Bioconversion in an aqueous two-phase system using a smart biocatalyst: casein hydrolysis by a α-chymotrypsin derivative

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Abstract
Casein hydrolysis was carried out in PEG-dextran two-phase system by using a α-chymotrypsin immobilized on a water soluble polymer Eudragit S-100. This biocatalyst along with the substrate casein was predominantly found to partition into the PEG phase. Under these optimized conditions, the product was found to partition (84%) in the dextran phase. Removal of dextran phase at an appropriate time interval and replacing it with fresh dextran phase led to considerable enhancement of casein hydrolysis. The biocatalyst could be separated from the PEG phase by lowering the pH to 3.8 and again dissolved in PEG phase by increasing the pH to 7.6. Thus, this smart biocatalyst could be reused for casein hydrolysis in PEG-dextran two-phase aqueous system.

Keywords: α-Chymotrypsin; Casein hydrolysis; Smart biocatalyst; Aqueous two-phase bioconversion

1. Introduction
Extractive bioconversion in aqueous two-phase systems has been frequently employed for degradation of macromolecular substrates like polysaccharides and proteins [1,2]. This technique integrates bioconversion with the removal of the product by two-phase extraction. Mukataka et al. [1] have described the hydrolysis of casein by α-chymotrypsin in PEG-dextran system. In the present work, we describe the hydrolysis of casein in the same two-phase system by a smart biocatalyst prepared by covalently linking α-chymotrypsin to a reversibly soluble insoluble polymer Eudragit S-100. Use of smart biocatalyst allows the recovery of the enzyme catalyst after reaction as well as reuse of costly PEG-phase.

2. Materials and methods

2.1. Materials

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Sigma (St. Louis, MO, USA). Chymotrypsin was procured from SRL (Sisco Research Laboratories), Mumbai, India. All other chemicals were of analytical grade.

Eudragit S-100 was purchased from Rohm Pharma GmbH, Weiterstadt, Germany. Dextran T 500 was purchased from Pharmacia Fine Chemicals, Sweden. Polyethylene glycol 6000 was obtained from E. Merck, Mumbai, India.

3. Methods

3.1. α-Chymotrypsin derivative preparation

Preparation of bioconjugate of α-chymotrypsin with Eudragit S-100 by EDC coupling by following the method described by Arasaratanam et al. [3]. The derivative (Eud-C) could be precipitated by lowering the pH to 4.5 just like Eudragit S-100 and the enzyme retained 94% of its caseinolytic activity upon conjugation with the polymer (MN Gupta, unpublished results).

3.2. Determination of proteolytic activity and protein

The proteolytic activity of chymotrypsin and its derivative was estimated using casein as the substrate [1]. Amount
of protein was estimated according to Bradford [4] using bovine serum albumin as the standard protein.

3.3. Preparation of aqueous two-phase systems containing the enzyme (or its derivative) and the substrate

To 10 ml of the graduated centrifuge tubes, the desired phase components in the order of PEG solution (1.5 ml from a stock solution of 4.5% (w/v) polyethylene glycol 6000 in 50 mM phosphate buffer, pH 7.6), dextran solution (1.5 ml stock of 7% dextran T-500 (w/v) in 50 mM phosphate buffer, pH 7.6), enzyme (27 U containing 0.15 mg or its conjugate (27 U containing 0.13 mg protein) and casein (150 ml of 100 mg/ml in 50 mM phosphate buffer, pH 7.6) were added. After vortexing for a minute, two distinct phases were formed within 5 min at 37 °C. The upper phase (PEG) was 1.6 ml and the lower dextran phase was 1.4 ml. The amount of hydrolysis products formed were estimated in the lower dextran phase.

3.4. Casein hydrolysis

Casein hydrolysis was studied at 37 °C using PEG-dextran aqueous two-phase system formed in 50 mM phosphate buffer, pH 7.6. The amount of product formed was measured as a function of time by removing an aliquot (by piercing) from the lower dextran rich phase. As a control, casein hydrolysis was also studied with free a-chymotrypsin in 50 mM phosphate buffer, pH 7.6.

