Honeybee Corpses as an Available Source of Chitin

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ABSTRACT: Corpses of naturally died honeybees were used as a raw material for chitin isolation. Process of deproteinization of the powder made from clean bee corpses was carried out in the presence of 1*M* NaOH at 80°C. Influence of time of alkaline treatment on the yield and molar mass of chitin was studied and optimal conditions of proteins removal were found. Process of final depigmentation of protein-free remainders was carried out using oxidization–reduction reagents. Dependences of the yield of reaction and molar mass of the obtained chitin samples from concentration of oxidizing agent $KMnO_4$ and from time of discoloring treatment were determined. Final product—high quality chitin with molar masses in range from 318×10^3 to 424×10^3 Da—was obtained in amount of 18% from initial mass of honeybee corpses. Chemical structure of chitin was determined in ^{1}H NMR investigation. It was found that honeybee chitin has high degree of acetylation of about 96%. FTIR spectra of honeybee chitin did not differ from FTIR spectrum of control sample of shrimps chitin with degree of acetylation about 95%. Results of quantitative determination of isolated chitin and its molar characteristic showed that applied treatment of honeybee corpses allowed to acquire successfully chitin of high quality in wide range of molar masses. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 1974–1981, 2008

Key words: honeybee chitin; isolation; chemical structure; molar masses

INTRODUCTION

Chitin is the second most abundant polysaccharide, next to cellulose. This naturally renewed polymer with excellent biological properties is composed of β-(1-4)-linked 2-acetamido-2-deoxy-D-glucopyranose units. It occurs as an important constituent of the exoskeleton of many organisms (particularly crustaceans, insects, and mollusks) and in the cell walls of most fungi and some algae. Currently, main source of chitin production is focused on the chitin isolation from marine invertebrates like crab, krill, shrimp, lobster and others (ca. 79%) and fungi (ca. 21%).[1] However, exists a new natural source of chitin, untouched up to present days: insects, and honeybee bodies are among them.[2] At first glance this source is not suitable for industrial use because of small content of chitin (ca. 10–15%), however, some species of insects can accumulate a large amount of chitin containing material and could be good enough to serve as a raw material for chitin production.[3–5] These could be insects which can be reared artificially (e.g., honeybees, silkworms, bumblebees and synanthropic flies).

It is assumed that Polish beekeepers abolish every spring about 200 tons of bodies of naturally died bees. Thus, this source could deliver yearly about 30 tons of chitin of excellent quality.

Insect cuticles are composite materials containing chitin fibrils conjugated with various proteins, typical for particular species.[6] In the cuticle chitin is covalently bound to protein as well as to melanin, brown polymeric pigment of high molecular weight in range from 2×10^3 to 200×10^3 Da.[7] Melanin, collection of phenolic biopolymers, due to presence of large amount of unsaturated double bonds plays role as a photo protector against damaging UV light and various types of ionizing radiation. Melanin serves also as a sponge and absorbs toxic free radicals. It is suggested that chitin–melanin complex could be used as a valuable biopolymeric material for medical applications.[8] However, practice usage of chitin requires preparing of chitin without any proteins which could be allergens, and needs to resolve a problem of isolation of protein free chitin. Procedures of purification of chitin, which is produced from commonly used raw materials are known very well.[9] There are widely described also chitin isolates obtained after demineralisation and deproteinisation treatment, which properties vary depending on the source material and used methods of purification.[10–13]

However, there is misery information about effective chitin evaluation from insects and honeybee corpses. The only publication[14] presents results of determination of optimal condition for isolation of water soluble chitosan from bee corpses as a proposal for its large-scale manufacturing.

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Thus, investigation of chitin evaluation from honeybee bodies, purification of obtained chitin and comparison of efficiency of used treatment with treatment usually applied for isolation and purification of crustacean chitin delivers new data and improves knowledge about chitin.

In the present work first results of deproteinization kinetic by treatment of honeybee bodies with NaOH and melanin removal kinetic in the oxidization-reduction reaction applied to chitin/melanin complex have been described. Certain parameters of characteristic of obtained chitin are also given.

