Structural Characterization of Chitin and Chitosan Obtained by Biological and Chemical Methods

ARTICLE *in* BIOMACROMOLECULES · AUGUST 2011

Impact Factor: 5.75 · DOI: 10.1021/bm200750t · Source: PubMed

7 AUTHORS, INCLUDING:

Miquel Gimeno
Universidad Nacional Autónoma de México
47 PUBLICATIONS **452** CITATIONS
SEE PROFILE

Eduardo Bárzana
Universidad Nacional Autónoma de México
78 PUBLICATIONS **1,085** CITATIONS
SEE PROFILE

Laurent David
French National Centre for Scientific Research
226 PUBLICATIONS **3,322** CITATIONS
SEE PROFILE

Keiko Shirai
Metropolitan Autonomous University
64 PUBLICATIONS **904** CITATIONS
SEE PROFILE

ARTICLE

pubs.acs.org/Biomac

Structural Characterization of Chitin and Chitosan Obtained by Biological and Chemical Methods

Neith Pacheco,[†] Mónica Garnica-Gonzalez,[†] Miquel Gimeno,[‡] Eduardo Bárzana,[‡] Stéphane Trombotto,[§] Laurent David,[§] and Keiko Shirai*[,†]

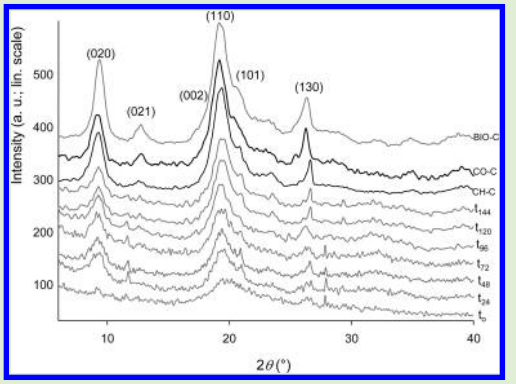
[†]Universidad Autonoma Metropolitana, Biotechnology Department, Laboratory of Biopolymers. Av. San Rafael Atlixco No. 186. Col. Vicentina, C.P. 09340, Mexico City, Mexico

[‡]Dpto. Alimentos y Biotecnología. Facultad de Química, Universidad Nacional Autónoma de México, Ciudad Universitaria, México D.F., 04510, Mexico

[§]Université de Lyon, Université Claude Bernard Lyon 1, Ingénierie des Matériaux Polymères (IMP) UMR CNRS 5223, 15 Bd Latarjet, 69622 Villeurbanne, Cedex France

Supporting Information

ABSTRACT: Chitin production was biologically achieved by lactic acid fermentation (LAF) of shrimp waste (*Litopenaeus vannameii*) in a packed bed column reactor with maximal percentages of demineralization (D_{MIN}) and deproteinization (D_{PROT}) after 96 h of 92 and 94%, respectively. This procedure also afforded high free astaxanthin recovery with up to 2400 μg per gram of silage. Chitin product was also obtained from the shrimp waste by a chemical method using acid and alkali for comparison. The biologically obtained chitin (BIO-C) showed higher M_w (1200 kDa) and crystallinity index (I_{CR}) (86%) than the chemically extracted chitin (CH-C). A multistep freeze−pump−thaw (FPT) methodology was applied to obtain medium M_w chitosan (400 kDa) with degree of acetylation (DA) ca. 10% from BIO-C, which was higher than that from CH-C. Additionally, I_{CR} values showed the preservation of crystalline chitin structure in BIO-C derivatives at low DA (40−25%). Moreover, the FPT deacetylation of the attained BIO-C produced chitosans with bloc copolymer structure inherited from a coarse chitin crystalline morphology. Therefore, our LAF method combined with FPT proved to be an affective biological method to avoid excessive depolymerization and loss of crystallinity during chitosan production, which offers new perspective applications for this material.



INTRODUCTION

Chitin, the poly(β-(1-4)-*N*-acetyl-D-glucosamine), and its deacetylated derivative chitosan are biorenewable, biodegradable, biofunctional, and nontoxic biopolymers with envisaged biomedical applications owing to their biological properties, such as in tissue engineering; wound healing, or excipients for drug delivery, among others.[1−3] Chitin is present in crustaceans as ordered crystalline microfibrils forming a complex structure with proteins, minerals, and lipids[4] and possesses three polymorphic forms (α, β, λ), where α is the most common structure, corresponding to a tightly compacted orthorhombic cell of alternate sheets of parallel and antiparallel chains.[5] In general, chitosans have lower molecular weights, and they are less crystalline than chitin precursors and thereby suitable candidates for chemical or physical modifications.[6] Commercial chitin is effectively isolated from crustacean shells after chemical treatments;[1] however, these methodologies do not allow for the recovery of added value and sensitive byproducts such as protein hydrolyzates and pigments.[7−9] Moreover, the chemical procedures imply the generation of undesirable corrosive side products and reduction in chitin molecular weights.[2] Alternatively, biological approaches for chitin recovery have been proposed,[7,10,11] and among them, lactic acid fermentations (LAFs) are promising because minerals (calcium carbonate) are solubilized in situ and endogenous proteases are adequately activated for deproteinization.[7,8,12]

