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ENZYMATIC SYNTHESIS OF CYCLOOLIGOSACHARIDE ESTERS

Abstract

The aim of this study was to prove the possibility of obtaining amphiphilic esters of β-cyclodextrin in the way of its enzymatic modification involving lipase from porcine pancreas and fungal (Candida Antarctica) lipases. The study was focused to direct esterification processes of carboxylic acids of varying chain lengths and degrees of unsaturation, and indirect esterification using vinyl ester of fatty acids. Also an attempt was done in order to perform transesterification of edible oils (rapeseed and olive oil). The study found that it is possible to obtain low-substituted ester cyclodextrin mainly in the direct esterification. Transesterification of vinyl esters and vegetable fats lead to saponification of the output esters mainly and the degree of cyclodextrin substitution is small. Construction of esters obtained was confirmed using infrared spectroscopy. It was also found that the main limiting factor for the esterification process is a cyclic structure, cyclodextrins, as well as the ability to substrates complexation (acyl donor).

Introduction

Cyclodextrins (CD) are macrocyclic oligosaccharides, composed of residues of α-D-glucose, linked together by α-1-4 glycosidic bonds. There are a broad group of compounds, of which the most known natural homologues include: α, β, γ cyclodextrin. They consist of six to eight anhydroglucose units (AGU), but also higher homologs cyclodextrins are known [1].

The most important feature of CDs is the ability of cyclodextrins to form inclusion complexes of varying stoichiometries. In complexes CD play the role of a host, and the molecule penetrating the CD cavity without creating with him on the covalent bonds is a guest. Change in the properties of this molecule included in CD is obserwed by means of: solubility, resistance to temperature/radiation, reduction in volatility, the processes of light absorption, fluorescence and many others [2].

Cyclodextrin derivatives now have a wider application than the natural cyclodextrins. This is mainly because of changes in the solubility and stability of the complexes which are obtained by chemical modification of the cyclodextrin molecule by reacting by means of the esterification or etherification of the hydroxyl group by electrophilic attack [6].

CDs hydroxyl groups are located on the outer side of molecule on carbon C2, C3, and C6 and differ in reactivity. In the reactions of substitution usually a mixture of various substituted cyclodextrins is obtained. This can cause problems in reproducibility of different manufacturing batches. Unfortunately, most methods based on multi-stage synthesis using specific, but low selective methods known in organic chemistry. The resulting products are a mixture of the derivative with a different degree of substitution and the pure derivative is obtained by laborious chromatographic separation [2].

Enzymatic esterification of the carbohydrate with mono- and di-saccharides is a well known and important technical transformation [15], [16]. Apart from the chemical methods for their preparation they are known, however, also enzymatic sugars esterification processes. They use enzymes from the group of hydrolases mainly thermo-lysine, and lipase. Lipase in recent years was successfully used in the synthesis of low molecular weight esters, mainly due to the low price, availability, no need for coenzyme and the wide possibilities of catalytic. In esterification processes involving lipases activation mechanism of the catalysis probably involves the type of Ping-Pong BiBi mechanism [17]. Since lipases are effective catalyst in an anhydrous or low-water content and carbohydrates are hydrophilic compounds soluble in water, the enzymatic esterification of sugars is mostly carried out in an solvents such as DMSO, DMF, *tert*-butanol, pyridine, ionic liquids or supercritical CO_2. The largest number of reports on the use of lipase in the esterification of sugars refers to the glucose, fructose and sucrose [16]. Enzymatic esterification is also subjected to polysaccharides including starch [18]–[21] and cellulose [22]. On the other hand there is a little research conducted while the possibility of enzymatic modification of cyclodextrins. So far the only known transesterification processes of vinyl esters and CD in the presence of enzymes from the group of proteases was presented. These reactions are carried out in a medium of DMSO Niewiele badań jest natomiast prowadzonych nad możliwością enzymatycznej modyfikacji cyklodekstryn. Jak dotąd znane są jedynie procesy transestryfikacji estrów winylowych i cyklodesktryn w obecności enzymów z grupy proteaz. Reakcje te prowadzono w środowisku DMSO [23]. Development of a universal method for preparing amphiphilic cyclodextrin derivatives by enzymatic and low cost is an important task of modern synthetic chemistry.

