

Synthesis and characterization of binary complexes of furcellaran with gelatin and bovine serum albumin

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DOI: dx.doi.org/10.14314/polimery.2018.6.2

Abstract: Furcellaran (FUR) complexes with albumin (BSA) and gelatin (GEL) were investigated. The zeta potential (ζ) values of furcellaran, gelatin and their mixtures at weight ratios 2 : 1, 1 : 1, and 1 : 2 (w/w) were measured over a pH range 2.0–10.0. FUR/GEL and FUR/BSA complexes were prepared by the electrolysis of aqueous solutions of both components taken in the 1 : 1 (w/w) ratio. The results of the elemental analysis, FT-IR spectroscopy and thermal analysis confirmed the formation of the complexes. The investigated complexes differed in their susceptibility to enzymatic hydrolysis and solubility. At room temperature, the solubility of FUR/GEL and FUR/BSA complexes was 0.055 ± 0.021 g/100 cm³ H₂O and 0.031 ± 0.020 g/100 cm³ H₂O, respectively. Different structures of FUR/GEL and FUR/BSA complexes, determined by SEM studies, can explain differences in both solubility and hydrolytic susceptibility. Enzymatic studies showed that the furcellaran/gelatin and furcellaran/bovine serum albumin complexes are biodegradable. The complexes were not physical mixtures of the components.

Keywords: albumin, furcellaran, gelatin, polysaccharide-protein complexes.

Otrzymywanie i charakterystyka binarnych kompleksów furcellaranu z żelatyną i albuminą surowicy bydlęcej

Streszczenie: Badano kompleksy furcellaranu (FUR) z albuminą (BSA) oraz żelatyną (GEL). Wartości zeta potencjału (ζ) furcellaranu, żelatyny oraz ich mieszanin w stosunku masowym 2 : 1, 1 : 1, 1 : 2 określono w zakresie pH 2,0–10,0. Kompleksy FUR/GEL oraz FUR/BSA otrzymano metodą elektrosyntezy przy stosunku masowym 1 : 1. Metodami analizy elementarnej (EA), spektroskopii w podczerwieni z transformacją Fouriera (FT-IR) oraz analizy termicznej (DSC, TGA) potwierdzono powstanie kompleksów FUR/GEL i FUR/BSA. Otrzymane kompleksy różniły się podatnością na hydrolizę enzymatyczną i rozpuszczalnością. W temperaturze pokojowej rozpuszczalność kompleksów FUR/GEL i FUR/BSA wynosiła, odpowiednio, $0,055 \pm 0,021$ g/100 cm³ H₂O i $0,031 \pm 0,020$ g/100 cm³ H₂O. Obserwowana na zdjęciach SEM odmienna struktura kompleksów może tłumaczyć różnice w ich rozpuszczalności oraz podatności na hydrolizę enzymatyczną. Analiza przebiegu hydrolizy wskazywała na biodegradowalność badanych kompleksów. Potwierdzono, że otrzymane kompleksy nie są fizycznymi mieszaninami składników.

Słowa kluczowe: albumina, furcellaran, żelatyna, kompleksy polisacharydowo-białkowe.

Combining biopolymers such as proteins and polysaccharides results in the formation of natural complexes see, for instance, review [1]. Factors determining the formation of the complexes are size (molecular weight) and type of biopolymers, that is, their chain conformations, flexibility, localization of reaction sites and charge density over their pH, ionic strength and temperature dependent solubility, polysaccharide to protein ratio, and overall biopolymer concentration [2]. However, the anionic characteristics of polysaccharides combined with proteins are the most crucial

factors. Thus, recent reports on the formation of such binary complexes involve anionic polysaccharides such as potato starch [3–5], phosphorylated starches [6, 7], carboxymethyl cellulose [8–10], carrageenans [11–13], furcellaran [14], various polysaccharide carboxylates such as pectins [15–18], hyaluronic acid [19, 20], xanthan gum [21, 22], chitosan [23] and other polysaccharide carboxylates [24, 25]. The formation of the complexes is based on bringing the blends of the components to an isoelectric point providing the complexation of the ionized components. Reaching the isoelectric point can involve either regular titration [5–7, 13, 23] or electrosynthesis [3, 4, 8–12, 17, 22]. The complexation of natural anionic polysaccharides with proteins, provides potentially biodegradable either covalent or electrostatic, hydrophobic or non-polar complexes. For the formation of such complexes,

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polysaccharides sourced from plant materials such as distillery corn grain [26], corncobs [27], sawdust [28], and agricultural waste [29] can be utilized with soy protein to provide a biodegradable material with mechanical properties resembling styrene-butadiene synthetic polymers.

