Sonication-Assisted Extraction of Chitin from North Atlantic Shrimps (*Pandalus borealis*)

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The influence of sonication during extraction of chitin from North Atlantic shrimp (NAS) shells (*Pandalus borealis)* on chitin yield, purity, and crystallinity was investigated. Shells were peeled, washed, lyophilized, ground, and suspended for 4 h in 0.25 M HCl (1:40) at 40 °C followed by ultrasonication at 41 W/cm² for 0, 1, and 4 h, respectively. Demineralized shells were lyophilized, resuspended in 0.25 M NaOH (1:40), and ultrasonicated at 41 W/cm² for 0, 1, and 4 h to remove proteins. The yield and mineral and protein contents were determined after each processing step. The purity of extracted chitin was determined from the total amount of glucosamine. The crystallinity index and size of crystals were calculated from wide-angle X-ray scattering measurements. Scanning electron microscope images were recorded to evaluate morphological changes in samples. The yield of chitin from NAS decreased from 16.5 to 11.4% for 0 and 1 h sonicated samples, respectively, which was attributed to increased concentrations of depolymerized materials in the wash water. Sonication did not enhance the removal of minerals. The application of ultrasound enhanced the removal of proteins from 39.8 to 10.6, 8.3, and 7.3% after 0, 1, and 4 h of sonication treatments. The crystallinity index of chitin decreased from 87.6 to 79.1 and 78.5% after 1 and 4 h of sonication, yielding chitosans with crystallinity indices of 76.7, 79.5, and 74.8% after deacetylation, respectively. Fourier transform infrared spectroscopy scans indicated that the degree of acetylation of chitins was unaffected by sonication. Comparison of the extraction results of NAS with that from freshwater prawns indicated that more impurities were left in NAS chitin, suggesting that composition and structural arrangement of chitin in shells influence the efficiency of ultrasound-assisted extraction.

KEYWORDS: North Atlantic shrimp; chitin; extraction; ultrasound; sonication

INTRODUCTION

Chitin or poly-*N*-acetyl-D-glucosamine is a major structural polysaccharide in the exoskeleton of arthropods and the cell wall of fungi. Chitin is a linear chain molecule typically composed of several hundred 1 → 4-linked 2-acetamido-2-deoxy-*β*-D-glucopyranose units. The polymer molecules can be aligned in antiparallel fashion (α-chitin) to yield highly crystalline, durable structures, a form that is predominantly found in crustacean shells and fungal cell walls. The parallel *β*-chitin configuration yields softer and more pliable structures and is dominant in squid pans (*1*). Both α- and *β*-chitin are insoluble in water, dilute acid and base solutions, and most organic solvents because of the large number of intermolecular hydrogen bonds that favors the formation of dense, crystalline structures (*2*).

It is estimated that crustaceans, mollusks, insects, and fungi synthesize about 100 billion tons of chitin annually (*3*), making chitin one of the most abundant biopolymers. Chitin can be readily obtained by simple extraction. Presently, the industrial production of chitin is primarily based on the recovery of the biopolymer from crustaceans shells that accumulate as a byproduct in the seafood industry but fungi has recently emerged as an alternative source (*4*). On a dry weight basis, crustacean shell waste consists approximately of 40% protein, 35% minerals, 20% chitin, and 5% lipids (*5*). The actual chitin content varies depending on species, health of the animals, harvesting season, and geographical location. For example, the chitin content in crab shells may be as high as 32% as compared to less than 20% in shrimp shells (*5, 6*). The production of chitin begins with removal of minerals, mainly calcium and magnesium phosphates and carbonates, usually by submersion of shells in hydrochloric acid. The demineralized shell fragments are then treated with an alkaline solution to remove proteins. Studies have shown that the order of the two steps may be reversed

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with no adverse effect on quality and yield of the final product (*6*). Generally, the industrial yield of chitin from North Atlantic shrimp (NAS), *Pandalus borealis*, is less than 17% on a dry weight basis (*5*). Shrimp waste contains large concentrations of protein, which stem primarily from the skeletal tissue and to a lesser extent from remaining muscle tissue. In the skeletal tissue, protein and chitin combine to form a protein−chitin matrix, which is then extensively calcified to yield hard shells (*7*). The waste may also contain lipids from the muscle residues and carotenoids, mainly astaxanthin and its esters, associated with proteins in the epithelial layer of the exoskeleton (*8*). If the recovery of lipids and/or carotenoids is desired, the compounds can be extracted prior to the demineralization and deproteinization using a mixture of organic solvents, e.g., chloroform, methanol, and water (1:2:4) at 25 °C (*5*). If the recovery of carotenoids is not required, they may instead be bleached with potassium permanganate, NaOCl, or H_2O_2 in a final processing step to complete the chitin purification (*1*, *9*).

Processing conditions used in industry vary widely and differ in type and concentration of acid and base and time and temperature of the treatments. Demineralization is commonly performed in 0.25−2 M HCl at 0−100 °C for 1−48 h, while deproteinization is done in 0.125−2.5 M NaOH solution at 65−100 °C for 1−72 h (*8*, *10*, *11*). The major concern in chitin production is the quality of the final product, which is a function of the molecular weight (average and polydispersity) and the degree of acetylation (DA). Harsh acid treatments can reduce the molecular weight of polymers due to hydrolysis while high concentrations of NaOH and high deproteinization temperatures can cause undesirable deacetylation and depolymerization of chitin. However, chitin may be deliberately deacetylated in hot concentrated ($\geq$10 M) NaOH for several hours or at lower temperatures for up to 2 days to obtain chitosan, a highly bioactive chitin derivate (*9*, *12*).