Experimental

Synthesis of CD ester by means of enzymatic protocol

CD was pre-dried at 105 °C until dryness. Then the 5g (30mmol) of CD was dissolved in 100 ml of solvent (DMSO/DMF). After dissolution, 30mmol of carboxylic acid (lauric - LA, stearic - SA, oleic - OA, linolenic - LNA, erucic - EA), vinyl ester (laurate - LAW and vinyl stearate - SAW) or 1 g of vegetable oil was added. Once dissolved, to the mixture the appropriate amount of enzyme (ppl- lipase from porcine pancreas II - 700 mg (activity as the number of moles of p-nitrophenol released from the

ester of palmitic acid: 0,76 μmol·(min·mg)$^{-1}$), lipase CAL- Candida Antarctica - 100mg (activity as the above number of moles of 7,65 μmol·(min·mg)$^{-1}$) was added . Reactions were carried out in a water bath at 60 °C±1°C for 12 h. aided by mechanical stirring. By-product (water) was perceived from the reaction with 5 g of molecular sieves (4A, 8-12 mesh). After the reaction was finished, the mixture was centrifuged (20 min, 5000rpm). The solid (enzyme) was separated and the supernatant treated with acetone (250ml) to precipitate the product. After precipitation the precipitate was separated by centrifugation (20 min, 5000rpm) and washed with water (250ml). The system was centrifuged again and the solid (crude) product was dried and weighed.

Określenie liczby kwasowej w tłuszczach

Samples from the reactor (1 g) was weighed and transferred to a conical flask at intervals of every 60 minutes. To the samples 10 ml of ethanol were added to denature the enzyme. To the solution a few drops of phenolphthalein was added and the mixture was stirred. The titration solution using 20 mM NaOH was performed until color changes persisting longer than 30 seconds. The relative degree of hydrolysis (H) is determined as a percentage by weight of free fatty acids present in the sample relative to the total content of these acids in the sample [26].

FTIR Spectroscopy

Analysis was performer using Mattson/Unicam 1000 IR (Perkin Elmer) spectrophotometer, in the range of 400-4000cm^{-1} and resolution of 4cm^{-1}. The sample was prepared using KBr as a matrix.

Degree of substitution - DS_M

Degree of substitution (DS_M) was analyzed Rusing Miladinov saponification method [27].

Chromatographic analysis

Chromatographic analysis was performer using HPLC system Knauer (Germany) equipped with refractive index detektor as well as UVVIS detektor (Knauer, Germany). Analysis of CD and CD esters was performed using ACN/water 87:13(v/v) with 1 ml/min flow rate, NP-NH_2 Lichrosphere (250×4 mm, 5 μm) (Knauer, Germany) column and using 20 μl injection loop. Analysis of fatty acids was done according to literature [29].

Results and discussion

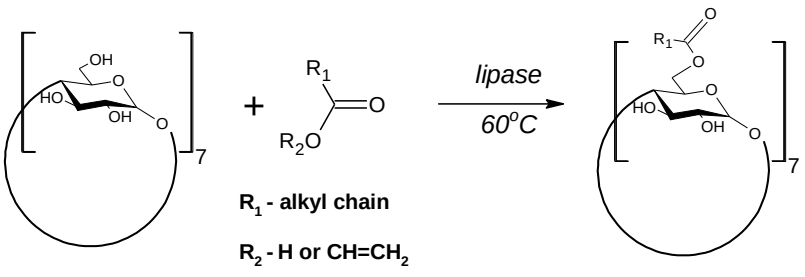

Figure 1. General scheme of CD direct esterification.

