product of

the esterification) was removed using a 10.3 g molecular sieve (4 Å, 8 - 12 mesh; 0.2 – fold weight equivalent of saccharide and fatty acid). The reaction was performed with constant shaking (300 rev/min) at 60 °C for 28 days. Afterwards, the mixture was washed with acetone and, next, filtered. The sediment from the filter was dried at a room temperature and placed in an air-tight container. The resultant starch oleinate was the subject of subsequent research. Simultaneously, an identical reaction of starch esterification with fatty acid without using lipase was performed as a test reaction to demonstrate the effect of enzyme on the process of starch esterification.

Determining the esterification level with the use of a 1H NMR method (Nuclear Magnetic Resonance)

The starch oleinate was subject to analysis of 1H NMR spectrum using an Avance AMX 300 spectrometer (Bruker Co.). Prior to analysis, the samples were dissolved in a blend of DMSO and water (ratio of 9:1). The analysis was carried out at 70 °C. The degree of substitution was determined as a 1/3 ratio of the signal surface of the final CH_3 group of oleic acid, at 0.85 ppm to 1/7 of the surface of proton signals of the glucose ring in the starch chain, ranging from 3.1 to 3.9 ppm, and at 5.1 ppm.

Analysing the functional groups present in the tested specimens by means of an FTIR absorption spectroscopy

In order to confirm the existence of an ester bond, the pills of the tested material compressed with KBr were analysed using an FTIR absorption spectroscopy with a Mattson FTIR-300 spectrometer.

Determining the thermal characteristics of the substance using an 822e DSC (Mettler – Toledo)

The starch oleinate portions of 5 mg (in conversion to dry matter) were placed in 100 μl aluminum containers, to which distilled water was added, and its quantity was three times higher than that of the ester. The samples underwent a conditioning process in closed containers at a room temperature for 30 minutes. Afterwards, they were placed in a DSC furnace and kept at 30 °C for 5 minutes. The measurements were made while heating them to 100 °C at a rate of 4 °C/min.

The measurements comprised the initial and final temperatures of changes observed at different phases, the temperatures of an extrapolated peak centre, and the heat appearing specific for phase changes.

Determining the viscosity of solutions with a Haake RS 50 Rheostress rheometer

50 cm^3 portions of 2 % suspensions of starch and starch oleate were prepared. They were analysed in a rotational mode using a measuring unit type DG 41. The 6.3 cm^3 of the above-mentioned suspensions were put into a cylinder.

The viscosity of the solutions was measured under the following conditions: shear force 200 s^{-1}; range of temperature between 30 °C and 85 °C, at a heat rate of 1.4 °C/min.

Results and discussion

The activity of the lipase, obtained from the *Candida Antarctica* yeast according to the procedure as described above, was 0.1 U/mg and it was sufficient to efficiently perform the starch esterification using this enzyme. The product obtained during the starch esterification with the use of lipase was a white powder containing two fractions of varying solubility values. One of them was soluble in a blend of DMSO (dimethyl sulfoxide) and water. The highest solubility was found at a DMSO-to-water ratio of 9:1, at 70 °C . The second fraction was neither soluble in the DMSO-water blends prepared from different quantities of water nor in the chloroform at a temperatures in various temperature ranges. The above two solvents were used pursuant to the ^{1}HMNR analysis requirements.

The product obtained was then analysed using MNR (Fig. 1).

The analysis of the ^{1}HNMR spectrum showed that the product obtained was an ester (of potato starch and oleic acid). Moreover, the FTIR analysis confirmed the existence of an ester bond (signal at 1715 cm^{-1}) in the product tested, which was an explicit proof of the occurrence of enzymatic reaction.

The degrees of starch substitution were calculated with regard to the spectra obtained [3]. The degree of substitution shows the number of hydroxyl groups in a glucose molecule, which have been substituted for fatty acid. Theoretically, its maximum value is around 3 [9, 10]. The degree of starch substitution with oleic acid determined with regard to ^{1}HNMR spectrum was 0.13. This means that each eighth glucose molecule in a starch chain possesses one hydroxyl group substituted with fatty acid. No data are available in the reference literature, which would make it possible to compare them with the results obtained under the present study. So far, no data have been reported on potato starch esterification with a long-chain fatty acid using an enzymatic method.

