# Purification of Lysozyme with Congo Red Immobilized Cryogel Discs

# Kongo Kırmızısı İmmobilize Kriyojel Disklerle Lizozim Saflaştırılması

Research Article

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**ABSTRACT**

In this study, congo red immobilized poly(hydroxyethyl methacrylate) [CR-PHEMA] cryogel discs were prepared for purification of lysozyme. CR-PHEMA cryogel discs were characterized by swelling test, elemental analysis and scanning electron microscopy. Lysozyme adsorption was performed from aqueous solutions and maximum lysozyme adsorption capacity of CR-PHEMA cryogel discs was found to be 136.6 mg/g. Effects of initial lysozyme concentration, pH and ionic strength on lysozyme adsorption capacity of CR-PHEMA cryogel discs were investigated. Maximum adsorption was obtained at pH 8.0. In the last step of this study, lysozyme was purified from egg white using CR-PHEMA cryogel discs. Egg white fractions were observed on sodium dodecyl sulfate polyacrylamide gel electrophoresis.

**Key Words**
Congo red, lysozyme, PHEMA, cryogel.

**ÖZET**

Bu çalışmada, lizozim saflaştırılması için congo kırmızısı immobilize edilmiş poli(hidroksietil metakrilat) [CR-PHEMA] kriyojel diskler hazırlanmıştır. CR-PHEMA kriyojel diskler şişme testi, elementel analiz ve taramalı elektron mikroskobu ile karakterize edilmiştir. Lizozim adsorpsiyonu sulu çözeltilerden gerçekleştirilmiş ve CR-PHEMA kriyojel disklerin maksimum lizozim adsorpsiyon kapasitesi 136.6 mg/g olarak bulunmuştur. Başlangıç lizozim derişiminin, pH'ın ve iyonik şiddetin CR-PHEMA kriyojel disklerin lizozim adsorpsiyon kapasitesine olan etkileri araştırılmıştır. Maksimum adsorpsiyon pH 8.0'de elde edilmiştir. Bu çalışmanın son basamağında, lizozim CR-PHEMA kriyojel diskler kullanılarak yumurta beyazından saflaştırılmıştır. Yumurta beyazı fraksiyonları sodyum dodesil sülfat poliakrilamid jel elektroforezinde incelenmiştir.

**Anahtar Kelimeler**
Congo kırmızısı, lizozim, PHEMA, kriyojel

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## INTRODUCTION

Reactive dyes using in dye affinity chromatography are alternative ligands to traditional biological ligands. Dye affinity ligands are stable, cheap and can be easily immobilized. They have charged groups to interact with proteins. When they were compared with expensive and unstable biological ligands, dye ligands with high capacity were preferred for many protein purification processes [1-3].

Cryogels are new generation polymeric systems preparing under sub-zero temperatures. Macropores provide important advantages in purification of macromolecules such as protein, DNA and different kinds of cells. Cryogels modified by different ligands have been used successfully in adsorption and purification studies. When compared with conventional polymeric beads, cryogels have no back pressure problem because of their macroporosity. In addition, cryogels are cheap and can be easily prepared [4-7].

Lysozyme as an antibacterial protein has a great importance in pharmacy and food industry. Lysis of bacterial cell wall is the main function of lysozyme. Therefore, lysozyme can prevent bacterial growth on foods and provides longer storage time. Use of lysozyme in eye drops and anticancer drugs is also very common. Main source of lysozyme is egg white. Egg white contains many proteins such as ovalbumin, ovotransferrin and ovomucoid. Lysozyme constitutes only 3.5% of egg white proteins. Many methods to purify lysozyme were reported in the literature. Ion exchange chromatography and dye affinity chromatography are main methods applied for purification of lysozyme [8-11]. This study presents a dye affinity chromatography system. Congo red was immobilized on PHEMA cryogel discs and lysozyme adsorption efficiency of CR-PHEMA cryogel discs was investigated. After characterization studies, effects of pH, initial lysozyme concentration and ionic strength were observed. Purification of lysozyme from egg white was performed and sodium dodecyl sulfate gel electrophoresis (SDS PAGE) was used to evaluate the results.

