Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2008; 33 (4) ; 148–153.

Research Article [Araştırma Makalesi]

Yayın tarihi 23 Ocak, 2009 © TurkJBiochem.com

[Published online 23 January, 2009]

Glutaraldehyde Cross-Linked Agarose Carriers: Design, Characterization and Insulin Release Behaviour

[Glutaraldehit ile Çapraz Bağlanmış Agaroz Taşıyıcılar: Sentezi, Karakterizasyonu ve İnsulin Salım Davranışı]

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[Received: 04 June 2008, Accepted: 01 Eylül 2008]

Kayıt tarihi: 04 Haziran 2008, Kabul tarihi: 01 September 2008

ABSTRACT

Purpose: Insulin is commonly used in the treatment of diabetus mellitus. In this study, controlled release of insulin was investigated and glutaraldehyde crosslinked agarose films examined as a insulin carrier. Swelling ratios and surface properties of agarose films were investigated for the characterization of the carrier materials.

Materials and Methods: In vitro insulin release studies were carried out in physiological saline solution. The amount of released insulin was determined with spectrophotometric analyzing and the effects of film composition and insulin loading on release profile were investigated.

Results: The swelling ratio of the agarose films was 93% and approximately 1.63 times decreased with glutaraldehyde crosslinking. The insulin release ratio was decreased with increasing crosslinking agent and the maximum insulin release was obtained by using non-crosslinked agarose films with a cumulative release of 91.5% within 10 h. By increasing the glutaraldehyde concentration in agarose film structure, the release rate of insulin were decreased but the release time was rised to 18 h.

Conclusion: In diabet treatments, a slow-prolonged stable insulin release profile is preferable. So from these results it can be concluded that glutaraldehyde crosslinked agarose films might be a new candidate carrier for the controlled insulin release systems.

Key Words: Agarose, controlled release, crosslinking, insulin

ÖZET

Amaç: Insulin diabetus mellitus tedavisinde yaygın bir şekilde kullanılmaktadır. Bu çalışmada insulinin kontrollü salımı çalışılmıştır ve glutaraldehit ile çapraz bağlanmış agaroz filmler insulin taşıyıcı sistem olarak test edilmiştir. Agaroz jellerinin karakterizasyonu için şişme ve yüzey özellikleri incelenmiştir.

Materyel ve Metod: In vitro insulin salımı fizyolojik tuz çözeltisi içerisinde gerçekleştirilmiştir. Insulin salım miktarı spektrofotometrik analiz ile belirlenmiş ve salım profili üzerine film komposizyonu ve insulin yükleme parametrelerinin etkisi de incelenmiştir.

Bulgular: Agaroz filmlerinin şişme oranı yaklaşık %93 olarak bulunmuştur ve çapraz bağlama ile birlikte şişme oranının 1.63 kat azaldığı belirlenmiştir. Çapraz bağlayıcı oranının artması ile insulin salım oranının azaldığı gözlenmiş ve maksimum insulin salımı çapraz bağlayıcı içermeyen filmlerle 10 saat içerisinde %91.5 olarak elde edilmiştir. Agaroz film yapısında glutaraldehit konsantrasyonunun artması ile insulin salım oranının azaldığı fakat salım süresinin 18 saate çıktığı belirlenmiştir.

Sonuç: Diabet tedavilerinde yavaş, uzunsüreli ve sabit bir salım profili tercih edilmektedir. Bu çalışmada elde edilen verilerden glutaraldehit ile çapraz bağlı agaroz filmlerinin kontrollü insulin salım sistemleri için kullanışlı bir taşıyıcı olduğu gözlenmiştir.