Regarding chitosan, the conditions employed for deacetylation of chitin, such as temperature, alkali concentration, time, and raw material properties, affect its properties and consequently its further applications.[1,13] Chemical deacetylation conducted under heterogeneous conditions at high temperature during a short period of time is faster in amorphous regions,[14,15] whereas homogeneous deacetylation, at relatively low temperatures and extended time, results in random distribution of deacetylated residues in the polymer backbone. In both cases, high deacetylation can be successfully achieved but with remarkable reduction in molecular weight.[1,14] It has been suggested that the initial crystalline structure of the chitin is an important parameter during deacetylation and affects final chitosan structure as well

Received: June 2, 2011
Revised: July 22, 2011
Published: July 26, 2011

as molecular mass and the DA.[6,16] Therefore, this will influence its solubility, reactivity for chemical modification as well as biological, mechanical, and rheological properties.[2]

In this work, LAF of shrimp waste (*Litopenaeus vanameii*) was evaluated as a biological method for chitin and astaxanthin recoveries. In addition, the results on DA, molecular weight (M_w), crystalline index (I_{CR}), and apparent crystallite size (D_{ap}) from the heterogeneous deacetylation of our biologically obtained chitins (BIO-C) by a multistep FPT methodology were compared with those on chitin recovered by chemical means.

EXPERIMENTAL SECTION

Materials. Cephalothoraxes of the species *Litopenaeus vannameii* were kindly supplied by Biopolímeros Acuícolas S.A. de C.V. (Sinaloa, Mexico). Samples were shipped to the laboratory (−9 °C) after separation of the edible parts, then minced in a Torrey 32-3 apparatus (Mexico) to homogeneous particle size (1 mm) and stored at −20 °C until use. Commercial astaxanthin standard (all-trans isomer, 98% purity) was supplied by Sigma and stored at −78 °C until use. *N*,*N*-Dimethylacetamide (98% purity) and lithium chloride were supplied by Sigma and kept at room temperature. Commercial α-chitin was purchased from Mahtani PVT (India). *Lactobacillus plantarum* was cultivated and maintained in Man, Rogosa, and Sharpe (MRS) agar at 30 °C. The inoculum was prepared in MRS broth by deposition from the bacterial slants and incubated at 30 °C for 24 h up to a cell count of 10^8 colony-forming units per milliliter (CFU/mL).

Biological and Chemical Chitin Production and Their Deacetylation. LAF procedure was similar to that reported by Cira et al.;[8] shrimp waste was mixed with sucrose (10% w/w) and the bacterial starter (5% v/w). This mixture (3.5 kg) was placed in a 5 kg column reactor, which was previously described by Pacheco et al.[17] The reactor was incubated for 144 h at 35 °C. Sample aliquots were withdrawn from liquid and solid fractions every 24 h for further analyses. The product obtained after 144 h of fermentation was treated under mild conditions with HCl and NaOH[8] to eliminate the remaining minerals and proteins, hereafter named BIO-C sample. Chemical extraction of chitin was carried out according to Percot et al.;[18] for demineralization, 1 g of shrimp waste was submerged in an excess of 0.25 M HCl (40 mL) for 20 min at vigorous agitation. Deproteinization was carried out with 15 mL of 1 M NaOH per 1 g of sample at 70 °C during 24 h with agitation. After aqueous neutralization, samples were washed and lyophilized for further characterization. Heterogeneous deacetylation of chitins was carried out according to the freezing−degassing−thawing (FPT) method, as described by Lamarque et al.[16] Samples were extensively washed with deionized water at pH 8.5 until the conductivity reached that of water and then lyophilized prior to M_w determination.[19]

Determination of pH, Total Titratable Acidity, and LA Production. pH and total titratable acidity (TTA) were determined following the procedure reported by Pacheco et al.[17] LA production was determined by high-performance liquid chromatography (HPLC). Sample (10 g) was diluted in water in a 1:10 (w/v) ratio and centrifuged; the supernatant obtained was filtered through a 0.45 μm membrane and injected into a chromatograph (Perkin-Elmer 250) equipped with a photodiode array detector with an Aminex (HPX-87H) column. Sulfuric acid solution 0.008 M was used as mobile phase in 0.06 mL/min flow rate at 50 °C.

Lactic Acid Bacteria and Coliforms Growth. The bacterial growth in fermented shrimp waste was determined by colony enumeration using MRS agar for lactic acid bacteria (LAB) and eosin methylene blue (EMB) agar for coliforms. The results were expressed as CFU/mL.

Free-Astaxanthin Determination. Determination of free-astaxanthin content was carried out using acetone as extractive solvent in both solid and liquid fractions of each LAF following the procedure reported elsewhere.[9]

Modeling the Acidification Process and Growth of LAB. LAB growth and acidification rates were estimated by the Gompertz model using the nonlinear regression software (STATISTICA (StatSoft)) according to eq 1.

$$y(t) = a \exp(-b \exp(-kt)) \quad (1)$$

where $y(t)$ is the LA produced (TTA in mmol/g) or log of CFU/mL at time (t), a is the maximum product concentration (mmol/g) or log of CFU/mL at $t \rightarrow \infty$, b is a constant related to the initial conditions when $t = 0$, then $y(t) = y_0 = a \exp(-b)$, and k is the acidification rate constant or bacterial growth rate (h^{-1}). When y is the LA concentration, the maximum rate of acid production (V_{max}) was calculated from parameters of the model as $V_{max} = 0.368ak$.

