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## LF NMR STUDIES OF MICROWAVE MODIFIED STARCH WITH LYSOZYME

ANALIZA METODĄ LF NMR MIKROFALOWO ZMODYFIKOWANEJ SKROBI Z DODATKIEM LIZOZYMU

### Abstract

**Background.** This article presents the results of novel research focusing on the influence of the power of the electromagnetic wave on changes in starch-water interactions in potato starch powders as well as in 5% potato starch gels and 5% starch-lysozyme gels. Lysozyme is applied as an additive in various products thanks to its bacteriostatic and antibacterial properties. The aim of the investigation presented in this paper was to analyse water binding by starch subjected to physical modification. Through to its antibacterial properties the resulting compound can be also applied as an additive in meat processed articles or employed in medicine to treat bacterial, viral as well as fungal infections. Formulations prepared in the form of gels/ointments can be applied onto mucous membranes of the mouth, throat or applied on the skin.

**Material and methods.** Changes taking place at the molecular level were observed employing the low-field NMR method. The strength of microwaves used during the modification process was changed from 50 to 200 W/g of the sample for the period of 2 min.

**Results.** It was found that, as a result of action of the electromagnetic field, starch changed its hygroscopic properties. Application of the electromagnetic waves of power exceeding the value of 175 W/g of starch led to changes in the starch granule structure which permanently altered its hydrophobic-hydrophilic properties. On the basis of NMR results, the spin-lattice and spin-spin interaction energy has been calculated.

**Conclusions.** In comparison to the starch lattice, the starch-protein lattice was characterized by smaller energy value returned to the surroundings and higher energy returned to neighbouring spins. The recorded differences were small, probably due to a small share of the protein lattice in the system.

**Keywords:** lysozyme, modified potato starch, microwave, low-field NMR, interaction energy

## Introduction

Starch is one of the most universal and multifunctional raw materials utilized in food industry (Fortuna et al., 2004). In Poland, potato starch is most commonly used but starches manufactured from maize, wax maize, tapioca as well as wheat or rice are also employed. This biopolymer is utilized, primarily, in food industry as a functional additive to food articles, as a thickening, stabilizing or texture-forming agent (Singh et al., 2007). Native starch is characterized by restricted possibilities due to its limited rheological stability. That is why starch is frequently subjected to a wide range of chemical, physical or enzymatic modification methods or their combinations obtaining preparations of modified starch (Fortuna et al., 2004). The aim of starch modifications is to improve its specific functional properties or to obtain a product which is characterized by desired, pre-programmed properties (Fortuna et al., 2004; Kucharczak et al., 2015). Native starch gels show a tendency to retrogradation and, consequently, to syneresis. The applied modifications can prevent these phenomena and, additionally, improve properties of the produced gels. Due to its unique chemical structure, starch can react with different chemical compounds. Chemically modified starch preparations can be characterized by various properties depending on the applied reagent and reaction (Fortuna et al., 2004). Among the most frequent chemical modifications, the most common ones include cross-linking and stabilization (Krysińska et al., 2008). The simplest method of starch physical modification is thermal or pressure-thermal treatment. In the result of heating, starch grain structure is destroyed and partial gelatinization takes place; hydrogen bonds which stabilize tertiary and quaternary macromolecular conformation structure undergo disruption (van den Einde et al., 2003; Yang et al., 2017). For this purpose, various forms of drying, extrusion or high hydrostatic pressure are applied (Lewandowicz, 2001; Mitrus et al., 2010). Another form of physical modification is microwave radiation. This method allows very rapid penetration of heat deep into the material in comparison with traditional heating techniques. Microwave technique finds increasingly wide application in food processing due to its considerable energy savings and speed of the thermal treatment (Lewandowicz, 2001).