Recent studies describing the use of high-intensity ultrasound to extract a number of polysaccharides from a variety of sources including pectin (*13*), hemicellulose (*14*), and starch (*15*) suggested significant reductions in time and solvent requirements leading to cost savings and increased production rates. Because of time, temperature, and solvent conditions required to produce chitin, the use of ultrasound to improve the efficiency of the extraction process could lead to cost and time savings. The goal of this study was therefore to evaluate whether the use of high-intensity ultrasound during the extraction may benefit chitin production. Specifically, our objective was to determine effects of ultrasonication during demineralization and deproteinization on yield and quality of chitin extracted from *P. borealis.* Results were compared to that of a previous study where freshwater prawns (*Macrobrachium rosenbergi*) were used as a source of raw materials (*16*).

MATERIALS AND METHODS

Raw Material Preparation. Byproducts from NAS (*P. borealis*) were kindly provided by Primex (Reykjavík, Iceland). Shells were extensively washed under running hot water (40 °C) to remove any visible tissues and impurities. Clean shells were freeze-dried, weighed, and ground (Wiley Mill, Thomas Scientific, Swedesboro, NJ) to obtain a fine powder with an average particle size of 60 μm.

Extraction Procedure. Chitin was extracted from NAS shells following the method of Percot et al. with slight modifications (*16*). NAS shell powder (4 g) was suspended in 160 mL of 0.25 M HCl (1:40 solid-to-solvent ratio, w/v) at 40 °C for 4 h. The control treatment that simulated a conventional extraction process did not involve sonication, and the suspension was occasionally stirred during extraction of minerals and proteins. Sonication treatments were conducted in a

double mantle cylindrical reaction vessel composed of borosilicate (Pyrex glass) (89 mm × 32 mm, height × radius) with cooling liquid circulating through the double mantle to keep the sample temperature at 40 ± 2 °C. The temperature of the samples was continuously recorded using a digital thermometer. The sonicator probe was submerged 6.4 cm into the reactor (1.7 cm from the bottom) to minimize foaming initially observed during deproteinzation. Samples were sonicated for 1 and 4 h at 41 W/cm² (Ultrasonic Processor, model 501, Cole Parmer, Vernon Hills, IL). The ultrasonic wave intensity (*I*) was determined calorimetrically by measuring the initial rise in temperature (d*T*/d*t*$_a$) and the slope of the heat loss after the sonicator was turned off (d*T*/d*t*$_b$) under adiabatic conditions (*17*):

$$I = \frac{mc_p}{\pi r^2}\left[\left(\frac{dT}{dt}\right)_a - \left(\frac{dT}{dt}\right)_b\right] \tag{1}$$

where *m* is the sample mass (164 g), c_p is the heat capacity of the solvent (4.19 J g^{-1} K^{-1}), and *r* is the radius of the ultrasonic probe (0.0065 m).

Samples that were sonicated for 1 h were left in the acid suspension for an additional 3 h at 40 °C with occasional stirring. The demineralized powder was extensively washed with deionized water until neutral pH, lyophilized, weighted, and milled again. For deproteinized samples, powders were suspended in 0.25 M NaOH (1:40 solids-to-solvent, w/v) at 40 °C and sonicated for 0, 1, and 4 h. Demineralized and deproteinized powders were again extensively washed to neutral pH, lyophilized, weighted, and milled. It should be noted that the concentration of NaOH used during the deproteinization was lower as compared to that used in most industrial processes. However, at higher solvent strengths, a rapid deterioration of the sonicator's probe tip occurred. For example, in 0.5 M NaOH, the intensity decreased by as much as 50% after as little as 30 min of sonication. Hence, to determine the maximum degree of deproteinization of the material, the samples were additionally soaked in 1 M NaOH for 2 h.

Chitin Deacetylation. To determine differences in the susceptibility of ultrasonically assisted extracted chitin to subsequent conversion processes, chitin was deacetylated to chitosan by a traditional method in the absence of sonication. The samples were mixed with 12.5 M NaOH at a 1:20 solid-to-solvent ratio and stirred for 4 h at 100 °C (*18*). After conversion, samples were extensively washed with deionized water, lyophilized, and milled.

Compositional Analysis. Freeze-dried powered samples were kept in desiccators at room temperature until used. The yield of each treatment was determined gravimetrically, immediately following lyophilization of the treated samples. Each sample was subjected to mineral, protein, and chitin content analysis, and DA and crystallinity were determined. The mineral content (ash) was assessed gravimetrically after ashing at 600 °C for 6 h. Proteins from freeze-dried samples were solubilized in 1 M NaOH at 40 °C and quantified by the Lowry method (*19*) using bovine serum albumin as a standard. The chitin content was determined as total glucosamine content following the method of Tsuji et al. (*20*) after acid hydrolysis (*21*). Powdered material was hydrolyzed with 6 N HCl at 110 °C and neutralized with sodium acetate (*22*). Liberated glucosamine was determined spectrophotometrically at 650 nm after reaction with 3-methyl-2-benzothiazolone hydrazone hydrochloride and $FeCl_3$ (HP 84528A spectrophotometer, Hewlett-Packard, Ramsey, MS).

DA. A Nexus 670 Fourier transform infrared (FTIR) spectrometer (ThermoNicolet Co., Mountain View, CA) with attenuated total reflection accessory and ZeSe crystal was used to record infrared spectra of samples between 4000 and 700 cm^{-1} with 64 scans at a resolution of 4 cm^{-1}. DA (%) was calculated using the OMNIC 6.1 software (ThermoNicolet) from absorbance areas of the bands at 1655 and 3450 cm^{-1} (*23*):

$$\% \text{ acetylation} = \left(\frac{A_{1655}}{A_{3450}}\right) \times \frac{100}{1.3} \tag{2}$$

Samples were scanned at least three times, and the average DA values were reported.

