Purification of Native and Modified Enzymes Using a Reactive Aqueous Two-Phase System

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Abstract: We describe the purification of native and modified enzymes using a reactive aqueous two-phase system. The two-phase systems consist of a hydrophilic AKM copolymer and dextran. Cellulase was modified with the AKM copolymer; the degree of modification is directly proportional to the number of maleic acid anhydride groups present in the AKM copolymer. Partitioning of the native and modified cellulases was also investigated in the aqueous two-phase AKM copolymer/dextran systems; the partition coefficient increases as the degree of modification in creases. The AKM-0531 copolymer is enzyme selective; the modified enzyme always shows separation that is better than that of the native enzyme.

Keywords: aqueous two-phase system, partitioning, enzyme, copolymers

Introduction

The stability of many industrial aqueous systems is governed by the interactions between the macromolecules. Phase separation is the most common phenomenon occurring in aqueous polymer/polymer systems; it arises from thermodynamic incompatibility [1,2]. Because of easy phase separation, aqueous two-phase systems (ATPS) are frequently adapted in the recovery of proteins and biomolecules. These ATPS have been studied in some detail over the past 30 years and, more recently, the application of ATPS for the extraction of recombinant proteins has been exploited by industry [3-6]. Enzymatic conversion of waste cellulosic materials to lower-molecular-weight chemicals is a very promising process because cellulose is the most abundant renewable resource. Enzymatic hydrolysis has the advantages of sparing energy and avoiding the use of toxic substances or corrosive acids because of its relatively mild reaction conditions. Therefore, cellulose hydrolysis catalyzed by cellulase has been investigated widely [7], but one of the major problems associated with this process is the high cost and consumption of the cellulolytic enzyme [8]. Park [9] has modified cellulase with synthetic copolymers, including polyoxalkylene derivatives. These modified cellulases are soluble and stable in the presence of organic solvents and retain their activity over wide ranges of temperature and pH. It may be expected that cellulases modified with copolymers would be useful for separating the cellulase into the copolymer phase when using the reactive aqueous two-phase system.

In this study, we first modified the, native enzyme with various copolymers to make it less sensitive to physical and chemical factors, such as pH and temperature, and then we conducted a partitioning study of the native and modified enzymes in these ATPS.

Experimental

Materials

Enzyme Y-NC (Yacult Co., Japan) from Aspergillus niger was used in this work; its protein content was 21.1% and impurities, such as reducing sugars, were 32.4%. The activity of this cellulase was 0.19 FPase. The FPase activity was assayed using the method reported by Mandel and coworkers and applying FP-SC (Tokyo Roshi Ltd., Japan) as a standard substrate. An activity of 1 unit (1 FPase) is defined as the amount of enzyme required to
Table 1. Characteristics of Synthetic Copolymers.

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>EO/AO (wt%)</th>
<th>n</th>
<th>k</th>
<th>MW</th>
<th>CPT (°C)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKM-0531</td>
<td>100</td>
<td>10</td>
<td>30</td>
<td>18,000</td>
<td>100 &gt;</td>
</tr>
<tr>
<td>AKM-1015</td>
<td>100</td>
<td>19</td>
<td>14</td>
<td>14,000</td>
<td>100 &gt;</td>
</tr>
<tr>
<td>AKM-1511</td>
<td>100</td>
<td>33</td>
<td>10</td>
<td>16,000</td>
<td>100 &gt;</td>
</tr>
<tr>
<td>AKM-2010</td>
<td>100</td>
<td>41</td>
<td>11</td>
<td>21,000</td>
<td>100 &gt;</td>
</tr>
</tbody>
</table>

*Cloud point temperature (CPT) measured using a 10 mg/mL aqueous solution of copolymer.