MATERIALS AND METHODS

Raw material

Corpses of honeybees (*Apsis mellifera*) collected at spring as a natural fall in the beehives were used as a raw material. Prior to further use corpses were washed in boiled water with a detergent and next several times with pure water to remove any mechanical pollute. Then honeybee corpses were treated twice with ethanol with a solvent-to-solid ratio of 2 dm^3/100 g at ambient temperature to remove waxes covered corpses. Cleaned and dried corpses were ground into powder with particles size below 200 μm. The powder was demineralized in 1*M* HCl (with ratio 2 dm^3/100 g of powder) during 1 h at ambient temperature. The reaction medium was then filtered off and demineralized powder was washed with distilled water to pH = 7 and vacuum dried at 60°C. Such honeybee powder was used as a starting material for deproteinization and depigmentation processes.

Other materials

All reagents, solvents and auxiliary materials of laboratory grade were used without additional purification.

Shrimp chitin with degree of acetylation over 95% and molar mass of 455×10^3 Da was kindly delivered by FRANCE-CHITINE, F-13008 Marseille, France. This sample of chitin was used as a control sample.

Deproteinization process

Deproteinization process of honey bee starting material was carried out under heterogeneous condition at 80°C in 1*M* NaOH water solution without stirring with a solution-to-solid ratio of about 1.5 dm^3/100 g. Time of alkaline treatment was kept at intervals from 6 to 64 h. Solid products of deproteinization step were subtracted by filtration, washed to neutrality and dried at 60°C under reduced pressure.

Deproteinization kinetics was monitored by two methods. The first one was conductometric titration method of the reaction medium with 0.1*M* HCl following the changes in amount of NaOH being consumed with released proteins. The second method was gravimetric method, when amount of chitin/melanin complex and amount of chitin fully soluble in dimethylacetamide containing 5% of LiCl (DMAc/5%LiCl) were determined. Investigation of deproteinization kinetic was completed with measurements of intrinsic viscosity values of chitin soluble in DMAc/5%LiCl solution, obtained after different time of alkaline treatment.

Process of depigmentation

Final depigmentation of still brown samples purified from proteins and partly from melanin during deproteinization stage was carried out under heterogeneous conditions using oxidizing-reducing reagents. Dilute water solutions of potassium permanganate ($KMnO_4$) with concentration of 1, 0.5, and 0.1% were used at 20°C as oxidizing medium with solid-to-solution ratio of about 100 g/1 dm^3 during different time of treatment. Oxalic acid $C_2H_2O_4$ with concentration of 1% in water was used as a reducing agent. Treatment with oxalic acid solution was carried out at 60°C during 1 h with solution-to-solid ratio of ca 1 dm^3/100 g. Solid product was filtered off and washed with distilled water to neutrality and then vacuum dried at 60°C. Yield of final depigmentation was evaluated from gravimetric measurements.

Analyses of obtained products

Characteristics of three kinds of products have been determined:

- pure chitin free from proteins and pigments using viscometry measurements, ^1H NMR and FTIR investigation,
- chitin/protein/melanin and protein/melanin complexes using FTIR investigation.

The samples of pure chitin were obtained after filtration of the product solutions in DMAc/5% LiCl, when all nonsoluble complexes were removed. Chitin was isolated from the solution in form of film or precipitated with water.

Chitin/protein/melanin complex was obtained as a product of demineralization process of honey bee corpses (starting material).

Protein/melanin complex was obtained from the alkaline filtrate collected in the process of deproteinization of the starting material, after neutralization of the filtrate with HCl-solution and isolation of dark brown solid product in the operation of filtration.

Viscometry measurements

Intrinsic viscosity of chitin was determined by viscometry method using dilute solutions of chitin in DMAc/5% LiCl in range of concentrations from 0.04 to 0.01 g/100 cm^3. Initial solution with the greatest polymer concentration was filtered through a glass G3 filter, further solutions were obtained in the process of controlled dilution of obtained solution with above solvent. The precise concentration of the initial solution was determined from the weight of the dry polymer in form of the film prepared from 10 mL of filtered polymer solution. Above solution of chitin was poured out on the Petri dish with diameter of 7.5 cm. After about 24 h solution of polymer was coagulated and disc of chitin gel was formed. The solvent was washed off successfully from swollen disc of chitin with water first and ethanol finally and obtained film was dried. Lack of LiCl in washing water was confirmed using solution of $AgNO_3$, lack of DMAc—using UV-spectroscopy at the wave length of 260 nm. The concentrations of three other diluted solutions were calculated. Measurements were performed using Ubbelohde viscometer at 25°C, viscosity average molar mass of chitin was calculated using Mark-Howink equation with values of the constants K and a determined experimentally for chitin-DMAc/5%LiCl system: $[\eta] = 2.1 \times 10^{-4} \times M_v^{0.88}$.[15]

NMR investigations

^{1}H NMR spectra of honey bee chitin were obtained using a Bruker DPX 250 MHz spectrometer. Chitin samples free from proteins and pigments were dissolved in DCl/D_2O (20% w/w) with vigorous stirring for 8 h at 50°C.

