A Simple and Reliable Method for the Determination and Localization of Chitin in Abalone Nacre

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We collected test reactions which can be applied in an easy and reproducible way to the chemical composition of the abalone organic matrix. Several chemical and biochemical test reactions were applied to the interlamellar organic matrix of abalone nacre to study the content and nature of polysaccharides. The preparation of the polysaccharide matrix was examined in parallel by light microscopy. The polysaccharide core is covered by a honeycomb-like structure, which can be completely removed by the protease subtilisin under release of the hydrophobic amino acid proline. The honeycomb pattern is in its size and its shape exactly the inverse matrix of the aragonite tablets, which are well-known to build up the nacreous layer of abalone shells. With this protocol we proved and verified in a straightforward and simple way the polysaccharides in abalone to be chitin. In addition, ^{1}H and ^{13}C NMR analysis of the interlamellar organic matrix of abalone nacre confirmed that it consists to a high extent of the polysaccharide chitosan or its partially/completely *N*-acetylated derivative chitin (β-(1→4)-2-acetamido-2-deoxy-D-glucose or *N*-acetyl-D-glucosamine).

Introduction

The molluscan shell in general and nacre in particular are examples of extremely sophisticated mineralized structures.[1,2] Especially nacre is a biogenic material with remarkable mechanical properties such as extreme stability, stiffness, and strength combined with elastic properties, reducing the brittleness of the mineral phase.[3,4] Nacre, which represents the iridescent inner layer of the molluscan shell, consists of single aragonite tablets of 10−15 μm diameter and 500 nm height.[5,6] These tablets are arranged in vertical stacks and form sheets of lamellae like a brickwall.[7] The mortar is represented by an organic matrix, which forms interlamellar sheets, connects these sheets to each other,[8] and is also present in the aragonite crystals, so-called intracrystalline proteins.[9] The interlamellar sheets have a detailed structure and consist of water-soluble and -insoluble material, mainly polysaccharide and protein layers.[8] The polysaccharide matrix functions as the framework for insoluble proteins, which seem to cover the polysaccharide very tightly by the formation of a dense layer.[1,10] Although the amount of organic matrix is relatively low in nacre,[11,12] it seems to have a key role in mineral nucleation and growth[9,13−15] and to be responsible for the unique and characteristic properties of this composite material.[16,17] There are special demands for the organic matrix in terms of permeability because the nacre tablets grow parallel in different stages in their compartments:[18] large amounts of inorganic ions as well as organic macromolecules (matrix precursors and catalytic devices) have to be transported to their destination across the interlamellar sheets. Schäffer et al. suggested that mineral bridges keep nacre tablets connected to each other,[10] which might contribute to the formation of a single crystal in the vertical direction.[12] Anyway, the organic matrix must tolerate large numbers of holes without losing its rigidity. A detailed structural and chemical analysis of this material, which has to fulfill the requirements of

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both stability and permeability, is of great interest. The polysaccharide core might be responsible especially for structural features of the organic interlamellar sheets of abalone nacre, but it could also act in a more functional way like proteins do. We describe here for the first time purification techniques for the several different molecular constituents of abalone nacre. For systematic analysis of the organic matrix of abalone nacre, it is necessary to completely purify the molecules and localize them in the shell to investigate its chemical and structural composition in detail. It was already proposed by Weiner[19] that the polysaccharide found in the shell of certain molluscs is chitin, but the study was done with a more or less complete mixture of organic material. The suggestion of Weiner was supported by X-ray data. We decided to confirm this idea by a detailed analysis of the interlamellar sheet of abalone nacre. After the purification of the different species of organic material, we performed chemical assays and light microscopy and nuclear magnetic resonance (NMR) measurements to localize and characterize the molecules. If the polysaccharide sheet also has an effect on crystal formation by assembling certain ionic species, it will be helpful for in vitro crystal growth experiments[15,20] to know exactly the substance the surface must be coated to mimic the native structure. Furthermore, it is necessary to establish an assay for a fast and easy investigation of the organic matrix extracted out of abalone flat pearls[21] using atomic force microscopy (AFM) to correlate the imaged region with the natural composite material. Combining AFM with NMR and biochemical assays will be helpful for the elucidation of biomineralization processes, an important aspect of material science. In this paper, we introduce a series of chemical and biochemical test reactions suitable for the analysis of chitin and its deacetylated form chitosan. We applied these test reactions to chitin extracted out of natural abalone shell material and determined the chemical identity as well as the degree of purification of the isolated natural product by ^{1}H and ^{13}C NMR spectroscopy, which allowed the detailed determination of the degree of deacetylation.

Materials and Methods

Preparation of the Interlamellar Organic Matrix. Shells of *Haliotis rufescens* were cleaned by a sand blaster, and the remaining nacre was further purified by shortly rinsing it with concentrated sodium hypochlorite solution. Finally, it was washed several times in deionized water. For the storage of nacre a Tris buffer, pH 7.4, 0.02% sodium azide, was used. Interlamellar organic sheets were prepared according to a modified method of Schäffer.[10] The nacre pieces were crushed with a bench vice and dialyzed against 10% acetic acid until the pH of the solution stabilized at pH 3.4. The remaining organic matrix was washed in 100 mM Tris buffer, pH 8, treated with a Waring blender, and ultrasonicated. Protease digestion of the raw extract was achieved with 1 U/mL subtilisin (Sigma P-5380, Sigma-Aldrich, Deisenhofen, Germany) in 4 times the volume buffer containing 100 mM Tris, 10 mM calcium chloride, and 0.02% sodium azide, pH 8, at room temperature under gentle mixing for 18 days. The remaining insoluble organic matrix was centrifuged at 17000*g* for 1.5 h and separated into two distinct phases. The two phases were washed twice with deionized water, complete removal of subtilisin was checked by SDS−PAGE, and the two phases were stored. The two phases were lyophilized separately.

