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Studies on Decolorization Process for rhEGF as Cosmetic Ingredient

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Abstract A decolorization process using ion exchange chromatography was developed to refine rhEGF as a cosmetic ingredient. A macroreticular resin (D314) was selected, with respect to its high decolorization rate and recovery yield of rhEGF, and the operational conditions of the decolorization process optimized. The optimum conditions were as follows: the rhEGF effluent was ion exchanged at a flow rate of 60.0 mL/h, with an effluent pH 5.0, using a chromatographic column (i.d. 16 mm) packed with D314, with a 7.5 cm in bed height. The decolorization process was carried out under the optimum conditions, and halted when the effluent volume reached 350 mL, giving a decolorization rate and recovery yield of rhEGF higher than 67 and 80%, respectively. When the decolorization rate exceeded 67%, the final product turned out to be white or light yellowish, which was to the satisfaction of the cosmetic standard.

Keywords: recombinant human epidermal growth factor (rhEGF), decolorization, ion exchange chromatography, macroreticular resin, D314

INTRODUCTION

Human epidermal growth factor (hEGF), which is considered to be structurally and functionally identical to human urogastrone [1], is a single chain polypeptide, with a molecular weight of about 6,200 Da comprised of 53 amino acid residues. hEGF stimulates the proliferation of a number of cell types, including epithelial and epidermal tissues, enhances the syntheses of protein and RNA, and inhibits the secretion of gastric acid [2-7]. These biological properties have led to many therapeutic and cosmetic applications, which promise the development of high-quality hEGF production on a large-scale.

In order to achieve high hEGF productivity, gene recombination techniques are extensively used. Many previous studies have succeeded in addressing the technical challenges encountered [8-11]. Most of these studies have focused on the upstream processes of rhEGF production; however, the downstream processes have not been fully reported [12]. Nevertheless, the isolation and purification of rhEGF in adequate amounts is still a formidable task because properties, such as the isoelectric point of many heterogeneous proteins, some of which are pigments, in the fermentation broth of rhEGF are similar to those of rhEGF [11].

The requirements of different consumers for different applications would be satisfied by rhEGF with different purities, and rhEGF of moderate purity can be acceptable as a cosmetic ingredient. The purer the final product, the greater the production costs. An easily-operated and low-cost downstream process reduces the price, and promotes the application and competence of rhEGF. It is obvious that a less pure final product contains more heterogeneous proteins, which bring about its darkness. The cosmetic application of rhEGF is confined by the darkness of the final product, which has necessitated the development of decolorization processes for rhEGF as a cosmetic ingredient, but no process has ever been reported.

We constructed an hEGF-expressing recombinant *Escherichia coli*, with high stability and productivity [10, 13,14], and developed a procedure for the isolation and purification of rhEGF as a cosmetic ingredient, which recovered more than 66% of the target protein, with a final product purity higher than 32%: the rhEGF-containing supernatant from the recombinant *E. coli* culture, obtained by low-temperature and high-speed centrifugation, was salted out, dialyzed and ion exchanged with DEAE Sephadex-A50, and the effluent vacuum lyophilized to dryness. However, there was still a problem; both the rhEGF and some pigments in the fermentation broth were ion exchanged with DEAE Sephadex-A50, so that they were eluted simultaneously, making the final product brown.

Decolorization is very important to refine the final product and can be performed by physical, chemical or biological strategies. For biological active proteins, the major concern is the activity, and therefore the denaturation should be avoided during the decolorization. Adsorption

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is the versatile technique for the decolorization of protein solutions [15-17], and is already used successfully for the isolation and purification of rhEGF [1,11,12,18,19].

In this work, the focus was on the decolorization process using adsorption/ion exchange chromatography, as well as its optimization, to refine rhEGF to the satisfaction of the cosmetic standard.

MATERIALS AND METHODS

Materials

The effluent from the chromatographic column packed with DEAE Sephadex-A50 had a rhEGF concentration range of 4.42~5.44 g/L.

