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Preparation of highly active α-chymotrypsin for catalysis in organic media

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Abstract—A simple one step process for the preparation of free α-chymotrypsin, using an organic solvent to precipitate the enzyme from a buffered solution, followed by washing with organic solvents, is described. This preparation gave 132 times greater esterification activity than lyophilized powder.

Enzymes are being increasingly used in organic synthesis.[1,2] One major concern in this regard has been their low activity in organic media where most of the synthetic applications are carried out. This is especially so in the case of commonly employed lyophilized enzyme powders. It is now well established that the major reason for this low activity is the inactivation during lyophilization.[3] In order to circumvent the lyophilization step, Partridge et al.[4] had described PREP (propanol-rinsed enzyme preparation) in which enzymes are immobilized on silica gel and 'dried' (water removed) by washing repeatedly with *n*-propanol. Thus, α-chymotrypsin PREP gave activity, which was 'two orders of magnitude better than the freeze-dried powder'.[4] Here, we demonstrate that by a similar modification of the method, it is possible to obtain the enzyme preparation with similar level of activity even in the free form (without immobilization).

The procedure used is essentially a combination of three different approaches: (a) precipitation of enzymes by alcohols,[5] (b) 'drying' of the precipitate by rinsing with *n*-propanol[4] and (c) use of high amount of salt during co-precipitation. This follows the rationale of using high concentration of KCl during lyophilization of enzymes.[6]

The precipitation of the enzyme was carried out from a solution at a pH corresponding to the pH optimum of the enzyme for its activity in aqueous buffers. pH tuning/pH memory is a well known phenomenon in nonaqueous enzymology.[7] Enzymes 'dried' from such pH have been shown to possess higher activity in low water containing organic solvents. The enzyme was precipitated by addition of *n*-propanol and water was also removed by repeatedly rinsing with the same water-miscible solvent. Partridge et al.[4] have mentioned *n*-propanol as one of the most suitable solvents for the purpose for obtaining active and dry enzyme precipitates. Table 1 shows the esterification activity of α-chymotrypsin preparation (in *n*-octane containing 0.3%, $v\,v^{-1}$, water) obtained with the enzyme precipitated and rinsed with *n*-propanol (EPRP). This level of water is

Table 1. Activity of α-chymotrypsin obtained as EPRP

Preparation	Initial reaction rate ($\mu mol\,h^{-1}\,mg^{-1}$) (esterification in *n*-octane, 0.3% $v\,v^{-1}$ water)
pH tuned/lyophilized	144
untuned/lyophilized	48
EPRP	6183

For pH tuning, the enzyme (100 mg) was dissolved in 10 mL of 20 mM Tris HCl, pH 7.8 and the solution was lyophilized. For EPRP, the enzyme (100 mg) was dissolved in 10 mL of 20 mM Tris HCl, pH 7.8 and the solution was cooled to 4 °C. This was followed by the slow addition of ice cold and dry *n*-propanol (at 4 °C) (in a volumetric ratio of 1:4) till precipitation of the enzyme occurred. After allowing the suspension to stand for 30 min, the suspension was centrifuged (12,000*g*, 5 min) at 4 °C. The precipitate was washed three times with 10 mL each of *n*-propanol (containing 0.3%, $v\,v^{-1}$, water) at room temperature, the supernatant liquid was carefully pipetted out and replaced with the organic solvent of choice. After washing the EPRP once with the desired solvent, the reaction was carried out.

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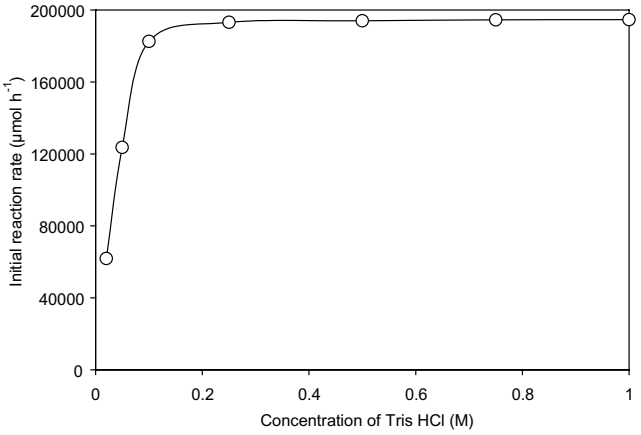

