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Enzymatic Modification of Potato Starch

Abstract
This article presents investigations into the modification of potato starch with the use of pullulanase, an enzyme which hydrolyses the α-1,6-glycoside bonds. We tested the effect of the enzymatic reaction time and the amount of the enzyme used on the amylose content in biomodified starch, and investigated the molecular characteristics of starch. The structural changes which occurred in the starch were identified by FTIR and NMR spectroscopy. We established that an increase in the modification time and in the enzyme/substrate module causes an decrease in the average molecular weight, as well as increases in polydispersion and in the amylose content in biomodified starch. Both native and biomodified starch are characterised by a B-type crystalline structure. Biomodified starch is characterised by a lower share of the ordered phase in comparison to native starch. Biomodified potato starch with an increased amylose share in relation to native starch may find application in manufacturing films and fibres.

Key words: *starch, amylose, pullulanase.*

Introduction

Starch and cellulose are the two most common natural polymers occurring in nature, with chains of D-glucose units differentiated only by the type (α or β) of the joining glycoside bond.

Starch granules are three-dimensional objects with diameters within the range from 2 μm to 100 μm, composed of two D-glucose homopolymers differentiated by the chain structures of amylose and amylopectin. Potato starch contains commonly about 20-25% of amylose and 75-80% of amylopectin. Amylose is a linear polymer with a small amount of side branches (from 9 to 20 per each macromolecule) which contain up to 6,000 glucose residues joined by α-1,4-glycoside bonds. The molecular weight of amylose is within the range of 10^5 to 10^6 g/mol. Amylopectin is characterised by a molecular weight about 1000 times greater, of 10^7 to 10^9 g/mol, and a strong branched main chain. The side branches are formed thanks to the α-1,6-glycoside bonds [1]. The distance between the adjacent branches is commonly equal to 20-25 units of α-D-glucose [2].

The starch granules are characterised by a high degree of radial arrangement, which can be proved by observing the interferential Maltese crosses in a microscope, in polarised light. The amylose molecules and the amylopectin regions with chain branches form amorphous regions in native starch, whereas the outer amylopectin linear chains coiled into double helices form crystalline lamellae [3, 4].

Different kinds of polymorph forms, called A, B, C, and V were observed in the crystalline regions of starch depending on the orgin. The A-form appears commonly in starch from cereals, whereas the B-type can be found in starch from tuber plants. The C-structure, rather rare, is a mixture of the A and B types, and appears in starch isolated from peas. The crystalline structures of the A and B types are composed of parallel placed double helices with a hexagonal arrangement [5]. The elementary crystalline cells in both systems include 12 glucose residues, where the more densely packed structure of the A-type additionally contains 4 water molecules in the elementary cell, whereas the B-structure contains 36 water molecules. The crystalline structure of the V-type, which is composed of singular helices, has been observed in amylose complexed by lipids or other compounds [6].

The starch structure is destroyed by heating in water or processing with aqueous solutions of reagents, which cause the decomposition of hydrogen bonds and crystalline regions inside the granules. Starch granules integrated in an aqueous solution are called gelanitised starch. Starch solutions are unstable at lower temperatures. In diluted solutions, the macromolecules form aggregates which precipitate, whereas concentrated solutions form gels. This process is known as retrogradation. As a result of this, the system of macromolecules dispersed in the solution is transformed into a more arranged state, which is thermodynamically more stable. The retrogradation of amylose is a quick process, which consists in spirally coiling the chains, creating systems of double helices, and next forming crystallites. In strongly-branched polymers, such as amylopectin, the parallelisation and aggregation of macromolecules is greatly impeded, and therefore its retrogradation is slow and proceeds only in solutions of great starch concentration or at a reduced temperature.

The ease of amylose retrogradation can be used in the processes of forming starch fibres and fibrids from alkali solutions. In these cases, obtaining fibrous forms of starch consists in extruding an alkali starch solution into a coagulation bath [7 – 9]. Such fibres and fibrids may be applied in paper production. Adding starch in the form of fibres and fibrids to the paper pulp causes their high retention, comparable with that of cation starch, and allows papers of increased strength to be manufactured, better resistance to fats, and decreased air permeability [10]. Considering the high cost of cation starch, investigations into obtaining different starch fibrous forms, which are non-soluble in water, were carried out. Only starches with a high amount of amylose are suitable for this purpose [11 – 13].

