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Characterization of chitin and chitosan extracted from shrimp shells by two methods

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Abstract: Shrimp shells from *Penaeus Vannamei* species were hydrolyzed for chitin extraction by a chemical and a papain enzymatic method. Composition of shells was analyzed and their microstructure was characterized before and after hydrolysis by microscopy. Chitin fibers arrangement in the tissue was preserved after chemical extraction, but after papain hydrolysis the tissue presented structural disarrangement indicating that papain reacts indistinctly with peptidic and *N*-acetyl linkages. Although chemical purification is very effective, by-products are not recoverable. Conversely, papain hydrolysis yields partially purified chitosan but permits aminoacids isolation, which is important in food industry. This method has other advantages such as low cost and easy accessibility of papain. Chitin and chitosan were characterized by thermogravimetric analysis, infrared spectrophotometry and capillary electrophoresis. Degree of N-acetylation (DA) was determined by cross-polarization magic angle spinning nuclear magnetic resonance (CPMAS ^{13}CNMR) or potentiometry and crystallinity was measured by X ray diffraction.
Keywords: Shell microstructure, chitin, chemical hydrolysis, papain

Introduction

Chitin is a natural biopolymer found in crustaceans' shells, exoesqueletons of insects and fungi cell walls. It is a linear polysaccharide containing β-(1-4)-2-deoxy-2-acetamido-D-glucopyranose repeating units. Its partiallly deacetylated derivatives are named chitosan. Because of its solubility in acidic, neutral and alkaline solutions, chitosan is preferred over chitin for a wide range of applications. Chitin and chitosan are biodegradable, biocompatible and non-toxic biopolymers, properties that have allowed their widespread use as films, hydrogels and semi permeable membranes, mainly for the food industry and biomedicine [1-3]. They show some biological activities such as immunological, antibacterial, wound healing activity, drug delivery and has been proposed for tissue engineering applications [4, 5].

The main source of commercial chitin is the extraction from shrimp shells, due to the world wide extensive production of shrimps, around 51 millions of ton in 2006. According to statistics published by the Food and Agriculture Organization, FAO, [6] shrimp world production of *Penaeus Vannamei* species surpassed 210 kTon in 2006.

This is a significant source for chitin, considering that around 45% of the shrimps mainly the heads and shells are discarded.

Chitin is found in shrimp shells in the form of a natural biocomposite, which provides mechanical support and protection. Chitin in structural tissues is intimately associated with other materials such as fibril proteins, lipids, calcium carbonate, and pigments. The most characteristic feature of these tissues is the hierarchical organization with various structural levels as described by Raabe et al. [7]

The first level is composed by chitin and its anti-parallel alignment, which leads to α-chitin crystals. Chitin crystals are forming an orthorhombic unit cell of two adjacent antiparallel N-acetyl-D-glucosamine chains heavily linked with hydrogen bonds, which provide stiffness and chemical stability. [1]

The second level is composed by the arrangement of α-chitin crystals into fibrils and wrapped by fibril proteins of about 50-100 nm in diameter. Proteins have numerous functions mainly as matrix structure, and have been identified by several researchers as tachycitins and crustacyanins [6-8]. Tachycitins are the chitin binding proteins; though the precise nature of the chitin-protein interaction has not yet been solved; it can briefly be said that the region comprising an antiparallel β-sheet and a helical turn in the C-domain of tachycitin serves as an essential chitin binding site, which projects the side-chains of the functional residues, Asn-47, Tyr-49, and Val-52 [8, 11]. Crustacyanins do not interact with chitin but are part of the structural support of the tissue and are the bases for biological camouflage because they are closely associated with astaxanthin, a carotenoid pigment which gives the coloration of marine crustaceans. [9]

The third level is formed by the clustering of these fibrils into chitin-protein fibres whose spaces are filled with other proteins and biominerals. The fourth level is formed by these fibers that shape into layers. The fifth level is the helicoidal stacking sequence of the former layers that builds a twisted plywood or Bouligand pattern; their density of stacking then will form the cuticle layers, which form the shells. [7, 12]

Chitin can be isolated from the cuticle by a series of decalcification and protein extraction steps using acid and alkaline treatments. Enzymatic methods and biological processes using microorganisms have also been implemented [13, 14]. Chemical hydrolysis is an easy low cost process which has been generally applied for obtaining commercial chitin.

Enzymatic hydrolysis can be a more clean process for which many types of enzymes have been used - mainly proteases-, which hydrolyze the proteins allowing the separation of chitin. Enzymatic methods allow the use of the protein hydrolysis supernatant and other by-products such as lipids and pigments, which are not recoverable otherwise [9, 15-19]. Variation in preparation methods result in differences in chitin acetylation degree and molecular weight. These variations influence its solubility and other properties that will define its future applications. [16]

Papain is a cystein protease which has been used for protein hydrolysis of crustacean shells. It is of general use in food industry because it has broad specificity and can be easily obtained from papaya latex. The stability of papain is good in a wide range of pH, being optimum from 5 to 7, which depends on the substrate. It is exceptionally resistant to high temperatures and shows high activity in various media [13]. The molecular weight of papain is 20900 g/mol and consists of a single

polypeptide chain which contains 212 aminoacids. It has a catalytic triad formed by three aminoacids: cysteine 25, hystidine 159, and asparagin 158- [20]

In this research, chitin is extracted from shells of *Peaneus Vannamei* species by chemical hydrolysis and enzymatic extraction with papain. Chemical composition of shells is quantified and microstructure of shells is studied to give an insight on how chitin is affected through the different extraction treatments. Highly purified chitin and chitosan are obtained, which is observed through characterization by several techniques. The advantages of combining both methods for shells purification are shown.

Results and Discussion

Structure and Composition of Shells

The main components of shells in weight percent are reported in Table 1. The total nitrogen content percentage measured by Kjedahl analysis corresponds to chitin plus proteins. It was not possible to quantify each one of them separately by the available techniques.

Tab. 1. Estimated shells composition of *Paneus Vannamei* species (on dry basis).

Constituents	Percentage (%)	Total (%)
α-chitin fibres plus Proteins: (crustacyanins and tachycitin)	76	76
Lipids:		8
Methyl palmitate	1.6	
Methyl oleate	1.3	
Methyl linoleate	1.4	
Methyl estherate	0.6	
Other lipids and pigments (astaxanthin)	3.1	
Ashes		16
CaO	8.0	
P_2O_5	4.0	
K_2O	1.0	
MgO	1.0	
Na	0.2	
Zn, Fe and Al	1.8	

Lipids were extracted with petroleum ether and quantified by gas chromatography, although their amount is highly dependent on the shrimps' nourishment and their stage of growth [21]. They are mostly present in the epicuticle or outer skin which acts as a diffusion barrier for the surroundings [7].

