Conference paper

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Hydrolysis of chitin and chitosan in low temperature electron-beam plasma

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Abstract: Hydrolysis of natural chitin and chitosans was performed in the electron beam plasma (EBP) of oxygen, by means of specially designed electron beam plasmachemical reactor (EBPR). Low molecular water-soluble chitin oligosaccharides with weight-average molecular mass 800–2000 Da and polydispersion index 1.5–2.5 were produced due to action of active oxygen species formed in the EBP. By optimizing the treatment conditions the 95% yield of chitin oligosaccharides was obtained after 2 min whereas the conventional chemical hydrolysis usually takes several days. The studies of the antimicrobial activity of low molecular products formed due to EBP-stimulated degradation showed that they inhibit the multiplication of various mycelial and yeast-like fungi. The technique involved is likely to be promising for the production of bioactive low molecular chitin oligosaccharides and the EBP-stimulated hydrolysis appears to be competitive with technologies conventionally used in the industry.

Keywords: bioactive oligosaccharides; chitin; chitosan; electron-beam plasma; EUCHIS-12; ICCC-13; plasma-stimulated hydrolysis.

Introduction

Natural renewable biopolymers chitin (linear heterocopolymers of β-1,4-linked 2-amino-2-deoxy-D-glucopyranose and 2-acet-amido-2-deoxy-D-glucopyranose units) and, its deacytelaited derivative chitosan, are very promising for technological and industrial applications such as agriculture, food processing, cosmetics production and others [1, 2]. Despite unique properties (high biocompatibility with living tissues, biodegrability, ability to the complexation, low toxicity, and etc.) these biopolymers, by themselves, have limited applications in industry, agriculture and biomedicine because of their insolubility in most solvents. In medicine and pharmaceutics water-soluble low molecular weight chitin oligosaccharides (< 10 kDa) are usually required. These substances can be used as immune response-modulating or antibacterial agents, sorbents, radioprotectors, and for the production of microcapsules, thin films, and substrates for cell cultures [1, 2].

To produce low molecular weight oligosaccharides several techniques, including chemical, enzymatic, and radiation treatment by γ-irradiation and high-energy electron beams (with energies MeVs) have been suggested [3–5].

Simple and rather low-cost chemical hydrolysis in concentrated acids or alkalis at high temperature is a conventional method. The toxic wastes and environment contamination are inherent in chemical processing

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of polysaccharides. Besides, the chemical methods are very time consuming and usually take several hours. The radiation treatment is also complicated because of limited controllability of treatment conditions, high power consumption, and operation complexity of electron accelerators and γ-radiation isotope sources.

Thus, the development of effective techniques for quick and environment friendly polysaccharides degradation is the burning issue of the day. The plasmachemical technologies based on non-equilibrium low temperature plasmas could be a promising alternative to the hydrolysis methods mentioned above.

The novel approach to the water-soluble low molecular weight oligosaccharides production based on the electron beam plasma (EBP) is considered in present paper. The EBP is generated by injecting an electron beam (EB) into a gaseous medium. Under typical conditions of the EBP generation (medium pressure $1 < P_m < 10$ kPa and moderate EB power $N_b < 1$ kW) plasma is strongly non-equilibrium and cold. The advantages of the EBP with respect to gas discharge plasmas commonly applied for the modification of polysaccharides thin films and solutions [6–8] have been considered in our previous papers [9, 10].

Objectives of present study were as follows:

- To experimentally prove the possibility of the EBP-stimulated hydrolysis of native polysaccharides and formation of water-soluble low molecular weight products by plasmachemical processing.
- To characterize both the structure of low molecular weight products of the plasmachemical treatment and their bioactivity.
- To obtain a high yield of the low molecular weight products by optimizing the treatment conditions.

The procedure of polysaccharide EBP-stimulated hydrolysis

Crab shell high molecular weight chitin (viscosity-average molecular weight, $M_v = 1000$ kDa) obtained form red king crab *Paralithodes camtschaticus* and chitosans ($M_v = 200–500$ kDa) with the degree of deacetylation 85%–98% and polydispersity index 1.5–2.5, were used as the original substances for the further EBP-treatment. Chitin was preliminary deproteinated, demineralized and discolored by standard chemical methods. The chitosans were produced by means of chemical chitin hydrolysis in 40% NaOH solution for 24 h at 100 °C. All substances were not water-soluble.

