

HONEYBEE (*Apis mellifera*) CHITOSAN: PURIFICATION, HETEROGENEITY AND HEMOCOAGULATING ACTIVITY

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The aim of the research was to elaborate the method of chitosan preparation from honeybee corpses. It includes the following stages: 1) washing of bee corpses with hot water; 2) delipidation of powdered material with petrol ether; 3) decalcification by EDTA at pH 4; 4) deproteination by 3 fold treatment with 5% NaOH 1 h at 70 °C; 5) bleaching of chitin with sodium hypochlorite; 6) deacetylation of chitin in 40% NaOH solution at 115 °C for 3 h; 7) purification of chitosan by its dissolving in 3% acetic acid and precipitation with ammonia at pH 8,5; 8) separation into three fractions precipitated at pH 6.4, 7.0 and 8.6. The yield of chitosan from dry bee powder was 8.5–10.0%. A distinct diversity in the molecular mass of different chitosan fractions was revealed in the range of 80–320 kDa. Heterogeneity of chitosan samples was studied using gel permeation chromatography on Acrylex P-150 and electrophoresis in a slab of polyacrylamide gel with a stepwise gradient of acrylamide concentration 5, 10, 15, 20% in pH 4.5 buffer system. High molecular mass chitosan possessed blood coagulating activity, while low molecular mass fractions were poorly active. The rate of blood clot formation induced by active honeybee chitosan was 3 times lower comparing with that of chitosans obtained from crabs or shrimps.

Key words: honeybee, chitosan, heterogeneity.

Chitin and chitosan are substances of natural origin which have been recently intensively introduced into different fields of science and techniques [1–3]. These substances are produced on a great scale commercially, usually from shells of marine crustaceans (crabs, oysters, krill, shrimps) which are available in big amount as a waste product of food industry. In the terrestrial organisms, chitin is widespread in insects, however, it is much less studied. There are several publications and patents devoted to the obtaining of chitin, chitosan and chitosan-melanin complex from social insects, such as honeybees (*Apis mellifera*) [4–8], silkworm pupas (*Bombyx mori*) [9], locust (*Schistocerca gregaria*) and beetles *Calosoma rugosa* [8], bumblebee (*Bombus terrestris*) [10], fly pupas (*Musca domestica*) [11]. The main attention was paid to the honeybee

due to common availability of bee corpses as a waste product of the apiculture. Some preparations obtained from honeybee corpses are used in nontraditional or folk medicine and a commercial product from bee corpses is proposed recently as a source of the biologically active substances [12].

It can be suggested that some properties of insect chitin and chitosan differ from that of marine organisms and might be of interest in biomedical view. In this communication, we presented our version of chitosan preparation from honeybee corpses and described its physico-chemical and biological properties, namely the hemocoagulating, activity. A rapid and convenient procedure for depigmentation of the insect chitin as well as original approach for the electrophoretic analysis of chitosan molecules distribution according to their molecular mass are also proposed.

Materials and Methods

Honeybee corpses of local origin (Lviv region, Ukraine) were obtained during hives cleaning. The material was cleaned from foreign particles just after obtaining and washed by suspending in a boiling water. After a spontaneous cooling, the liquid was squeezed through a cloth and discarded. Washing of the solid residue with boiling water was repeated 3–4 times, and finally the rest of liquid was eliminated by press machine. Wet material was dried at ambient temperature and stored in hermetically closed vessel with a piece of cotton moistened with chloroform for preventing mould growth.

Standard samples of chitosan were purchased from “Aldrich” (middle molecular weight), chitosan “Tyanshi”, dietary supplement (Tiens Group Co. Ltd., China; Tiens.in.ua), “Organica” (Health Products Inc., Canada) used as a dietary supplement for management of blood cholesterol level. Chitosan from the last source was purified by an extraction of powder with 3% acetic acid, the extract was clarified by centrifugation at 4,000 g and chitosan was precipitated with ammonia at pH 8.0. This process was repeated twice.

Solution of sodium hypochlorite used for bleaching of bee chitin was prepared according to [13]. Briefly, 40 g of calcium hypochlorite (chlorine content 25–28%) was minced with 40 ml of water in a mortar. To this slurry 28 g of sodium carbonate in 60 ml of water was added and mass was thoroughly steered to liquefaction and left for several hours in a closed vessel. Thereafter, it was filtered through a thick cloth and obtained liquid (about 70 ml) was filtered using a filter paper. Concentration of chlorine in solution was 7.0–7.5% (determined by iodometrical analysis) [14]. Indicated proportions have to be saved if larger quantities are processed.

