Middle-East Journal of Scientific Research 20 (3): 396-403, 2014
ISSN 1990-9233

DOI: 10.5829/idosi.mejsr.2014.20.03.11466

Catalytic Efficiency of Trypsin Immobilized onto Macroporous Poly (Epoxy-Acrylamide) Cryogels

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Abstract: The application of trypsin immobilized onto macroporous poly(epoxy-acrylamide) monolithic cryogels for flow-through protein digestion is reported. Thermally pre-treated bovine serum albumin (BSA) was used as the high molecular weight model protein. The poly(epoxy-acrylamide) cryogel based immobilization preparation could degrade fully denatured BSA using a column of six monoliths at 40°C and pH 9.0 at both low and high flow rates. The immobilization preparation was re-used 20 times in a flow-through system with retention of about 90% of its initial (maximum) capability. The poly(epoxy-acrylamide) cryogel immobilized trypsin achieved a catalytic efficiency of about 40% relative to the free trypsin for the given conditions.

Key words: Immobilized trypsin · Poly(epoxy-acrylamide) cryogel

INTRODUCTION

Proteolytic cleavage is the breaking of the peptide bonds between amino acid units in proteins by the action of the enzymes referred to as peptidases, proteases, proteinases or proteolytic cleavage enzymes. Proteolytic cleavage is very useful for protein analyses that involve specific peptide fragmentations instead of random cleavage of the entire protein chains [1]. Proteases have found valuable industrial applications in food and dairy industries as well as detergent additive for cleavage of proteinaceous stains in clothing [2, 3].

Trypsin, one of the three principal digestive proteinases besides pepsin and chymotrypsin, is a widely used proteolytic enzyme and the most discriminating of all the proteolytic enzymes in terms of the restricted number of chemical bonds it affects [3-5]. Trypsin (EC 3.4.21.4) is a 23.8 kDa pancreatic serine protease found in the digestive system [3, 6]. The hydroxyl group of the serine has a strong nucleophilic character that it is able to react with electrophilic groups like carbonyl groups in peptide bonds or ester bonds. Trypsin cleaves only peptide bonds in which the carboxyl group is contributed by the positively charged lysine or arginine residues, regardless of the length or amino acid sequence of the chain [3, 5]. The enzyme has a wide range of applications such as amino acid analysis, protein sequencing, mapping and structural studies besides removal of adherent cells from tissue culture flasks [3].

Immobilized trypsin can replace free trypsin virtually in any application. Immobilized trypsin is in most cases more useful than the free trypsin in many ways [3, 7-9]. Immobilized trypsin practically eliminates both autolysis and contamination of the product with the protease. In addition, it enables the control of digestion by simply removing the immobilized enzyme. Immobilized trypsin is more stable against heat-induced denaturation, resulting in longer maintenance of activity. Furthermore, the use of immobilized trypsin enhances re-usability and good reproducibility, suitable for trace-level samples, amenable to high-throughput automation and overall cost-effectiveness.

Cryogels are macroporous heterophase gels in which polycrystals of frozen solvent act as porogens during gel formation [10, 11]. Monolithic cryogels have adequate osmotic, chemical and mechanical stabilities and are characterized by interconnected systems of macropores and the unique sponge-like morphology that can permit unhindered diffusion of solutes of practically any size [10]. By and large, the cryogels have found different applications in the medical, biotechnological and pharmaceutical areas [10, 12, 13]. They can be used as

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smart matrices for chromatography of large biomaterial such as protein aggregates, membrane fragments and viruses. Cryogels can also be used to immobilize biocatalysts operating in both aqueous and organic solvents [10].

Hydrophilic epoxy-modified polyacrylamide monolithic cryogels (Epoxy-MPAAGs) have been synthesized and adequately studied [12-15]. These supermacroporous cryogels have successfully been used to covalently immobilize the model enzyme bovine pancreatic trypsin through ethylenediamine-glutaraldehyde spacer arm [16]. This report subsequently assesses the catalytic efficiency of the immobilized trypsin over the high molecular weight substrate bovine serum albumin (BSA) in flow-through system. The efficiency of the immobilized trypsin is compared with that of free trypsin in batchwise digestion of the BSA. The degradation of the BSA is analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the TNBS assay. Retention of the catalytic activity of the enzyme while it is being used repeatedly and continuously is among the important parameters for the assessment of the efficiency of immobilized enzymes [6]. Thus reusability capacity of the immobilized trypsin onto the cryogels is also assessed and discussed.