Polysaccharide-protein complexes formed in food systems play a role in improving the functional properties of foodstuffs. Such complexes can be used as meat and fat replacers, nanocapsules and edible coatings. Many polysaccharide-protein complexes have found applications in the food industry, medicine, pharmacy and cosmetics [30].

Furcellaran is a negatively charged polysaccharide extracted from red algae *Furcellaria lumbricalis*. It is composed of (1→3)- β -D-galactopyranose and its 4-sulfate modification, and (1→4)- α -D-galactopyranose [31]. For structural and functional properties, furcellaran can be related to κ -carrageenan. In the food industry, furcellaran is used as a thickener, stabilizer and emulsifier (E 407).

In the present paper, the electrochemical syntheses of anionic polysaccharide – furcellaran (FUR) – binary complexes with either bovine serum albumin (BSA) or gelatin (GEL) as the protein component are presented.

The electrochemical synthesis applied for the preparation of furcellaran/protein complexes appeared reproducible for the preparation of stoichiometric polysaccharide/protein complexes, which separated on the anode, being a suitable method for the preparation of a wide range of polysaccharide/protein complexes.

The complexes are environmentally benign as they enzymatically hydrolyze primarily into individual amino acids [32, 33] and monosaccharides [34, 35], and then into ammonia, carbon dioxide and water.

The objectives of the study were:

- to determine the zeta potential of FUR/GEL and FUR/BSA complexes [36] under different concentrations and pH;
- to describe the process of electrosynthesis of FUR/GEL and FUR/BSA binary complexes.

EXPERIMENTAL PART

Materials

- Furcellaran (FUR) was purchased from the Est-Agar Company (Karla Village, Estonia);
- Gelatin (GEL) was isolated from Canadian fish skin (Sigma-Aldrich, Poland, catalogue no. G7765);
- Bovine serum albumin (BSA), fraction V, lyophilized powder, 99 % was purchased from Sigma-Aldrich, Poland;
- Trypsin from porcine pancreas, lyophilized powder, Bio-Reagent, suitable for cell culture, 1000–2000 BAEE units/mg solid (Sigma-Aldrich, Poznan, Poland), [\sim 270 BAEE units correspond to 1 international unit (U) at 25 °C];
- Glucoamylase OPTIDEX[®] L-400 401-04122-001 (Genencor International, USA) isolated from fungus with activity of 365 U/g at pH 4.0–5.5 and 35–40 °C.

All materials were used without further purification. The distilled water used in all experiments was produced with the three-stage Millipore Direct-Q 3UV purification system. The experiments were performed at room temperature [22 °C (295 K)].

Complex formation

15 cm² platinum electrodes were placed in a beaker (200 cm³) with a 2.5 cm distance. The beaker was filled with aqueous solutions of FUR (1 %, w/w) and protein (1 %, w/w). Electrodes were connected to the feeder (DC POWER A 605D, PowerLab). The electrosynthesis was conducted at room temperature and 20 V with an initial current intensity of 0.18 \pm 0.1 A.

The complex covered the anode and it was removed after each 5 min period, washed twice on the filter with an acetone : water (2 : 1) solution (20 cm³), and then with acetone (20 cm³) and left to dry at room temperature. The complex formation was conducted in triplicate.

Enzymatic degradation

FUR/GEL and FUR/BSA complexes, as well as the individual components of those complexes, were separately digested with glucoamylase and with trypsin. Both the protein and polysaccharide components of the complexes were subjected to enzymatic digestion. The polysaccharide component of the complex was hydrolyzed by glucoamylase. Glucoamylase (0.25 cm³ of commercial product) was introduced to the aqueous suspension of the complex (0.1 g/200 cm³ H₂O) and the flask was placed in a shaker at 37.7 °C. The reaction course was monitored spectrophotometrically by controlling the level of the reducing sugar by the Miller method utilizing 3,5-dinitrosalicylic acid [37].

The reaction course of the protein digestion by trypsin was monitored by titration according to Sørensen (the formol method) [38]. The trypsin solution was prepared (0.1 g in 10 cm³ H₂O) and added to the complex (0.1 g/190 cm³ H₂O). The mixture was incubated in a water bath at 37.7 °C. Immediately after mixing, 2 cm³ of the mixture was collected and transferred to a measuring flask (50 cm³) containing 4 cm³ of formalin (control test). The control test was titrated with 0.02 M NaOH solution against phenolphthalein. Samples collected at 10 min intervals were analyzed identically. The amount of consumed titrant was recalculated for the amount [μ mol] of protons of carboxylic groups (1 cm³ of 0.02 M aq. NaOH solution is equivalent to 20 μ moles of COOH groups).