The *N*-acetylation degree for chitin sample was determined from the ratio of the integral intensity, I_{CH3}, of the *N*-acetyl protons to the sum of integral intensities, $I_{H2\text{-}H6}$,of the H2, H3, H4, H5, and H6 as reported by Hirai et al.[16] and Megia et al.[17] Intervals 2.00 ÷ 2.50 ppm and 3.2 ÷ 4.2 ppm were selected for integration.

The DA was calculated from integration ratios using followed eq. (1):

$$DA_{1_{\text{H NMR}}}(\%) = \left(\frac{1/3 \times \text{I}_{\text{CH}_3}}{1/6 \times \text{I}_{\text{H2}-\text{H6}}}\right) \times 100 \qquad (1)$$

FTIR investigations

FTIR investigations for pure chitin samples were carried out using thin films prepared from the part of the product soluble in DMAc/5%LiCl, IR spectra of the chitin/protein/melanin and protein/melanin complexes were recorded using disks of KBr containing those complexes.

Pure chitin films for FTIR investigation were prepared from filtered chitin solution in DMAc/5%LiCl with concentration of polymer of about 0.01 g/100 mL. Method of film preparation is described above. Thin film of polymer was additionally dried directly before spectrum recording during 10 min at 110°C.

KBr disks with complexes as above were prepared as follows: 2 mg of the sample of chitin/protein/melanin complex and the sample of protein/melanin complex respectively, were grounded together with 200 mg of KBr into the fine powder with particles size below 5×10^{-6} m and compressed to form clear disk.

Spectra of the samples were recorded using spectrometer Perkin–Elmer FTIR 2000 in the range of frequency from 4000 to 400 cm^{-1}.

RESULTS AND DISCUSSION

Deproteinization process

One of the major problems related to isolation of pure chitin from raw materials meets a necessity to maintain a structure of it as close as possible to the native form, i.e., minimization of chitin deacetylation and chain degradation caused by demineralization, deproteinization and bleaching stages, applied during processes of chitin purification.[8]

According to Ref. 18 honeybee corpses contain small amount of minerals (ca. 2–3%) and their removing from the raw material does not require of long-time lasting acid treatment. That it was the reason why the stage of demineralization in the medium of 1*M* HCl at ambient temperature was shorten to 1 h. Such treatment allowed chitin preventing from significant degradation, which could occur under influence of strong acid.

It was found that above procedure applied to use of dry powder of honeybee corpses resulted in loss of about 4% of its initial weight.

The aim of the basic treatment of the samples remained after acid treatment was removal of proteins in the reaction of alkaline hydrolysis and reduction of content of melanin partly soluble in alkaline solution.

In the publication of Nemtcev et al.[14] process of deproteinization of dry corpses of bees with alkali was widely described. Authors used solutions of NaOH with concentration from 0.25 to 19% at temperatures from 22 to 99°C during up to 180 min and determined optimal condition for manufacturing of water-soluble chitosan of low molar weight.

Preliminary investigation of alkaline hydrolysis conditions applied to the honeybee corpses used as a present raw material showed that alkaline treatment

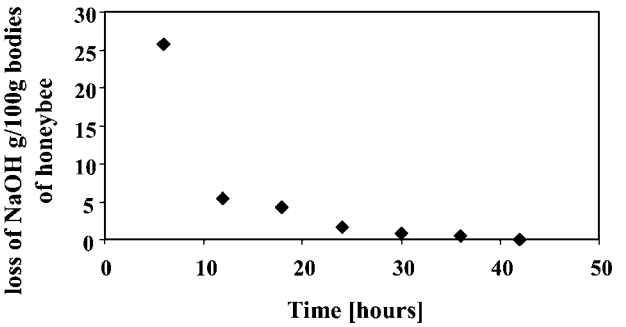

Figure 1 Consumption of NaOH during deproteinization process applied at 80°C to 100 gs of honeybee corpses.