Crystallinity. The crystallinity of freeze-dried samples was determined from X-ray diffraction patterns obtained with a wide-angle X-ray diffractometer (Philips PW1729 Diffractometer, Philips Electronics, Almelo, Netherlands) using Kα Cu radiation. The 2θ angle was scanned from 5.025 to 54.975° at a counting time of 2 s with an angle step width of 0.05°. The operating voltage and current of the cathode tube were 45 kV and 40 mA. The crystallinity index (CrI$_{peak}$) was calculated by a method of Segal (*24*) originally proposed for cellulose and subsequently applied to chitosan (*25*):

$$CrI_{peak} = \frac{(I_{110} - I_{am})}{I_{110}} \qquad (3)$$

where I_{110} is the maximum intensity (arbitrary units) of the (110) lattice diffraction pattern at $2\theta = 20°$ and I_{am} is the intensity of amorphous diffraction in the same units at $2\theta = 16°$.

Sizes of Crystallites. The apparent crystal size, D_{app} at $2\theta = 20°$ in the direction perpendicular to the (110) crystal plane, was calculated using Scherrer's equation (*26*):

$$\Delta(2\theta) = \frac{k\,\lambda}{B \times \cos(\theta_B)} \qquad (4)$$

where $\Delta(2\theta)$ is the average crystallite size (nm) in the direction perpendicular to the $2\theta = 20°$ plane; λ is the wavelength of the Kα Cu (1.5405 Å); B (in radians) is the full with of half the maximum of the reflection corrected for instrumental broadening (fwhm measured after a correction by subtracting the baseline for the amorphous region); k is a constant indicative of the crystalline perfection, here assumed to be 0.9 (*26*); and $2\theta_B$ is the peak angle (radian). The fwhm due to the equipment (source diffraction) was measured on boron lanthanum and was subtracted from the experimental values.

Morphology. The influence of high-intensity ultrasound on the morphological structure of lyophilized shrimp shells was investigated using scanning electron microscopy (scanning electron microscope, LEO 1455 VP, LEO Electron Microscopy, NY). Samples were coated with gold to ensure sufficient electron refraction. A series of images were taken after each processing step at magnification factors of 100×, 500×, 1000×, 2000×, and 5000×.

Statistical Analysis. To determine possible correlations between the different extraction steps and treatments, results were subjected to statistical analyses using SAS, version 8 (SAS Institute Inc., Cary, NC). For yield, mineral, and glucosamine measurements, a mixed procedure (proc mix) was used. Proc mix was also used to determine the interaction between sources (*M. rosenbergii* and *P. borealis*) and extraction steps and treatments. A general linear model procedure (proc glm) was used to determine correlations between sonication time and amount of protein in samples. Mean separation was achieved using orthogonal polynominal contrast.

RESULTS AND DISCUSSION

Effect of Sonication on Yield and Composition. Relative and true yields of all extraction steps are shown in **Table 1**. The "true yield" is expressed as a percent of the extracted material from the material that was used for the extraction in each step while the "relative yield" shows the amount of extracted material (g) per initial 100 g shell (dry weight basis). On the basis of our definition, relative and true yields are identical after the first step. The application of sonication during demineralization with 0.25 M HCl decreased the true yield from 58.3 to 51.8 and 47.4% for 0, 1, and 4 h of sonication, respectively (**Table 1**). The removal of minerals from the shells in the first step is based on the chemical reaction of the acid with minerals, primarily $CaCO_3$, which results in the neutralization of the acid to produce soluble salts that can be washed out in a rinsing step. Statistical analysis verified that the differences in yield depending on the duration of the sonication treatment were significant ($P < 0.0001$), and prolonged sonication during

Table 1. Yield of NAS Shells after Subsequent Extraction Steps

| sonication time (h) | true yield | | |
	demineralization (% of initial NAS shell powder)[a]	deproteinization (% of demineralized NAS powder)[a]	additional NaOH treatment (% of deproteinized NAS powder)[b]
0	58.3 ± 2.72	36.1 ± 2.02	79.9 ± 4.14
1	51.8 ± 1.89	30.2 ± 2.47	85.4 ± 2.62
4	47.3 ± 0.98	26.8 ± 2.15	88.8 ± 2.92
significance[c] ($P \leq F$)	<0.0001	<0.0001	<0.0001
linear	<0.0001	<0.0001	<0.0001
quadratic	0.0021	<0.0001	0.0074

| sonication time (h) | relative yield | | |
| | grams of extracted material from initial 100 g of NAS shell powder | | |
	demineralization	deproteinization	additional NaOH treatment
0	58.3 ± 2.72	20.7 ± 0.87	16.6 ± 0.72
1	51.8 ± 1.89	16.1 ± 0.56	13.8 ± 0.47
4	47.3 ± 0.98	12.8 ± 0.24	11.4 ± 0.22

[a] Means ± standard deviation ($n = 9$). [b] Means ± standard deviation ($n = 6$).
[c] Linear = significance of linear relationship; quadratic = significance of adding the quadratic arguments to the linear relationship.

