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New Composite, Poly(Hydroxyethylmethacrylate-Expanded Perlite), for Single step Separation of Egg White Lysozyme

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Abstract. In this study, we investigated adsorption behaviour of lysozyme on poly(hydroxyethylmethacrylate-expanded perlite) [P(Hema-Ep)]. For this purpose, we synthesized P(Hema-Ep) composite by bulk polymerization and it was characterized by SEM, FTIR and swelling ratio. The fresh chicken egg white lysozyme was used as a model protein, had an isoelectric point (pI) 10.7 and molecular weight (MW) 14 kDa. Adsorption capacity was found 51 mg g^{-1} at room temperature and in 0.1 M pH=8.0 phosphate buffer. Experiments on desorption and reusability were also performed. It appears that P(Hema-Ep) composites can be used ten times without lost of efficiency. Lysozyme purification, without causing any denaturation, can be performed from chicken egg white by using this new synthesized composite in a single step.

***Keywords*:** Hydroxyethylmethacrylate, Lysozyme, Perlite, Purification.

Poli(Hidroksietilmetakrilat-Genişletilmiş Perlit) Kompoziti ile Yumurta Akından Lizozimin Tek Basamakta Saflaştırılması

Özet. Bu çalışmada poli(hidroksietilmetakrilat-genişletilmiş perlit) P(Hema-Ep) kompozit materyali kullanılarak sulu çözeltiden lizozim adsorpsiyonu çalışıldı. Bu amaçla öncelikle P(Hema-Ep) kompoziti sentezlendi ve SEM, FTIR ve şişme testleri ile karakterizasyonu yapıldı. İsoelektrik noktası 10.7 (pI) ve molekül kütlesi 14 kDa olan tavuk yumurta beyazı lizozimi, enzimi model protein olarak kullanıldı. Oda sıcaklığında ve 0.1 M pH:8.0 fosfat tamponunda gerçekleştirilen deney sonucunda adsorpsiyon kapasitesi 51 mg g^{-1} olarak bulundu. Desorpsiyon ve tekrar kullanılabilirlik deneyleri yapıldı. Sentezlenen P(Hema-Ep) kompoziti verimini kaybetmeden on kere tekrar kullanılabilir olduğu saptandı. Sentezlenen bu yeni kompozit ile lizozim enzimi tek basamaka saflaştırıldı.

***Anahtar Kelimeler*:** Hidroksietilmetakrilat, lizozim, perlit, saflaştırma.

1. INTRODUCTION

Lysozyme (EC 3.2.1.17), damages bacterial cell walls by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins [1]. Lysozyme can be found in a number of secretions such as tears, saliva, human milk, mucus and large amounts are found in egg white [2]. Because of cell damaging properties, lysozyme is used as a cell disrupting agent, antibacterial agent, food additive, drug and many additional applications in the literature [3]. There are wide range of methods that are used to isolate lysozyme. Some of these methods are: Aqueous biphasic system for lysozyme separation studies [4], affinity cryogel and composite cryogel for lysozyme purification [5-7]. In addition, dye-

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attached nanoparticles were used for lysozyme seperation [8].

An amorphous volcanic rock, perlite, has water content as 5% and silicate content more than 70%. This naturally occuring volcanic rock has a property of expansion after heating between 850 and 900 ^{o}C as it loses the water trapped within its structure and becomes a relatively light, white mineral called expanded perlite [9]. Perlite mainly composed of aluminium silicate and the silanol groups formed by silicon atoms on the surface of perlite that gives the adsorptive properties to perlite. Its surface's charge can be changed (more negative or positive) according to the pH of the medium. Thus surface charge depent on the pH [10]. In addition, its surface can be modified by functional groups for high protein adsorption.

Perlite have got some advantages such as, low cost, resistance to microbial attacks, mechanical stability, high surface area in comparison with inorganic adsorbents such as silica gels, alumina and zeolites [11]. Because of nice adsorptive features, expanded perlit is effectively used in the food industry such as processing of vegetable fat, juice and beer [10]. Moreover, it is also used as a high performance fillers, fitlers for chemical industry [9]. Silica are used as a matrix for protein separation and enzyme immobilization [12-14]. Although there are plenty of studies about lysozyme adsorption on silica containing materials [15, 16]. Our study describe for the first time, lysozyme adsorption is studied on expanded perlite (natural material that contain silica groups) composite.