Molecular masses (weight-average M_w, number-average M_n, and z-average mass M_z) of the EBP-treatment products were determined by the size exclusion chromatography on a LC-20 Prominence HPLC system (Shimadzu, Japan) equipped with refractometric detector RID-10A. The chromatographic column was Ultrahydrogel 500 7.8×300 mm (Waters, USA). Other analysis conditions were as follows: the mobile phase – buffer containing 0.05 M acetic acid and 0.15 M ammonium acetate (pH = 5.1); the flow rate – 0.5 ml/min; temperature – 30 °C; volume of the injected sample – 40 µl; duration of the analysis – 25 min. Dextrans (Sigma, USA) were used as standard samples for mass scale calibration. Individual *N*-acetylglucosamine oligomers were determined by the reversed-phase chromatography using the Asahipak NH2P-LF (Shodex, Japan) chromatographic column. The column was calibrated with model mixture of *N*-acetylglucosamine oligomers with the polymerization degree n = 1–6.

For the controllable EBP-stimulated hydrolysis of polysaccharides a special electron beam plasmachemical reactor (EBPR) was used. The EBPR, its operation modes and optimization of the biomaterial treatment regimes were described in detail in [11].

Figure 1 illustrates the design and operation of the EBPR used for the biomaterials modification. The focused EB 3 generated by the electron-beam gun 1 that is located in the high vacuum chamber 2 is injected into the working chamber 5 filled with the plasma generating gas through the injection window 4. In passing through the gas (pure oxygen or vapor of bidistilled water) the EB is scattered in elastic collisions and the energy of fast electrons gradually diminishes during various inelastic interactions with the medium (ionization, excitation, dissociation). The main plasmachemical reactions occurring in the oxygen EBP are summarized in Table 1. As a result, the EBP cloud 10 is generated, all plasma parameters being functions of x, y, and z coordinates (z is the axis of the EB injection).

The electromagnetic scanning system 12 placed inside the working chamber near the injection window is able to deflect the injected EB axis in x and y directions and, therefore, to control the spatial distribution of

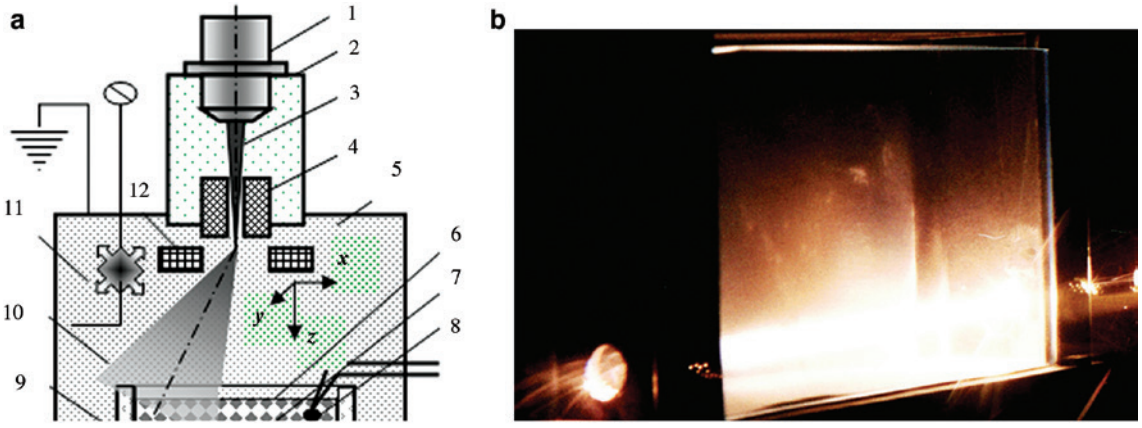

Fig. 1: The plasmachemical reactor design and treatment procedure of polysaccharides powders (a); the mixing layer of polysaccharides powders in EBP (b).
1, electron beam gun; 2, high vacuum chamber; 3, EB; 4, injection window; 5, working chamber; 6, mixing layer of the powder to be treated; 7, piezoceramic plate; 8, temperature sensor; 9, glass container; 10, EBP cloud; 11, water evaporator; 12, scanning system.

Table 1: The main plasmachemical reactions in oxygen EBP.