the demineralization decreased the amount of material that could be collected after the extraction. In the deproteinization step, shells were soaked in 0.25 M NaOH (1:40) while simultaneously being treated with high-intensity ultrasound. True yields decreased from 36.1 to 30.1 and 26.8% for 0, 1, and 4 h of sonication, respectively. In general, less than one-third of the shell mass that was used in the deproteinization remained and more than 66% of the material after the demineralization consisted of alkali soluble compounds such as proteins. Despite what appears to be an efficient removal, an additional 1 h of 1 M NaOH treatment was required to remove the remaining tightly bound alkali soluble compounds decreasing the total mass by another 20.1, 14.6, and 11.2% for 0, 1, and 4 h, respectively. Calculated on the initial dry shell basis, the relative chitin yield after completion of the entire extraction was 16.6, 13.8, and 11.4% for nonsonicated, 1, and 4 h sonicated samples, respectively. Relative chitin yields in industrial extractions generally range from 5 to 8% (*1*), and our results are somewhat higher than one would expect. This may be attributed to differences in raw material preparation. In our experiments, we chose to follow a procedure that was previously used under laboratory conditions, extensively cleaning the shells to remove attached meat fragments followed by drying and grinding to obtain a fairly "clean" powder (*10*). Under industrial conditions, shell fragments are often used "as is"; they still contain a large amount of proteins and lipids that is part of the meat that seafood manufacturers were unable to remove (*9, 12*). While the lowest chitin yield obtained in our experiment (11.4% after 4 h of sonication) was still higher than the highest yield obtained under industrial conditions, this constituted an almost 5% decrease from the yield obtained in the absence of sonication. At first glance, the reduction in yield could be explained by an improved removal of proteins and mineral, suggesting that more proteins and minerals may remain within the chitin that was extracted in the absence of sonication. However, results shown in **Tables 2** and **3**, discussed later, indicate that this is not the case. We postulate that the decreased yield is due to a dissolution of chitin itself. The application of high-intensity ultrasound may have

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Table 2. Mineral Content in NAS Material after Subsequent Extraction Steps

sonication time (h)	NAS powder		NAS powder after additional NaOH treatment
	demineralized	deproteinized	
% ash in material			
0	2.5 ± 0.1	2.5 ± 0.3	3.2 ± 0.2
1	3.3 ± 0.2	2.7 ± 0.1	3.9 ± 0.5
4	3.5 ± 0.5	4.4 ± 0.3	5.4 ± 0.4
significance[a]	0.0152	0.0095	0.9615
($P \le F$)			
linear	0.0647	0.0031	0.7793
quadratic	0.5752	0.8072	0.6444
ash (g) remaining from 100 g of NAS initial shell powder			
0	2.5 ± 0.1	0.45 ± 0.02	0.53 ± 0.02
1	3.3 ± 0.2	0.44 ± 0.03	0.53 ± 0.03
4	3.5 ± 0.5	0.56 ± 0.07	0.61 ± 0.08

[a] Linear = significance of linear relationship; quadratic = significance of adding the quadratic arguments to the linear relationship.

Table 3. Protein Content in NAS Material after Subsequent Extraction Steps

sonication time (h)	% protein in material		
	NAS powder		NAS powder after additional NaOH treatment[b]
	demineralized	deproteinized	
0	33.4 ± 0.3	10.6 ± 0.8	2.8 ± 0.2
1	28.8 ± 0.1	8.3 ± 0.4	3.3 ± 0.3
4	29.1 ± 2.8	7.3 ± 0.8	2.5 ± 0.3
significance[a]	0.4654	0.5719	0.9717
($P \le F$)			
linear	0.6264	0.0137	<0.0001
quadratic	0.6147	0.0659	0.0019

sonication time (h)	proteins (g) remaining from 100 g of initial NAS shell powder		
	after demineralization	after deproteinization	after additional NaOH treatment
0	33.4 ± 0.3	2.2 ± 0.02	0.5 ± 0.04
1	28.8 ± 0.1	1.3 ± 0.02	0.5 ± 0.02
4	29.1 ± 2.8	0.9 ± 0.07	0.3 ± 0.03

[a] Linear = significance of linear relationship; quadratic = significance of adding the quadratic arguments to the linear relationship.

produced solvent-soluble chitin fragments (oligosaccharides) that were washed out with the solvent and could not be recovered. Several studies have shown that application of ultrasound especially at high ultrasonic intensity levels (>10 W cm^{-2}) induces depolymerization of stiff, linear chain molecules (*17*, *27−29*). The decrease in molecular weight of polymers was attributed to strong shear stresses that are exerted during growth and collapse of cavitational bubbles generated in the wake of the acoustic field (*30−34*). With respect to chitosan, Baxter et al. recently demonstrated an almost 10-fold decrease in average molecular weight after application of 20 W cm^{-2} ultrasound for less than 60 min (*35*). In our experiments, ultrasound was applied twice for 4 h. Unfortunately, we did not analyze the wash water to determine the presence and concentration of chitin oligosaccharides, an experiment that we are planning to include in a future study.

A comparison of the yields obtained from freshwater prawn shells (FWP) determined in an earlier study (*16*) and NAS shells showed significant differences between the two species ($p <$ 0.0001), where relative and true yields obtained after each step varied greatly between the two species. The initial demineral-

ization removed between 30 and 40% of acid-soluble compounds from FWP as compared to 40−50% from NAS. In the deproteinization step, the mass of NAS material was reduced by 64−73% while in FWP, the mass was reduced by as much as 85−90%. Regardless of sonication treatment, the results suggest possible differences in mineral and protein contents and/ or a different architecture and polymer composition due-to-different ratios of α- and β-chitin in the shells of the two species. Generally, α-chitin forms more dense, closely packed structures as compared to β-chitin. Kurita et al. showed that processing of β-chitin from squid proceeded more rapidly than the processing of α-chitin (*36*). The greater and faster mass reduction in FWP during demineralization and deproteinization may thus be indicative of a higher β-chitin content. The ratio of α/β-chitin in shells also depends on the age of crustaceans. The α-chitin content typically increases with increasing age of crustaceans while β-chitin content decreases (*9*). FWP prawns were harvested after 6 months, but the age of NAS likely varied since they were not farm-raised.

