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Effect of Polymer Shielding on Elution of G3PDH Bound to Dye-ligand Adsorbent

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Abstract Batch binding experiments were performed to assess the recovery performance of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) bound to the unshielded and polymer (polyvinyl pyrrolidone, PVP)-shielded dye-ligand (Cibacron Blue 3GA) adsorbent. The adoption of a polymer-shielded, dye-ligand technique facilitated the elution efficiency of bound G3PDH. It was demonstrated that the recovery of G3PDH using polymer-shielded dye-ligand adsorption yielded higher elution efficiency, at 60.5% and a specific activity of 42.3 IU/mg, after a low ionic strength elution (0.15 M NaCl). The unshielded dye-ligand yielded lower elution efficiency, at 6.5% and a specific activity of 10.2 IU/mg.

Keywords: dye-ligand, Cibacron Blue 3GA, G3PDH, elution yield, low ionic strength elution

Initially, chlorotriazine dyes such as Cibacron Blue 3GA, Procion Red H-E7B, Procion Green H-4G, and Yellow H-E3G, were designed for use in the textile and imprinting industries. The chlorotriazine dyes are known to show affinities for several classes of proteins, including dehydrogenase and phosphatransferase [1]. As a result, the chlorotriazine dyes have been widely exploited as ligands in affinity chromatography for the purification of protein products [2,3]. Cibacron Blue 3GA is one of the most extensively studied chlorotriazin dyes; it has been successfully immobilised on polysaccharide matrices, including agarose [4-6] and cellulose [7], for fluidised bed adsorption of protein products. Chase and Draeger [5] investigated the purification of phosphofructokinase from unclarified yeast homogenate by exploitation of Sepharose Fast Flow as the base adsorbent. Gilchrist [7] studied the adsorption of G3PDH from brewer's yeast disruptate to an enhanced density cellulose matrix. Here, a significantly low degree of cell debris fouling on the dye-ligand adsorbent was reported.

The term "pseudo-affinity" has been typically used to describe the dyes [8] because the dyes interact with target proteins based upon a rational mimicking of natural ligands (*i.e.* no biological relations to the desire protein). Typical dissociation constants, (K_d), of dye ligands commonly fall in the range of 10^{-6} to 10^{-7} M [9,10], which are intermediate between ion exchange, 10^{-4} to 10^{-6} M [11] and truly biospecific ligands, 10^{-8} to 10^{-12} M [12].

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Desorption of bound protein can be achieved by means of unspecific elution (*e.g.* NaCl, KCl, and KSCN), which perturbs the molecular conformations, and thus weakening the interactions between the proteins and dyes. It has been claimed that unexpected low recovery yields in dye-ligand adsorption and high ionic strength elution (*e.g.* 1.0~1.5 M NaCl or KCl) were required [13]. A high ionic strength condition may promote subunit dissociation of the protein and result in product inactivation [14].

In the present communication, a comparative study of unshielded and polymer-shielded dye-ligand recovery of intracellular proteins from unclarified yeast extract was performed in a batch binding study. The purification of an intracellular enzyme, glyceraldehyde 3-phosphate dehydrogenase (G3PDH), was selected as a suitable target for comparative testing of adsorption systems.

Baker's Yeast and Cell Disruption

Baker's yeast (1 kg, package size) was obtained from British Fermentation Products (BFP). The frozen baker's yeast (50% frozen wet weight per volume, w/v) was thawed overnight at 4°C in buffer A (10 mM Tris/HCl containing 1 mM EDTA, pH 7.5). Cell disruption of baker's yeast [15] for the release of intracellular enzyme (G3PDH) was performed by bead milling in a Dyno Mill KDL-I (Willi A. Bachofen AG, Switzerland). The resultant yeast homogenate was used immediately, or was stored at 20°C. It was then diluted to the required biomass concentration with buffer A (10 mM Tris/HCl containing 1 mM EDTA, pH 7.5) for subsequent use.

