Hydrolysis of chitin by Pectinex™

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Abstract
It has been shown that a commercially available pectinase, Pectinex™, hydrolyzes chitin fairly efficiently with a K_m of 1.1mg/ml^1 of chitin and V_max equal to 6.5nmol/min^1 mg^1. The pH optimum range (5.0-6.0) and temperature optimum range (55-65 °C) for this unusual activity are more in agreement with the corresponding known values for chitinases rather than the values observed in case of its pectinase activity. The half lives (of chitinolytic activity) of 81 and 76 min at 55 and 65 °C indicate that the chitinolytic activity survives much better than the pectinase activity of the molecule. The occurrence of a carboxyl group in both pectinases and chitinases suggests that similar active site designs may be responsible for this unusual and useful activity of Pectinex™.

Keywords: Biomass conversion; Chitinase; Chitin hydrolysis; Pectinase; Pectinex™

1. Introduction
Chitin [(1 -> 4)-2-acetamido-2-deoxy-(3-D-glucan] is the main structural component of mollusks, insects, crustaceans, fungi, algae and marine invertebrates like crabs and shrimps [1-3]. Worldwide, the solid waste from processing of shellfish, crabs, shrimps and krill constitutes large amounts of chitinaceous waste. Chitin can be degraded by endochitinases to oligomers of N-acetylglucosamine. Chitinases are widely distributed among bacteria and have also been reported to occur in plants, protozoans, nematodes and arthropods. However, commercial preparations of chitinases are fairly expensive.

In the present work, we show that a relatively inexpensive commercial preparation (Pectinex™ from Novozymes) of pectinase is able to hydrolyze chitin. Although there are a few reports that pectinase and many other enzymes are able to hydrolyze chitosan [4,5]; this, we believe is the first report of a pectinase exhibiting chitinolytic activity towards insoluble chitin. The level of chitinolytic activity was quite significant and considerably exceeded the ‘chitinase’ activity shown by lysozyme [6] or even by chitinase of one of its best sources, S. marcescens [2]. The activity was not due to an impurity in the commercial preparation as the purified pectinase preparation showed simultaneous increase in specific activity of the enzyme towards chitin hydrolysis.

1.1. Materials and Methods

1.1.1. Materials
Chitin (Cat. No. C-7170) was purchased from Sigma (St. Louis, MO, USA). Pectinex™ Ultra SP-L (a highly purified preparation of pectolytic enzymes from a selected strain of Aspergillus niger) was from Novo Nordisk, Switzerland and lysozyme was obtained from Sisco Research Laboratory, Mumbai, India. All other chemicals used were of analytical grade.

The present work also reports kinetic parameters concerning chitin hydrolysis by pectinase.

2. Materials and methods

2.1. Materials
Chitin (Cat. No. C-7170) was purchased from Sigma (St. Louis, MO, USA). Pectinex™ Ultra SP-L (a highly purified preparation of pectolytic enzymes from a selected strain of Aspergillus niger) was from Novo Nordisk, Switzerland and lysozyme was obtained from Sisco Research Laboratory, Mumbai, India. All other chemicals used were of analytical grade.

2.2. Method
2.2.1. Preparation of chitin suspension
Chitin suspension was prepared by adding 1 g of chitin in 100 ml of 0.01 M acetate buffer, pH 5.0 and stirring it for 3 h at 25 °C. The suspension was stirred again before each use. Acid swollen chitin was prepared by adding 0.5 g of chitin in 50 ml of dilute aqueous acetic acid (pH 2.5) and stirring it for 4 days at 4 °C. Finally, the pH of the suspension was adjusted to 5.0 by adding 1N NaOH.

2.2.2. Determination of enzyme activity
Enzyme activity using chitin as substrate was determined as described by Pegg [7], using a slight modification. One

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unit of enzyme activity using chitin as substrate is defined as the amount of enzyme required for the formation of 1 nmol of the product (estimated as N-acetylglucosamine) per minute. The amount of reducing sugar was estimated by dinitrosalicylic acid method [8].

2.2.3. Properties of the enzyme

2.2.3.1. pH optima. Chitin suspension was prepared in 0.05 M acetate buffer (for a pH range of 4.0-6.0) and in 0.05 M Tris buffer (for a pH range of 6.0-8.0). The assay was carried out at these respective pH values.

2.2.4. Temperature optima and thermal stability

An appropriate aliquot of Pectinex Ultra SP-L was incubated with 1 ml of 1% chitin at different temperatures. After 30 min, the amount of reducing sugar was estimated by dinitrosalicylic acid method [8]. Thermal stability was determined as given in Fig. 3.

2.2.5. Determination of kinetic parameters

The Michaelis constant was calculated using Hanes-Woolf plot, which is a graphical presentation of the substrate concentration divided by reaction velocity against substrate concentration divided by reaction velocity divided by reaction velocity.