The reuse of Eud-C was checked as follows. After every half an hour the lower dextran phase was removed and tested for the amount of product formed. Simultaneously, the upper PEG phase was precipitated by lowering the pH to 3.8 (such that both the biocatalyst as well as the unhydrolysed substrate precipitates) and separated by centrifuging at 11,570 x g for 10 min. Thereafter, the precipitate was again dissolved in the PEG phase (4.5%, pH 7.6) and the procedure was repeated as before till no product could be detected in the supernatant.

4. Results and discussion

Table 1 gives the optimized conditions (of PEG (%), dextran (%), and buffer molarity and pH) under which the substrate casein predominantly partitions into the PEG phase and the product (represented by) tyrosine partitions into the dextran phase. a-Chymotrypsin shows the partition coefficient of unity in these phases. The last is in agreement with the observations of Mukataka et al. [1]. The conjugate of a-chymotrypsin with Eudragit (Eud-C), however, partitions mostly (94%) in the PEG phase. Thus, highly favorable partitioning of the Eud-C (catalyst) and casein (substrate) in the PEG and that of tyrosine (product) in the dextran phase constitute a useful design for the hydrolysis of casein.

![Fig. 1](image_url)  
**Fig. 1.** Casein hydrolysis. Casein hydrolysis was studied at 37 °C using PEG (4.5%) and dextran (7%) in 50 mM phosphate buffer, pH 7.6. The amount of product formed was measured spectrophotometrically at 280 nm as a function of time. An aliquot (100 ml) was removed from the aqueous buffer and two-phase system. For the two-phase system, the lower dextran phase was pierced to remove the aliquot.

<table>
<thead>
<tr>
<th>Tyrosine (%)</th>
<th>Casein (%)</th>
<th>a-Chymotrypsin (%)</th>
<th>Conjugate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG</td>
<td>15</td>
<td>80</td>
<td>50</td>
</tr>
<tr>
<td>Dextran</td>
<td>84</td>
<td>9</td>
<td>50</td>
</tr>
</tbody>
</table>

The pH optimum (for casein hydrolysis) of the Eud-C in the PEG phase was found to be in the same range (pH 7.8) as the pH optimum of the free a-chymotrypsin in the aqueous buffer (data not shown). The temperature optimum of Eud-C in the PEG-dextran phase as well as in aqueous buffer was found to be 37 °C (data not shown). Hence, this temperature was chosen for carrying out casein hydrolysis in the two-phase system. Fig. 1 gives the time course for the hydrolysis of casein by Eud-C in the two-phase system described above. The hydrolysis by a-chymotrypsin in aqueous buffer is also shown for comparison. It can be seen that Eud-C was as efficient in PEG as it was in the simple aqueous buffer.

One way of increasing the product yield is to replace product phase with a fresh one [5]. The advantage of doing that is shown in Fig. 2. In this case, the dextran phase was removed every 30 min, the product concentration estimated by absorption measurement at 280 nm. Simultaneously, fresh dextran was added to continue the hydrolysis in the two-phase system. The absorbance reading at each time
Fig. 2. Bioconversion (O): The casein hydrolysis was carried out as in the case of Fig. 1. However, in this case, after every 30 min, (a) the amount of product formed was determined in the dextran phase and fresh dextran phase was added to continue product extraction. The absorbance reading at each time interval represents the total product formed till that point. Reusability experiment (A): This was carried out as above except that after every 30min, the pH of the PEG phase was lowered to 3.8. The precipitate formed was resuspended in the same PEG phase and the hydrolysis was continued.

While extractive bioconversion has been extensively studied for hydrolysis of starch [6] and cellulose [7], protein hydrolysis using this approach has been examined only in a limited fashion [1].

The Eud-C used in the present study shows more favorable partitioning in PEG phase as compared to PEG-chymotrypsin conjugate reported by Mukataka et al. [1]. More important, ‘smart biocatalyst’ used here allows its separation from the reaction system and is reusable. Use of such a smart biocatalyst in two-phase format, thus, constitutes a novel and attractive strategy for bioconversions.

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References