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ADSORPTION OF PEPSIN WITH POLYETHYLENEIMINE

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ABSTRACT

Polyethyleneimine is being increasingly applied for immobilizing enzymes employing its primary, secondary and tertiary ionisable amino groups. In this study, we attempt to immobilize pepsin on Sepabeads functionalized with Polyethyleneimine (PEI) polymers of varying molecular sizes (M_w 600, 1200, 10,000 $gmol^{-1}$). The stability and activity of pepsin bound to PEI was found to be dependent on PEI molecular weight and also on the ionic strength of medium, used for immobilization and protein hydrolysis. Immobilized pepsin exhibited good storage and operational stability which was investigated for generating F $(ab')_2$fragments from hydrolysis of immunoglobulins. Digestion of IgG by soluble pepsin and immobilized pepsin showed the same electrophoresis profile (of F $(ab')_2$ and Fc fragments) indicating no modification in pepsin specificity after immobilization on PEI activated Sepabeads support. The above method of immobilization presents an efficient means to immobilize pepsin onto a solid support wherein the said preparation would be free from autolysis and can be reused for multiple cycles.

Key words: Polyethyleneimine, Pepsin, Sepabeads, Immobilization

INTRODUCTION

Pepsin (EC 3.4.23.1), has generated lot of interest due its ability to remain active at acidic pH. This animal protease has potential industrial applications in cheese industry, wine industry and digestion of IgG to produce F $(ab')_2$fragments which has several therapeutic uses[1-4]. Pepsin belongs to protease group of enzymes which have the inherent propensity to undergo autolysis causing self destruction thus decreasing their potential as an industrial enzyme. Immobilization of pepsin stabilizes the enzyme by increasing its half life leading to reutilization of the biocatalyst[5].Modified catalyst would have advantage of ease of handling i.e. simple filtration for separation of products from reaction mixture[6] and enzyme recycling. Additionally, use of biocatalyst i.e. enzymes eliminates the use of hazardous chemicals such as acid (which is conventionally used for protein hydrolysis) thus empowering green chemistry which is the need of the time[7], enabling the formation of products *via* safer route[8]. In order to produce F $(ab')_2$ fragments for therapeutic purposes it is necessary to obtain them in active form which can be done using enzymes, due to their regio-selectivity and mild operating conditions[9].

Earlier, Pepsinogen was immobilized and converted to active pepsin by refolding it on hydrophobic or hydrophilic polymeric surfaces; this entire renaturation process takes 300hr, making it unfeasible[10]. Frýdlová *et al.*, have derivatized Sepharose with α-acetyl phenylalanine and iodinated tyrosine that binds to pepsin[11]. This approach involves multiple preparative steps, making it unsuitable for large-scale production. Covalent immobilization chemistries like glutaraldehyde activation and divinylsulphone methods are effective in neutral or alkaline conditions, however, X-ray diffraction analysis have shown pepsin to denature under alkaline conditions restricting the methodologies that can be used for pepsin immobilization[12].

Ionic adsorptions of proteins on matrices are preferred alternative when harsher irreversible covalent techniques cannot be applied[13, 14]. Direct modification with polymer prior to enzyme attachment preserves the functionality of protein[15]. PEI is widely used as enzyme carriers for monomeric as well as multimeric enzymes like penicillin G acylase, lipases QL and CALB glutarylacylase, glutamate, dehyrogenase *etc.*[16]. The hydrophilic microenvironment and multipoint attachment sites provided by PEI has a positive

influence on the stability and activity of immobilized enzyme[17-19].PEI is also reported to prevent oxidation, aggregation and interaction with surfaces.
Pepsin contains abundantly distributed negative residues and few positively charged moieties on the surface[20]. Polyethyleneimine, a cationic polymer, therefore permits interactions of polymeric amine groups with the acidic enzyme. These polymeric amine groups will react with unsaturated double bond of epoxy group which form stable framework[21]onto which the enzyme can be entrapped. The hydrophilic side groups on the polymer branches surround the enzyme surface acting like scaffold providing multipoint localization and also flexibility to protein molecule that are essential for the preservation of enzyme's activity[14]. High density charge on the synthetic polymer is also reported to provide an ideal micro-environment for the enzyme to bind to the support[22]. In this study, we report immobilization of pepsin onto a macro porous support, Sepabeads® EC-EP coated with positively charged PEI.