Studies on direct esterification (Figure 1) was performed using five carboxylic acids. As a result of experiments a range of products with different degrees of substitution (Table 1) was obtained. Considering the effectiveness of the process for the conversion of the acylating agent has been found that between the used acids, there are no significant differences in effectiveness. Conversion of the acyl donor in all cases was in the range of 7 - 12%. The only exception to this rule is the reaction in DMF (PPL) system with the participation of EA (5.4%). On the other hand, the highest conversion was found for the LA regardless of the enzyme used. The change does not affect the degree of conversion of different chain length of the acyl donor and the reaction medium. Discussed in the literature, the impact of the type of biocatalyst here is also small. From the literature data suggest that, in the esterification of saccharides fungal lipase (CAL) has a much higher activity than the lipase PPL [30]. The phenomenon was not reflected the results of the tests. Taking into account the results obtained it can be concluded that the limiting factor of the process is the molecular construction of a substrate specific cyclodextrin. Cyclooligosaccharides have a stiffening structure at the molecular level significantly restricting the conformational freedom [31]. In the case of enzymatic reaction it can lead to a reduction of matching substrate of the enzyme molecule at the binding step with CD. Another limiting factor may also be the ability to complex with CD-carboxylic acids.

The yields of crude esterification process (Table 1) do not exceed 25%. For acid ≥ C17 they are smaller than for the C11 acid. In all cases, greater efficiency is observed when the reaction is carried out in DMF as the reaction medium.

Tabela 1. The field of CD esterification by acids.

Acyl donor	Solvent	Yield, %	/Product purity ,%	Conversion, %	Degree of Substitution, %	Degree of Substitution based on Conversion, %	Yield, %	/Product purity ,%	Conversion, %	Degree of Substitution, %	Degree of Substitution based on Conversion, %
	PPL						CAL				
LA	DMSO	16	90	$9,9^{a}$ ±0,1	$3,24^{a}$ ±0,35	3.30^{a}±0,06	11	95	$8,7^{a}$ ±0,2	$2,93^{a,b}$ ±0,03	2.93^{a} ±0,14
	DMF	28	91	$11,6^{b}$ ±0,1	$3,69^{b}$ ±0,07	3.87^{b}±0,04	19	93	$9,2^{a}$ ±0,2	$2,91^{a,b}$ ±0,06	2.90^{a} ± 0,03
SA	DMSO	14	86	$7,9^{c,d}$ ±0,2	$2,59^{c}$ ±0,13	2.63^{c}±0,04	15	91	$7,6^{b}$ ±0,5	$2,51^{c}$ ±0,01	2.53^{b} ± 0,01
	DMF	19	89	$8,5^{c}$ ±0,2	$2,69^{c}$ ±0,42	2.83^{c}±0,21	17	87	$8,8^{a}$ ±0,4	$2,89^{a}$ ±0,03	2.95^{a} ± 0,03
OA	DMSO	15	81	$8,1^{c}$ ±0,2	$2,55^{c}$ ±0,01	2.70^{c}±0,42	18	87	$8,1^{b}$ ±0,1	$2,67^{b}$ ±0,07	2.70^{c} ± 0,03
	DMF	22	86	$8,7^{c}$ ±0,4	$2,84^{c,a}$ ±0,08	$2.90^{a,c}$±0,10	20	89	$9,1^{a}$ ±0,2	$3,01^{d}$ ±0,04	3.03^{a} ± 0,04
EA	DMSO	12	80	$7,1^{d}$ ±0,9	$2,83^{c,a}$ ±0,08	2.82^{c}±0,17	14	74	$6,7^{c}$ ±0,1	$2,21^{e}$ ±0,03	2.66^{c} ± 0,08
	DMF	15	80	$5,4^{e}$ ±0,8	$2,10^{d}$ ±0,21	2.14^{d}±0,20	15	85	$6,8^{c}$ ±0,2	$2,20^{e}$ ±0,04	2.70^{b} ± 0,06
LNA	DMSO	16	82	$7,9^{c,d}$ ±0,1	$2,59^{c}$ ±0,08	2.63^{c}±0,10	18	89	$8,0^{b}$ ±0,1	$2,42^{c}$ ±0,06	2.67^{c} ± 0,06
	DMF	19	85	$8,1^{c}$ ±0,2	$2,70^{c}$ ±0,06	2.70^{c}±0,07	21	86	$8,0^{b}$ ±0,1	$2,45^{c}$ ±0,07	2.67^{c} ± 0,03