The pigments were identified from the petroleum ether extract by thin layer chromatography. The retention factors (Rf) were equivalent to those reported for carotenoid pigments in marine crabs. The experimental Rf were 0.37, 0.45 and 0.72 which correspond to astaxanthin, astaxanthin monoester, and astaxanthin diester respectively. [22]

The ashes were analyzed for quantification of minerals. The quantification was repeated several times and did not show highly reproducible results, due to the intrinsic heterogeneity of the sample. Nonetheless, there was a tendency, which allowed the identification of the major components and an estimated quantification. These minerals provide rigidity to the biocomposite, and are found in the shells filling in the spaces between the chitin-protein networks.

Calcium carbonate - observed as calcium hydroxide after calcination - is the main component of the minerals of the shells as it was concluded by XRD and FTIR analyses, even though an exact identification was not possible because of the diversity of minerals that were found. As it is generally agreed, calcium carbonate in the tissue is found in the crystalline form of calcite [12, 23, 24].

Shells structure before and after hydrolysis

Two of the cuticle internal layers, named endo and exocuticle were observed by optical and electronic microscopy, as having different stacking densities of the chitin-protein fibrous sheets, Figure 1A and 1B.

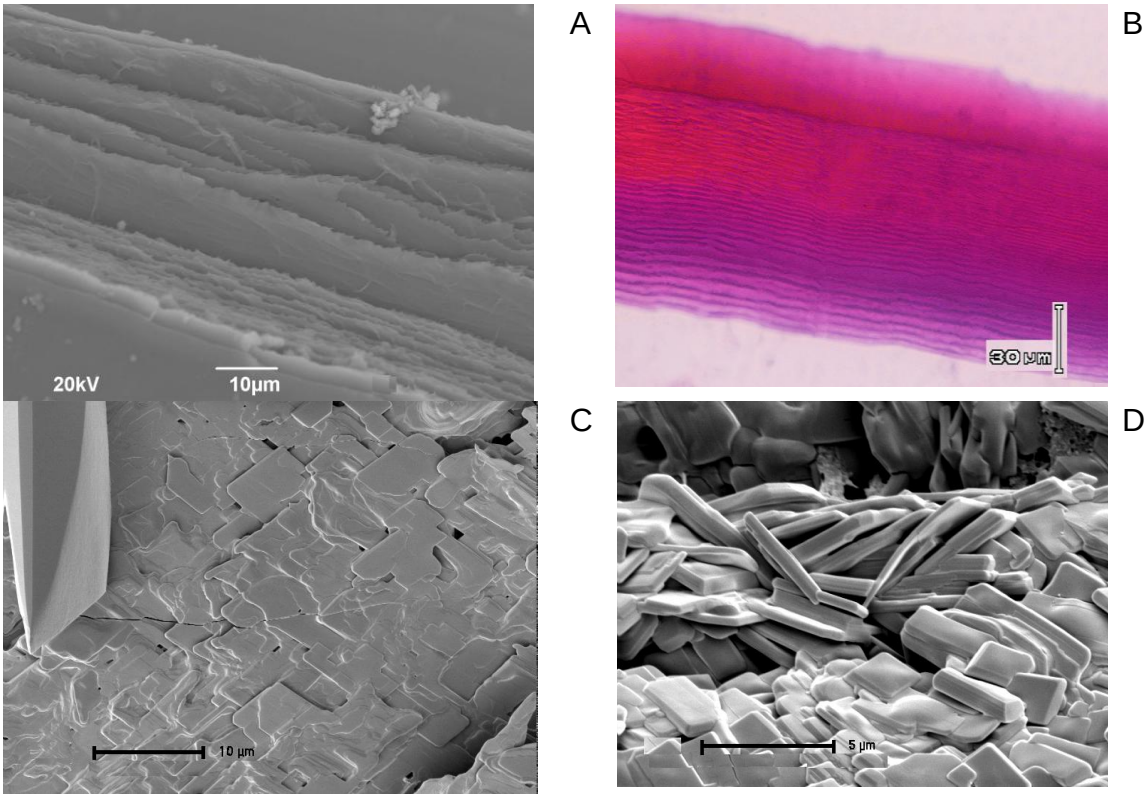


Fig. 1. Micrographs of raw shells before hydrolysis of *Paneus Vannamei*: Cross section of the organic chitin–protein matrix of the cuticle. (A) Two layers are visible: endocuticle and exocuticle (10 µm). (B) The same section by optical microscopy (20 µm). The epicuticle is also observed. (C) Characteristic matrix of a shell: plywood pattern of the α-chitin-protein layers (10 µm). (D) Exfoliation in sheets or layers of the fibrous chitin-protein pattern (5 µm).

The plywood or Bouligand pattern is observed in the SEM micrograph, Figure 1C. This pattern is formed by the chitin oriented in both directions: one given by the parallel alignment of chitin crystals to the surface, the other has some

interpenetrating fibers perpendicular to the surface, with fibers wrapped by proteins covalently bonded to chitin OH. [25] A large mineral crystal, most likely calcite, is observed in the upper left side of this view. Figure 1D is a higher magnification of the layers; exfoliation may be due to the sample treatment.

The plywood arrangement has an important mechanical effect on this biocomposite, as explained by Vincent & Wegst. [12] Chitin takes the load and the proteins act as mechanical energy stores, being able to recover completely after deformation, because of the presence of covalent crosslinks. These crosslinks also account for the insolubility of proteins. The orientation of chitin varies to be able to carry the stresses and to avoid stress concentrations. The general effect of plywood arrangement is to lower the module, making the material less stiff and isotropic. [25]