EXPERIMENTAL

Materials

Polyethyleneimine High Molecular Weight Mw ~10,000 (water free), Mw ~1,200 (50% water) and Low Molecular Weight ~600 were procured from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sepabeads EC-EP-EP04 (particle size150 - 300 µm, porous diameter >1000A°) was obtained as a gift from Resindion S.R.L (Mitshubishi Chemical Corporation, Italy). Bovine pepsin (EC 3.4.23.1), bovine serum albumin (BSA), trichloroacetic acid, tyrosine and sodium borohydride of biochemistry grade were procured from SD Fine Chemicals (India).All other chemicals were of biochemistry grade and purchased from local suppliers.

Methods

Preparation of PEI-Sepabeads supports

The PEI-Sepabeads support was prepared with some changes as described elsewhere[14]. 10ml of wet packed volume of EC-EP04 Sepabeads with epoxide group density of 110µmol/g of support were suspended in 90ml of 10% (v/v) Polyethyleneimine (Mw ~10000, Mw ~1200 and Mw ~600) at pH 10.5 and gently stirred for 3h, at room temperature. Then solid sodium borohydride up to a final concentration of 10mg/ml was added and left for stirring for 2h. The PEI-Sepabeads were then filtered and sequentially washed with 100mM sodium acetate buffer pH 4.0, 100mM sodium borate buffer, and pH 9.0 and finally with excess of distilled water to eliminate any molecule of PEI adsorbed on the support.

Adsorption of Pepsin on PEI coupled supports

1ml of support coupled with different molecular weight of PEI was equilibrated separately with 0.01M buffer pH 2.0 for 30min. After removal of buffer the beads were mixed with 1mg/ml pepsin (prepared in 0.01M buffer pH 2.0) at 30°C for 2hrs with stirring at speed of 150rpm and finally washed till free pepsin was not observed. The immobilized pepsin preparation was stored at 8°C.

Assay for soluble and immobilized pepsin activity

Proteolytic activity of pepsin was estimated by determining the amount of hydrolysis of denatured BSA solution by enzyme after incubation for 10min at 30°C. One unit (U) enzyme activity was taken as the amount of enzyme which liberated 1µmole of tyrosine (min/ml) at 30°C in 10min. 1ml of diluted pepsin sample (or 1ml of immobilized pepsin) was added to 10ml of 0.2% (w/v) BSA at 30°C and stirred at 200rpm for 10min. The reaction was terminated by adding 1ml of 30% (w/v) TCA followed by centrifugation at 10000rpm for 15min. The absorbance of released tyrosine was measured spectrophotometrically at 280nm for immobilized and free pepsin.
The amount of immobilized enzyme was estimated by subtracting the amount of protein determined in the supernatant after immobilization from the initial loaded protein. The protein content in solutions was determined by using Bio-Rad protein assay kit using the micro-assay procedure and bovine serum albumin as standard.

Leaching of pepsin during storage and operation was assessed. The immobilized enzyme (1ml) was suspended in 0.01M buffer of pH 3 (10ml) and incubated for 2hr. Aliquots of supernatant were collected at regular intervals and the protein content of the solution was analyzed.

Optimization of operating parameters

Activities of free and immobilized pepsin were analyzed in the pH range of 2.0–7.0. The immobilization of the enzyme was carried out in respective buffer and activity was determined at indicated pH values using 0.2% (w/v) BSA solution as mentioned in section above.

Temperature profiles of free and bound enzyme were determined at varying temperatures 10, 20, 25, 30, 35 and 40°C using 0.2% (w/v) BSA solution as discussed earlier.

Thermal stability of free and immobilized pepsin was calculated by measuring the activity of the residual enzyme after exposure to 40°C for 40min in presence of pH 3.0 buffer.

Stability studies

Retention of the immobilized enzyme activity was tested, after each reaction run; the enzyme preparation was washed with buffer of pH 2.0. The washed immobilized enzyme was then reintroduced into fresh reaction medium and enzyme assay was done till the activity of pepsin was lost.

Free and immobilized pepsin were stored in buffer of pH 2 at 8°C. The activity of the consecutive fresh samples taken from the store at uniform time interval was determined periodically in a batch operation mode under same experimental conditions.