An alternative to obtaining high-amylose starch by the genetic modification of plants, such as corn and potatoes, is to use enzymes which eliminate chain branches in amylopectin [14]. These include enzymes occurring in plants, including those of the pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68) groups. Pullulanase is a 1,6-α-glucosidase, which statistically impacts the linear α-glucan, a pullulan which releases maltotriose oligomers. This enzyme also hydrolyses α-1,6-glycoside bonds in amylopectin and dextrines when their side-chains include at least two α-1,4-glycoside bonds. Isoamylase is an enzyme which totally hydrolises α-1,6-glycoside bonds in amylopectin, glycogen, and some branched maltodextrins and oligosaccharides, but is characterised by low activity in relation to pullulan [15].

The aim of our research work was to investigate the process of enzymatic modification of potato starch to a form

Table 1. *Selected properties of the native potato starch.*

Amylose content	wt%	20.4
Amylopectin content	wt%	79.6
Moisture	wt%	18.6
pH of the aqueous extract	-	6.3
Average numerical molecular weight, $\bar{M}n$	kDa	272
Average gram molecular weight, $\bar{M}w$	kDa	1295
Polydispersion Pd	-	4.8

characterised by increased amylose content. Research works published [7 – 13] indicate the possibility of applying such a starch for manufacturing films and fibres by spinning from a solution. The authors will devote their next article to methods of obtaining films and fibres from enzymatic modified potato starch and to characterisation of their properties.

Experimental part

Materials

'Superior' potato starch produced by the Enterprise of Potato Industry in Trzemeszno (Poland) was used for our investigations. Selected properties of the native starch are listed in Table 1.

The modification process was carried out with the use of thermostable Optimax L-300 pullulanase from Genencor Int. Inc., of 270 U/cm^3 activity.

Methods

The gelatinisation process was conducted by heating an aqueous suspension of starch with a concentration of 10% wt. in a glass reactor placed in a water bath, and continuously mixing the suspension with a paddle agitator at a speed of 100 rpm for 30 minutes, at a temperature of 90 °C.

The geletinised starch was gently cooled down in the water bath to a temperature of 60°C. While continuously mixing with a paddle agitator at a speed of 100 rpm, the pH of the reaction medium was set to pH 4.5 with the use of a 0.1 M solution of acetic acid, and the enzyme solution was added. An enzyme/substrate module within the range of 5 U/g to 50 U/g of starch was set, and the reaction time was changed from 10 minutes to 300 minutes. After the end of the reaction, the enzyme was deactivated by heating the reactor together with the reaction mixture for 20 minutes in the water bath at boiling temperature.

The pullulanase activity was determined on the basis of the procedure [16]. It uses the hydrolysis reaction of the α-1,6-glycoside bonds in the pullulan, releasing maltotriose as product. Pullulan from the *Aureobasidium pullulans* group (from Sigma) was used as a substrate. The reaction was conducted at a temperature of 50 °C for 10 minutes. After the end of the reaction, the reducing sugars formed were determined by the colorimetric method using a reaction with 3,5-dinitrosalicylic acid (DNS). Absorbance measurements were carried out at a wavelength of 540 nm. The activity unit of pullulanase causes the formation of reducing sugars which are equivalent to 1 mg of anhydrous maltose under the reaction conditions.

The amylose content in starch was determined by the colorimetric method using iodine according to the method devised by McGrance et al. [17]. A solution of iodine in potassium iodite was added to the solution of starch in DMSO, and the absorbance was measured at a wavelength of 600 nm. The content of amylose was assessed on the basis of a standardisation curve prepared for mixtures of amylose and amylopectin containing 0, 10, 25, 50, and 100% of amylose.

The distribution of the starch's molecular weight was determined by gel chromatography (GPS/SEC). The GPS/SEC system was composed of a DG 700 degasifier from Viscotek, a HP 1050 pump from Hewlett Packard, a manual sample injector from Rheodyne, a set of three PLgel Mixed A columns preceded by a PLgel guard protective column (all columns from Polymer Laboratories Ltd.), a HP 1047 differential refractometric detector, and PL Caliber GPC software from Polymer Labratories. DMAc/0.5% LiCl was used as the mobile phase, which was vacuum filtered before use (by a PTFE filter of 0.45 μm from Sartorius) and degasified. The mobile phase flowed at a rate of 1 ml/min; the column temperature was 80 °C, and the volume of the injected solution was 150 μl. Eight pullulan standards (from Polygen) of molecular weights Mp within the range from 5,600 to 1,520,000 g/mol and polydispersion Mw/Mn < 1.2 were used for calibration.