## EXPERIMENTAL

### Materials

Lysozyme (chicken egg white, E.C. 3.2.1.7), congo red and ammonium persulfate (APS) were purchased from Sigma (St. Louis, MO, USA). 2-hydroxyethyl methacrylate (HEMA), N,N,N′,N′-tetramethylene diamine (TEMED) and N,N′ -methylene-bis(acrylamide) (MBAAm) were purchased from Fluka A.G. (Buchs, Switzerland). Water used in the experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed-bed system. All laboratory glassware rinsed with deionized water before use.

### Synthesis of PHEMA cryogel discs

Preparation of PHEMA based cryogels was presented before in the literature [12, 13]. Monomer HEMA and crosslinker MBAAm were dissolved in pure water under deoxygenated atmosphere. After stirring on an ice bath, APS and TEMED were added to the reaction mixture and the mixture was poured between two glass plates. Reaction was continued at -16°C for 24 hours. Then, PHEMA cryogel was thawed at room temperature and cut into circular discs (0.8 cm in diameter). In order to remove unreacted monomers, PHEMA cryogel discs were washed many times.

### Congo red immobilization

Covalent immobilization of congo red on PHEMA cryogel discs was achieved according to the method given below [14]. Congo red solution was heated on a water bath and PHEMA cryogel discs were added to this solution. Incubation of PHEMA cryogel discs in congo red solution was continued in basic conditions at 70°C. Then, congo red immobilized PHEMA cryogel discs (CR-PHEMA) were washed with water to remove unreacted dye. In order to test dye leakage, CR-PHEMA were washed with all the buffers used in this study.

### Characterization of PHEMA cryogel discs

Swelling properties of PHEMA cryogel discs were characterized by calculating the swelling degree (S).

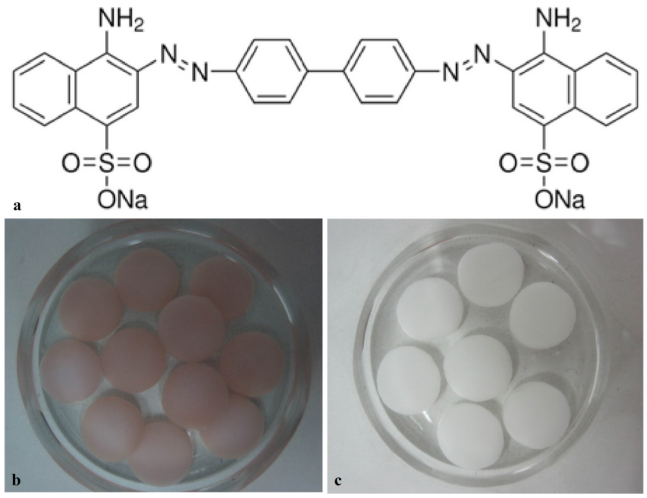


**Figure 1.** a) Molecular structure of congo Red. b) Optical photo of CR-PHEMA cryogel discs. c) Optical photo of PHEMA cryogel discs.

$$S=(m_{wet\ gel} - m_{dry\ gel}) / m_{dry\ gel} \quad (1)$$

Here, $m_{wet\ gel}$ is the weight of the wet cryogel discs and $m_{dry\ gel}$ is the weight of dried cryogel discs.

The bulk structure of PHEMA cryogel discs was investigated by using scanning electron microscopy (SEM). Gold-palladium (40:60) coated congo red immobilized cryogel discs were examined using JEOL JSM 5600 SEM (JEOL, JSM 5600, Tokyo, Japan). In addition, amount of congo red immobilized on PHEMA cryogel discs were found by elemental analysis (Leco Elemental Analyzer, Model CHNS-932, USA) using the sulphur stoichiometry.

### Adsorption studies

Lysozyme adsorption from aqueous solutions was performed in small beakers on a laboratory mixing rotator. 5 pieces of cryogel discs were added to 5 mL adsorption medium. Effects of pH, ionic strength and initial lysozyme concentration on adsorption of lysozyme on CR-PHEMA cryogel discs were tested. Protein amount was spectrophotometrically measured at 280 nm.