Anahtar Kelimeler: Agaroz, kontrollü salım, çapraz bağlama, insulin

Introduction

Over the past several decades, biotechnology has developed extensively and led to a significant increase in the number of bioengineered products especially protein drugs like hormones and their analogues used in treatment of many diseases, such as diabetes. Most of these products are generally administered via the parenteral route instead of oral application because of the protein inactivation by acid and proteases of the gastrointestinal tract (1,2). A promising strategy to increase the therapeutic efficacy of these proteins is the use of controlled release system that releases the drug at a rate or at a location continuously. Several controlled release systems have been tested for their ability to sustain the release of drugs such as insulin (2,3), theophylline(4), bromocresol green (5), platelet derived growth factor (6) and interferon (7).

A range of materials have been employed to control the release of drugs and other active agents. Most of the materials used in controlled release systems are based on hydrogels, which are hydrophilic polymeric networks capable of imbibing large quantities of water (8), And also Hydrogels are stabilised by cross-links between the polymeric units that form the backbone of the gel (9,10).

In this study glutaraldehyde crosslinked agarose hydrogel carriers were devoleped and examined in controlled release system. Insulin was chosen as a model drug/protein agent for incorporation in the polymer matrix. For this purpose, insulin loaded agarose films were prepared and characterization, insulin release studies were conducted in-vitro. In these studies the effects of crosslinking agent concentration and insulin loading ratio on the release profile were determined.

Material and Methods

Preparation of agarose films

Agarose films with a concentration of 2.0% (w/w) were prepared as follows: The desired weight of agarose type VII (Sigma, St. Louis, MO) and different concentrations of glutaraldehyde solutions (0.5-1.5%) were added to distilled water, and then the mixture was heated to the boiling temperature of the solution for complete dissolution of the agarose. After that, the resulting agarose solutions were cooled to room temperature. Before the agarose solution gelled insulin (50 IU/ml, Humalog, Eli Lilly, USA) was added and to determine the effect of pH on insulin loading capacity, the soaking medium pH was adjusted between 4.0-5.5. The effect of initial insulin concentration on loading efficiency was determined on AGF-4 films with three concentration of insulin (25, 50, 100 IU/ml). All prepared solutions were poured into a plane petri-dish and after gelation period agarose films were cut into circular pieces (diameter 1.0 cm, thickness:0.06 cm) with a perforator. The resultant circular films were washed with water and phsiological buffer to removal the non-polimerizad monomers. All the AGF formulations prepared in this study was summarized in Table 1.

To investigate the glutaraldehyde leakage from the agarose preparations, AGF-2, AGF-3 and AGF-4 films were soaked in physiological buffer for 48 h. After this period films were removed ant the buffer was analyzed for glutaraldehyde leakage. Glutaraldehyde concentration was determined by a process based on formation of the glutaraldehyde-bisulfite complex described by Frigerio et al. (11)

The surface morphology of the AGF-1 and AGF-4-50 films were observed by scanning electron microscopy (SEM). The dried film were coated with gold under reduced pressure and their scanning electron micrographs were obtained using a JEOL (Model JSM 5600; Japan).

Determination of loading efficiency

The loading efficiency of films were determined as follows: 1 mg agarose films were hydrated with 0.5 M HCl for 6 h under magnetic stirring at room temperature. After 24 h, the supernatants were filtered and centrifuged 15 min (MinispinPlus, Eppendorf). Insulin concentration was determined spectrophotometrically using the Bradford assay (12). Briefly, supernanat samples and Bradford reagent were mixed at 1:1 (v/v) ratio and incubated at room temperature for 15 min. The absorbance was measured at 595 nm and the incorporation efficiency (%) determined by insulin released as percentage of initial amount used in formulation.

Table 1: AGF formulations with different crosslinking agent concentration

Carrier matrix	Agarose (%)	Glutaraldehyde (%)	Drug loading
AGF-1	2.0	0.0	50 IU/ml
AGF-2	2.0	0.5	50 IU/ml
AGF-3	2.0	1.0	50 IU/ml
AGF-4-25	2.0	1.5	25 IU/ml
AGF-4-50	2.0	1.5	50 IU/ml
AGF-4-100	2.0	1.5	100 IU/ml

Swelling properties

AGF films (0.1g) were carefully weighed before being placed in 50 ml vials containing physiologic buffer. The vials were placed in a waterbath (37°C) for 4 h. The samples were removed periodically every 15 min and weighed. The swelling ratio of AGF films were calculated by using Equation 1.

