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Chitin deacetylation by enzymatic means: monitoring of deacetylation processes

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Abstract

A method for monitoring enzymatic deacetylation processes of natural or artificial chitin substrates as well as *N*-acetylchitooligosaccharides by the direct determination of the acetate released is described. Furthermore, a new assay is presented for the determination of chitin deacetylase activity employing hexa-*N*-acetylchitohexaose [$(GlcNAc)_6$] as substrate and measuring the acetate released enzymatically. The K_m value for $(GlcNAc)_6$ has been determined as well as the pH and temperature dependence of activity and the thermostability of the enzyme. Finally, initial studies on the effectiveness of the enzyme on various chitin and chitosan substrates are presented.

Keywords: Chitin; Chitosan; Chitin deacetylase; *Mucor rouxii*; Chitin deacetylation

1. Introduction

Chitin, a homopolymer of β-(1 → 4)-linked *N*-acetyl-D-glucosamine, 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 as chitosan [3,4].

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Chitosan is a biopolymer with unique properties favourable for a broad variety of industrial and biomedical applications [5–7]. Presently, chitosan is produced by the thermochemical alkaline deacetylation of crab chitin. To develop an alternative, controlled, nondegradative and well-defined enzymatic process for chitosan production, a study of fungal chitin deacetylases has been initiated. Furthermore, the degree of deacetylation of chitosan has been found to influence the physical and chemical properties as well as the biological activities of chitosan [8]. Thus, monitoring of deacetylation processes is of particular importance.

Chitin deacetylase (CDA) from *Mucor rouxii* has been purified to homogeneity [9,10]. The enzyme is an acidic glycoprotein of ~ 75 kDa with 30% (w/w) carbohydrate content. Further biochemical characterization revealed that the enzyme has a very narrow specificity for chitinous substrates. Furthermore, a cDNA to the *Mucor rouxii* mRNA-encoding chitin deacetylase was isolated, characterized, and sequenced [11]. Protein sequence comparisons revealed significant similarities of the fungal chitin deacetylase to rhizobial nodB proteins, suggesting functional homology of these evolutionary-distant proteins.

A radioactive assay employing *O*-hydroxyethylchitin (glycol chitin), radiolabelled in the *N*-acetyl group, as substrate has been reported for the determination of chitin deacetylase activity [12]. Although this method is very sensitive, it is not easy to evaluate (*i*) the extent and distribution of derivatization (*O*-hydroxyethyl groups) in glycol chitin commercially available and (*ii*) the effect of derivatization on enzyme activity. Furthermore, this method cannot be used for monitoring deacetylation processes of nonradiolabelled natural substrates.

In this study we report a new method for monitoring enzymatic deacetylation processes, using either natural or artificial chitin substrates, and determining chitin deacetylase activity.

2. Experimental

Materials.—Enzymes and reagents for acetate determination were obtained from Boehringer Mannheim Biochemica. *N*-Acetylchitooligosaccharides were purchased from Accurate Chemicals (USA). Glycol chitosan was purchased from Sigma. Acetylated chitosans (28% and 42%) from *Euphasia superba* were a gift from Dr B. Focher (Stazione Sperimentale Cellulosa, Carta e Fibre Tessili, Milan, Italy). Amorphous and crystalline chitin from shrimp and crab were a gift from Dr K.M. Vårum (Norwegian Biopolymer Laboratory, Institute of Biotechnology, Trondheim, Norway). Hyaluronan was purchased from Sigma. *Mucor rouxii* (ATCC No 24905) was obtained from the American Type Culture Collection. Chitin deacetylase from *Mucor rouxii* was grown in-house and purified to homogeneity [1] [10]. All other chemicals were of the highest purity commercially available.

[1] The chitin deacetylase technology is protected by patents issued to the Institute of Molecular Biology and Biotechnology.

Enzyme activity assays.—Acetate released by the action of chitin deacetylase on various chitinous substrates was determined by the enzymatic method of Bergmeyer [13] via three coupled enzyme reactions:

$$\left(\text{Chitin} \xrightarrow{\text{CDA}} \text{Chitosan} + \text{Acetate}\right)$$

$$\text{Acetate} + \text{ATP} + \text{CoA} \xrightarrow{\text{ACS}} \text{Acetyl} - \text{CoA} + \text{AMP} + \text{PPi}$$

$$\text{Acetyl} - \text{CoA} + \text{Oxaloacetate} + \text{H}_2\text{O} \xrightarrow{\text{CS}} \text{Citrate} + \text{CoA}$$

$$\text{Malate} + \text{NAD}^+ \overset{\text{MDH}}{\leftrightarrow} \text{Oxaloacetate} + \text{NADH} + \text{H}^+$$

where CDA = chitin deacetylase, ACS = acetyl-CoA synthetase, CS = citrate synthase, MDH = malate dehydrogenase.

