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Bioconversion of chitin to chitosan: Purification and characterization of chitin deacetylase from *Mucor rouxii*

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ABSTRACT **Chitin deacetylase, the enzyme that catalyzes the hydrolysis of acetamido groups of *N*-acetylglucosamine in chitin, has been purified to homogeneity from mycelial extracts of the fungus *Mucor rouxii* and further characterized. The enzyme exhibits a low pI (≈3). Its apparent molecular mass was determined to be ≈75 kDa by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and ≈80 kDa by size-exclusion chromatography, suggesting that the enzyme exists as a monomer. Carbohydrate analysis of purified chitin deacetylase revealed that the enzyme is a high-mannose glycoprotein and that its carbohydrate content is ≈30% by weight. Chitin deacetylase is active on several chitinous substrates and chitin derivatives. The enzyme requires at least four *N*-acetylglucosamine residues (chitotetraose) for catalysis, and it is inhibited by carboxylic acids, particularly acetic acid. When glycol chitin (a water-soluble chitin derivative) was used as substrate, the optimum temperature for enzyme activity was determined to be ≈50°C and the optimum pH was ≈4.5.**

Chitin, a homopolymer of β(1 → 4)-linked *N*-acetylglucosamine, is one of the most abundant, easily obtained, and renewable natural polymers, second only to cellulose. It is commonly found in the exoskeletons or cuticles of many invertebrates (1) and in the cell walls of most fungi and some algae (2). Chitin exists in several *Zygomycetes* species in its deacetylated form referred to as chitosan (3, 4).

Chitin, is an extremely insoluble material and has yet to find an important industrial use, whereas chitosan is water soluble and a much more tractable material with a large number and a broad variety of reported applications (5–7). At present, chitosan is produced by the thermochemical deacetylation of chitin. An alternative or complementary procedure exploiting the enzymatic deacetylation of chitin could potentially be used, especially when a controlled, nondegradative, and well-defined process is required.

Chitin deacetylase, the enzyme that catalyzes the conversion of chitin to chitosan by the deacetylation of *N*-acetylglucosamine residues, was first identified and partially purified from extracts of the fungus *Mucor rouxii* (8). Since then, the presence of this enzyme activity has been reported in several other fungi (9, 10) and in some insect species (11).

Studies performed to understand the way chitosan biosynthesis proceeds in fungi suggest that chitin synthase is operating in tandem with chitin deacetylase, the first enzyme synthesizing chitin by the polymerization of *N*-acetylglucosaminyl residues from uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc), and the second enzyme hydrolyzing the *N*-acetamido groups in the chitin chains, acting more efficiently on nascent than on microfibrillar chitin (12, 13).

Initiating a study to elucidate further the molecular and cellular aspects of chitosan biosynthesis in fungi and evaluate the potential use of an enzymatic process for the deacetylation of chitin substrates, we have purified chitin deacetylase to homogeneity from its best-characterized source, the dimorphic fungus *Mucor rouxii*, and further characterized it.

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MATERIALS AND METHODS

Materials. *Mucor rouxii* (ATCC no. 24905) was obtained from the American Type Culture Collection. Glycol chitosan, concanavalin A (type IV), and glass beads (425–600 μm) were purchased from Sigma. [^{3}H]Acetic anhydride was obtained from DuPont/New England Nuclear. Enzymes and reagents for acetic acid determination were purchased from Boehringer Mannheim. All chromatography media (phenyl-Sepharose CL-4B, Q Sepharose fast flow, S Sepharose fast flow, Sephacryl S-200 HR) and molecular weight markers were obtained from Pharmacia, whereas ultrafiltration membranes were from Amicon. N-Acetyl chitooligoses were purchased from BioCarb (Lund, Sweden). All other chemicals were of the highest purity commercially available.

Growth of *Mucor rouxii*. *Mucor rouxii* was grown as described by Bartnicki-Garcia and Nickerson (14) in a minimal medium containing glutamic acid as the nitrogen source in 16-liter batches. Medium was inoculated with 2×10^8 spores per liter and was stirred and aerated with sterile air for 24 hr at 28°C. Mycelia were harvested at mid-growth phase by filtration through a sintered glass funnel; ≈20 g of mycelia (wet weight) per liter was obtained.