Analyzing the distribution of the degree of substitution depending on type of agent acyl it is worth noting that the highest value of DS was obtained for lauric acid, and slightly lower for the group of C17 fatty acids. The lowest values were found for erucic acid. This relationship confirms that the complexion of the CD is not a factor inhibiting progress of the reaction. Considering the differences within the group C17 one can, however, noted that the degree of unsaturation, measured in terms of multiple bonds has no effect on the DS. Differences in the degree of substitution between the two applied enzymatic systems, namely CAL and PPL are not great. It appears that the more important in the analyzed context is the environment of the reaction. Reactions were conducted in DMF environment characterized by a greater degree of substitution. It may indicate a higher diffusion coefficients of carboxylic acids in DMSO than DMF. The values of these coefficients are not known. It is also worth noting that the obtained degree of substitution are close to the value of the conversion as determined by HPLC. Conversion of carboxylic acids with comparable effectiveness of two key enzyme systems (PPL and CLA) combined with significant differences in the price of these enzymes indicates rather PPL as a potential tool for further research and possible use of the process on a larger scale.

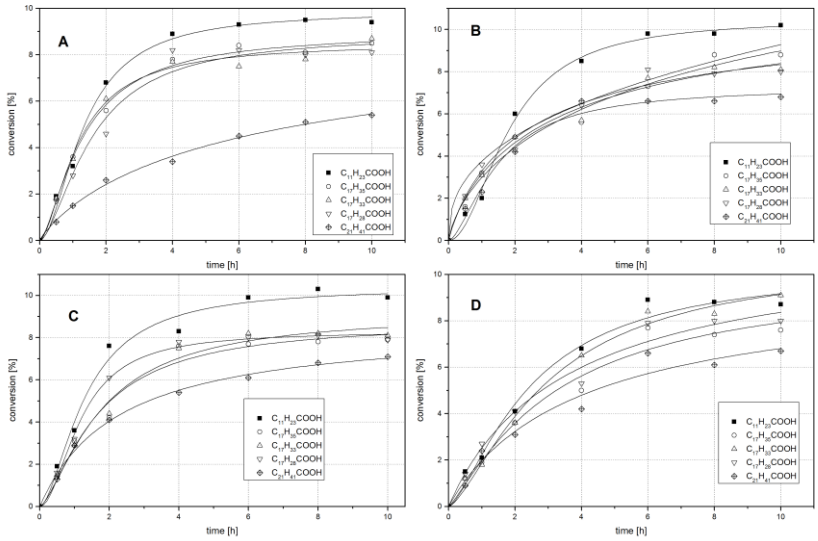

Figure 2. Dynamics of CD esterification: A - DMF/PPL; B-DMF/CLA; C-DMSO/PPL; D-DMSO/CLA

The study was also carried out experiments showing the dynamics of the esterification reaction as the change of the acyl donor over time (Figure 2). On this basis it can be stated that a C17 group of fatty acids shows similar behavior throughout the process. This applies to systems DMF/PPL, DMF/CLA and DMSO/PPL. Conversion of carboxylic acid in the process has a form characteristic of equilibrium processes. The establishing of equilibrium holds for the reaction with the PPL 6 (DMSO) or up to 4 hours (DMF). In the arrangement of CLA is slightly longer and starts after six hours, regardless of the solvent used.

