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Comparison of polymer induced and solvent induced trypsin denaturation: The role of hydrophobicity

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ABSTRACT

Trypsin adsorption from aqueous buffer by various copolymers of allyl glycidyl ether-ethylene glycol dimethacrylate (AGE-EGDM) copolymer with varying crosslink density increases with increasing crosslink density and the effect slowly wears off after reaching a plateau at 50% crosslink density. The copolymer with 25% crosslink density was reacted with different amines with alkyl/aryl side chains to obtain a series of copolymers with 1,2-amino alcohol functional groups and varying hydrophobicity. Trypsin binding capacity again increases with hydrophobicity of the reacting amine and a good correlation between $\log P_{octanol}$ of the amine and protein binding is observed. The bound trypsin is denatured to the extent of 90% in spite of the presence of hydrophilic hydroxyl and amino groups. The behavior was comparable to that in mixtures of aqueous buffer and water-miscible organic co-solvents where the solvent concentration required to deactivate 50% of the enzyme (C_{50}) is dependent on $\log P_{octanol}$ of the co-solvent.

1. Introduction

Protein separation and purification by hydrophobic interaction chromatography is now a well established technique [1] and several methodologies of predicting chromatographic separation of proteins have been developed with a fair degree of success [2,3]. However, in case of enzymes and bioactive proteins such as trypsin inhibitors, it is not enough to separate the enzymes from a protein extract by chromatography; they should also be catalytically active. Our previous studies on the relationship between surface hydrophobicity of allyl glycidyl ether-ethylene glycol dimethacrylate (AGE-EGDM) copolymers and that of enzymes such as α-chymotrypsin, alcohol dehydrogenase (ADH), glucose dehydrogenase(GDH) and alkaline phosphatase (CIAP) have shown that surface characteristics of both protein and the polymer play equally important roles in defining the adsorption efficiency as well as protein denaturation [4a,b]. Adsorption and denaturation of proteins follow the order YADH > α-chymotrypsin > GDH » CIAP although the molecular weights of proteins follow the order YADH > CIAP > GDH > α-chymotrypsin. These observations were quite similar to those observed in binary mixtures of aqueous buffers and water miscible organic co-solvents [5]. In light of our observations on the pattern of adsorption induced enzyme denaturation, it was interesting to investigate whether polymer induced denaturation and solvent induced denaturation follow similar trends. This would help in designing polymer supports for protein immobilization and chromatographic separation. With this aim, we have chosen trypsin as a model enzyme and AGE-EGDM epoxy activated polymer as a model copolymer. Since the copolymer is uncharged, it was also interesting to study the effect of introduction of hydrophilic and electrostatic interactions during protein binding and its denaturation. Towards this goal, the copolymer was modified by reaction with different amines to obtain a series of copolymers possessing amino groups with alkyl/aryl side chains (Scheme 1). At operating pH of 7–8.5, the substituted amino groups would be mostly protonated and hence the copolymers would possess a net positive charge. Trypsin with pI 10.5 would also possess a net positive charge and direct electrostatic interaction, which would complicate matters, would be avoided. At the same time, opening of epoxy group would generate a hydroxyl group that would introduce extra hydrophilicity, while the alkyl/aryl side chain on the substituted amine would contribute an additional hydrophobic residue. Thus a series of similar polymers with changing hydrophobicity would be generated. Herein we present our results on binding and denaturation of trypsin to these polymers

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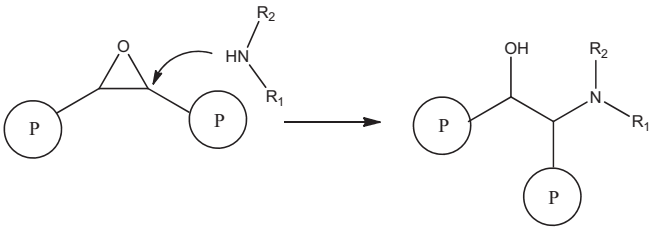

Scheme 1. Preparation of amine substituted polymers.

and compare the results with those obtained from trypsin activities in binary mixtures of several water-miscible organic solvents with varying hydrophobicity.

2. Materials and methods

2.1. Materials

Trypsin (cat.no.C7762), Nα-benzoyl-L-arginine ethyl ester (BAEE), N_α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA), 1-cyclohexyl-2-pyrrolidinone (CHP) and 2-butoxyethanol (2-BE) were obtained from Sigma–Aldrich, USA. CHP and 2-BE were redistilled under vacuum before use. All other reagents were of analytical grade obtained from HiMedia, India and used as received. Curve fittings were performed with Origin version 8.0, Microcal Software Inc. USA and GraphPad Prism Version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. All experiments were performed 3 times and were reproducible within ±5%.

Copolymers of allyl glycidyl ether (AGE)-ethylene glycol dimethacrylate (EGDM) with varying mole ratios of ethylene glycol dimethacrylate to allyl glycidyl ether were synthesized by suspension co-polymerization as described earlier [4a]. The copolymers were of average particle size of 100–150 microns and average pore diameter of 100–150 Å.