### Desorption and reusability

It is important that the materials using in protein purification are cheap and reusable. Desorption of lysozyme from CR-PHEMA cryogel discs were achieved by using 1 M of potassium thiocyanate (KSCN) solution. Reusability of CR-PHEMA cryogel discs was evaluated by using the same cryogel discs six times in adsorption-desorption cycle. After each desorption CR-PHEMA cryogel discs were washed with 1 M of NaCl and water.

### Purification of lysozyme from egg white

Lysozyme was also purified from a natural source such as chicken egg white. Egg white was diluted in phosphate buffer (pH 7.0) and then centrifuged at 4000 rpm for 30 min. Supernatant was used as the lysozyme source and the method applied for adsorption studies were performed to purify lysozyme from chicken egg white. Chicken egg white extract and eluted sample was analysed by SDSAGE (SE 600 Ruby, Amersham Biosciences) with 12% separating gel.

## RESULTS AND DISCUSSION

### Characterization of CR-PHEMA cryogel discs

Swelling degrees of PHEMA and CR-PHEMA were found to be 9.6 and 9.4 $g_{H2O}/g_{cryogel}$, respectively. Both PHEMA and CR-PHEMA cryogel discs were spongy and dye immobilization did not affect the swelling properties of cryogel discs. Molecular structure of congo red was presented in Figure 1a. Covalent bonds were formed between aromatic amine groups of congo red and hydroxyl groups of HEMA [1]. Optical photos of CR-PHEMA and PHEMA cryogel discs were given in Figure 1b and Figure 1c. Color difference because of dye immobilization is apparent. Also, the amount of immobilized congo red was determined by elemental analysis and the value was found to be 25.4 μmol/g. No dye leakage was detected in desorption or adsorption buffers used in this study. Pore sizes ranged from 10 and 100 μm of CR-PHEMA cryogel discs can be

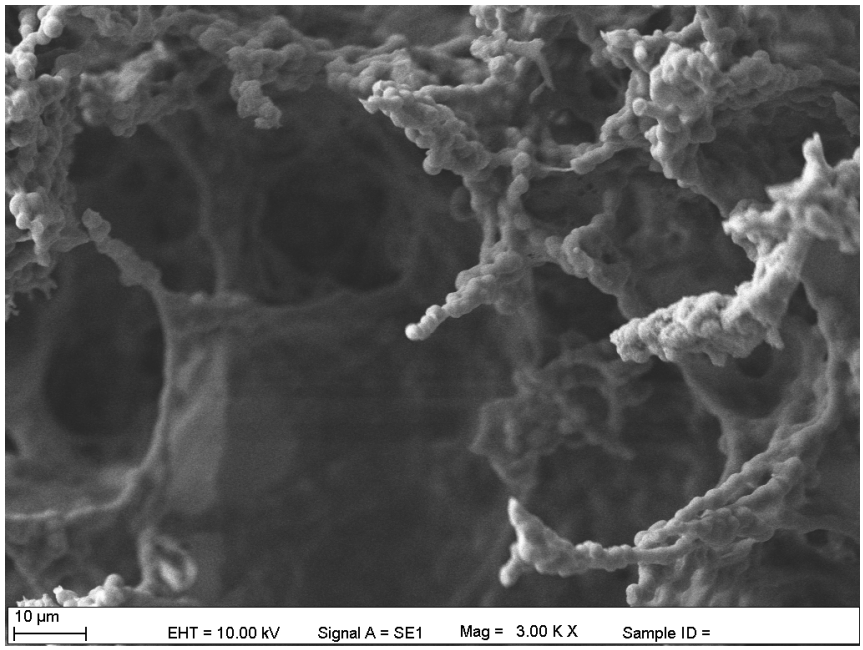


**Figure 2.** SEM image of CR-PHEMA cryogel discs.

clearly seen in SEM image presented in Figure 2. Due to interconnected macropores, lysozyme molecules can easily enter the pores without any diffusion limitations.

## Adsorption studies

### Effect of pH

Effect of pH on lysozyme adsorption on CR-PHEMA cryogel discs was investigated and the results were given in Figure 3. Maximum adsorption capacity was obtained at pH 8.0. Because the isoelectric point of lysozyme is around 11.0, it is positively charged at pH 8.0 [15]. Therefore, electrostatic interactions occur between positively charged lysozyme molecules and negatively charged congo red molecules.