Deacetylation of Chitin. Each phase was transformed according to the method of Campbell.[22] The sample was incubated with 10 times the weight of a saturated solution of potassium hydroxide and heated under constant stirring at 160 °C for 20 min in a rear flow system. To prevent the sample from boiling, some tablets of potassium hydroxide were added to the solution. After 2 h, the reaction was stopped and the mixture was cooled slowly to 70 °C. The product was isolated by filtration and then washed in ethanol/water and finally in deionized water several times. The resulting product was dried at 70 °C and cooled to room temperature under vacuum.

Chemical and Biochemical Test Reactions for Chitosan. For each of the test reactions, 10 mg of all substances were used and all reactions were performed in 1.5 mL Eppendorf vials. A couple of reference substances were tested as listed in the following: chitosan (Aldrich 41,941-9, Sigma-Aldrich, Deisenhofen, Germany), self-converted chitosan, deacetylated from chitin (Fluka 22720, Fluka, Neu-Ulm, Germany), chitin (Fluka 22720), gelatin (Fluka 48720), cellulose (Sigma C-6288), casein (Sigma C-5890), polyamide (Aldrich 19,101-9), poly(vinyl alcohol) (Serva 33364, Serva-Novex, Heidelberg, Germany), poly(vinylpyrrolidone) (Sigma PVP-360), agar (Fluka 05040), dextran (Sigma D-5501), Amberlite (Sigma A-2523), D(+)-glucose (Merck 8342, Merck, Darmstadt, Germany), methyl-α-(D)-glucose (Sigma M-9376), methyl-α-D-mannopyranoside (Sigma M-6882), agarose (Serva 11397), D(+)-raffinose (Fluka 83400). The natural samples prepared out of abalone shells (dark and light phases) were tested each as raw samples and deacetylated to chitosan.

Solubility Assay (Test 1). Samples were incubated with 150 μL of 3% acetic acid. If the substances were solubilized or gel formation occurred, the addition of 100 μL of 1% sulfuric acid should give a white precipitate (chitosan sulfate).[22]

Iodine Complex Formation (Test 2). A 0.2% iodine/potassium iodide solution was prepared (150 μL per sample), which should give a brown color due to complex formation for chitosan samples. The sample was washed in 1 mL of deionized water and incubated with 150 μL of 1% sulfuric acid, which led to a purple-brown to black color in the case of a positive result, but the color has to disappear on the addition of 300 μL of 75% sulfuric acid as the object goes slowly into solution.[22]

Periodic Acid/Schiff Reaction (Test 3). Chitin samples were oxidized by 150 μL of 10% periodic acid to form polyaldehydes, which were detected by 250 μL of Schiff's reagent. The positive samples formed a red to purple color after 5 min.[22−24]

Anthrone Reaction (Test 4). The samples were incubated with 50 μL of deionized water, 25 μL of 1% sodium nitrite, and 25 μL of 20% acetic acid solution for 10 min under constant stirring. Then a 6% ammonium sulfamate solution was added, and the test tubes were incubated on ice for 10 min. Finally, 300 μL of anthrone reagent (50 mg/25 mL of concentrated sulfuric acid) was added, and the mixture was vortexed. A positive result should give a blue- or brown-green color at an absorption maximum around 580−590 nm after 10 min.[25−27]

Light Microscopy. The interlamellar organic matrix samples were ultrasonicated for 5 min in a bath ultrasonicator and investigated by light microscopy. For differential interference contrast microscopy, an inverted microscope, Axiovert 135 TV (Carl Zeiss, Jena, Germany), equipped with a 40× Fluar