Characterization of obtained products

Average molecular mass of chitosan was determined by the viscometry method [15] as described in [16]. Viscometer of Ubbelohde type VPZh-4, “Soyuznauchpribor”, USSR, with a diameter of capillary 0.56 mm was used. Chitosan solutions were prepared in a solvent of 0.17 M acetic acid: 0.2 M NaCl (1:2). The initial concentration of chitosan was 1 mg/ml, and for low molecular mass chitosan (below 100 kDa) it was 2 mg/ml. The starting solutions were filtered through a Schott filter and in course of measurement they were diluted sequentially with above solvent. Measurements were conducted at 25 °C.

Deacetylation degree (DD) was determined by the potentiometric titration as described earlier [17].

UV/Vis spectra of chitosan solutions in 3% acetic acid were measured using spectrophotometer SF-42 (LOMO, Russian Federation).

Fourier transformed infrared (FTIR) spectra were investigated using Bruker FTIR spectrometer (Germany), Vertex X70 model. Discs with samples of chitin or chitosan were prepared by the compression of the fine powdered mixture of tested sample (2 mg) and 200 mg of KBr prepared by a thorough grinding in the agate mortar. Spectra were recorded in the range of frequency from 4 000 to 500 cm^{-1} . Experiments were conducted at the Department of Inorganic Chemistry of Duisburg-Essen University (Germany) under a courtesy of prof. M. Epple.

Characterization of chitosan powder also included determination of bulk density, swelling in water and in Tris-buffered saline, pH 7.4, a time of clot formation in a probe of the heparinized blood as described in [17].

Gel permeation chromatography (GPC) was conducted in a column filled with the polyacrylamide gel Acrylex P-150 (Reanal, Hungary) using 0.05 M ammonium-acetate buffer, pH 6.0 as eluent. Chitosan in the eluate was detected by an adjustment of the tube content to $\text{pH } 9.0 \pm 0.3$ with several drops of 10% ammonium hydroxide and measuring the turbidity at 600 nm after 30 min. Distinct fractions of chitosan were obtained from the combined eluates by collecting precipitated at pH 9.0 material using centrifugation, dissolution of the sediment by an adjustment of pH to 6.0–6.5 and drying the solution in a thin layer on a glass plate. Solid residue was collected and stored in a dessicator over anhydrous CaCl_2 .

Electrophoretic analysis of chitosan was performed in a slab of polyacrylamide gel using Reisfeld buffer system (β -alanine – acetic acid, pH 4.5) [18]. The original approach was developed on a basis of the method described by Audy & Asselin [19]. Our modification consists in using polyacrylamide gel with a stepwise gradient of acrylamide concentrations 5, 10, 15 and 20%. This approach permits achieving resolution of chitosan sample into several peaks corresponding to a distribution of chitosan molecules according to their molecular mass. The quantitative interpretation of the electrophoregrams was performed using “Image J” program.

Results and Discussion

Preparation of honeybee chitosan

The washed raw material was grinded to a rough powder (particle size less than 1 mm) using coffee mill and sieve. Thereafter, it was treated with petrol ether by the percolation in the column for elimination of fatty material and wax. 3–4 changes of the petrol ether, each time 1 volume of solvent per 1 volume of material was applied. Finally, the solvent was drained from the column by suction, material was evacuated from the column and residual solvent was eliminated by the evaporation. Petrol ether in the extract (yellow colored) can be recuperated by a distillation leaving yellow soft waxy residue.

The material was further grinded to particle size less than 0.25 mm. The process was conducted in water suspension, as it was revealed that dust of honeybee bodies possess an irritative properties for the respiratory ways and its inspiration may be hazardous. The defatted honeybee material was suspended in a tap water, thick slurry was grinded in the homogenizer (MPW, Poland) with a knife of “scissors” type and sieved in water through a sieve 60 mesh (0.25 mm). The passing particles were collected by a spontaneous sedimentation in water for 60–90 min. The process was manifold repeated in order to accumulate a sufficient quantity of material. Finally, it was collected on a Buchner funnel, washed with water, and dried in a thin layer or stored as a paste in a closed vessel. In the last case, the percentage of solid residue in the paste was determined.

Decalcification was performed by suspending a distinct quantity of powder (for instance 100 g) or wet paste with an equivalent amount of solids in 2.5–3.0% solution of EDTA (400–500 ml), suspension adjusted to pH 3.5–4.0, and left at room temperature for 24 h. Thereafter solid residue was collected by filtration on a Buchner funnel through a thick polyamide cloth as filter material and washed with water. The obtained material was weighted and the amount of retained water was calculated. This material was suspended in 400 ml of distilled water at 80 °C. The calculated quantity of NaOH was added to reach its final concentration 5%. The suspension was heated on a water bath to 70 °C with an occasional steering and left for a spontaneous cooling. The mixture was centrifuged at 2,000 rpm for 15 min, the dense black sediment was retained, while brown turbid supernatant was discarded. Sediment

was washed with 3 volumes of distilled water and centrifuged for 15 min at 2,000 rpm. This washing procedure was repeated 3 times. The treatment of the sediment with 5% NaOH at 70 °C and its washing was repeated once again and a resulting sediment was collected by filtration on a Buchner funnel using polyamide cloth as a filter material. Obtained material was of black color due to presence of melanin which cannot be eliminated completely by the above treatment with a diluted alkali.