The results of pH, temperature, reusability and storage stability of free and immobilized pepsin are presented in a normalized form, with the highest value of each set being assigned the value of 100% activity.

Biotransformation of IgG using immobilized pepsin

Sepabeads PEI bound Pepsin was used for production of F $(ab')_2$ antibody fragment from serum antibodies by enzymatic hydrolysis. 1ml of immobilized pepsin was contacted with 20ml of antibody solution/ equine serum containing antibodies (15mg/ml). Reaction was carried out in batch mode at 37°C for 4hrs in buffer (pH 3.25). Intermittent aliquots were withdrawn and antibody hydrolysis was analyzed by SDS-PAGE method.

RESULTS AND DISCUSSION

Preparation of PEI coupled supports

Pepsin is an acidic protease which has varied applications in many fields such as food processing and mainly in therapeutics. Due to its ability to produce F $(ab')_2$ fragments employed in several antigen detection methods and/or treatment of the diseases. However, the enzyme is highly unstable in solution either due to unfolding of the enzyme molecule, or chemical modification of one or more peptide bonds or critical residues with respect to enzyme mechanism[4] and also spontaneous self-hydrolysis. There is a need to stabilize pepsin en route for harnessing its potential as an efficient proteolytic system.

Pepsin is a single chain proteolytic enzyme with 324 residues of which anionic residues predominate on the surface. The enzyme is active at an acidic pH and is particularly known for its capacity to hydrolyze the Fc region of antibodies with great specificity. Traditional methods of protein immobilization cannot be used for pepsin as the protein is denatured under alkaline conditions. Simple physical adsorption or acidic medium based covalent coupling methods have also resulted in low immobilization efficiencies and therefore it was decided to study ionic polymer mediated immobilization of pepsin. The chemical surface topology for pepsin is predominantly negatively charge and can therefore be efficiently used for mediating enzyme interactions with positively charged ionic polymers via stabilized salt bridges. In this study, we attempt to immobilize pepsin on Sepabeads functionalized with Polyethyleneimine (PEI) polymers of varying molecular sizes (M_w 600, 1200, 10000 $gmol^{-1}$).

Adsorption of Pepsin on PEI coupled supports

Molecular weight and branching of the functionalized polyethyleneimine has been shown to influence its applications for protein sorption. Multipoint salt bridge formations between protein and the layers of PEI have been used successfully by researchers for immobilization of enzymes. Efficiency of pepsin in terms of protein sorption and relative enzyme activity was studied by using PEI of three different molecular weights, namely, 600, 1200 and 10,000 $gmol^{-1}$. As seen from Table 1, immobilized preparation with PEI (Mw 600) exhibited 72% of the free enzyme activity and 96% protein adsorption under 0.01M ionic strength. Pepsin immobilized on PEI (Mw 1200) displays 54% activity and 94% protein binding while PEI (Mw 10000) exhibits 26% activity and 92% protein binding. During protein attachment, higher branch structures (1200 and 10,000) did not permit favorable interactions of pepsin molecule (34kDa). This may be attributed to the steric hindrances by the intense branches in high molecular weight PEI resulting in distortion of the protein structure causing low enzyme activity. PEI (Mw 600) derived matrices with lower branching points offer optimal microenvironment for anchorage and salt bridge formations, thereby mediating non-distorting, stabilizing ionic interactions of pepsin. The PEI (Mw 600) derived matrix offers higher activity for immobilized pepsin in comparison to the other molecular weight polymers and was therefore selected for further studies.

However, continuous stirring of immobilized enzyme in the presence of 0.1M buffer results in 35% loss of protein from PEI immobilized preparation. This indicates that pepsin bound onto the surface of hydrophilic PEI could dissociate and cause leaching, thereby leading to loss of bound activity over a period of time. Leaching of pepsin can be due to disruption of salt bridges that are formed between the protein and the ionic polymer under operating conditions for the immobilized preparation.

Proteins usually adsorb to ion exchangers at low ionic molarity[23].Under these conditions, salt bridges hold the protein to the functionalized matrix. Counter ions in the buffer solution during immobilization are essential for maintaining PEI-pepsin interactions. An optimal concentration of ions is essential for high protein sorption and low desorption[20].

