Acta Scientific Biotechnology

Volume 2 Issue 4 July 2021

Research Article

Immobilization of Trypsin onto Porous Support Matrix Pre-activated with Cyanogen Bromide

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Received: May 18, 2021

Published: June 30, 2021

Abstract

Covalent immobilization of molecules like proteases especially trypsin is of paramount importance as its leaching off can have detrimental effects on the target molecule which is to be manufactured. Residual trypsin will not only cleave the target molecule but will also put tremendous burden on chromatographic and/or ultrafiltration step. Use of scalable matrix for the effective immobilization of trypsin and optimization of effective parameters would solve the purpose and can be implemented in bio manufacturing processes. Cyanogen Bromide (CNBr) activated sepharose is a challenging matrix for easy coupling of trypsin onto it. It provides ease of separation, upscalability and one step covalent coupling of biomolecules on it. The proposed study includes the parameter optimization for effective immobilization of biomolecules onto CNBr activated sepharose. Various coupling parameters like coupling time, temperature, pH and protein density was optimized and the esterolytic activity of trypsin was assessed using assay and the optimized conditions were selected to develop stable, non-leachable immobilized trypsin. Due to amine coupling the immobilization was found to be effective in alkaline conditions at higher coupling densities. This proves that the effective immobilization of trypsin occurs at pH near to its pI value, suggesting the contribution of ionic groups and the immobilization event.

Keywords: Immobilization; Covalent Coupling; Trypsin; CNBr Activated Sepharose

Introduction

The first covalent attachment of biologically active proteins to insoluble carriers dates back to the early fifties. The widespread use of artificially immobilized enzymes began with the advent of a simple and straightforward method resulting in high efficiency of binding and the preservation of most of the activity. The introduction of cyanogen bromide (CNBr) activation of insoluble polysaccharides for the coupling with ligand molecules filled this gap [1].

Cyanogen bromide-activated agarose matrices are used for preparation of resins for affinity chromatography. Processed agarose has a primary structure consisting of alternating residues of D-galactose and 3-anhydrogalactose and these sugars provide an uncharged hydrophilic matrix. Cross-linked agarose is usually preferred over the non-crosslinked version for most affinity applications that require harsh activation or usage conditions.

Cyanogen bromide in base reacts with hydroxyl groups on agarose to form cyanate esters or imidocarbonates (Figure 1). These groups react readily with primary amines under very mild conditions; the net result is covalent coupling of a ligand to the agarose matrix. The preferred resultant structure is an imidocarbonate, which has no net charge. The isourea bond formed between the activated support and amine ligand is a bit unstable, so a slight but constant leakage of coupled ligand may occur. Isourea derivatives may also act as weak anion exchangers, causing nonspecific binding, especially when small ligands are immobilized.

Advantages of cyanogen bromide-activation:

- Many matrices contain -OH groups.
- The pH conditions needed for coupling are mild enough for many sensitive biomolecules.
- The procedure is relatively simple and reproducible.
- The coupling works for large and small ligands. Although for very small ligands, a spacer may be used to reduce steric hindrance.

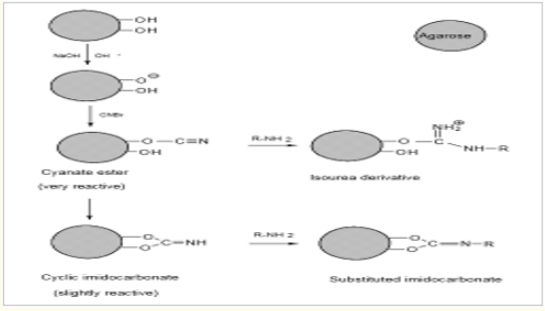

Figure 1: Reaction Scheme for coupling of ligand on CNBr activated bead.

CNBr-activation chemistry has immense use in the preparation of specific adsorbents, and with the introduction of a simplified activation procedure the popularity of sepharose-conjugated materials will undoubtedly increase [2]. Cyanogen bromide-activated sepharose is a readily available commercial product. Immobilization of proteins like Bovine lactoperoxidase, Bovine serum albumin, antibodies, Glucose oxidase and Glucoamylase using CNBr activated sepharose have been mentioned in the literature [2,3]. As multistep immobilization process yields less activity of the enzyme, one step covalent immobilization of enzymes onto matrix such as CNBr activated sepharose would yield better immobilization and improved enzyme activity.