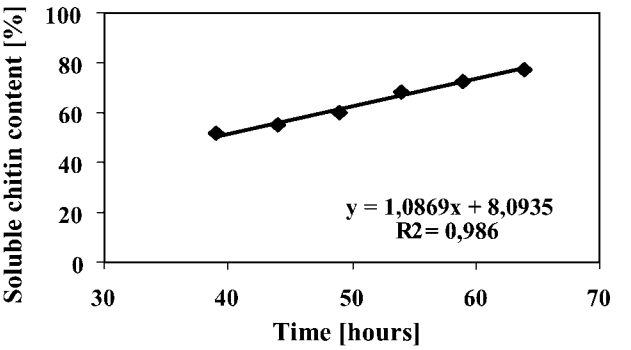

Figure 2 Chitin content in the solid product of alkaline treatment of mineral-free honeybee corpses, with 1*M* NaOH at 80°C.

of them at ambient temperature and using NaOH solution with concentration less than 1*M* (ca. 3.8%) did not cause effective removal of proteins. Alkaline treatment of used honeybee corpses with 1*M* NaOH during 1 month resulted in loss of only about 15% of their initial weight. For that reason it was decided to accomplish proteins hydrolysis in the presence of 1*M* NaOH at the temperature of 80°C.

Yield of proteins removal was estimated by the loss of NaOH amount (in grams in 1 dm^3 of 1*M* NaOH) during alkaline hydrolysis taking place at the presence of 100 g of honeybee corpses purified from minerals. The conductometric titration of the portions of alkaline solution 5 cm^3 withdrawn from reaction mixture after assumed intervals of time and diluted with 100 cm^3 of water was carried out using 0.1*M* HCl solution. Results of the conductometric titration are presented in Figure 1.

As it has been observed, after about 40 h NaOH was practically not consumed from alkaline bath.

Thus, 40–44 h of alkaline treatment with 1*M* NaOH at 80°C seemed to be long enough time for almost full removing of proteins. Together with proteins was removed also a certain amount of melanin, and color of dry sample obtained after deproteinization stage changed from black to brown. In the process of alkaline treatment carried out as above during 40 h sample lost about 77.4% of its initial weight.

Chitin bound to complexes with proteins and melanin is nonsoluble in the solvent of pure chitin—DMAc/5% LiCl. Alkaline treatment of used honeybee powder caused destruction of chitin-protein-melanin conjugates and dissolution of released chitin. The solubility of the solid samples remained after deproteinization stage in DMAc/5% LiCl increased with increase of the time of alkaline treatment, which was continued up to 64 h.

In the Figure 2 there is shown influence of the time of alkaline treatment on amounts of chitin soluble in DMAc/5% LiCl in the solid product of deproteinization. The yield of soluble chitin increased linearly and reached the value of about 77.2% after 64 h.

Alkaline treatment time influences also on molar mass of chitin causing its decrease. Influence of above alkaline treatment on intrinsic viscosity values of released chitin, determined in dilute DMAc/5% LiCl solutions at 25°C, is shown in Figure 3.

Molar masses of chitin samples obtained after alkaline treatment of demineralized honeybee corpses were calculated using equation $[\eta] = 2.1 \times 10^{-4} \times M_v^{0,88}$ recommended by Terbojevich and Cosani.[15] In this equation $[\eta]$ is the intrinsic viscosity value of chitin sample determined at 25°C using dilute chitin solutions in DMAc/5%LiCl, M_v is viscosity average molar mass of chitin, 2.1×10^{-4} and 0.88 are the constants determined for chitin-DMAc/5%LiCl system. Obtained results are collected in Table I.

It was noticed that molar mass of chitin started to decrease more considerably when the time of alkaline treatment was elongated after 44 h, but in every case chitin with high molar mass was obtained.

Depigmentation and characteristics of obtained samples

On the stage of alkaline treatment all proteins and a certain amount of melanin were removed from

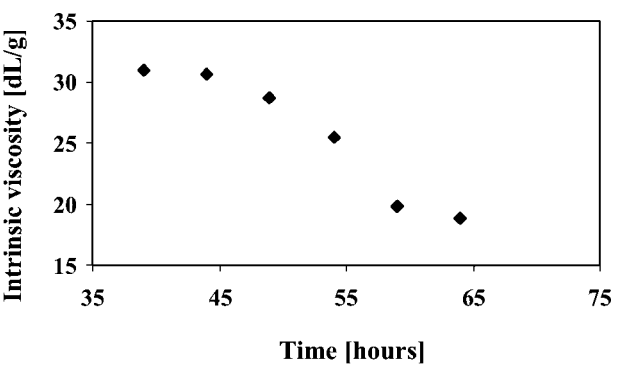

Figure 3 Intrinsic viscosity of chitin released after alkaline treatment of mineral-free honeybee corpses with 1*M* NaOH at 80°C.