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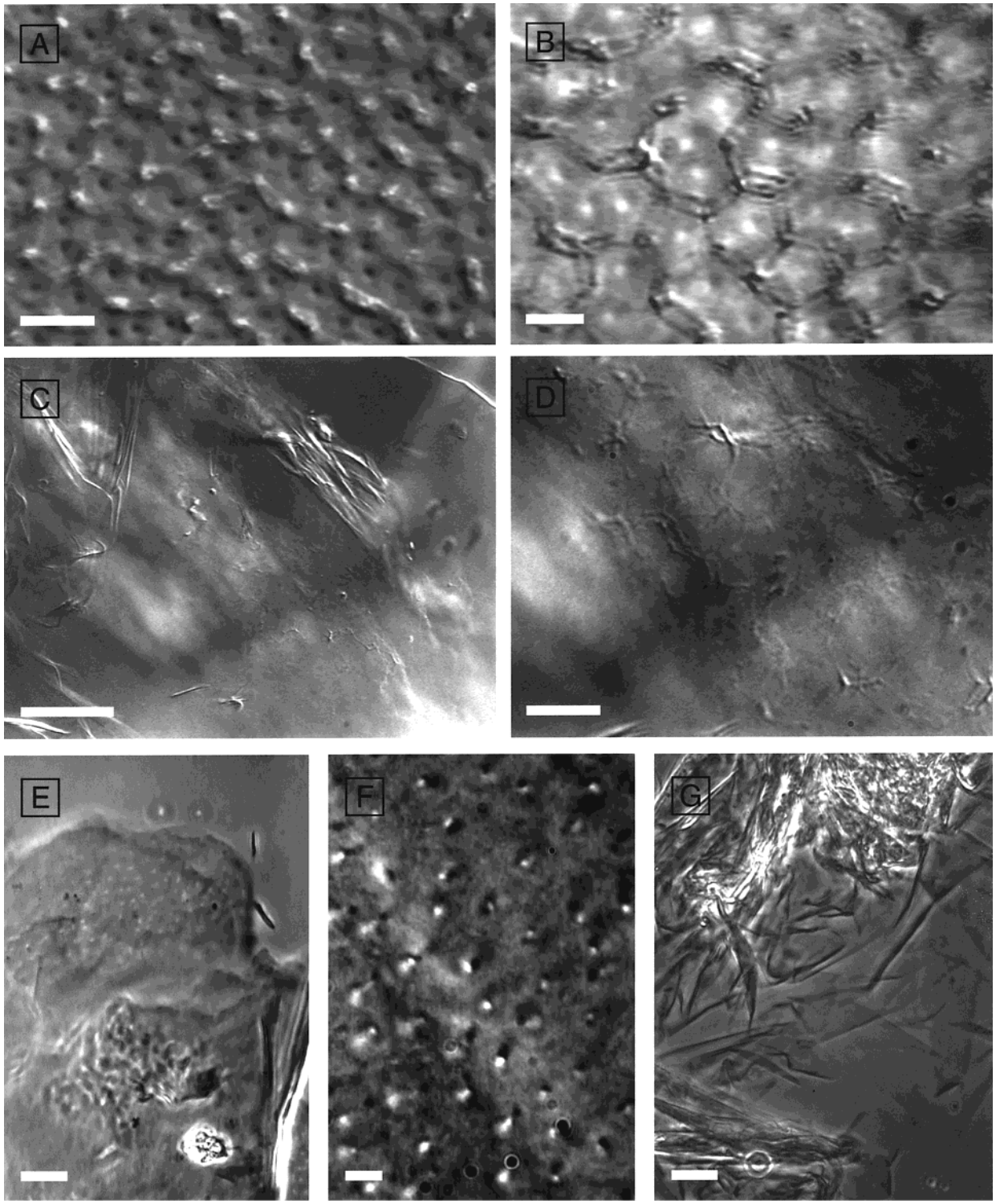

Figure 1. Light microscopy images of different stages in the preparation of chitin extracted out of abalone nacre. (A) DIC microscopy image of the insoluble crude extract of organic matrix after solubilization of the mineral parts of abalone nacre. This image was taken with 1.6× additional optical magnification. Scale bar = 10 μm. (B) DIC microscopy image of the same sample as in (A), but with an additional optical magnification of 2.5. There is a honeycomb-like structure clearly visible which resembles the form of the aragonite tablets. Scale bar = 5 μm. (C) DIC image of the subtilisin-treated extract after 12 days of incubation without additional optical magnification. The sample seems partly like a very hydrophobic and very thin sheet comparable to "cellophane", but in the more stable regions one can still detect remnants of the honeycomb-like structures. Scale bar = 20 μm. (D) Magnification (1.6×) of the honeycomb-like structure in (C). The image shows a significant degradation of the structure by the protease subtilisin. Scale bar = 10 μm. (E) Phase contrast microscopy image of the subtilisin-treated sample after 18 days of incubation. There is no additional optical magnification, but it is clearly visible that no honeycomb-like structures surround the regular arranged little holes as in (A) and (B). Scale bar = 20 μm. (F) DIC image of the same sample as in (E) with 1.6× additional optical magnification. The regular arrangement of holes in the matrix core is shown rather clearly. Scale bar = 5 μm. (G**)** Phase contrast microscopy image of the same sample as in (E) and (F) showing the second characteristic structure after subtilisin treatment, that is, a more cellophane-like one. This structure was found mainly in the light phase of the organic matrix extract, whereas the compact structures shown in (E) and (F) are mainly present in the dark phase. Scale bar = 20 μm.

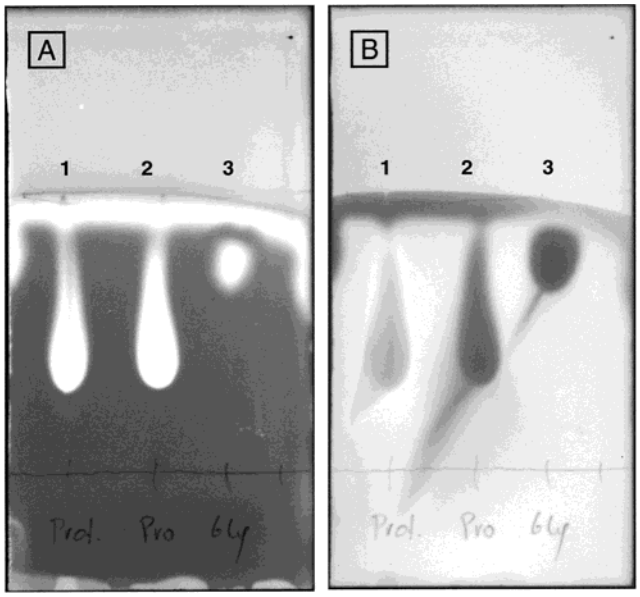

Figure 2. Thin-layer chromatography for amino acid analysis of the interlamellar organic matrix of abalone nacre. This test shows the predominant amino acid of proteins which can be removed from the insoluble matrix by subtilisin. Lane 1 is the supernatant after subtilisin treatment, lane 2 represents the standard amino acid proline, and lane 3 contains the standard amino acid glycine. The sample shows retention behavior nearly equal to that of proline in both detection methods. (A) Detection of the spots by UV light. (B) Spots are colored by the ninhydrin reaction.

oil immersion objective with a numerical aperture of 1.30 was used. There were additional optical magnifications of 1.6 and 2.5. The samples were also imaged in phase contrast (20× Achrostigmat phase contrast objective). The images were projected onto the chip of a 1/3 in. CCD camera (C5403, Hamamatsu, Hamamatsu City, Japan). The camera controller unit was of the C2400 series (Hamamatsu), allowing analogue background and contrast adjustment. Image processing was done by contrast enhancement using the software NIH image (public domain, National Institutes of Health, Bethesda, MD).