Determination of Chemical Composition. Moisture and ash contents were determined in raw shrimp wastes and solid fraction from the LAF by weight difference following the Association of Official Analytical Chemists (AOAC) procedure.[20] Total nitrogen contents were measured by Kjeldahl in automated equipment (Buchi, Switzerland). The nitrogen contents in chitins were obtained from purified samples according to Black and Schwartz method.[21] Corrected protein contents were calculated by the subtraction of the chitin nitrogen from the total nitrogen content and multiplied by 6.25. Lipid content was measured by Soxhlet using petroleum ether in homogenized samples, previously hydrolyzed and dried at 103 °C. After 1 h of reflux, the extracts were dried and weighed. The results were expressed as total fat in grams per 100 g of wet sample.

Determination of Demineralization (D_{MIN}) and Deproteinization (D_{PROT}) Percentages. D_{PROT} % and D_{MIN} % for the fermented samples at 24 h intervals of LAF were obtained using eq 2.

$$Y(\%) = \frac{[(X_0 \times S_0) - (X_R \times S_R)]}{(X_0 \times S_R)} \times 100 \quad (2)$$

where Y is D_{PROT} % or D_{MIN} % and X_0 and X_R are the protein or ash content percentages in raw and fermented samples, respectively. S_0 and S_R are raw and fermented samples weights (g), respectively.

Determination of Proteolytic Activity. Proteolytic activity was evaluated according to the method described by Anson.[22] Hemoglobin and casein were used as substrates for activity determination. Hemoglobin was dissolved in universal buffer (0.05 M) at pH 5. Casein solutions were prepared in phosphate buffer at pH 6 and 7. Fermented solid (1 g) was homogenized in 10 mL of distilled water and centrifuged at 4 °C for 30 min at 11 500 rpm. The supernatant (150 μL) was added to 1 mL of substrate (1% w/v) in buffer and incubated for 1 h at 35 °C. Reaction was quenched by the addition of 5% v/v of trichloroacetic acid and then centrifuged. The absorbance of supernatant was measured at 280 nm. One unit of enzymatic activity was defined as the amount of enzyme that produces an increment of 0.001 of absorbance units per minute under the assay condition. Proteolytic activity was expressed as activity units per total protein (specific activity).

Physicochemical Characterization: Determination of M_W, DA, I_{CR}, and D_{ap}. Chitosans were solubilized in acetate buffer and analyzed by size exclusion chromatography (SEC) according to Pacheco et al.[17] However, the low solubility of chitins in acetate buffer precluded SEC analyses, and their molecular weight distributions were obtained by viscosimetry using an automatic capillary viscometer (Viscologic TI 1 SEMATech). Chitin samples were dissolved in *N*,*N*-dimethylacetamide containing lithium chloride (5% w/v), and its viscosimetric average M_w was calculated using Mark−Houwink−Kuhn−Sakurada (MHKS) equation $[\eta] = KM_w^{\alpha}$. The parameters employed were $\alpha = 0.69$ and $K = 2.4 \times 10^{-4}$ L/g, as reported by Lamarque et al.[16] DA was calculated according to the procedure described by Hirai et al.[23] using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy in a Bruker

(AC 200) at 200 MHz in DCl/D_2O with 3-(trimethylsilyl) propionic acid as internal reference. X-ray diffraction measurements were carried out in a diffractometer (Bruker D8 Advance) with an incident radiation Cu Kα and wavelength of λ =1.5418 Å in the range of 2θ = 4.5 to 50 °C with steps of 0.02°. I_{CR} of the samples was determined according to the method reported by Focher et al.[24] using the intensities of the (110) peaks at around $2\theta \cong 20°$ (corresponding to the maximal intensity) and at $2\theta \cong 16°$ (corresponding mainly to the amorphous halo contribution) according to eq 3. The values of D_{ap} were determined by the Scherrer eq 4.

$$I_{CR} = \frac{I_{110} - I_{am}}{I_{110}} \times 100 \quad (3)$$

$$D_{ap[110]} = \frac{K\lambda}{\beta_0 \cos\theta} \quad (4)$$

where K is a constant close to 0.9, λ (angstroms) is the wavelength of the incident radiation, β_0 (rad) is the width of the crystalline peak at half height, and 2θ (rad) is the scattering angle of the [110] diffraction line.