Because the functional group of DEAE Sephadex-A50 is 2-(diethylamino)ethyl, the rhEGF and impurities in the effluent should be negative charged. In order to remove the pigments from the effluent, base ion exchange resins were to be investigated to evaluate their decolorization ability. Mild-polar and apolar adsorbents were also to be investigated in consideration of the diversity and complexity of the pigments.

The absorbents to be investigated for the decolorization were as follows: macroreticular resin D314 (Weak-base, Zhengguang Resin Co, Ltd., Hangzhou, China), macroreticular resin D750 (Strong-base, Zhengguang Resin Co, Ltd., Hangzhou, China), macroreticular resin HZ-806 (Mild-polar, Huachang Polymer Co, Ltd., Shanghai, China), macroreticular resin LSA-20 (Apolar, Lanshen Hydronium Exchange Resin Co, Ltd., Xi'an, China) and granular active carbon (Apolar, Active Carbon Co, Ltd., Hangzhou, China).

The absorbents to be investigated were rinsed with double distilled water, kept in a double volume of ethanol for 2~3 h, with stirring, to remove the impurities and cause complete swelling, and then rinsed with double distilled water until there was no ethanol odor. The rinsed absorbents were packed slowly into a chromatographic column for the decolorization process until the double distilled water of a designated bed height was displaced. The saturated adsorbents were regenerated by 1% (v/v) HCl-ethanol solution [20] after adsorption.

rhEGF Assay

A double antibody sandwich enzyme-linked immunoabsorbent assay, for the quantitative determination of rhEGF [21], was performed using a human EGF ELISA Development Kit (Catalog No 900-K05, PeproTech, Inc., Rocky Hill, USA).

Darkness Determination

So far, no specific method for the determination of darkness has been developed, mainly due to the diversity and complexity of pigments. Spectrophotometry was used to determine the darkness of the rhEGF effluent in this work.

The pigments in the rhEGF effluent have no characteistic absorbance peak. Therefore, the relationships between the absorbance and darkness were examined within the visual spectrum corresponding to the complementary color of brown (410~480 nm), as the primary rhEGF effluent looks brown. The primary effluent was diluted serially with double distilled water. The darkness of the primary effluent was defined as 100% and that of double distilled water as 0%. The results are shown in Fig. 1. A wavelength of 430 nm was chosen as this showed the most linear relationship, as in Fig. 2:

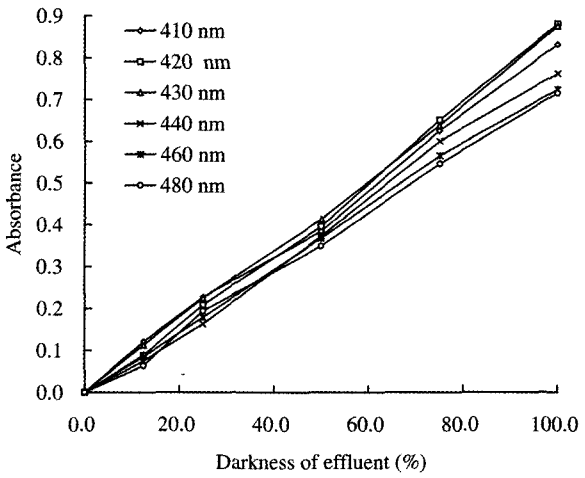

Fig. 1. Relationships between spectrophotometrical absorbance and darkness of the rhEGF effluent. The absorbance of the rhEGF effluents with different darkness was examined between 410~480 nm. The darkness of the primary rhEGF effluent was defined as 100% and that of double distilled water as 0%.