Amino Acid Analysis. The supernatant occurring after subtilisin treatment was analyzed by thin-layer chromatography according to the methods of Presek[28] and Hunter and Sefton.[29] Proline and glycine were used as amino acid references. A 750 μL sample of the supernatant after subtilisin treatment was concentrated in a speed vac concentrator at 45 °C for 3 h to a 20 μL final volume. The proline reference was prepared to a final concentration of 0.4 μg/μL in deionized water, and the glycine reference to a final concentration of 75 μg/mL in 1 M hydrochloric acid. The mobile phase consisting of 1 mL of acetic acid and 100 μL of pyridine was diluted in 20 mL of deionized water. A cellulose F thin-layer ready-to-use plate (Merck, Darmstadt, Germany) was used as the stationary phase. A chromatogram was developed in ascending direction in 25 min. The spots were detected by both UV light and ninhydrin reaction (Merck 1-06705) after the plate was heated to 200 °C.

Nuclear Magnetic Resonance. For NMR analysis, two references (chitin, Fluka 22720, converted into chitosan as described above out of two different conversion charges) and two samples prepared out of raw interlamellar sheets as described above (dark and light phases) were dried at 90 °C for 1 h and cooled to room temperature in an evacuated desiccator. A 4 mg portion of the reference and sample was solubilized in 1 mL of 1% trifluoroacetic acid in deuterium oxide and incubated in a rotary mixer for 2 days. All the samples were centrifuged at 3000*g* for 50 min at room temperature to separate insoluble material from the liquid, and the supernatants were transferred into NMR tubes.

Table 1

substance	test 1	test 2	test 3	test 4	result
chitosan (Aldrich 41,941-9)	+	+	+	+	4
deacetylated chitin (Fluka 22720), no. 1	+	+	+	+	4
deacetylated chitin (Fluka 22720), no. 2	+	+	+	+	4
deacetylated dark phase	+	+	+	+	4
deacetylated light phase	+	+	+	+	4
chitin (Fluka 22720)	−	−	−	−	0
raw extract dark phase	−	−	−	−	0
raw extract light phase	−	−	−	−	0
gelatin (Fluka 48720)	−	−	−	−	0
cellulose (Sigma C-6288)	−	−	−	+	1
casein (Sigma C-5890)	+	−	+	−	2
polyamide (Aldrich 19,101-9)	−	−	−	−	0
poly(vinyl alcohol) (Serva 33364)	−	+	−	−	1
poly(vinylpyrrolidone) (Sigma PVP-360)	−	−	−	−	0
agar (Fluka 05040)	−	+	−	+	2
dextran (Sigma D-5501)	−	−	+	+	2
Amberlite (Sigma A-2523)	−	−	+	−	1
D(+)-glucose (Merck 8342)	−	−	−	+	1
methyl-α-(D)-glucose (Sigma M-9376)	−	−	−	+	1
methyl-α-D-mannose (Sigma M-6882)	−	−	−	+	1
agarose (Serva 11397)	−	−	(+)	+	(2)
D(+)-raffinose (Fluka 83400)	−	−	−	+	1

NMR Experiments. NMR experiments were carried out at a proton frequency of 500.13 MHz on a Bruker DRX500 spectrometer equipped with a triple-resonance probehead. To obtain ^{1}H and ^{13}C resonance assignments, two-dimensional DQF-COSY experiments,[30] 2D NOESY experiments[31] with a mixing time of 200 ms, and ^{1}H−^{13}C HMQC experiments were performed at temperatures between 10 and 90 °C. Quadrature detection in indirectly detected dimensions was obtained by the TPPI method.[32] The residual HDO signal was suppressed in a very weak presaturation field (<10 Hz). The shifts were referenced against TSP and reported at 60 °C: ^{1}H NMR δ(H1) 4.91 ppm, δ(H2) 3.24 ppm, δ(H3) 3.94 ppm, δ(H4) 3.95 ppm, δ(H5) 3.77 ppm, δ(CH_2) 3.97/3.82 ppm, δ(CH_3) 2.09 ppm; ^{13}C NMR δ(C1) 100.8 ppm, δ(C2) 59.0 ppm, δ(C3) 73.3 ppm, δ(C4) 80.1 ppm, δ(C5) 77.9 ppm, δ(CH_2) 63.4 ppm. The degree of acetylation was determined for each sample by integration of well-resolved ^{1}H resonances. The integral of the signal at 2.09 ppm, corresponding to the three protons of the acetyl group, was divided by three and compared to the integral of a resolved signal corresponding to one proton of the ring (H1 or H2). As an internal check, the integrals of different ring protons were compared and found to be of the same size. Due to overlap the H5 signal could only be integrated together with one of the CH_2 signals, and H3, H4, and the other CH_2 signal also had to be included in one integral. The resulting integration values corresponded to two and three protons, respectively.

Results and Discussion

For an exact characterization of the organic matrix of abalone nacre, purification of the contents is an imperative step. We show here for the first time a protocol which allows the purification, localization, and characterization of some of its molecular species. The purification steps have been followed by thin-layer chromatography and light microscopy to localize the purified molecules in parallel to their biochemical and NMR characterization.