Water uptake ratio %=(W_S - W_0)/ W_0 x 100 (1)

W_0 and W_S are the weights of films before and after uptake of water, respectively.

In vitro release studies

Insulin release studies were carried out in a continuous release system. The continuous release system consisted of a column with 17 cm length and 0.9 cm diameter. The system temperature was controlled by circulating water through the jacket part of reactor. The insulin-loaded carriers (1.9g) were placed in the release cell and the physiological buffer was introduced into the release cell at a flow-rate of 0.1 ml/min using a peristaltic pump (Longerpumup BT100-1J) through the lower inlet. At defined time intervals, samples were collected and assayed for insulin release. Insulin concentration was determined spectrophotometrically using Bradford Assay (12). Briefly, samples obtained from release media and Bradford reagent were mixed at 1:1 (v/v) ratio and incubated at 25°C for 15 min. The amount of released insulin was measured at 595 nm with UV-Vis spectrophotometer. All the results were made in triplicate.

The following Equation 2 could be used to evaluate the Fickian and non-Fickian mechanism used for the analysis of data of Fickian or non-Fickian diffusional release parameters (13).

$Mt/M\infty = Kt^n$ (2)

Where *Mt* is the amount of insulin released at time *t*, $M\infty$ is the total amount of insulin released and *K* is a constant including structural characteristics of the carrier system and the drug, and *n* is a constant which relates to the transport mechanism.

Insulin integrity

The protein integrity of insulin released from the AGF-4-50 films was analyzed by Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The samples were treated with dithiothreitol and boiled for 3-5 minute to reduce the disulfide bridges. The separating gel of 20% acrylamide and the stacking gel of 5% acrylamide was used. The sample buffer and running buffer are same as those of the standard Laemmli method (14). The samples (20 ml of loading volume per well) were applied to wells with a microsyringe. For band detection, the gel was stained with 0.1% Coomassie brilliant blue G-250 in 10% acetic acid and 45% methanol for 15 to 20 min and then destained in 10% methanol and 10% acetic acid.

Data are presented as ± standard error of mean. Statistical evaluation was performed with a one-way ANOVA followed by a Dunnett multiple comparison test. A $p<0.05$ was taken as the criterion of significance.

Results and Discussion

Agarose is a polysaccharide obtained from red algae of the *Rhodophyceae* class which can be considered as an alternating copolymer of β-D-galactopyranosyl and 3,6-anhydro-α-L-galactopyranosyl units. The surface SEM photographs of the AGF films are shown in Figure 1. It was suprising that after crosslinking, bearing formations were observed on the surface structure of films. These new creations can be explained by the decreased molecular mesh size with increasing croslinking density. Studies aimed at detecting leakage of glutaraldehyde from AGF-2, AGF-3 and AGF-4 films revealed no leakage in any of the release media.