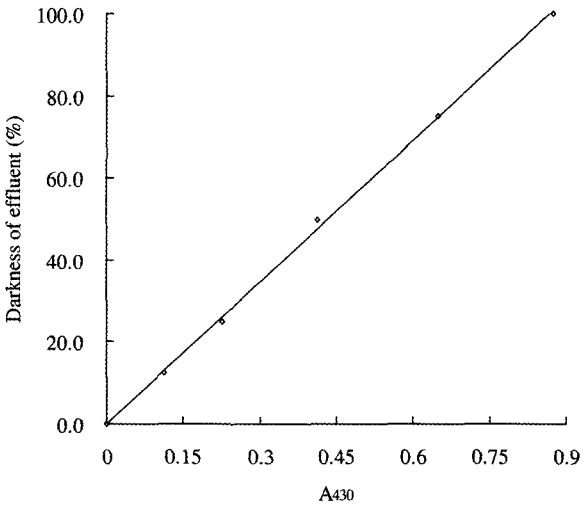

Fig. 2. Linear regression of the relationship between the spectrophotometric absorbance and darkness of the rhEGF effluent at the optimum wavelength of 430 nm.

$$D = 1.147 \times 10^2 A_{430} \qquad (R^2 = 0.9983) \qquad (1)$$

where D represents the darkness of the rhEGF effluent

and A_{430} the absorbance at 430 nm, the optimum wavelength.

The decolorization rate (*DC*) was calculated as follows:

$$DC\ (\%) = [(1 - A_{430\ \text{after decolorization}}) / A_{430\ \text{before decolorization}}] \times 100\% \quad (2)$$

Decolorization Process

The decolorization process was carried out on a chromatographic column (XK 16/20, Amersham Pharmacia, Inc., Uppsala, Sweden) at room temperature. The primary rhEGF effluent was loaded downward onto the chromatographic column packed with the absorbents to be investigated. The fractions from the chromatographic column were collected to determine the rhEGF concentration and darkness of the effluent. The decolorization process was halted when the absorbents were saturated. The decolorized rhEGF effluent was pooled and vacuum lyophilized to dryness.

Different types of absorbent were investigated. The effects on decolorization of the operational factors (flow rates, effluent pHs and column bed heights) were examined by comparing the decolorization rates and recovery yields of rhEGF.

The recovery yield (*RY*) was calculated as follows:

$$RY\ (\%) = (C_{\text{rhEGF after decolorization}} / C_{\text{rhEGF before decolorization}}) \times 100\% \quad (3)$$

where C_{rhEGF} is the rhEGF concentration of the effluent.

RESULTS AND DISCUSSION

Selection of Absorbent

The decolorization process was carried out at a flow rate of 60.0 mL/h, with an effluent of pH 5.0 and column bed height of 7.5 cm.

As showed in Fig. 3, HZ-806 and D314 showed the highest *DC*, at 69.7 and 68.1%, respectively. The *RY* of HZ-806 was the lowest (11.5%), which must have been that both the pigments and rhEGF were absorbed onto HZ-806. D314 manifested the highest *RY* (78.5%), suggesting that D314 was specific to the pigments and the pigments were ion exchanged preferentially to rhEGF under the described conditions during chromatography.

In this work, the macroreticular resin D314 was selected as the ion exchanger for the decolorization process.

Pigment Breakthrough Curve and Elution Curve on D314

The breakthrough curve of the pigments on D314 under the conditions described above is shown in Fig. 4. When the effluent volume reached 360.0 mL, the ion exchangers became saturated.

In our proceeding investigations, the decolorization process would be halted when the effluent volume reached