MATERIALS AND METHODS

Materials: Bovine serum albumin (BSA) ≥ 96% electrophoresis grade and 5% (w/v) 2,4,6-trinitrobenzenesulphonic acid (TNBS) solution in methanol, 99% electrophoresis grade, both from Sigma-Aldrich; salt-free bovine pancreatic trypsin (EC 3.4.21.4) from Novozymes Denmark, calcium chloride dehydrate of analytical grade, tris-HCl salt and ethanol ≥ 95% from Merck (Darmstadt, Germany) were used as received.

Equipments: A Watson-Marlow peristaltic pump (0-99 rpm) was used for circulating the substrate, BSA, solution through the cryogel immobilized trypsin packed in cylindrical glass column in flow-through digestion system. SDS-PAGE system was used for qualitative estimation and analysis of BSA degradation by both the immobilized and free trypsin in flow-through and batch systems, respectively. Ultrospec 1000 UV-VIS-Spectrophotometer (Pharmacia Biotech) was used for quantitative estimation of BSA digestion by the immobilized trypsin.

BSA Degradation by the Immobilized Trypsin: A 5 mg/ml BSA stock solution was prepared by dissolving 100 mg BSA into 20 ml of 0.3 M tris-HCl buffer pH 9.0 enhanced with 0.1 mM $CaCl_2$ solution. The BSA solution was thermally pre-treated by heating at about 95°C for about 15 minutes and allowing gradual cooling to room temperature (~ 23°C). A glass column was packed with six trypsin immobilized monolithic Epoxy-MPAAGs cryogels (each of at least 0.7 cm internal diameter and about 0.5 ml volume obtained from a recipe with 10% initial monomer concentration (10% Epoxy-MPAAGs cryogel-trypsin conjugates). The conjugates were synthesized as described by the present authors [16]. The monolithic column of immobilized trypsin was pH equilibrated with about 5 ml of the tris-HCl buffer and then thermally equilibrated over water bath at 40°C for at least 15 minutes; concurrently the BSA solution was warmed to 40°C before being pumped through the column, marking the onset of degradation reaction. The effects of flow rate (residence times) and recycling times on the degradation of the BSA were investigated.

Re-Usability Capability of the Immobilized Trypsin: A column of six 10% Epoxy-MPAAGs cryogel-trypsin conjugates was equilibrated both in pH using tris-HCl buffer and temperature (40°C) as described in the preceding section. About 5 ml portions of thermally treated 5 mg/ml BSA were then recycled through the column at a flow-rate of 0.32 ml/min (corresponding to a residence time of 7.9 min) while being recycled for both 30 min and 1 hour. Before recycling, the column was saturated with the BSA solution flowing at the rate of 0.32 ml/min by allowing about 3 ml to pass out as an effluent. Several 1 ml samples were collected at the end of each recycling time and kept at 4°C for further electrophoresis analyses. The column was re-used 20 times while being washed with about 9 ml of tris-HCl buffer at 40°C before the subsequent run. The entire re-usability investigation took 3 days. Between analyses, the thoroughly washed immobilized trypsin column was kept at 4°C.

BSA Degradation Studies by the Free Trypsin: Typically 0.1 mg/ml of aqueous trypsin solution was prepared by dissolving 1 mg of the enzyme into 10 ml of 0.3 M tris-HCl buffer enhanced with 0.1 mM $CaCl_2$. Then 125 mg of fresh BSA were dissolved into 20 ml of the same tris-HCl buffer to give a 6.25 mg/ml BSA solution. The BSA solution was heated at about 95°C for 15 minutes [17]. Both the BSA and trypsin solutions were warmed at 40°C for at least 15 minutes; then for batch reaction, 2 ml of the trypsin

solution and 8 ml of the heat-treated BSA solution were mixed. The reaction temperature was maintained at 40°C using a thermostated water-bath. 1 ml samples were collected after 1, 2, 3, 4, 5 and 30 minutes and quickly mixed thoroughly with 77 µl of 4 M HCl to inhibit further reaction. BSA standard control was also prepared by mixing 800 µl of the heat-treated 6.25 mg/ml BSA with 200 µl of 0.3 M tris-HCl buffer followed by addition of 77 µl of 4 M Hcl.