Methods of testing

Zeta potential measurements

A Zetasizer Nano ZS Malvern (Worcestershire, UK) was used to determine the zeta potentials for FUR and GEL solutions and FUR+GEL mixtures in 0.005 M NaCl.

The initial concentration of FUR and GEL was 1000 ppm. Zeta potential analyses were conducted in triplicate.

Solubility

The complex was dried for 5 h at 100 °C. Then, 0.2 ± 0.0001 g sample was placed in distilled water (100 cm^3). The suspension was left at room temperature without stirring. After 24 h, the residues were filtered and dried at 100 °C for 2 h. Additionally, solubility tests in DMSO and 7 M aqueous urea at room temperature were conducted. The precipitate was weighed, the solubility of the product was determined. The estimations were triplicated.

Elemental analysis

The elemental analysis for C, H and N was performed with an EURO EA 3000 Elemental Analyser (EuroVector, Italy).

FT-IR spectra

FT-IR spectra of complexes and of all components were examined in the range of $4000\text{--}500 \text{ cm}^{-1}$ in KBr discs (3 mg in 300 mg KBr) using a Mattson 3000 FTIR spectrophotometer (Pye-Unicam, Cambridge, UK).

Differential scanning calorimetry (DSC)

DSC experiments were performed with a Mettler-Toledo 821e (Columbus, Ohio, USA) calorimeter equipped with an Haake intracooler (Apeldoorn, Nederland) under a constant flow of argon ($80 \text{ cm}^3/\text{min}$) within the temperature range of $25\text{--}500 \text{ °C}$ at the heating rate of $10 \text{ °C}/\text{min}$. Samples (2–3 mg) were placed in 40 mm^3 hermetically closed aluminum pans. DSC analyses were conducted in triplicate.

Thermogravimetric analysis

Thermogravimetric analysis was performed using a thermogravimetric analyzer TGA701 (LECO Corporation, St. Joseph, MI, USA). Samples ($\sim 0.2 \text{ g}$) were placed in ceramic crucibles and heated from 25 °C to 650 °C at $2 \text{ °C}/\text{min}$ under nitrogen ($3.5 \text{ dm}^3/\text{min}$). Weight loss (TG) and first derivative of weight loss (DTG) in time dependence on temperature were recorded.

Table 1. The physicochemical properties of furcellaran, gelatin and bovine serum albumin

Sample	Molecular weight kDa	Isoelectric point
Bovine serum albumin (BSA)	67.4	4.8
Gelatin (GEL)	29.6	4.0–8.0
Furcellaran (FUR)	255	–

Surface morphology

The surface morphology of complexes was observed by the scanning electron microscope JEOL JSM-7500F (Japan).

RESULTS AND DISCUSSION

Effect of the component ratio on the furcellaran/protein complex

The physicochemical properties of BSA, GEL and FUR are shown in Table 1.

Zeta potential manifests the presence of charged molecules in the system. The change of its value upon the formation of complexes can be a convenient indicator in studying the complexation. Thus, zeta potentials of the individual components and the FUR+GEL mixtures at varying polysaccharide : protein concentration ratio were investigated. The results are shown in Fig. 1.

The measurements of zeta potential of FUR+BSA mixtures were previously reported. The formation of FUR/BSA complexes at varying ratios and pH were monitored by changes of zeta potential. The optimum conditions for FUR/BSA complex formation as a core of nanocapsules were determined [23].

GEL showed a net positive charge because, below the isoelectric point, proteins carry a net positive charge. When the pH decreased, an increase in the zeta potential values might contribute to the increasing number of positively charged amino groups, while the dissociation of the carboxyl groups was suppressed. Negative charges for FUR were observed in a wide range of pH from 2 to 10. As shown in Fig. 1, the net charge of FUR+GEL mixture in the 2 : 1 (w/w) ratio remained negative, indicating