RESULTS AND DISCUSSION

Biological Chitin Extraction. The initial pH of 8 showed a decrease during 96 h of LAF to 5.8 and 4.2 in the solid and liquid fraction, respectively; then, it remained constant until the end of the experiment (144 h). LA production initially showed a gradual increase, reaching the steady state between 72 and 96 h in both fractions. (See the Supporting Information (section I) for pH, TTA, LA production, LAB, and coliform growth, D_{MIN} and D_{PROT} during LAF.) The estimated parameters by Gompertz model ($R^2 > 0.97$) displayed a k of 0.036 h^{-1} with a V_{max} of 0.006 g/mol h and a maximum product (solid fraction) concentration (a) of 0.42 mmol/g. The liquid fraction showed k = 0.056 h^{-1}, V_{max} = 0.016 g/mol h, and a = 0.78 mmol/g, which were higher than those previously reported by Cira et al. and Pacheco et al. using shrimp waste.[8,17] The colony enumeration of LAB showed an increase from 7.8 to 9.5 log CFU/mL after 48 h of LAF, and growth rate estimated by the Gompertz model ($R^2 > 0.96$) was 0.125 h^{-1}, which was also higher than those reported elsewhere.[8,17] The initial count of coliforms was 7.5 log CFU/mL, although it increased during the first 48 h to 8.7 log CFU/mL. Afterward, the growth decreased drastically to <6.3 log CFU/mL after 72 h, which might be due to the presence of LAB. The absence of acetic acid, according to the HPLC analysis, indicated growth of homofermentative bacteria, thus preventing spoilage. It is worth mentioning that the short handling time of the shrimp waste from the source well may have decreased postharvest contamination, then favoring the development of LAB and the consequent production of LA.

D_{MIN} of 30% was observed after 24 h, and a maximum of 92% was reached at 96 h according to V_{max} in solids. However, significant differences were not found during the final LAF time ($p \pm 0.05$), which can be explained by the absence of carbon source after 72 h, thus compromising the acid production. D_{MIN} of shrimp waste by LAB has been reported by other authors; Cira and coworkers claimed a D_{MIN} of 87% after 144 h of fermentation using a mixture of species of shrimp waste. More recently, Rao and Stevens reported D_{MIN} of 89% via addition of acetic acid under continuous agitation.[25] In a related work, 72% D_{MIN} was claimed by Bhaskar et al. using only D-glucose as a carbon source.[12] According to the results, the high D_{MIN} obtained associated with high LA production might be attributed to the quality of shrimp waste and species as well as the mincing of the sample to homogeneous small particle size, which enhanced the acid diffusion in our designed reactor, thereby improving the separation of the liquid fraction rich in soluble minerals.[17] Nonetheless, this value is not the highest reported because Xu et al. reported D_{MIN} above 94% in a complex system based on a two-stage fermentation process that involved cultures from the native microflora of Indonesian shrimp and proteolytic bacteria from ground meat.[26] As a comparison herein, the maximal D_{MIN} attained suggests a process improvement because the optimal LAF time was ca. 96 h, which is faster than previous reports.[8,17,26]

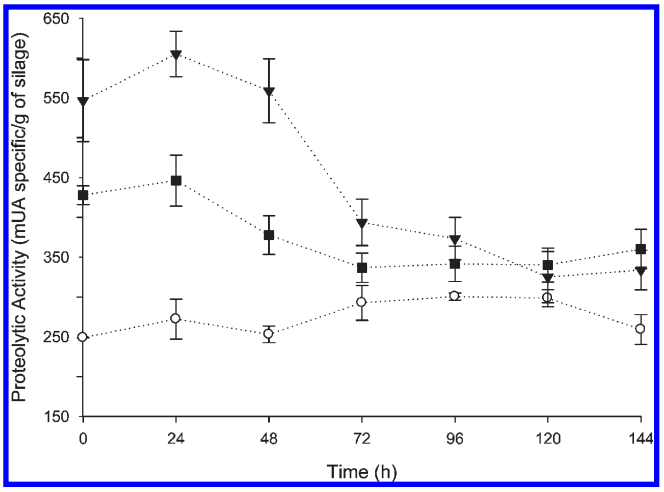


Figure 1. Time course of proteolytic activity in solid fractions of LAF carried out at 35 °C determined at pH 5 (○), 6 (■), and 7 (▼).

Regarding D_{PROT}, the highest rate was observed during the first 48 h (D_{PROT} = 70%), and it increased more slowly afterward to the highest D_{PROT} of 94% (96 h). Previous reports suggest that D_{PROT} of shrimp waste during LAF is carried out by endogenous enzymes as well as those produced by the LAB.[7,10,17] In agreement with that, experiments conducted at pH 7 showed a maximal protease activity (600 mUA) at 24 h (Figure 1), whereas samples collected after 72 h displayed values lower than 400 mUA. Similar behavior was observed at pH 6, with highest activity of 430 mUA at 24 h. The lowest protease activity was found at pH 5 (ca. 250 mUA) without significant variations upon LAF time. Several digestive enzymes such as trypsin, cathepsin, collagenase chymotrypsin, and elastase from shrimp hepathopancreas have been reported with proteolytic activities over a wide pH range (5−8).[27,28] In our case, proteases with high activity at pH 7 were favored at 24 h (pH 6.8 in solids), which is confirmed by the detection of D_{PROT} above 50%. In addition, the deproteinization process persisted until 48 h, which indicates the presence of protease activities also at pH 5 to 6. It is noteworthy that the value of D_{PROT} obtained in this study was higher than those previously reported without the need for pretreatment or two-stage fermentation process.[8,25,17,29] Our results on D_{PROT} may be attributable to (i) the collateral D_{MIN} process favoring the availability of proteins from the crustacean shells, (ii) the combination of an adequate pH in the solid fraction, thus allowing the activity of proteolytic enzymes, (iii) small waste particle size, which promotes the action of proteases, and (iv) reactor design, which promotes the protein separation.[17]