Increasing concentrations of NaCl (25, 75, 100, 150, 200 and 500mM) was added to 0.01M buffer that was used for immobilization to study if desorption could be contained during operating conditions. The strength of binding was monitored at the end of 2hrs (Fig.-1). Highest binding strength was observed in presence of 100mM salt as seen in Figure1. Retention of 90% relative activity of pepsin was observed in the presence of 25mM, 75mM and 100mMsalt. The increase in activity is mainly due to counter ions present throughout the polymer and enzyme surface which help in achieving equilibrium between attractive Vander-Waal and repulsive ionic interactions. Adsorption at 100mM ionic strength stabilizes enzymes by dominant multipoint electrostatic interactions while concentrations greater than 100mM NaCl disrupt salt bridges between protein and PEI by forming networks with PEI themselves[24]. In absence of NaCl, the protein adsorption is random and can involve any area on the protein with few negative charges, mediating weak ionic immobilization. Optimal ionic concentration of 100mM NaCl was found to be essential for promoting strong multipoint attachment of pepsin to PEI and this was also observed to have a profound effect on the desorption of protein during operating conditions. It was observed that under reaction conditions for more than 2hrs, there was only 3% loss of pepsin. This validates earlier reports and substantiates that enzyme immobilization on PEI coupled supports are optimum at 100mM ionic strength. Increased ionic strength can be used for desorbing inactive pepsin from the matrix allowing efficient reuse of the PEI-coupled support for sorption of enzyme.

Operational studies

Pepsin is an acidic protein and shows high enzymatic activity at lower pH values, optimum pH for pepsin is from 1 to 3[25]. At lower pH values, PEI coated beads are charged positively because hydrogen ions can bind to free amino groups. This leads to stronger binding with negative pepsin and bound pepsin's optimum pH shifts to 3 from optimum pH of 2 of free enzyme (Fig.-2).Shift in optimum pH after immobilization is a common phenomenon. The cationic environment of the support tends to shift the pH optimum towards the basic side. The activity of immobilized pepsin changed with variation in pH and exhibited a better stability at medium acidity than free pepsin. The stabilizing effect of PEI was observed

at pH 3. The isoelectric point (pI) of pepsin is 1. Thus at pH 3, the negatively charged pepsin could be immobilized due to electrostatic interaction with positively charged PEI-Sepabeads.

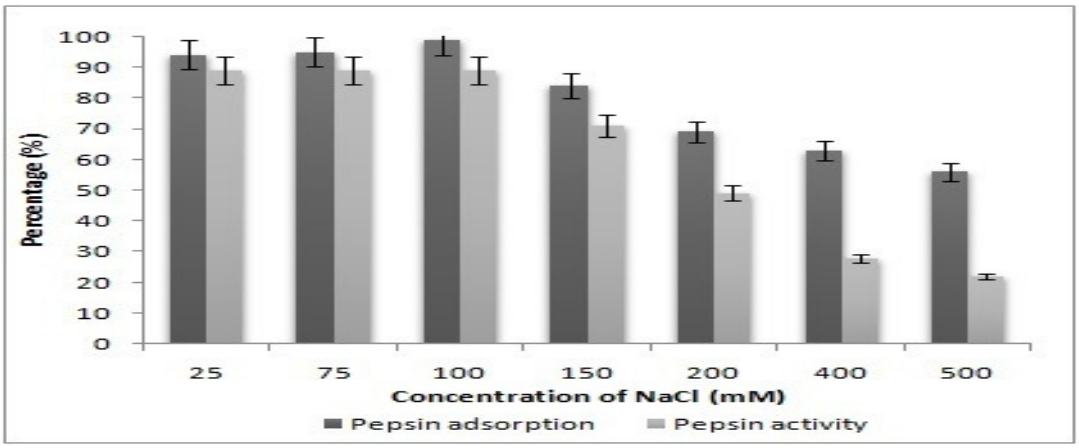

Fig. -1 Adsorption and activity of pepsin on PEI coated Sepabeads in presence of different NaCl concentrations