In recent years, protein enriched products have become increasingly popular in food industry. Depending on the type of the employed protein, its introduction into food articles can improve nutritional properties of a given product (Ribotta et al., 2012). Among proteins most widely utilized recently is lysozyme, which belongs to the system of the non-specific, humoral immunological response. Lysozyme is a protein, which occurs in the form of a single peptide chain developed from 129 amino acid residues. Muramidase is characterized by properties which allow its use in food article conservation as well as to develop packaging, wrappings and covering agents used in processed food articles (Gajda and Bugla-Płoskońska, 2014; José Fabra et al., 2014). Through to its bacteriostatic, bactericidal and antibacterial properties, lysozyme is also applied as an additive in meat processed articles and for antimicrobial packaging for foods (Ozer et al., 2016). In addition, muramidase is also employed in medicine to treat bacterial, viral as well as fungal infections. Formulations prepared in the form of gels/ointments can be applied onto mucous membranes of the mouth, throat or applied directly on the skin (Gajda and Bugla-Płoskońska, 2014).

The aim of the investigation presented in this paper was to analyze water binding by starch subjected to physical modification. Additionally the water binding in starch and starch-lysozyme gels were study

Changes taking place at the molecular level were observed employing the low-field NMR method. This method is the best to analysis a molecular properties of water in biopolymer systems (Han et al., 2014; Jia et al., 2019). It is noninvasive and nondestructive. The samples can to be storage and analysis repeatedly. In this important is that sample preparation is not complicate. By low-field NMR can to be analysis biopolymer gels. (Baranowska et al., 2015; Kucharczak et al., 2015; Walkowiak et al., 2018)

## Material and methods

### Material

The experimental material was Superior Standard potato starch (PPZ Trzemeszno, Poland) and lysozyme derived from chicken egg white (Sigma Aldrich L-7001).

#### *Starch modification*

The initial starch moisture content of 32% was established by water addition. In order to carry out physical modification in a vacuum-microwave drier, starch prepared in this way was placed in small containers (Pawlak et al., 2013). The dried material was placed inside a rotating vacuum drum, which was situated in the microwave chamber.

It was possible to reduce air pressure inside the drum to the value of approximately 3% of atmospheric pressure (30 hPa). The strength of microwaves used during the modification process was changed from 500 to 2000 W. The power of transmission system made it possible to change the drum rotations from 11 to 19 rpm. Due to the system of relocation employed inside the rotating dielectric drum, no harmful effect of local material overheating took place (Pawlak et al., 2013).

The potato starch used in the experiment was exposed to the action of electromagnetic waves emitting radiation of 2450 MHz frequency for the period of 2 min.

#### *Starch powder hydration*

Starch samples modified using microwaves were subjected to hydration for the period of 14 days in a desiccator with water in order to determine the amount of water absorbed by a given sample. The correct hydration time was assumed to be the time in which samples did not change their mass for two consecutive days. After hydration, all NMR tubes were closed using parafilm (Makowska et al., 2017).

Drying method was applied to determine water loss caused by the action of electromagnetic waves and the quantity of water per 1 g of starch after hydration from the gaseous phase. Starch samples after modification and after hydration were dried for 3 hours at the temperature of 120°C. The quantity of water per 1 g of the sample ( $M_H$ ) after hydration were determined and expressed as the mass of water with reference to 1 g of dry matter. Additionally, the relative humidity  $H_c$  of the modified starch were determined.

*Starch and starch-lysozyme gels samples*

Selected samples of native and physically modified starch were used to prepare gels adding to them lysozyme at appropriate concentrations. The prepared suspensions of 10 ml volume and 5% concentration consisted of: 0.02 g lysozyme and 0.48 g native starch or modified starch at identical proportions supplemented with water to the volume of 10 ml. Additionally, suspensions of 5% concentrations were prepared consisting, exclusively, of native starch or modified starch. The prepared solutions were mixed using a magnetic mixer and left for 24 h. In order for the gelatination process to take place, all samples were heated for 60 minutes at the temperature of 90°C.