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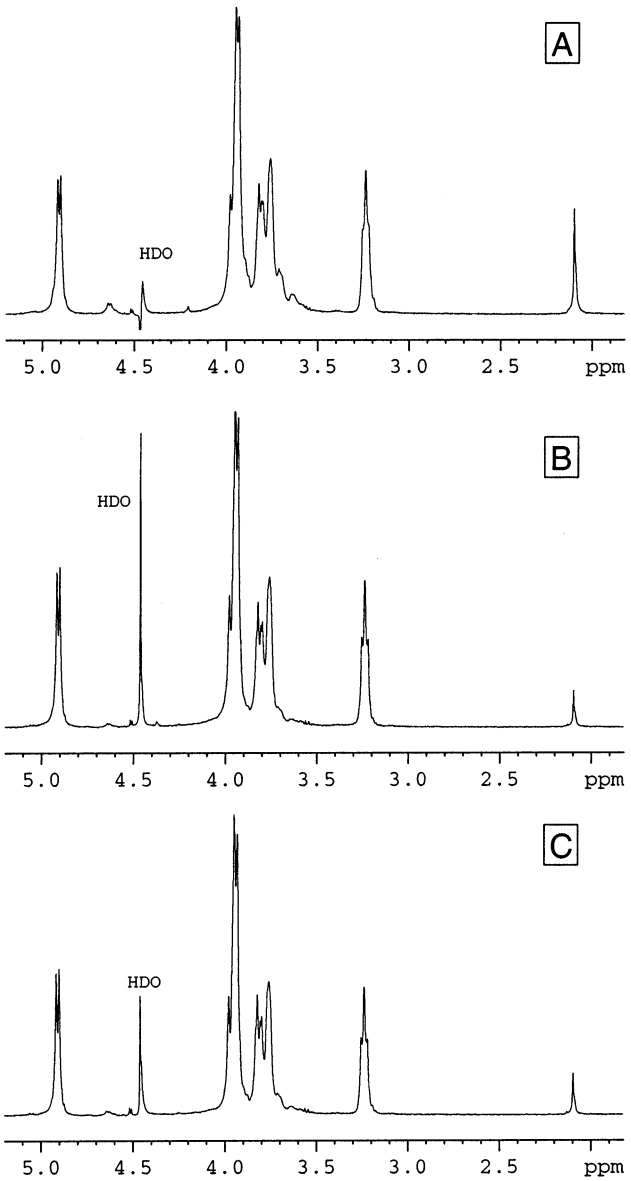

Figure 3. 1D ^{1}H NMR spectra of chitosan samples. The spectra are almost identical for the chitosan reference and the samples of the natural shells. Thus, the abalone nacre contains chitosan or its acetylated form chitin, which was isolated to a high degree of purity according to these spectra. (A) Chitosan reference sample prepared by deacetylation of commercial chitin. (B) Deacetylated dark phase prepared from natural abalone nacre. (C) Deacetylated light phase extract of natural abalone shells.

Preparation of Interlamellar Organic Sheets. Shells of *H. rufescens* were cleaned with a sand blaster, which has proved to be the best method for removing the calcitic parts from the aragonitic nacre. For preparation of the interlamellar organic sheets the aragonite pieces were best crushed if braced against each other in a bench vice. It was found that for a complete solubilization of calcium carbonate the best method is dialysis against 10% acetic acid until the pH of the solution becomes stabilized at a value of pH 3.4. For subtilisin (protease) treatment, which removes the proteinaceous part of the organic matrix, 10 mM calcium chloride was necessary as a cofactor. The treatment with protease was repeated until light microscopy showed complete removal of the proteinaceous structure. To get the protease in better contact with the single organic

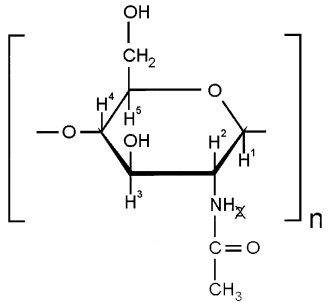

Figure 4. Structural formula of chitosan with the atom labels for assigning the NMR peaks of Figures 5 and 6.

sheets, the extract was also treated with a Waring blender and ultrasonicated. The remaining insoluble organic matrix showed separation into two distinct phases when centrifuged, a light one and a dark one. Each phase was transformed according to the deacetylation method of Campbell.[22] Hardly any biopolymer is resistant to hydrolysis under the conditions of this deacetylation reaction, so the remaining brown-colored solid particles in the mixture represented chitosan. This can also be considered a preliminary test. The product was isolated by hot filtration to prevent the saturated potassium hydroxide solution from crystallizing, which would make it difficult to separate chitosan. The filter cake was washed with an ethanol/water solvent mixture and finally deionized water several times.

Light Microscopy of Interlamellar Sheets. It was convenient to ultrasonicate the samples, so the aggregates of the organic matrix were divided into single sheets, which were thin enough for light microscopy imaging. The preparation steps of the interlamellar organic sheets from abalone shells could be followed by light microscopy because a very characteristic structure of these sheets was clearly visible, namely, the honeycomb pattern, which is a negative structure to the aragonite tablets, which have already been visualized by electron microscopy.[33] Parts A and B of Figure 1 show differential interference contrast (DIC) microscopy images of a sample of raw extracts of decalcified abalone nacre. The same results were obtained by phase contrast microscopy. The size of the honeycombs ranging between 7 and 12 μm in diameter fits very well to the size of aragonite tablets known from other studies.[5,8,21] Parts C and D of Figure 1 show a sample after treatment with the protease subtilisin after 12 days of incubation. Remnants of the honeycomb pattern can be observed, but after a repeated subtilisin treatment, nearly all of the structures are removed as shown in Figure 1E−G. Schäffer proposed that collagen is part of the proteins connected with the structure.[10] We could show that subtilisin removed collagen from the interlamellar matrix. This is a remarkable result as collagen is believed to be nondegradable. This can only be achieved by specialized proteases such as collagenases.