Overall, the large differences in yield obtained after each stage from FWP and NAS indicated that extraction of chitin from NAS is more difficult, and under the same processing conditions, more proteins remain bound to the chitin backbone. Moreover, α-chitin has a higher crystallinity than β-chitin, which may reduce access of solvents to the polymer chains (*37*, *38*). Because of the higher crystallinity, NAS shells have apparently increased chemical resistance and improved mechanical properties, which may be required for shrimp to survive in the cold North Atlantic seawater. Growing conditions for FWP are less harsh, and FWP used in our study were grown under optimal conditions over a period of 4 months in stationary 15−20 °C ponds.

Morphology of Shell Fragments during Sonication-Assisted Chitin Extraction. The removal of minerals and proteins in NAS by traditional extraction left the stacked layer structure of chitin in shell fragment largely intact (**Figure 1a**). Application of 1 h of sonication during demineralization and deproteinization did not fracture the chitin sheets (**Figure 1b**), but after 4 h of sonication, the material showed signs of perforation (**Figure 1c**). Comparing the effects of sonication duration on the morphology of NAS and FWP shells, it becomes apparent that the FWP shells are much more susceptible to ultrasonically induced forces than NAS. The FWP and NAS shells were morphologically similar after the traditional extraction, where chitin fibers were staked in layers (**Figure 1a,d**). Sonication altered the structure of shells, and after 1 h of sonication, the chitin sheets in FWP became shattered (**Figure 1b**) but remained largely intact for NAS shells (**Figure 1e**). After 4 h of sonication of FWP, the shells became highly fractured and spongy (**Figure 1c**). The effect was much less dramatic for NAS shells (**Figure 1f**).

Effect of Ultrasonication on Mineral Content. The mineral content in NAS shells was initially 25.8%, and 4 h of 0.25 M HCl treatment absent of sonication reduced it to 2.5%. With 1 and 4 h of sonication, 3.3 and 3.5% of minerals remained, respectively (**Table 2**). While the additional alkali treatments used to remove remaining alkali-soluble compounds appear to increase the content of minerals in chitin, this is really due to a further removal of proteins and reduction of total mass and not because of an accumulation of minerals during the extraction. Differences in mineral content after 0, 1, and 4 h of sonication were not statistically significant ($p < 0.1176$). Percot et al. (*10*) found in their studies that almost all minerals were removed from shrimp shells in less than 15 min at room
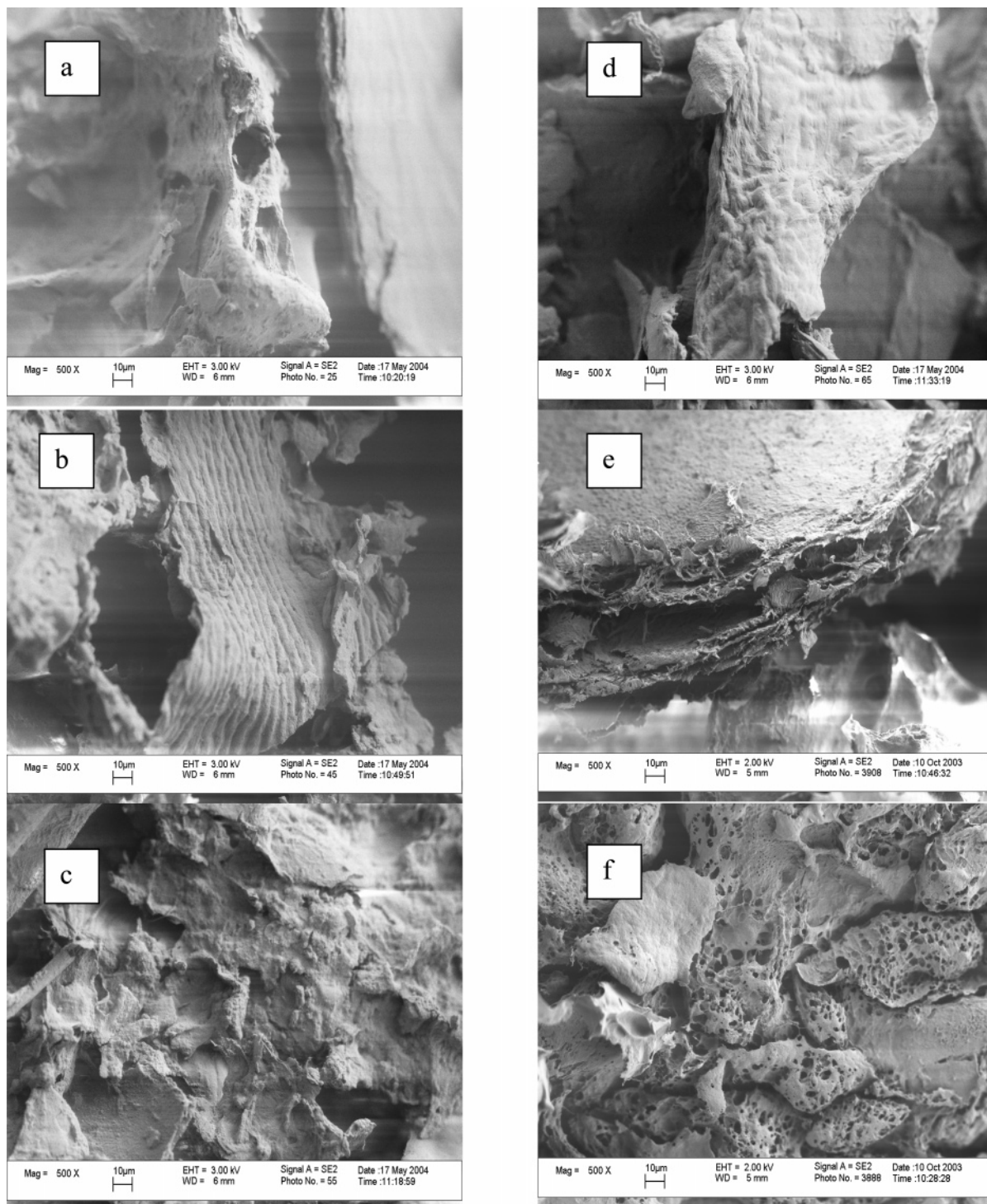
Figure 1. Scanning electron micrographs of lyophilized NAS (**a**–**c**) and FWP (**d**–**f**) shell powder after (**a**,**d**) traditional extraction, (**b**,**e**) 1 h of sonication-assisted extraction, and (**c**,**f**) 4 h of sonication-assisted extraction.