Trypsin (EC 3.4.21.4) is a serine protease found in the digestive system of many vertebrates where it hydrolyses proteins. Trypsin is used for numerous biotechnological processes such as for the production of insulin, where it is used in combination with carboxypeptidase B for processing of an insulin precursor such as human proinsulin into active insulin. Various attempts were made to immobilize trypsin onto vast range of matrices that involves ionic, adsorption as well as covalent type of immobilization. It includes immobilization on divinyl sulfone (DVS) activated agarose [4], low cost lignocellulosic support [5], Styrene-methacrylic acid in combinations crosslinked with p-DVB [6] etc.

Covalent coupling of proteins onto activated matrices is dependent on several parameters viz. pH of the reaction, temperature, coupling density of the ligands, coupling time and to certain extent ionic strength of the reaction buffer.

The pH plays two important roles in the immobilization procedure. For the reaction to occur with the activated ester, the ligand has to be uncharged and should be brought in close proximity with the surface by a process called pre-concentration. This surface matrix-linked process is achieved by electrostatic attraction between the oppositely charged remaining surface carboxyls and the amino group of the ligand [7].

Materials

Bovine serum albumin (BSA), and N-alpha-Benzoyl-l-arginine ethyl ester (BAEE) were obtained from Sigma Aldrich , Trypsin (3.4.21.4), CNBr activated sepharose 4B were obtained from Sigma Aldrich, Sodium carbonate, Sodium bicarbonate, Monosodium phosphate, Disodium phosphate, Sodium chloride, Tris-HCl, Glycine were obtained from Merck, β-mercaptoethanol, and Coomassie brilliant Blue were obtained from Loba chemicals, Sodium dodecyl sulphate, Acryalamide, Bisacryalamide and Ammonium Persulfate were obtained from Ameresco. Trypsin stock 10 mg/mL, BSA stock 1mg/mL, Coupling buffer (0.1 M Carbonate, pH-9.5), PBS (50 mM Phosphate, 150 mM NaCl, pH7.4). The instrument and equipments used for this study were UV-visible spectrophotometer from Shimadzu 1800, LabIndia 3000+, Biorad SDS-PAGE apparatus, pH meter from EuTech, Rocker, and Stirrer from Acensions Innovation, vortex from Remi.

Methods

- **Matrix activation:** 15 mg of CNBr activated Sepharose resin was activated with chilled 1 mM HCl for 15 mins. The resin was washed with Distilled water and then equilibrated with coupling buffer.
- **Covalent coupling of protein:** BSA and Trypsin were quantitated and kept ready for coupling, further coupling reaction was carried out. After incubation, the resin was allowed to settle and the supernatant was separated and collected. Further the resin was washed twice with coupling buffer, after every wash resin was allowed to settle and supernatant was separated and collected.
- **Blocking of unreacted sites:** To block non-reacted sites resin was washed with Blocking buffer (1 M Tris, pH 8.0). The supernatant was separated and collected.
- **Washing:** The resin was washed with Low pH buffer (0.1 M Acetate buffer + 0.5M NaCl, pH 4.0) and high pH buffer (0.1 M Phosphate Buffer +0.5 M NaCl, pH 8.0) containing 0.5 M NaCl to remove nonspecifically bound proteins from the resin.
- **Spectroscopic analysis:** All the collected washes were analyzed using spectrophotometer and residual protein was quantitated using extinction coefficient method.

- **SDS PAGE analysis:** All the collected washes were then analyzed on SDS PAGE and visualized using silver staining protocol [8].
- **Activity based analysis:** All the collected washes were assayed using BAEE substrate assay and residual trypsin was quantitated [9].

Initial screening parameters for coupling of proteins were pH, temperature of coupling, time of coupling, initial protein density for coupling and the measurements were done spectrophotometrically.

Results and Discussion

Although, the immobilization process seems to be simple but it is affected by multiple parameters. Thus, active immobilization of enzyme is dependent on various parameters. Changes in coupling pH, coupling time and protein density give rise to variation in the magnitude of protein binding. The current study focuses on observations of various coupling parameters and its combinatorial effect on protein coupling. Different proteins vary in number of functional groups, thus differ in extent of binding at particular coupling conditions.