Bleaching of honeybee chitin. Process was conducted in a plastic vessel which can be closed hermetically and containing a tube with a valve or cock for introduction of reagents without opening the vessel. Plastic food bottles with such a valve tube installed in the lid are the most convenient.

40 g of wet material obtained after treatment with NaOH were placed in the bottle, mixed with 20 ml of distilled water thereafter 80 ml of sodium hypochlorite solution was added and bottle was closed. During mixing the mass starts warming and it is necessary to cool the vessel by wet ice in order to prevent temperature rise over 40 °C. After 20 min, 2.5 ml of 2 N HCl was added into the bottle through a valve with a syringe, and the mass was mixed. The addition of 2.5 ml of HCl was repeated 2 times within 20 min interval. 20–30 min after the last addition of HCl, the mass was filtered with a suction using the polyamide filter and washed with distilled water. Dark brown filtrate was discarded, and residue on the filter was returned to the reaction bottle and mixed with 15 ml of distilled water and 40 ml of sodium hypochlorite solution. In a course of 15–20 min the reaction mass was changed its color from brown to light yellow and was filtered as above. Insoluble residue was washed with distilled water, followed by 0.2% acetic acid (pH 3.0–3.5). Care should be taken for protection against chlorine, which can be released from the washed hypochlorite due to acidification of filtrate.

The elimination of residual chlorine was achieved by suspending product in distilled water and titration with 5% solution of sodium sulfite under monitoring of pH. The initial addition of sodium sulfite leads to acidification of suspension up to pH 2.0 due to a production of HCl during chlorine reduction. After exaggeration of chlorine further addition of sulfite caused a shift of pH to alkaline range. After achievement of pH 6.0 the suspension was left for several hours, thereafter chitin particles were collected by filtration, washed

with 0.2% acetic acid, ethanol and dried in a dish at room temperature.

Bleaching of melanin in the chitin of insect origin can be achieved by treatment with different oxidants, such as hydrogen peroxide [4], potassium permanganate [6], or hypochlorites [11, 20]. We compared the efficacy of these substances and additionally investigated the effect of the hypobromite, iodine, potassium ferricyanide as the oxidants. The best results were obtained with sodium hypochlorite. The advantage of hypochlorite is based on its action in relatively mild conditions (temperature less than 40 °C), acceptable rate of the reaction and possibility for visual controlling the process. The residual active chlorine retained in chitin due to absorption was completely eliminated by the treatment with sodium sulfite at pH monitoring. Treatment of chitin with hypochlorite does not abrogate the hemocoagulating activity of subsequently obtained chitosan. Hypobromite was acting more slowly as compared with the hypochlorite, and complete decoloration of honeybee chitin lasted for about 48 h instead of 3 h with hypochlorite.

Iodine as Lugol or alcohol solutions, as well as potassium ferricyanide, were not effective. Permanganate provided satisfactory results, however, the process was not available for visual control. Treatment with hydrogen peroxide performed in more harsh conditions (3% H₂O₂ at 75–80 °C for 1 h, as recommended in [4]) provided a completely decolorized chitin, but the obtained from it chitosan exhibited a lower hemocoagulating activity, apparently, due to partial degradation of the polysaccharide chains.

Deacetylation of chitin by alkaline hydrolysis

The obtained chitin was powdered to particle size less than 0.25 mm using coffee mill and 60 mesh sieve. 10 g of powder was mixed with 80 ml 40% NaOH in a mortar, transferred into flask and closed with a Teflon stopper. Hydrolysis was conducted at 115 °C during 2.5–3.0 h. After cooling the mixture was diluted with 2 volumes of distilled water and centrifuged for 15 min at 4,000 rpm. Brown supernatant was collected and saved for further treatment, while the sediment was suspended in 2 volumes of distilled water, mixture was adjusted to pH 10 with concentrated acetic acid and centrifuged for 15 min at 4,000 rpm. The sediment was washed once more and was defined as the main chitosan part. Supernatants were combined, adjusted to pH 10 with concentrated acetic acid and

centrifuged for 15 min at 4,000 rpm. Obtained sediment was defined as additional chitosan part, and it served for obtaining the additional amount of chitosan (see below).