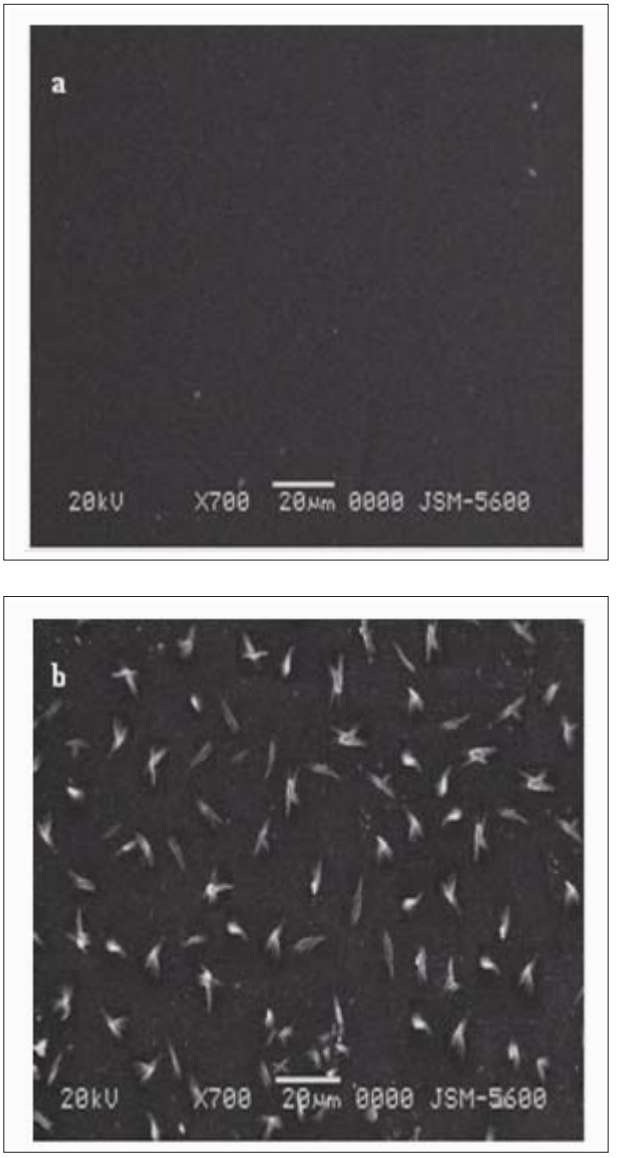

Figure 1. Scanning electron micrographs of the a. AGF-1 and b. AGF-4 films

Swelling studies

The swelling properties of AGF-1-4 formulations are shown in Figure 2. The swelling ratio of the agarose films was 93% and the minimum swelling ratio was obtained with AGF-4 as 57%. The ionize groups of the agarose introduced positive and negative charges into the polymeric structure, and should be caused more water uptake. Crosslink reactions are frequently performed to avoid the solubility of the carrier in aqueous environments. The swelling ratio of AGF films decreased with the increased croslinking agent concentration. The water content of the crosslinked material decreased about 1.63 times with respect to uncrosslinked counterpart. This observation can be explained by the reducing the molecular mesh size of the agarose for water diffusion. Alike, Bajpai (5) studied the controlled release of bromocresol green as a model drug from casein crosslinked polyacrylamid carriers and noted that with increasing the crosslinker concentration in polymer structure, the swelling ratio of polyacrylamid hydrogels decreased. And also it can be observed from the Figure 2, the swelling of the films were quite rapid approximately within 2 h.

Loading efficiency

pH has an important effect on insulin loading capacity of AGF-1-4 films. As seen from the Figure 3, the maximum loading capacity was observed at pH 4.5 for AGF1 and at pH 4.0 for AGF-2, AGF-3 and AGF-4 carriers as 77.6±2.9%, 81.3±3.1%, 87.5±2.1% and 93.2±1.9% of the initial amount of insulin (50 IU/ml), respectively. Insulin has an isoelectric point around 5.3 and insulin would be cationic at pH values below this point (15). At pH values below 5.3, electrostatic attractions between hydroxyl groups (-OH) of agarose and oppositely charged insulin may occur high incorporation efficiency. These clarifications may be explained the higher loading capacities obtained at pH 4.0 and 4.5. The optimal pH values for AGF-2, AGF-3 and AGF-4 films shifted down from 4.5 to 4.0. After crosslinking process, the surface charges of agarose polymer might be screened so the electrostatic attaractions between insulin and carrier shifted acidic regions. Reis et al.(16) studied alginate–dextran nanospheres for insulin release and reported an incorporation efficiency of 82.5%. Liu et al.(17) examined poly(dl-lactic acid)/poly(dl-lactic-co-glycolic acid) carriers for insulin release and which achieved an incorporation efficiency of 92.21%. Comparison of these results shows that the loading efficiency obtained in our study is in agreement with the amounts noted in literature.