Astaxanthin Extraction. The highest pigment recovery (2400 μg of free astaxanthin/g of silage) was attained in the

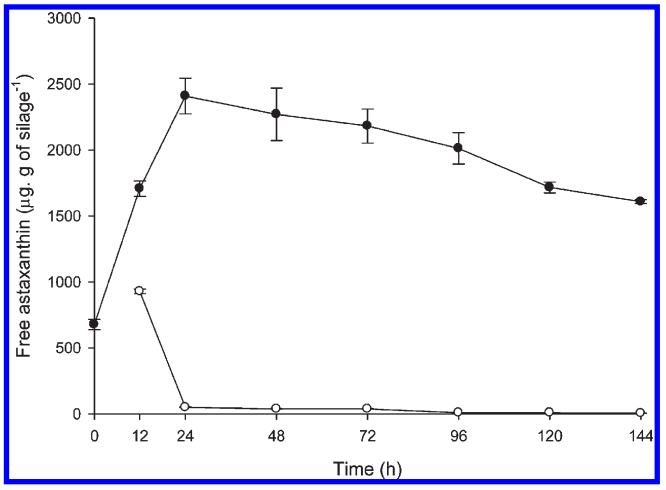


Figure 2. Time course of free astaxanthin concentration in fractions of LAF carried out at 35 °C: solid (●) and liquid (○) fractions.

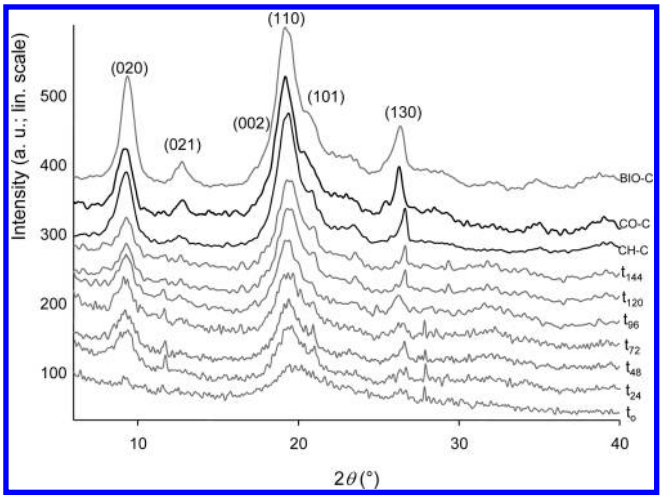


Figure 3. X-ray diffraction pattern of the samples obtained during fermentation (t_0, t_{24}, t_{48}, t_{72}, t_{96}, t_{120}, and t_{144} h), BIO-C, CH-C, and CO-C. The diffractograms are shifted for clarity.

solid fraction after 24 h of LAF (Figure 2). Although the concentration of free astaxanthin extracted decreased after 48 h, the values obtained outweigh widely those determined in previous works, such as 43 μg/g reported by Sachindra et al.,[30] 115 μg/g by Gimeno et al.,[9] or 225 μg/g claimed by Pacheco and coworkers in 2009.[17] This high pigment content is attributed to the large amount of astaxanthin present in the *Litopenaeus vanameii* species as well as meticulous waste handling that prevented oxidation during shipment and storage after its collection. The content of free astaxanthin extracted between 48 and 96 h was not significantly different ($p \leq 0.05$), but at 120 h, the carotenoid decreased significantly by 35%, which suggests from the standpoint of pigment recovery the convenience of 24 h of LAF time. The pigment recovery in the liquid fraction was ca. 900 μg/g in 12 h LAF; afterward, it decreased below 50 μg/g according to astaxanthin oxidation (Figure 2).

Characterization of Chitins. The chitin from LAF and BIO-C and the chemical extraction from the shrimp waste, CH-C, and a commercial chitin (CO-C) were characterized in terms of protein and ash content as well as in terms of their intrinsic viscosity ($[\eta]$), M_w, and DA. (See the Supporting Information (section 2) for results from the analyses of protein and ash content in dry basis, $[\eta]$, M_W, DA, I_{CR}, and D_{ap} from raw material, samples obtained during several LAF times, BIO-C, CH-C, and CO-C.) The fat content was determined in samples before and after LAF (144 h), being 27 ± 1.5 and 23 ± 2%, respectively. Lipids were previously removed by acetone extraction to avoid interferences in mineral and protein content determinations. The results indicated a reduction in the percentage of protein and mineral content from raw material until 96 h of LAF. Further protein content did not change significantly ($p \leq 0.05$) within samples. Ash content in samples diminished gradually; the lowest value obtained in fermented samples was observed at 96 h, and it did not differ significantly ($p \leq 0.05$) between 120 and 144 h. Similar ash contents were determined for BIO-C, CH-C, and CO-C. The $[\eta]$, which indirectly provides the M_W of the biopolymer, showed the highest values above 4100 mL/g (1400 kDa) in BIO-C samples, although with slight $[\eta]$ reduction after the post-LAF mild chemical treatment $[\eta]$ > 3700 mL/g (1200 kDa). CH-C and CO-C showed $[\eta]$ ± 3000 mL/g corresponding to M_w ± 900 kDa. Viscosity values below 3300 mL/g after chemical chitin extraction have been reported by Percot et al., referred to partial hydrolysis of the biopolymer owing to the contact with relatively severe acid a or alkali conditions.[18] These results indicate that our LAF process for chitin extraction minimizes the degradation of the biopolymer, thus preserving viscosity values and therefore the relatively high molecular weight of the material. In addition, the process mass yield was 45%, which is remarkably higher than that attained by the chemical method (37%). DA values were similar among all samples (94%) and close to those reported for α-chitins (91−93%) from shrimp waste.[18,31]