The Fourier-transform infrared spectra were performed with the use of a FTIR spectrometer from Unicam, controlled by the Winfirst ATI Mattson software. The spectra were recorded within the wavelength number of 4,000-400 cm^{-1}, at a resolution of 4.0 cm^{-1}. The preparations were tested in the shape of pressed pellets with KBr.

NMR investigations were carried out with a Avance DMX 300 spectrometer from Bruker using an Magic Angle Spinning (MAS) probe at a resonance

Table 2. *Influence of the reaction time on the amylose content and the molecular characteristics of biomodified starch; module E/S = 20 U/g starch.*

Kind of starch	Reaction time	Amylose content	$\bar{M}n$	$\bar{M}w$	Pd	Fraction content	
						Mw < 500 kD	Mw > 500 kD
	min	%	kD	kD	–	%	%
native	0	20.4	272.0	1295.0	4.8	40.4	59.6
biomodified	10	25.4	218.2	817.9	3.7	46.7	53.3
	30	30.3	163.4	694.3	4.3	53.2	46.8
	60	34.8	130.0	640.8	4.9	54.7	45.3
	120	36.1	103.7	591.6	5.7	57.5	42.5
	240	37.9	85.3	501.7	5.9	62.5	37.5
	300	48.2	35.0	313.5	9.0	80.8	19.2

Table 3. *Influence of the enzyme/substrate module on the amylose content and the molecular characteristic of biomodified starch; reaction time 15 min.*

Type of starch	Reaction time	Amylose content	$\bar{M}n$	$\bar{M}w$	Pd	Fraction content	
						Mw < 500 kD	Mw > 500 kD
	min	%	kD	kD	–	%	%
native	0	20.4	272.0	1295.0	4.8	40.4	59.6
biomodified	5	24.4	218.5	843.0	3.8	47.8	52.2
	10	27.6	168.3	689.2	4.1	51.0	49.0
	20	30.7	130.4	703.7	5.4	53.3	46.7
	30	46.2	45.8	713.7	15.6	55.4	44.6
	50	56.0	18.5	441.7	23.9	78.9	31.1

frequency of 75.47 MHz. The samples were placed in a rotor of ZrO_2 with a diameter of 4 mm. The spectra obtained were decomposed into component peaks using the producers' professional program, and next the percentage shares of the particular peaks of the spectrum were calculated.

Research results and discussion

Testing the influence of the reaction time on the biomodification of starch

The influence of the enzymatic reaction time on the amylose content and molecular characteristic of the biomodified starch was tested. The starch gelatinisation at 90 °C for 30 minutes was applied as the preliminary thermal processing. The enzyme/substrate module was equal to 20 U/g starch, the reaction medium maintained a pH of 4.5, and the reaction temperature was 60 °C. The results are presented in Table 2 and Figure 1.

On the basis of the results presented in Table 2, it can be indicated that the enzymatic reaction time at constant module enzyme/substrate (E/S = 20 U/g starch) influences the process of starch biomodification. Increasing the biomodification time from 10 min to 300 min caused an increase in the amylose content in biomodified starch, an increase in the share of the fraction with molecular weight below 500 kD, and an increase in the polymer's polydispersion (Pd). At the same time, drops in the average numeric molecular weight and the average gram molecular weight occurred. The graphs of the function of molecular weight distribution of biomodified starch (Figure 1) indicate a two-modal character with a shift towards the fraction of smaller molecular weight at increasing reaction time.

Testing the influence of the enzyme/substrate module on biomodified starch

The influence of the enzyme/substrate module on the amylose content and the molecular characteristic of biomodified starch were tested. The enzymatic reaction time was 15 min, the reaction medium maintained pH 4.5, and the reaction temperature was 60 °C. The results are presented in Table 3 (see page 101).

The data listed in Table 3 presents the influence of the enzyme amount used in the reaction, expressed by the number of enzymatic activity units per 1 gram of starch on the proceeding of the biomodification process. For the same reaction time, increasing the enzyme/substrate module causes an increase in the amylose content, a decrease in the average molecular weights of starch, an increase in polydispersion and an increase in the