![Figure 1](image)

Figure 1. Modification of cellulase.

produce 1 µmol of reducing sugar from the substrate per minute. The reducing sugar content was determined by the dinitrosalicylic acid (DNS) method using glucose as a standard [9]. Dextran having a MW of 505,000 was used with AKM to form the ATPS. Four synthetic copolymers of AKM-series (Nippon Oil & Fats Co., Japan) were used in this study; their characteristics are shown in Table 1. These are alternating copolymers that are derived from the reaction of polyoxyethylene glycol (PAG) with maleic acid anhydride (MA). Here, k is the degree of copolymerization and n is the number of alkylene oxide (AO) units, which represents the total number of ethylene oxide (EO) and propylene oxide (PO) units in the PAG chain. All of these AKM copolymers consist of 100% EO in the PAG chains, but they have different values of k and n. The value of k indicates the number of MA units and n is a measure of the hydrophobic or hydrophilic character of the copolymer. As n increases, the hydrophilic nature of the copolymer increases.

Cloud Point Temperature (CPT) Measurement
The cloud point temperature (CPT) is the temperature at which phase separation begins. At this temperature the solution becomes turbid/cloudy because of the formation of polymer-rich emulsion droplets. For CPT measurement, we dissolved 10 mg of AKM copolymer in 1 mL of water. The CPTs were measured by immersing a capped glass tube containing the polymer solution in to a thermostable bath. The temperature was increased slowly (1°C increments) and the CPT was recorded at the first sign of turbidity. The CPT values of all these AKM-series of copolymers in copolymer solutions were > 100°C, as was reported also by Persson and coworkers [10].

Modification of Cellulase
The modification of cellulase was carried out by reacting a cellulase solution with AKM copolymer for 2 h at pH 8.0 ~ 8.2 and at a temperature of 0°C [11]; the reaction scheme is shown in Figure 1. As the reaction proceeds, the pH decreases, as a result of the production of carboxylic acid, which we controlled by the successive addition of 0.2 N NaOH. After the modification reaction, the degree of modification (DM) was calculated by measuring the concentration of amino groups of the native and modified cellulases using the trinitrobenzenesulfonic acid (TNBS) method [12] with UV-2401. The DM of the modified cellulase is defined as

\[
DM = \frac{[\text{Unmodified } \text{NH}_2 \text{ units of modified cellulase}]}{[\text{Total } \text{NH}_2 \text{ units of native cellulase}]}
\]

Two-Phase Partition of Native and Modified Cellulases
Native cellulase was immobilized with AKM copolymer using the modification reaction shown in Figure 1.
Figure 2. Working methods of two phase partition.

The partitioning of the native and modified cellulases was conducted using the approach shown in Figure 2. Case 1 and Case 2 describe the methods used to determine the moving ratio of the modified and native cellulases, respectively. First of all, dextran and AKM copolymer (20% w/v each) were mixed and the native or modified cellulase (1 mL) was added. This mixture was then shaken at 300 rpm for 10 min and separated after centrifugation at 3000 rpm for 10 min. A portion (1 mL) of samples from the top and bottom layers were then separated and acetic acid buffer (pH 5.2) was added to both samples for saccharification. Two samples (1 mL) were selected again; in the first sample, a filter paper substrate was added, and the mixture was shaken at 50 rpm for 30 min in a water bath. Finally, the concentrations of glucose were determined in both this mixture and in the second sample by using the DNS method suggested by Miller [13].

Results and Discussion

Cellulase was modified with AKM copolymer and the degree of modification and activity of this modified cellulase were determined; the values are plotted with respect to the polymer concentration in Figure 3. We found that DM increases upon increasing k and decreasing n. This result occurs because a higher value of k means that a greater number of MA groups are available for modi-
Purification of Native and Modified Enzymes Using a Reactive Aqueous Two-Phase System

Figure 4. Phase diagram of dextran 505000 and AKM copolymer.

Figure 5. Partition coefficients of enzymes vs the chain length of polyethylene oxide (n).

Figure 6. Partition coefficient of the modified enzyme vs the concentration of AKM-0531.

ification and a lower value of n represents fewer AO groups, which may offer a smaller degree of steric hindrance to cellulase and favor the modification reaction. Thus, DM is higher for AKM-0531. The behavior of the activity vs the copolymer-to-cellulose weight ratio is opposite to that of the DM with respect to this weight ratio. In this case, the activity increases with decreasing k and increasing n. The activity of the modified cellulase is inversely proportional to the DM. The activity is 80% when the DM is 58%, and it increases to 90% when the DM is 43%. The activity of the modified cellulase decreases initially, but it becomes almost constant with increases in the copolymer/cellulose weight ratio. This observation implies that cellulase becomes less sensitive to changes in the physical and chemical conditions after its immobilization with the copolymer.