Units of chitin deacetylase activity were estimated using 166 nmol of hexa-*N*-acetylchitohexaose in a total volume of 500 μL of 25 mM sodium glutamate buffer (pH 4.5). Incubation time was 15 min at 50°C, and the reaction was terminated by heating at 100°C prior to acetate determination.

We define one unit of chitin deacetylase activity as the amount of the enzyme required to produce 1 μmol of acetate per min when incubated with hexa-*N*-acetylchitohexaose as described above. As a compromise between sensitivity and speed, 50°C and 15-min incubation were chosen as the standard assay conditions for chitin deacetylase.

Protein concentration.—The protein concentration was determined by the method of Lowry et al. [14] using bovine serum albumin (BSA) as standard.

Preparation of artificial chitin derivatives.—Carboxymethylchitin was prepared as previously described [15], while glycol chitin was prepared according to Araki and Ito [12].

Determination of acetamido groups.—The *N*-acetyl group content of water-soluble chitin substrates and *N*-acetylchitooligosaccharides was measured by the spectrophotometric method of Muzzarelli and Rocchetti [16].

3. Results and discussion

Monitoring of deacetylation processes of chitin substrates.—By the direct determination of the acetate released it is possible to monitor deacetylation processes using either natural or artificial chitinous substrates.

Presently, chitosan is produced from chitin of crustacean shells by thermochemical alkaline deacetylation [17]. This process leads 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 specific physical and chemical properties is required. Furthermore, the degree and distribution of deacetylation of chitosan has been found to influence the physical and chemical properties as well

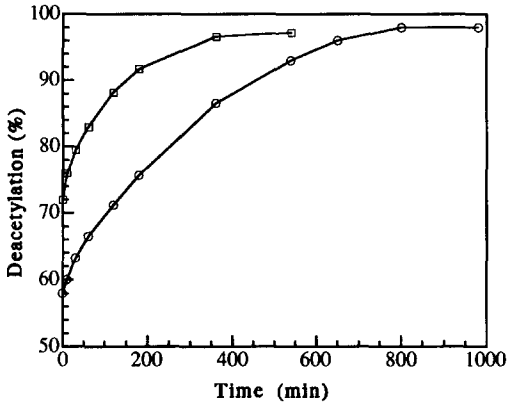

Fig. 1. Time course of deacetylation of partially acetylated chitosans (42%, ○; 28%, □) by chitin deacetylase. The enzymatic reactions were performed using 1 mL of 0.5% chitosan solution in glutamate buffer (pH 4.5) and the mixtures each containing 3 mU of CDA were incubated at 50°C. Aliquots of 0.1 mL were taken out at different time intervals. Samples were measured in duplicate. From the amount of acetate released, which was determined enzymatically, the % degree of deacetylation was estimated.

as biological activities of this polymer, for example, the adsorption ability of metal ions [18], the tensile strength of chitosan films [19], the enzyme binding ability [20], and immunological activity [21]. Thus, monitoring the degree of deacetylation of chitosan substrates is of particular importance. The development of a controllable process using the enzymatic deacetylation of chitinous substrates presents an attractive alternative, while it can potentially result in the preparation of novel chitosan polymers.

In order to test chitin deacetylase effectiveness in deacetylating chitin and chitosan substrates, two crystalline and two amorphous chitin samples, as well as two chitosan samples of different deacetylation degrees, were incubated with the enzyme under the standard assay conditions. The enzyme deacetylated a 28% acetylated chitosan within 6 h and a 42% acetylated chitosan within 13 h almost completely (97 and 98%, respectively), demonstrating the enzyme's effectiveness in deacetylating almost all GlcNAc units of the polymers (Fig. 1). The degrees of deacetylation of the final products determined with this method were in good agreement with the values obtained by the spectrophotometric method of Muzzarelli and Rocchetti [16] (95 and 96%, respectively). The method gives reproducible results, and hence will be useful for an easy and rapid determination of the degree of deacetylation of chitin and chitosan polymers during deacetylation processes. When crystalline chitin and its chemically modified form, amorphous chitin, were incubated with the enzyme for 20 h, approximately 0.5% and 9.5% deacetylation was achieved respectively (Table 1). This indicates that the enzyme is not very effective in deacetylating insoluble chitin substrates and that pretreatment of crystalline chitin substrates prior to enzyme addition is necessary in order to improve the accessibility of the acetyl groups to the enzyme and therefore enhance the yield and rate of the deacetylation reaction. The mechanism of action of the enzyme on chitinous polymer substrates is under investigation.