The main chitosan part was suspended in 3% solution of the acetic acid by stirring suspension for 1 h with the mechanical stirrer. Small insoluble material was eliminated by centrifugation for 20 min at 4,500 rpm. Clear supernatant was treated with gaseous NH₃ until pH 9, and voluminous sediment was collected by filtration through polyamide filter. Sediment was suspended in 2 volumes of distilled water, the mixture was homogenized by several strokes of pestle in Potter-Elvehjem homogenizer, adjusted to pH 4.8 with 10% acetic acid, and left for at least 1 h for a complete dissolution of chitosan. Thereafter, chitosan was purified by fractional precipitation at distinct pH values. Changes in pH were achieved by an addition of 10% solution of the ammonium hydroxide. 3 chitosan fractions were obtained: fraction A precipitated at pH 6.4, fraction B at pH 7.0, fraction C at pH 8.6. Sediments were collected by centrifugation 10 min at 1,000 g, washed with water at pH of their precipitation, spread as a thin layer on a glass plate and dried by evaporation. Dry material was collected, grinded to powder of particles size less than 0.25 mm (60 mesh sieve) and stored in the dessicator over anhydrous CaCl₂. The yield of fractions was as follows: A 0.43 g, B 3.5 g, C 0.47 g.

Purification of chitosan from the additional part of chitosan was performed by similar way as from the main part. Obtained fractions were noted as A+, B+, C+, and their quantities were 0.05g for A+, 2.07 g for B+, 0.026 g for C+. The overall yield of chitosan was 6.5 g from 10 g of chitin.

Samples obtained from additional chitosan part differed from that of the main chitosan part by better solubility and lower viscosity of solutions.

Obtained chitosan fractions were further purified as follows. 3% solution of distinct chitosan fraction in 3% acetic acid was prepared by steering overnight. The solution was diluted with 2 volumes of distilled water producing 1% chitosan in 1% acetic acid. An equal volume of 0.2 M solution of NaCl was added, and after standing for 12 h solution was filtered through glass Schott filter. Clear filtrate was adjusted to pH 8.0–8.5 with 10% ammonium hydroxide, the mixture was centrifuged for 10 min at 1,500 g. The sediment was adjusted to pH 6.0–6.5 with 10%

acetic acid, the liquefied mixture was spread on a glass plate and dried by evaporation. Dry material was collected, grinded to a fine powder and stored in a dessicator over the anhydrous CaCl_2 .

Physicochemical characteristics of honeybee chitosan

Average molecular mass of the obtained chitosan samples as determined by viscometry ranged from 80 to 320 kDa that depended on the type of the fraction (Table 1). Samples precipitated at higher pH values showed a lower molecular mass. Samples obtained from the additional chitosan part exhibited significantly lower average molecular mass due to their enrichment with 15–30 kDa components, as was revealed by electrophoretic analysis. The values of higher molecular mass of our principal chitosan fractions agree with value of 257 kDa, referred by Nemtsev et al. [4]. An existence of a series of chitosan fractions with low molecular mass suggest a considerable heterogeneity of honeybee chitosan that depended upon the conditions of its isolation.

The mean value of DD was in a range of $85 \pm 6\%$ with a trend to enhancement in fractions precipitated at higher pH values.

Solubility of honeybee chitosan preparations. Dry chitosan powder readily dissolves in 10–50 mM ammonium acetate buffer at pH 5.0, sparingly and slowly at pH 6.0. Stable solutions in neutral pH region can be obtained after chitosan dissolving at pH 4.8–5.0 and subsequent adjustment

Table 1. Molecular mass of honeybee chitosan fractions compared with several reference chitosans

Samples of chitosan	Mol. mass (kDa)
Honeybee chitosan, fractions A and B	280 ± 30
Honeybee chitosan, fraction C	125 ± 17
Honeybee chitosan, fractions A+, B+	100 ± 12
Honeybee chitosan, fraction C+	80 ± 9
Chitosan of crab shells	620 ± 55
Chitosan of “Celox” preparation	500 ± 55
Chitosan from “Aldrich” (medium mol. mass)	360 ± 30
Chitosan “Organica” (Canada)	150 ± 14

of pH with 10% ammonium hydroxide to a point of the first appearance of precipitation (usually at pH 6.4). A specific property of chitosan obtained by the described method is that particles of powder applied onto the surface of water in the tube are retained on the surface, swell and gradually dissolve, thus, causing an increase in the viscosity. Taking this into account, our preparations resemble “Celox”, while particles of the commercial chitosans rapidly settle down to the bottom of the tube, and poorly absorb water. Such property depends on a porosity of the material that corresponds to its bulk density. This index in our chitosan preparations was on the level of $0.31\text{--}0.40 \text{ g/cm}^3$ for fraction A and $0.46\text{--}0.52 \text{ g/cm}^3$ for fractions B and C. For comparison, bulk density of “Celox” was 0.56 g/cm^3 , of “Aldrich” chitosan — 0.59 g/cm^3 , and of “Tyanshi” chitosan — 0.33 g/cm^3 .

Honeybee chitosan exhibited hemocoagulating effect in heparinized blood differing to a great extent in different fractions (Table 2).

Distinct hemocoagulating activity was manifested by chitosan fractions of higher molecular mass (A, B), while those with low molecular mass (C and all the rest obtained from the additional part of chitosan) were active poorly. Fractions of A-type which were precipitated at outset showed less reproducible results apparently due to the presence of absorbed impurities. Obtained data demonstrate a distinct weakening of the hemocoagulating activity of the honeybee chitosan with a decrease of its molecular mass.