2.2. Methods

2.2.1. Modification of epoxy groups

AGE-25 (2 g) was stirred with amine solutions (50 mL, 25% (w/v)) in methanol–water (50% v/v) overnight. The polymer was then thoroughly washed successively with methanol and distilled water till pH of washing was neutral and did not show any absorbance in the UV region (210–230 nm) in case of aromatic amines. The polymers were then finally washed with methanol and dried under vacuum.

2.2.2. Protein adsorption experiments

The polymer (25 mg) was shaken with enzyme solution (2.4 mg/mL, 1 mL in aqueous buffer) in closed Eppendorf tube and shaken on an orbital shaker at 80 rpm for 1 h. Polymer was separated from the supernatant by centrifugation at 5000 rpm for 10 min. Supernatant was filtered through 0.2 μ filter for protein estimation by measuring its absorbance at 280 nm. Difference in absorbance of control (A_0), and that of the supernatant (A_s), gave the value for adsorbed enzyme. Same solution was used for enzyme activity assay. All experiments were repeated 3 times and were reproducible within±5%.

2.2.3. Trypsin assay

Activity of trypsin dissolved in buffer and aqueous binaries was measured at 25 °C on Cary 100 spectrophotometer using N_α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) as substrate in Tris–HCl buffer (50 mM, 1 mM $CaCl_2$, pH 8.2). Enzyme stock solution (10 mg/mL) was prepared in 1 mM HCl and stored in cold. In the final assay solution (2.5 mL) substrate concentration was 2.5 mM and the enzyme concentration was 1.66 μM. Change in absorbance was monitored at 410 nm ($\Delta\varepsilon$ 8800 M^{-1} cm^{-1}).

Activity of immobilized enzyme could not be measured by spectroscopic method due to adsorption of *p*-nitroaniline and N_α-benzoyl-L-arginine on polymer surface. It was hence measured by titrimetry using N_α-benzoyl-L-arginine ethyl ester as substrate in a temperature controlled double walled stirred vessel containing substrate solution (15 mL, 50 mM in 10 mM Tris–HCl buffer containing 0.1 M $CaCl_2$, pH 8.2, 25 °C). Enzyme solution or polymer (25 mg) bearing immobilized enzyme was added with vigorous magnetic stirring. Acid produced during hydrolysis was continuously titrated with 0.1 N NaOH maintaining the pH at 8.2 for 10 min. Enzyme activity units are expressed in terms of μmoles of NaOH consumed in 1 min. Native trypsin had activity of 60 units/mg.

3. Results and discussion

In our earlier studies on protein denaturation in aqueous binaries, we have demonstrated that Frank's classification of water miscible organic solvents into structure making "typically aqueous (TA)" and structure breaking "typically non-aqueous (TNA)" solvents [5] should be taken into account while predicting enzyme denaturation in aqueous binaries. Polar solvents such as dimethyl sulfoxide, acetonitrile and 1,4-dioxane form 'typically non-aqueous (TNA)' solutions in which solution thermodynamics is governed by the polar part of the molecule. In such solutions, hydrophilic co-solvents show specific hydrogen-bonding interactions between their polar part and water. On the other hand, solvents like alcohols, ethers, glycol ethers, and amines belong to 'typically aqueous (TA)' class. These solvents contain polar groups, but their dipole moments are rather small. They predominantly exhibit most of the features of hydrocarbons in water [6]. In water rich region, several properties such as sound velocity, ultrasonic absorption, compressibility and molar volume show anomalous behavior [7]. Extrema in activation parameters $\Delta H^{\#}$ and $\Delta S^{\#}$ have been observed in water catalyzed hydrolytic reactions in binary mixtures of water and organic solvents [8,9]. The solvent concentration at which a sharp change in properties is observed has been defined as 'critical hydrophobic interaction concentration' (CHIC) by Engberts and Haak [9]. It was suggested that at CHIC, the solvent molecules start aggregating. In our earlier studies on protein denaturation in aqueous binaries, we have observed that in case of TA solvents, the onset of aggregation of solvent molecules with large a polar parts at CHIC causes a fairly distinct change in enzyme activity due to hydrophobic interactions between the co-solvent and protein. Since the AGE-EGDM copolymer also possesses a large hydrophobic backbone along with polar ester and epoxy groups, the polymer can be viewed as a solidified TA solvent. Introduction of 1,2-amino alcohol function would increase its hydrophilicity and trypsin with its isoelectric point of pH 10.5 [10] would possess a net positive charge in the pH range of 7–8. This would minimize the complications of electrostatic interactions between the amino groups of the polymer and trypsin and also this might make the polymer to resemble a TNA solvent.

3.1. Polymer hydrophobicity and trypsin binding

Protein adsorption from aqueous buffer by various copolymers (AGE-5 to AGE-150) was studied under a standard set of conditions keeping constant protein concentration (2.4 mg/mL), polymer weight (25 mg), reaction volume (1 mL), temperature (30 °C) and contact time (1 h) in 50 mM Tris–HCl buffer containing 1 mM $CaCl_2$, pH 8.2. Protein adsorption was measured by monitoring the absorbance of the supernatant at 280 nm. It was observed that