In this work, P(Hema-Ep) composite was used for lysozyme adsorption studies which was performed at aqueous solution in batch system. For this purpose, firstly P(Hema-Ep) composite was synthesized by bulk polymerization and characterized by SEM (Scanning electron microscopy,) FTIR and swelling ratio. Then composites were used for adsorption studies. Following adsorption experiments, desorption and resorption studies were performed.

2. MATERIALS AND METHODS

2.1. Materials

Lysozyme (chicken egg white, EC 3.2.1.17), ethylene glycol dimethacrylate (EGDMA), TEMED were obtained from Sigma-Aldrich. Expanded perlite (Ep) was obtained from Etibank (Izmir, Turkey). Ammonium persulfate (APS) were purchased from Merck (Germany).

2.2. Preparation of P(Hema-Ep) composite

After washing step the mineral was dried for 24 h at 110 ^{o}C and then prepared in 100 mesh-size [17]. To prepare P(Hema-Ep) composite by bulk polymerization following experimental procedure was applied: 2 g of Ep and 10 mL Hema solution (1/3 Hema (v/v)), 8 mL EGDMA as a crosslinker, 500 µg APS as a starter, and 200 µL TEMED as a accelerator were mixed. The obtained mixture was stirred for 4 h at 25 ^{o}C. The product, 25 g of P(Hema-Ep) composite, was washed and then dried.

2.3. Characterization of P(Hema-Ep) composite

For screening surface morpholgy of the P(Hema-Ep) composites SEM analysis was used. The composite was coated with a thin layer of gold under reduced pressure, and their surface morphology was visualized using SEM (JEOL/JSM-6335F).

Chemical structure of P(Hema-Ep) was characterized by using FTIR spectrometry (Mattson 1000, UK). The composite was prepared as KBr pellet and spectra were taken five times using 4 cm^{-1} resolution and 400–4000 cm^{-1} frequency range.

Similar particle size of newly synthesized P(Hema-Ep) composite was prepared by using Tyler Standard sieves (Quantachrome Instruments).

Swelling ratio of Ep, P(Hema) and P(Hema-Ep) composite were determined as following procedure: 1.0 g of dry composite was placed into distilled water and kept at 25 ± 0.5°C. Swollen composite was taken and weighed. The weight ratios of dry and swollen samples were recorded.

The swelling ratio of the swollen composite was calculated using the equation 1.

$$\text{Swelling ratio}(\%) = [(W_f - W_o)/W_o] \times 100 \quad [1]$$

Where Wo and Wf are the weight of the composites, before and after swelling, respectively.

All experiments were always performed in triplicate.

2.4. Adsorption

To investigate adsorption behaviour of lysozyme on the P(Hema-Ep) composite; 10 ml of lysozyme solution were incubated with composite which magnetically stirred at 100 rpm at 25°C during 2 h (i.e.,equilibrium time), in a flask. The initial concentration effects of lysozyme on adsorption capacity was performed by changing the initial concentration of lysozyme between 0.3 and 3.0 mg/ml values. Bradford reagent was used for determination of lysozyme concentration at 595 nm. Following equation was used for determination of adsorption capacity:

$$Q = (C_o - C) \times V/m \quad (1)$$

Q ; mg adsorbed lysozyme per gram of adsorbent, Co and C ; initial and final concentration of lysozyme (mg/ml), respectively. V; medium volume (ml); m; the amount of adsorbent (g).

Effects of pH, temperature and ionic strength on lysozyme adsorption on to P(Hema-Ep) composites were studied. The acidity of the solution was changed between 6.0 and 11.0 by using different buffer systems, phosphate buffer (0.1 M pH 6.0–8.0), and carbonate buffer (0.1 M pH 9, 10, 11); The temperature effect on lysozyme adsorption capacity was carried out at three different temperatures, 23-33-43°C, in 0.1 M pH 8.0 phosphate buffer containing 1.0 mg/ml lysozyme solution. The ionic strength effect on lysozyme adsorption capacity was studied in the range of 0.0–1.0 M NaCl containing solution.

2.5. Desorption and resorption

To investigate the resorption cyle number of the P(Hema-Ep) composite, resorption experiments were performed ten times by using the same composites after adsorption and desorption which is performed in 1.0 M NaCl containing aqueous solution as desorption agent. Lysozyme adsorbed P(Hema-Ep) composites were placed in the desorption medium and stirred for 1 h at 25 °C at 100 rpm. The lysozyme concentration within the desorption medium was determined by method in section 2.4.