In the study, experiments were also performed with the possibility of receiving laurates and stearates CD by transesterification according to the scheme shown in Figure 3.

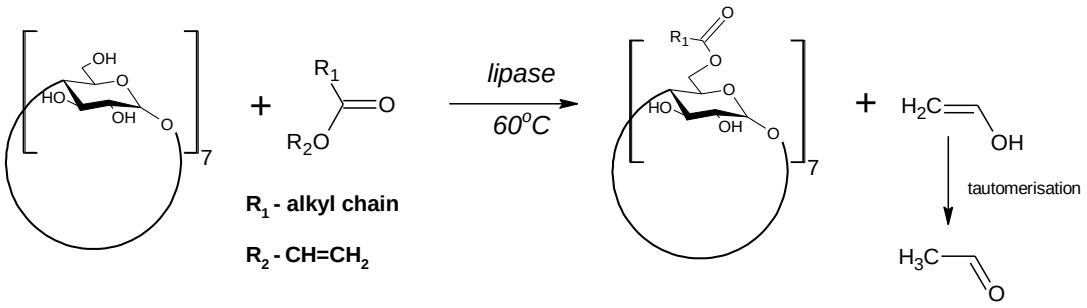

Figure 3. Transestrification of CD using vinyl esters

The degree of conversion of vinyl esters is much higher than for reactions involving carboxylic acids and are in most cases more than 50% (Table 2). At the same time the degree of substitution determined by saponification - D_{SM} is much lower than that of carboxylic acids does not exceed 2.5%. The highest degree of substitution was obtained in the case of vinyl laurate for both studied systems of catalytic amounts to 2.4% (PPL) and 2.2% (CAL). However, as before, in the case of the transesterification system of DMF/PPL has a higher efficiency compared to the DMSO/CAL. On the basis of the data obtained it can be concluded that the reaction is a series of subsequent reactions. In the first stage it comes to the hydrolysis of the vinyl ester. The products of this reaction are carboxylic acid and vinyl alcohol/acetaldehyde, and it proceeds with an acceptable efficiency (reacting up to 25%). In a further step, the reaction of a carboxylic acid with CD. This reaction also involved a lipase as a catalyst.

Tabela 2. Transestryfication of CD using vinyl esters

Acyl donor	Solvent	Yield, %	Product purity ,%	Conversion, %	Degree of Substitution, %	Degree of Substitution based on Conversion, %	Yield, %	/Product purity ,%	Conversion, %	Degree of Substitution, %	Stopień Degree of Substitution based on Conversion, %
		PPL					CAL				
SAW	DMSO	87	90	16,5[a] ±0,8	1,5[a] ±0,03	4,50[a] ±0,04	82	93	25,6[a] ±0,1	1,3[a] ±0,03	8,53[a] ±0,04
	DMF	86	92	18,9[b] ±0,4	1,7[a,b] 0,10±	6,30[b] ±0,03	88	90	23,8[b] ±0,1	1,3[a] ±0,07	7,93[b] ±0,11
LAW	DMSO	87	89	18,7[b] ±0,2	1,76[b] ±0,06	6,23[b] ±0,04	81	79	26,4[a] ±0,8	1,8[b] ±0,11	8,80[c] ±0,08
	DMF	84	85	19,9[b] ±0,1	2,4[c] ±0,11	6,63[c] ±0,10	83	90	20,1[c] ±0,2	2,2[c] ±0,08	6,70[d] ±0,07

In conclusion it can be stated that the systems are less suitable to give ester derivatives of the changes occurring on the same substrate hydrolysis more (vinyl ester), which does not increase the esterification process CD.

The study also attempted to transesterification of natural fats in the presence of lipase and CD. This reaction (Figure 4) as intended should lead to a statistical mixture of esters of cyclodextrins. It is also possible a formation of mixed and homogeneous polysubstituted systems.