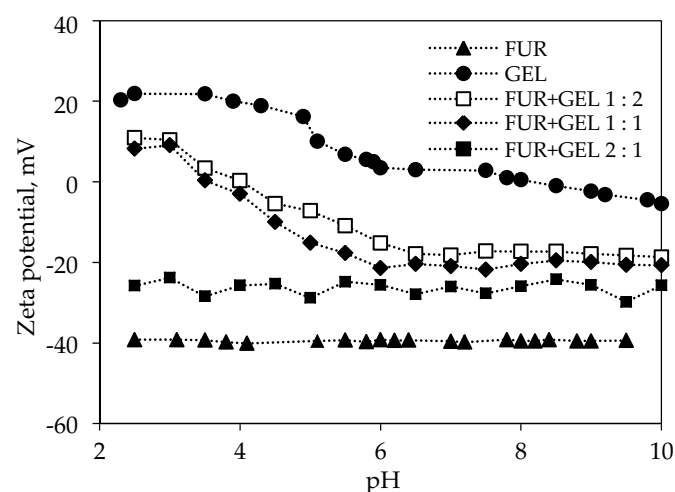


Fig. 1. The zeta potential measurement of individual FUR, GEL and FUR+GEL mixtures in different ratios as a function of pH, measurements in 0.005 M NaCl; FUR – furcellaran, GEL – gelatin, FUR+GEL 1 : 2 – furcellaran + gelatin mixture in ratio 1 : 2, FUR+GEL 1 : 1 – furcellaran + gelatin mixture in ratio 1 : 1, FUR+GEL 2 : 1 – furcellaran + gelatin mixture in ratio 2 : 1

that the positively charged GEL did not neutralize all of the negative FUR groups. A similar result was observed in the FUR+BSA mixture of the 2 : 1 ratio [23]. At pH below the isoelectric point of the proteins, the net positive charge of proteins interacted with the anionic groups of the polysaccharide. For a high protein to FUR ratio, the zeta potential of such mixtures was slightly lower than that for individual proteins. The measured net charge of all mixture FUR+GEL = 1 : 2 were positive and similar to those for protein, indicating an excess of protein.

Electrochemical synthesis and characterization of FUR/protein complexes

The FUR/protein complexes were prepared through electrosynthesis. The complexes collected from the anode were colorless, gummy materials, which gradually solidified into a hard sponge. The 1 : 1 FUR blends with either GEL or BSA yielded corresponding complexes. The formation of the FUR/BSA complex was slower than the formation of the FUR/GEL complexes. After 60 minutes, the yield of the complexes was 55 and 65 %, respectively. In the reaction course, the initial pH changed over time stabilizing within the first 20 min. On synthesis of the FUR/GEL complex, the initial pH of 5.60 increased to 10.55 whereas on synthesis of the FUR/BSA complex, the pH rose from pH 6.95 to 11.30. This showed that, due to discharge of the cations of the IA and IIA groups present in the reagents, the isoelectric point specific for the formation of water insoluble complexes was reached at the anode. Electrosynthesis automatically adjusted the pH of the reaction mixtures in a range corresponding to an electrical compatibility of components.

The FUR/GEL and FUR/BSA complexes were prepared by electrosynthesis from aqueous solutions of both components taken in the ratio 1 : 1. This proportion was chosen after the estimation of the zeta potential and yield measurements of complexes. At component ratios differ-

ent from 1 : 1, an increase in the protein or polysaccharide content in the solution was paralleled by an increase in the protein or polysaccharide content in the complex. The results of the elemental N, S analysis (Table 2) confirmed the stoichiometric 1 : 1 ratio for the FUR/GEL and FUR/BSA complexes.

Based on the nitrogen content in the complexes, it was found that both complexes contained 59 % protein and 41 % FUR, whereas the estimations based on the determination of the sulfur content gave 53 % and 47 %, protein and FUR, respectively.

The investigated complexes differed in their solubility. At room temperature, the solubility of FUR/GEL and FUR/BSA complexes was 0.055 ± 0.021 g/100 cm³ H₂O and 0.031 ± 0.020 g/100 cm³ H₂O, respectively. Moreover, DMSO (dimethyl sulfoxide) and 7 M aq. urea solution did not dissolve the tested complexes. A low aqueous solubility of complexes is typical for the products of the combination of anionic polysaccharides and proteins [10, 15].

The characteristic bands on the FT-IR spectra of FUR, BSA and GEL, as well as the FUR/BSA and FUR/GEL complexes are presented in Table 3.

The spectral pattern of the FUR/protein complexes might be considered as a superposition of the spectra of both components. The amide I band (~ 1659 cm⁻¹, stretching carbonyl vibrations) and amide II band (~ 1535 cm⁻¹, stretching C-N vibrations), characteristic for GEL (~ 1662 and ~ 1547 cm⁻¹, respectively), and the band at ~ 1072 cm⁻¹ ascribed to C-O stretching vibrations, characteristic for FUR (1064 cm⁻¹), were present in the spectrum of the complex, but they were shifted with respect to the position of the corresponding bands for the pure components. These shifts indicated that the electrosynthesis led to complex formation, and these spectral changes confirmed the character of protein-polysaccharide interactions.

