Affinity precipitation of *Aspergillus niger* pectinase by microwave-treated alginate

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Abstract

Affinity precipitation is a simple, single plate separation process in which the complex of a smart macroaffinity ligand with the target protein (from a crude broth) can be selectively precipitated by application of a suitable stimulus. Alginate is a copolymer of guluronic acid and mannuronic acid residues and precipitates with Ca$^{2+}$ ions. It was found to bind to pectinase present in a commercial preparation of *Aspergillus niger*, Pectinex Ultra-SPL. Microwave pretreatment of alginate at 75 °C was found to enhance the selectivity of the affinity precipitation. Using microwave-treated alginate, 83% of the enzyme activity with 20-fold purification could be recovered. SDS-PAGE upon silver staining confirmed the enhanced selectivity of affinity precipitation when microwave-treated alginate was used.

**Keywords:** Affinity precipitation; Affinity ligands; Alginate; Microwave pretreatment; Pectinase; Protein purification

The purification of pectinase by affinity precipitation was one of the early applications of alginate as a macroaffinity ligand [1]. Subsequently, alginate was also found useful in affinity precipitation of lipase [2], peanut phospholipase D [3], and wheat germ amylase [4]. Affinity precipitation is a simple, scalable, and non-chromatographic technique which exploits the affinity of a smart macroaffinity ligand with the target protein present in the crude broth [5]. The complex of the target protein-macroaffinity ligand is precipitated by applying a suitable stimulus which is known to make macroaffinity ligand insoluble. One limitation of affinity precipitation is that being a single plate process, it tends to give lower fold purification than affinity chromatography. This is especially so when one is using an affinity interaction (like between alginate and pectinase) which is really not a case of true biospecific molecular recognition. Recently, microwave pretreatment of chitin was found to increase $V_{max}=K_m$ of chitinase, which was almost totally due to reduction in $K_m$ value [6]. Scanning electron microscopy (SEM) and powder X-ray diffraction revealed definite morphological changes in chitin. This indicated that it may be possible to obtain subtle changes in interactions between a polymeric affinity ligand and various proteins (including target protein pectinase) present in the crude broth.

The present work evaluates the changes in performance of alginate (in the purification of pectinase by affinity precipitation) as a result of its microwave pretreatment at a constant temperature. It is shown that the approach led to substantial improvement in the extent of purification of the enzyme without reducing the recovery of the biological activity. Pectinase is extensively used in food processing industry [7] and in hydrolysis of cellulosic biomass [8]. The improved affinity precipitation design described here would be useful in its purification.

**Materials and methods**

**Materials**

Protanal LF 10/60 (from brown seaweed, containing high guluronic acid content, i.e., 65-75%) was a product...
of Protan (Drammen, Norway). Polygalacturonic acid was purchased from Sigma (St. Louis, MO, USA). Pectinex Ultra SPL (a commercial preparation of pectolytic enzymes from a selected strain of Aspergillus niger) was from Novo Nordisk, Denmark, and was obtained from Arun and Co., Mumbai, India. All other chemicals used were of analytical grade.

Methods

Determination of enzyme activity
Pectinase activity was estimated by taking polygalacturonic acid as substrate [1]. One enzyme unit is defined as the amount of enzyme which liberates 1 lmol of reducing sugar (calculated as galactouronic acid) per minute at 30 °C. The amount of reducing sugar produced was estimated by the 3,5 dinitrosalicylic acid method [9].

Determination of protein
Protein was estimated by the dye binding method using bovine serum albumin as the standard [10].

Preparation of alginate solution
Alginate (2%, w/v) was dissolved in 0.05 M acetate buffer, pH 5.0. The solution was stored at 4°C and diluted with buffer for further use.

Microwave treatment of alginate
Protanal alginate solution (20 mL, 2%, w/v) was taken in a beaker and placed in the microwave oven (Model RM2001, Plazmatronika, Wroclaw, Poland, operating frequency 2.5 GHz). The oven had built-in magnetic stirrer, which was used to stir the alginate solution. The irradiation was carried out for 30 min at different temperatures (40, 50, 60, 75, and 90 °C). A control was heated in a water bath (under the same conditions as for the microwave-irradiated sample) in an identical fashion but without microwave irradiation.