Gel-Electrophoretic Determination for the Digested BSA Fragments: For the gel electrophoretic determination of molecular weights of the digested BSA fragments, the Laemmli SDS-PAGE procedure was adopted [18]. The running gel had 12.5% (w/v) acrylamide, suitable for the separation range of 30-70 kDa; while the stacking gel was of 3% (w/v) acrylamide. Sample buffer with 4% (w/v) SDS was mixed with the tryptic fragments (digested BSA) in 1:1 volume ratio and heated for about 10 min and then cooled for 2 min over ice. Control sample from the original undigested BSA stock was equally treated. Maximum loading volume was 20 µL per well. Precision Plus Protein Standards, all blue, from Bio-Rad Laboratories Inc. with ten protein bands from 10 – 250 kDa were used. Running conditions were 100 V for 15 min + 200 V for 40 - 45 min. Protein staining of the gels was done either with the Coomassie Brilliant Blue for about 1 hr and thereafter de-stained for about 4 hrs; or with silver staining using the SilverXpress(R) Silver Staining Protocols by Invitrogen Life Technologies. Quantification of the SDS-PAGE bands was done by using the Gel Doc Scanner by Bio-Rad Laboratories Inc.

TNBS Spectrophotometric Analysis of Tryptic Fragments: Standard procedure for the TNBS (2,4,6-trinitrobenzenesulphonic acid) assay was followed [19]. Generally, weekly fresh solution A (0.1 M Na_2SO_3) and solution B (0.1 M NaH_2PO_4) were prepared from their respective salts in deionized distilled water (ddH_2O). Then solution C (0.1 M $Na_2B_4O_7$ in 0.1 M NaOH) and a daily fresh solution D made up from 1.5 ml A + 98.5 ml B were prepared. For the assay, typically 0.1 ml sample of the degraded BSA solution + 0.25 ml of solution C + 10 µl TNBS were thoroughly mixed. After 5 min, 1.0 ml of solution D was added into the mixture to stop the reaction. The reaction was conducted at room temperature (~ 23°C). The product was then diluted (n = 5) in tris-HCl buffer pH 9.0 and immediately replicate measurements of absorbance at 420 nm against the similarly treated BSA standard blank (set reference) were obtained.

RESULTS AND DISCUSSION

BSA Degradation by the Immobilized Trypsin: The effectiveness of immobilized trypsin for the degradation of BSA was analysed by SDS-PAGE and the results are shown in Figure 1 for the degradation experiments carried out with long recycling times (two hours) at relatively low flow rates and in Figure 2 for the degradation reaction at relatively high flow rates with relatively short recycling times (30 min and 1.0 hr). The results in both Figures 1 and 2 indicate that the immobilized trypsin onto the 10% Epoxy-MPAAGs cryogel can digest the high molecular weight BSA (~ 67 kDa) successfully. The pre-heated BSA samples were completely degraded after two hrs, at both fast and slow flow rates (Lanes 4 and 5 in Figure 1). In figure two are the results of BSA degradation by the immobilized trypsin at two different recycling times, i.e., 30 min and 1 hr, with variable residence times ranging from 1.1 to 7.9 minutes. The results indicate that complete degradation of the BSA was attainable within 30 min. The degradation efficiency tended to improve with decreasing flow rates (increasing residence time). It was generally noted that, the longer the residence time the more the produced free amino groups; implying higher BSA degradation efficiency with lower flow-rates. The observed good performance of the poly(epoxy-acrylamide) cryogel immobilised trypsin on degradation of BSA is, in addition to the appropriate flow-rates, also attributed to the combined effect of pre-heating the BSA and proper choice of temperature and pH conditions, i.e., 40°C and pH 9.0. The use of a lower flow-rate or a longer immobilized-enzyme column, i.e., more enzymes, for complete consumption of BSA is also reported elsewhere [5].