### Effect of ionic strength

Concentration of NaCl in adsorption medium was changed between 0 and 0.1 M. Results related to the effect of ionic strength on lysozyme adsorption capacity of CR-PHEMA cryogel discs were given in Figure 4. It is clear that the increase in ionic strength cause a decrease in adsorption capacity. Amount of adsorbed lysozyme was 88.3 mg/g

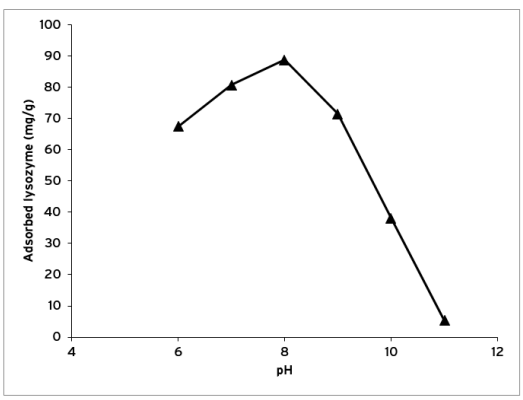


**Figure 3.** Effect of pH on lysozyme adsorption capacity of CR-PHEMA cryogel discs. Concentration of lysozyme: 1 mg/ml; T: 25°C.

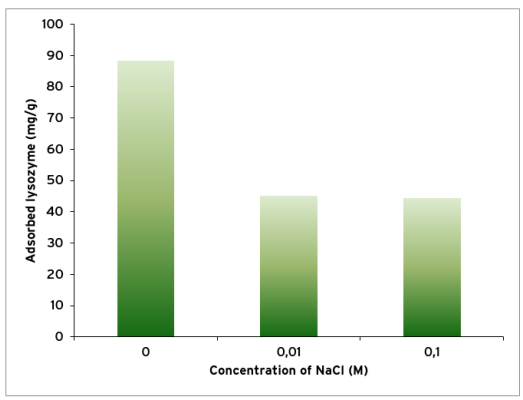


**Figure 4.** Effect of ionic strength on lysozyme adsorption capacity of CR-PHEMA cryogel discs. Concentration of lysozyme: 1 mg/ml; Phosphate buffer, pH 8.0; T: 25°C.

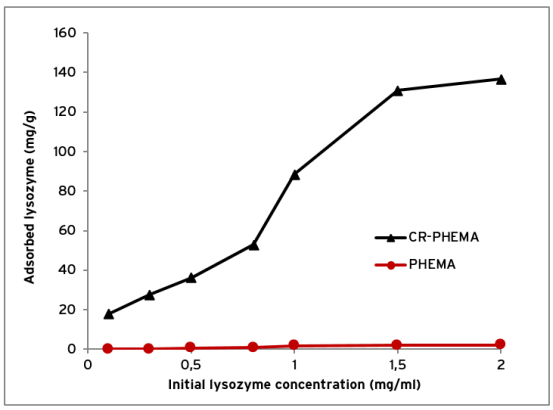


**Figure 5.** Effect of initial lysozyme concentration on lysozyme adsorption capacity of CR-PHEMA cryogel discs. Phosphate buffer pH 8.0; T: 25°C.

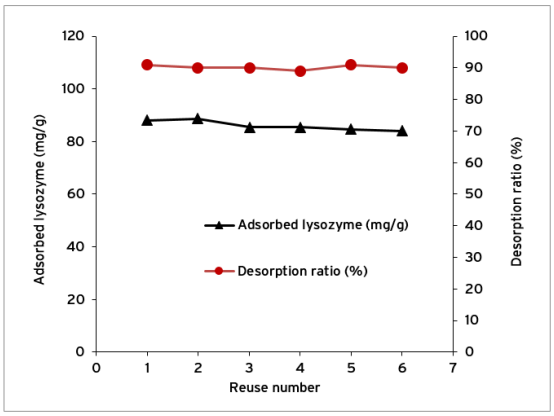


Figure 6. Reusability and desorption ratios of CR-PHEMA cryogel discs for lysozyme adsorption. Concentration of lysozyme: 1 mg/ml; phosphate buffer pH 8.0; T: 25°C.

when there was no NaCl in adsorption medium. But this value decreased to 44.3 mg/g when concentration of NaCl increased to 0.1 M. Salt molecules prevent the electrostatic interactions between lysozyme and congo red molecules.