Spectral characteristics of chitosan. The UV-Vis absorption spectra of studied chitosan specimens are presented in Fig. 1.

Table 2. Blood coagulating activity of bee chitosan fractions

Chitosan fractions	Time of clot formation, min ($M \pm m$)
A	4.1 ± 0.8
B	4.8 ± 0.5
C	30.0 or more
A+	15.0 ± 2.0
B+	8.5 ± 1.0
C+	30.0 or more

Note. 7 mg of powdered chitosan was introduced into 0.2 ml of human heparinized blood, and time of clot formation in min was registered.

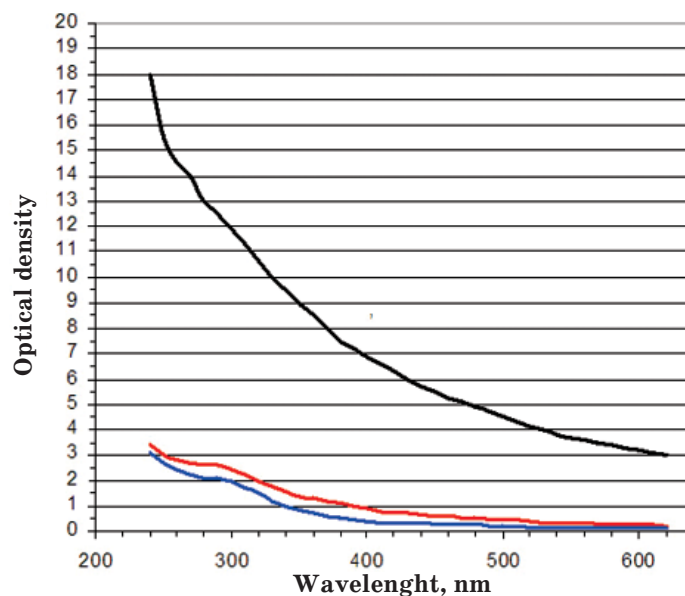


Fig. 1. UV-Vis spectra of chitosan specimens:
 — bee chitosan, — crab chitosan, — chitosan-melanin complex.
 10 mg/ml solutions of chitosan in 1% acetic acid were used

Chitosans exhibit a weak light absorption in 620–400 nm range, and for obtaining reliable values of absorption a relatively high concentrations of chitosan 10 mg/ml were used. Honeybee chitosan after an extensive elimination of melanin showed a monotone increase in light absorption from 0.22 to 0.9 OD units in range from 620 to 400 nm. We used chitosan extinction at 400–420 nm for determination of the purification of chitosan specimens from melanin. The best samples of chitosan showed $E_{400}^{1\%} 0.87 \pm 0.07$, twofold exceeding the analogous values of samples obtained from the marine organisms. The same index for chitosan samples obtained without elimination of melanin was 7.2 ± 0.4 . Thus light absorption in visible region of spectrum can serve as an objective index of melanin contamination level in chitosan of insect origin.

In the UV region, a well expressed rise in light absorption up to 230 nm with a small shadow hump at 280–300 nm was observed in both, honeybee and crab, chitosans. We suppose that this hump might be caused by traces of polypeptides tightly bound with chitosan. As expected, samples contaminated with melanin showed a sharp increase in the UV absorption.

The data of UV-Vis spectroscopy of chitin/chitosan were discussed in detail in the review of Kumirska et al. [21]. The principal light absorption of chitin/chitosan occurs in far UV at 190–215 nm that is used in structural

investigations of chitin/chitosan. The transparency of chitosans in 230–400 nm range is convenient for investigation of its binding with ligands possessing subsequent chromophore groups.

FTIR spectrum of honeybee chitosan tightly resembled spectra of chitosan of crab and shrimp origine, referred in [22] (Fig. 2).

Heterogeneity of honeybee chitosan according to molecular mass, as revealed by determinations of average molecular mass, was investigated in more detail using the polyacrylamide slab gel electrophoresis (PAG) and GPC on Acrylex P-150.

In the first publication on electrophoretic investigation of chitosan by Audy and Asselin [19] it was revealed that PAG and acidic buffer system pH 4.5 are suitable for chitosan resolution. However, the principal problem was that its high molecular mass fractions (over 500 kDa) do not enter in PAG, while fractions with lower molecular mass migrate in the PAG as smooth uniform track without distinct resolution on the bands. Here we partially solved this problem modifying the above mentioned method by conducting electrophoresis in a slab gel with a stepwise gradient of acrylamide concentration — 5, 10, 15 and 20%, and using Reisfeld buffer system (β -alanine acetic acid, pH 4.5). Chitosan fractions with a proper molecular mass are retarded and accumulated on the borders between gradient steps, thus, forming distinct peaks.