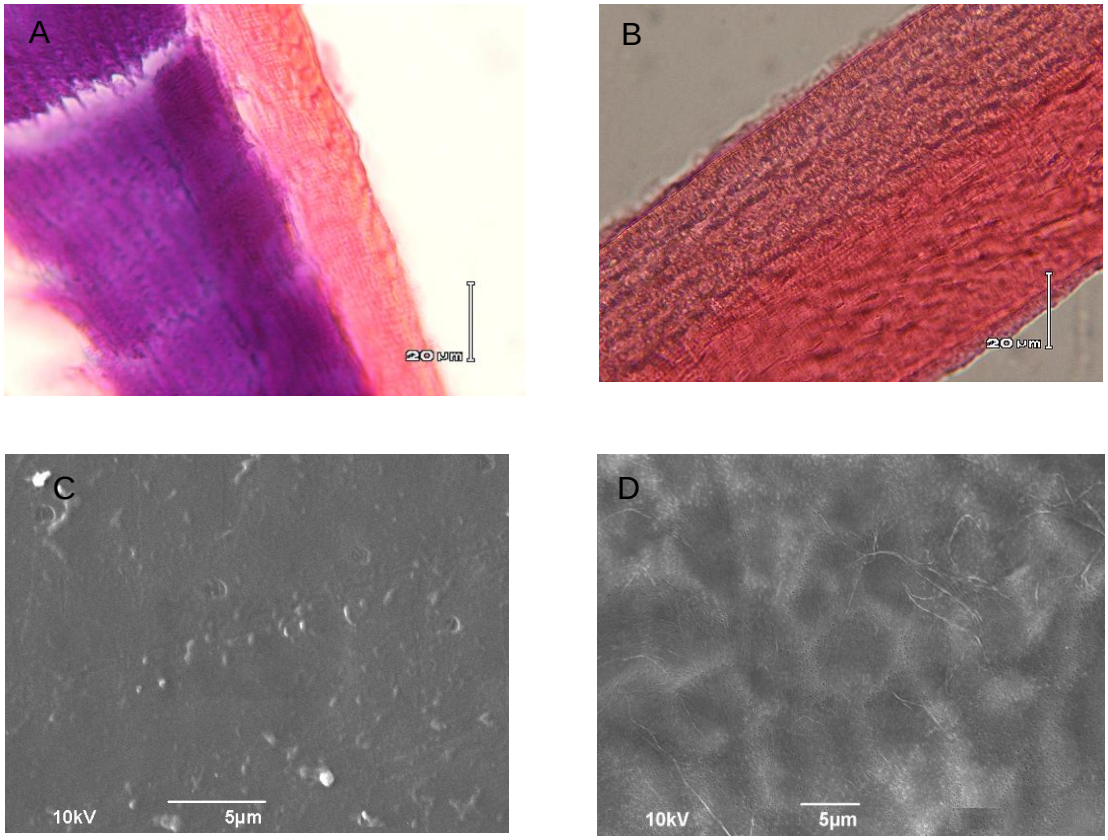


Fig. 2. Micrographs of shells after hydrolysis: (A) Cross section of the cuticle by optical microscopy (20 µm), after 3 hours of papain hydrolysis. (B) Cross section of the tissue after 12 hours of basic hydrolysis (20 µm) (C) SEM picture of the cuticle after papain hydrolysis (5 µm). (D) SEM picture of the cuticle after chemical hydrolysis (5 µm).

Micrographs obtained after both hydrolysis revealed the changes in cuticle layers. After basic hydrolysis the chitin arrangement is preserved, even after the complete extraction of proteins, as it is shown in Figure 2B. The partially destroyed cuticle after papain hydrolysis, leads us to the conclusion that despite an incomplete protein extraction, papain also reacts with chitin acetyl groups, causing a total structural damage, Figure 2A. The plywood arrangement of chitin maintains through chemical hydrolysis, Figure 2D, but it is completely absent after the papain hydrolysis, Figure 2C.

Raw shells were colored for optical microscopy observation, to contrast the different layers of the cuticle (Figure 1B). The exocuticle reveals a denser stacking of fibrous matrix of chitin-protein layers and the coloration is due to eosine (orange). Hematoxyline purple basic colorant stains the endocuticle where the layers present a coarser and thicker distribution [7]. A possible explanation for this behavior is that the basic colorant reacts with the acidic proteins, more available in the endocuticle.

After the exhaustive chemical extraction of proteins, purple coloration is not observed because there are no residual acidic proteins. However, tissues structure is preserved, because chitin structural arrangement remains. Conversely, after papain hydrolysis, the tissues structure is partially damaged and due to the incomplete extraction of proteins, there is still contrast coloration caused by the interaction of hematoxyline with the remaining proteins (Figures 2A and 2B).

Hydrolysis and Characterization Results

-Papain hydrolysis

Papain hydrolysis allows the isolation of the polymer in addition to by-products that are possibly useful.

Shells supernatant after papain treatment contain a large and varied presence of aminoacids (reported in Table 2), which are formed after protein hydrolysis of shells. These aminoacids may be used as a source of animal food, among many other applications.

Tab. 2. Amino acids content from protein hydrolysis with papain, quantified by capillary electrophoresis: Average of two kinetic studies.

Aminoacid (ppm)	Time 0	1 hour	2 hours	3 hours
Asp	42.6	83.0	140.6	131.3
Glu	199.1	188.1	315.1	310.9
Tyr	148.8	146.7	224.9	220.4
Gly	368.2	370.0	518.7	550.8
Ala	262.8	232.0	344.4	369.8
Ser	77.2	57.9	100.4	69.8
Thr + Pro	678.4	606.8	759.7	898.2
Ile+Leu	173.8	168.6	248.2	235.5
Phe	165.0	155.3	186.7	196.2
Lys	890.5	1000.3	1107.7	1284.9
Trp	43.9	34.2	32.7	37.2
Total	1481.2	1548.2	1587.2	1934.2

Kinetics was followed by the increase of aminoacids content. The quantification was performed by capillary electrophoresis of the supernatant. The hydrolysis with papain was held for three hours, since it was observed that beyond this time the aminoacids were degraded, perhaps due to the presence of microorganisms.

It is worth mentioning that it was not possible to follow this kinetics through a colorimetric method like ninhydrin, as it was intended initially. Ninhydrin reacts with peptides primary amine groups but also with those of chitosan, yielding false results

on this particular analysis. Actually, methods using ninhydrin have been reported for DA determination [26, 27].