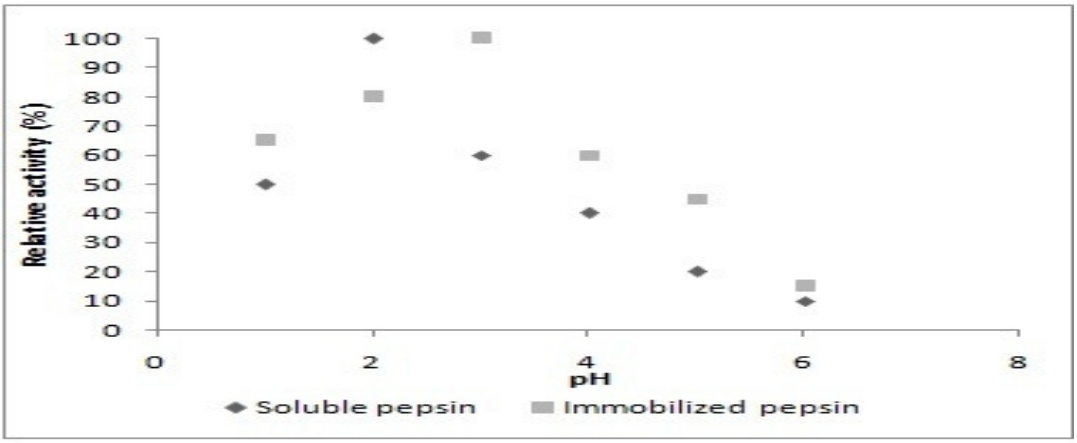

Fig. -2 Effect of pH on pepsin activity

The temperature dependence of the activities of the free and immobilized pepsin was studied in 0.01M buffer (pH 3.0) in temperature range of 20–70°C and temperature profiles are shown in (Fig.-3). The optimum temperature for free and immobilized pepsin was found to be 30°C. Severe loss in pepsin activity was observed above 40°C for both free & immobilized preparation. At 40°C, PEI coupled pepsin showed maximum activity. Enzyme activity was monitored for free and immobilized preparations in presence of 0.01M buffer (pH 3.0) at 10min intervals. After 30min, the immobilized and free enzyme activity reduced to 23% and 0% of the original value respectively (Fig.-4). Thus, immobilized enzyme showed increased tolerance to temperature as compared to free enzyme.

Table-1: Immobilization of pepsin on different molecular weight PEI coupled supports

PEI Mw ($gmol^{-1}$)	Sample loaded	Unbound fraction (supernatant + wash)	Immobilization yield (%)*	Bound protease activity (%)
600	1289	52	96	72
1200	1206	72	94	54
10000	1311	105	92	26

*Based on protein binding

Stability studies

Operational stability represents the persistence of enzyme activity under processing conditions. The operational stability of immobilized pepsin in the current study was evaluated in repeated batch process. The immobilized pepsin could be used for nine cycles. A gradual fall in activity was observed during these nine cycles. However, for a relatively unstable autocatalytic enzyme like pepsin, the stabilization for continuous use over nine cycles is significant. The pepsin immobilized on PEI beads retained 30% relative activity after 180h of storage at 30°C, whereas soluble pepsin lost all its activity after 80h(Fig.-5).