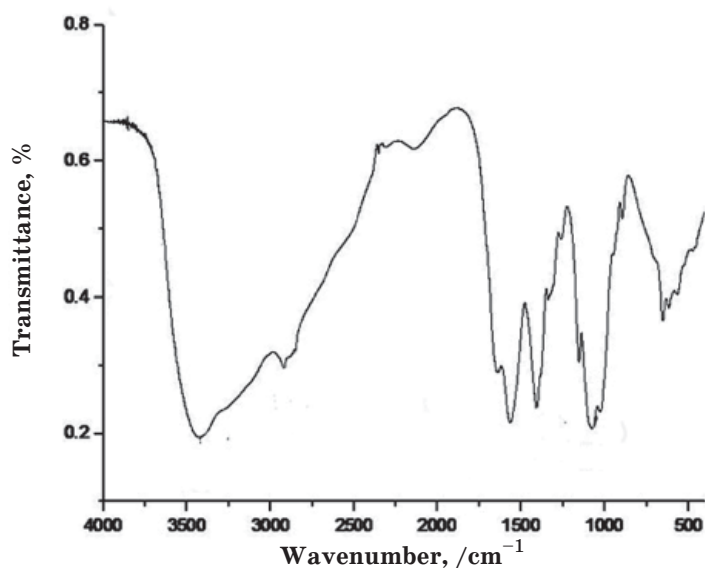


Fig. 2. FTIR spectrum of honeybee chitosan:

IR spectral investigations were conducted at Department of Inorganic Chemistry of Duisburg-Essen University (Germany) under courtesy of prof. M. Epple

An example of electrophoretic pattern of honeybee chitosan specimens, as well as “Aldrich” commercial sample used for comparison, is shown in Fig. 3, A. Quantitative distribution of chitosan between the electrophoretic bands, as evaluated from the densitometric curves, is shown in Table 3.

Thus, chitosan from “Aldrich” is rich in high molecular mass fractions that were deposited in the bottom of the slot, not entering even concentrating gel (bands 1 and 2). Fractions with a lower molecular mass were retarded in 4.8–5.0% gel. Minor fraction of the middle size chitosan molecules was retarded in 10% gel, while low molecular mass fractions were practically missing. Honeybee chitosan from the main part contains predominantly middle size molecules that are concentrated in 10 and 15% gel. A small amount of high molecular mass chitosan was detected as a deposit on the bottom of slots. Samples obtained from additional part of chitosan (A+, B+, lanes 4 and 5) were distinctly enriched with low molecular mass fractions and lacked high molecular mass ones.

Fractionation of chitosan by GPC was conducted on a column filled with Acrylex P-150. A typical chromatogram is presented in Fig. 4. Collected fractions as indicated with bars were analyzed by electrophoresis (Fig. 3, B) and quantitative interpretation of electrophoretogram is presented in Table 4.

The first two fractions eluting just after free volume of the column represented the main quantity of material corresponding to high molecular mass components, and showed a similar electrophoretic pattern (lanes 8 and 9). Fraction 3 was partially enriched with low molecular mass compounds (lane 10) while a minor tail fraction 4 demonstrate a distinct separation of low molecular mass chitosan from its high molecular mass forms. Our attempts to use chromatographic materials based on dextran or agarose were less satisfactory due to chitosan adhesion to these chromatographic media and considerable trailing during migration (data not shown).

In general, physicochemical properties and hemocoagulating activity of chitosan from honeybee corpses were studied. Chitosan was obtained using an original version of purification, which includes improved stages of chitin bleaching with sodium hypochlorite and fractional precipitation in distinct pH values. The characteristic feature of obtained preparations was a considerable heterogeneity of their molecular mass which was in a range of 80–320 kDa. A modification of electrophoretic analysis of chitosan molecules distribution according to their molecular mass was proposed. Honeybee chitosan possesses better solubility and produce more stabile solutions in water media at pH near 7 as compared with chitosans from crabs or shrimps. Hemocoagulating activity was

