# COMPARATIVE VALUE OF CHITOSAN TEST AND ENZYMATIC METHOD FOR CHITIN DETECTION

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## ABSTRACT

In order to check their relative value for the specific identification of chitin on minute pieces of skeletal material, the chitosan test of Campbell (1), readapted by Krishnan and Sundara-Rajulu (2) and the enzymatic method of Jeuniaux (3) using purified chitinases, were applied simultaneously to the same material.

In most cases, both methods gave similar results, the chitosan test being more rapid and useful to locate chitin in composite structures.

In two specific cases, these methods yielded contradictory results. Fragments of Rhabdopleura sp exoskeleton (Pterobranchia), after alkali treatment, gave a more or less typical colour with chitosan test, but remained unaltered in pure chitinase solutions without N-acetyl-D-glucosamine production. In the same manner, hooks of Cloeosiphon sp ( Sipunculida ) gave a positive reaction to chitosan test but failed to dissolve in pure chitinase, after repeated incubations at 37°C.

It is concluded that, in some cases, undetermined organic substances, which do not correspond to true chitin, may be interpreted as chitin with chitosan test.

## INTRODUCTION

It is well known that chitosan or deacetylated chitin in acidic medium develops a violet or reddish colour with iodine. The application of this reaction for chitin detection was elaborated by van Wisselingh (4), modified by Campbell (1) and more recently readapted by Krishnan and Sundara-Rajulu (2). This so called "chitosan test" was currently used by many authors but its lack of specificity was suspected by some others (for instance, see Richards (5)).

The high specificity of chitin hydrolysis by purified chitinases led one of us (3,6) to improve an enzymatic method for chitin detection and quantitative estimation. This quantitative method was adapted for chitin qualitative detection in minute pieces of material. After alkali treatment, the remaining organic material, eventually stained with Congo Red in order to facilitate further observations, is washed in water and brought into chitinase solution at pH 5.2, where it melts more or less rapidly if made of chitin (3,7).

However, some discrepancies are known between these two methods when used

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to detect chitin in minute pieces of skeletal material. In two specific cases at least, these methods yielded contradictory results: the exoskeleton or "tubes" of some Pterobranchia colonies as well as hooks of Sipunculida introvert were thought to be chitinous in nature by one of us using chitosan test (8) while no chitin could be detected by enzymatic method on the same structures of related species (9,10).

In order to check their relative value for chitin detection in such cases, both methods were applied simultaneously to the same material. The results of these comparative experiments are given in the present paper.

## EXPERIMENTAL

Fragments of Rhabdopleura sp exoskeleton (Pterobranchia) and hooks of the introvert of Cloeosiphon sp (Sipunculida) were boiled during 1/2 hour in a saturated NaOH solution. The remaining material was then immersed in a drop of 0.2% iodine in potassium iodide solution on a glass slide. The excess iodine solution was removed and replaced with 2% sulfuric acid. The developing colour is immediately noted.

Fragments of the same exoskeleton of Rhabdopleura sp (30.2 mg) were decalcified in 1N HCl, at room temperature, washed with distilled water and then treated during 6 hours with 0.5N NaOH at 100°C. to set chitin free from its glycoprotidic complexes (11).

After washings with water, the fragments were incubated during 6 hours in a 1 mg/ml solution of commercial chitinase (Koch Light) or in a 600 nephelometric units / ml (according to Jeuniaux (1)) solution of chitinase purified in our laboratory according to the method of Jeuniaux (13), from microbial cultures of Streptomyces antibioticus in chitin medium (12).

After incubation in the presence of chitinase, the supernatants were incubated in the presence of chitobiase ( addition of lobster serum diluted 10 times) Acetylglucosamine was then estimated by the method of Reissig et al. (14).

