

SUMMARY AND CONCLUSIONS

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The present investigation was undertaken to characterize a chitosanase detected in the extracellular fluid of *Rh. gracilis* CFR-1, which is a locally isolated strain. The identity of the culture was confirmed by National Collection of Yeast Cultures, USA.

By several criteria studied, the chitosanase was found to be a novel enzyme hitherto not reported in literature. Thus it was of interest to study its properties in some detail and design experimentation to understand its biological role.

Most of the procedures for the assay of chitosanase are based on the estimation of the reducing groups generated (76), glucosamine formed (78) or by determination of reduction in viscosity of chitosan solution resulting from enzyme catalysed hydrolysis (37,42,53).

In the present case however, the only possible method of assay was by determination of viscosity reduction, because of the barely measurable levels of glucosamine or reducing groups. A new assay procedure for chitosanase was developed in the present work taking advantage of the ability of chitosan to complex with calcofluor white, resulting in a product with absorption maximum at 406 nm.

The growth pattern of the yeast and the production of extracellular chitosanase in three media namely, YEPD, MS + peptone and PD were studied in order to evaluate the behaviour of this organism vis-a-vis enzyme production.

In all the three media enzyme production appeared to be growth associated. Typical cell morphology was found in YEPD and MS + peptone media, but in PD broth the cells were found to contain large inclusion bodies presumed to be fat globules. In media having high C: N ratio, greater than 16 *Rhodotorula* yeasts are known to accumulate excessive amounts of lipid, and PD broth has high C:N ratio (120-122).

The purification of the chitosanase was carried out starting from the culture fluid of the yeast grown in all the three media in separate experiments. From the YEPD grown culture a 3 fold purified enzyme with 34% recovery having a specific activity of 200 units/mg protein was obtained. From the MS + peptone grown culture, a 12 fold purified enzyme with a recovery factor of 43% having specific activity of 538 units/mg protein was obtained. From the PD grown culture a 17 fold purified enzyme with 30% recovery and specific activity 1,870 units/mg protein was obtained.

Data obtained from chromatofocussing of the crude enzyme preparation from PD grown culture suggested that the

enzyme protein has been fragmented to segments having PIs 7.4, 5.75, 5.25 and 4.25 all retaining the catalytic activity.

From all the three media, very low levels of protein was available for processing. This posed considerable problem in enzyme purification. Furthermore diffused elution patterns in gel permeation and ion exchange chromatography and highly streaking pattern of protein bands in PAGE were generally observed during enzyme purification.

The chitosanase of *Rh. gracilis* CFR-1 was found to be optimally active at pH 4.5 and at 45°C. The enzyme was most stable at pH 5.0 and at 4°C. The apparent molecular mass of the enzyme was determined to be 100 kDa by gel filtration. Manganese chloride was found to have a slight stimulatory effect on enzyme activity, while mercuric chloride and copper sulphate were inhibitory. Among the other effectors the tryptophan reagent, NBS and the thiol reagent, pCMB inhibited the enzyme. The enzyme was specific for chitosan. It did not hydrolyze chitin, glycol chitosan, CMC, starch or pectin. The K_m and V_{max} values of the chitosanase were determined as 0.83 mg of chitosan/ml and 142 units of enzyme/mg protein respectively.

A detailed characterisation of the products of hydrolysis of chitosan catalysed by the chitosanase was necessary owing to the product being unusually large.

The enzyme catalysed products of chitosan hydrolysis were separated by Biogel P-100 fractionation revealing a 9.5 kDa fragment as the major product, while the unhydrolysed chitosan was composed of predominantly 36 kDa sized molecules. Thus the enzyme was found to have caused a limited hydrolysis of the substrate chitosan. These results were corroborated by analytical ultracentrifugation (Fig.3.25). The hydrolysed chitosan sedimented at 2.1 S as against 3.54 S for the unhydrolysed sample. Similar conclusions were drawn from electrophoretic separation of the chitosan hydrolysis products, performed by the procedure of cathodic disc PAGE (Fig.3.23) (115). The hydrolysed product seen as a single band had a higher mobility than the unhydrolysed sample. The electrophoresis on cellulose acetate paper also gave similar data on the hydrolysis of chitosan by enzyme (Fig.3.24).