Enzyme Purification. Frozen mycelia (≈400 g) and 600 g of glass beads were added to 700 ml of 50 mM Tris·HCl, pH 7.5/100 mM NaCl/0.2 mM phenylmethylsulfonyl fluoride and blended for 1 hr over ice. After the extraction was completed, glass beads were settled and removed, and the extract was centrifuged for 30 min at 8000 × *g* at 4°C. The supernatant was incubated in a waterbath at 50°C for 30 min, and the precipitated material was removed by centrifugation at 8000 × *g* for 30 min at 4°C.

Ammonium sulfate was added to the supernatant to a final concentration of 2.1 M, and precipitated proteins were removed by centrifugation at 10,000 × *g* for 45 min. The supernatant was then applied onto a phenyl-Sepharose CL-4B column (44 × 230 mm) equilibrated with 20 mM Tris·HCl, pH 7.5/2.1 M ammonium sulfate (buffer A). The column was washed with buffer A, and the retained proteins were subsequently eluted with a linear gradient of ammonium sulfate concentration (2100 ml; 2.1–0 M) in buffer A, at a flow rate of 250 ml/hr. Fractions (14 ml) containing chitin deacetylase activity were pooled and concentrated by ultrafiltration.

The sample from the latter preparation was dialyzed against 20 mM Tris·HCl, pH 8 (buffer B), and applied onto a Q Sepharose fast flow column (26 × 340 mm) previously equilibrated in buffer B. The column was washed with buffer B and subsequently developed with a linear gradient of NaCl (2000 ml; 0–0.75 M) in buffer B at a flow rate of 300 ml/hr. Fractions (11.5 ml) containing enzyme activity were pooled.

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Table 1. Purification of chitin deacetylase

Step	Total protein, mg	Total enzyme activity, units*	Specific activity, units/mg	Yield, %	Fold purification
Crude extract	10,380	317	0.0305	100.0	1
50°C treatment	4,719	221	0.048	69.7	1.6
Phenyl-Sepharose	1,374	138	0.100	43.5	3.3
Q Sepharose	279	73.6	0.263	23.2	8.6
S Sepharose	12.6	37.4	2.968	11.9	97.3

*One unit of enzyme activity is defined as the amount of the enzyme required to produce 1 μmol of acetic acid per min when incubated with 16.6 nmol of hexa(*N*-acetyl)chitohexaose under optimum pH (4.5) and temperature (50°C) conditions.

Pooled fractions were dialyzed against 25 mM sodium formate buffer, (pH 3.5; buffer C), and the sample was loaded onto an S Sepharose fast flow column (26 × 280 mm) equilibrated in buffer C. The column was washed with buffer C and subsequently developed with a linear gradient of NaCl (2000 ml; 0–1.2 M) in buffer C at a flow rate of 250 ml/hr. The majority of chitin deacetylase activity was detected in the flow-through fractions, while a small part of the activity was retained by the column and eluted at ≈0.8 M NaCl. Fractions (12 ml) containing the major enzyme activity were pooled and stored at −20°C.

Enzyme Activity Assays. (*i*) Chitin deacetylase activity was estimated by using as substrate partially *O*-hydroxyethylated chitin (glycol chitin) radiolabeled in *N*-acetyl groups. The substrate was prepared according to Araki and Ito (8). Enzyme assays were performed as described earlier (8) with the following modifications: 25 mM sodium glutamate (pH 4.5) was used as buffer, and bovine serum albumin (0.1 mg/ml) was added to the assay mixture. Incubation time was 30 min at 50°C. (*ii*) Acetic acid released by the action of chitin deacetylase on various chitinous substrates was determined by the enzymatic method of Bergmeyer (15) via three coupled enzyme reactions. Units of enzyme activity were estimated by using 166 nmol of hexa(*N*-acetyl)chitohexaose in a total volume of 500 μl of 25 mM sodium glutamate buffer (pH 4.5). Incubation time was 10 min at 50°C, and the reaction was terminated by heating at 100°C prior to acetic acid determination.