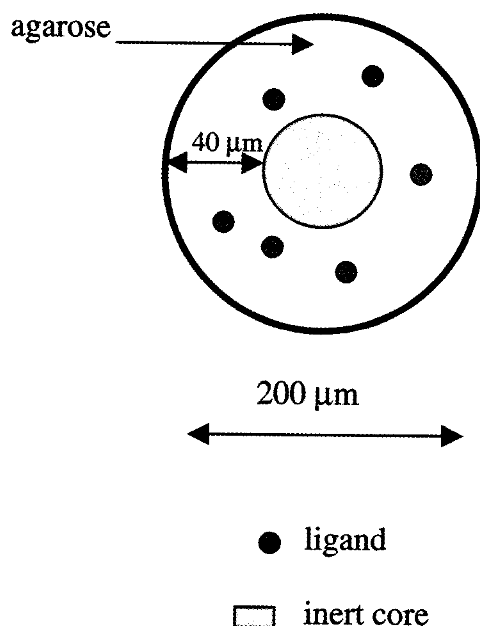

Fig. 1. The representation of structural characteristics of UpFront steel-agarose adsorbent. UpFront steel-agarose (151~323 μm) representing a pellicular adsorbent particle comprises an inert core (stainless steel) coated with a thin layer of porous agarose [15]. The diagram is not drawn to scale and the particle size of 200 μm is shown here as a representative example.

Adsorbent

UpFront steel-agarose (Fig. 1) is a pellicular expanded bed matrix with a size range of 151~323 μm [15]. It was obtained from UpFront Chromatography A/S, Denmark. The adsorbent comprising 6% cross linked agarose and stainless steel core. The Cibacron blue 3GA was immobilized onto UpFront adsorbent as described in a previous publication [15], and the dye-ligand concentration was estimated based upon a method described by Chambers [16]. The polymer-shielding of dye-ligand adsorbent was carried out according to the procedure described by Galaev *et al.* [17].

Desorption of Bound G3PDH Using Neutral Salt

The screening of elution conditions for bound G3PDH was performed using unclarified feedstock comprising 50% (w/v original cells) yeast disruptate at pH 6.1. Six universal bottles containing 9 mL of unclarified feedstock were prepared and 1 mL settled volume of unshielded UpFront Cibacron Blue 3GA was distributed into each bottle. The reaction bottles were rolled for 40 min at room temperature. After the batch adsorption, 1 mL of sample was removed from each bottle. These 1 mL samples were centrifuged at 10,000 g for 5 min prior to G3PDH activity assay. The depleted feedstock was decanted and the G3PDH-loaded adsorbent was washed thoroughly with buffer A. The washing protocol was repeated 3 times in order to remove unbound or loosely bound materials. Following the washing steps, the adsorbent was subjected to 9 mL of NaCl at various concentrations (0.15~1 M) in buffer A. The washing protocol was repeated 3 times in order to remove unbound or loosely bound materials. Following the washing eteps, the adsorbent was subjected to 9 mL of NACl at various concentrations (0.15~1 M) in buffer A. The reaction bottles were rolled for 40 min at room temperature. Elution samples (1 mL) were assayed for G3PDH activity and total protein concentration [15]. The unit of enzyme activity was expressed as the number of μmol of NADH produced per minute at 25°C, pH 8.5 in 1 mL of solution (IU/mL). Enzyme-specific activities were expressed as units of enzyme activity recovered per unit mass of total protein recovered (IU/mg). Elution yield (relative to bound activity after washing) of G3PDH was calculated using Eq. 1 [(G3PDH recovered in elution/Bound G3PDH) × 100%].

Comparative Performance of Batch Binding of PVP-shielded and Un-shielded Adsorbents

Two Pyrex reaction vessels containing 45 mL of unclarified feedstock were prepared. Five mL of PVP-shielded adsorbent particles were then dispersed into each vessel. The reaction vessels were batch incubated with rolling for 40 min at room temperature. After batch adsorption, 1 mL of sample was removed from each vessel. These 1 mL samples were centrifuged at 10,000 g for 5 min prior to G3PDH assay. The depleted feedstock was decanted and the adsorbent was thoroughly washedwith buffer A. The washing protocol was repeated 3 times in order to remove unbound or loosely bound ma terials. The adsorbent particle was subsequently loaded into a BRG (10 mm i.d.) contactor. Fixed bed elution of bound G3PDH was carried out in a two-step proce dure usingelution 1 (0.15 M NaCl in buffer A, 75 cm/h) and elution 2 (1 M NaCl in buffer A, 30 cm/h). The non-shielded

Table 1. Effect of increasing NaCl concentration upon elution of G3PDH

Concentration of NaCl in buffer A (M)	Total eluted activity (IU)	Total eluted protein (mg)	Elution yield (%)	Enzyme specific activity (IU/mg)
0.15	74.0	8.90	7.4	9.75
0.20	181.4	10.10	18.1	17.93
0.35	334.3	14.00	33.4	21.86
0.50	431.5	16.20	43.2	26.72
0.75	564.3	18.44	56.4	30.60
1.00	695.2	19.13	69.5	39.80

Table 2. Effect of PVP-shielding on Elution of G3PDH from UpFront Cibacron Blue 3GA.