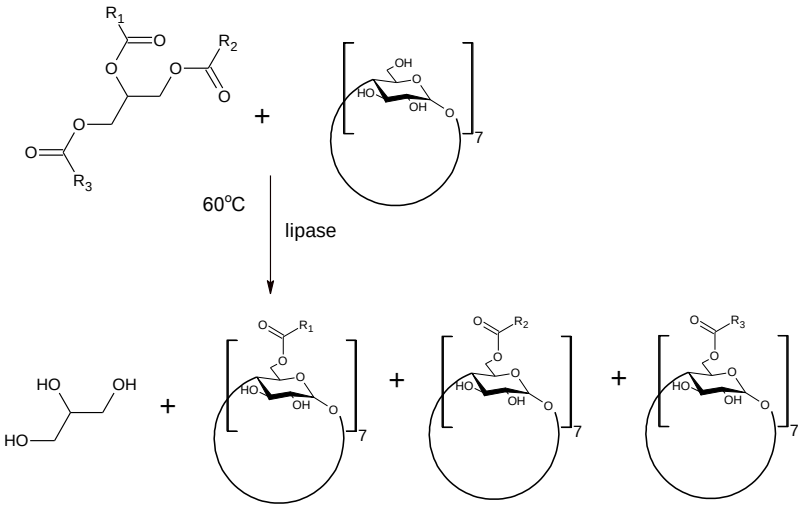

Figure 4. Natural fat tranesterification

Due to the possible side reaction or hydrolysis of triglyceride to free fatty acids and alcohol, studies on transesterification of fats were carried out by means of two way. First fat hydrolysis is monitored by determining the acid number (H). On the other hand the average degree of CD substitution was investigated (after separation from the reaction medium) by saponification method - D_{SM}. The test results are shown in Table 3 and in the form of fat hydrolysis of the progress curve over time (Figure 5).

Based on research carried out on two selected vegetable fats we found a high degree of conversion of triglycerides into free fatty acids. Depending on the enzyme used, after the process (10 hours), it extends on approximately 35% (PPL), and 44% (CAL) - rapeseed oil and 38% (PPL), and 43% (CAL) - for oil respectively. Analyzing the dynamics of the glyceride conversion (Figure 5), it was found that at the initial stage (about 4 hours) the reactions take place in a similar manner, regardless of the catalyst system. At a deeper stage, the efficiency of the hydrolysis reaction of the CAL increases to a greater extent than the reaction with PPL. For more efficient catalysis of the reaction medium PPL defense DMF, while in the presence of CAL processes are more efficient in an DMSO. These differences show the effect of solvent on the enzymatic transformation of fats.

Tablea 3. Transesterification of commercial fats

Fat	Solvent	Yield, %	Conversion, %	Degree of Substitution, %	Yield, %	Conversion, %	Degree of Substitution, %
			PPL			CAL	
Rapeseed oil	DMSO	9	33,5[a]±0,7	-[4]	11	45,9[a] ±0,1	0,8
	DMF	12	36,9[b]±0,3	0,9	13	43,9[b]±0,3	-[4]
Olive oil	DMSO	11	37,7[b]±0,5	0,8	14	46,2[a]±0,1	0,5
	DMF	12	39,0[a]±0,1	-[4]	12	40,9[c]±0,8	-[4]