2.2.6. Purification of pectinase

Pectinase was purified from Pectinex Ultra SP-L by affinity precipitation technique using the procedure described by Gupta et al. [11]. A 650U of pectinase was added to 0.3% of alginate (Protanal) and precipitated by adding 0.1 M CaCl2. The polymer-bound precipitate with 0.05 M acetate buffer, pH 5.9 (containing 0.5 M NaCl and 0.2 M CaCl2) till no enzyme activity could be detected by adding dinitrosalicylic acid method [8]. Thermal stability was determined using the Leonora software program [9]. The values of kinetic parameters were calculated using Hanes-Woolf plot, which is a graphical representation of the substrate concentration divided by reaction velocity against substrate concentration divided by reaction velocity.

2.2.7. Hydrolysis of chitin

For sequential hydrolysis, Pectinex Ultra SP-L (0.55 mg, in 1 ml of 0.05 M acetate buffer, pH 5.0) was incubated with 1 ml of 1% chitin (in 0.05 M acetate buffer, pH 5.0) at 37 °C for 1 h. After 30 min, the unhydrolyzed chitin was removed by centrifugation and the amount of reducing sugar formed was estimated in the supernatant (X). The unhydrolyzed chitin was then incubated with 2.75 mg of lysozyme (in 1 ml of 0.05 M acetate buffer, pH 5.0) at 37 °C for 2 h. The amount of reducing sugars produced was estimated (Y). The total reducing sugars generated by sequential hydrolysis was obtained by adding X + Y.

For synergistic action, 1% chitin was incubated with both the enzymes, Pectinex Ultra SP-L and lysozyme (5 mg), in 1 ml of 0.05 M acetate buffer, pH 5.0 at 37 °C for 2 h. The hydrolysis of chitin by lysozyme alone was monitored by incubating 1 ml of 1% chitin with 2.75 mg of lysozyme (in 1 ml of 0.05 M acetate buffer, pH 5.0) at 37 °C for 2 h.

3. Results and discussion

Fig. 1 shows the pH optimum for chitinolytic activity of pectinase. It shows somewhat broader pH optimum in the range of 5.0-5.5 as compared to the sharper pH optimum of 5.0 for pectinase activity [12]. It is interesting to note that the chitinases in general also have a broad pH optimum in the range of 5.0-6.0 [2,13].

The data regarding temperature optimum are quite interesting (Fig. 2). The chitinolytic activity has quite broad temperature optimum in the range of 55-65 °C. The pectinase activity of Pectinex Ultra SP-L has temperature optimum of 30 °C [14]. The temperature optimum of chitinases is reported to vary with the source and generally lies between 30 and 50 °C [7,15].

Fig. 3 shows the thermal stability of pectinase (at 50 and 55 °C) and chitinolytic (at 55 and 65 °C) activities. The half lives of chitinolytic activity work out to be 81 and 76 min, respectively, at 55 and 65 °C. The “thermostable chitinase” from Bacillus sp. BG-11 was stable at 50 °C for more than 2 h [18]. Half lives of the enzyme at 60, 70 and 80 °C are reported to be 90, 30 and 20 min, respectively.

The kinetic parameters for chitinolytic activity were calculated from Hanes-Woolf plot using the Leonora software program [9]. The Km was found to be 2.2 mg ml−1 for native chitin and Vmax was calculated to be 6.5 nmol min−1 mg−1. The Km value is in the same range as reported for chitinases from various sources. Chitinase from Lycopersicon esculentum has Km value of 10.5 mg ml−1 [7], from Bacillus sp. BG-11 has Km of 0.55 mg ml−1 (against swollen chitin) [16]. Recently, Xia et al. [13] have reported a novel chitinase from A. faigatus YJ-407 with a Km value of 1.12 mg ml−1 (against swollen chitin).

Fig. 4 shows the time course hydrolysis of chitin at 37 °C. It may be added that pectinase showed the same activity towards acid-swollen chitin as the untreated chitin (data not shown).
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Fig. 1. pH optimum of chitinase activity in Pectinex™ Ultra SP-L. Pectinex™ Ultra SP-L (1ml, containing 247U of pectinase activity) was incubated in 0.05 M acetate (O)/Tris (A) buffer at different pH values. After 30min incubation at 37°C, enzyme activity was determined using 1% chitin as a substrate. The enzyme activity at pH 5.0 is taken as 100%

At this point, it was thought prudent to check whether the chitinolytic activity of pectinase originates in some contaminating chitinolytic activity present in this commercial preparation. Hence, pectinase was purified by an earlier reported procedure based upon affinity precipitation by alginate. This method is reported to result in eight-fold purification (the specific activity of pectinase increases from 327.7 U mg⁻¹ to 2622 U mg⁻¹ on purification) and a preparation which shows single band on SDS-PAGE [11]. It was found that the purified preparation showed specific activity of 72 U mg⁻¹ as compared to 11 U mg⁻¹ of the crude preparation (with chitin as substrate). Thus, this protocol results in 8-fold purification for pectinase activity and 35-fold purification for chitinase activity. Two possible explanations could be: (a) the pectinase and chitinase activities reside on different populations of molecules present in Pectinex™; (b) the purification removes some inhibitor of chitinase activity. In view of the fact that affinity precipitation with alginate is a fairly selective