X-ray Diffraction Analyses. The X-ray diffraction profiles displayed five crystal reflections corresponding to the α-chitin according to the model proposed by Minke and Blackwell identified as 020, 110, 120, 101, and 130 (Figure 3).[5] The degree of crystallinity was determined from the more intense peak corresponding to 110 located at 2θ = 19 to 20° according to Lavall et al.[32] Samples obtained after the early days of fermentation (24, 48, and 72 h) displayed three reflections of lower intensity, located at 2θ = 12, 27.5, and 29°. The last two reflections might correspond to the mineral fraction present in the waste (calcium carbonate).[33] I_{CR} values were lower in fermented samples than those obtained from BIO-C, CH-C, or CO-C. This is due to the protein, lipid, and mineral present in the raw material remaining in fermented samples that contribute to the amorphous fraction, thus reducing relative peak intensities (110). Nevertheless, a chitin crystallization process occurring during the removal of proteins and minerals is not ruled out. BIO-C showed higher I_{CR} (86.4%) than CH-C and CO-C. According to this result, the relatively mild treatment of the semibiological extraction process preserved the crystallinity, whereas a slight deacetylation of the polymer and a decrease in the I_{CR} value can be observed for the other two samples. Stawski et al. reported I_{CR} up to 85% in an α-chitin obtained by chemical method.[31] The gradual increase in the apparent size of crystallites (Dap (020)) and (Dap (110)) during fermentation could be considered to be an estimation of chitin purification because it apparently triggers a subsequent crystallization, crystalline perfection, or both.

First Stage of Chitin Deacetylation. BIO-C and CH-C samples were deacetylated in one stage by FPT process during

20, 40, 60, and 80 min at 100 °C. The results showed that during the first 40 min of reaction, both samples showed DA ca. 55%. Further on, the steady state was reached at 60 min with DA of 70 and 60% for CH-C and BIO-C, respectively. (See the Supporting Information (section 3) for DA, I_{CR}, and D_{ap}, corresponding to the two principal reflexions of α-chitin (020 and 110) after the first deacetylation process.) The M_W results indicated a loss of 40% in both types of chitins after 20 min of reaction. However, BIO-C showed a higher M_W than CH-C until 60 min of reaction. Previous reported works on the deacetylation process suggest that M_W decreases faster during the first 60 min of reaction, followed by a gradual decline.[13,34] The increase in reaction time can also reduce M_W when acetyl groups start to decrease as the reaction slows down, leaving more alkali available for the oxidative degradation.[16] Relatively low losses in M_W have also been reported using the FPT process in oxygen-reduced atmospheres with the largest M_W preservation in β chitin, and it is more reactive and favors the reaction rate.[16]

The lowest final DA reached after one deacetylation stage may be influenced by the initial structure of the molecule. Chitin rigidity and crystallinity affect chemical reactions of functional groups, and it is reported that heterogeneous deacetylation takes place faster in the amorphous region than in the crystalline regions.[14,16,35] The highest value of I_{CR} in BIO-C would then affect the first stage of deacetylation, thus being slower than in CH-C. The I_{CR} values determined from the (110) reflection peak intensity indicated a decrease in crystallinity ratio related to the increase in deacetylation. This is in agreement with that obtained by Zhang and coworkers,[15] who determined a method of quantifying DA in chitin and chitosan samples as a function of I_{CR}. In our case, the apparent crystallites size showed a similar decrease, with low values obtained for CH-C during the first 40 min, although crystallite sizes are relatively similar for both chitins. X-ray diffractograms obtained from BIO-C deacetylated samples with DA ∼40% showed characteristic peaks corresponding to chitin due to heterogeneous deacetylation process that produces a block deacetylation of intermediate DA.[16] The preservation of chitin structures in relatively chitosan samples (DA 40%) suggests a deacetylation in blocks, which induced its partial solubilization in acidic solutions. As the deacetylation increased (DA ± 40%), the I_{CR} and also the reflection peak for 020 decreased; meanwhile, the peak corresponding to 110 found in $2\theta = 19°$ in the chitin and partially deacetylated samples was shifted slightly to $2\theta = 20°$.

Second "FTP" Cycle of Chitin Deacetylation. A second heterogeneous FPT deacetylation proceeded in samples obtained from BIO-C after 20 min of a first cycle of deacetylation, with initial DA of 65%, $M_W = 720 \times 10^3$ g/mol, and I_{CR} of 74.8%. During the first 40 min of reaction, the DA decreased 10% every 10 min until a minimum of 10%. However, experiments conducted for 60 and 80 min did not decrease the DA. M_W gradually decreased from (720 to 400) $\times 10^3$ g/mol after 40 min and to 250 $\times 10^3$ g/mol after 80 min. (See the Supporting Information (section 3) for DA, I_{CR}, and D_{ap} corresponding to the two principal reflexions of α-chitin (020 and 110) after the second deacetylation process.) The use of BIO-C in FPT produced chitosans with medium M_W and DA of 10%, whereas, for the same M_W, CH-C showed DA of 60% after 20 min of a first FPT deacetylation cycle.