2.6. Purified lysozyme activity

Preparing egg white was described as the following experimental procedure was applied for preparing chicken egg white. Chicken egg white was separated from fresh eggs and mixed with a phosphate buffer (50% (v/v), 100 mM, pH 8.0). The prepared egg white was homogenized in an ice bath and centrifuged at 4°C at 10,000 rpm for 30 min. 100 mg P(Hema-Ep) microcomposites were stirred with 10 ml of prepared egg white solution for 1.5 h in a container magnetically at 150 rpm. The P(Hema-Ep) composites were next washed and removed unbound proteins centrifugation. Then, for desorption of lysozyme from lysozyme adsorbed P(Hema-Ep) composite, it was placed in the desorption medium and stirred for 1 h at 25 °C at 100 rpm. The desorption of lysozyme from P(Hema-Ep) composite was carried out in 1.0 M NaCl. The lysozyme activity was determined by using optical density measurements at 450 nm by spectrophotometer, and the rate of decrease of culture of *Micrococcus lysodeikticus* cells suspended in the phosphate buffer (0.1 M, pH 8.0) was followed for 5 min after addition of lysozyme. One enzyme unit (U) is the amount of enzyme that decrease of absorbance as 0.001 units per minute (at pH 8.0, 25°C) in OD 450.

3. RESULTS AND DISCUSSION

3.1. Characterization of P(Hema-Ep) composite

3.1.1 Interaction sites of P(Hema-Ep) *composite*

Isomorphous substitution is a replacement that replacing divalent ions with tetravalent silicon in the tetrahedral sheet and obtain a net negative charge on the clay surface [18-21]. Positive charged groups or ions can be adsorbed onto the

negative charged Ep surface due to the ionic interactions (Fig.1). Although "Hema", by itself, had inert features for adsorption, it might have increased the adsorption capacity of ''Ep'' in P(Hema-Ep) by enabling the expansion basal spacing of ''Ep''[17 ,22].

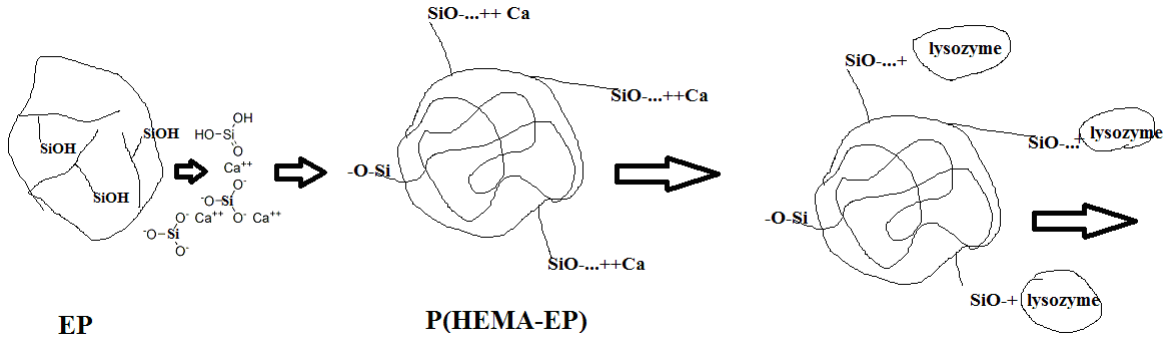

Figure 1. Presentation of interaction between P(Hema-Ep) and lysozyme.

P(Hema-Ep) was containing 1:3(w/w) of the Ep. The swelling ratio of Ep and P(Hema) compounds were found as 121% and 98%, respectively. A hypothetical expansion value of 179% was estimated for P(Hema-Ep). According to this result, hydrophilic nature of the composite P(Hema-Ep) was enhanced in comparison to its individual components that might be attributed to the hydrophilic contribution of silanol groups of Ep in P(Hema-Ep) to P(Hema). This large internal surface area may also provide a high ion-transfer rate. Particle size of newly synthesized P(Hema-Ep) composite was prepared by using Tyler Standard sieves and sieved to a particle size of 150 mesh, and stored in a polypropylene container.