The degradation of fresh untreated BSA was less successful. The BSA is a high molecular weight protein containing several disulfide bonds and hydrogen bonds and is frequently glycosylated making it more difficult to digest [5]. Denaturation process breaks up the firmly three-dimentional protein molecular structure that involves hydrogen bonding and other secondary forces [5]. This can be brought about by various means including heating, using chemical agents, e.g., urea and dithiothreitol and by changing the pH [5, 17, 20]. In this work, denaturation of the BSA by heating was adopted since it does not affect the immobilized trypsin and is environmental friendly. In that regard it was necessary to pre-heat the BSA before degradation experiments so as to break up the 3D structure of the protein and thereby facilitate easier accessibility to the cleavage sites by

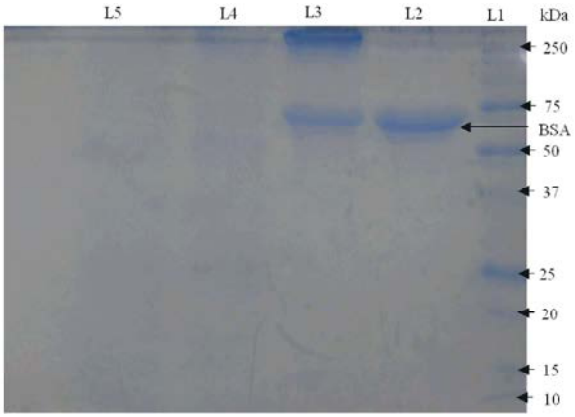

Fig. 1: SDS-PAGE images for tryptic BSA fragments in sequential high residence times and high recycling times.

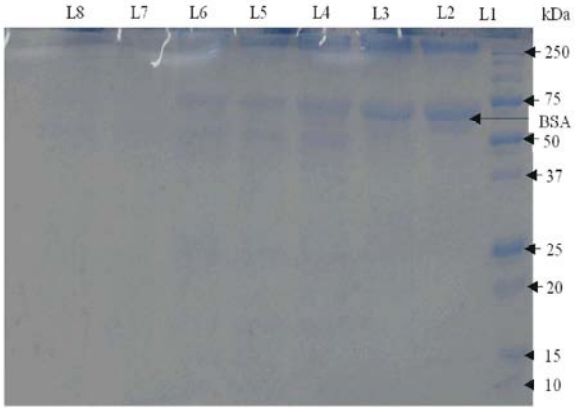

Fig. 2: SDS-PAGE images for tryptic BSA fragments in sequential low residence times and low recycling times.

Key for figure 1:
L1: Protein standards (molecular weight markers)
L2: Fresh BSA (5 mg/ml, unheated)
L3: BSA standard control (5 mg/ml, pre-heated at 94°C for 15 min)
L4: Digested BSA, residence time 7 min and recycled for 2 hrs
L5: Digested BSA, residence time 60 min and recycled for 2 hrs

Key for figure 2:
L1: Protein standards (molecular weight markers)
L2: BSA standard control (5 mg/ml, pre-heated at 95°C for 15 min)
L3 & L4: Digested BSA, residence time 1.1 min; recycled for 30 min & 1 hr respectively
L5 & L6: Digested BSA, residence time 2.5 min; recycled for 30 min & 1 hr respectively
L7 & L8: Digested BSA, residence time 7.9 min; recycled for 30 min & 1 hr respectively

the active sites of the immobilized trypsin. On comparing the degradation of thermally treated and untreated BSA samples (Lanes L2 and L3 of SDS-PAGE images in Figure 1), it is observed that there was slight deformation of the native structure; giving a trace product in the region about 250 kDa. Most likely this effect is due to self-induced association/coagulation of some BSA molecules. However, even this trace high molecular mass product was completely digested by the immobilized trypsin, an indication of its potential to degrade even larger molecules than the BSA.

Re-Usability Capability of the Immobilized Trypsin: The trypsin digested BSA fragments obtained from 20 runs (uses) of contacting the substrate with the immobilized enzyme for 30 min and 1 hr recycling times, respectively, were analysed in two SDS-PAGE gel plates, i.e., fragments from the first to the tenth run in one plate and those from the eleventh to twentieth run (use) in the second gel plate (Figures 3-6). From the SDS-PAGE results (Figures 3-6), the re-usability trend appeared to be similar for both 30 min and 1 hr recycling times, i.e., the BSA band had almost completely disappeared in the first