Release studies

The data obtained from experimental studies show that the structural differences of carriers are significantly affected the release behaviour of materials. This phenomen is supported by Figure 4 which depicts the release profiles of insulin from agarose materials. When the swelling graphs and the release rates of the AGF-1-4 materials are examined, a rapid release profile was observed at the beginning of the insulin release. Initial burst release (39% in the first hour) from AGF-1 could probably be attributed to the diffusion of the insulin from the surface of the films. This result could be attributed to the the use of hydrophilic agarose polymers which rapidly takes up the water molecules leading to rapid initial release (burst) of insulin. This may give rise to difficulty in providing a true control of drug release and minimal inter-patient variability in insulin plasma levels. After crosslinking process initial burst release was decreased a range of 25-10%. The penetration of the solvent molecule was hindered or decreased due to crosslinking leading to slow drug release for a prolonged period.

The effect of different ratios of glutaraldehyde/agarose on the release profiles of insulin was studied. For the formulations of AGF1-4 the release percents were found 91.5, 85.6, 79.3, 66.0% within 10 h., respectively. In addition, insulin release from crosslinked films demonstrated statistical differences in comparison to non-

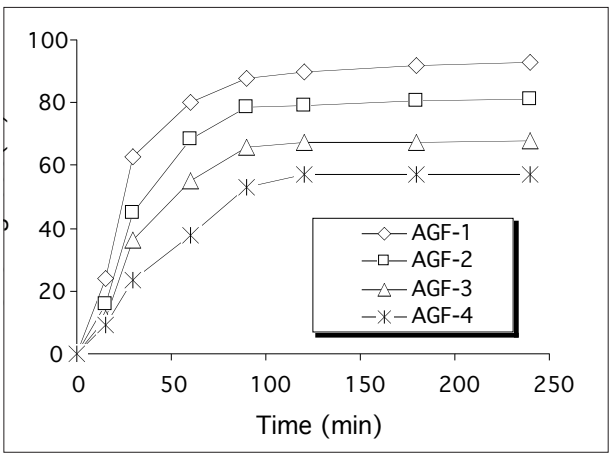

Figure 2. Swelling degrees of AGF-1-4 films over a period of 240 min with a temperature of 37oC.