The thermal properties (DSC) of the physical mixtures of the components and relevant complexes are demonstrated in Fig. 2a and Fig. 2b, for FUR-GEL and FUR-BSA systems, respectively. The diagrams showed that the products collected from the anode could not be the physical mixtures of the components. Some down shifts of the thermal effects could suggest some degradation of the materials under alkaline conditions generated in the course of the complex formation. Formation of the polysaccharide/protein complexes was also confirmed by the thermogravimetric analysis (TG and DTG, Fig. 3a and Fig. 3b).

The TG lines in diagrams of the complexes showed different patterns from the pattern in the diagrams for the physical mixtures of the components. In the thermogram of FUR+GEL mixture and FUR/GEL complexes, very fast decomposition occurred at 183 °C and 215 °C, respectively. The DTG peaks were very sharp. The FUR+BSA mixture and FUR/BSA complex decomposed with an initial loss of 15 % water as manifested by a peaks with its maximum at 64 °C. Then, the FUR+BSA mixture decomposed gradually in at least three steps with the main decom-

Table 2. Nitrogen and sulfur content in complexes and in their components

Symbol fo sample	Nitrogen, %	Sulfur, %
BSA	14.66	1.64
GEL	16.63	0.50
FUR	-	4.14
FUR/GEL complex	10.00	2.81
FUR/BSA complex	8.86	2.24

Table 3. Characteristic bands of FT-IR spectra of complexes and their components

Wave number (cm ⁻¹) and band assignment				
FUR	BSA	GEL	FUR/BSA	FUR/GEL
1064 $\delta_{C=O}$	-	-	1069	1072
-	1533 δ_{C-N}	1547 δ_{C-N}	1533	1535
-	1659 $\gamma_{C=O}$	1662 $\gamma_{C=O}$	1663	1659

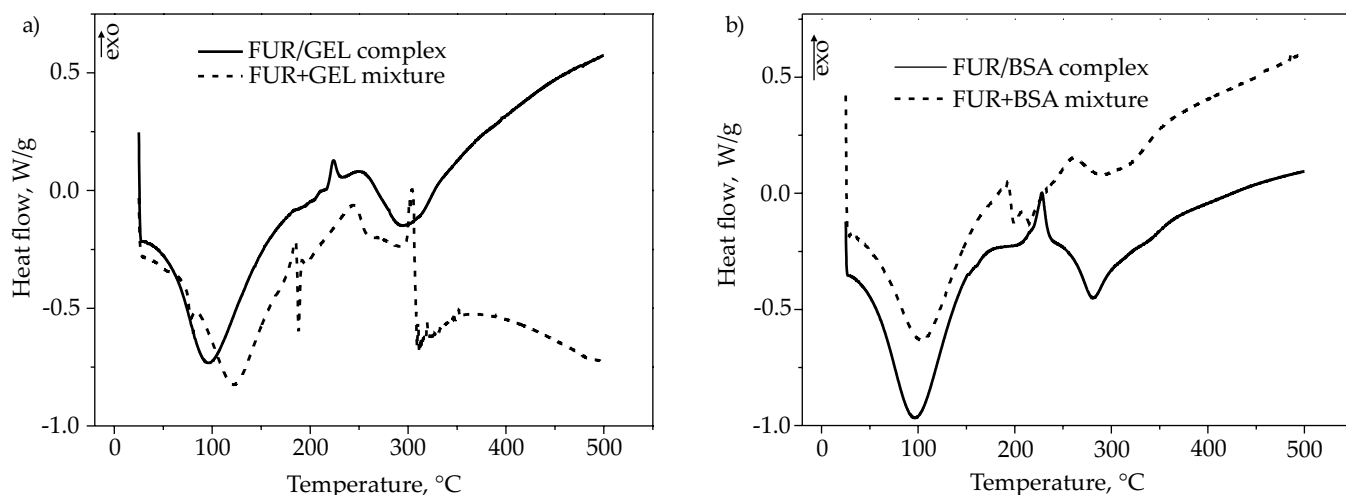


Fig. 2. DSC curves of: a) FUR/GEL complexes and FUR+GEL mixtures, b) FUR/BSA complexes and FUR+BSA mixtures