Each AKM copolymer (0531, 1015, 1511, and 2010) and dextran were mixed for phase separation; phase diagrams of these mixtures are shown in Figure 4. The lower region of the line represents a single phase where no separation occurs. The upper region represents the two-phase region. Several researchers have made use of a theory developed by Flory and Huggins to describe the thermodynamics that lead to phase separation. According to their theory, water does not play a key role in determining the phase separation; rather, it is the polymer interaction that controls the phase separation [14]. Thus, a high concentration of polymer is also required to achieve phase separation when a copolymer having a low value of n is used.

Mixtures of each AKM polymer and dextran were used as ATPS to study the phase separation of native and modified cellulase; the partition coefficient curves are shown in Figure 5. The partition coefficient of the native and modified cellulases decreases as the n value of the AKM copolymer increases; the modified cellulase always shows a higher partition coefficient than does the native one. We also observed that AKM-0531 shows the best separation of cellulase thus, it displays the highest value of partition coefficient among the four AKM copolymers. Therefore, we selected the AKM-0531 copolymer to study the effect of the concentration of the copolymer on the partition coefficient of the modified cellulase; the results are shown graphically as a partition-coefficient-vs-AKM-0531-concentration curve in Figure 6. As the concentration of AKM increases, cellulase moves more effectively to the AKM copolymer phase. However, the partition coefficient does not improve substantially for AKM-0531 concentrations > 11 w/v%. This finding may be due to the volume difference between the top and bottom layers. When 6% of the copolymer is used, the volume of the top layer is very small and cannot provide enough space for cellulase to move into it. Upon adding more the copolymer, the volume of top layer increases
and, thus, partition coefficient increases sharply. As the concentration of the AKM-series copolymer increases, the modified enzyme moves into the AKM-copolymer layer because the affinity of the modified enzyme increase. Upon adding more AKM copolymer, the partition rate also increases. Thus, phase separation of cellulase is effective at higher concentrations of the AKM polymer. This observation is inconsistent with the Flory and Huggins theory. A two-phase method of glucose partition was performed and the results (partition coefficient data) are presented in Table 2. The same definition of partition is used for both cellulase and glucose. Thus, partition of glucose is better when the partition coefficient value is low. Therefore, it is clear from Table 2 that glucose moves to the dextran layer effectively in case of the AKM-0531/dextran system. Thus, partition of cellulase is better in AKM-0531 copolymer, which, therefore, is cellulase selective. Consequently, a relatively high concentration of cellulase appeared, rather than a high glucose concentration, in the AKM layer.

<table>
<thead>
<tr>
<th>n</th>
<th>Native Glucose PC</th>
<th>Modified Glucose PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.50</td>
<td>0.33</td>
</tr>
<tr>
<td>19</td>
<td>0.39</td>
<td>0.55</td>
</tr>
<tr>
<td>33</td>
<td>0.44</td>
<td>0.70</td>
</tr>
<tr>
<td>41</td>
<td>0.78</td>
<td>0.98</td>
</tr>
</tbody>
</table>

**Conclusions**

Reactive two-phase separation of native and modified enzymes has been studied. We used AKM copolymer to modify the enzyme, and dextran was used to form two phases with the AKM copolymer. In the modification reaction, the activity decreases as the degree of modification increases, and the degree of modification of the enzyme increases as the number of maleic acid functional groups of the polymer increases. The partition coefficient of the enzyme is greatly affected by the type of AKM copolymer used in the reactive two-phase separation. The partition coefficient of the enzyme decreases as the length of the EO chain of the polymer increases, and it increases with increases in the value of k. Thus, AKM-0531 copolymer is enzyme selective and the modified enzyme always shows better partitioning than does the native one.

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**References**