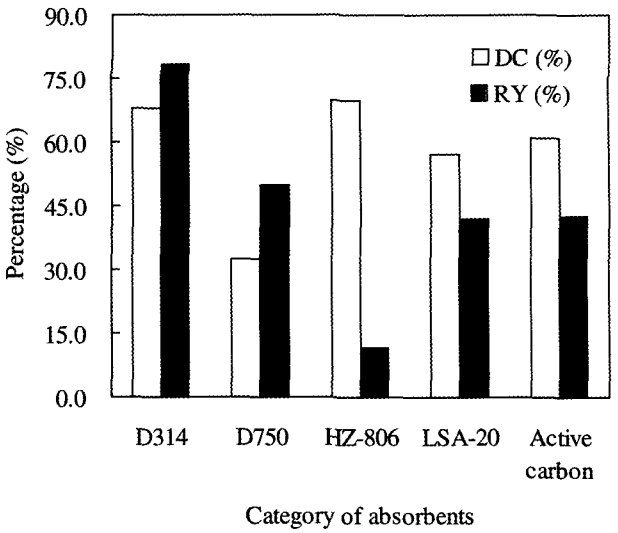

Fig. 3. Effects of the absorbent on the decolorization rate and recovery yield of rhEGF. The decolorization process was carried out at a flow rate of 60.0 mL/h, effluent pH 5.0 and column bed height of 7.5 cm.

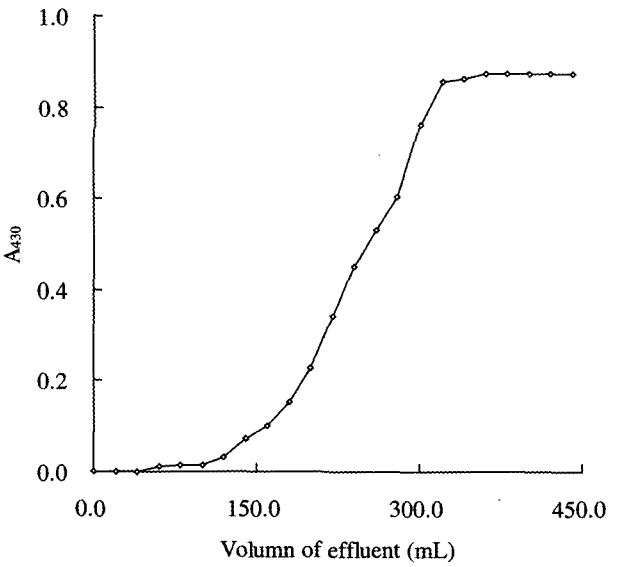

Fig. 4. Breakthrough curve of pigments on D314. The decolorization process was carried out at a flow rate of 60.0 mL/h, effluent pH 5.0 and column bed height of 7.5 cm.

350.0 mL.

The saturated ion exchangers were regenerated using a 1% (v/v) HCl-ethanol solution at a flow rate of 60.0 mL/h. As showed in Fig. 5, the pigments were washed out completely when the eluate volume reached 80.0 mL.

Effect of Flow Rate on Decolorization

The decolorization process was carried out with an effluent pH of 5.0 and column bed height of 7.5 cm. The

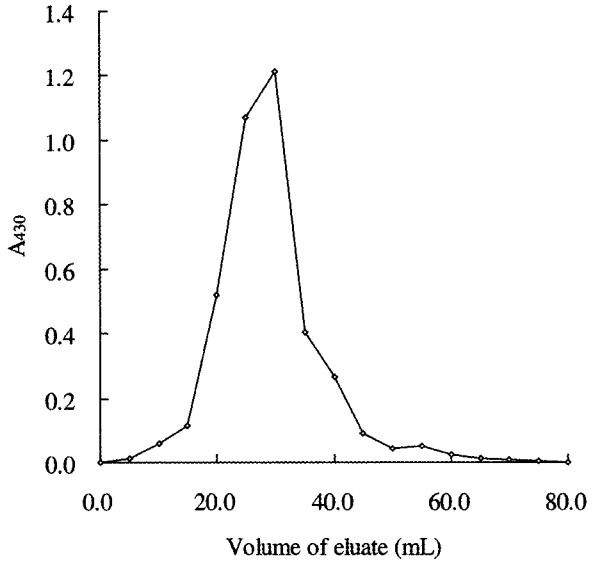

Fig. 5. Elution curve of pigments on D314 by 1% (v/v) HCl-ethanol solution. The elution process was carried out at a flow rate of 60.0 mL/h.