Table 1
Deacetylation of chitin substrates by CDA[a]

Substrate	Acetate released (nmol)	Deacetylation (%)
Crystalline chitin (shrimp)	126± 2	0.50±0.01
Amorphous chitin (shrimp)	2383±30	9.5 ±0.1
Crystalline chitin (crab)	120± 2	0.48±0.01
Amorphous chitin (crab)	2347±29	9.4 ±0.1

[a] Duplicate enzyme incubations (plus control reactions) were carried out in a final volume of 2 mL including 5 mg of substrates, 25 mM glutamate buffer (pH 4.5), and 123 mU of CDA. Reactions were performed at 50°C for 22 h and stopped by heat inactivation (10 min at 100°C).

Finally, for extensive deacetylation processes the stability of chitin deacetylase to thermal denaturation is an important factor. After incubation of the enzyme at 50°C for 23 h, less than 30% decrease in activity was observed (Fig. 2). Therefore, the enzyme is quite stable at its incubation temperature and could potentially be employed for long incubations with various chitinous substrates.

Substrate specificity and kinetic properties.—Deacetylated *N*-acetylchitooligomers exhibit unique properties (water-soluble, easily derivatisable, biologically active) favourable for many biomedical and industrial applications. In order to test the enzyme's effectiveness in deacetylating *N*-acetylchitooligosaccharide substrates we overincubated several *N*-acetylchitooligomers with the enzyme (Table 2). When hexa-*N*-acetylchitohexaose [$(GlcNAc)_6$] was used as substrate an increased rate of deacetylation was observed in comparison to $(GlcNAc)_4$ and $(GlcNAc)_5$, while the enzyme was inactive on *N*-acetyl-D-glucosamine, $(GlcNAc)_2$, and $(GlcNAc)_3$. The mechanism of action of the enzyme on *N*-acetylchitooligomers is under investigation.

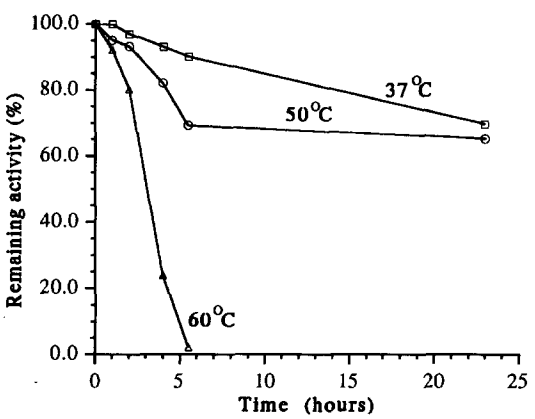

Fig. 2. Thermal stability of CDA. Thermostability of CDA was tested after preincubation of 2 mU of the enzyme for different time periods at 37, 50, and 60°C. After cooling on ice, each sample was assayed under standard conditions to determine the remaining activity.

Table 2
Deacetylation of *N*-Acetylchitooligosaccharides by CDA[a]

Substrate	Acetate released (nmol)	Deacetylation (%)
N-Acetyl-D-glucosamine	0	0
$(GlcNAc)_2$	0	0
$(GlcNAc)_3$	0	0
$(GlcNAc)_4$	152 ± 2	23.0 ± 0.3
$(GlcNAc)_5$	330 ± 4	40.0 ± 0.5
$(GlcNAc)_6$	480 ± 6	48.5 ± 0.6

[a] Duplicate enzyme incubations (plus control reactions) were carried out with the following components in a final volume of 200 μL: 165 nmol of substrates, 25 mM glutamate buffer (pH 4.5), and 3.6 mU of CDA. Reactions were performed at 50°C for 48 h and stopped by heat inactivation (10 min at 100°C).