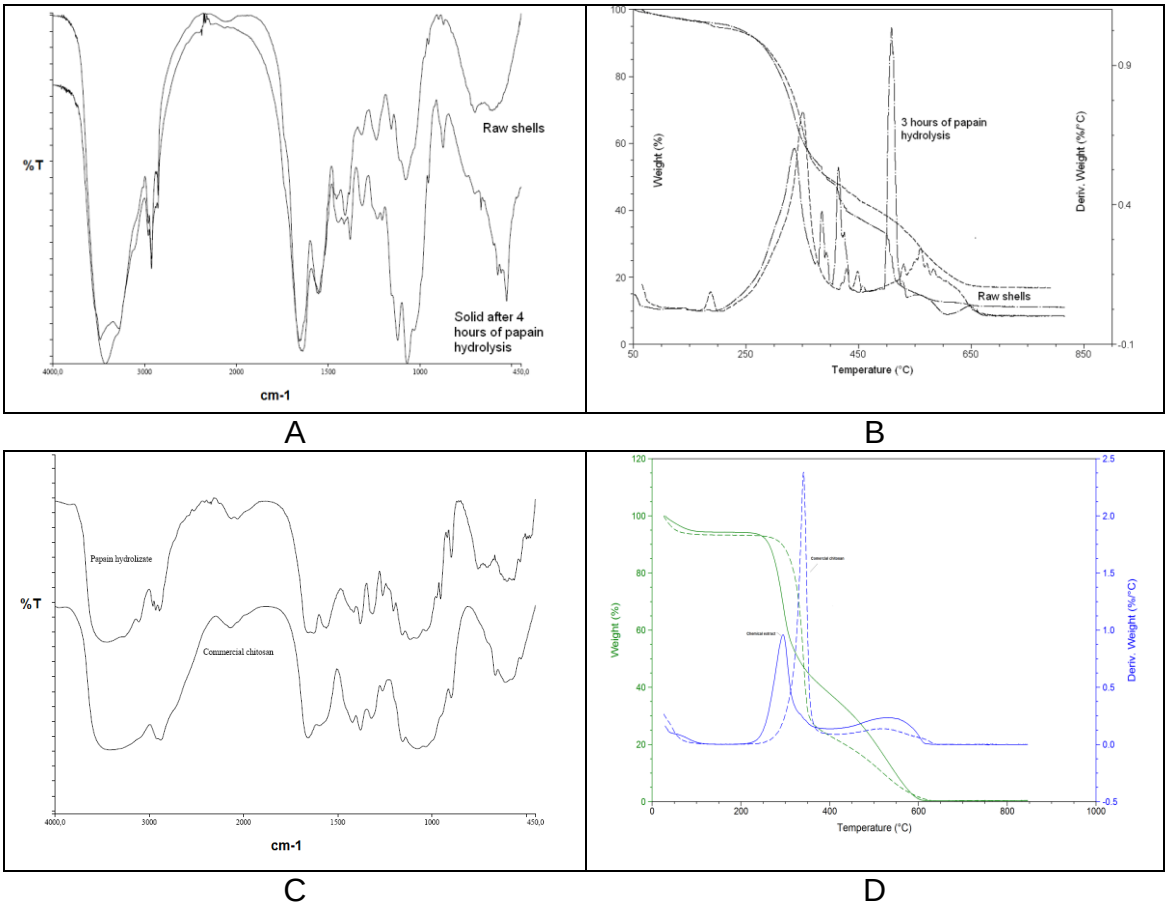


Fig. 3. FTIR spectra and TGA plots after 3 hours of papain hydrolysis: (A) Spectra comparison between raw shells and shells after hydrolysis. (B) Overlay of the TGA curves of raw shells and shells after hydrolysis. (C) Spectra comparison of commercial chitosan and the purified extract. (D) TGA plot of purified extract.

TGA plots of raw and hydrolyzed shells are compared in Figure 3B. The thermogram of raw shells shows several thermal decompositions from the diverse components that are in the shell. After papain hydrolysis, there are also several decompositions due to an incomplete purification. FTIR spectrum after papain extraction showed some changes when compared with the spectrum of a raw shell, but it does not correspond to the one of pure chitin, as it is shown in Figure 3A. Through these results, it was found that chitin obtained by papain hydrolysis needs to be further purified.

The purification of the papain extract is explained in the experimental part. TGA plot of the purified product showed only two thermal degradations and low percentage of residues (an average of 2%), Figure 3D. FTIR spectrum of the polymer obtained after this additional purification step is very similar to the spectrum obtained for a commercial chitosan, Figure 3C.

The spectrum shows the typical infrared absorption bands of chitosan, whose assignments are: stretching vibrations of NH primary amines and OH between 3500 and 3000 cm^{-1}, NH primary amines in plane bending at 1654 cm^{-1}, C-C bonding

vibrations of glucopyranose rings at 1205 cm^{-1}. Between 1157 and 1027 cm^{-1} there are many bands assigned to C-O alcohol stretching and tension. This spectrum also presents the characteristic absorption bands of chitin but in lower intensity: C=O stretching at 1659 cm^{-1} overlapped with NH bending, 1560 cm^{-1} (amide II band), CH bending of CH_3 groups at 1378 cm^{-1}, CH torsion at 1314 cm^{-1}, C-N flexion in secondary amines at 1261 cm^{-1} (amide III band) [28, 29].

The non-selectivity of papain which breaks indistinctly peptidic linkages of proteins and N-acetyl linkages of glucopyranose rings will probably deacetylate chitin. N-Deacetylated chitin leads to a structural disorder that might cause the rupture of glycosidic bonds, as well. This fact has been studied for purification of shells using papain and also for depolymerization and deacetylation of chitin [30-32]. Figure 4 shows a proposed mechanism for deacetylation of chitin with papain, on the basis of the mechanism of cleavage of a peptidic bond by a cysteine protease. Although the catalytic triad is confirmed by Cys, Hys and Asn, the latter is not shown. Its function is to maintain the stereo specificity of Hys ring.

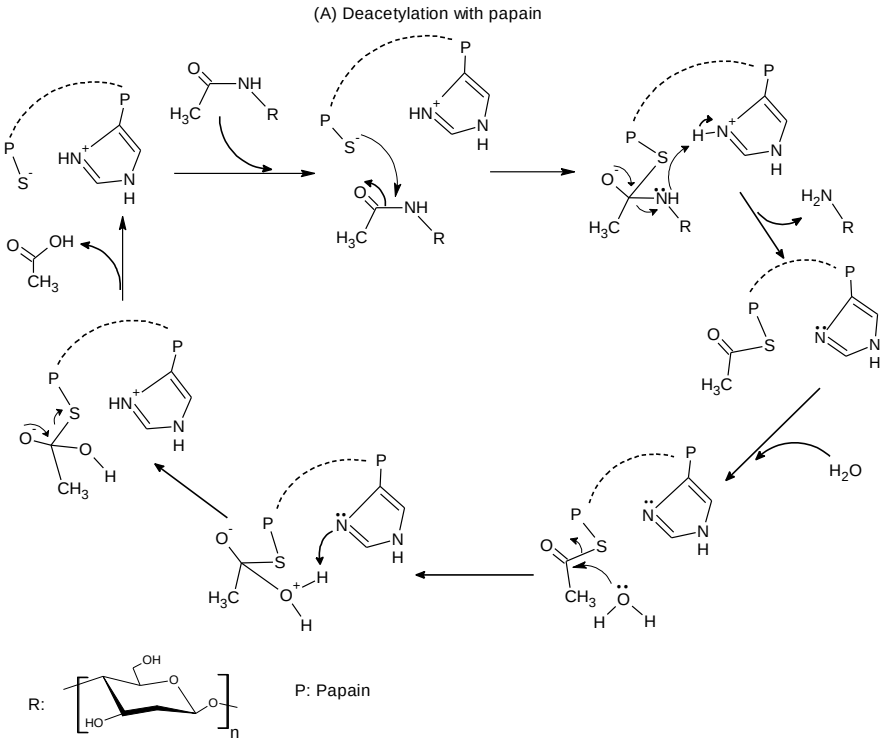


Fig. 4. Deacetylation mechanism of chitin with papain.