Reaction	The energy (eV (*)) or the rate constant ($cm^3\ s^{-1}$ or $cm^6\ s^{-1}$ (**))
$e_b + O_2 \rightarrow e_b + O + O$	20,2 (*)
$O + 2O_2 \rightarrow O_3 + O_2$	$6.9\times10^{-34}(300/T_g)^{1.25}$ (**)
$O + O_3 \rightarrow O_2 + O_2$	$2\times10^{-11}\exp(-2300/T_g)$
$O + O + O_2 \rightarrow 2O_2$	$6.7\times10^{-33}(300/T_g)^{0.63}$ (**)
$O_3 + O_2 \rightarrow O + 2O_2$	$1.65\times10^{-9}\exp(-11400/T_g)$

the plasma particles over the plasma bulk. The working chamber is preliminary evacuated to pressure ~1 Pa and then filled with the plasma generating media.

In the experiments the powder of polysaccharides partially filled the glass container 9 over the thin plate 7 made of piezoelectric ceramics that is at the container bottom. Being fed with AC-voltage the plate vibrates, throws up the powder particles and forms the mixing layer 6 of the treated material inside the container (Fig. 1b).

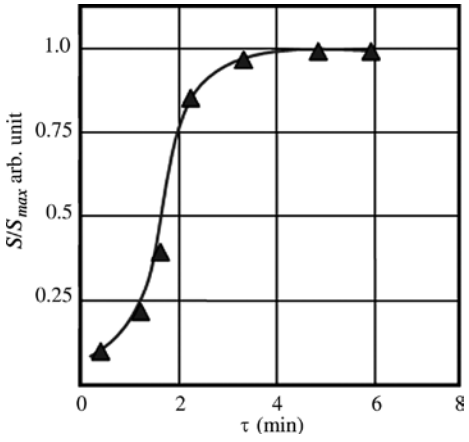

Fig. 2: The yield of the water-soluble low molecular weight products (S/S_{max}) from the chitosan treated by the oxygen EBP as a function of the treatment time (τ).

The experimental conditions were as follows:

- Plasma generating gasses – pure oxygen or water vapor.
- The pressure of the plasma generating gas – 670 Pa.
- The distance between the injection window and sample surface – 250 mm.
- The EB scanning mode – concentric circles with maximal diameter – 130 mm.
- Treatment time τ was varied from 1 to 20 min.
- To prevent thermal destruction of the material all samples were processed at material temperature < 70 °C. The sample temperature was monitored during the treatment by miniature thermo-sensor 8 (Fig. 1a). The sample temperature was controlled by selecting the EB current I_b ($1 < I_b < 100$ mA).

Characterization of chitin oligosaccharides produced by the EBP-stimulated hydrolysis

The original chitin and chitosan were not water-soluble and the EBP-treatment increased their solubility in water due to the low molecular weight chitosans and chitin oligosaccharides (COS) formation. The 90%–95% yield of low molecular weight EBP-treatment products was attained by optimizing the treatment procedure (for example the treatment duration or the composition and the pressure of the plasma generating media).

The variation of the low molecular weight products yield as a function of the treatment time $S(\tau)$ is shown in Fig. 2. At the shortest τ the dependence $S(\tau)$ increases smoothly, then – steeply close to $\tau = 2$ min after which the yield of the water-soluble products does not change. Similar $S(\tau)$ relations were found for chitin.

Weight-average (M_w) and number-average (M_n) molecular masses of COS produced by the EBP-stimulated degradation were characterized using the exclusion chromatography. The mechanism of the EBP-stimulated hydrolysis of polysaccharides was studied mainly for chitosan due to its solubility in aqueous media in a protonated form. Thus the detection of low molecular weight chitosan forms is possible in a wide M_w range whereas only short fragments (polymerization degree $n \leq 7$) can be identified in the case of chitin.

The exclusion chromatography of the EBP-treated chitosans treated in the EBP of both oxygen and water vapor for 1–3 min revealed the formation of a number of COS with $M_w = 800$–2000 Da and polydispersion index 1.1–3.9, which corresponds to the formation of chitosan fragments with degradation degree varied from dimers to pentamers. The chitosan destruction terminated with the prolongation of the treatment time up to 5–7 min (Fig. 3). When the treatment duration exceeded 10 min the condensation of chitin oligosaccharides occurred, which resulted in the high molecular products formation due to the reaction between aldehyde and amino groups contained in chitosan chemical structure and the loss of solubility.

Similar low molecular weight products formed by the EBP-stimulated hydrolysis of chitin for 1–5 min had $M_w = 1000$–2000 Da. Thus the same mechanism of both chitin and chitosan EBP-stimulated destruction can be assumed.