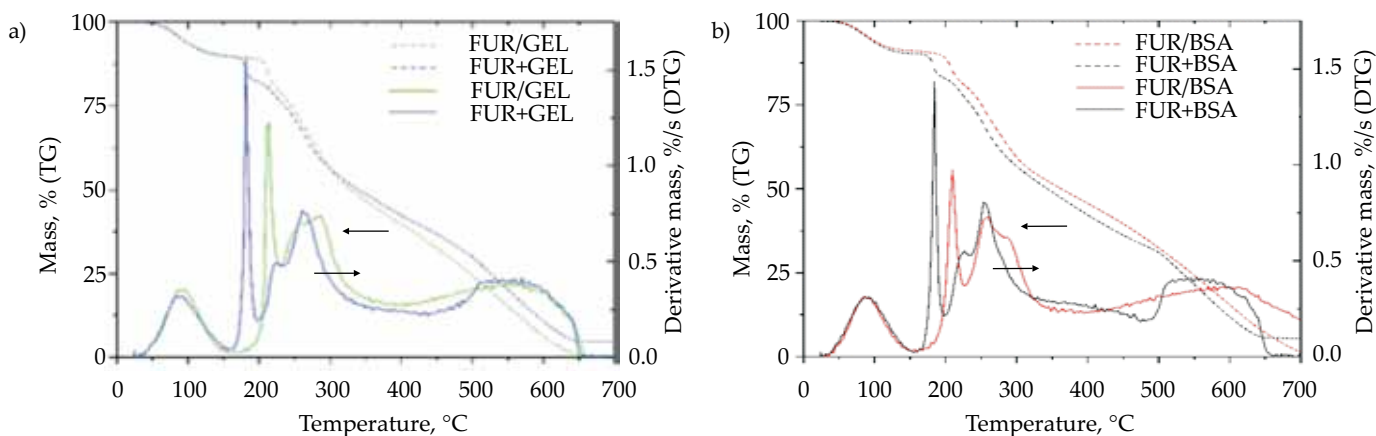


Fig. 3. Thermograms (TG and DTG) of: a) FUR/GEL complexes and FUR+GEL mixtures, b) FUR/BSA complexes and FUR+BSA mixtures

position at 179 °C, and decomposition of the FUR/BSA complex occurred at 220 °C. The TG curves of FUR/BSA and FUR/GEL complexes in the range of 200–650 °C were very steep.

Using glucoamylase, enzymatic hydrolysis was performed on FUR in physical mixtures of proteins as well as in FUR/GEL and FUR/BSA complexes. This series of

experiments delivered further arguments speaking for the formation of complexes. Figures 4a and 4b demonstrate the course of hydrolysis of 1 : 1 physical mixtures of FUR and its complexes with GEL and BSA, respectively.

FUR in the complexes was digested within three steps, but in physical mixtures it hydrolyzed in four stages. In every step, there was a linearity of the reducing sugar

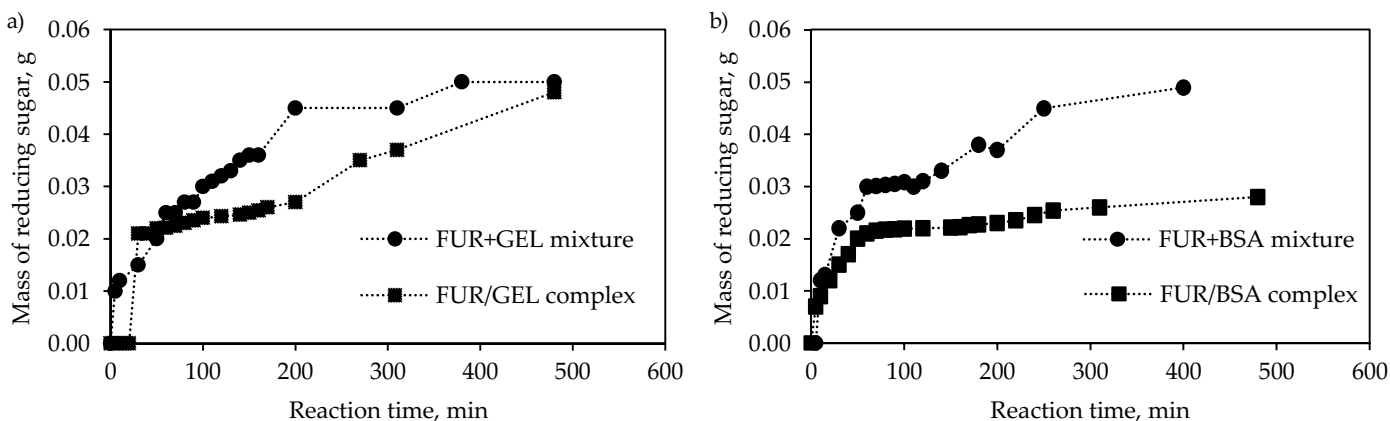


Fig. 4. Time-dependent level of reducing sugar in materials digested with glucoamylase: a) furcellaran and gelatin and their complex, b) furcellaran and albumin and their complex