temperature. Shorter and less intense treatments may be sufficient to effectively reduce mineral fractions in the final product since the total mineral content was less than 0.5 g from 100 g NAS shell powder regardless of treatment.

Effect of Ultrasonication on Protein Content. The initial protein content in NAS shells was 39.8%. Conventional demineralization reduced the protein content by approximately 6% while 4 h of sonication-assisted demineralization resulted in a >10% reduction (**Table 3**). A fraction of proteins was (unintentionally) removed during the demineralization step, and this elimination was increased by the application of high-intensity ultrasound. The deproteinization significantly decreased

the protein content by more than 90% resulting in 10.6, 8.3, and 7.3% after 0, 1, and 4 h of sonication, respectively. While this is a substantial reduction, a protein concentration of >10% is typically deemed unacceptable for pure chitin. Apparently, 0.25 M NaOH for 4 h was insufficient to remove all proteins from the material, although the application of ultrasound yielded better results and reduced the total protein content to 7.3%. Only after an additional 2 h of treatment with 1 N NaOH was the final protein content in the conventionally extracted material in an acceptable range of less than 3.5%.

Assessment of Purity of Ultrasonically Extracted Chitin. The total glucosamine content (TGA) increased in the shell

Table 4. Total Glucosamine Content in NAS Material after Subsequent Extraction Steps

	% glucosamine in material		
	NAS powder		NAS powder after additional NaOH treatment[b]
sonication time (h)	demineralized	deproteinized	
0	26.0 ± 1.6	59.0 ± 0.3	66.7 ± 2.8
1	25.0 ± 3.0	58.4 ± 2.0	67.6 ± 2.0
4	27.9 ± 1.7	62.7 ± 4.7	59.1 ± 3.0
significance[a] ($P \leq F$)	0.3993	0.1225	0.0014
linear	0.2091	0.1235	0.0064
quadratic	0.4124	0.5037	0.2029

|	glucosamine (%) remaining from 100 g of initial NAS shell powder		
sonication time (h)	after demineralization	after deproteinization	after additional NaOH treatment
0	26.0 ± 1.6	12.2 ± 0.72	11.0 ± 0.71
1	25.0 ± 3.0	9.4 ± 1.01	9.3 ± 1.12
4	27.9 ± 1.7	8.0 ± 0.53	6.7 ± 0.52

[a] Linear = significance of linear relationship; quadratic = significance of adding the quadratic arguments to the linear relationship.

Table 5. Crystallinity Index of Lyophilized NAS Powder

	crystallinity index		
	NAS powder		NAS powder after additional NaOH treatment
sonication time (h)	demineralized	deproteinized	
0	60.6	54.7	87.6
1	65.8	78.7	79.1
4	55.9	80.2	78.5

material after each step in the extraction process (**Table 4**). For conventional extraction, TGA increased from 26.0 to 59.0 and 66.7% in demineralized, deproteinized material and in NAS chitin after additional NaOH treatment, respectively. Similar results were obtained with high-intensity ultrasound, and TAG increased from 25 to 58 and 67.6% and from 27.9 to 62.7 and 59.1% for 1 and 4 h sonicated samples, respectively. On the basis of the accuracy of the measurements, the differences were not considered statistically significant. The total amount of glucosamine in samples was reduced with ultrasonication, which may be attributed to the previously discussed reductions in total yield.