Polyacrylamide Gel Electrophoresis. Slab-gel electrophoresis using gradient or homogeneous polyacrylamide gels and discontinuous buffer systems under denaturing and reducing conditions were performed as described by Laemmli (16). Nondenaturing polyacrylamide gels were prepared by the method of Davis (17). Protein bands were visualized by staining with Coomassie brilliant blue R. The pI of the enzyme was determined by isoelectric focusing and electrophoretic titration curve analysis on 3-9 Pharmalyte-generated pH gradients (18).

Protein Determination. Protein content was determined by the method of Peterson (19) with bovine serum albumin as standard.

Coupling of Concanavalin A to CNBr-Activated Sepharose. Concanavalin A was coupled to CNBr-activated Sepharose CL-4B in the presence of 200 mM α-methyl mannoside, according to manufacturer's instructions. The concentration of immobilized concanavalin A was ≈5 mg/ml of gel.

Amino Acid Analysis. Amino acid analysis was performed after protein hydrolysis with 6 M HCl at 110°C for 24 hr by the method of Spackman *et al.* (20). Half cystine was estimated by the method of Hirs (21), and tryptophan was determined by the method of Beaven and Mildner (22).

Carbohydrate Analysis. Carbohydrates were analyzed after hydrolysis in 4 M trifluoroacetic acid at 100°C for 4 hr as described by Sawardeker *et al.* (23). Sialic acid was determined by the method of Jourdian *et al.* (24).

Determination of Acetamido Groups. *N*-Acetyl group content of water-soluble chitinous substrates and determination of *N*-acetyl chitooligoses was performed by the spectrophotometric method of Muzzarelli and Rocchetti (25).

RESULTS

Purification and Molecular Weight Determination of Chitin Deacetylase. The purification of chitin deacetylase from mycelial extracts of *Mucor rouxii* is summarized in Table 1. Enzyme purification was achieved in three chromatographic steps with an overall yield of 11.9% and a purification factor of 97.3. Chromatography profiles are presented in Figs. 1–3. The enzyme purified with this procedure was judged to be electrophoretically homogeneous, as tested by both native PAGE and SDS/PAGE. In gradient (5–20%) SDS/PAGE, the enzyme band migrated at a position corresponding to a molecular mass of ≈75 kDa (Fig. 4). Enzyme activity assays using the gel slices obtained from gradient (5–20%) native PAGE exhibited chitin deacetylase activity coinciding with the visualized protein band. Isoelectric focusing analysis on Pharmalyte-generated pH gradients indicated that chitin deacetylase migrated as a single band at a position corresponding to a pI of ≈3. Pure chitin deacetylase subjected to gel filtration on Sephacryl S-200 HR was eluted as a single peak with an apparent molecular mass of ≈80 kDa (chromatogram not shown), indicating that the native enzyme exists as a monomer.

Carbohydrate Content. Several pieces of evidence indicate that chitin deacetylase is a glycoprotein. After electrophoresis, the enzyme band was stained with periodate/Schiff reagent on polyacrylamide gels. The enzyme was retained on a concanavalin A-Sepharose 4B column and eluted with a linear gradient of α-methyl mannoside (0–0.3 M) at a position

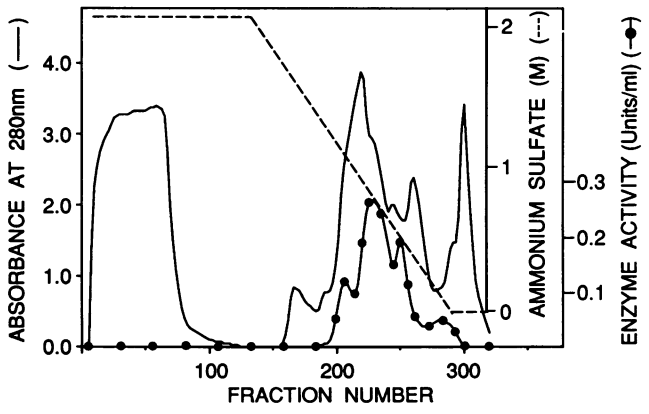

FIG. 1. Purification of chitin deacetylase on phenyl-Sepharose CL-4B column. A sample (850 ml; 3020 mg of protein) of partially purified chitin deacetylase from ammonium sulfate fractionation was applied onto a phenyl-Sepharose CL-4B adsorbent (350 ml; 44 × 230 mm). The column was washed with irrigating buffer (2.1 M ammonium sulfate/20 mM Tris·HCl, pH 7.5) and then developed with a linear gradient of ammonium sulfate (2100 ml; 2.1–0 M) at a flow rate of 250 ml/hr. Fractions of 14 ml were collected. Chitin deacetylase activity appeared in fractions 195–295, which were pooled for further purification. The protein content was followed by a UV monitor at 280 nm.