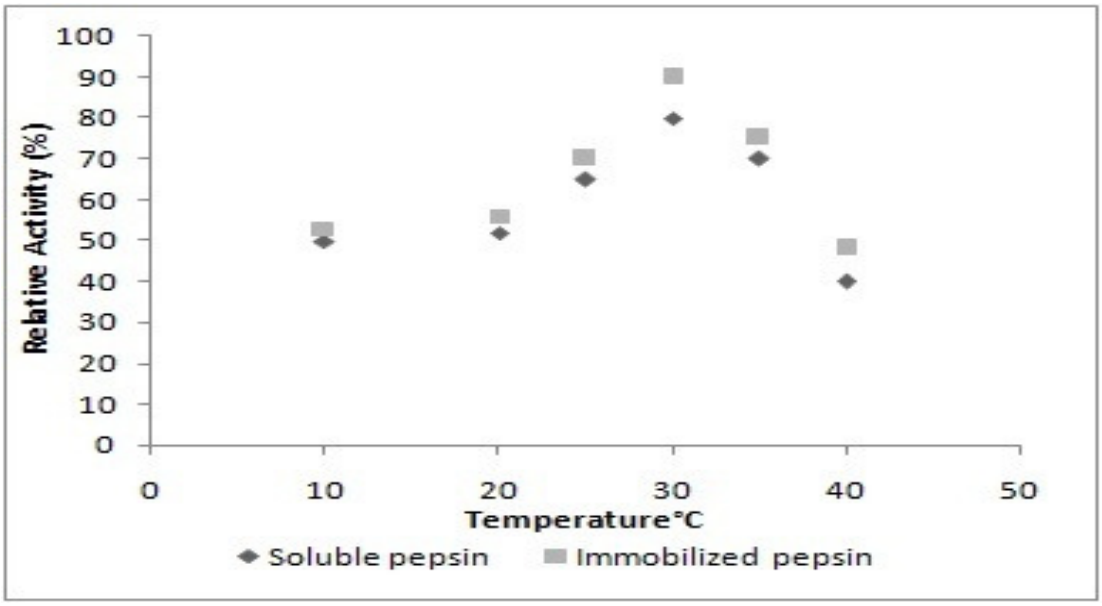

Fig. -3 Effect of temperature on activity

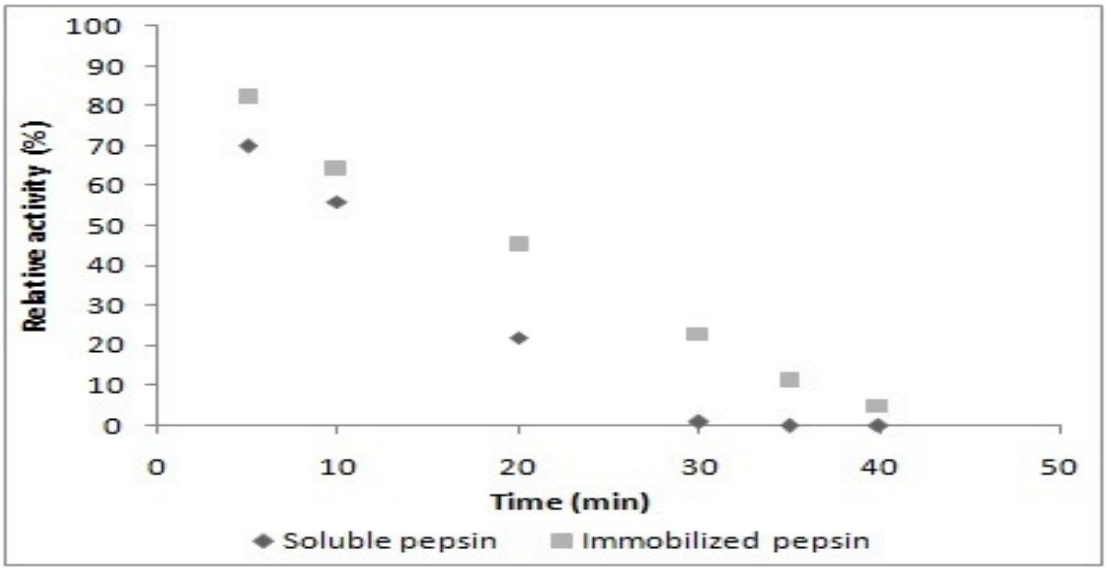

Fig.-4Thermo stability of enzymes at 40°C

Biotransformation of IgG using immobilized pepsin

Human immunoglobulins (IgG) were hydrolyzed using soluble and immobilized pepsin preparation. The SDS-PAGE profile of hydrolysates was same for both immobilized and soluble pepsin which showed presence of F $(ab')_2$ fragment (Fig.- 6). These results indicated that there was no modification in pepsin specificity after adsorption to PEI activated support.