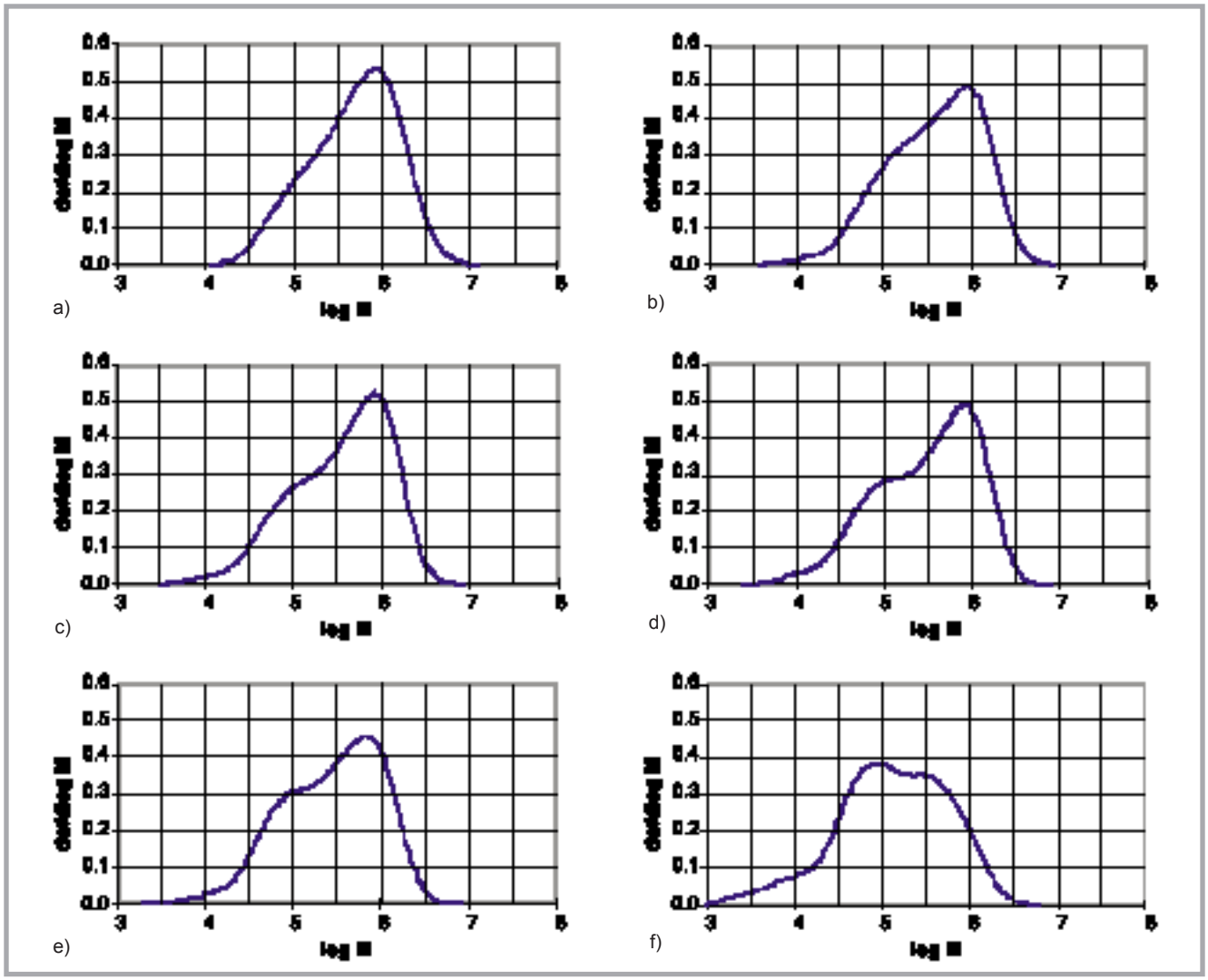

Figure 1. *Function of the molecular weight distribution of starch at different time periods (module E/S = 20U/g): a) 10 min, b) 30 min, c) 60 min, d) 120 min, e) 240 min, f) 300 min.*

share of the fraction with average molecular weight below 500 kD. An increase in the E/S module above 30 U/g causes a very significant drop in the average molecular weight and an increase in the polymer's polydispersion.

Investigating the structure of starch by the FTIR spectroscopy method

In the spectrum of native potato starch obtained by the FTIR method, the following bands can be distinguished [18, 19]:

- 2,900 – 3,000 cm^{-1} (stretching vibration of CH),
- 1,150, 1,124, and 1,103 cm^{-1} (stretching vibration of CO, CC, and COH),
- 1,047, 1,022, 994, and 928 cm^{-1} (bending vibrations of COH and CH_2),
- 861 cm^{-1} (symmetric stretching vibrations of COC and deformation vibrations of CH).

