A PATHWAY OF CHITOSAN FORMATION IN MUCOR ROUXII: ENZYMATIC DEACETYLATION OF CHITIN

Yoshio Araki and Eiji Ito

Department of Chemistry, Faculty of Science
Hokkaido University, Sapporo 060, Japan

Received December 5,1973

Summary An enzyme which hydrolyzes the acetamido groups of N-acetylglucosamine residues in chitin was partially purified from Mucor rouxii. The enzyme deacetylates also N-acetylchitooligoses, whereas it is inactive toward bacterial cell wall peptidoglycan, N-acetylated heparin, a polymer of N-acetylgalactosamine, di-N-acetylchitobiose, or N-acetylglucosamine. The enzyme shows a pH optimum of 5.5 and is markedly inhibited by acetate. The occurrence of this enzyme accounts for the formation of chitosan in fungi.

Some polysaccharides produced in microorganisms are known to contain poorly or nonacylated amino sugar constituents, whereas, in most of natural polysaccharides which contain amino sugars, the amino sugar residues are believed to be entirely acylated at their amino groups. Chitosan, a nonacylated glucosaminoglycan, has been found in cell walls of Phycomyces blakesleeanus (1) and Mucor rouxii (2), although its biosynthesis remained unknown. In view of the established pathway of biosynthesis of chitin from the precursor UDP-GlcNAc (3), it seems most likely that chitosan is produced through deacetylation of chitin. The occurrence of a specific deacetylase which acts on cell wall peptidoglycan was recently described (4), and accounts for the formation in some bacteria of peptidoglycan unacetylated at glucosamine residues (5, 6). The present communication describes

Abbreviations: GlcNAc, N-acetylglucosamine; CM, carboxymethyl; Ac, acetyl; DNP, dinitrophenyl; TES, N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; MurNAc, N-acetylmuramic acid; GalNAc, N-acetylgalactosamine.

the partial purification and properties of chitin deacetylase which is believed to be responsible for the formation of chitosan in Mucor rouxii.

Materials and Methods Mucor rouxii AHU 6019, furnished by Dr. S. Takao, Hokkaido University, was grown under the conditions suitable for a filamentous growth (2). Mycelia (wet weight 73 g) harvested by filtration from 10 liters of culture were disrupted with glass powder (50 g/25 g mycelia) in a mortar at 2° for 1 hr. After dilution with 20 mM Tris-Cl, pH 7.2 (400 ml per 25 g mycelia), the homogenate was centrifuged at 2500 x g for 5 min, and the supernatant was further centrifuged at 20,000 x g for 20 min. The resulting supernatant (total 1 liter) was used as the crude enzyme. The enzyme was partially purified as follows: A portion of the crude enzyme (200 ml) was brought to 63 % saturation with solid ammonium sulfate and centrifuged. The supernatant was further brought to 85 % saturation. The precipitate collected by centrifugation was dissolved in and dialyzed against 5 mM sodium acetate, pH 5.3, and chromatographed on a 20 ml column of CM-cellulose in the same buffer. The column was washed with 30 ml of the same buffer and then eluted with a linear gradient (200 ml) of sodium acetate, pH 5.5, from 5 mM to 100 mM. The active fractions eluted at about 50 mM were collected, concentrated to 1 ml by filtration through a collodion bag, and chromatographed on a 3 ml column of DEAE-cellulose in 20 mM Tris-Cl, pH 7.2. The column was eluted successively with 40 ml of a linear gradient from 0 to 0.3 M NaCl, 15 ml of 0.3 M NaCl, and 30 ml of 0.45 M NaCl in the same buffer. The active fractions (0.45 M NaCl eluate) were collected and used as the purified enzyme.