### Effect of initial lysozyme concentration

Effect of initial lysozyme concentration on lysozyme adsorption capacity of CR-PHEMA cryogel discs was investigated. As clearly seen in Figure 5, amount of adsorbed lysozyme increases with increasing of initial lysozyme concentration. However, after 1.5 mg/ml initial lysozyme concentration, the graph reaches a plateau. Therefore, CR-PHEMA cryogel discs have maximum adsorption capacity on this point. Maximum adsorption capacity of CR-PHEMA cryogel discs was 136.6 mg/g which is obtained at 2 mg/mL initial lysozyme concentration. In addition, lysozyme adsorption on PHEMA cryogel discs is very low. Therefore, immobilization of congo red increases the lysozyme adsorption capacity

### Reusability

91% of desorption ratio was obtained using 1 M of potassium thiocyanate (KSCN) solution for lysozyme desorption from CR-PHEMA cryogel discs. Figure 6 presents the reusability of CR-PHEMA cryogel discs. Desorption ratios was also included for each repeat. According to the results the decrease in adsorption capacity is less than 5% after six adsorption-desorption cycle. In addition, desorption ratio is almost the same for each repeat. CR-PHEMA cryogel discs can be used many times without a significant decrease in lysozyme adsorption capacity.

### Purification of lysozyme from egg white

Lysozyme purification from egg white was performed and SDS PAGE results of egg white fractions were presented in Figure 7. Egg white proteins which are ovotransferrin (77 kDa), ovalbumin (45 kDa), ovomucoid (28 kDa) and lysozyme (14 kDa) can be seen on Lanes 2, 3 and 4. There is a single lysozyme band on Lane 5. This single band indicates that lysozyme was purified from egg white successfully using CR-PHEMA cryogel discs.

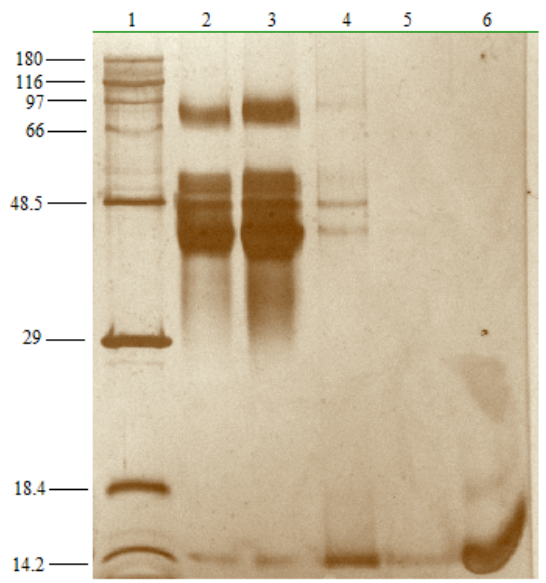


**Figure 7.** SDS PAGE of egg white fractions. Lane 1: Sigma marker, Lane 2: Egg white after adsorption to CR-PHEMA cryogel discs, Lane 3: Egg white before adsorption to CR-PHEMA cryogel discs, Lane 4: Washing solution after adsorption, Lane 5: Eluted sample from CR-PHEMA cryogel discs, Lane 6: commercial lysozyme (14 kDa).

## CONCLUSION

Antibacterial properties of lysozyme make it important in drug and food industry. The study presented here proposes an easy and cheap way to purify lysozyme from egg white extract. Macropores of PHEMA based cryogel discs are advantageous for lysozyme purification as lysozyme molecules can easily reach ligand molecules on the surface of CR-PHEMA cryogel discs. Easy immobilization of congo red and high capacity of CR-PHEMA cryogel discs should also be noted. In conclusion, as it is showed in SDS PAGE analysis, CR-PHEMA cryogel discs are useful materials to purify lysozyme from complex egg white extract.

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