It is likely that the substitution degree of a fraction insoluble in a solvent is much higher than that of the soluble fraction. Similarly, the acetylated starch (DS 2.97) and its products, obtained through chemical esterification [3], were hardly soluble (the degree of substituting the acetyl-caprylic starch with caprylic acid was DS = 0.51 and with acetic acid DS = 2.51).

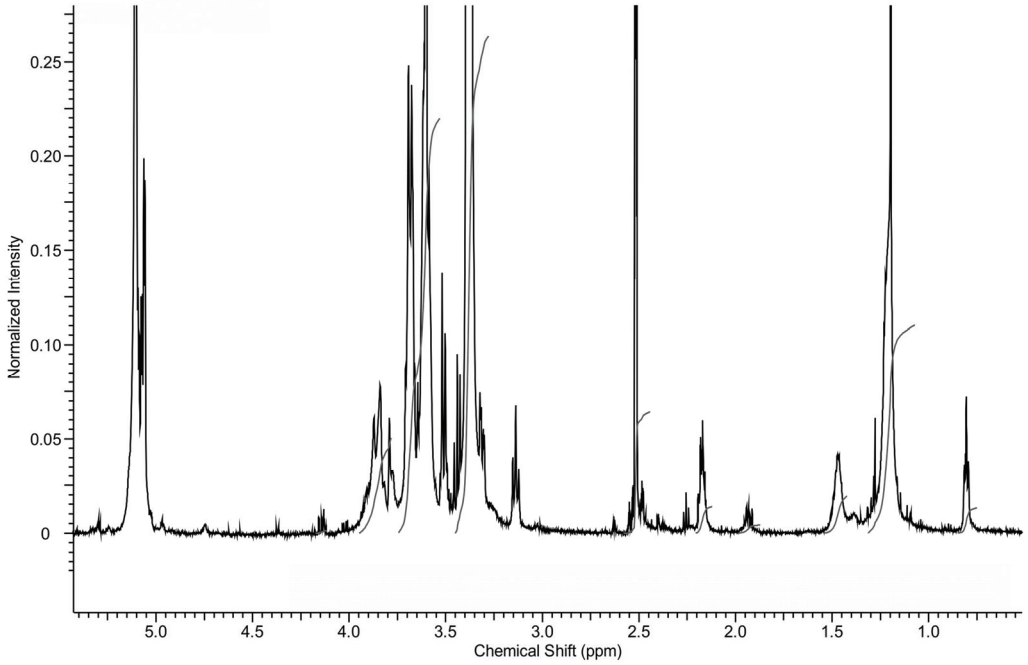

Fig. 1. ^{1}HNMR spectrum of starch oleinate.
Rys. 1. Widmo ^{1}HNMR oleinianu skrobi.

The ^{1}HNMR and FTIR analyses of the reaction product without using enzyme did not show any chemical changes in the starch chain. It means that the starch esterification reaction with fatty acid takes place only in the presence of the lipase enzyme obtained from the *Candida antarctica*.

Starch modification causes changes in the thermal characteristics of the subsequent product determined with the use of a differential scanning calorimeter (DSC). The analysis included a starch oleinate obtained by the enzymatic method and a natural starch (for comparison) used as a substrate during the ester production. The measurements comprised the initial and final temperatures of changes observed at different phases, the temperatures of the extrapolated peak centre, and the heat appearing specific for phase changes (Fig. 2).

The phase changes in the natural starch occurred at the temperatures similar to those occurring when the starch was modified to ester. A pasting process of the starch ester, soluble in water containing DMSO (presumably with a low DS), began at 60.23 ºC and was accomplished at 71.16 ºC. The similar temperatures were observed when pasting the natural starch granules, which were not substituted with fatty acid. However, the heat appearing specific for the phase changes in the starch oleinate was about three times lower (4.31 $J \cdot g^{-1}$) compared to the native starch (12.07 $J \cdot g^{-1}$). This

finding confirms that the starch was substituted with oleic acid; the same was also proved by HNMR.