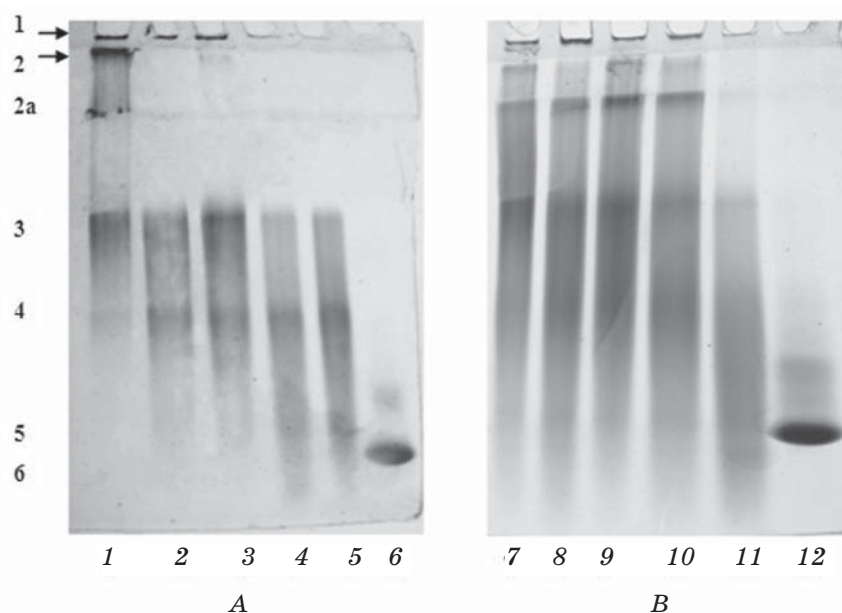


Fig. 3. Electrophoretograms of honeybee chitosan fractions (A) and fraction B after GPC on Acrylex P 150 (B):

1 — chitosan from “Aldrich”; 2 — bee chitosan, fraction A; 3, 7 — bee chitosan, fraction B; 4 — bee chitosan, fraction A+; 5 — bee chitosan, fraction B+; 6, 12 — cytochrome c, used as a molecular mass 12 kDa marker; 8–11 — chitosan chromatographic fractions 1, 2, 3, 4 as noted in Fig. 4. Electrophoretic bands are numerated from the left

Table 3. Quantitation of electrophoretic bands of commercial (“Aldrich”) and honeybee chitosan samples

Band number	Percentage of electrophoretic bands in chitosan samples				
	“Aldrich” (medium mol. mass)	Honeybee chitosan fractions			
		A	B	A+	B+
1	5.0 ± 0.6	4.6 ± 0.5	4.6 ± 0.5	1.9 ± 0.2	1.4 ± 0.2
2	21.0 ± 3.0	2.0 ± 0.3	3.6 ± 0.3	1.4 ± 0.2	0.8 ± 0.1
2a	8.1 ± 0.9	0.4 ± 0.1	2.2 ± 0.2	1.6 ± 0.2	0.9 ± 0.1
3	54.1 ± 4.5	44.5 ± 4.5	43.0 ± 4.5	23.8 ± 2.9	23.3 ± 2.9
4	11.8 ± 1.2	38.5 ± 4.0	38.2 ± 4.0	47.6 ± 4.6	43.0 ± 4.5
5	–	10.0 ± 1.1	8.3 ± 1.0	19.3 ± 2.0	25.5 ± 2.2
6	–	–	–	4.4 ± 0.5	5.1 ± 0.5
Total	100.0	100.0	100.0	100.0	100.0

revealed in high molecular mass bee chitosan fractions. However, the rate of blood clot formation was about 3 times lower as compared with chitosan of crabs or shrimps, that induce blood clot formation in 45–90 s. This circumstance together with problems of large scale production of honeybee chitosan makes its use for stemming the acute hemorrhages rather questionable, as yet. However, honeybee chitosan like other chitosans exhibits also other types of biological activity which

might be of interest for practical application. These include the antibacterial effect [23, 24], wound dressing application [25], drugs delivery [26], adjuvant effect in vaccines [27, 28], etc. These aspects are prospective and need further special investigations.

Authors express a sincere gratitude to professor Mathias Epple (Duisburg-Essen University, Germany) for an opportunity to perform investigations on IR spectra of chitosans.

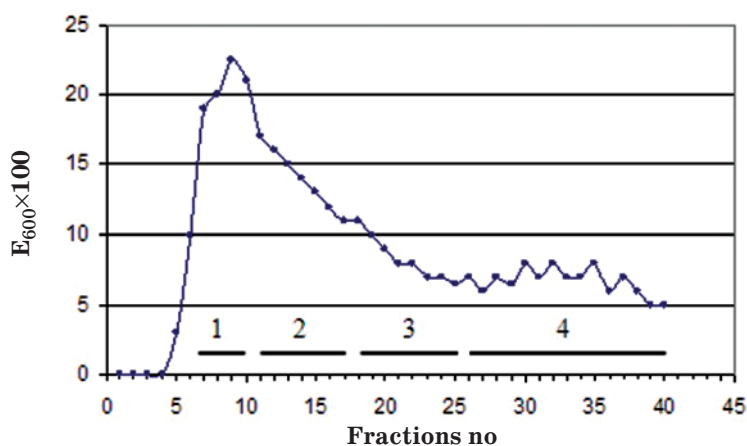


Fig. 4. Gel permeation chromatography of honeybee chitosan (fraction B) on Akrylex P 150: column 2.8×100 cm, bed volume 615 ml, V_0 155 ml, solvent 0.05 M ammonium acetate, pH 6.0. 50 mg of chitosan in 4 ml of buffer was applied, fractions volume 7 ml. Collected fractions are marked with numerated horizontal bars