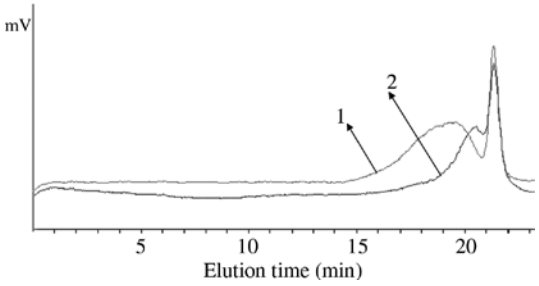

Fig. 3: The exclusion chromatograms of chitosan treated in the oxygen EBP for $\tau = 2$ min (curve 1; $M_n = 2258$ Da, $M_w = 8755$ Da, and $M_w/M_n = 3.88$) and $\tau = 5$ min (curve 2; $M_n = 542$ Da, $M_w = 818$ Da, and $M_w/M_n = 1.51$).

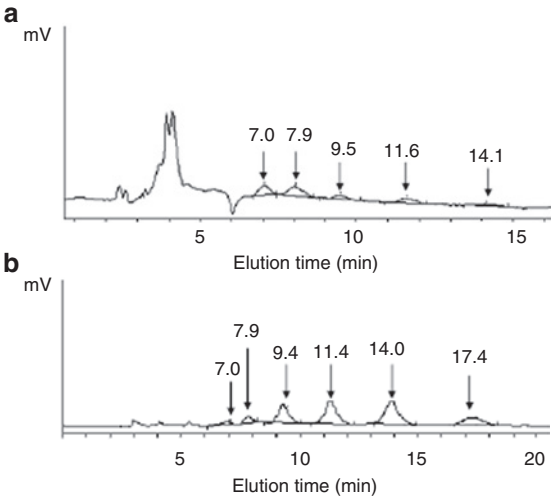

Fig. 4: The reversed-phase chromatography of (a) water soluble products of the chitin treatment in the EBP of oxygen ($M_n = 461$ Da, $M_w = 1110$ Da, and $M_w/M_n = 2.38$) and (b) a model mixture of the individual *N*-acetylglucosamine oligomers with the degrees of polymerization n = 1–6. The numbers at arrows indicate the elution times of the compounds.

The reversed-phase chromatography (within the sensitivity of the technique) showed that retention times of water soluble COS produced during chitin EBP-treatment and a model mixture of the individual *N*-acetylglucosamine oligomers were approximately equal (Fig. 4). The lack of significant deacetylation was also proved by the FTIR-spectroscopy. So the unmodified acetylated chitin oligomers can be obtained by the plasmachemical hydrolysis.

The degradation of the original polymer is due to the effect of free radicals formed in the EBP. Active oxygen species (O, O$^{\cdot}$, singlet oxygen) that are produced in plasmachemical processes (Table 1) and the products of the water plasmolysis (e.g. OH$^{\cdot}$) seem to be the most important. These chemically active particles break the β-1,4-glycosidic bound and decrease the polysaccharides molecular weight. Figure 5 illustrates the possible degradation mechanism [12]. The oxidation changes in the polysaccharides molecules with the