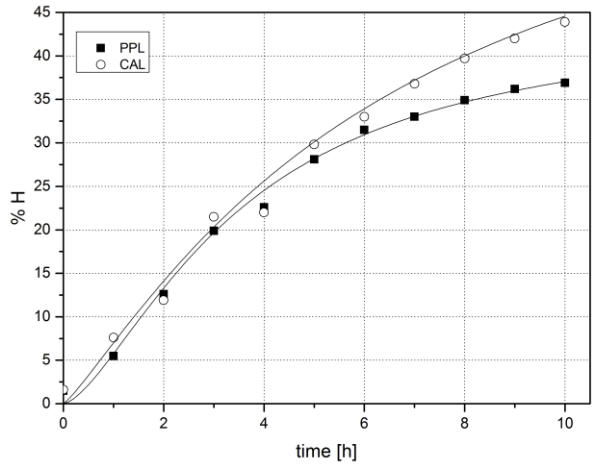

Figure 5. Dynamics of rapeseed oil hydrolysis in PPL and CAL system

Unfortunately the high degree of progress of hydrolysis is not associated with increased esterification CD in these reaction conditions. Degrees of substitution were obtained by the saponification are small, and in some cases impossible to determine. The highest value obtained for DS by reaction with rapeseed oil in DMF using PPL - 0.9%. Comparable degrees of substitution were also found for olive oil (DMSO/PPL) and rapeseed oil (DMSO/CAL). In the latter process, while showing increased lipase CAL enzyme compared to the PPL. This phenomenon is consistent with the literature data [33]. From the results obtained cannot also request the superiority of any of the catalyst systems used, the substrate (dietary fat) or reaction medium. One should assume that the proposed transesterification process does not take place, and the only products are fatty acids derived from the enzymatic hydrolysis of glycerides.

In conclusion, the group of cyclooligosaccharides are less susceptible to the process of introduction of acyl groups comparing to simple sugars. During the tests it was found that substitution of the hydrogen atoms of the acyl groups in enzymatic systems can take place only with the use of carboxylic acids. Phenomena that can be responsible for this must be included: the ability to complex with the substrate (the acylating agent), steric hindrance at the stage of adjustment of the enzyme/substrate (carboxylipase/cyclodextrin), and a low equilibrium constant of the process. Based on the results it can be concluded that the main factor influencing the efficiency of the process is most likely a problem of geometric mismatch of substrate and enzyme. The formation of complexes: acylating agent, the CD is a phenomenon ordering system in the thermodynamic sense. During the studies, however, both the high susceptibility of the ester to enzymatic hydrolysis in the presence of lipase. While considering in detail the possibility of steric hindrance resulting from the construction of the substrates to be noted that, in accordance with the mechanism of action of lipases in the first stage, a state transition of a carboxylipase - E1 [17]. It appears that the formation of the investigated systems involves no effect hindering the process. Steric hindrance may take place at a later stage, when E1 is to create an intermediate complex with a molecule of alcohol - CD. CD molecule composed of seven residues AGU system is relatively rigid, wherein the hydroxyl groups, primary and secondary are on the outer edges of the molecule. Their number (7 - 1^{O} and 14-2^{O}) can cause significant difficulties in the rapprochement of both molecules and the formation of a E1B. In other oligo and polysaccharides chain structure (eg. starch) is more flexible (in solution) and the density of

functional groups is much smaller. This facilitates E1 contact with an alcohol and complex formation E1B, which in subsequent steps cleaves to form the ester.

The structure of the product was confirmed using infrared spectroscopy. FTIR analysis was performed for the samples having the highest degree of substitution, in its group.

Conclusions

In the study, it was found possible to obtain esters with cyclodextrins and selected aliphatic acids by direct enzymatic esterification and transesterification of vinyl esters. It has been found that for the proposed reaction systems, it is possible to obtain esters with low degrees of substitution. It follows that the compounds from the group of cyclooligosaccharides are less susceptible to the process of introduction of acyl groups. Depending on the reaction conditions (solvent, temperature conditions for the enzyme) it was also found a small effect of alkyl chain length on the efficiency of the process. Of the two solvents tested DMF has a slightly greater efficiency. The study of the structure and properties of calls received unambiguously confirmed the course of the esterification of identifying the product ester bond (FTIR). The research is an important element of enriching knowledge of enzymatic transformation of saccharides. They received degrees of substitution, however, point to the need to seek new methods for the preparation of esters of cyclic oligosaccharides and optimization of selected reaction systems.

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