Some of the bonds are sensitive to changes of the arrangement degree at molecular level. The intensity of the 1,022 cm^{-1} band increases with the drop in crystallinity, and is characteristic of freshly-prepared hot starch solutions. The band 1,047 cm^{-1} is composed of two mutually overlapping bands at 1,040 cm^{-1} and 1053 cm^{-1}..During starch retrogradation, the 1,040 cm^{-1} band is created over a period of some hours, whereas the 1053 band takes a significantly longer time to appear. The intensity of the 1,047 cm^{-1} band increases with the increase in the crystallinity of starch. The ratio of the 1,047 cm^{-1} and 1,022 cm^{-1} peaks' height express the relation of the orderd phase to the amorphous phase in starch, whereas the ratio of the 1,047 cm^{-1} and 1,035 cm^{-1} peaks' height is a measure of the amount of the orderd phase [20].

FTIR investigations were carried out for native and biomodified starch (E/S = 20 U/g, pH 4.5, temperature 60 °C, reaction time 30 minutes). In order to obtain the spectra, the ratio of absorbances $A_{1,047}$ /$A_{1,022}$ and $A_{1,047}$ /$A_{1,035}$ were calculated. The results are presented in Figure 2 and Table 4.

On the basis of Figure 2 and the data listed in Table 4, it appears that the share of the orderd phase in biomodified starch is smaller than in native starch. This is certified by the absorbances ratio $A_{1,047}$ /$A_{1,022}$ and $A_{1,047}$ /$A_{1,035}$ which are lower than those for initial starch.

Investigating the starch structure by the method of magnetic nuclear resonance spectroscopy ^{13}C CP-MAS in a solid state

Characteristic bands attributed to the particular carbon atoms in the glucose ring [21] can be distinguished in the starch spectrum obtained by the ^{13}C CP-MAS NMR technique. Signals within the regions of 99-104 ppm, 81-84 ppm, and 59-62 ppm come from the atoms C-1, C-4, and C-5, respectively. The broad band within the range of 70-73.3 ppm is the result of overlapping signals from the atoms C-2, C-3, and C-5. Chemical shits can be observed in the C-1 carbon signal, which are characteristic for each of the three crystalline conformations of starch [22]. In the case of the A-type crystalline structure, whose elementary cell includes three different glucose residua, the C-1 signal is characterised by a triplet, whereas in the case of the B-type crystalline structure, whose elementary cell includes two glucose residua, a doublet is visible in the C-1 signal. The crystalline structure of the V-type can be distinguished by the singular signal within the range of 103-104 ppm, and a characteristic band for the atom C-4 within the range of 82-83 ppm.

Tests were carried out for native and biomodified starch. The spectra obtained were computer-processed in order to decompose the spectra into component peaks, and then the percentage shares of each particular peak in the spectrum were calculated. On the basis of the percentage shares of the area of peaks related to the crystalline phase [23], the relative crystallinity of the particular samples was evaluated. The research results are presented in Figures 3 and 4, and in Table 5 (see page 104).

On the basis of the results obtained (Figure 3 and 4), we can state that native and biomodified starch are characterised by the B-type crystalline structure. In the case of biomodified starch, the peaks characteristic for the crystalline phase are accompanied by a peak attributed to the presence of an amorphous phase, which causes a broadening of the C-1 atom band. The relative share of the crystalline phase is lower for biomodified starch in comparison with native starch (Table 5).

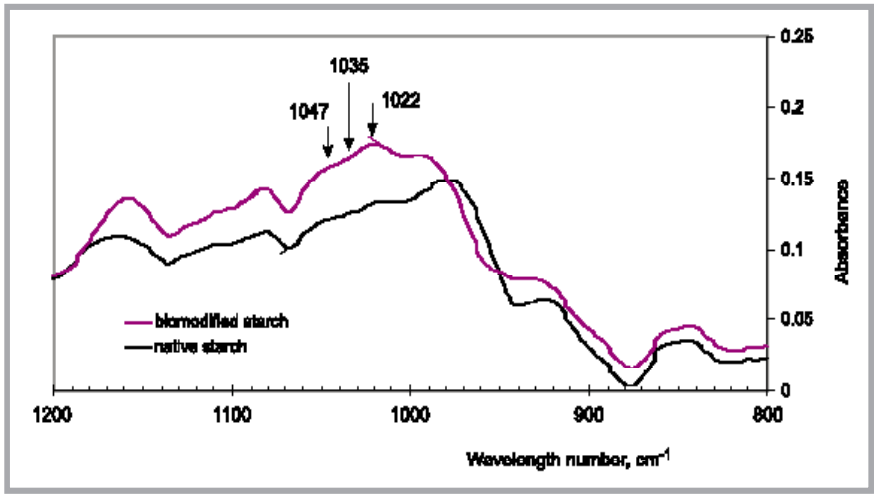

Figure 2. *FTIR spectra of native and biomodified potato starch.*

Table 4. *Values of the absorbance of the bands 1,022 cm^{-1}, 1,035 cm^{-1}, and 1,067 cm^{-1}, and the ratio of absorbances $A_{1,047}$ /$A_{1,022}$ and $A_{1,047}$ /$A_{1,035}$.*