The available amounts of hooks of Cloeosiphon being not sufficient to apply the enzymatic quantitative method, the presence of chitin was searched by a qualitative enzymatic test. After treatment with hot alkali, as described above, the remaining material was incubated at 37°C. in purified chitinase solution (2400 nephelometric units / ml according to Jeuniaux (12)) during 24 hours: a further incubation was performed in a fresh chitinase solution. During the incubation period, the material was observed from time to time under binocular lenses and the eventual modifications were carefully noted.

## RESULTS

After alkali treatment, the fragments of Rhabdopleura sp exoskeleton gave a more or less blue-green or violet colour with the chitosan test.

Neither the incubation in commercial chitinase (Koch Light) nor in chitinase purified in our laboratory from Streptomyces cultures did alter the mate-

rial. Moreover, successive incubations in chitinase and chitobiase at 37°C. and pH 5.2 did not release any acetylglucosamine.

In the same manner, hooks of Cloeosiphon introvert gave a positive reaction with the chitosan test but failed to dissolve in pure chitinase after repeated incubation at 37°C.

In the case of Rhabdopleura exoskeleton, the remaining material after prolonged incubation in chitinase solution was subjected again to chitosan test. The development of a blue-green or violet colour was observed again.

## DISCUSSION

These observations confirm that, even when applied simultaneously to the same material, the chitosan test (according to Krishnan and Sundara-Rajulu (2)) and the enzymatic method (according to Jeuniaux (3)) can give rise to contradictory results.

The activity of the chitinases used during these experiments is not in question. These chitinases were indeed from different origin; they were tested on reference samples ( purified chitin from crustacean cuticles ) and gave positive results i.e. rapid dissolution of chitin and characterization of hydrolytic products as N-acetyl-D-glucosamine by colorimetric method.

Moreover, in the case of the fragments of Rhabdopleura exoskeleton, the chitosan test remained positive after incubation in hot 0.5 N NaOH followed by prolonged chitinase incubation, treatments ensuring chitin breakdown and hydrolysis if present.

It is thus concluded that positive chitosan test were given by two kinds of exoskeleton organic material of animal origin, in which chitin could not be identified with the aim of purified chitinase.

Chitosan test presented also some other disadvantages:

- the reproductibility of this method was not always warranted. Using chitosan test, Meenakshi et al. (15) failed to detect chitin in the shell of Monoplacophoran Mollusks, in the case of Neopilina ewingi as well as in that of Neopilina bacescui. On the contrary, Poulicek and Jeuniaux (16), using the same method, were able to detect the presence of chitin in the calcified layers of the shell of Neopilina galatheae.This latter conclusion was confirmed by the specific enzymatic method.
- the chitosan test also lacks sensibility. Peters (17) did not detect chitin in the calcified layers of the shells of many Gastropods using chitosan test. The enzymatic method however revealed appreciable amounts of chitin in the shells of the same species (18). As far as the shell of Agriolimax reticulatus is concerned, the same author (18), using chitosan test, was able to point out chitin in specimens collected at the end of summer or in autumn (when the amounts of chitin are at the highest) but failed to detect chitin in the shells of the specimens collected in spring (when the amounts of chitin are at the lowest).

The chitosan test remains however not only the most rapid method for a preliminary attempt at chitin identification but also a usefull method for the

localization of chitin inside skeletal structures to which it can be applied more easily than the chitinase method. In the case of the shell of Neopilina galatheae, for which only minute fragments were available, the localization of chitin, previously identified by enzymatic method, was performed using the chitosan test (16). Chitin was shown to be located in nacreous and prismatic layers, according to the clear cut violet colour given with the chitosan test.

## CONCLUSION

As already stated by some authors, the chitosan test is no longer entirely reliable as a tool for chitin identification. In some cases indeed, when chitin is present in low amounts, the chitosan test may give negative results. In some other cases, as probably Pterobranchia exoskeletons and Sipunculid introvert hooks, some unidentified organic material may withstand alkali degradation and give more or less typical colour with the chitosan test, despite the absence of true chitin.

It thus appears that enzymatic method is more reliable. The use of both methods simultaneously can however be strongly recommended especially for chitin topographic studies.

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