TABLE I
Molar Masses of Purified Chitin Samples

Time of alkaline treatment with 1*M* NaOH at 80°C (h)	Part of the remained sample soluble in DMAc/5%LiCl (chitin) (%)	Chitin intrinsic viscosity [η] determined at 25°C in DMAc/5%LiCl (dL/g)	Calculated viscosity average molar mass M_v (Da)
39	51.0	30.6	738,806
44	55.0	29.8	716,213
49	59.8	28.7	685,463
54	68.5	25.5	598,494
59	72.5	19.9	452,136
64	77.2	18.9	426,407

honeybee corpses. However, remained solid samples were still light-brown-colored. It means that part of chitin was still bound up in chitin/melanin complex present in the product of deproteinization. For destroying of chitin/melanin complex there is recommended to use one from the several bleaching medium e.g.: sodium hydrosulfite,[19] H_2O_2,[20] $NaClO_2$[21] or dilute solutions of potassium permanganate ($KMnO_4$).[22] Usage of $KMnO_4$ needs to carry out next operation: removal of the products of oxidation with oxalic acid ($H_2C_2O_4$).

Our preliminary attempts to remove all pigments from the solid product remained after deproteinization stage using sodium hydrosulfite showed that this remedy is not effective for honeybee chitin bleaching. Also H_2O_2, which caused significant degradation of chitin, was not used. It was decided to investigate depigmentation kinetic using $KMnO_4$ solutions and next solution of $H_2C_2O_4$.

A new batch of chitin free from minerals and proteins was prepared for investigation of efficiency of honeybee chitin bleaching. Initial sample of chitin/melanin complex was obtained after treating of clean honeybee chitin corpses with 1*M* HCl during 1 h at ambient temperature (demineralization stage) and further treatment with 1*M* NaOH at 80°C during 48 h (deproteinization stage). Dry sample brown-colored free from protein and partly from melanin was used in further operations.

Influence of treatment it with solutions of potassium permanganate $KMnO_4$ of different concentrations and with 1% solution of oxalic acid $H_2C_2O_4$ on molar mass and solubility of obtained product was investigated.

Previously a consequences of apart long-time treatment of initial sample with 1% $KMnO_4$ (oxidizing agent) or with 1% $H_2C_2O_4$ (reducing agent) were checked. Obtained results are presented in Table II.

The dry samples remained after treatment with $KMnO_4$ and $H_2C_2O_4$ solutions were still brown-colored, and their solubility in DMAc/5%LiCl solution practically did not improve, but intrinsic viscosity of chitin released from $KMnO_4$-treated sample strongly decreased.

Results obtained after apart treatment with reagents of oxidization-reduction system showed that the main factor determining molar mass of released chitin is $KMnO_4$. Treatment with 1% $H_2C_2O_4$ at 60°C even during 5 h did not cause significant degradation of chitin. For that reason it was decided to investigate an influence of concentration of $KMnO_4$ solutions and treatment time on intrinsic viscosity and molar mass of released chitin.

Solutions with $KMnO_4$ concentration of 0.1, 0.5, and 1% were used for oxidizing treatment of honeybee chitin/melanin complex at 20°C in time from 10 min to 1 h. All samples were finally treated during 1 h with 1% $H_2C_2O_4$ at 60°C, washed with water to pH = 7 and vacuum-dried at 60°C.

Concentration of $KMnO_4$ influenced on the solubility of the solid samples remained after depigmentation stage in DMAc/5%LiCl. Amounts of fully soluble chitin in obtained solid samples are shown in Figure 4.