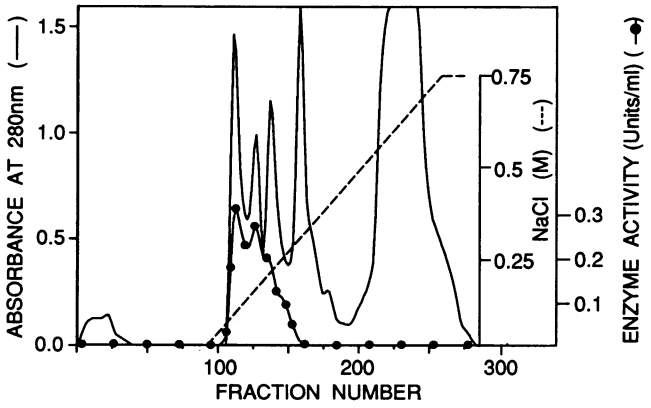

FIG. 2. Purification of chitin deacetylase on Q Sepharose fast flow column. A sample (230 ml; 1374 mg of protein) of partially purified chitin deacetylase from a phenyl-Sepharose column was applied onto a Q Sepharose fast flow adsorbent (180 ml; 26 × 340 mm). The column was washed with irrigating buffer (20 mM Tris·HCl, pH 8.0) and then developed with a linear gradient of NaCl (2000 ml; 0–0.75 M) at a flow rate of 300 ml/hr. Fractions of 11.5 ml were collected. Chitin deacetylase activity appeared in fractions 105–150, corresponding to ≈0.13 M NaCl. The protein content was followed by a UV monitor at 280 nm.

corresponding to ≈25 mM. Direct carbohydrate and amino acid analysis of the enzyme revealed that the carbohydrate content is ≈30% by weight of the protein. The carbohydrate part of the protein consists of mannose, *N*-acetylglucosamine, and fucose (Table 2). Sialic acid and other sugars were not detected in significant amounts.

Enzyme Activity Characterization. The pH optimum of the enzyme was estimated to be ≈4.5 and the temperature optimum was ≈50°C with glycol chitin as substrate. Chitin deacetylase was active on carboxymethyl chitin (a water soluble derivative), microcrystalline chitin (colloidal chitin), chitosan, and chitooligoses with more than three *N*-acetylglucosamine residues (Table 3). The enzyme did not require any metal ions for activity, and it was not inhibited by 1–10 mM EDTA or EGTA. Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, and Cu^{2+}, tested as chlorides, were inhibitory at concentrations exceeding 10 mM. Chitin deacetylase was inhibited by carboxylic acids, particularly acetic acid (Fig. 5). When 83%

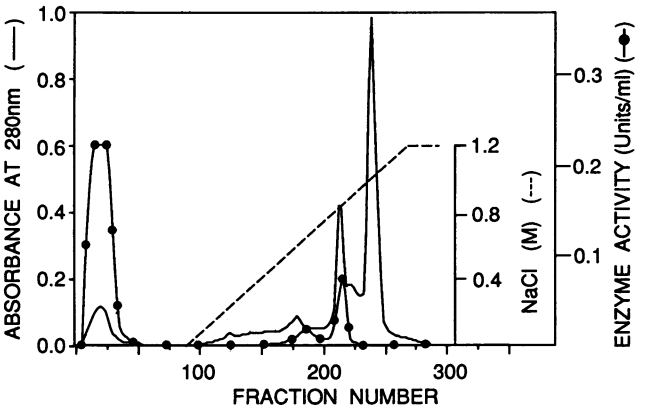

FIG. 3. Purification of chitin deacetylase on S Sepharose fast flow column. A sample (200 ml; 270 mg of protein) of partially purified chitin deacetylase from a Q Sepharose column was applied onto an S Sepharose fast flow adsorbent (150 ml; 26 × 280 mm). The column was washed with 25 mM sodium formate (pH 3.5) irrigating buffer and then developed with a linear gradient of NaCl (2000 ml; 0–1.2 M) at a flow rate of 250 ml/hr. Fractions of 12 ml were collected. The majority of chitin deacetylase activity appeared in the nonretained protein fractions. The protein content was followed by a UV monitor at 280 nm.