3.1.2 Scanning Electron Microscobic (SEM) analysis of **P(Hema-Ep)** *composite*

The SEM picture of P(Hema-Ep) composite (Fig. 2) shows surface of the composite. The homogeneous view of P(Hema-Ep) composite was seen clearly in Fig. 2. The presence of polyhydroxyethylacrylamide in the perlite structure behaves as a filler between two perlite layers and by incorporation of polymeric filler, the foliated structure of perlite is lost and the perlite gains a rough surface [17].

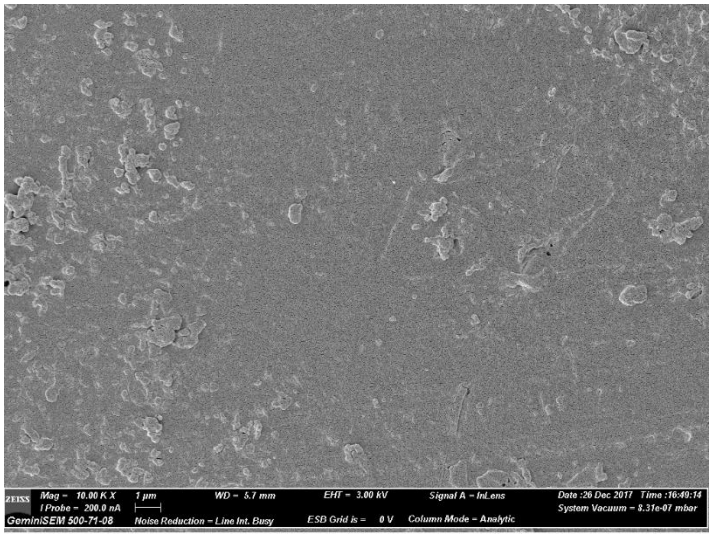

Figure 2. Scanning electron micrograph of P(Hema-Ep) composite.

3.1.3 FTIR

The FTIR spectra of P(Hema-Ep) is shown in Fig. 3. The characteristic structure of the FTIR spectra of P(Hema-Ep) composite shows peaks at 3,190 cm^{-1} as --Si–OH stretching vibrations. 1,170 cm^{-1}, and 1,650cm^{-1} (--Al–OH and --AI–2(OH) stretching vibrations) due to the presence of predominant silanol groups and hydroxyl groups, respectively. The surface silicon atoms try to keep on their tetrahedral coordination with oxygen. Silanol groups are formed by attachment to monovalent hydroxyl groups, at room temperature [9]. It was reported that amino acids which were positively charged, interacts with perlite via inner and outer layer through hydrogen bonding and cationic exchange interactions [23].

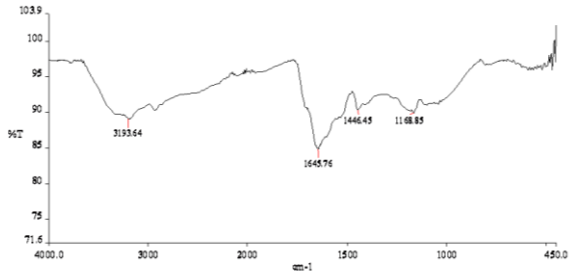

Figure 3. FTIR analysis of p(hema-ep) composite.

3.2 Lysozyme adsorption

3.2.1 Effect of pH

Lysozyme adsorption on to the P(Hema-Ep) composite was pH dependent as illustrated in Fig. 4. It increased from pH 6.0 to 8.0 and decreased after this pH value (Fig.4), means that the adsorption capacity of lysozyme was maximum at the pH values 8.0 which affects the end surface charge of the Ep and the degree of ionizable groups of lysozyme. Owing to the strong electrostatic interaction between negatively charged surface of P(Hema-Ep) composite and the positively charged lysozyme, the high adsorption capacity was obtained at pH 8.0. As the pH of the solution increased up to 8.0, interaction increased between the positive charges on the surface of lysozyme and the negative charges on the surface of P(Hema-Ep) composites. After pH 8.0, ionizable basic groups of lysozyme ionized and because of decreasing pozitive charges of lysozyme, interaction between adsorbent and protein was decreased.

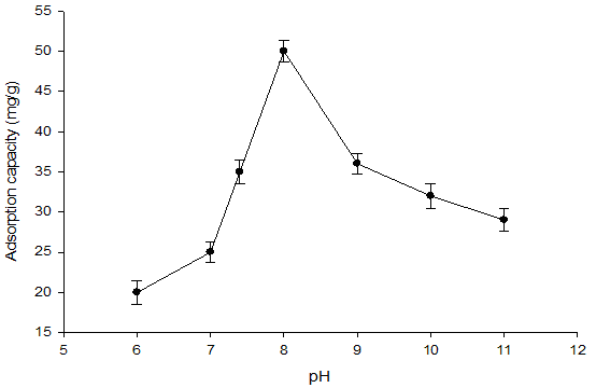

Figure 4. Effect of pH on adsorption. Lysozyme concentration: 1.0 mg/ml, T= 23 ºC.