TABLE II
Results of Chemical Treatment on the Honeybee Chitin/Melanin Complex

Initial sample obtained after demineralization and deproteinization stages		Sample after additional treatment with 1% $KMnO_4$ at 20°C during 5 h		Sample after additional treatment with 1% $H_2C_2O_4$ at 60°C during 5 h	
Content of the part soluble in DMAc/5%LiCl (chitin) (%)	[η] of chitin (dL/g)	Content of the part soluble in DMAc/5%LiCl (chitin) (%)	[η] of chitin (dL/g)	Content of the part soluble in DMAc/5%LiCl (chitin) (%)	[η] of chitin (dL/g)
51.4	30.1	55.5	11.9	53.1	27.1

[η]—intrinsic viscosity value determined in DMAc/5%LiCl solutions at 25°C.

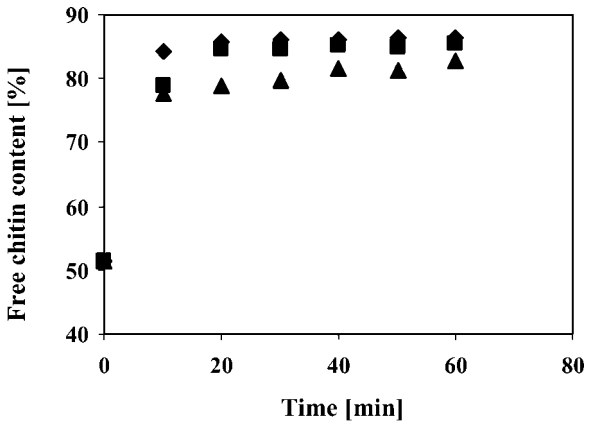

Figure 4 Influence of $KMnO_4$ concentration and time of bleaching on solubility of the solid products remained after depigmentation process: ▲1% $KMnO_4$, ■ 0.5% $KMnO_4$, ◆ 0.1% $KMnO_4$.

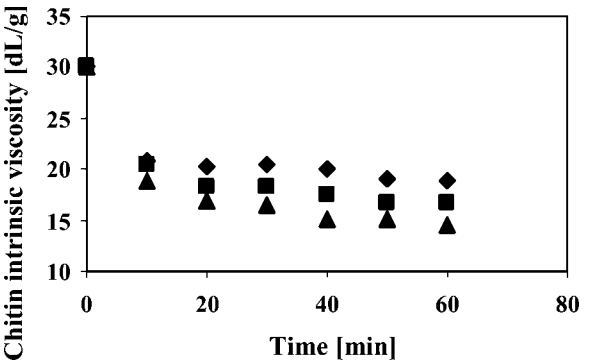

Figure 5 Influence of concentration of $KMnO_4$ and time of its treatment during depigmentation process on chitin intrinsic viscosity: ▲1% $KMnO_4$, ■ 0.5% $KMnO_4$, ◆ 0.1% $KMnO_4$.

Similar products of bleaching were obtained after oxidizing treatment of chitin/melanin complexes: with 1% $KMnO_4$—during 10 min, with 0.5% $KMnO_4$—during 30 min and with 0.1% $KMnO_4$—during 1 h.

Part of the product, being chitin still bounded with melanin, was not soluble in the solvent of pure chitin. Amounts of such nonsoluble remainders from 18.6 to 13.5% were depended on bleaching condition.

Results of intrinsic viscosity measurements made for obtained chitin samples are presented in Figure 5. As there was shown, destroying of chitin/melanin complex and melanin removal during $KMnO_4$/$H_2C_2O_4$ treatment caused considerable decrease of chitin intrinsic viscosity, especially at the initial stage of oxidizing reaction with $KMnO_4$. Usage of the higher concentration of oxidizing agent caused faster destroying of glycosidic links in chitin chain and its deeper degradation.

Determined intrinsic viscosity values and values of molar masses calculated from Ref. 15 of all chitin samples obtained after depigmentation process are listed in Table III.

Full removal of pigments from the product carried out during further bleaching caused about 19% loss of weight of the initial protein-free sample.

Results of quantitative determination of chitin isolated from honeybee corpses and its molar characteristic showed that reagents used for chitin purification are very effective ones. Applied treatment of honeybee corpses allowed to successfully acquire the chitin in wide range of molar masses.