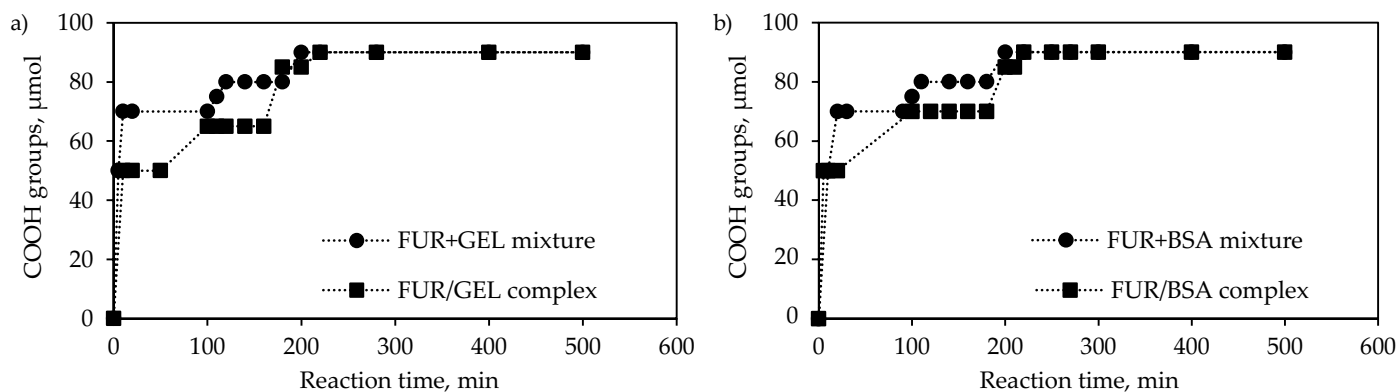


Fig. 5. Time-dependent level of reducing protein in materials digested with trypsin of: a) furcellaran and gelatin and their complex, b) furcellaran and albumin and their complex

Table 4. Reaction rate constants of the hydrolysis with glucoamylase of furcellaran in FUR/GEL and FUR/BSA complexes and 1 : 1 physical mixtures and rate constants of the hydrolysis with trypsin of the protein components in FUR/GEL and FUR/BSA complexes and 1 : 1 physical mixtures

Rate constants (k) of the hydrolysis with glucoamylase of furcellaran in complexes and 1 : 1 physical mixtures g/min					The reaction time of total hydrolysis of FUR, min
	k_1	k_2	k_3	k_4	
FUR/BSA	$3.0 \cdot 10^{-4}$	$6.1 \cdot 10^{-5}$	$2.9 \cdot 10^{-5}$	–	–
FUR/GEL	$4.8 \cdot 10^{-4}$	$5.5 \cdot 10^{-5}$	$6.3 \cdot 10^{-5}$	–	> 480
FUR+BSA	$6.7 \cdot 10^{-4}$	$4.3 \cdot 10^{-5}$	$14.5 \cdot 10^{-5}$	$1.3 \cdot 10^{-5}$	380
FUR+GEL	$3.9 \cdot 10^{-4}$	$10.0 \cdot 10^{-5}$	$32.0 \cdot 10^{-5}$	$1.3 \cdot 10^{-5}$	380
Rate constants (k) of the hydrolysis with trypsin of the protein in complexes and 1 : 1 physical mixtures µmol/min					The reaction time of total hydrolysis of protein, min
	k_1	k_2	k_3	k_4	
FUR/BSA	10	0.4	0.6	0.1	225
FUR/GEL	10	0.2	0.3	0.1	480
FUR+BSA	10	1.8	0.7	0.4	180
FUR+GEL	10	1.0	1.0	0.7	180

content against time, indicating the zero-order of the reactions induced by the maximum saturation of the substrate with the enzyme. Reaction rate constants for every hydrolysis reaction step are reported in Table 4.