Initial screening for coupling of proteins:

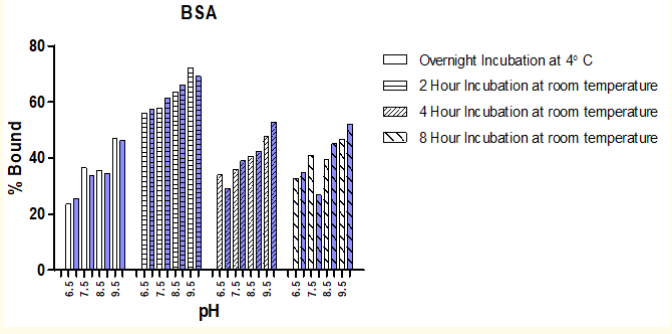

Figure 2: Graphical representation of % Bound of BSA obtained at different pH, temperature and interaction time.

It was observed that pH influenced protein immobilization by varying extent at low and high pH values. Figure 2 and 3 are showing the % bound of respective proteins at different immobilization parameters.

pH plays crucial role in covalent coupling via carboxyl groups. The density of the unprotonated amines on the proteins is directly

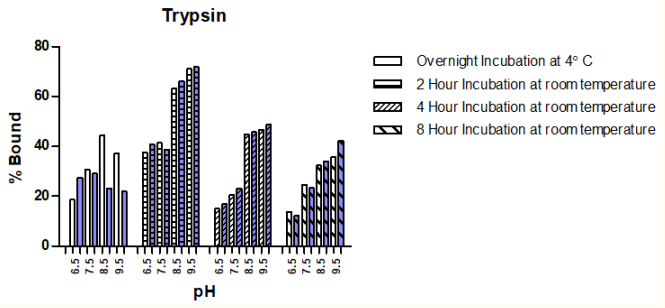

Figure 3: Graphical representation of % Bound of Trypsin obtained at different pH, temperature and interaction time.

proportional to the pH. The aldehydic group on the support are attacked by the unprotonated amines as a nucleophile, and hence pH augments the possibilities of the enzyme to multi-interact with the activated support. As pH increases, these possibilities do and therefore the stabilization of the derivatives must be greater [10].

As per the observations, pH and interaction time shows impact on the immobilization efficiency. Though, the studies performed for immobilization of enzymes shows that the alkaline pH reduces the relative activity of the immobilized enzymes [11], but it varies from protein to protein and the reactive groups used for the immobilization. In current study, alkaline conditions present the high binding of trypsin on CNBr activated sepharose which works on amine coupling chemistries.

Optimization of trypsin coupling density

Initial protein loading concentrations has greater impact on the stability and activity of enzyme post immobilization. The low coupling densities tend to result in more stable immobilized preparations, but at high coupling densities use of some organic solvents can have positive impact to provide elevated stability for immobilized preparations [12].

The critical observation and analysis of the initial screening values suggests that there is significant increase in the binding of proteins as pH shifts to more alkaline range. It has been previously reported that the nucleophilic attack is more efficient in highly deprotonated environment due to which coupling is strong. Based on the data obtained, trypsin binds with maximum binding for the pH range of 8.5 to 9.5 at room temperature within 2 hours of coupling time. Although these values suggest the binding of proteins onto

CNBr activated sepharose, one cannot ignore the leaching of proteins on further washing and reusability and the active protein immobilization.

Protein coupling density optimization

	% Immobilization	
Sample ID	**By UV Quantitation**	**By Activity assay**
C1	77.7	92.8
C2	78.0	92.8
C3	82.4	95.9
C4	86.4	97.6
C5	85.6	97.1

Table 1: % Immobilization by UV quantitation and by activity assay.

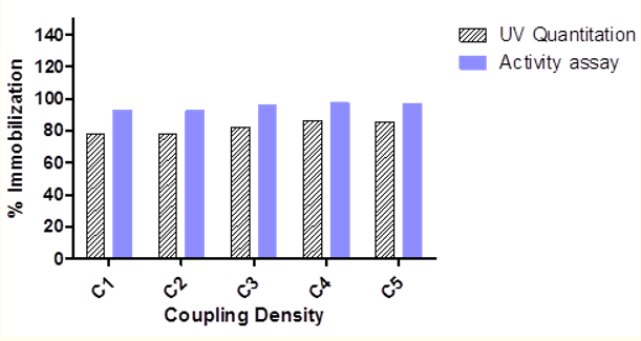

Figure 4: Graphical representation of % immobilization at different coupling densities.