Type of starch	Absorbance for wavelength number			Absorbance ratio	
	1022 cm^{-1}	1035 cm^{-1}	1047 cm^{-1}	A_{1047}/A_{1022}	A_{1047}/A_{1035}
native	0,1324	0,1250	0,1215	0,918	0,972
biomodified	0,1750	0,1646	0,1582	0,904	0,961

Conclusions

1. We indicated that an increase in the enzymatic reaction time and an increase in the E/S module increases the amylose content in biomodified starch, the fraction content of molecular weight below 500 kD and the polydispersion (Pd), and also causes a drop in the average molecular weight of starch.

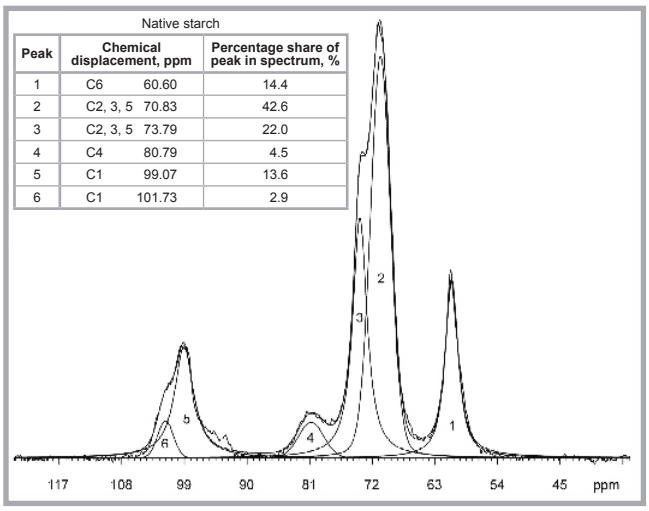

Figure 3. *^{13}C CP-MAS NMR spectrum of native starch.*

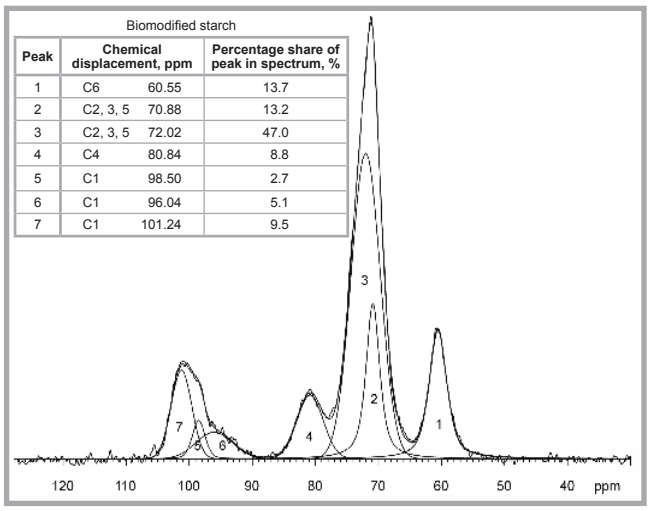

Figure 4. *^{13}C CP-MAS NMR spectrum of biomodified starch.*

Table 5. *Chemical shifts and percentage share of crystalline phase for native and biomodified starch.*

Type of starch	Chemical shifts for carbon atom in the glycoside ring of starch, ppm				Percentage share of crystalline phase, %
	C1	C2, 3, 5	C4	C6	
Native starch	99.07 101.73	70.83 73.79	80.79	60.60	16.5
Biomodified starch	96.04 98.50 101.24	70.88 72.02	80.84	60.55	12.2

2. On the basis of the FTIR investigations, we indicated that the percentage share of the ordered phase in biomodified starch is smaller in comparison to native starch. The NMR spectroscopy in a solid state proved that native, as well as biomodified starch are characterised by the B-type crystalline structure. Investigations by the NMR method confirmed that the relative content of the crystalline phase in biomodified starch is lower than that in native starch.

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