-Chemical hydrolysis

For chemical hydrolysis it was obtained a chitin yield of 12%. The experimental design for chemical hydrolysis guaranteed that proteins extraction and demineralization of shells will take place without depolymerization or deacetylation of chitin.

To avoid the rupture of N-acetyl bonds during protein extraction, mild concentration of alkali and medium temperature were used. When N-C bonds of the acetyl group of chitin are broken, a less stable tertiary radical will be developed, which needs higher energy to be formed. For demineralization, even lower concentration of acid and ambient temperature were used to prevent the rupture of C-O-C glycosydic bonds,

which are more labile and have a lower activation energy to undergo scission. On the other hand, nitrogen atmosphere prevented any oxidation side reactions.

TGA plots for the polymer obtained by chemical hydrolysis showed two thermal degradations, similar to the one obtained for a commercial chitin (Figure 5A). After performing three replicates, the average residue was 0.8%, which shows the efficiency of this method for shell purification.

Chitin and chitosan display two thermal degradations. The first thermal event occurs between 200 and 400 °C when chitin is heated under oxidative atmosphere, while under nitrogen it has a higher starting degradation temperature. This event is primarily due to depolymerization with the formation of volatile low molecular weight products produced from scission of glycosidic bonds at weak points. These weak points are due to the presence of radicals in the glucopyranose ring after the cleavage of amino or N-acetyl groups. Also the glycosidic bonds linked with acetylated side groups are more stable than the non-acetylated ones [33]. The first thermal decomposition accounts for a weight loss of approximately 80% (weight percent).

The second decomposition peak, which appears between 350 and 550 °C under oxidative atmosphere, may result from the thermal degradation of the new cross-linked material that possibly had been formed in the first stage of the degradation process, and accounts for a weight loss of approximately 20%.

The maximum temperatures of degradation for commercial chitosan and papain extract were shifted to lower temperatures than those for commercial chitin and chemical extract. This behavior is attributed to a decrease in thermal stability as a consequence of a decrease in acetyl content. Another reason for this behavior is the reduction in the crystalline order as a result of N-deacetylation of chitin. The relationship between thermal effects and the degree of N-deacetylation has been reported by Garcia Alonso et al [34]. However, this relation should also consider the molecular weight of chitin, which undoubtedly affects the degradation temperatures, as have been demonstrated [35]. Table 3 shows the maximum degradation temperatures for commercial chitosan and chitin and for both the chemical and the purified papain extracts.

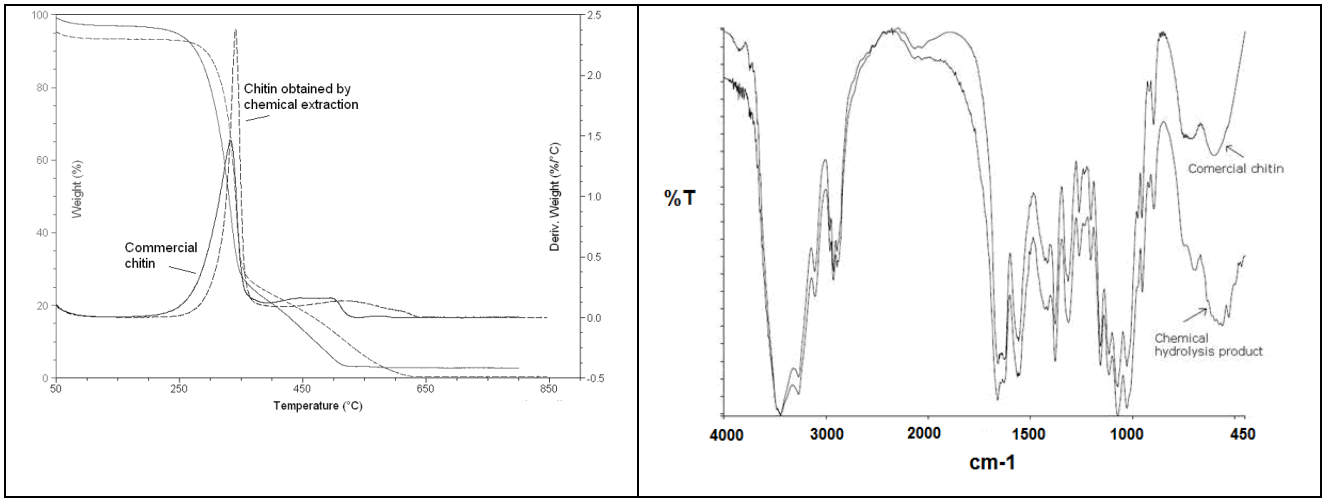
Tab. 3. Maximum degradation temperatures in TGA. Average of three replicates.

Maximum temperature (°C)	Chemical extract	Commercial chitin	Commercial chitosan	Papain extract
Event 1	327.0	327.2	295.3	316.5
Event 2	519.7	500.6	535.2	511.5

The changing trend with acetyl content was not so clearly observed for the second thermal degradation, which occurs under oxidative atmosphere. However, it is possible to deduce that the lower the acetyl content, the denser the crosslink network. This network is formed during the first thermal event, which may lead to higher degradation temperatures. The lower degradation temperature for papain extract, although it has a lower DA, might be explained by a decrease in molecular weight as a result from the non-selective hydrolysis of papain. Another reason is the higher percentage of mineral residues, which may catalyse the second oxidation

lowering the degradation temperature. This explanation is also valid for commercial chitin.

Chitin obtained by chemical hydrolysis is highly pure and the FTIR spectrum is similar to the one obtained for a commercial chitin, as can be seen in Figure 5B. The spectrum shows the typical infrared absorption bands of chitin, which have been previously designated.



A B

Fig. 5. TGA thermograms and FTIR spectra of the product obtained after chemical hydrolysis (A) Comparison of thermograms for commercial chitin and chitin obtained by hydrolysis. (B) Overlay of spectra for commercial chitin and chitin obtained after chemical extraction.

Chitin Characterization

-NMR measurements

The CPMAS ^{13}CNMR spectra for chemically hydrolyzed chitin and commercial chitin and chitosan are shown in Figure 6. The well-resolved spectrum for both types of chitin shows the high crystallinity of these samples. Chitosan spectrum shows broader bands and overlapping signals proving either low crystallinity or the formation of another crystalline structure [1, 28].