As per the optimized immobilization parameters, trypsin coupling was performed at pH 9.5 for 2 hours at room temperature and the trypsin immobilization efficiency was found to be 97.6% for trypsin coupling concentration 5 mg/mL. There isn't much significant difference in the percentage of immobilization efficiency for other concentrations. The immobilization efficiency was assessed by UV quantitation as well as activity assay for the residual trypsin post coupling.

SDS-PAGE analysis of post coupling washes to check leached trypsin

Figure 5 shows analysis of trypsin in the washes and the residual trypsin can be seen till 4th wash post coupling when sensitive staining technique was adapted to visualize. This indicates there is no loss of trypsin during washes and the coupling might be efficient.

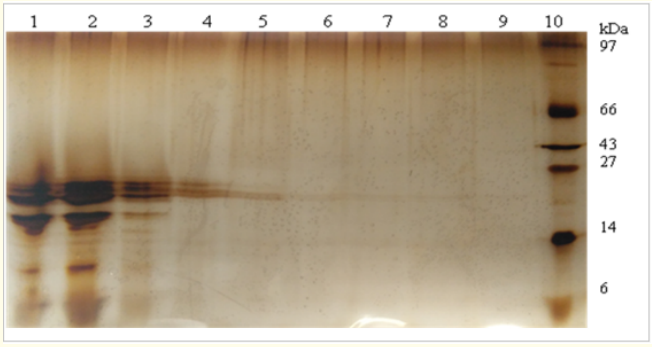

Figure 5: 15% PAGE, silver stained under non-reducing conditions, showing post coupling washes for analysis of washed off trypsin in the washes. 1st lane shows the Std. trypsin, lane 2-9 shows the washes collected post coupling from supernatant, coupling buffer wash 1 and 2, blocking buffer wash, low pH and high pH wash 1 and 2 respectively. Lane 10 shows the low range molecular weight protein marker.

Various coupling densities for trypsin immobilization results into optimum immobilization of trypsin onto CNBr activated sepharose. The term immobilization is not only referred for the amount of protein coupled on the matrix but also to retain the inherent activity of the enzyme, thus it can be modified to achieve elevated enzyme activity. Figure 5 implies that coupling of trypsin is higher at all coupling densities but cannot comment on % immobilization until the activity of the same is monitored. The activity of the immobilized enzyme might be hampered due to multipoint attachment with masking or alteration of catalytic site of the enzyme. The release of the single point/fixed enzyme could lead to leaching of the matrix. The defect of covalent type of method is that it often causes the low activity recovery, which is resulted from the destruction of enzyme active conformation during immobilization reaction, the multipoint attachment to the supports, steric hindrance of enzyme, or the strong strength of the covalent binding [13].

As trypsin digestion is crucial step in proteomics, its control over the reaction is achieved using immobilized trypsin. On the other hand, leaching of the enzyme will lose the control over proteolytic digestion resulting in over digestion and unintended peptide generation. In insulin biosynthesis, the controlled tryptic digestion of pro form of insulin is of paramount importance. In order to prevent non-leachable immobilized trypsin, a condition needs to be optimized to retain and enhance the activity.

Conclusion

The current study describes the process optimized for effective, non-leachable covalent immobilization of trypsin onto a commercially available upscalable matrix. The study presents data for effect of various immobilization parameters on the magnitude of protein coupling onto matrix. The selection of prectivated sepharose matrix would facilitate the extension of prototype into commercial columns. Although the behavior of immobilized trypsin would not be similar as in the reaction tube, the conditions for proteolytic digestion or conversion of zymogen form of other enzymes or proteins into active form need to optimize as per the industry requisites. In this study BSA acted as a model protein to check the effectiveness of the matrix. Whereas trypsin was a test protein to be immobilized on the matrix by covalent means. The immobilized trypsin can be further studied for its kinetic parameters, its reusability. For its various applications, the process parameters such as amount and time of incubation need to be optimized.

Conflict of Interest

The authors declare no conflict of interest.

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