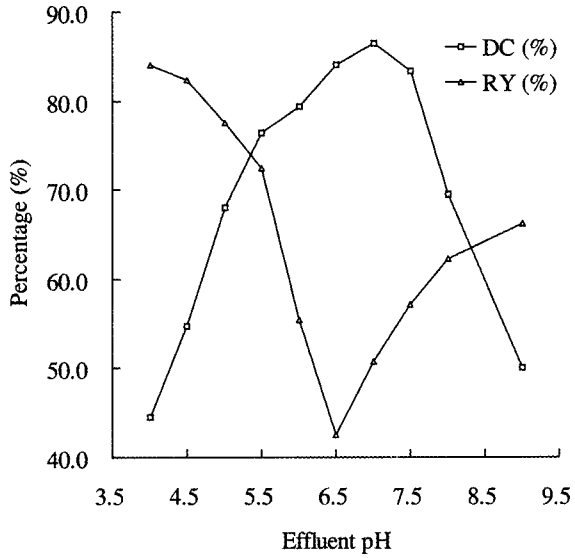

Fig. 7. Effect of effluent pH on the decolorization rate and recovery yield of rhEGF. The decolorization process was carried out at a flow rate of 60.0 mL/h and column bed height of 7.5 cm.

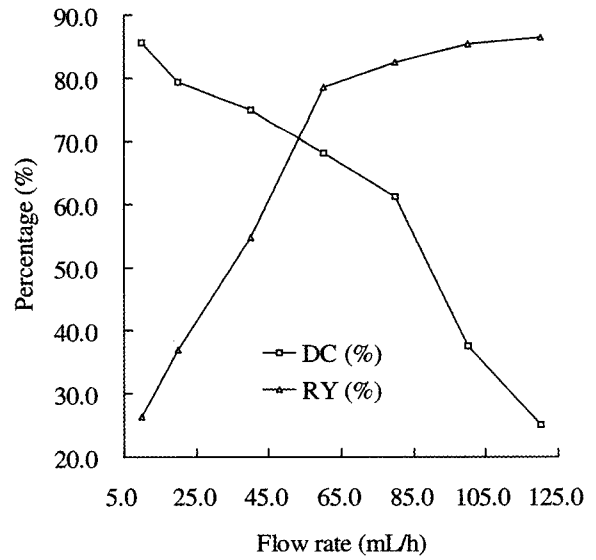

Fig. 6. Effect of flow rate on the decolorization rate and recovery yield of rhEGF. The decolorization process was carried out with an effluent pH of 5.0 and column bed height of 7.5 cm.

effect of the flow rate (levels of 10.0, 20.0, 40.0, 60.0, 80.0, 100.0 and 120.0 mL/h) on the decolorization was also examined.

As showed in Fig. 6, the *DC* initially decreased relatively slowly (<60.0 mL/h), but then sharply (>80.0 mL/h), and the *RY* initially increased sharply (<60.0 mL/h), and then very slowly (>60.0 mL/h) with increasing flow rate. This was due to the full interaction between the ion exchangers and the pigments and rhEGF at lower flow rates [18].

Effect of Effluent pH on Decolorization

In order to investigate the effect of the effluent pH on decolorization, different pHs between 4.0 and 9.0, at the interval of 0.5, were tested under the following operational conditions: flow rate of 60.0 mL/h and column bed height of 7.5 cm.

Both the *DC* and *RY* can be markedly affected by changes in the charge properties of the pigments, rhEGF and the ion exchangers; pH changes the charge properties. As showed in Fig. 7, the changing modes of *DC* and *RY* were rather complicated under different effluent pHs.

The *DC* initially increased, but then decreased with increasing effluent pH, with pH 7.0 as the turning point. The reason for this may have been as follows: Both the pigments and ion exchangers were cations under acidic conditions; therefore, the pigments would find it hard to approach the ion exchangers as they had the same charge. The pigments were transformed into anions, which are easily ion exchanged by D314 as the effluent pH increased, resulting in a higher *DC*. When the effluent pH was further increased, the ion exchangers became undissociated as the *DC* decreased.