Affinity precipitation of pectinase with alginate [1]
Different aliquots of Pectinex Ultra SPL (containing 4032 U/mL of pectinase activity) were incubated with 0.4 mL of microwave-irradiated alginate (stock solution of 2%, w/v) and the final volume was made up to 4 mL with 0.05 M acetate buffer, pH 5.0. The pH of the reaction mixture was adjusted to 3.8 with 3 M acetic acid, since this is reported to be the optimum pH for binding of the enzyme to alginate [1]. The solution was then incubated at 25 °C for 1 h. The polymer-enzyme complex was precipitated by addition of 0.4 mL of 1 M CaCl2 (final concentration of CaCl2 in the solution was 0.1 M). The precipitate was separated by centrifugation (10,000 rpm for 10 min) and washed thrice with 4 mL of 0.05 M acetate buffer, pH 5.0, containing 0.1 M CaCl2. The difference in the total enzyme activity loaded and the activity present in the supernatant and washings represented the amount of enzyme bound to the polymer.

The bound enzyme was recovered by following a slight modification of the procedure described earlier [1]. The polymer-enzyme complex was dissolved in 6 mL of 0.05 M acetate buffer, pH 5.0, containing 1 M NaCl and incubated at 4°C for 18 h. Thereafter, the polymer was precipitated and removed as described before. The salts (NaCl and CaCl2) present in the eluate were removed by passing the eluate through a PD-10 column (prepacked Sephadex G-25 column, Amersham Biosciences AB, Uppsala, Sweden) before estimating the recovered enzyme activity and protein.

A similar protocol was carried out with conventionally heated control and untreated alginate.

Polyacrylamide gel electrophoresis
SDS-PAGE of protein samples was performed using 12% gel according to Hames and Rickwood [11] on a Genei gel electrophoresis unit (Bangalore Genei, Bangalore, India) and standard molecular mass markers (Bangalore Genei). Silver staining of the gel was done according to the method described [11].

Results and discussion

One critical issue in deciding about any process design is that it should be possible to ensure proper control
of conditions. With the availability of commercial microwave reactors with temperature control, it is now possible to carry out microwave irradiation in a controlled and reproducible fashion. Microwave pretreatment has been found to be useful in hydrogenolysis of lignins [12] and hydrolysis of cellulosic waste [13]. Its usefulness in chitin hydrolysis has already been mentioned [6]. The microwave irrigation in the present work was carried out with a microwave reactor equipped with a non-contact infrared feedback controller which allowed fixing a constant temperature during microwave irradiation. To distinguish the effect of microwave irradiation per se, a control was also run wherein the alginate was also kept at the corresponding temperature in a conventionally heated incubator for the same amount of time.

Fig. 1 shows the amount of alginate precipitated vs Ca\(^{2+}\) concentration. It is known that 'the diaxially linked guluronic acid residues in alginate form cavities that function as binding sites for ions. Bonds formed...
between such sites and similar sequences in other polymer chains give rise to junction zones in the network [14]. Higher content of guluronic acid residues is known to require less Ca\textsuperscript{2+} concentration for complete precipitation [14]. The data in Fig. 1 show that microwave treatment results in reduction in availability of guluronic acid residues for interaction with Ca\textsuperscript{2+} ions. Thus, whereas alginate required only 0.02 M CaCl\textsubscript{2}, microwave-treated alginate required 0.1 M CaCl\textsubscript{2} for complete precipitation. This is in agreement with morphological changes obtained in chitin by SEM upon microwave treatment [6].

Fig. 2 shows the variation in pectinase activity and total protein bound (to alginate) with changes in the temperature of microwave irradiation. While no significant variation in binding of either was seen, 75 °C pretreatment gave the best selectivity (i.e., maximum pectinase activity and least amount of total protein bound to alginate). Hence, microwave irradiation at 75 °C for 30 min was used for carrying out further work.

Fig. 3 gives the variation in the selectivity at the binding stage with changes in the amount of pectinase activity which was initially added to the alginate solution. It was seen that the total protein bound to
microwave pretreated alginate was less than the total protein bound to untreated and alginate kept at 75 °C by conventional means (i.e., water bath, no microwave irradiation) at all activity loads. As far as binding of enzyme activity was concerned, untreated, heat-treated, and microwave-treated alginate showed similar pattern of behavior at various activity loads (Fig. 3).