Collagen: A Component of the Interlamellar Matrix. As a byproduct of the preparation of the interlamellar matrix, the supernatant occurring after subtilisin treatment was used to prove the hypothesis that the interlamellar matrix contained collagen.[10] To obtain hints about the presence of collagen, one of the

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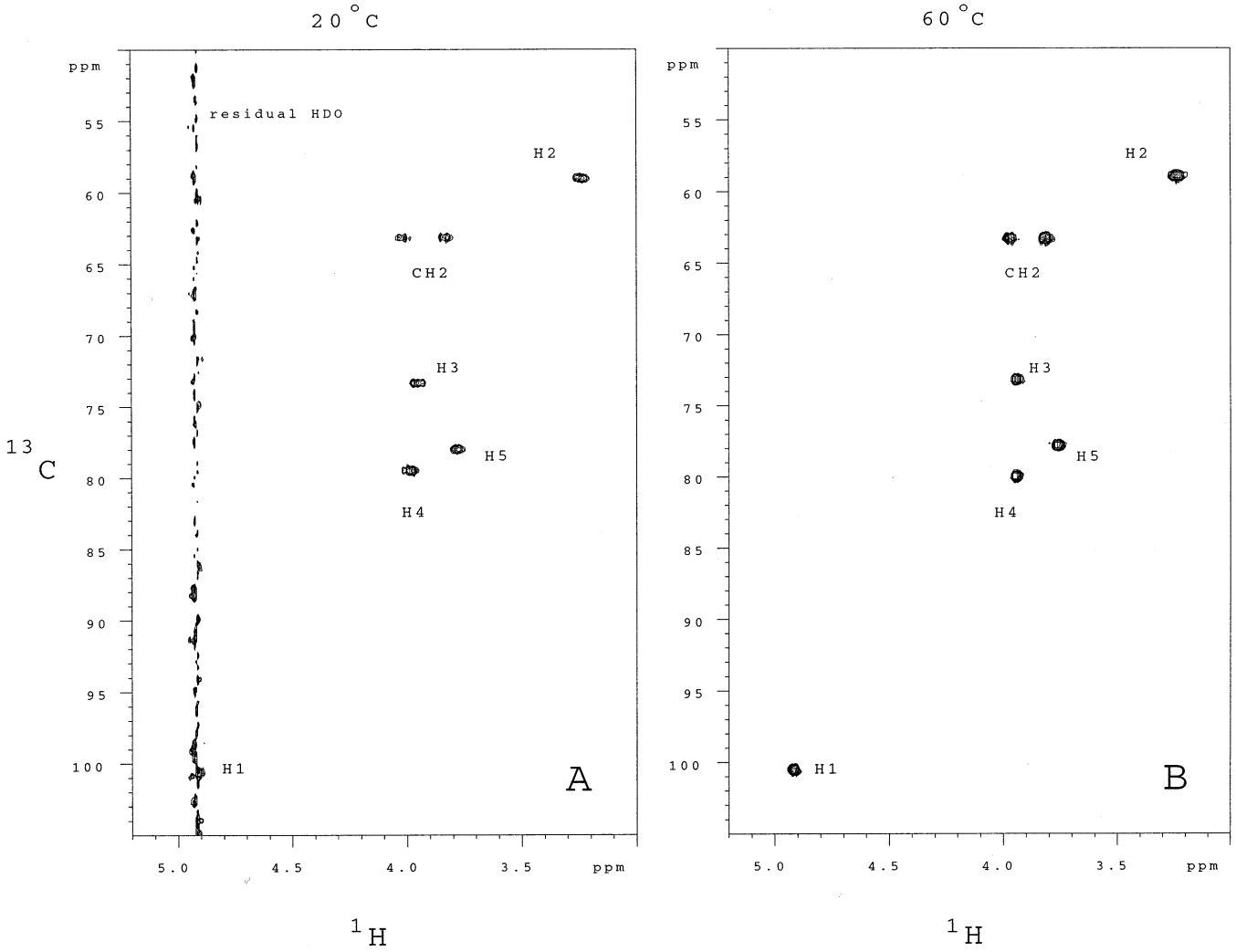

Figure 5. 2D $^{1}H-^{13}C$ HMQC spectra of the deacetylated dark phase prepared from natural abalone nacre recorded at 20 °C (A) and 60 °C (B). Labels indicate the assignment. At 20 °C suppression of the residual signal from water protons was much poorer, resulting in a noise ridge at a 4.9 ppm proton chemical shift.

major amino acids of collagen should be detected in the supernatant of the subtilisin extract. A simple method for detecting amino acids in solution is thin-layer chromatography.[28,29] The supernatant was tested against two amino acid standards which are present in natural collagen to a large extent, proline and glycine. The thin-layer chromatogram is shown in Figure 2. The subtilisin supernatant samples show a retention behavior similar to that of proline. It seems to be characteristic for proline to smear a little bit, so the amino acids are the same in both the sample and the proline standard solution. Surprisingly, proline was the only amino acid detected in the solution, although a mixture of at least three dominant amino acids was expected. It seems, however, that proline is the very dominant amino acid, and all the others are not detectable above the background noise. Polyproline[34] is a remarkable homopolypeptide with enormous conformational restriction due to its cyclic pyrrolidine side chain and the inability of hydrogen bond formation. Nevertheless, it has been shown to build up a left-handed α-helix with all-trans peptide bonds, a very stiff secondary structure. This structure is similar to that of polyglycine, where conformational restrictions are minimal. The structural motif of collagen is also present in polyproline, but for the collagen triple helix with its characteristic tensile strength, glycine is necessary to connect the single chains by hydrogen bonding and to promote dense packing. Whether the protein in the interlamellar sheets of nacre consists of a collagen-like or a polyproline-like structure still remains unclear, but a stabilizing function of the organic matrix due to insoluble proteins can be assumed in any case.