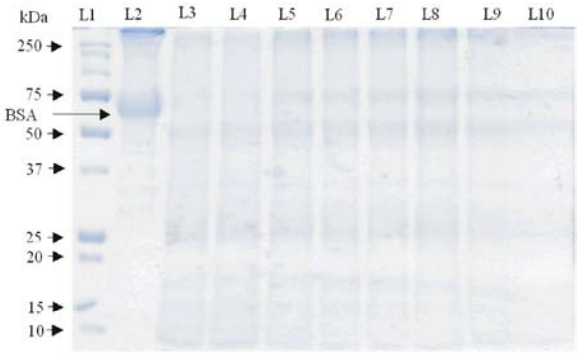

Fig. 3: SDS-PAGE image for tryptic fragments for the first 10 runs; residence time 7.9 min; recycled for 30 min.

2 runs (uses). The intensity of the BSA band appeared to increase gradually with the number of runs (uses) up to about the 10th use. Between the 11th and the 20th runs, the intensity of the BSA band remained almost constant (saturated).

The relative strength of bands (band densities) of the SDS-PAGE images of tryptic BSA fragments obtained with 7.9 min residence time and 1.0 hr recycling time

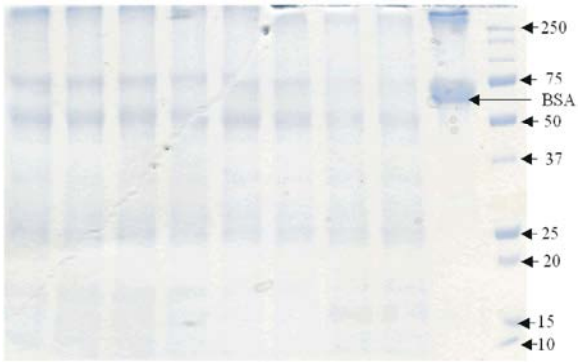

Fig. 4: SDS-PAGE image for tryptic fragments for the first 10 runs; residence time 7.9 min; recycled for 1 hr.

Key for figures 3 and 4:
L1: Protein standards (molecular weight markers)
L2: BSA standard control (5 mg/ml, pre-heated at 94°C for 15 minutes) L3: 1st run;
L4: 2nd run; L5: 3rd run; L6: 5th run; L7: 6th run; L8: 8th run; L9: 9th run; L10: 10th run

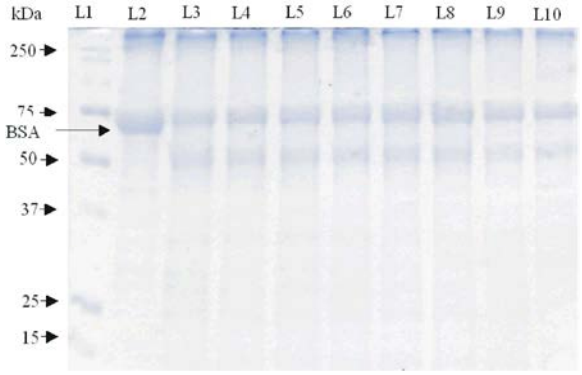

Fig. 5: SDS-PAGE image for tryptic fragments for the second 10 runs; residence time 7.9 min; recycled for 30 min.

(Figures 4 and 6) were estimated with the aid of Gel Doc Scanning software. The relative band densities for BSA standards from both gel plates were used to normalize the strengths of other bands. The percentage of degraded BSA was estimated from the difference between the band density of the BSA control standard and the band density at that point of use. The strength of 1st run band was considered to represent the maximum (100%) degradation capability at that particular flow condition. From the results of the estimation of the% degradation from band densities (Figure 7), it is clear that the immobilized trypsin could be re-used for 20 times while retaining about 90% of its maximum capability. The trend still promises for the

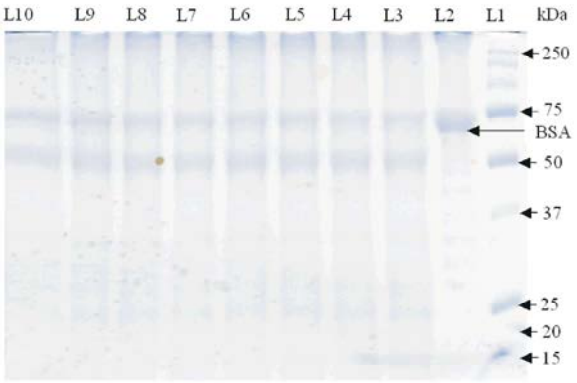

Fig. 6: SDS-PAGE image for tryptic fragments for the second 10 runs; residence time 7.9 min; recycled for 1 hr.