TABLE III
Molar Masses of Honeybee Chitin Samples Purified from Proteins and Melanin

Nr	History of sample treatment	Solubility in DMAc/5%LiCl (chitin content) (%)	[η] of chitin at 25°C (dL/g)	M_v^* of chitin (Daltons)
1	1 h 1*M* HCl and 48 h 1M NaOH, 80°C	51.4	30.1	723,584
2	As above, next 10 min, 1% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	84.2	18.9	426,407
3	As above, next 20 min, 1% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	85.8	16.9	375,514
4	As above, next 30 min, 1% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	86.2	16.4	362,914
5	As above, next 40 min, 1% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	86.1	15.2	332,893
6	As above, next 50 min, 1% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	86.5	15.1	330,405
7	As above, next 60 min, 1% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	86.3	14.6	318,000
8	As above, next 10 min, 0.5% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	79.0	20.4	465,068
9	As above, next 20 min, 0.5% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	84.6	18.3	411,058
10	As above, next 30 min, 0.5% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	84.6	18.2	408,507
11	As above, next 40 min, 0.5% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	85.1	17.5	390,700
12	As above, next 50 min, 0.5% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	85.0	16.7	370,468
13	As above, next 60 min, 0.5% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	85.4	16.7	370,468
14	As above, next 10 min, 0.1% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	77.6	20.8	475,444
15	As above, next 20 min, 0.1% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	78.9	20.2	459,890
16	As above, next 30 min, 0.1% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	75.7	20.4	465,068
17	As above, next 40 min, 0.1% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	81.5	20.0	454,719
18	As above, next 50 min, 0.1% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	81.4	19.1	431,539
19	As above, next 60 min. 0.1% $KMnO_4$ 20°C, 1 h 1%$H_2C_2O_4$ 60°C	82.9	18.8	423,845

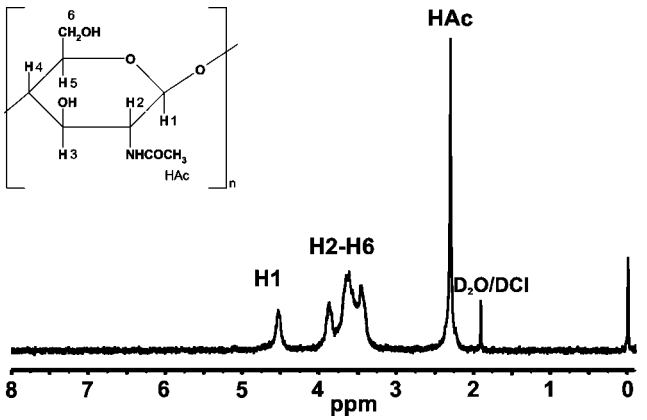

Figure 6 ^{1}H NMR of obtained honeybee chitin sample with $M_v = 318 \times 10^3$ Da.

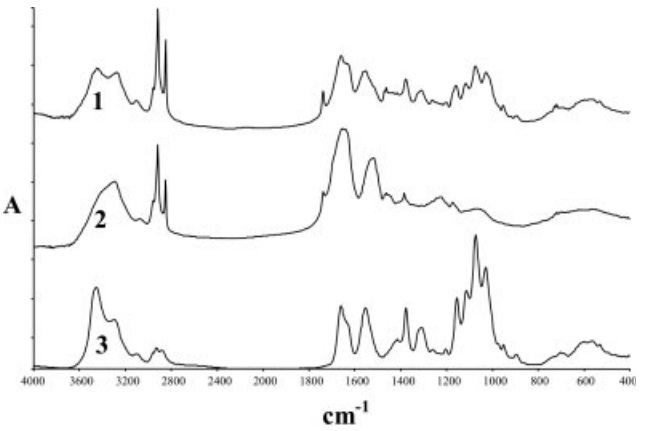

Figure 7 FTIR spectra: of the sample containing proteins, melanin and chitin (1), protein/melanin complex (2) and pure chitin released from honeybee corpses (3).

^{1}H NMR spectroscopy

^{1}H NMR investigation was carried out using four pure chitin samples: numbers 1, 2, 4, and 7 listed in Table III.

In Figure 6 is shown the ^{1}H NMR spectrum of the sample 7 with the smallest intrinsic viscosity value of 14.6 dL/g. The spectra of the other samples were similar ones.

In presented spectrum proton signals were assigned to the signal from protons in the Ac-H group at the range 2.00–2.50 ppm, to the glycosyl ring protons H2, H3, H4, H5, and H6 at the range of 3.2–4.2 ppm and to the signal from glycosyl ring proton of H1 at the range of 4.4–4.8 ppm.

Degree of acetylation calculated based on ^{1}H NMR spectra for samples received after each stage of treatments shows that honeybee chitin soluble in DMAc/5% LiCl have value 96% ± 2%.