Identification of Chitin in the Interlamellar Sheets. The major interest of this work lays in the characterization of the core of the interlamellar sheets. The core sheets are considered structure-forming and -stabilizing polysaccharides, presumably chitin. Pieces of the organic matrix which were left after successful protease treatment with subtilisin were converted into an analyzable form, namely, chitosan (in the case of chitin present in the original structure). The problem with chitin is that it is soluble only in some concentrated inorganic acids such as concentrated hydrochloric acid, concentrated sulfuric acid, 78−97% phosphoric acid, and anhydrous formic acid.[35] A method had to be found to obtain chitin in an analyzable form, such as chitosan, which represents the partly or totally deacetylated form

(34) Voet, D.; Voet, J. G. *Biochemistry*, 2nd ed.; John Wiley & Sons: New York, 1995; p 141.

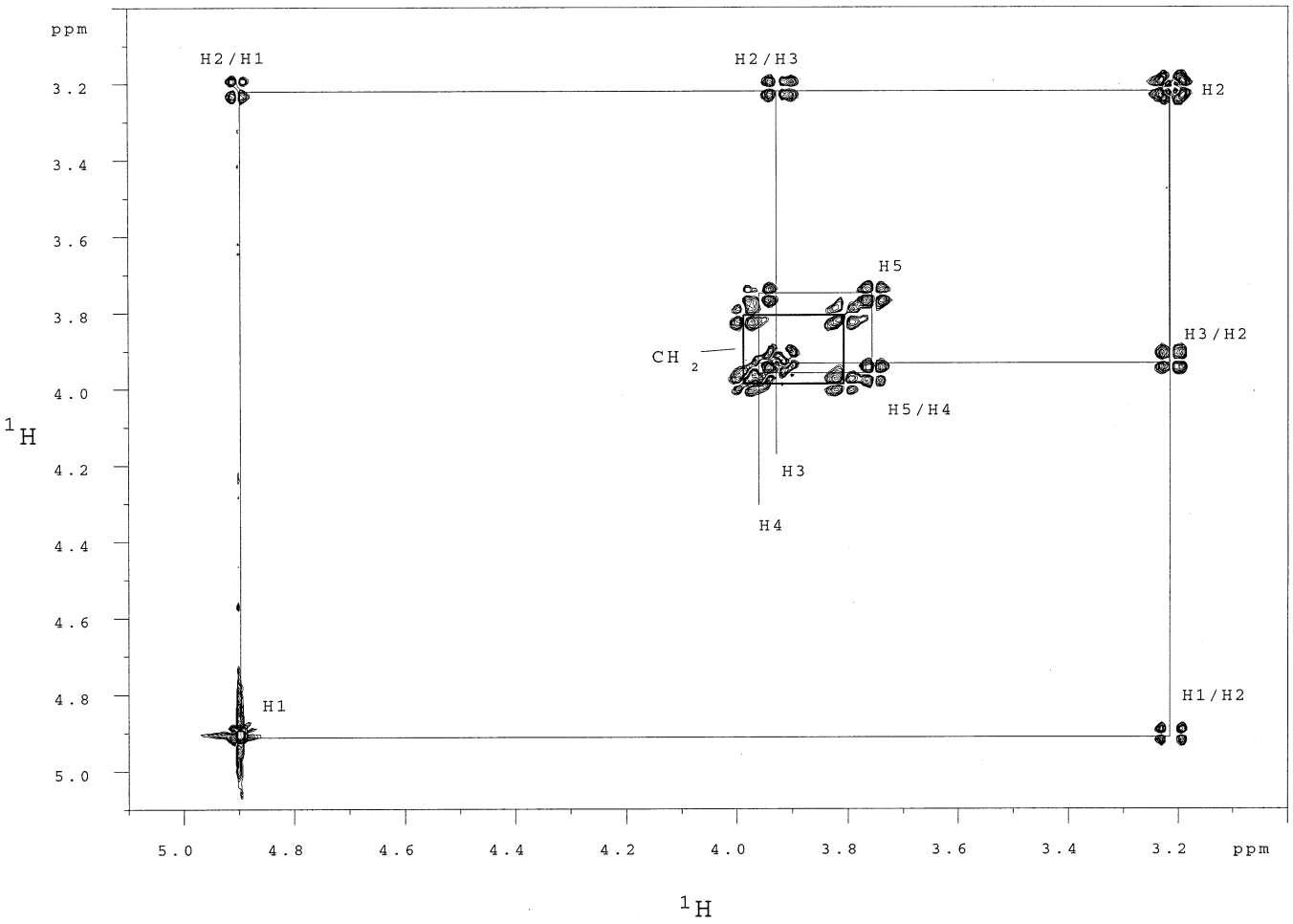

Figure 6. Section of the 2D $^1H-^1H$ DQF-COSY spectrum of the deacetylated dark phase prepared from natural abalone nacre recorded at 20 °C. Cross-peak assignments are shown.

of chitin. Depending on the degree of deacetylation, chitosan is soluble in aqueous solutions or forms gel-like solutions.[36] Therefore, a method was used to convert chitin and also the natural product extracted from the interlamellar matrix into chitosan by a deacetylation reaction described as Campbell's method.[22] The solubility pattern gives a hint of the presence of chitin since the natural samples were solubilized neither in aqueous solutions nor in organic solvents. But with Campbell's method it was possible to do some further test reactions to verify the contents of chitin and of chitosan. Only four test reactions out of seven were appropriate for the detection of chitosan, and according to tests with similar substances, these test reactions were only useful in combination with each other.