The I_{CR} values for the second FPT deacetylation cycle of partially deacetylated BIO-C showed a behavior similar to that of the first FPT cycle. I_{CR} gradually decreased as well as the DA, and the values of crystallite size also decreased during the first 30 min of reaction (Supporting Information, section 3). The peak corresponding to the (020) reflection in the α-chitin structure disappeared with DA <22% (i.e., the chitin crystalline structure was preserved for DA values >22%), and the (020) peak of the hydrated ("tendon") polymorph of chitosan appeared in the 2θ range above 10° (Figure 4). Samples obtained during the first 30 min of reaction produced X-ray diffractograms with the characteristic peaks of the α-chitin crystalline structure. These results are due to the highly crystalline structure of BIO-C chitin with relatively large crystallites, the crystalline structure of which is kept in chitosan down to a critical DA close to 22% despite the usual destructive effect of FTP cycles on the crystalline phase.

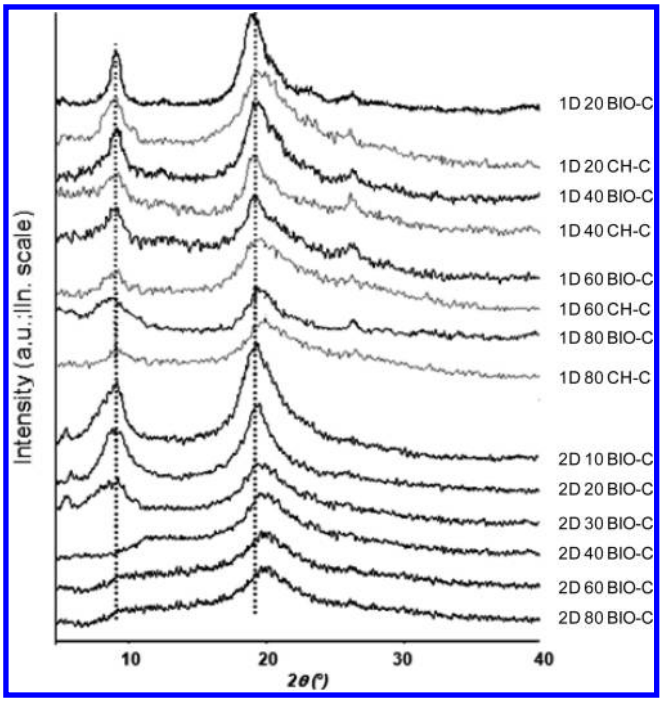


Figure 4. X-ray diffraction patterns of BIO-C and CH-C at different reaction times during first (1D) and second (2D) heterogeneous FPT deacetylation cycles.

CONCLUSIONS

LAF of shrimp waste (*Litopenaeus vanameii*) is an effective biological method for chitin and astaxanthin recovery. High LA production and lower particle size increased mineral solubilization (92%). Proteolytic enzymes with a wide pH range of activity and the increase in protein availability caused by mineral removal enhanced D_{PROT} (94%) and pigment extraction (2400 μg/g). The decreased fermentation time (±96 h) might improve the biological process that also prevents astaxanthin oxidation. The preservation of M_W and I_{CR} of BIO-C makes this biomaterial a promising starting material that couples to heterogeneous FPT deacetylation, produces highly crystalline chitosan with extended use in biomedical applications due to the increase in its biodegradation rate and the immune activity, and also favors mechanical and rheological properties in various physical forms.

ASSOCIATED CONTENT

Supporting Information. pH, TTA, LA production, LAB, and coliforms growth, D_{MIN} and D_{PROT} during LAF. Results from

the analyses of protein and ash content in dry basis, $[\eta]$, M_W, DA, I_{CR}, and D_{ap} from raw material, samples obtained during several LAF times, BIO-C, CH-C, and CO-C. DA, I_{CR}, and D_{ap} corresponding to the two principal reflexions of α-chitin (020 and 110) after the deacetylation processes. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: (525) 5804-49-21. Fax: (525) 5804-47-12. E-mail: smk@xanum.uam.mx.

ACKNOWLEDGMENT

We gratefully thank CONACyT (project no. 105628) and Institute of Science and Technology of Mexico City (ICyTDF) for the research funding and to the PCP-Program (N.P.). We thank Ruben Vera for assistance during the diffraction experiments (University Claude Bernard Lyon 1 Diffractometry Center).