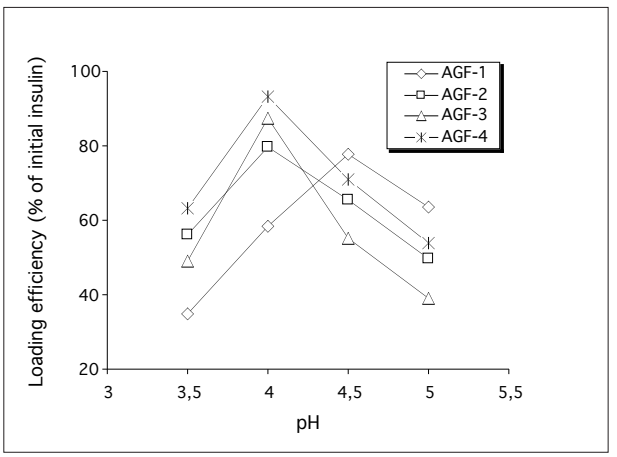

Figure 3. Effect of pH on the insulin loading efficiency of AGF-1-4 films

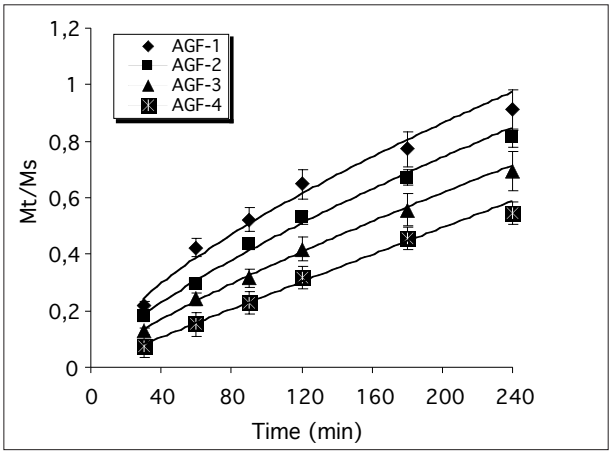

Figure 4. Amount of drug released in a period of 240 min as a function of time

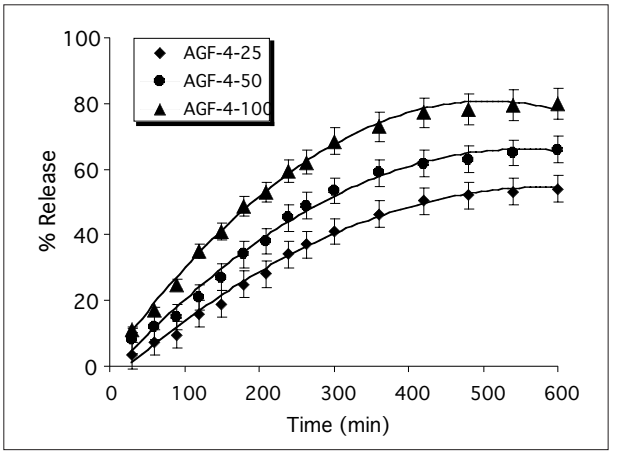

Figure 5. Release behaviour of insulin from AGF-1-4 films with different initial insulin concentrations

crosslinked counterparts (p <0.03). By increasing the glutaraldehyde concentration in agarose film structure used to crosslink the agarose, the release rate of insulin were decreased but the release time was rised to 18 h. Increasing the cross-linking concentration decreases the volume swelling and drug release rate that is attributed to the decrease of diffusion coefficient of drug and solvent (10).

For a perfectly Fickian process where the rate of solvent penetration is the slowest and hence is the rate limiting step, the value of n=0.50. When both the diffusion and polymer relaxation control the rate of water uptake, the diffusion mechanism is non-Fickian (0.5<n<1.0). The corresponding values of the K and the n for all carrier systems, are shown in Table 2. The n values reached in AGF systems ranging from 0.6 to 0.9. And also a value up to 0.98 was obtained from our results for $Mt/M\infty$ and 0.03 for K values. The values of release parameters n and k are inversely related. A higher value of k may suggest burst drug release from the carrier and also the n values of all formulations are within the limits of the non-Fickian transport mechanism. From all these results it is clear that the non-Fickian release mechanism takes place in all formulations.

The loading capacity of target molecule is very important in release profile. In diabet treatment a high value of insulin may be resulted a sharp decrease of glucose levels which can be undesired. So in this study the effect of drug loading on release profile was studied with AGF-4 films which provide a slow-prolonged controlled release of insulin. Three different drug loadings as 25, 50, 100 IU/ml were used and the carriers were coded as AGF-4-25, AGF-4-50 and AGF-4-100, respectively. The release profiles of insulin from three different drug loading AGF-4 materials are given in Figure 5. The release ratio of AGF-4-25, AGF-4-50 and AGF-4-100 were 55.6, 66.0, 79.8% within 18 h., respectively. AGF-4-100 with high dose insulin loading release a significantly higher (p<0.05) percentage of their content than low-dose counterpart as AGF-4-25 . An increase in drug concentration in the soaking medium of the gels also increases the loading efficiency significantly. A higher drug concentration provides an important driving force to overcome all mass transfer resistances between the gel structure and model drug. And also the increased loading capacity triggers the diffusion process and accelerates/prolongs the release process.