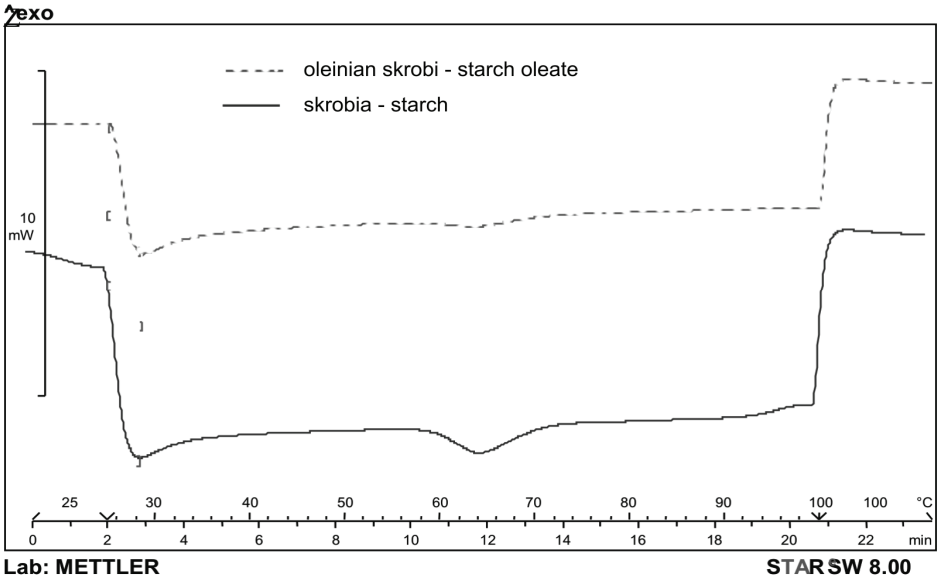

Fig. 2. Thermal characteristics of aqueous solutions of modified starch and its ester as measured by DSC.

Rys. 2 Charakterystyka termiczna DSC wodnych roztworów skrobi modyfikowanej oraz oleinianu skrobi.

Fig. 3 and 4 show changes in the viscosity of starch pastes and starch oleinate suspensions in water, depending on temperatures.

The heating of 2 % starch pastes slightly reduced their viscosity, when the temperature was increased to 70 ºC whereas their viscosity increased at temperatures >70 ºC. A contrary dependence was found as for the starch oleinate that exhibited an increasing viscosity at the beginning of the heating treatment, but, after some time, its viscosity began to decrease. The maximum viscosity of starch oleinate was $7.09 \cdot 10^{-3}$ Pas at 47 ºC. The viscosity of the starch at the same temperature was twice as low ($3.44 \cdot 10^{-3}$ Pas). This characteristic of esters could be utilized to produce densifiers, used, for example, in a variety of „convenience food" (desserts, instant soups, etc.). The reagents and solvents used in the enzymatic starch esterification with fatty acids are totally removed after the process is accomplished, i.e. during the extraction and steaming.

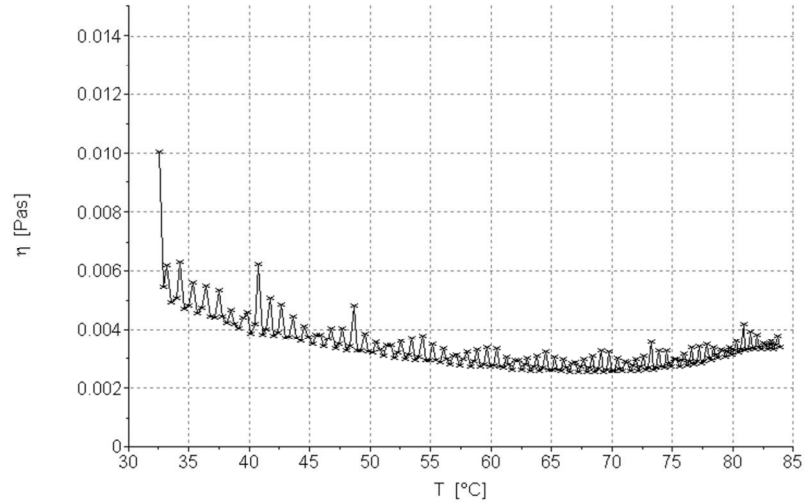

Fig. 3. Dependences between viscosity of 2 % starch paste and temperatures.
Rys. 3 Zależność lepkości od temperatury 2 % kleiku skrobiowego.