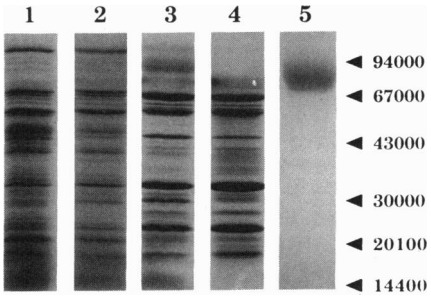

FIG. 4. Qualitative presentation of chitin deacetylase purification scheme. Samples representing the five stages of purification were electrophoresed on a 5–20% polyacrylamide gradient gel under denaturing and reducing conditions. Protein bands were visualized by staining with Coomassie brilliant blue R. Identities and amount of protein loaded were: ≈100 μg of crude extract (lane 1), ≈80 μg from 50°C treatment (lane 2), ≈80 μg of the phenyl-Sepharose eluate (lane 3), ≈80 μg of the Q Sepharose eluate (lane 4), and ≈30 μg of the S Sepharose flow-through (lane 5).

deacetylated chitosan was used as substrate, it was converted by the action of the enzyme to a highly deacetylated product (97.5% deacetylated), as estimated by the spectrophotometric determination of acetamido groups (25).

DISCUSSION

In this report, we describe the purification of the enzyme chitin deacetylase from the fungus *Mucor rouxii* to apparent electrophoretic homogeneity.

The enzyme is a glycoprotein and exists in its native form as a monomer of 75–80 kDa. Carbohydrate analysis revealed that chitin deacetylase is a high-mannose glycoprotein (Table 2). The carbohydrate content of chitin deacetylase was estimated from the molar ratio of carbohydrates to amino acids to be ≈30% by weight.

Although several other purification schemes have been also examined in our laboratory resulting in a final enzyme preparation of comparable purity, the one we describe here has been chosen for being simple (it can be completed within 4 days with standard protein purification media and equipment), economic (expensive adsorbents have been omitted), and easy to scale up.

As it is evident from the quantitative summary of purification (Table 1) and from the SDS/PAGE analysis of the purification steps (Fig. 4), the step that substantially increased enzyme purity was cation-exchange chromatography on S Sepharose, which exploited the exceptionally low pI of the enzyme. In this step, isoforms of the enzyme that appear in previous steps (Figs. 1 and 2) are retained by the column and eluted effectively by a salt gradient. One of these isoforms (10–15% of the enzyme activity) exhibits an apparent molecular mass of ≈38 kDa; it is also a glycoprotein and proved to be immunochemically similar to the ≈80-kDa enzyme activity when tested by Western blot. In preliminary

Table 2. Carbohydrate composition of chitin deacetylase

Monosaccharide	Monosaccharide per sample		
	μg	nmol	nmol %
Fucose	1.4	8.3	5.4
Mannose	21.1	117.0	75.5
N-Acetylglucosamine	6.5	29.6	19.1
Sialic acid	0.0	0.0	0.0
Total	29.0	154.9	100.0

A sample of purified enzyme (≈100 μg) was hydrolyzed in 4 M trifluoroacetic acid at 100°C for 4 hr. Monosaccharide analysis was carried out by gas–liquid chromatography and gas–liquid chromatography/mass spectrometry.