Binding Capacity (IU/mL)	Unshielded	Shielded (1% w/v PVP)
	556.2	267.8
Two-step elution:		
Step 1 (0.15 M NaCl in buffer A)		
Elution yield (%)	6.5	60.5
Enzyme-specific activity (IU/mL)	10.2	42.3
Step 2 (1.00 M NaCl in buffer A)		
Elution yield (%)	61.2	25.3
Enzyme-specific activity (IU/mL)	45.2	21.5
Total elution yield (%) (Step 1 + Step 2)	67.7	85.8

adsorbent particles were treated in an identical manner to the shielded adsorbent particles. Elution yield (relative to bound activity after washing) of G3PDH was calculated using Eq. 1 [(G3PDH recovered in elution/Bound G3PDH) × 100%]

Elution with Neutral Salt

The dye-ligand concentration immobilized within the UpFront adsorbent was estimated based upon an adapted method described previously by Chambers [16], and yielded a value of 9.4 μmol/mL. The relatively high immobilized dye-ligand concentration was attributed to the high proportion of adsorbent volume occupied by the agarose (78%) in the UpFront particle [15]. The application of a non-specific elution method was adopted because preliminary screening of specific elution method employing NAD^+ failed to obtain satisfactory elution efficiency [6].

A step change of the ionic strength of buffer containing various concentrations of NaCl in buffer A was performed at a batch scale with G3PDH-loaded adsorbents according to previously described procedures. The results obtained here are summarised in Table 1, and it was apparent that 0.15 M NaCl in buffer A could only achieve a 7.4% elution yield (relative to bound activity after washing). The G3PDH has four nucleotide binding sites [18], and the relatively high dye-ligand concentration immobilised in the UpFront adsorbent particles might contribute to the multi-site interaction. It has been reported previously that the degree of multiple interaction increased as the immobilized dye-ligand concentration increased [19]. For example, it has been described that the apparent binding strength for dehydrogenase enzymes increased with increasing dye-ligand concentrations immobilised onto support matrices [9]. Hence, the elution of bound protein was more difficult to achieve.

The effect of multi-site interaction [19,20] might lead to restricted enzyme elution (*i.e.* increase of binding strength as a result of specific interactions). The relatively low elution recovery observed here might also be attributed to the non-specific interactions [17]; it has also been noted that the effect of the interaction was additive. Analysis of the results depicted in Table 1 indicates that 0.15 M NaCl in buffer A could only be used to remove weakly-bound contaminant proteins from UpFront Cibacron Blue 3GA. The highest elution yield of G3PDH (69.5%) was achieved by 1 M NaCl in buffer A. The increase of elution yield and specific activity of eluted enzyme indicated that the adsorption force between the dye-ligand and G3PDH was decreased with increasing salt concentration in buffer A. However, the specific activity of eluted enzyme would expected to be reduced with increasing hold-up period (*i.e.* storage time) since the product modification and/or inactivition by system antagonists including harsh physical condition (*e.g.* high salt concentration) and proteases is time-dependent [14,21].

Comparative Performance of PVP-shielded and Unshielded Adsorbents

The impact of the application of water-soluble polymer, PVP-40, in the recovery of G3PDH was studied in this component of study. The comparative elution performance of PVP-shielded and unshielded UpFront Cibacron Blue 3GA is summarised in Table 2. It was demonstrated that the elution yield with 0.15 M NaCl in buffer A increased considerably when the adsorbent was shielded by pre-treatment with 1% (w/v) PVP. The increased elution yield seen here might indicate that treating adsorbent with PVP decreased the apparent binding strength of G3PDH and/or reduced the non-specific interactions [17] (*i.e.* binding sites away from the specific binding domains). In contrast, the target enzyme was bound more strongly to the unshielded adsorbent and a salt concentration as high as 1 M was required to achieve a satisfactory recovery yield. The specific activity of the enzyme eluted from unshielded adsorbent at 1 M NaCl was slightly higher (<10%) than the shielded adsorbent at 0.15 M NaCl was due to the more enzymes bound to unshielded adsorbent. The decreased of enzyme binding capacity in polymer shielded adsorbent was probably due to reduce of non-specific interactions [17]. The specific activity recorded for unshielded adsorbent would expect to be reduced with increase of storage time since high ionic strength condition promoted enzyme inactivation [14].

The present study demonstrated clearly that treating the dye-ligand adsorbent with PVP resulted in an improvement in elution yield using low ionic strength elution condition. This is an important finding, since a high

ionic strength was proven to diminish enzyme stability [21]. The elution condition identified in this batch binding study can be used to optimize expanded bed operation [22], and further investigation is required.

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