**Methods**

Experiments were conducted with the assistance of a nuclear magnetic resonance (NMR) using for this purpose an impulse spectrometer operating at 15 and 30 MHz at controlled temperature of 20°C. Sample volumes were 0.15 and 1.5 cm<sup>3</sup> respectively. Experiments were carried out in 3 replications and the results are presented as mean values.

The samples of the starch gels were placed in NMR tubes, cooling to room temperature and sealed using Parafilm.

The inversion-recovery ( $\pi$ -TI- $\pi/2$ ) impulse sequence (Brosio and Gianferri, 2009) was applied for measurements of the  $T_1$  relaxation times.

For hydrated starch powders samples distances between impulses (TI) were changed within the range from 4 to 800 ms and the repetition time was from 15 s. Each time, 32 FID signals and 119 points from each FID signal were collected.

For starch and starch-lysozyme gels samples distances between impulses (TI) were changed within the range from 4 to 800 ms and the repetition time was from 15 s. Each time, 32 FID signals and 119 points from each FID signal were collected. Measurements of the  $T_2$  spin-spin relaxation times were taken using the pulse train of the Carr-Purcell-Meiboom-Gill spin echoes ( $\pi/2$ -TE/2-( $\pi$ )<sub>n</sub>) (Brosio and Gianferri, 2009). The distance between  $\pi$  (TE) impulses amounted to 1ms. The repetition time was 15 s. The number of spin echoes (n) amounted to 50. This accumulation signals were employed.

Calculations of the spin-lattice relaxation time values were performed with the assistance of the CracSpin program (Węglarz and Harańczyk, 2000). Program for calculating relaxation parameters from experimental data using “spin grouping” approach. Marquardt’s method of minimization has been applied for fitting multiexponential decays. The accuracy of the relaxation parameters has been estimated with the standard deviation. Time changes of the current value of the FID signal amplitude in the employed frequency of impulses are described by the following formula:

$$M_z(TI) = M_0 \left( 1 - 2e^{-\frac{TI}{T_1}} \right) \quad (1)$$

where:

- $M_z(TI)$  – the actual magnetisation value,
- $M_0$  – the equilibrium magnetisation value,

$TI$  – the distance between impulses,  
 $T_1$  – the time of relaxation.

A monoexponential magnetisation recovery was found, which means that the system relaxes with one  $T_1$  spin-lattice relaxation time.

To calculate the spin-spin relaxation time values, the authors applied the adjustment of values of the echo amplitudes to the formula (Baranowska, 2011):

$$M_{x,y}(TE) = M_0 \sum_{i=1}^n p_i e^{-\frac{TE}{T_{2i}}} \quad (2)$$

where:

$M_{x,y}(TE)$  – the echo amplitude,  
 $M_0$  – the equilibrium amplitude,  
 $TE$  – the distance between  $\pi$  impulses,  
 $p_i$  – the fraction of protons relaxing with the  $T_{2i}$  spin-spin time.

The calculations were performed by using the dedicated software by apply non-linear least-square algorithm. The accuracy of the relaxation parameters has been estimated with the standard deviation. The presence of two proton fractions was determined for all analysed systems.

## Results and discussion

The purpose of starch physical modification was to improve its functional properties. The analysis of the effect of modification with the assistance of microwaves on starch hydration possibilities revealed that, together with the increase of strength of the applied microwaves, the amount of water hydrating 1 g of biopolymer declined (Table 1). This translated itself into a decline in final moisture content value after hydration from the gaseous phase.

Table 1. Water binding capacity by modified starch

Sample	Composition	The power of microwave per 1 g of the sample (W)	$M_H$ (g)	Hc (%)
01	Native starch + water	0	0.41	100
1	Modified starch	50	0.35	84
2		100	0.29	73
3		175	0.24	57
4		200	0.22	55

It was found that, as a result of action of the electromagnetic field, starch changed its hydroscopic properties. The above phenomenon required an analysis of water-polymer

interaction at molecular level. For this purpose, the authors used the method of low-field NMR in order to measure the  $T_1$  spin-lattice relaxation time of water hydrating the modified starch. Figure 1 shows spin-lattice relaxation rates changes  $R_{1H}$  ( $=1/T_1$ ) of the hydration water depending on the final relative moisture content.