Studies of different polymeric films, insulin release rates and applications have been reported. Liu et al.(17) examined the effect of NaCl on insulin release profile of poly(dl-lactic acid)/ poly(dl-lactic-co-glycolic acid) and reported that the incorporation efficiency increased from 65.61% to 92.21% and the initial release on the first day decreased from 34.43% to 6.33% when NaCl concentration increased from 0 to 5.0 wt.%. In a study which examined aminated gelatin microspheres as a insulin carrier system, a cumulate release of 75.1% within 8 h was reported (18). Kim et al. (17) were studied the permeation of riboflavin and insulin from poly (vinyl alcohol) (PVA) and chitosan blend membranes and they reported that the permeation behavior was pH dependent and also discussed in terms of water content and water structure inside of the swollen membrane. Portero et al (20) developed a chitosan glutamate films which released insulin with a release rate of 80% in 6h. In another study, Kim and Park (21) were used the glucose-sensitive hydrogel membrane as insulin delivery device and noted that the insulin release rate decreased as the glucose concentration was reduced to 1 mg/ml. Comparison of these results shows that the insulin release rate and loading efficiency of AGF-1-4 systems in our study is in agreement the datas reported in literature. In addition a desired release rate can be attained when the structural characteristics and the enviromental conditions are varied.

To determine the integrity of released insulin, SDS-PAGE was applied to native insulin and released insulin from AGF-4-50. It was shown that fron the Figure 6, the two bands of native and released insulin were similar and clear. This result shows that the release conditions

Table 2: Fit of release data in Equation (2).

Carrier type	K	N	Correlation coefficient
AGF-1	0.0307±0.014	0.6170±0.007	0.9657
AGF-2	0.0161±0.005	0.7172±0.021	0.9924
AGF-3	0.0087±0.003	0.8014±0.068	0.9975
AGF-4	0.0035±0.001	0.9192±0.087	0.9889

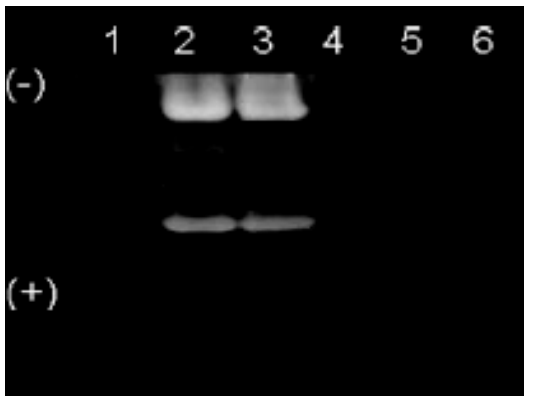

Figure 6. SDS-PGE of line 2: native insulin and line 3: released insulin from AGF-4-50

does not change the electric nature, mobility and molecular weight of the peptides so the activity of insulin should have been protected.

In this study we identify the ideal characteristics of the crosslinked agarose films. By choosing the film with the suitable characteristic properties and slowest release rate in vitro, we hope to obtain the slowest relative release rate in vivo at same conditions, despite the differences between the in vivo and in vitro systems. Due to physiological and anatomical barriers parenteral administration are less useful so the transdermal delivery is an attractive route for systemic administration. Several techniques have been developed to provide rate control over the release and transdermal permeation of drugs such as membrane-modulated systems and adhesive diffusion controlled systems. By using the transdermal system, the therapeutic agent is protected against enzymatic degradation in vivo thus allowing for prolonged release of the protein. The probable limitation in transdermal preparations is the extreme impermeability of the skin. To overcome this problem, permeability enhancer agents can be used to increase penetration through the skin. So insulin may be transported across the skin with a relatively high degree of efficiency when incorporated into a carrier. The release rate of insulin typically relies on diffusion protein through skin in transdermal applications. In general, this diffusion-controlled mechanism reduces the potential for higher burst release obtained in vitro studies.

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