Based upon earlier work [1], the elution of the enzyme activity from the alginate-pectinase complex was carried out by exposing the complex to 1 M NaCl solution in acetate buffer, pH 5.0 (details given in Materials and methods). Again while enzyme activity eluted did not change much with the initial activity applied to the polymer in all the three cases of untreated, heat-treated, and microwave-treated alginate, eluted protein profile showed useful variation (Fig. 4). Less protein could be eluted in the case of microwave-treated alginate.

Fig. 5 shows that as a consequence of this behavior, maximum fold purification (i.e., fold purification of 20) was obtained with microwave-treated alginate when initial activity load was around 400 U.

Fig. 6 shows the UV difference spectra of protein bound to alginate initially and after elution with 1 M NaCl. The spectral data confirm that greater amount of protein remains bound in the case of microwave-treated alginate (Fig. 6). Table 1 provides the corresponding data on the extent of protein bound to untreated and microwave-treated alginate at various stages of affinity precipitation. The data also show that in the case of microwave-treated alginate, a larger percentage of protein remains bound by hydrophobic forces and thus is eluted with 0.2% (v/v) Triton X-100. Microwave treatment has been reported to lead to dielectric loss and

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**Table 1**

Protein (present in Pectinex Ultra-SPL) bound to alginate at various stages of affinity precipitation

<table>
<thead>
<tr>
<th>Steps</th>
<th>Protein (%)</th>
<th>Untreated alginate</th>
<th>Microwave-treated alginate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein loaded</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Protein bound to polymer after affinity precipitation</td>
<td>48</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td><strong>Elution</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein eluted with 1 M NaCl in 0.05 M acetate buffer, pH 5.0, at 4 °C, 18 h</td>
<td>8</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Protein eluted with 0.2% (v/v) Triton X-100 at 4 °C for 18 h</td>
<td>10</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

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**Table 2**

Purification of Pectinase (from Pectinex Ultra-SPL) with untreated and microwave-treated Protanal alginate

<table>
<thead>
<tr>
<th>Steps</th>
<th>Activity (U)</th>
<th>Protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Untreated alginate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>400</td>
<td>1.34</td>
<td>298</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Supernatant + washing of precipitate</td>
<td>0.0</td>
<td>0.71</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Elution</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 M NaCl 0.05 M acetate buffer, pH 5.0, at 4 °C, 18 h</td>
<td>324</td>
<td>0.11</td>
<td>2945</td>
<td>81</td>
<td>10</td>
</tr>
<tr>
<td><strong>Microwave-treated alginate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>400</td>
<td>1.34</td>
<td>298</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Supernatant + washing of precipitate</td>
<td>0.0</td>
<td>0.90</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Elution</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 M NaCl in 0.05 M acetate buffer, pH 5.0, at 4 °C, 18 h</td>
<td>333</td>
<td>0.056</td>
<td>5946</td>
<td>83</td>
<td>20</td>
</tr>
</tbody>
</table>
increase in non-polarity of medium [15]. Table 2 is the purification table using these optimized conditions. Recovery (83%) of enzyme activity along with 20-fold purification was obtained by affinity precipitation with microwave-treated alginate. In comparison, affinity precipitation with untreated alginate led to 10-fold purification with 81% recovery of enzyme activity (Table 2). Fig. 7 shows the SDS-PAGE of the starting preparation along with pectinase preparation obtained after affinity precipitation with untreated, heat-treated, and microwave-treated alginate. The use of microwave-treated alginate led to the preparation with much higher purity level. The main protein band (indicated by an arrow) corresponds to the molecular weight of 55kDa which agrees with the reported molecular weight of pectinase from this source [1].

While it would be necessary to evaluate this strategy with larger number of systems, it appears that microwave treatment may be a useful approach to enhance the selectivity of the macroaffinity ligands in affinity precipitation.

It may be relevant to briefly comment about the effect of microwaves per se on chemical substances. There are two conflicting views about the way microwaves affect chemical changes [16]. Some workers believe that there are no non-thermal effects and the so-called non-thermal effect is due to superheating of solvents, others believe that the increase in the rate of reactions is so high that it cannot be accounted for by heating effects alone. The results in the present work suggest that microwave irradiation does cause changes which are non-thermal in origin.

Acknowledgments

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