Table 3. Deacetylation of *N*-acetyl chitooligoses

Substrate	Substrate, mM	Acetic acid released, nmol	Relative activity, %
GlcNAc	5.00	0	0
$(GlcNAc)_2$	2.50	0	0
$(GlcNAc)_3$	1.67	0	0
$(GlcNAc)_4$	1.25	8.41	3.9
$(GlcNAc)_5$	1.00	87.43	40.3
$(GlcNAc)_6$	0.83	216.82	100.0

The concentration of the substrates was adjusted with respect to their content in *N*-acetyl residues. Pure enzyme preparation (≈0.43 milliunit) was used in each of the assays, which were performed in a total volume of 500 μl of 25 mM sodium glutamate buffer at pH 4.5. Incubation time was 10 min at 50°C and the reaction was terminated by heating at 100°C prior to acetic acid determination. The acetic acid released by the action of chitin deacetylase was determined via three coupled enzyme reactions (15).

studies, SDS/PAGE analysis of enzyme preparations obtained from a single-step immunoaffinity-chromatography procedure exhibited a single band corresponding to ≈80 kDa, indicating that the ≈38 kDa enzyme form is most likely a degradation product of chitin deacetylase.

We have used two different assays for the determination of chitin deacetylase activity. A radiometric assay using radiolabeled glycol chitin as substrate proved to be a rapid and sensitive way of screening chromatographic fractions, whereas the estimation of released acetic acid by an enzymatic method (15) was used for monitoring the deacetylation process of nonradiolabeled substrates.

Glycol chitin has been used as a model substrate for the determination of chitin deacetylase activity (8). Since it is not easy to evaluate (*i*) the extent and distribution of derivatization (*O*-hydroxyethyl groups) in glycol chitin available commercially and (*ii*) the effect of derivatization on the enzyme activity, we report the use of hexa(*N*-acetyl)chitohexaose as a model substrate for the determination of the enzyme activity.

Chitin deacetylase appears to exhibit a very stringent specificity acting only on *N*-acetylglucosamine homopolymers (8). The enzyme requires at least four *N*-acetylglucosamine residues for catalysis, and the rate of the deacetylation is higher the longer the oligosaccharide is (Table 4). The requirements for substrate recognition and the mechanism of enzyme action need to be further studied. Preliminary

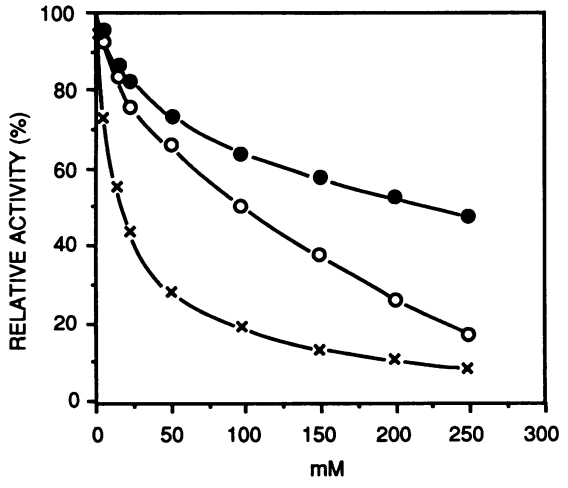

FIG. 5. Effect of carboxylic acids on chitin deacetylase activity. Chitin deacetylase activity was estimated at various carboxylic acid concentrations by using radiolabeled glycol chitin as substrate. Formic (●), acetic (×), and propionic (○) acids were used—all adjusted to pH 4.5 with NaOH.

experiments demonstrate that the enzyme can be effective in converting chitosans to more highly deacetylated products.

Chitin deacetylase activities from *Colletotrichum* species have been reported to exhibit different pH optimum and inhibition profiles (9).

Even though enzymology and cytology of chitin biosynthesis in fungi has been extensively studied (26, 27), very little information exists on chitin deacetylase and chitosan biosynthesis. It has been suggested that chitin deacetylase is a secreted enzyme and that its function is localized in the periplasmic space (13). It is expected that direct evidence for the spatial organization of chitosan biosynthesis will be obtained by the immunolocalization of chitin deacetylase in the fungal cell. It is also expected that further information for the number of the existing genes, the glycosylation, and the possible existence of zymogens will be obtained after cloning the chitin deacetylase gene.

Presently chitosan is produced from chitin of crustacean shells by thermochemical deacetylation. This process is not only subject to seasonal variation of starting material but is leading to a product having a broad range of molecular weights and a heterogeneous extent of deacetylation as well. However, for many high-value biomedical applications, uniform material with very specific physical and chemical properties is required. The development of a controllable process using either the biosynthesis of chitosan or the enzymatic deacetylation of chitinous substrates presents an attractive alternative.

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