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[14] Quantitative Determination of "Free" and "Bound" Chitin

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Introduction

Chitin (*1*), a polymer of 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine), may be found in three different crystallographic forms, α, β, and γ (*2*). Whatever the crystallographic form, all chitins analyzed so far appear to be composed of (1→4)-linked *N*-acetyl-β-D-glucosamine units. The isolation of chitin in a pure state, from an arthropod cuticle, for example by removing all the inorganic and organic constituents, may induce some deacetylation, mainly during treatments of hot alkali. The question remains whether some non-acetylated D-glucosamine (2-amino-2-deoxy-D-glucose) residues occur in the native chitin linear chain (*3–5*).

To identify chitin without ambiguity, one cannot rely on classical histochemical methods. The well-known "chitosan test" (*6*) lacks specificity and can lead to erroneous interpretations with different kinds of morphological structures (*7–9*). X-ray crystallography gives more accurate results, at least when appreciable amounts of chitin are present, but cannot give quantitative information.

A quantitative and specific enzymic method detection and estimation of chitin has been proposed (*8–10*). This method is based on the use of a purified chitinase (EC 3.2.1.14) as a highly specific reagent. By addition of chitobiase (or *N*-acetyl-β-D-glucosaminidase: EC 3.2.1.20), the original hydrolytic products (chitobiose, chitotriose) are split into *N*-acetylglucosamine, which may be measured accurately by a colorimetric method (*11*; Vol. I [140]). The chitin value, calculated from the amount of *N*-acetylglucosamine, may be somewhat lower than the actual value, to the extent that some non-acetylated glucosamine could exist in the chitin chains, at least after isolation.

When this method is applied to preparations of chitin isolated by treatment with hot alkali, all the chitin may be readily accessible to hydrolysis cata-

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TABLE I
"Free" and Total Chitin Estimated by the Enzymic Method with the Use of a Purified Chitinase (8, 16)

Material	*Mode of preservation*	*Preliminary treatments*	*Chitin[a] (% of dry weight[b])*	*"Free" chitin (% of total chitin)*
Silkworm (*Bombyx mori*)				
Larval cuticle	Freshly dissected	None	1.7	5.5
Larval cuticle	Freshly dissected	0.5 M NaOH, 6 h, 100°	30.8	—
Larval cuticle	25% Alcohol	None	1.5	5.08
	25% Alcohol	0.5 M NaOH, 6 h, 100°	29.5	—
Lobster (*Homarus vulgaris*)				
Branchiostegal	Freshly dissected	None	0.12	0.8
Outside (calcified) cuticle	Dried, ground	0.5 M HCl (decalcified)	8.5	57.04
		0.5 M HCl, 0.5 M NaOH, 6 h, 100°	14.9	—
Lobster (*H. vulgaris*)				
Branchiostegal	Freshly dissected	None	2.96	8.0
Inside (noncalcified) cuticle	Dried, ground	0.5 M NaOH, 6 h, 100°	37.24	—

[a] Calculated on the basis of the amount of *N*-acetyl-D-glucosamine liberated by successive hydrolytic action of purified chitinase and chitobiase.
[b] Dry weight: uncalcified (A and C) or calcified (B).

lyzed by the chitinases. However, when applied to chitinous structures in a "native state" (i.e., without any preliminary treatment), hydrolysis is far from complete. With the materials studied to date, the proportion of chitin that can be hydrolyzed without preliminary treatment represents only a fraction of the total chitin (Table I). These observations were interpreted (*8, 12*) as being the result of some form of protection given to chitin molecules by other organic components in native structures, mainly proteins with which these chitin molecules are complexed (*13*). The chemical nature of the relationship between chitin and proteins has not yet been elucidated. The existence of covalent bonds has been postulated (*13–15*).

Chitin molecules linked in some way to proteins within a complex have been defined as "bound" chitin, whereas chitin molecules readily accessible to hydrolysis by purified chitinases have been interpreted as "free" chitin. It has recently been shown that calcification also may protect chitin from enzymic hydrolysis (Table I) (*16*). Thus, the concept of "free" chitin has to be restricted to noncalcified or decalcified structures.

Procedure

Principle

When applied simultaneously to native material and to the same material previously decalcified and treated with hot alkali, the enzymic method for chitin detection (*8, 10*) provides an indirect estimation of the degree of bonding (of whatever kind). As far as is known, the proportion of free chitin has little to do with the function of the structure, which seems to be related to taxonomy (as evidenced in the unusually high amount of free chitin in all chitinous structures of mollusks) (*17*). In other zoological groups, calcified chitinous structures seem to contain higher proportions of free chitin than do noncalcified structures, with the remarkable exception of peritrophic membranes in arthropods.

Techniques

Chitinases

Chitinases may be produced and purified from culture filtrates of *Streptomyces antibioticus* (*18, 19*), *Streptomyces griseus* (*20, 21*), and *Aspergillus niger* (*22, 23*). Good chitinase preparations may be purchased from some commercial firms, but other commercial preparations have insufficient purity and/or activity.

Purified chitinases used for free chitin determination must be devoid of any proteolytic activity, at least at pH 5.2.

Preparation of Material

The morphological structures submitted for estimation of free and bound chitin are dissected, cleaned in distilled water, and stored in 30% ethanol. After desiccation over phosphorus pentaoxide and determination of constant dry weight, the material is decalcified with 0.5 M hydrochloric acid at 20–25°, washed, dried, and weighed ("organic matter"). One part of the material is treated with 0.5 M sodium hydroxide at 100° for 6 h, then washed until neutral pH is reached; it is used for total chitin determination. The remainder of the material is used for determination of free chitin.

Chitin Assay

The material (containing less than 6 mg of chitin), which has been previously treated (A) or not treated (B) with hot alkali, is sliced, suspended in a mixture of 1.0 mL of distilled water, 20 mL of buffer, and 1.0 mL of chitinase, and allowed to stand overnight at 0°. The buffer is 0.1 M citric acid–Na_2HPO_4, pH 5.2. The chitinase solution contains ~500 nephelometric units/mL of purified *Streptomyces* chitinase (*18*, *24*) or 2 mg/mL of commercial purified chitinase.

Test tubes A and B are incubated on a shaker in a water bath at 37° for 6 h in the presence of thymol; then they are centrifuged. A 1.0-mL aliquot of the supernatant is incubated with 1.0 mL of chitobiase solution (a solution of lobster serum diluted 10 times is a suitable source of chitobiase) at pH 5.2 for 1 h at 37°. The *N*-acetyl-D-glucosamine which is liberated is then measured (*11*; Vol. I [140]).

The centrifuged residual material is suspended again in 2.0 mL of buffer and incubated 6 h at 37°; the *N*-acetyl-D-glucosamine liberated is measured following the same procedure. The chitin in test tube A is generally completely hydrolyzed; however, in some cases, further chitinase incubation is required to achieve complete hydrolysis. The remaining material in test tube B (not previously treated) is made up of chitin bound to proteins or other substances.

The difference between the amount of *N*-acetylglucosamine in test tubes A and B corresponds to the amount of free chitin expressed as *N*-acetyl-D-glucosamine (Table I).

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