-Degree of N-acetylation, DA

DA was calculated from the ratio of intensities or integrated areas, (I) between methyl group (δ 23.2 ppm) and the D-glucopyranosyl ring carbons, C1 to C6 (δ 50–120 ppm), through the expression given below. [29]

$$DA\% = \frac{I_{CH_3}}{(I_{C1} + I_{C2} + I_{C3} + I_{C4} + I_{C5} + I_{C6})/6} \times 100$$

Chitin content of N-acetyl groups (measured as DA) is strongly dependent on the extraction method as it has been mentioned before. DA result for the chemically extracted chitin is 91.6%, similar to DA result for commercial chitin: 90.6%. Commercial chitosan has a DA value of 31.4%. DA for papain extract, determined by a titration method, has a value of 19.1%. The low value for DA obtained shows that

papain strongly deacetylates chitin, while the chemical method yields a highly acetylated chitin.

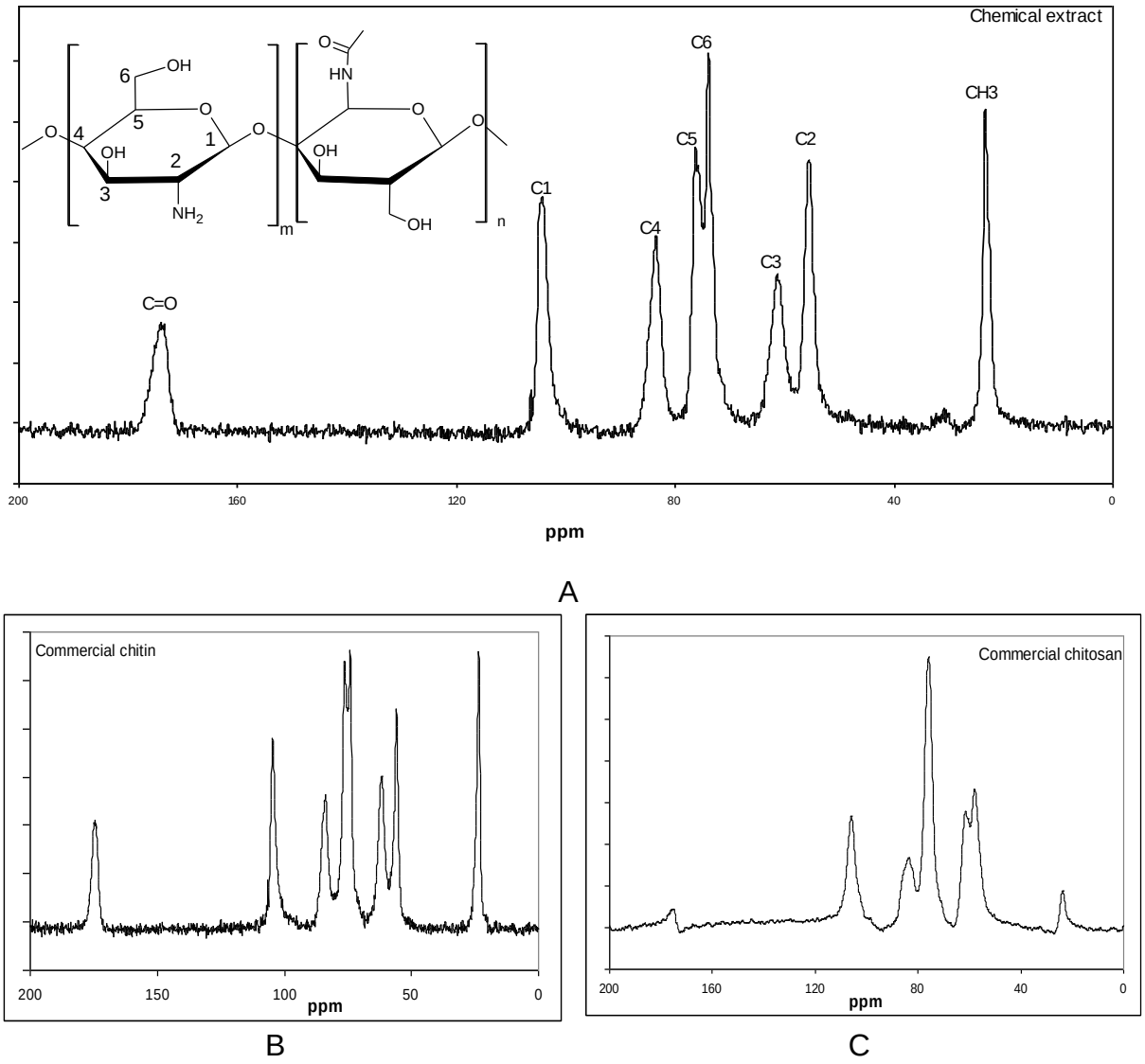


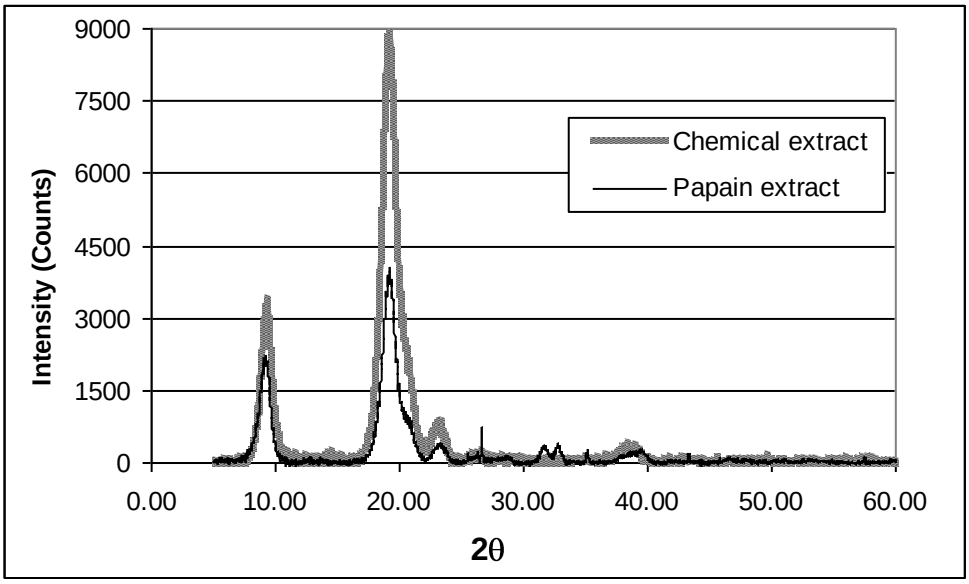
Fig. 6. ^{13}C CP/MAS solid state spectra (A) Chitin obtained by chemical hydrolysis (B) Commercial chitin (C) Commercial chitosan.