It could be seen that the FUR/GEL complex hydrolyzed with a higher rate possibly for the structure of the complex being more adjustable for the hydrolysis with the polysaccharide enzyme. The lower rate constant for initial k_1 and final k_3 stages of the hydrolysis were characteristic for the hydrolysis of the FUR/BSA complex. It could be controlled by a difficult access of the enzyme to the appropriate reaction sites of the complex. The hydrolysis of FUR in the FUR+GEL and FUR+BSA mixtures was completed within about 380 minutes. In turn, the total hydrolysis of FUR in the FUR/GEL and FUR/BSA complexes proceeded with a lower rate.

Trypsin was applied for the hydrolysis of protein components of the complexes and their physical mixtures with FUR. Progress in the protein hydrolyses with trypsin was accompanied with an increase in the content of the carboxylic groups determinable with the formol Sørensen method [25]. The carboxylic groups neutralized

by ammonia could not be directly titrated with alkali. In order to liberate the blocked carboxylic groups, ammonium cations were reacted with formaldehyde to give a methylene derivative of the peptide. The course of the titration of FUR/GEL and FUR/BSA complexes and corresponding 1 : 1 physical mixtures of the components are presented in Figs. 5a and 5b, respectively.

The relevant hydrolysis rate constants for each stage of the hydrolysis are collected in Table 4. These diagrams and determined rate constants indicated that the hydrolysis of GEL in both samples proceeded in four stages. Initially the hydrolysis was fast then, already in the second stage, it significantly slowed down. BSA hydrolyzed in four stages and the initial stage was very fast.

The complete hydrolysis of GEL in the complex took about 480 min whereas in the mixture it lasted only about 180 min. BSA fully hydrolyzed within 225 and 180 min when it resided in the complex and the physical mixture, respectively.

The morphology of the dry complexes was examined by scanning electron microscopy (SEM). The examples of SEM images are presented in Figs. 6a and 6b.

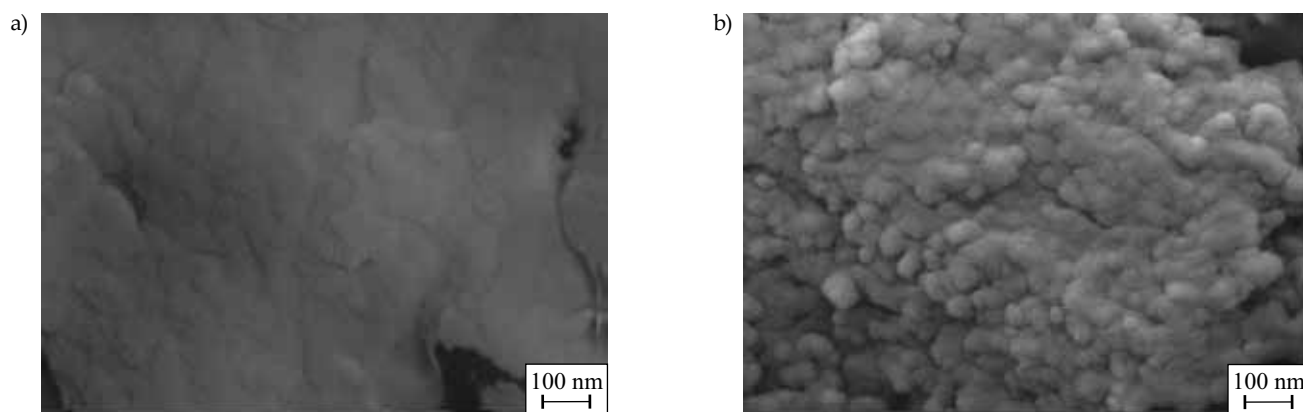


Fig. 6. Scanning electron microphotographs of: a) the FUR/GEL complex, b) the FUR/BSA complex

The surface structure of the FUR/GEL complex was homogeneous, in contrast to the granular structure of the surface of the FUR/BSA complex. Differences in their structure could relate to the differences in solubility and hydrolytic susceptibility of both complexes. The FUR/GEL complex appeared to be a suitable component of biodegradable foils [14] and the FUR/BSA complex can be utilized as a core of nanocapsules [36].

CONCLUSIONS

The electrosynthesis appeared suitable for the formation of binary complexes of furcellaran with proteins such as gelatin and bovine serum albumin.

The formation of the complexes can be controlled by the individual components and by the reaction conditions. The results of the electrosynthesis presented in this paper showed that the polysaccharide/protein ratio and pH are influential factors in the zeta potential.

High yields of the formation of complexes were promising for the development of a high scale production. Enzymatic studies showed that the furcellaran/gelatin and furcellaran/bovine serum albumin complexes are biodegradable.

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Received 17 X 2017.

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