When purified nodB, an *N*-acetylchitooligosaccharide-specific deacetylase encoded by the *nodB* genes of *Rhizobia*, was incubated with various *N*-acetylchitooligomers it was observed that only the nonreducing residues of $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNAc)_4$ were deacetylated while *N*-acetyl-D-glucosamine was not modified [22]. Chitin deacetylase from *Mucor rouxii* and nodB, two evolutionarily distant proteins, show significant protein sequence similarities [11]. The similarities and differences between these enzymes make chitin deacetylases an attractive system for studying structure and function relationships in substrate recognition and catalysis. Comparative analysis of sequence similarities between these two proteins can provide the basis for developing a protein engineering strategy to modify the specificity of chitin deacetylases.

Since an increased rate of deacetylation was observed with $(GlcNAc)_6$, a more systematic study was performed with this *N*-acetylchitooligomer. Acetate was released linearly up to an incubation time of 30 min. All subsequent incubations were performed within this linear time range. Linearity of the method was observed as a correlation between enzyme activity and chitin deacetylase concentration up to 0.6 μg of CDA per mL. Incubation of chitin deacetylase with various concentrations (5×10^{-5} to 3×10^{-3} M) of substrate (Fig. 3) showed that the K_m value for $(GlcNAc)_6$, using the Lineweaver–Burk method, is 1.3 mM.

Finally, chitin deacetylase from *Mucor rouxii* appears to exhibit a very stringent specificity acting only on chitinous substrates. The enzyme was not active on hyaluronan, a copolymer of alternating β-D-glucuronic acid and β-*N*-acetyl-D-glucosamine residues. Furthermore, initial studies have shown that the effectiveness of the enzyme in deacetylating various chitinous substrates was in the following order: carboxymethylchitin > glycol chitin > amorphous chitin > crystalline chitin (data not shown), indicating different substrate specificity and/or susceptibility of chitin substrates to enzymatic deacetylation.

Three factors could mainly affect the enzymatic deacetylation of insoluble chitin and chitin derivatives: the properties and mechanism of action of CDA, the structural properties of chitin, and the mode of interaction between the enzyme and the chitin molecules. Pretreatment of chitin materials can modify the conformation of the parent

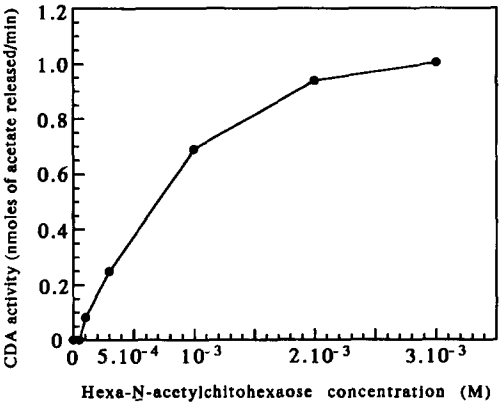

Fig. 3. Effect of substrate (hexa-*N*-acetylchitohexaose) concentration on CDA activity. Incubations were performed in a final volume of 0.5 mL containing 0.3 mU of CDA, $(GlcNAc)_6$ (5×10^{-5} to 3×10^{-3} M), and 25 mM sodium glutamate buffer (pH 4.5) at 50°C for 15 min. Samples were measured in triplicate.

polymer. As a result, the interaction between enzyme and substrate, as well as the subsequent deacetylation reaction, could be affected. The susceptibility of chitin derivatives to enzymatic deacetylation may vary with the type of substituent group as well as the pattern of substitution. The mechanism of enzymatic deacetylation of soluble chitin derivatives as compared to insoluble chitin is simpler because of the homogeneity of the reaction, but the substitution pattern and the degree of substitution of the substrate must be adequately taken into account.

Correlation between the structural features of chitinous substrates and extent of enzymatic deacetylation is required for a better understanding of the reaction mechanism. Identification of the appropriate substrate pretreatment requirements could potentially result in the production of novel chitosan polymers for various applications.

Assay of chitin deacetylase.—Determination of chitin deacetylase activity was performed using the enzymatic method of Bergmeyer [13]. Acetate released after the enzymatic deacetylation of hexa-*N*-acetylchitohexaose was measured via three coupled enzyme reactions according to the above method. Sensitivity of the assay is approximately 0.5 μg of acetate per sample volume.