The *RY* initially decreased, but then increased with increasing effluent pH; the turning point was pH 6.5. The isoelectric point of rhGEF is pH 4.6 [22]. When the effluent pH exceeded 4.6, the rhEGF was cationized, so the *RY* began to lower sharply. The ion exchangers became undissociated with further increases in the pH (higher than 6.5), so that the *RY* increased.

Effect of Column Bed Height on Decolorization

Different column bed height levels, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 cm, were investigated. The operational

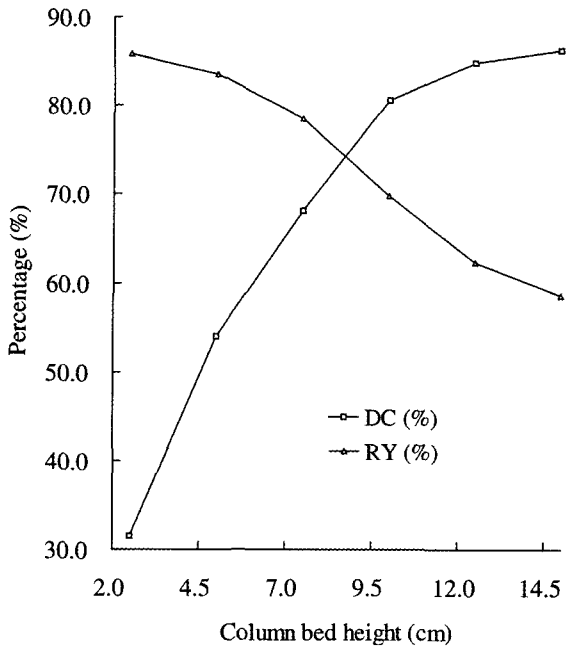

Fig. 8. Effect of the column bed height on the decolorization rate and recovery yield of rhEGF. The decolorization process was carried out at a flow rate of 60.0 mL/h and effluent pH of 5.0.

conditions were as follows: flow rate of 60.0 mL/h and effluent pH 5.0.

The effect of the column bed height on the decolorization is shown in Fig. 8. The *DC* increased and the *RY* decreased with increasing column bed height, as the ion exchange capacity of D314 increased with increasing column bed height.

Optimum Conditions for Decolorization Process

The decolorized effluents with different *DC*s were vacuum lyophilized to dryness. The results showed the final product to be a white or light yellowish, which was to the satisfaction of the cosmetic standard when the *DC* reached 67%. This suggested that the critical controlling point for the *DC* was 67%. Also, with respect to the *RY*, the optimum conditions for the decolorization process of rhEGF as a cosmetic ingredient were as follows: the rhEGF effluent was ion exchanged at a flow rate of 60.0 mL/h and effluent pH of 5.0, using a chromatographic column (i.d. 16 mm) packed with D314 to a bed height of 7.5 cm.

CONCLUSION

Ion exchange chromatography and the macroreticular resin, D314, are a suitable technique and ion exchanger, respectively, for the decolorization process of rhEGF as a cosmetic ingredient. The *DC* and *RY* of rhEGF were higher than 67 and 80%, respectively, and the final product turned out to be white or light yellowish, which was to the satisfaction of the cosmetic standard, when the decolorization process was carried out under the optimum conditions and then halted when the effluent volume reached 350.0 mL. The optimum conditions were as follows: the rhEGF effluent was ion exchanged at a flow rate of 60.0 mL/h and effluent pH of 5.0, using a chromatographic column (i.d. 16 mm) packed with D314 with a bed height of 7.5 cm.

The integration of the decolorization process with our previously developed procedure, for the isolation and purification of rhEGF used only ion exchange chromatography and achieved a final product with purity above 32%. The overall recovery yield of rhEGF was higher than 53%.

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