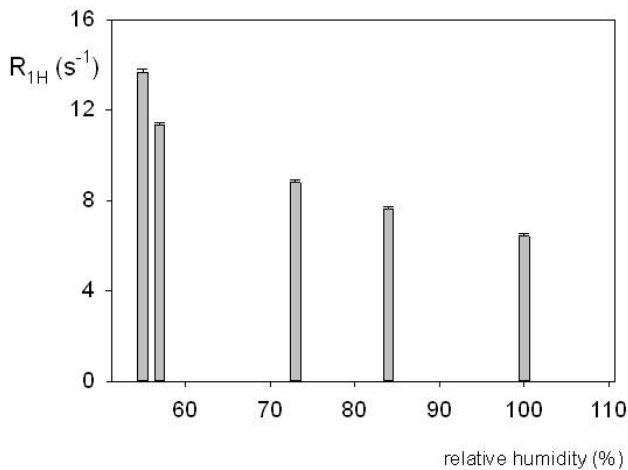


Fig. 1. Changes of spin-lattice relaxation rates  $R_{1H}$  of hydration water in potato starch modified using microwaves depending on the biopolymer final relative moisture content

The spin-lattice relaxation rate of hydration water increased with the decrease of starch final moisture content. The obtained result was not unequivocal. Relaxation rate increases as the quantity of water in the biological system decreases (Stangierski et al., 2012; Baranowska et al., 2011). Therefore, the presented result could only be the effect of restriction of the amount of bound water with modified starch. It is evident from the above that for the hydration water, changes in the spin-lattice relaxation rate  $R_1$  were a linear function of the strength of microwave radiation falling on 1 g of biopolymer dry matter (Fig. 2). However, the above relationship referred only to the power not exceeding 100 W/g of the starch.

The increase of the spin-lattice relaxation rate of hydration water molecules together with the power increase of microwaves modifying starch was observed up to approximately 175 W/g of starch. Above this value, a significant increase in the  $R_1$  value was recorded. It is probable that the high power of electromagnetic waves modified starch in such a way that the polymer lost its hydrophilic properties (Lewandowicz, 2001).

The applied method of biopolymer modification led to changes in water affinity. It is well-known that starch thermal treatment affects the value of gel viscosity (Baranowska et al., 2015). For that reason, experiments were undertaken to find how the modified biopolymer changed starch gel properties. In addition, the authors analysed gels prepared from mixtures of modified starch and lysozyme. Changes in the molecular water dynamics in starch and starch-protein gels are presented in Figure 3.

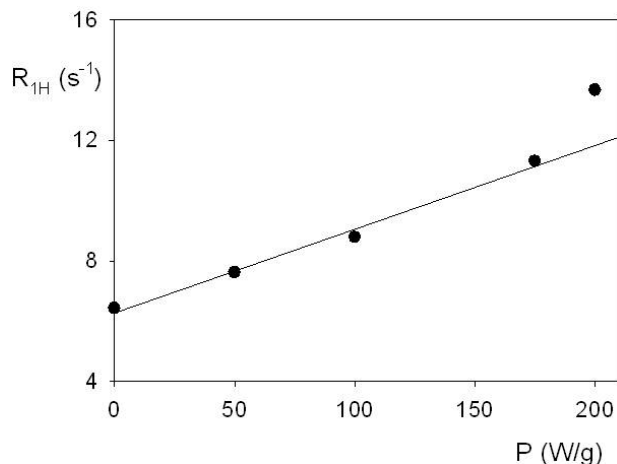


Fig. 2. Dependence of the spin-lattice relaxation rates  $R_1$  of hydration water on the power of electromagnetic waves