Crystallinity of Ultrasonically Extracted Chitin. The crystallinity index of the extracted materials was determined from the scattering intensity at two angles, the baseline at $2\theta = 16°$, and the maximum intensity at $2\theta = 19-20°$ (**Table 5** and **Figure 2**). Chitin exists in two crystal forms found between a 10 and a 25° angle. Form I has characteristic peaks at 11 and 18°, while form II has peaks at 15, 21, and 24°. Form I as a crystal structure that is characterized by unit lengths of $a = 7.76$ Å, $b = 10.91$ Å, and $c = 10.30$ Å and $\beta = 90°$. This form is larger than that of crystal II, whose unit cell is $a = 4.4$ Å, $b = 10.0$ Å, $c = 10.30$ Å, and $\beta = 90°$ (*39*). In all observed XRD diffractograms, the size of the peak area of crystal form II was greater than that of crystal form I, indicating that form II was predominantly present in all samples (**Figure 2**). Overall, the crystallinity of chitin obtained from NAS shells was lower than that of chitin from FWP; for example, the crystallinity index varied between 78.5 and 87.6% for NAS as compared to 80.6 and 91.6% for FWP (*16*). The application of ultrasound reduced
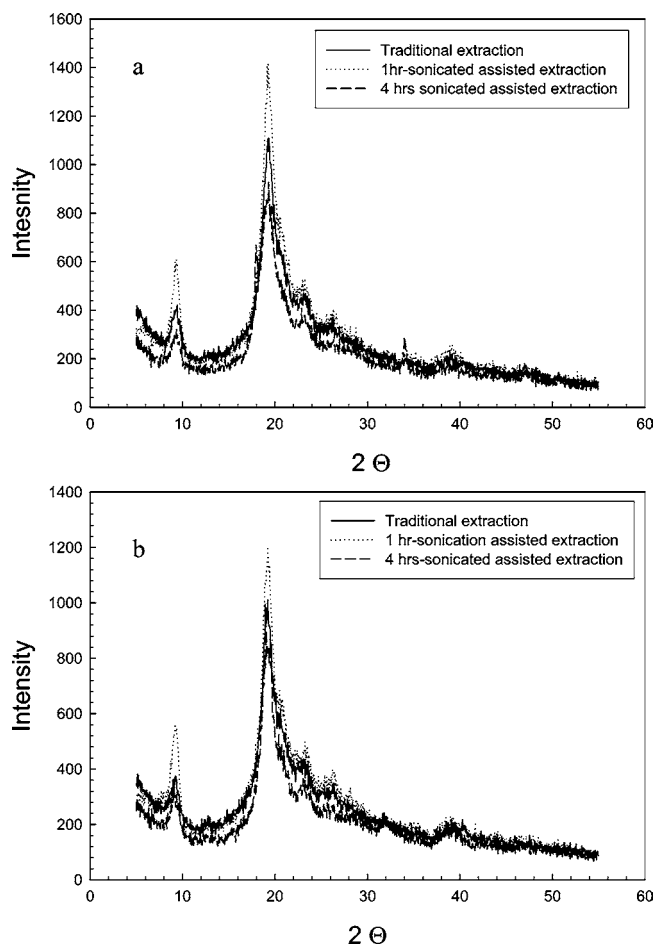
Figure 2. X-ray diffractographs of (**a**) NAS chitin and (**b**) chitosan produced from NAS chitin by deacetylation after traditional extraction (straight line), 1 h of sonication extraction (dotted line), and 4 h of sonication extraction (broken line).

Table 6. Size of Crystallites in Lyophilized NAS Powder

	size of crystallites (nm)		
	NAS powder		NAS powder after additional NaOH treatment
sonication time (h)	demineralized	deproteinized	
0	0.87	0.87	2.14
1	1.46	3.07	3.46
4	0.72	3.15	2.14

the crystallinity index in both FWP and NAS, and there was ~10% difference between 0 and 4 h sonicated samples (**Table 5**). A lower crystallinity of polysaccharides indicates disruption of intra- and intermolecular hydrogen bonds, which, in turn, provides the possibility for more efficient chemical modifications in subsequent processing steps (*40*). For example, chitin is commonly deacetylated to produce the highly bioactive chitosan, a reaction that may benefit from lower crystallinities to improve accessibility to reagents. Finally, X-ray diffractograms of chitin powder obtained from NAS and FWP showed that NAS chitin generally had broader peak areas than FWP chitin, which is indicative of impurities, again confirming that both the demineralization and the deproteinization were less efficient as compared to that of FWP (*41*).