Fig. 2. Temperature optimum of chitinase activity in Pectinex™ Ultra SP-L. Pectinex™ Ultra SP-L (1ml, containing 247U of pectinase activity) was incubated with 1ml of 1% chitin (in 0.05M acetate buffer, pH 5.0) at different temperatures. The enzyme activity at 55°C is taken as 100%
Fig. 3. (a) Thermal stability of Pectinex® Ultra SP-L using polygalacturonic acid as substrate. The enzyme was incubated at 50°C (x) and 55°C (O). Aliquots were withdrawn at regular intervals, cooled to assay temperature and polygalacturonase activity was determined. (b) Thermal stability of chitinase activity in Pectinex® Ultra SP-L. Pectinex® Ultra SP-L (1 ml, containing 247 U of pectinase activity) was incubated at 55°C (O) and 65°C (A) in 0.05 M acetate buffer, pH 5.0. Aliquots were withdrawn at various intervals of incubation and enzyme activity was measured using 1% chitin as a substrate.

separation protocol and the purified pectinase does show a single band on SDS-PAGE, the second possibility is more likely. Further, it was also of interest to examine whether chitin competes with pectin for the enzyme. Fig. 5 shows that increasing concentration of chitin inhibits pectinase activity. Some details about the design of this experiment may be worth mentioning. \( K_m \) of the enzyme is 1.6mg ml\(^{-1} \) for polygalacturonic acid as substrate whereas it is 2.2 mg ml\(^{-1} \) for chitin as substrate. Thus, the normal substrate polygalacturonic acid has lower \( K_m \) value for the enzyme. Also, under the conditions (temperature and time), no detectable hydrolysis of chitin by the enzyme is observed (data not shown). It should be added that without detailed structural work, it may not be easy to decide whether both substrates are competing for the same active site. Dixon and Webb [19] and Webb [20] have mentioned against the indiscriminate use of kinetic data in assuming the mechanistic details in such situations. The latter, in fact, points out that in the cases when ‘inhibitor’ is also a substrate, the inhibition kinetics is quite complex.

Yalpani and Pantaleone [21] have examined the hydrolysis of chitosan by simultaneous and sequential enzyme treatment (lysozyme + either a chitinase or a hemicellulase or a lipase). Such approaches with combined enzyme actions improved the extent of chitosan hydrolysis. Fig. 6 indicates the results of similar approach when pectinase treatment was either followed by lysozyme treatment or both enzymes were simultaneously used for chitin hydrolysis. The treatments with lysozyme and pectinase, in terms of amounts of
enzyme and time period, were the same as used by Yalpani and Pantaleone [21] for chitosan hydrolysis. While lysozyme alone yielded ~15 nmol of reducing sugar in 2h, pectinase gave 27.6 nmol of reducing sugar within 30 min. The 30 min pre-treatment with pectinase followed by 2 h treatment with lysozyme resulted in ~3.5 nmol of reducing sugars. Thus, not surprisingly, the sequential treatment gives much higher hydrolysis than the sum total hydrolysis by individual action of these enzymes. Incubation of chitin with the two enzymes together for 2 h also produced the same amount (~35 nmol) of reducing sugar.

Thus, PectinexTM alone or along with lysozyme appears to be an efficient biocatalyst for chitin hydrolysis. It may be interesting to speculate at the reasons behind this at the molecular level. Aspartate residue is implicated in the active site of pectinases as a conserved residue [22]. It is noteworthy

Fig. 4. Time course hydrolysis of chitin by PectinexTM Ultra SP-L. PectinexTM Ultra SP-L (1ml, containing 247U of pectinase activity) was incubated with 1ml of 1% chitin (in 0.05M acetate buffer, pH 5.0) at 37°C. Aliquots were withdrawn at various intervals of incubation and the enzyme activity was determined.

Fig. 5. Inhibition of polygalacturonase activity at different concentrations of chitin. Different aliquots of the inhibitor were added from a stock solution of 2% (w/v) chitin and polygalacturonase activity was measured at 30°C [14].
that a recent paper indicates that carboxyl groups are an essential group for chitinase from *A. fumigatus* [13]. It is not unlikely that both chitinases and pectinases have similar active site designs and similar hydrolytic mechanisms. This makes the unusual activity of pectinase possible. The convenient availability of a relatively inexpensive preparation of a chitinase activity should make chitin biotransformation to value-added oligomers/monomers an attractive proposition.

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