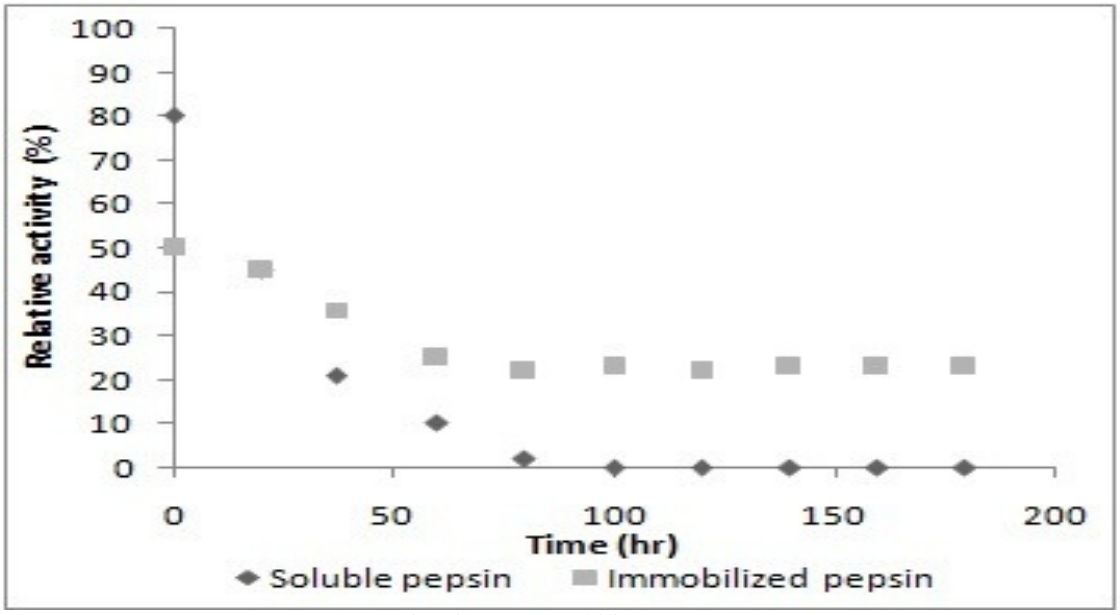

Fig.-5 Storage stability at 30°C

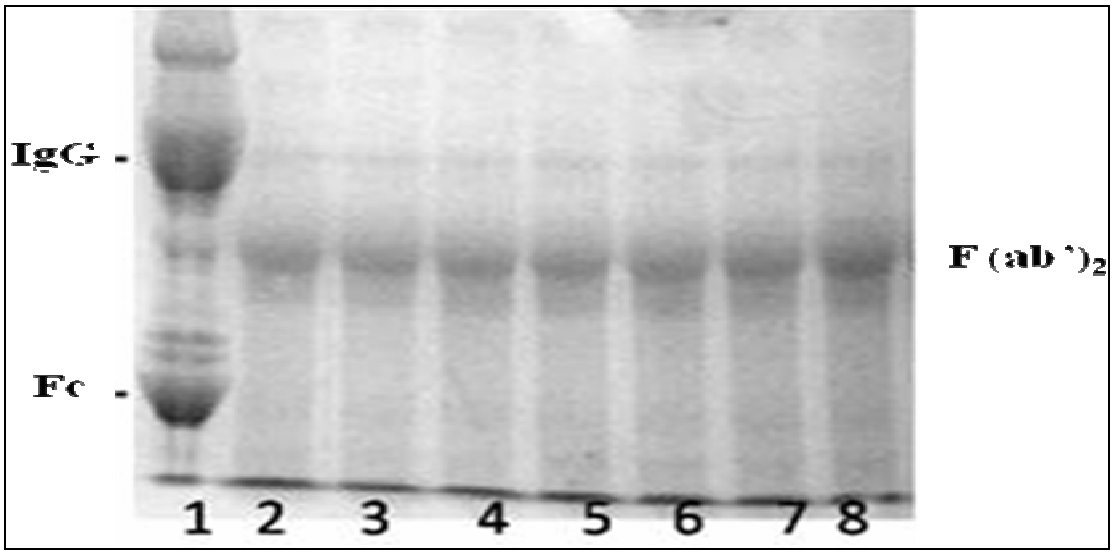

Fig.-6: Digestion of IgG using soluble and immobilized pepsin
Lane 1(Intact standard IgG), Lane 2(First fraction with immobilized pepsin), Lane 3 (Second fraction with immobilized pepsin), Lane 4 (Third fraction with immobilized pepsin), Lane 5 (Forth fraction with immobilized pepsin), Lane 6-8 (Hydrolysis Fraction with soluble pepsin).

CONCLUSION

Polyethyleneimine coated supports provide a powerful tool for immobilization and stabilization of pepsin under acidic conditions. This multipoint ionic interaction conserves the enzyme in its native non-distorted active-state, allowing its potential use in industrial applications. The operational and storage stability of immobilized pepsin increased considerably as compared to soluble enzyme, however additional work needs to be carried out. Immobilized pepsin was efficiently used for hydrolysis of IgG to obtain F (ab')$_2$ fragments without affecting its specificity. This immobilization technique opens a window towards the successful development of immobilization methods for pepsin.

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