Table 4. Quantitative evaluation of electrophoretic bands in fractions of bee chitosan after GPC

Band number	Percentage of electrophoretic bands in chromatographic fractions of bee chitosan				
	Fraction B, initial	Fraction 1	Fraction 2	Fraction 3	Fraction 4
1	3.3 ± 0.4	4.1 ± 0.5	1.3 ± 0.2	1.8 ± 0.2	–
2	3.1 ± 0.4	1.3 ± 0.2	3.3 ± 0.4	1.7 ± 0.2	–
2a	7.3 ± 0.8	8.8 ± 0.9	10.2 ± 0.9	8.8 ± 0.9	0.4 ± 0.1
3	49.7 ± 4.5	40.1 ± 4.0	37.7 ± 4.0	28.3 ± 3.2	8.1 ± 0.9
4	32.5 ± 4.0	38.2 ± 4.0	42.3 ± 4.0	48.4 ± 5.0	54.8 ± 5.0
5	4.1 ± 0.5	7.5 ± 0.8	5.2 ± 0.5	11.0 ± 1.0	19.8 ± 2.0
6	–	–	–	–	16.9 ± 2.0
Total	100.0	100.0	100.0	100.0	100.0

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ХИТОЗАН МЕДОНОСНОЇ БДЖОЛИ (*Apis mellifera*): ОЧИЩЕННЯ, ГЕТЕРОГЕННІСТЬ ТА ГЕМОКОАГУЛЯЦІЙНА АКТИВНІСТЬ

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Метою дослідження було розробити метод одержання хітозану з підмору медоносної бджоли, що передбачає такі етапи: 1) промивання підмору киплячою водою; 2) знежирювання висушеного порошку підмору петролейним ефіром; 3) декальцинація обробленням ЕДТА, рН 4,0; 4) депротеїнізація дворазовим обробленням 5% -м розчином NaOH по 1 год за 70 °С; 5) знебарвлювання хітину розчином гіпохлориту натрію за розбленою схемою; 6) деацетилювання хітину 40% -м NaOH 3 год за 115 °С; 7) очищення хітозану розчиненням у 3% -й оцтовій кислоті й осадження пропусканням аміаку до рН 8,5; 8) розділення хітозану на 3 фракції осадженням за рН 6,4; 7,0; 8,6. Вихід хітозану із сухого порошку підмору становив 8,5–10%. Отримані фракції хітозану суттєво відрізнялися за молекулярною масою, що була в межах 80–320 kDa. Гетерогенність препаратів хітозану досліджували методами гель-хроматографії на Acrylex P-150 та електрофорезу у пластині поліакриламідного гелю зі ступінчастим градієнтом концентрації акриламідну 5, 10, 15, 20% у буферній системі, рН 4,5. Високомолекулярні фракції бджолиного хітозану виявляли гемокоагуляційну активність, низькомолекулярні фракції були практично неактивні. Швидкість утворення кров'яного зсідка за дії активного бджолиного хітозану була у 3 рази нижча, ніж хітозану крабів або креветок.

Ключові слова: бджола медоносна, хітозан, гетерогенність.

ХИТОЗАН МЕДОНОСНОЙ ПЧЕЛЫ (*Apis mellifera*): ОЧИСТКА, ГЕТЕРОГЕННОСТЬ И ГЕМОКОАГУЛЯЦИОННАЯ АКТИВНОСТЬ

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Целью исследования была разработка метода получения хитозана из подмора медоносной пчелы, включая следующие этапы: 1) промывание подмора кипящей водой; 2) обезжиривание порошка подмора петролейным эфиром; 3) ЭДТА-декальцинирование при рН 4,0; 4) депротеинизация двукратной обработкой 5% -м раствором NaOH по 1 ч при 70 °С; 5) обесцвечивание хитина раствором гипохлорита натрия; 6) деацетилирование хитина 40% -м NaOH 3 ч при 115 °С; 7) очистка хитозана растворением в 3% -й уксусной кислоте и осаждением газообразным аммиаком при рН 8,5; 8) разделение хитозана на 3 фракции осаждением при рН 6,4; 7,0; 8,0. Выход хитозана из порошка подмора составлял 8,5–10%. Различные фракции хитозана существенно отличались по молекулярной массе, которая находилась в пределах 80–320 kDa. Гетерогенность препаратов хитозана исследовали методами гельхроматографии на Acrylex P-150 и электрофореза в пластине полиакриламидного геля со ступенчатым градиентом концентрации акриламида 5, 10, 15, 20% в буферной системе, рН 4,5. Високомолекулярные фракции пчелиного хитозана обладали гемокоагуляционной активностью, низкомолекулярные фракции — практически неактивны. Скорость образования кровяного сгустка при действии активного пчелиного хитозана была в 3 раза ниже, чем хитозана крабов или креветок.

Ключевые слова: пчела медоносная, хитозан, гетерогенность.