3.2.2 Effect of Initial Concentration

In Fig.5, adsorption capacities of P(Hema-Ep) composites were given as a function of lysozyme concentration. The lysozyme concentration in solution increases and adsorption capacity of the composites were increased (Fig.5). Maximum adsorption capacities of P(Hema-Ep) composites was determined as 51 mg g^{-1}. This is due to the saturation at concentrations around 2.0 mg g^{-1} of the binding sites on the P(Hema-Ep) composites. Shao et. al. were used the PAA-modified Fe3O4@silica microspheres for lysozyme separation, and they found that the maximum binding capacity was 127 mg g^{-1} [15]. H. Guo et al. were used lysozyme imprinted magnetic chitosan microspheres and the results showed that the maximum binding capacity was 129.8 ± mg g^{-1} [24].

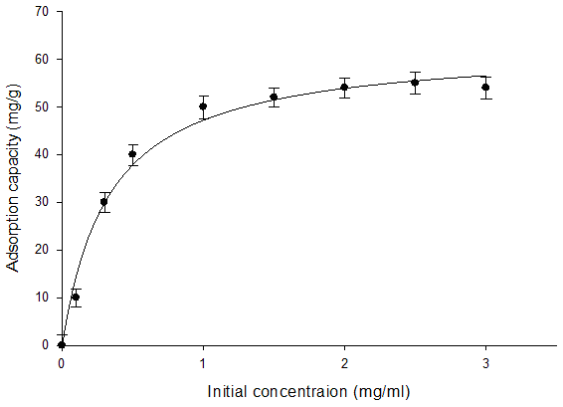

Figure 5. Effect of initial Lysozyme concentration. pH 8.0 (100 mM phosphate buffer), T= 23 ºC.

3.2.3 Effect of Temperature

Effect of temperature on lysozyme adsorption from aqueous solution is illustrated in Fig. 6. The effect of temperature on lysozyme adsorption was investigated at different temperatures, 23-33-43 ^{o}C, and as seen in Figure 6, lysozyme adsorption on to the P(Hema-Ep) composite is dependent on temperature. Maximum adsorption capacity was measured at 23 ^{o}C (Fig. 6). Adsorption capacity was decreased by increasing temperatures. This could be due to the surface silicon atoms which try to keep on their tetrahedral coordination with oxygen. Silanol groups are formed by attachment to monovalent hydroxyl groups, at room temperature [9]. The presence of predominant silanol groups and hydroxyl groups in the composite serves as an active binding site for metals and accordingly for positive charged protein molecules. At the higher temperatures, there is also adsorption on to the P(Hema-Ep) composites. This could be due to lysozyme stability. Some materials such as perlite were used for protein stabilization [25].

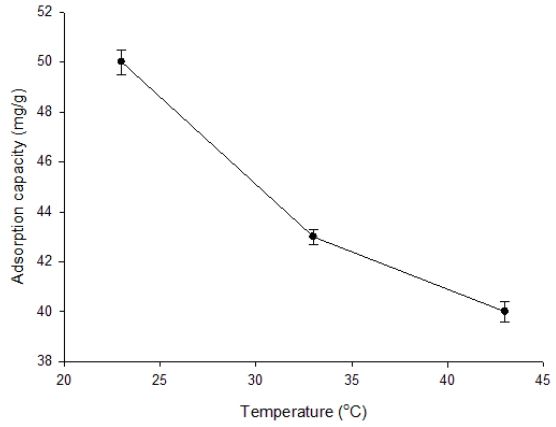

Figure 6. Effect of temperature. Lysozyme concentration: 1.0 mg/ml, pH 8.0 (100 mM phosphate buffer).