-Crystallinity

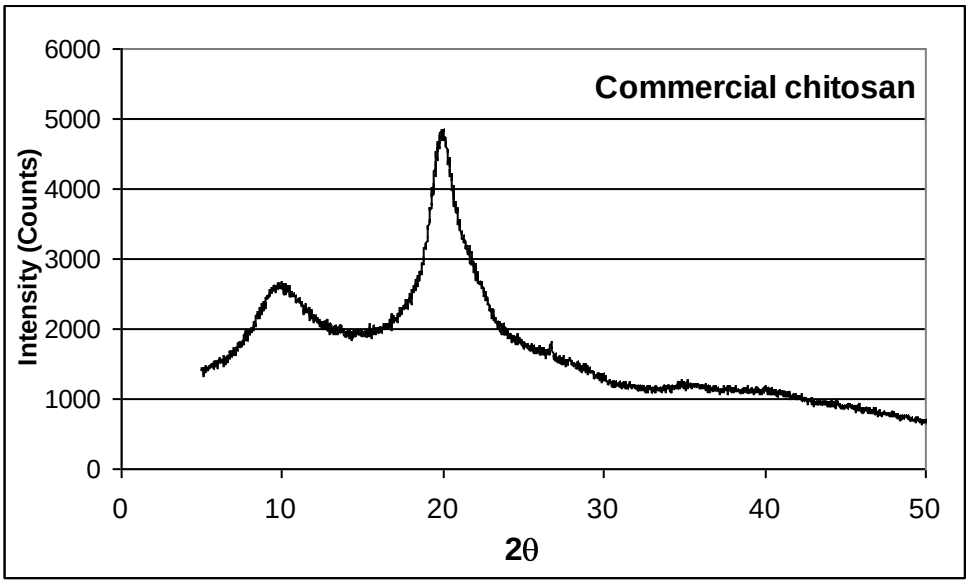
X ray diffractograms for chitin obtained by papain extract and chemical extraction are shown in Figure 7A. Four main crystalline reflections were observed in the range from 5–50° in 2θ. They are indexed in the literature as 020, 110, 120 and 130 reflections [36].

Both samples show high crystallinity. The intensity of reflection at 110 is higher for chemically extracted chitin. It is also noted that this reflection peak for papain extract moved to a higher angle (19.2° in 2θ or inter-planar distance in the crystal lattice, d-spacing 4.62 Å), when compared to chitin chemical extract, (19.0°, d-spacing 4.67 Å).

This may be a result of an increase in the unit cell dimension, due to differences in crystal sizes. The amorphous peak at 16.0° in 2θ is almost absent for both samples.



(A)



(B)

Fig. 7. XRD plots (A) chitin and chitosan obtained by chemical and papain extraction (B) commercial chitosan.

The reflection peak indexed as 020 at 9.31° (d-spacing 9.50 Å) for the chemically extracted chitin, and 9.23° (d-spacing 9.54Å) for papain extract, did not show a decrease in intensity as the reflection at 110 did. From these results, we conclude that deacetylation changes the crystalline order, as it was discussed previously. The chemical extraction method used preserved DA and, consequently, the crystallinity of the purified chitin.

For commercial chitosan, alkaline deacetylation hinders crystallization of chitosan as can be observed in Figure 7B, showing low intensity broad peaks at higher angles: at 19.9° in 2θ (d-spacing 4.44 Å) and at 9.55° in 2θ (d-spacing 9.25 Å). Although papain hydrolysis leads to a chitosan with lower crystallinity than the chemical extracted

chitin, its crystallinity is higher when compared with a commercial chitosan, which is alkali-deacetylated. It can be explained by a more ordered N-deacetylation reaction of papain that preserves the chains conformation within the unit cell. This might lead to homogenous deacetylation. This hypothesis should be confirmed by further NMR studies.

Conclusions

Shrimp shells are natural biocomposites with chitin fibers intimately related with proteins. Two methods were adapted for the extraction of chitin from the shells: a chemical method that uses alkali and acids for removal of proteins and demineralization and an enzymatic method with papain. It was shown that the applied chemical method can preserve chitin, as was demonstrated through morphology analysis of the shells and the characterization of chitin after hydrolysis. This chitin showed high degree of N-acetylation and high crystallinity.

Conversely, the enzymatic method with papain damaged the structural arrangement of chitin in the tissue. This disarrangement may be the result of the broad selectivity of papain that simultaneously hydrolyzes proteins and deacetylates chitin yielding chitosan which has to be further purified. The characterization showed lower crystallinity than the chitin obtained by chemical extraction. This polymer may be suitable for biomedical applications

The adapted chemical hydrolysis method is effective for obtaining highly purified chitin; however by-products are not useful. On the other hand, papain hydrolysis has the advantage of isolating valuable by-products such as the aminoacids which are important in food industry; in addition papain is an abundant and low cost enzyme.

Experimental Part

Shrimp shells characterization

Raw shrimp shells were dried and milled to a particle size between 0.2 to 200 μm, measured in a Malvern 2000 mastersizer.

For microstructural studies raw shells were used. For optical microscope analysis, shells were prepared following the common procedure for histological studies: chemical fixation in Bowin for preserving the chemical structure, followed by dehydration with alcohol in increasing percentages and clarification with xylene. Then the tissue was embedded in paraffin to assure enough toughness in order to facilitate the cutting with microtome. The tissue so sliced was fixed on a glass plaque to proceed with the coloration by contrast using hematoxylin/eosine, which eases the microscopical observation. For scanning electronic microscope (SEM) observation in a JEOL JSM 6490, tissues were gold sputtered after they were either fixed in Bowin and dehydrated with alcohols, or just freeze dried.

Chemical composition of shells was studied by Kjeldahl analysis for total nitrogen determination. To quantify lipids, extraction with petroleum ether (40 to 60° fraction) was performed, followed by gas chromatography analysis in an Agilent 6890 chromatograph, using a column DB 23. Pigment identification was performed in the petroleum ether extract by thin layer chromatography, eluted in mobile phase of acetone-hexane 25:75. Calcination in an oven at 850 °C was used to quantify the ashes; further analysis of these ashes were done by X ray diffraction (XRD) in a Phillips Panalytical X'PertPro MPD, with a wavelength of 1.542Å (Cu $K\alpha_1$ radiation);

spectra were recorded in a scattering range of 5-60° in steps of 0.013°. Ashes also were analyzed by Fourier transformer infrared spectrophotometry (FTIR) performed in a Perkin Elmer Spectrum One, and by atomic absorption analysis in a Perkin Elmer 2380.