To prepare a glycol ether of chitin labeled in N-acetyl

groups (glycol [acetyl-^{3}H]chitin), glycol chitosan purchased from Wako (40 mg) was treated at 4° for 24 hr in a mixture containing 400 mg of $NaHCO_3$ and [^{3}H]acetic anhydride (6.7 mCi, 200 μmoles) in a total volume of 4.5 ml, and the treatment was continued for further 24 hr with additional 200 μl of nonlabeled acetic anhydride. The product after thorough dialysis was used as substrate for the assay of chitin deacetylase. N-[^{3}H]Acetylgalactosamine polymer was similarly prepared from polygalactosamine isolated from culture of Aspergillus parasiticus (7). N-^{3}H-Acetylated heparin, in which N-sulfuryl groups were replaced by N-[^{3}H]acetyl groups, was prepared from heparin by the similar treatment with [^{3}H]acetic anhydride following desulfurylation with acid (8). Other labeled substrates were prepared as described previously (4). N-Acetylchitooligoses were prepared from chitin by partial acid hydrolysis according to the method of Rupley (9). Colloidal chitin was also prepared (10).

For the assay of chitin deacetylase, a reaction mixture, containing glycol [acetyl-^{3}H]chitin (47.8 μg, 114,000 cpm), 50 mM TES-NaOH buffer, pH 5.5, and enzyme in a final volume of 50 μl, was incubated at 30° for 10 min. After termination of the reaction by adding 20 μl of 0.2 M HCl, 5 μl of 1 M acetic acid, and 100 μl of water, the mixture was extracted three times with 0.5 ml of ethyl acetate. The ethyl acetate phase was combined and measured in toluene scintillator for radioactivity in a liquid scintillation counter.

Results and Discussion As shown in Table 1, the activity of an enzyme that catalyzes liberation of radioactivity from glycol [acetyl-^{3}H]chitin was found both in the 20,000 x g supernatant

Table 1. Distribution of chitin deacetylase

Fraction	Total activity	Specific activity
	cpm x 10^{-6}	cpm/μg protein
2500 x g sediment	69	618
20,000 x g sediment	28	147
20,000 x g supernatant	347	928
Culture filtrate	264	54

The assay of chitin deacetylase activity was carried out as described in the text. Total activity is expressed in radioactivity released per fraction from 1 liter of culture.

Table 2. Purification of chitin deacetylase

Fraction	Total protein	Total activity	Specific activity
	mg	cpm x 10^{-7}	cpm x 10^{-3}/μg protein
Supernatant (200 ml)	852	91.2	1.07
$(NH_4)_2SO_4$ precipitate	186	68.0	3.66
CM-cellulose	19.6	31.2	15.9
Collodion bag	8.24	16.5	20.0
DEAE-cellulose	0.52	7.38	142

The purification and assay of deacetylase were carried out as described in the text.

fraction and in the culture filtrate of Mucor rouxii. This enzyme was partially purified (about 140-fold) from the supernatant fraction as summarized in Table 2. The radioactive product, released through the enzyme reaction and extracted with ethyl acetate, was identified as acetate by paper chromato-

graphy in 1-butanol-1.5 M NH_4OH (1:1, upper phase) and in 1-propanol-1.5 M NH_4OH (7:3). The enzymatic deacetylation at the N-acetylglucosamine residues could also be confirmed by the assay of the liberated amino groups as DNP-glucosamine (see below, Table 4). Since colloidal chitin was deacetylated slowly as compared with glycol chitin, glycol chitin was used in the assay of this enzyme. In contrast with peptidoglycan deacetylase which has been shown to require Co^{++} or Mn^{++} for its full activity (4), chitin deacetylase was not stimulated by any metal ion. This enzyme has a pH optimum at 5.5. Under the standard assay conditions, it was markedly inhibited by acetate (50 % inhibition at 20 mM) and weakly by formate (10 %, at 100 mM).