REFERENCES

(1) Shahidi, F.; Aybounsayton, R. *Adv. Food Nutr. Res.* **2005**, *2*, 1043.
(2) Synowiecki, J.; Al-Khateeb, N. A. *Crit. Rev. Food Sci. Nutr.* **2003**, *43*, 145.
(3) Jayakumar, R.; Deepthy, M.; Manzoor, K.; Nair, S. V.; Tamura, H. *Carbohydr. Polym.* **2010**, *82*, 227.
(4) Rabea, E, I.; Badawy M, E.-T.; Stevens c, V.; Smagghe, G.; Steurbaut, W. *Biomacromolecules.* **2003**, *4*, 1457.
(5) Minke, R.; Blackwell, J. *J. Mol. Biol.* **1978**, *120*, 167.
(6) Rudrapatnan, N.; Tharanathan; Farooqahmed, S. K. *Crit. Rev. Food Sci. Nutr.* **2003**, *43*, 61.
(7) Shirai, K; Plascencia, M; Cira, L; Huerta S. IMPI no. 247295. Int. CI.8:A23J1/04; C08B37/08; C22B26/20, 2000.
(8) Cira, L. A.; Huerta, S.; Hall, G. M.; Shirai, K. *Process Biochem.* **2002**, *37*, 1359.
(9) Gimeno, M.; Ramírez-Hernández, J. Y.; Mártinez-Ibarra, C.; Pacheco, N.; García- Arrazola, R.; Bárzana, E.; Shirai, K. *J. Agric. Food Chem.* **2007**, *55*, 10345.
(10) Rao, M. S.; Muñoz, J.; Stevens, W. F. *Appl. Microbiol. Biotechnol.* **2000**, *54*, 808.
(11) Duarte, H. H.; Netto, F. M. *J. Food Sci.* **2006**, *71*, C298.
(12) Bhaskar, N.; Suresh, P. V.; Sakhare, P. Z.; Sachindra, N. M. *Enzyme Microb. Technol.* **2007**, *40*, 1427.
(13) Hwang, K. T.; Jung, S. T.; Less, G. D.; Chinnan, M. S.; Park, Y. S.; Park, H. J. *J. Agric. Food Chem.* **2002**, *50*, 1876.
(14) Nemtsev, S. V.; Gamzazade, A. I.; Rogozhin, S. V.; Bykova, V. M.; Bykov, V. P. *Appl. Biochem. Microbiol.* **2002**, *38*, 521.
(15) Zhang, Y.; Xue, C.; Li, Z.; Zhang, Y.; Fu, X. *Carbohydr. Polym.* **2006**, *65*, 229.
(16) Lamarque, G.; Viton, C. M.; Domard, A. *Biomacromolecules.* **2005**, *6*, 1380.
(17) Pacheco, N.; Garnica-Gonzáles, M.; Ramírez-Hernández, J. Y.; Flores-Albino, B.; Gimeno, M.; Bárzana, E.; Shirai, K. *Bioresour. Technol.* **2009**, *100*, 2849.
(18) Percot, A; Viton, C; Domard, A. *Biomacromolecules* **2003**, *4*, 12.
(19) Sorlier, P.; Denuzière, A.; Viton, C.; Domard, A. *Biomacromolecules.* **2001**, *2*, 765.
(20) *Official Methods of Analyses of the Association of Official Analytical Chemists*; Association of Official Analytical Chemists: Washington, DC, 1980.
(21) Black, M. M.; Schwartz, A. M. *Analyst* **1950**, *75*, 185.
(22) Anson, M. L. *J. Gen. Physiol.* **1938**, *22*, 79.
(23) Hirai, A.; Odani, H.; Nakajima, A. *Polym. Bull.* **1991**, *26*, 87.
(24) Focher, B.; Beltrame, P. L.; Naggi, A.; Torri, G. *Carbohydr. Polym.* **1990**, *12*, 405.
(25) Rao, M. S.; Stevens, W. F. *J. Chem. Technol. Biotechnol.* **2005**, *80*, 1080.
(26) Xu, Y.; Gallert, C.; Winter, J. *Appl. Microbiol. Biotechnol.* **2008**, *79*, 687.
(27) Oh, E.; Kim, D.; Kim, J.; Kim, H. *J. Food Biochem.* **2000**, *24*, 251.
(28) Gordon, O. J. *Western Indian Ocean. J. Mar. Sci.* **2005**, *4*, 109.
(29) Sini, T. K.; Santhosh, S.; Mathes, P. T. *Carbohydr. Res.* **2007**, *342*, 2423.
(30) Sachindra, N. M.; Bhaskar, N.; Siddegowda, G. S.; Sathisha, A. D.; Suresh, P. V. *Bioresour. Technol.* **2007**, *98*, 1642.
(31) Stawski, D.; Rabiej, S.; Herczynska, L.; Draczynski, Z. *J. Therm. Anal. Calorim.* **2008**, *2*, 489.
(32) Lavall, R.; Assis, O. B. G.; Campana-Filho, S. P. *Bioresour. Technol.* **2007**, *98*, 2465.
(33) Heredia, A.; Aguilar-Franco, A.; Magaña, C.; Flores, C.; Piña, C.; Velázquez, R.; Schaffer, T. E.; Buio, L.; Basiuk, V. A. *Mater. Sci. Eng.* **2007**, *27*, 8.
(34) Tsai, G.-J.; Su, W.-H.; Chen, H.-C.; Pan, C.-L. *Fish. Sci.* **2002**, *68*, 170.
(35) Chebotok, E. N.; Novikov, V. Yu.; Konovalova, I. N. *Russ. J. Appl. Chem.* **2007**, *80*, 1745.