Key for figures 5 and 6:1
L1: Protein standards (molecular weight markers)
L2: BSA standard control (5mg/ml, pre-heated at ~ 95°C for 15 min)
L3: 11th run; L4: 12th run; L5: 13th run; L6: 14th run; L7: 16th run; L8: 18th run;
L9: 19th run; L10: 20th run.

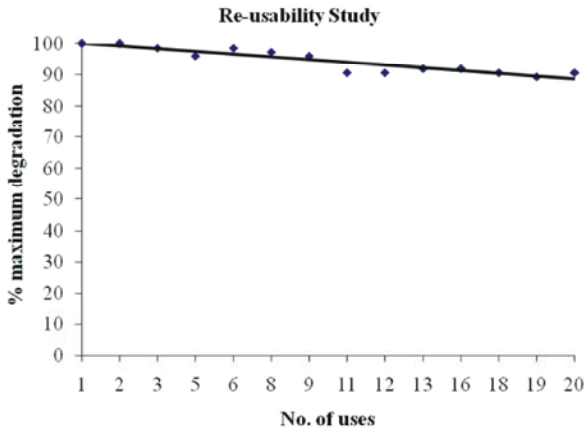

Fig. 7: Re-usability efficiency of the immobilized trypsin onto the 10% Epoxy-MPAAGs cryogels as a function of run number (no. of uses).

ossibility of re-using the system several more times with retention of above 50% of the maximum capability. This re-usability capability is much higher than the reported reusability capabilities of immobilized trypsin using different other carriers, i.e., less or at least 10 uses [5, 21, 22].

Loss of activity of the immobilization preparation in repeated use is a common phenomenon [23]. The loss of activity of poly(epoxy-acrylamide) cryogel immobilised trypsin observed in this study could probably be

attributed to little leakage or denaturation of the immobilized enzyme at some point in the BSA degradation process or during washing [21].

TNBS Assay of the Tryptic BSA Fragments: 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) reacts readily with the primary amino groups of amino acids, peptides or proteins in aqueous solution at pH 8 to form yellow adducts. The coloured derivatives are not formed with secondary amino acids. The coloured products of the reaction of TNBS with BSA fragments were monitored by spectrophotometric analysis at 420 nm for their quantitative estimation and the results are presented in Figure 8. It was generally noted that, the longer the residence time the more the free amino groups produced; implying higher BSA degradation efficiency with lower flow-rates.

TNBS is considered to be very selective reagent. In slightly alkaline conditions and at room temperature it couples only with primary amino group but neither with water nor other groups commonly encountered in protein and reaction terminates by lowering the pH [24, 25]. On the other hand, quantification of free amino groups by the TNBS method is not without limitations [21]. Sulfite is displaced from TNBS by nucleophiles such as primary amines and free amino groups and associates reversibly with trinitrophenyl-amino groups to form complexes which interact with each other, thereby decreasing the molar absorbances of these groups thus complicating their quantitative determination. It has also been reported that TNBS reacts slowly with hydroxyl ions causing increase of blank reading; this increase is stimulated by light [26]. As observed in this work, a spread of results from repeated analysis of the same material is also reported elsewhere [25]. Nevertheless the TNBS reaction fairly served as a useful method for quantitative estimation of protein digestion by the immobilized enzyme.

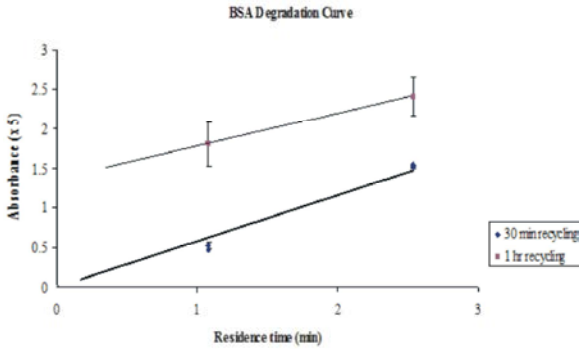

Fig. 8: TNBS reaction curves for the tryptic BSA fragments by the immobilized trypsin at various residence times and recycling times.