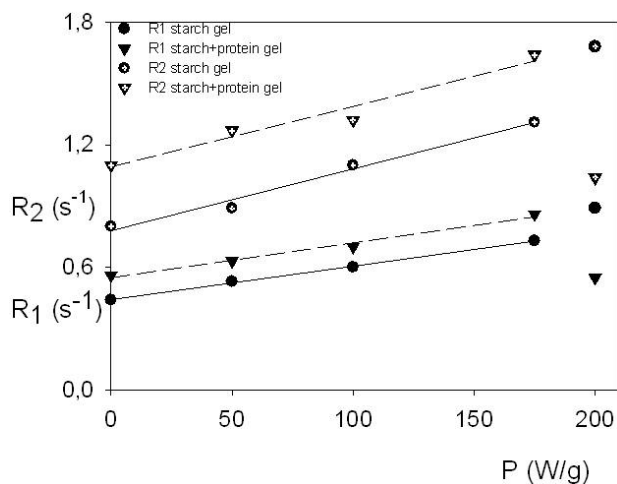


Fig. 3. Relaxation rates in two and three component biopolymer gels

Relaxation rate constitutes a parameter which reflects water molecular dynamics in gels (Baranowska and Rezler, 2015). The higher the velocity with which spins return energy to the environment ( $R_1$ ) or to the neighboring nuclear spins ( $R_2$ ), the greater are the re-orientation restrictions of water molecules which act as solvents for polymer networks. This results from macroscopic mechanic-textural properties of biopolymer gels (Baranowska et al., 2015). Starch gels are characterized by smaller relaxation rate in comparison with starch-protein systems. This can be taken as evidence indicating influence of starch-protein interactions on water molecule dynamics in gels.

Figure 3 shows that spin-lattice and spin-spin relaxation rates increased linearly within the range of the applied microwave power of up to 175 W/g. When greater microwave power was applied, a non-linear increase of spin-lattice and spin-spin relaxation rates in starch gels was observed. Similar changes were recorded analyzing spin-lattice relaxation rates of the hydration water (Fig. 2). This corroborates the fact that the application of the electromagnetic waves of power exceeding the value of 175 W/g of potato starch led to changes in the starch granule structure which permanently altered its hydrophobic-hydrophilic properties. It was, most probably, associated with changes in the crystalline structure of potato starch from B to A form reported by other researchers (Lewandowicz et al., 1997). In three component systems, a significant value decrease in the relaxation rate is observed caused by water-protein interactions. Losing its hydrophilic properties, starch is negligibly involved in the development of the polymer network.

Relaxation rate changes in a linear manner together with the change of microwave power used in the modification process, which can be described using the following dependence:

$$R_{1,2} = \frac{1}{E_{1,2}} \cdot P + R_0 \quad (3)$$

where:

- $E_{1,2}$  – the energy accumulated and transferred to the system, neighboring spin with  $P$ ,
- $P$  – a power per mass unit of starch,
- $R_0$  – constant of system.

On the basis of such dependence, the authors calculated the spin-lattice and spin-spin interaction energy per 1 g of modified starch. The results of these calculations are presented in Table 2.

Table 2 Values of spin-lattice and spin-spin interaction energy in biopolymer gels

Biopolymer gel	Interaction energy (kJ/kg)	
	Spin-lattice ( $E_1$ )	Spin-spin ( $E_2$ )
Starch	610,73 ± 2,35	330,25 ± 3,86
Starch-protein	585,34 ± 1,08	338,18 ± 4,17

Water-starch and water-starch-protein interactions were characterized by different spin-lattice energy values. Simultaneously, changes in energy values associated with interactions between spins of water-starch and water-starch-protein systems failed to change significantly. Therefore, it can be concluded that the presence of protein in the system altered the structure of the biopolymer network. In the starch gel, part of water molecules was bound directly with the biopolymer, mainly network nodes. The addition of protein caused that part of network nodes developed as a result of starch-protein instead of starch-water interactions. This explains the observed decrease both of the relaxation rates and energy of spin-lattice interactions.