This enzyme acts specifically on chitin and glycol chitin. As shown in Table 3, no appreciable deacetylation was observed

Table 3. Specificity for substrate

Substrate	Radioactivity released
	cpm
Glycol [Ac-^{3}H]chitin (113,000 cpm, 47 µg).....	32,000
[Ac-^{3}H]Peptidoglycan (*Bacillus cereus* cell wall, 318,000 cpm, 37 µg).....	0
N-^{3}H-Acetylated heparin (160,000 cpm, 22 µg)....	10
[Ac-^{3}H]GalNAc polymer (82,700 cpm, 4.6 µg).....	0
[Ac-^{3}H]GlcNAc-MurNAc (70,000 cpm, 1.4 nmoles)....	0
[Ac-^{14}C]GlcNAc (21,400 cpm, 2.1 nmoles).....	0
[Ac-^{14}C]GlcNAc-6-P (23,500 cpm, 2.3 nmoles).....	0
UDP-[Ac-^{14}C]GlcNAc (20,500 cpm, 2 nmoles).....	0

Enzymatic deacetylation was carried out as described in the text for 16 hr using the indicated substrate and the purified enzyme (0.19 µg protein).

Table 4. Enzymatic deacetylation of N-acetylchitooligoses

Substrate	DNP-glucosamine formed
	nmoles
GlcNAc, 2 μmoles	0.0
$(GlcNAc)_2$, 1 μmole	0.9
$(GlcNAc)_3$, 0.67 μmole	16.8
$(GlcNAc)_4$, 0.5 μmole	89.3
$(GlcNAc)_5$, 0.4 μmole	230
Colloidal chitin, 0.4 mg	35.3
Glycol chitin, 0.66 mg	244

The indicated amount of N-acetylchitooligose (about 2 μmoles as N-acetylglucosamine residue) or chitin was treated with the purified enzyme (1.92 μg protein) at 30° in 500 μl of 50 mM TES-NaOH, pH 5.5 for 48 hr. The reaction mixture was then subjected to treatment with fluorodinitrobenzene followed by acid hydrolysis and separation of DNP-glucosamine by paper chromatography in 1.5 M potassium phosphate, pH 6. The DNP-glucosamine area was eluted with 0.04 M NH_4OH. The eluate was lyophilized and the absorbance at 360 nm was measured in a 1 % $NaHCO_3$ solution.

with any of other polysaccharides examined as substrate. In addition, UDP-GlcNAc and other N-acetylglucosamine derivatives of low molecular weights proved to be inactive as substrate. It was also observed that lysozyme digests of glycol chitin were deacetylated at a much smaller rate than the intact polysaccharide. From these results, the enzyme appeared to act only on the chitin polysaccharide and oligosaccharides having more than a certain number of N-acetylglucosamine units. This was confirmed with purified chitooligoses. As shown in Table 4, the pentamer of N-acetylglucosamine was deacetylated as rapidly as glycol chitin, whereas the trimer was attacked much more slowly and the dimer was virtually inert as substrate.

The present results, together with those reported previously (4), indicate that some microorganisms have a novel class of deacetylases which act specifically on the N-acetylamino sugar components of particular polysaccharides. Thus, the formation of chitosan in Mucor rouxii is accounted for by the action of the specific deacetylase on chitin.

References

1. Kreger, D. R. (1954) Biochim. Biophys. Acta 13, 1-9.
2. Bartnicke-Garcia, S., and Nickerson, W. J. (1962) Biochim. Biophys. Acta 58, 102-119.
3. McMurrough, I., Flores-Carreon, A., and Bartnicki-Garcia, S. (1971) J. Biol. Chem. 246, 3999-4007.
4. Araki, Y., Fukuoka, S., Oba, S., and Ito, E. (1971) Biochem. Biophys. Res. Commun. 45, 751-758.
5. Araki, Y., Nakatani, T., Nakayama, K., and Ito, E. (1972) J. Biol. Chem. 247, 6312-6322.
6. Hayashi, H., Araki, Y., and Ito, E. (1973) J. Bacteriol. 113, 592-598.
7. Distler, J. J., and Roseman, S. (1960) J. Biol. Chem. 235, 2538-2541.
8. Silbert, J. E. (1967) J. Biol. Chem. 242, 5153-5157.
9. Rupley, J. A. (1964) Biochim. Biophys. Acta 83, 245-255.
10. Jeuniaux, C. (1966) Methods in Enzymology 8, 644-650.