When $(GlcNAc)_6$ was used as substrate an increased rate of deacetylation was observed in comparison to $(GlcNAc)_4$ and $(GlcNAc)_5$ (Table 2). Furthermore, since $(GlcNAc)_7$ is not commercially available and *N*-acetylchitooligomers of higher chain length are insoluble in aqueous solutions we have employed $(GlcNAc)_6$ as a novel substrate for the determination of chitin deacetylase activity. The temperature where maximum enzyme activity was observed without any significant enzyme denaturation is 50°C. A temperature range between 37 and 60°C was examined. An optimum pH of 4.5 was observed by measuring the rate of hexa-*N*-acetylchitohexaose deacetylation at a pH range between 3.5 and 8.0.

The new method presented is nonradioactive, fast, simple, and reproducible, and can be therefore used routinely for estimating chitin deacetylase activity.

References

[1] C. Jeuniaux, *Bull. Soc. Zool. Fr.*, 107 (1982) 363–386.
[2] J. Ruiz-Herrera, *Proc. Int. Conf. Chitin Chitosan*, 1st MIT Sea Grant Rep. 78–7, (1978) 11–21.
[3] S. Bartnicki-Garcia and W.J. Nickerson, *Biochim. Biophys. Acta*, 58 (1962) 102–119.
[4] K. Shimahara, Y. Takiguchi, T. Kobayashi, K. Uda, and T. Sannan, in G. Skjak-Braek, T. Anthonsen, and P. Sandford (Eds.), *Chitin and Chitosan*, Elsevier Applied Science, Barking, 1989 pp 171–178.
[5] C.K. Rha, D. Rodriguez-Sanchez, and C. Kienzle-Sterzer, in R.R. Colwell, E.R. Pariser, and A.J. Sinskey (Eds.), *Biotechnology of Marine Polysaccharides*, Hemisphere, Washington, DC, 1984, pp 283–311.
[6] D. Knorr, in R.R. Colwell, E.R. Pariser, and A.J. Sinskey (Eds.), *Biotechnology of Marine Polysaccharides*, Hemisphere, Washington, DC, 1984, pp 313–332.
[7] S. Hirano, in G. Skjak-Braek, T. Anthonsen, and P. Sandford (Eds.), *Chitin and Chitosan*, Elsevier Applied Science, Barking, 1989, pp 37–43.
[8] F. Nanjo, R. Katsumi, and K. Sakai, *Anal. Biochem.*, 193 (1991) 164–167.
[9] D. Kafetzopoulos, A. Martinou, and V. Bouriotis, *Proc. Natl. Acad. Sci. U.S.A.* 90 (1993) 2564–2568.
[10] A. Martinou, D. Kafetzopoulos, and V. Bouriotis, *J. Chromatogr.*, 644 (1993) 35–41.
[11] D. Kafetzopoulos, G. Thireos, J. Vournakis, and V. Bouriotis, *Proc. Natl. Acad. Sci. U.S.A.*, 90 (1993) 8005–8008.
[12] Y. Araki and E. Ito, *Eur. J. Biochem.*, 55 (1975) 71–78.
[13] H.U. Bergmeyer (Ed.), *Methods of Enzymatic Analysis*, 2nd ed., Vol 1, Verlag Chemie, Weinhein, 1974, pp 112–117.
[14] O.H. Lowry, M.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
[15] S. Hirano, *Methods Enzymol.*, 161 (1988) 408–410.
[16] R.A.A. Muzzarelli and R. Rocchetti, *Carbohydr. Polym.*, 5 (1985) 461–472.
[17] T. Sanna, K. Kurita, and Y. Iwakura, *Macromol. Chem.*, 176 (1975) 1191.
[18] K. Kurita, F. Sannan, and Y. Iwakura, *J. Appl. Polym. Sci.*, 23 (1979) 511–515.
[19] S. Mimas, M. Miya, R. Iwamoto, and S. Yoshikawa, *J. Appl. Polym. Sci.*, 28 (1983) 1909–1917.
[20] M. Hisamatsu and T. Yamada, *J. Ferment. Bioeng.*, 67 (1989) 219–220.
[21] K. Nishimura, S. Nishimura, N. Nishi, K. Nuwata, Y. Tone, S. Tokura, and I. Azuma, *Vaccine*, 3 (1985) 379–384.
[22] M. John, H. Röhrig, J. Schmidt, U. Wieneke, and J. Schell, *Proc. Natl. Acad. Sci. U.S.A.*, 90 (1993) 625–629.