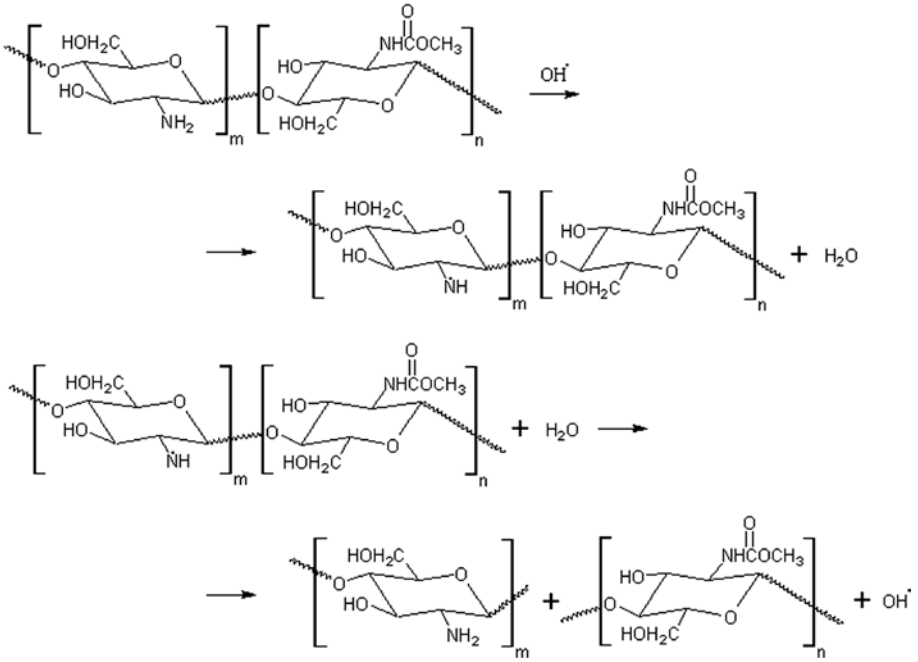

Fig. 5: The scheme of chitin degradation under hydroxyl radical action in the EBP of the water vapor [12].

significant increase of hydroxyl –OH, carbonyl C=O, and carboxyl –COOH groups due to the treatment in the EBP of oxygen and water vapor were detected using the FTIR-spectroscopy.

Biological activity of chitin oligosaccharides produced by the EBP-stimulated hydrolysis

The inhibition of the bacteria growth *in vitro* was measured to quantitatively characterize the bioactivity of low molecular weight chitin oligosaccharides, formed due to the EBP-stimulated degradation, yeast-like and filamentous fungi being used in these experiments.

The products obtained by the treatment of chitosan in the oxygen EBP for 5 min inhibited the growth of filamentous fungi *P. tardum*, *P. chrizogenum*, *A. flavus*, *P. betae*, and *C. herbarum* at final concentration 500 mcg/ml up to 99%. The most sensitive yeast-like fungi were *C. scotti* and *R. rubra*. We suppose that the antibacterial activity of the EBP-produced low molecular weight chitin oligosaccharides results from their interaction with cell walls of microorganisms.

Prospective applications of chitin oligosaccharides produced by the EBP-stimulated hydrolysis

Low molecular weigh chitin oligosaccharides produced by the EBP-stimulated hydrolysis can be used as:

- Active components for novel hybrid bioactive materials with combined properties (e.g. hemostatic/antibacterial).
- Bioderadable scaffolds for Regenerative Medicine with enhanced cell adhesion and growth.
- Active components for addressed drug delivery systems and systems for controllable drug release.
- Active components for plant biostimulators and fertilizers.
- Components for biosensors.
- Materials for effective sorbents, filters, and membranes (e.g. for wastewater treatment, hemodialysis, etc.).
- Materials for active packaging and food conservation.

The interesting application of chitin and chitosan (mostly of poriferan origin) is Extreme Biomimetics [13–15] which are the imitation of natural systems widely used in the in modern technology and medicine including extreme (psychrophilic and thermopilic) biomineralization, solvothermal and hydrothermal chemistry of metal oxides and nanostructured composites, bioinspired materials science, and biosensing [16]. The driving force for developing new chitin-based materials and chitin processing at high temperatures using the principles of hydrothermal synthesis and Extreme Biomimetics is its thermal stability up to 200 °C [13]. Though we considered the EBP-stimulated hydrolysis of polysaccharides at sufficiently low temperatures (≈ 70 °C), the temperature of this process can be increased by increasing the EB power, changing plasmagenerating media, and adjusting geometry of reaction volume. Furthermore many extreme factors, besides high temperature, act on the material treated in the EBP, namely the X-ray irradiation, irradiation by fast electrons of the electron beam, flows of chemically active plasma particles and some others. Note that the beams with electron energies up to 30 keV can be generated in the EBPR described in present paper if the operation pressure is reduced (at 10^{-3} Pa and lower). Therefore, the beam-plasma reactors can be used for Extreme Biomimetics engineering to model a variety of extreme processing conditions and to study the advanced chitin-based materials stability.

Conclusions

1. The possibility of the EBP-stimulated hydrolysis of chitin and chitosan with formation of water-soluble low molecular weight products was proved experimentally. The threshold relationship between polysaccharide destruction and the duration of EBP-treatment was found.
2. The 90%–95% yield of low molecular weight EBP-treatment products was attained by optimizing the treatment procedure. The highest yield of low molecular weight water soluble products was obtained at treatment time ~ 2 min whereas the traditional chemical hydrolysis usually takes several days.
3. The deacetylation of chitin oligomers did not occur during the EBP-stimulate hydrolisys.
4. The active oxygen species produced in plasmachemical reactions and the products of water plasmolisys were found to be responsible for the COS formation.

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