As shown in Table 1, only the chitosan samples and the deacetylated interlamellar sheets give positive results in each of the test reactions. The chitin samples do not react in any of the four tests positive as do the raw extract dark and light phases; however, polyamide and poly(vinylpyrrolidone) react in a similar manner. All other tested substances react in one or two test reactions positive, so one can group the substances. Mostly the anthrone reaction[25−27] gives additional positive results in the case of cellulose, glucose, methylglucose, methylmannose, and raffinose. Agarose also reacts partly with periodic acid/Schiff's reagent[22−24] and is, therefore, similar to dextran. Agar is also anthrone-positive, but is the only substance which is also positive in the iodine reaction.[22] Poly(vinyl alcohol) forms a colored complex with iodine, but is anthrone-negative. If the anthrone reaction is rather unspecific for the detection of chitosan, but might be helpful in a decision between chitosan and chitin, one can assume that the only substances which are similar to chitosan in this test are casein, poly(vinyl alcohol), and agar, because the periodic acid/Schiff reaction is known to be specific just for the aldehyde group, so dextran and Amberlite should not be taken into account. One of the problems of these solubility and color assays is that little is known about the reaction mechanisms on which the tests are based. A positive reaction pattern as observed in Table 1 is a strong indication in the identification of chitosan, but the result should be verified by further analytical methods. NMR spectroscopy and mass spectrometry are well suitable for exact identification of biomolecules; therefore, chitin or chitosan samples were analyzed by these methods as well.

^{1}H NMR Spectroscopy of Chitosan and Degree of Acetylation. The results obtained from NMR spectroscopy are in good agreement with chemical and biochemical analysis. From 1D ^1H NMR spectra (Figure

(35) Windholz, M.; Budavari, S.; Blumetti, R. F.; Otterbein, E. S. *The Merck Index—An Encyclopedia of Chemicals, Drugs, and Biologicals*, 10th ed.; Merck & Co., Inc.: Rahway, NJ, 1983; p 286.

(36) Vachoud, L.; Zydowicz, N.; Domard, A. *Carbohydr. Res.* **1997**, *302*, 169.

3) the purity of the test samples (>95%) was found to be as good as the purity of commercially available deacetylated chitin that was used as a reference. The methods used and developed in this study for the preparation of parts of the organic matrix from natural abalone shells prove to be very specific and to achieve excellent purity for the polysaccharide component chitin. The only detectable difference between the abalone organic matrix and commercially available chitin/chitosan was the degree of acetylation, which was around 5% for the test samples and between 6% and 11% for the reference samples. This can be traced back to either differences in the acetylation degrees of the crude materials from crab and abalone or slightly varying deacetylation conditions. The degree of acetylation of the NMR samples was determined by integration of the well-resolved CH_3 signal from the acetyl group and comparison with the integral of resolved ring proton signals in the 1D ^{1}H NMR spectrum (see the Materials and Methods). Assignment of all ^{1}H and ^{13}C resonances confirmed the identification of the test substance as chitosan, partially acetylated. For assigning the NMR peaks (Figures 5 and 6) a structural formula of chitosan is shown in Figure 4. Overlap present in the 1D ^{1}H spectrum for H3, H4, H5, and the protons of the CH_2 group could be resolved with the help of 2D spectra. Identification of the CH_2 group was simple in the 2D ^{1}H−^{13}C HMQC (heteronuclear multiple quantum coherence) spectrum, shown in Figure 5, as both protons share the same carbon chemical shift. No overlap occurred in the 2D ^{1}H−^{13}C HMQC spectrum, but at 60 °C two protons (H3 and H4) share the same chemical shift (Figure 5B). Lowering the temperature to 20 °C led to slightly different chemical shift values for H3 and H4 (Figure 5A). Although they still overlap strongly in the 1D ^{1}H spectrum, they could be separated and assigned with the help of 2D ^{1}H−^{1}H spectra. Figure 6 shows a section of the 2D DQF-COSY (double quantum filtered correlation spectroscopy) spectrum at 20 °C displaying cross-peaks between vicinal (and geminal) protons. ESI mass spectroscopy did not yield good spectra, probably due to fragmentation. Only weak signals corresponding to short chains of less than five monomers were found. On the contrary, the NMR line width as well as the positive sign of NOE cross-peaks (with respect to a positive diagonal) even at 90 °C points to the existence of mainly larger molecules of about 10 monomers in solution. However, the degree of polymerization is strongly influenced by the reaction conditions at deacetylation, as hot alkali might cause breakage not only at the ester bond of the *N*-acetyl residue but also at the glycosidic bonds between single *N*-acetyl-D-glucosamines and shorten the originally high molecular weight polymers. In terms of determining the chemical composition of abalone extracellular organic matrix polysaccharide by NMR, short oligosaccharides should be considered even more advantageous than long polymer chains. But one has to keep in mind that in the original nacreous structure chitin will be present as a long-chained polymeric form.

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