Chemical hydrolysis

The following method was adapted for chemical hydrolysis (Figure 8): dried and milled shells were suspended in NaOH, 2.5 M in a 1:10 weight/volume relation. The suspension was maintained at 60 °C for 6 hours under nitrogen atmosphere under constant stirring. This procedure was repeated for three times to assure a complete removal of proteins. After filtration and washing with water, the precipitate was solubilized in HCl 0.25 M for demineralization during 3 hours at room temperature while stirring. Chitin was washed thoroughly with water and acetone/ethanol mixture, and dried at 40 °C. It was characterized by thermo gravimetrical analysis (TGA) in a TA Instrument model Q100 under oxidative atmosphere and a heating rate of 10 °C/min and by FTIR.

Enzymatic hydrolysis

A solution of commercial papain was prepared in a phosphate buffer of pH 6.2, within a concentration of 0.25% (w/v). 4 mL of this solution were used to saturate 0.5 g of shells, to guarantee an adequate enzyme-substrate complex formation, which may undergo a high kinetics reaction. The protein hydrolysis was made at room temperature under constant orbital shaking (Figure 8).

Kinetics of reaction was followed through the quantification of the aminoacids in the supernatant solution at different reaction times, performed by capillary electrophoresis in a Hewlett Packard 3D-CE using an extended path light capillary, 25 kV of voltage and negative polarity. The solid was characterised by TGA and FTIR.

The additional stages of proteins and minerals extraction were carried out following the method already adapted for chemical hydrolysis performing only one step of proteins extraction. The degree of N-acetylation (DA) for this sample was measured by a potentiometric method, adapted from Tan et al. [27]. This method can be briefly described as the solubilization of chitosan in a known excess of standardized HCl of ionic strength 0.1M. The excess is back titrated with NaOH. Through the use of the appropriate equivalence volume, DA is calculated.

Chitin and chitosan characterization

The polymer was characterized by TGA and FTIR. For DA quantification, cross polarization magic angle spinning nuclear magnetic resonance (CP-MAS ^{13}CNMR), performed on a **Bruker** ASX 100 low field spectrometer at 25.2MHz, with high power 1H decoupling and ^{1}H-^{13}C cross polarisation was used. The Hartmann-Hahn matching condition was obtained by adjusting the power on ^{1}H channel for a maximum ^{13}C FID signal of adamantane. All spectra were acquired with contact time of 1ms, repetition time of 10 s and spinning speed 12.5 kHz. Crystallinity was measured by X-ray diffractometry (XRD). All necessary chemical reagents were analytical grade

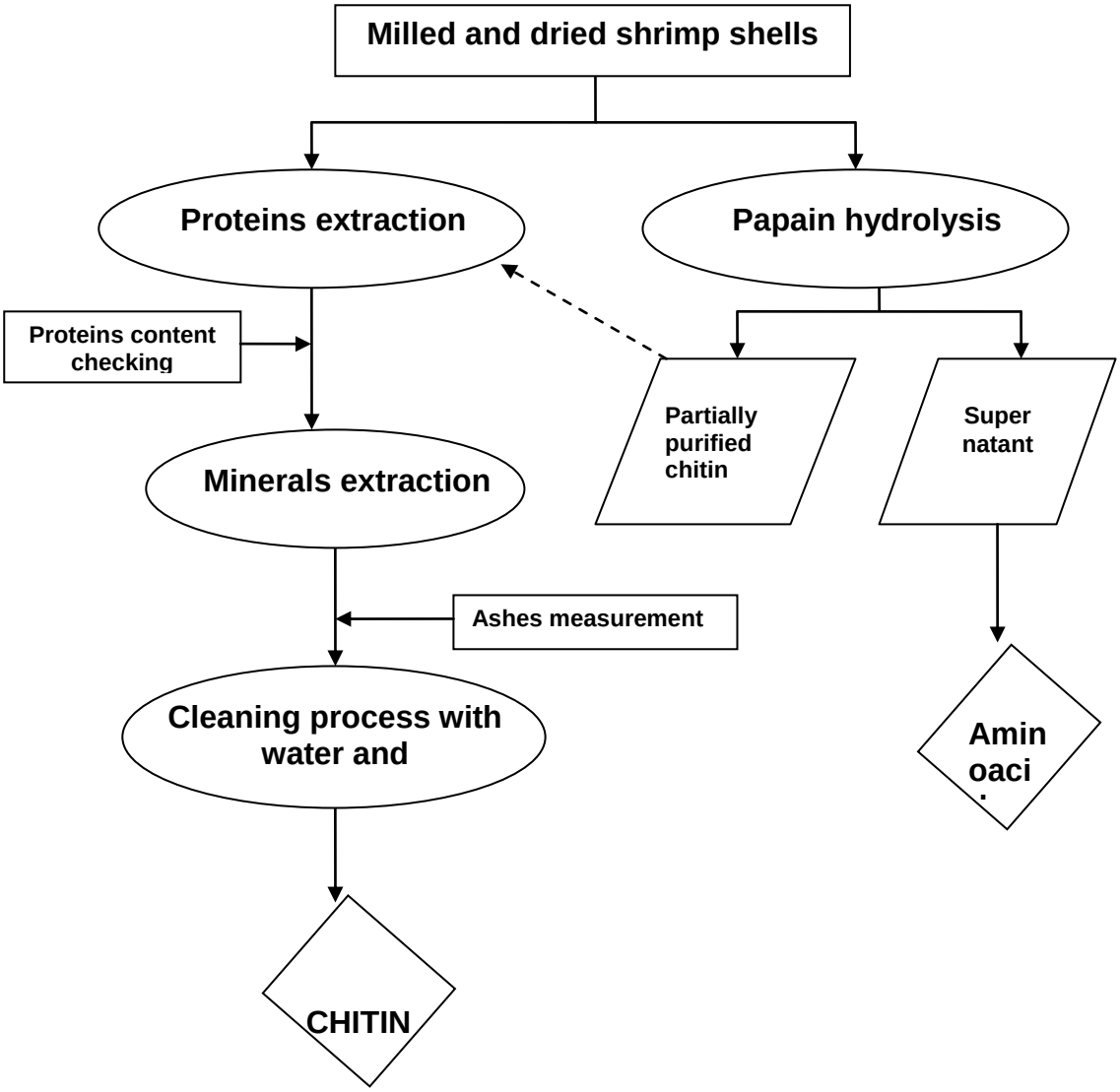


Fig. 8. Scheme of shrimp shells purification.

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