3.2.4 Effect of Ionic Strength

The studies of ionic strength effect on adsorption capacity of lysozyme on to the P(Hema-Ep) composites were investigated in the presence of different NaCl concentrations (from 0.0 to 1.0 mol L^{-1}). When the concentration of NaCl was increased from 0.0 to 1.0 mol L^{-1} the adsorption capacity of lysozyme decreased (from 50 to 5) mg g^{-1} as in mentioned in the literature [26-29], which shows that anions and cations affects the interaction between lysozyme and P(Hema-Ep) composites (Fig. 7). In fact, electrostatic interactions were the basic interaction between lysozyme and P(Hema-Ep) composites in the adsorption process. In addition, the adsorption process could be taking place on the surface of the P(Hema-Ep) composites. Thus, salt ions may interact with metal ions of composites at high salt concentrations and this could block the surface of the composites for interaction of lysozyme molecules.

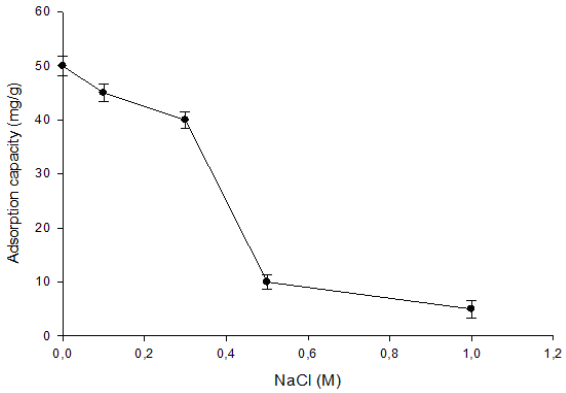

Figure 7. Effect of ionic strength. Lysozyme concentration: 1.0 mg/ml, pH 8.0 (100 mM phosphate buffer), T= 23 ^{o}C.

3.2.5 Desorption and Resorption

In this work, 97% of the adsorbed lysozyme molecules were desorbed easily from the P(Hema-Ep) composites during 1 h when 1.0 M sodium chloride was used as a desorption agent in the 100 mM pH 8.0 phosphate medium (Fig. 8). The desorption of lysozyme from the P(Hema-Ep) composites was performed in a batch system. On the basis of the research results (Fig. 8), it can be concluded that sodium chloride was a suitable desorption agent. To demonstrate the reusability of the P(Hema-Ep) composites, the adsorption/desorption cycle was repeated 10 times with the same P(Hema-Ep) composites from an aqueous lysozyme solution (Fig.8). There was no significant loss in the adsorption capacity of the composites after 10 cycles.

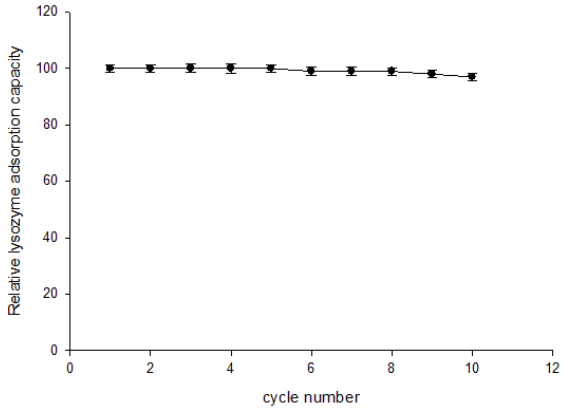

Figure 8. Reusability of P(Hema-Ep) composite.

3.3 Lysozyme activity

Chicken egg white's protein components were ovalbumin (pI:4.5), ovotransferrin (pI:6.1), ovomucoid (pI:4.5) and lysozyme (pI:10.7) [30,31]. The specific activity of the purified lysozyme with P(Hema-Ep) composite was 50 U mg^{-1} which was reported in literature [32].

4. CONCLUSION

In the last decade, there has been increasing interest in finding easily available, low-cost materials with high adsorption affinities such as bentonite, zeolite, chitosan, hydroxyapatite, and perlite. Due to large deposits of perlite in Turkey, perlite can be easily obtained. Perlite is one of the promising adsorbents but it was not used effectively for biomolecules. In this work, for the first time, it is used as a suitable and low-cost adsorbent for purification of lysozyme from egg white. The best efficiency of perlite is due to its rough structure and the presence of matrix of macro-pores in it that yields greater active surface area, enhancing adsorption. Composite materials were used in a various of protein purification applications in literature [33-36]. The inert behavior of P(Hema) enables it to act as a matrix (host) within the composite, expanding the adsorbent capacity of Ep (guest) particles. Hence, by using P(Hema-Ep) composites lysozyme could be purified in a single step from diluted egg white.

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