Size of Crystallites. The sizes of crystallites were calculated from diffraction at the $2\theta \sim 20°$ angle using the software Peak Fit to calculate the fwhm. The size of chitin crystals increased
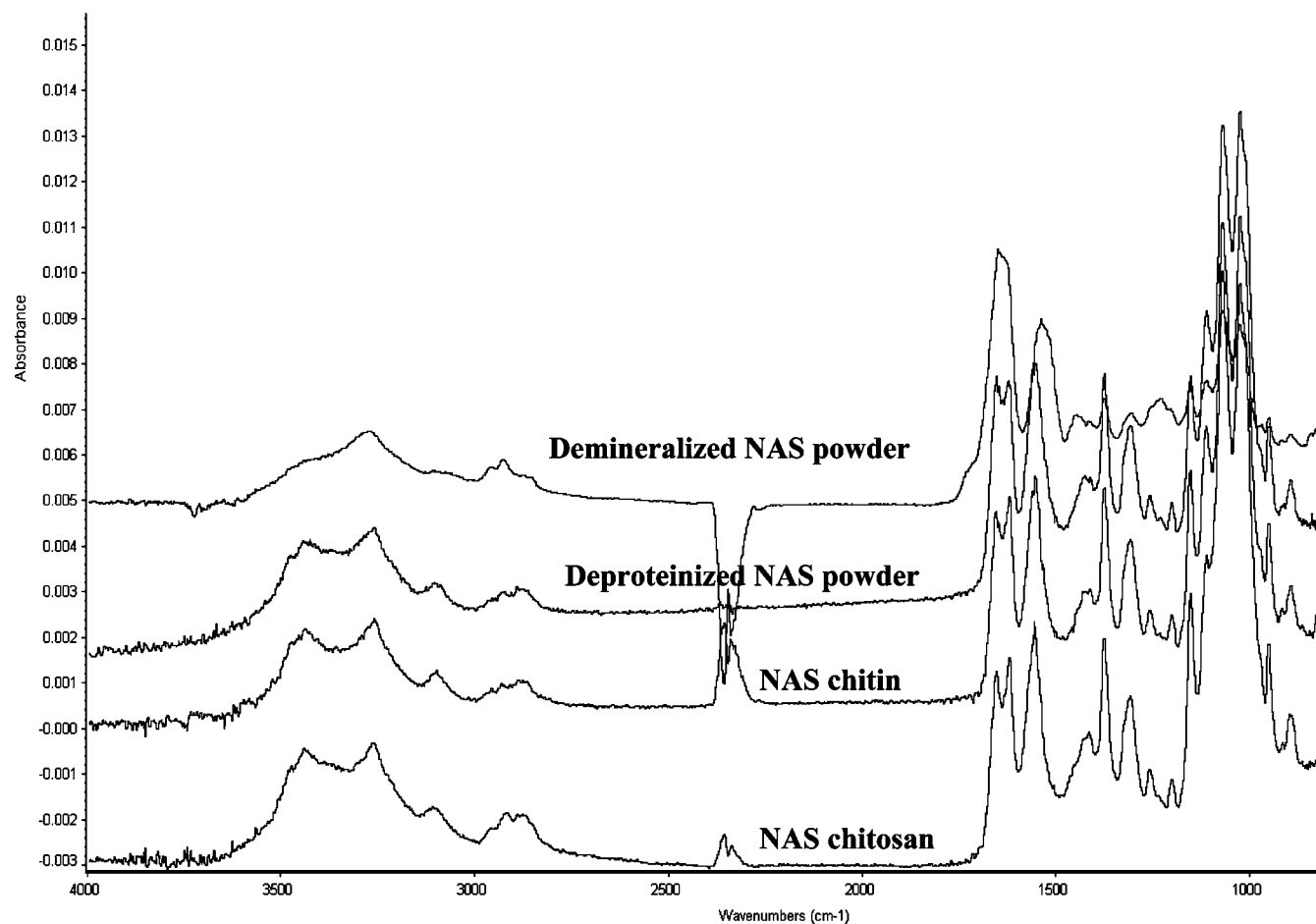
Figure 3. FTIR spectra of demineralized and deproteinized NAS shell powder and chitin and chitosan obtained during extraction.

during the processing, e.g., the size of crystals after demineralization was 0.87 nm but increased to well above 2 nm after the final NaOH treatment (**Table 6**). Statistically, ultrasonicated materials had larger crystal sizes, but there was no difference between the 1 and the 4 h treatment. The order of magnitude was in agreement with results of a study published by Jaworska et al. (*42*) who reported the size of chitin crystals from shrimps of 3.3 nm. However, the underlying assumption of having a single uniform crystal size in the powder is likely not correct since materials were not necessarily homogeneous. Calculation of the fwhm also depends on the chosen algorithm to deconvolute overlapping peaks, and errors in calculated sizes are commonly in the order of ±0.5 nm when this method is used.

Conversion of Ultrasonically Assisted Extracted Chitin to Chitosan. To assess the further impact of ultrasonically assisting the extraction of chitin, chitosan was produced by deacetylation with hot 12.5 M NaOH in the absence of ultrasound. The purity of the obtained material was further improved as indicated by the total glucosamine content that rose from 66.7, 67.6, and 59.1% to 70.4, 84.7, and 90.4% for samples that were sonicated for 0, 1, and 4 h during the extraction. The results seem to confirm the previously stated hypothesis that treatment with high-intensity ultrasound may improve the accessibility of the solvent in subsequent processing steps. Interestingly, differences in the FTIR scans between chitins and their corresponding chitosans were relatively small (**Figure 3**) and DA decreased from 89.2 ± 3.2, 87.0 ± 2.4, and 88.5 ± 9.5% for 0, 1, and 4 h sonication-assisted extracted chitins to 67.2 ± 6.5, 82.8 ± 4.7, and 74.6 ± 7.3% for corresponding chitosans. At the same time, the crystallinity index decreased from 87.6, 79.1, and 78.5 for the 0, 1, and 4 h sonicated chitins to 76.7, 79.5, and 74.8 in

their corresponding chitosans. Similarly to X-ray diffractographs of FWP shells, the conversion of chitin to chitosan caused disappearance of many peaks and only two distinguishable peaks were detected regardless of the treatment, one at $2\theta = 9-10°$ and the other at $2\theta = 19-20°$.

Conclusions. Results of this study indicated that sonication treatment may enhance protein extraction from shimp shells. However, sonication treatments did not improve the amount of minerals extracted from the shells, suggesting that application of ultrasound in the demineralization step is not particularly useful or may even be detrimental since some material may be solubilized and subsequently washed out with the reagents due to depolymerization. It should be noted though that at present one of the most problematic aspects of chitin production from crustaceans is the amount of proteins that is still left in the product. This issue may become even more important in the future since shellfish proteins have been associated with an increasing number of food allergen cases. Moreover, our studies suggest that high-intensity ultrasonication may be beneficial to the deacetylation reaction required to produce the highly bioactive chitosan possibly due to improved accessibility of acetyl groups to reagents in ultrasonically treated chitin. The application of high intensity ultrasound appears to provoke changes in the crystalline structure of the chitin and its morphology that are beneficial to conducting subsequent chemical reactions. Future studies should therefore emphasize the utilization of ultrasonically produced chitin in the production of chemical derivatives. Finally, comparison of the two raw materials NAS and FWP indicates large differences in the susceptibility to high-intensity ultrasound as shown by the

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morphological and structural changes observed after ultra-sonication of FWP and NAS by SEM and X-ray diffracto-metry.

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