Obtained results of degree of acetylation of honeybee chitin confirmed the usage of parameters from Mark-Howink equation $[\eta] = 2.1 \times 10^{-4} \times M_v^{0.88}$ proposed in[15] for calculation of molar masses of honeybee chitin samples.

FTIR spectroscopy

FTIR spectrum of the mineral-free initial sample containing proteins, melanin and chitin (1), FTIR spectrum of the sample of protein/melanin complex isolated from honeybee corpses (2), and FTIR spectrum of obtained pure chitin (3) are shown in Figure 7. Spectra (1) and (2) were recorded for KBr discs containing the samples, spectrum (3) was recorded for thin chitin film.

In the spectrum (1) (Fig. 7) there are strong absorptions at about 3443 cm^{-1} due to —OH groups present in chitin, proteins and melanin chains, at about 3280 cm^{-1} mainly to the groups of —NH_2 present in melanin/protein complex and to the —NH— groups of chitin and very intensive absorption in the range of about 3000 to 2800 cm^{-1} mainly due to =CH—, —CH_2—, and —CH_3 groups of protein/melanin complex. Band of absorption is present also at 1737 cm^{-1} due to =C=O unit from carboxylic groups of melanin as well as of proteins, which are absent in chitin chain. In the farther range of frequencies of the spectrum (1) appear simultaneously undivided absorption bands of all components due to their characteristic groups. In the spectrum (3) recorded for pure chitin, free from protein/melanin complex, there are absorptions due to the —OH, —NH—, —CH_2—, and —CH_3 groups present in chitin and appear intensive double absorption bands at about 1696 to 1607 cm^{-1}, assigned to amid I, and isolated band at about 1554 cm^{-1}, corresponding to amid II absorption. Spectrum of honeybee chitin exhibits detailed structure characteristic to structure of α-chitin.

In the Figure 8 there are shown FTIR spectra of pure honeybee chitin (1) and control sample of

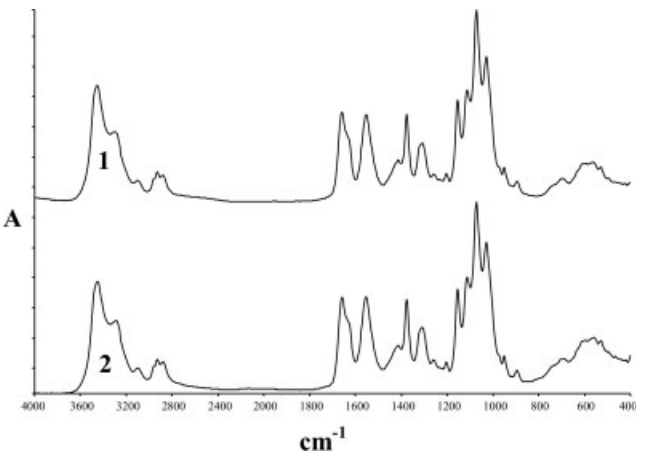

Figure 8 FTIR spectra: of pure honeybee chitin (1) and shrimps chitin (2) used as a control sample.

shrimp chitin with degree of acetylation over 95%, product of FRANCE-CHITINE, France (2), recorded for the thin films prepared using the same technique. Visually the same FTIR spectra of the samples confirmed identical chemical structure of the both polymers.

CONCLUSIONS

Results of investigations demonstrated that honeybee corpses, which were not utilized up to-day, might serve as a material for preparation of good quality chitin and protein/melanin complexes. Classical procedures usually applied to the commonly used chitin-containing waste material like exoskeletons of various crustaceans can be also adopted for removal of all impurities from honeybee corpses. However, strong association of honey bee chitin with proteins and pigments required much longer treatment with 1*M* NaOH in the process of deproteinization (ca. 40 h at 80°C without stirring of reaction mixture) and two-step treatment with potassium permanganate and oxalic acid in the process of honeybee chitin depigmentation. Various concentration of potassium permanganate and different time of oxidizing treatment resulted in isolation of pure chitin with varying molar masses with high degree of acetylation of about 96%.

It was found that dry mass of honeybee corpses pretreated with 1*M* HCl and used as a starting material contained about 18.3% of high-quality chitin, which could be used as itself or as the initial polymer for further modification and preparation of bioactive materials for medical usages. The other benefit of honeybee corpses utilization is acquirement of the next valuable biomaterial—protein/melanin complexes in amount of about 80%.

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