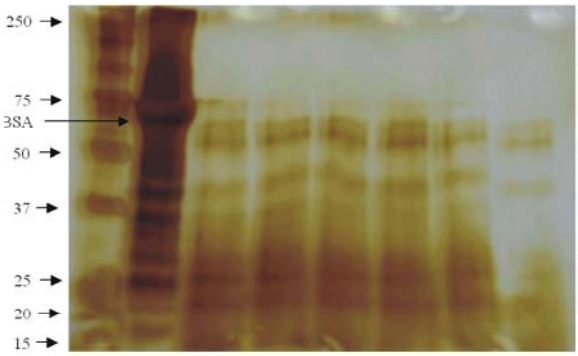

Fig. 9: Silver Stain images for free trypsin degraded BSA fragments at various reaction times

Key for figure 9:
L1: Protein standard (molecular weight markers)
L2: BSA standard control (5 mg/ml, pre-heated at 94°C for 15 min)
L3: Digested BSA, 1 min reaction time;
L4: Digested BSA, 2 min reaction time
L5: Digested BSA, 3 min reaction time;
L6: Digested BSA, 4 min reaction time
L7: Digested BSA, 5 min reaction time;
L8: Digested BSA, 30 min reaction time

Efficiency of the Immobilized Trypsin Relative to the Free Trypsin: The BSA degradation by free trypsin (Figure 9) was also investigated (in a batch mode) for comparison with the BSA degradation utilizing the immobilized trypsin at similar experimental conditions. It was observed that 0.2 mg of the free trypsin in solution were able to degrade efficiently 40 mg of BSA (5 mg/ml, 8 ml) in 30 min. under batch reaction; which is 0.005 mg-enzyme/mg-BSA. However, 12mg of immobilized trypsin (6 cryogel monoliths @ 2 mg protein) degraded 45 mg BSA (5 mg/ml, 9 ml) under flow-through condition (7 min residence time, 2 hrs recycling); which is 0.27 mg-enzyme/mg-BSA. This gives relative efficiency (free trypsin : immobilized trypsin) of about 2%, in a single use. However, from the re-usability study the immobilized trypsin could be re-used successfully for more than 20 times; making the overall efficiency attained over 40%. This is noteworthy, especially at industrial scale of applications where materials are in large quantities and the economic parameters of projects become more important.

Steric hindrance of substrate diffusion to enzyme active sites can affect the enzymatic efficiency [27]. Probably as a huge molecule the limited accessibility of

the BSA cleavage sites to the active sites of the immobilized trypsin, might have contributed to this low efficiency. However this efficiency could be highly improved by further optimization of significant conditions such as flow-rates/residence times, recycling times and making use of more porous Epoxy-MPAAGs cryogels carriers [28]. From these results, like in other reports, peptide mapping of the digested BSA fragments using gel permeation chromatography, reverse phase-HPLC or mass spectrophotometry is highly recommended [22, 27, 28].

CONCLUSION

The 10% Epoxy-MPAAGs cryogel-trypsin conjugates synthesized in this work are generally capable of degrading efficiently the high molecular weight substrate, BSA. Due to their excellent re-usability catalytic efficiency, the 10% Epoxy-MPAAGs cryogel-trypsin conjugates can significantly replace many other previously reported trypsin carriers. The catalytic efficiency of the 10% Epoxy-MPAAGs-trypsin conjugate is expected to improve further with appropriate optimization of flow-rates and porosity of the monoliths. Since the epoxy-MPAAGs monolithic supported trypsin are thermally and mechanically stable, there is great potential for the immobilised trypsin system, following the improved catalytic efficiency, to be upscaled and applied in the place of free trypsin in cost effective on-line industrial protein digestion and peptide analyses.

ACKNOWLEDGEMENT

Sincere gratitude for funding this study to the SIDA/SAREC-International Science Project, through a bilateral programme between the Biotechnology Department of Lund University (Sweden) and the Chemistry Department of the University of Dar es Salaam (Tanzania). The authors appreciate the support from the Dar es Salaam Maritime Institute of Tanzania.

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