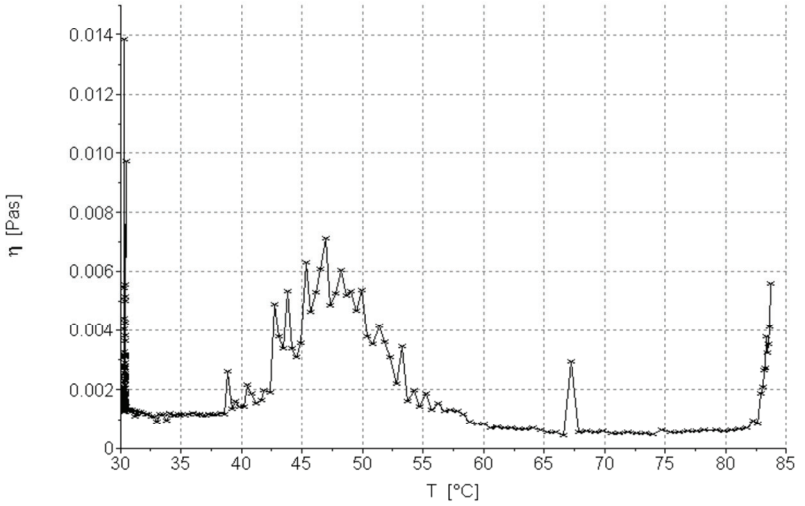

Fig. 4. Dependences between viscosity of 2% water suspension of starch oleinate and temperatures.
Rys. 4. Zależność lepkości wodnej zawiesiny oleinianu skrobi od temperatury 2 %.

An ester of the potato starch and oleic acid was the final product obtained when applying the method as developed and described above of the enzymatic esterification of the starch with the use of preparation of lipase from the Candida antarctica yeast. The degree of starch substitution (DS) with oleic acid in the ester fraction solvable in

the 9:1 mixture of DMSO and water, was 0.13 as determined based on the ^{1}HNMR spectrum. The reaction proceeded at a low temperature (60^o C) with non-toxic reagents and a non-toxic reaction medium. The product of this reaction, starch oleate, could be a constituent of thickening agents owing to its hydrophilic and hydrophobic properties.

Conclusions

1. The ester of potato starch and oleic acid was obtained with the use of a lipase isolated from the *Candida antarctica* yeast.
2. The degree of substituting the starch with oleic acid in the fraction of ester soluble in a 9:1 blend of DMSO and water was DS = 0.13.
3. The starch was esterified at a low temperature (60 ºC) with the use of non-toxic reagents and a non-toxic medium.

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OTRZYMYWANIE ESTRU SKROBI I KWASU OLEINOWEGO ORAZ JEGO WŁAŚCIWOŚCI

S t r e s z c z e n i e

Estry sacharydów i kwasów tłuszczowych otrzymuje się głównie metodami chemicznymi. Alternatywną metodą estryfikacji sacharydów kwasami tłuszczowymi jest sposób z użyciem biokatalizatora – lipazy.

Celem pracy było opracowanie metody enzymatycznej estryfikacji skrobi przy użyciu preparatu lipazy z drożdży *Candida antarctica*. Reakcja estryfikacji skrobi przebiegała w niskiej temperaturze (60 ºC), z zastosowaniem nietoksycznych reagentów oraz nietoksycznego środowiska reakcji. Otrzymany produkt – oleinian skrobi – dzięki właściwościom hydrofilowym oraz hydrofobowym może być składnikiem substancji zagęszczających.

Ester skrobi uzyskany za pomocą reakcji enzymatycznej estryfikacji poddano analizie HNMR. Stopień podstawienia skrobi kwasem oleinowym wynosił DS = 0,13. Analiza DSC wykazała, że modyfikacja enzymatyczna skrobi spowodowała zmiany właściwości termicznych uzyskanego produktu. Ciepło właściwe przemiany fazowej oleinianu skrobi było 3 razy mniejsze niż ciepło właściwe przemiany fazowej skrobi naturalnej. Określono również właściwości reologiczne zawiesin wodnych skrobi oraz oleinianu skrobi w zależności od temperatury. Oleinian skrobi charakteryzował się szybkim wzrostem lepkości w początkowej fazie ogrzewania i jej powolnym zmniejszaniem wraz z dalszym wzrostem temperatury.

Słowa kluczowe: skrobia, estryfikacja, lipaza, kwas oleinowy