Figure 1. Effect of concentration of buffer salt on the rate of esterification reaction catalyzed by EPRP. The reaction was monitored by HPLC, following the esterification of *N*-acetylphenylalanine with ethanol.[12] α-Chymotrypsin (10 mg) was added to 1.9 mL of dried *n*-octane (a measured volume of water was added so that the final concentration of water in the reaction mixture is 0.3%, $v\,v^{-1}$). This was followed by the addition of 118 μL of ethanol containing 4 mg of *N*-acetylphenylalanine. The amount of product formed was monitored by HPLC using a Beckman C_{18} column, eluted with water/acetonitrile/acetic acid 55:40:5, and detection of absorbance at 258 nm.

within the solubility limit of water in *n*-octane.[8] This preparation was obtained with the starting enzyme solution in 0.02 M Tris HCl buffer, pH 7.8. EPRP showed 43 times higher initial rate (esterification in *n*-octane) as compared to pH tuned and lyophilized enzyme. The rate was 130 times higher as compared to untuned lyophilized enzyme.

Figure 1 shows that molarity of buffer in which enzyme is initially dissolved, plays a critical role in the activity of EPRP. Use of about 0.2 M buffer enhanced the initial rate by another order of magnitude. Thus, the overall increase in the initial rate (over pH tuned and lyophilized enzyme) was 134 times. This increase is of the same order as reported for PREP (immobilized preparation) of α-chymotrypsin.[4] The mechanism of salt activation of lyophilized enzyme powders has been discussed earlier[9] and seems to involve a variety of factors such as polarity of the enzyme active site and kosmotropicity of the activating salt. It may be added that the concentration of the salt used in the salt activation strategy is much higher (98% $w\,w^{-1}$) than the buffer concentration used in the present work. This is rather important since very high level of salt compromises the usefulness of the biocatalyst in industrial applications.[9] Secondly, the absence of any immobilization matrix constitutes a more efficient biocatalyst design since reactor volumes can be much smaller. The EPRP showed consistently higher esterification rates in solvents of differing polarity (Table 2), as compared to pH tuned, lyophilized α-chymotrypsin.[10]

Finally it may be added that unlike enzyme coated microcrystals, the procedure for obtaining EPRP does not involve any excipient like K_2SO_4.[11] Scanning electron micrograph (Fig. 2) also shows that this preparation is very different from enzyme-coated microcrystals.[11] The tunnelling electron micrograph of enzyme-coated microcrystals showed crystalline structures with an average length of the order of 500 nm.[11] The SEM of EPRP, even at one order lower magnification (i.e., at 5 μm), does not show any crystalline structure. In this respect, relatively speaking, EPRPs are closer to salt activated enzymes. The simple and convenient procedure described here may find general applications in the preparation of highly active enzymes for use in organic media.

Table 2. Activity of EPRP in different organic solvents

Solvent	Initial reaction rate of EPRP ($\mu mol\,h^{-1}\,mg^{-1}$)	Initial reaction rate of pH-tuned lyophilized enzyme[10] ($\mu mol\,h^{-1}\,mg^{-1}$)
Octane	19,320	144
Toluene	1615	12
t-Amyl alcohol	782	6
Tetrahydrofuran	140	1
Acetonitrile	174	<1
Dioxane	8	<1

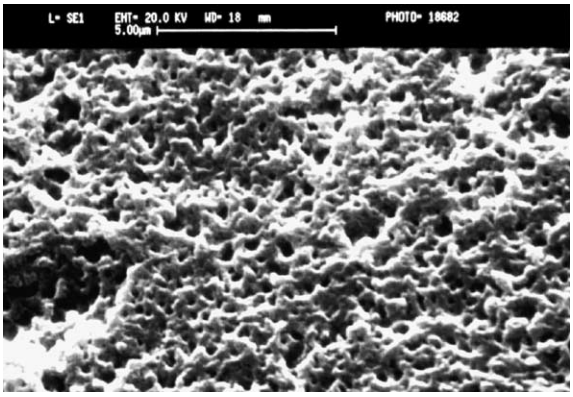

Figure 2. Scanning electron micrograph of EPRP at a magnification of 7500×. The bar represents 5 μm.

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