Chitosanases have been reported in a number of microorganisms and plants (1,12,34,37-64). In most of the microorganisms the enzyme is known to have a nutritional role for the utilization of chitosan for growth and reproduction (12,34,40-44,46,48,49,52,53). In plants the enzyme

apparently has a defense role against fungal pathogens and insect pests (56-59). The last mentioned role has however not been substantiated adequately. Since in the present case, the *Rh. gracilis* CFR-1 produced an extracellular chitosanase which catalyses hydrolysis of the substrate chitosan in a limited way, it was considered necessary to study a possible biological role for this enzyme.

The organism utilized several carbon and nitrogen sources for growth and production of extracellular chitosanase. It utilised sucrose, glucose with highest efficiency and xylose, mannitol, glycerol to a limited extent. It was not however able to utilise lactose, cellulose, starch or pectin. Most strikingly it was not able to utilise chitosan. The two major conclusions drawn from the above observations are (i) the chitosanase is produced constitutively by *Rh. gracilis* CFR-1 and (ii) the enzyme does not have a nutritional role.

The organism was also capable of producing the chitosanase, by growth on both inorganic and organic nitrogenous compounds. While testing the effect of the various components of a rich organic medium like YEPD, it was found that yeast extract does not contribute as significantly as peptone to enzyme production.

Since peptone is the enzymic digest of animal tissues and is composed predominantly of peptides and amino acids the effect of various amino acids on enzyme production was tested. Barring tryptophan all the amino acids tested were found to be conducive for enzyme production when the amino acids were incorporated in MSM at levels equivalent to nitrogen content (16.16%) of peptone (126). In the media containing leucine and cysteine a six fold higher level of chitosanase product ion was found than that in peptone containing medium under similar conditions of experiment.

The data obtained on the production of chitosanase in different kinds of nitrogenous media further substantiated the constitutive nature of the chitosanase in this organism.

Since the chitosanase in the present case did not appear to have a nutritional role, attempt has been made to find, if it will be involved in the budding process and reproduction of *Rh. gracilis* CFR-1. The culture was mutagenised with NTG to isolate chitosanase deficient mutants. The mutagenised culture did yield growth defective variants which produced small colonies on agar medium and grew weakly in broth culture. However, these variant cultures were not found to be deficient in chitosanase *per se* when the level of enzyme was calculated for unit biomass.

Thus attempts to study if the chitosanase is involved in the budding process was inconclusive, also because of the limited experimental approach.

Since the plant chitosanases and chitinases are suspected to be involved in defense mechanism (140-150), whether a similar role exists for the present chitosanase was tested. Forty cultures of zygomycetes were tested for antagonism by *Rh. gracilis* CFR-1 by the plate culture method. The growing culture of *Rh. gracilis* CFR-1 inhibited distinctly the growth of eleven fungal cultures, but the filter sterilised culture fluid (containing the chitosanase) did not show the inhibition. It is speculated that the enzyme concentration was too low to inhibit the germination of the fungal spores.

Since the growing culture of *Rh. gracilis* CFR-1 inhibited eleven of the zygomycete fungi, it was of interest to see if chitosanase production can be elicited by heat killed fungal mycelium. This test gave negative result, which however cannot be considered to discount the defense role of chitosanase. In some plants such as *Cucumis sativus*, the chitosanase produced without fungal infection has also been speculated as having a defense role (58).

Considering all the data obtained in the present work, the chitosanase of *Rh. gracilis* CFR-1 is likely to have a defense role. That this enzyme may be a vestigial entity was also considered, but an organism which makes a large enzyme of the size of 100 kDa and secretes into the extracellular medium would require tremendous resources of cell machinery and energy which is unlikely for a vestigial entity.

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