## Conclusion

Relaxation parameters provide information about how quickly the energy of excited spins is transferred to the surroundings ( $R_1$ ) and to neighboring spins ( $R_2$ ). Therefore, energy values determined from equation (3) can be treated as the amount of energy accumulated in the system and transferred to the surroundings ( $E_1$ ) or to neighboring spins ( $E_2$ ) during the return of the system to the state of equilibrium. Values of these energies changed only slightly in starch and starch-protein gels. In comparison with the starch lattice, the starch-protein lattice was characterized by smaller energy value returned to the surroundings and higher energy returned to neighboring spins. The recorded differences were small, probably due to a small share of the protein lattice in the system. The performed experiments demonstrated that the application of electromagnetic waves for potato starch modification required the use of power which did not exceed 175 W/g of the starch. The use of higher microwave power leads to a change in the hydrophilic properties of starch. The consequence of this is the deterioration of starch-water interactions. Analyzing the obtained parameters in starch-protein gels, a distinct influence of protein on the molecular structure of the polymer lattice was observed. This is important from a practical point of view. The analysis carried out indicates that mixtures of physically modified starch and lysozyme form compact structures. Therefore, they can be used to design new biopolymer materials.

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## ANALIZA METODĄ LF NMR MIKROFALOWO ZMODYFIKOWANEJ SKROBI Z DODATKIEM LIZOZYMU

### Abstrakt

**Wstęp.** W artykule przedstawiono wyniki nowatorskich badań dotyczących wpływu mocy fali elektromagnetycznej na zmiany interakcji skrobia-woda w proszkach skrobi ziemniaczanej, a także w 5-procentowych żelach skrobi ziemniaczanej i 5-procentowych żelach skrobia-lizozym. Lizozym jest stosowany jako dodatek w różnych produktach, dzięki właściwościom bakteriostatycznym i bakteriobójczym. Celem badania przedstawionego w niniejszym artykule była analiza wiązania wody przez skrobię poddaną fizycznej modyfikacji. Dzięki swoim właściwościom antybakteryjnym utworzony związek może być również stosowany jako dodatek w artykułach mięsnych lub wykorzystywany w medycynie do leczenia zakażeń bakteryjnych, wirusowych i grzybiczych. Preparaty przygotowane w postaci żelu/maści można nanosić na błony śluzowe jamy ustnej, gardła lub nakładać na skórę.

**Materiał i metody.** Zmiany zachodzące na poziomie molekularnym obserwowano za pomocą metody niskopolewego NMR. Moc użytych mikrofal podczas procesu modyfikacji została zmieniana w zakresie od 50 do 200 W na 1 g próbki w czasie 2 min.

**Wyniki.** Stwierdzono, że w wyniku działania pola elektromagnetycznego skrobia zmieniła swoje właściwości hydroskopowe. Zastosowanie fal elektromagnetycznych o mocy przekraczającej 175 W na 1 g skrobi doprowadziło do zmian w strukturze granulki skrobi, które trwale zmieniły jej właściwości hydrofobowo-hydrofilowe. Na podstawie wyników NMR obliczono energię oddziaływania spin-sieć i spin-spin.

**Wnioski.** W porównaniu do granulki skrobiowych związków skrobiowo-białkowy charakteryzował się mniejszą wartością energii zwróconą do otoczenia, a wyższa energia zwrócona do sąsiednich spinów. Zarejestrowane różnice były niewielkie, prawdopodobnie ze względu na niewielki udział sieci białkowej w całym układzie.

**Słowa kluczowe:** lizozym, modyfikowana skrobia ziemniaczana, mikrofała, low-field NMR, energia aktywacji

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