Optimal Conditions for Manufacturing Immobilized Metal Ion Affinity Membrane and Its Application in Enzymes Purification

Chih-I Chen, Chen-Tung Huang, Chia-Cheng Hwang

Abstract

The optimal reaction condition for preparing immobilized metal affinity membrane (IMAM) was established. In the process of coupling epichlorohydrin (EPI) to the regenerated cellulose membrane (RC membrane), NaOH concentration is critical. With lower NaOH concentration only a few amount of EPI was reacted to RC membrane. However, when NaOH concentration was higher, the membrane was found to be distorted and significant pressure drop was observed in flow through mode. An objective function is defined as the ratio of penicillin G acylase (PGA) activity adsorbed to the pressure drop increase in the modified IMAM. The reaction conditions with the highest objective function value were searched. According to this criterion, the optimal reaction conditions were found as follows: 20 ml, 1.4 *M* NaOH, 5 ml EPI, 24 ℃, 150 rpm for 14 h. Under this condition, the cupper ion adsorbed in the modified IMAM could reach 75.5±0.2 μmol/disc. When 10 pieces of IMAM was loaded into the cartridge and the flow operation was conducted at 18 ºC with 10 m*M*, pH 6 sodium phosphate buffer as loading buffer and 1.5 *M* NH_4Cl as elution buffer, a 21.3-fold purification in specific activity with 97.1% recovery for PGA purification was obtained.

Keywords: Penicillin G acylase, immobilized metal affinity membrane, Enzymes.

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投稿日期：980227 接受刊登日期：980518

固定化金屬親和薄膜之改質最適化條件探討及其在酵素純化應用

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摘 要

本研究探討製備固定化金屬親和薄膜最適化反應條件。以再生纖維素薄膜爲固體載體，在 *EPI (epichlorohydrin)*與再生纖維素薄膜接合反應程序中，*NaOH* 濃度是關鍵。使用低濃度 *NaOH*，僅小量 *EPI* 反應接合在 *RC* 膜上；相反地，*NaOH* 濃度高時，薄膜會有損傷且將其置於流動式試驗時亦出現較明顯的壓力降。將 *IMAM* 改質反應之目標函數設定爲吸附 *PGA* 活性值與增加壓力降之比值，這反應狀況將可找出最高目標函數之比值。根據這關鍵之比值，最適化反應條件爲 *20 ml 1.4* M *NaOH, 5 ml EPI, 24 ℃, 150 rpm, 14 h*。在此最適化方法的條件下，有 *75.5±0.2 μmol/disc* 銅離子吸附量。將 *10* 片固定化酵素膜置於流動式管柱中，在 *18℃*、*loading buffer (10 m*M*, pH6 sodium phosphate buffer)*與 *elution buffer(1.5* M *NH_4Cl)*操作條件下進行 *PGA* 純化試驗，可得到 *97.1%PGA* 之回收率與 *21.31* 倍純化倍率。

關鍵詞：盤尼西林醯胺酵素、固定化金屬親和薄膜、酵素。

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1. Introduction

Penicillin G acylase (PGA) is an important bioenzyme capable of hydrolyzing penicillin G into 6-aminopenicillanic acid (6-APA) which is widely used as the raw material for antibiotics. The preparation of highly active and stable PGA is thereby crucial for industrial 6-APA production. Generally, PGA is produced via fermentation of recombinant Escherichia coli, followed by a series of purification steps to obtain the enzyme from the cells [1].

Among the recently developed purification techniques, immobilized metal affinity chromatography (IMAC) is shown to be a promising technique for the purification of recombinant proteins [2] . IMAC is based on the affinity adsorption between certain metal-binding amino acids in the side chains of proteins (e.g. histidine , cysteine , tyrosine, etc.) [3, 4] and metal ions (e.g. Cu^{2+}, Ni^{2+}, Zn^{2+} etc.) chelated by the chelating agents (e.g. iminodiacetic acid, IDA) immobilized on the solid supports [5]. For a successful affinity adsorbent, the ligand should be attached to the solid supports in a way that its binding to the target protein would not be seriously disturbed.

In recent years, attempts were made to immobilize metal ions on membrane surface (i.e., immobilized metal ion affinity membrane, IMAM) and to apply it in enzyme purification processes [6-8]. In general, the membrane process could offer advantages such as no intra-particle diffusion, short axial-diffusion path, low pressure drop, no bed compaction, and easier scale up. But they are usually limited in the conventional packed-column systems. Up to now, the IMAMs have been successfully applied in isolating or purifying proteins such as enzymes, albumins, etc. [5, 9].

In our previous study [7], it was observed that when applying Cu^{2+}-IMAM to the purification of PGA, a remarkably high recovery of 90.25% with a purification fold of 9.11 was achieved. However, according to the report by Hu et al. [10], in the modification reactions to couple EPI, IDA, and immobilized metal ions onto regenerated cellulose membrane, the best results for each reaction were as follows: EPI (164.98 μmol /disc; 33.5%), IDA (23.69 μmol /disc; 14.4%) and metal ion capacity (2.75 μmol /disc; 11.6%). It is noticed that the utilization percentage (capacity obtained for the current reaction step/capacity for the earlier one step) for each reaction step was low. Therefore, there is a need to further study the IMAM-preparation procedures.

In this study, the reactions for

coupling EPI and IDA to the RC membrane were carried out. To quantitatively evaluate the coupling efficiency, an objective function, taking the binding PGA activity and the membrane pressure drop into account, was adopted to yield the highest chelating capacity in the RC membrane. Furthermore, to compare the purification effects, the obtained IMAM was applied to the PGA purification and the flow adsorption process using a membrane cartridge.

An ideal membrane carrier for protein and enzyme separations must have most of the following characteristics: high hydrophilicity and low nonspecific binding, fairly large pore size and a narrow pore size distribution, chemical and mechanical resistances, as well as enough reactive functional groups. RC membranes are considered to meet most of the required characteristics and should be a good choice for IMAM preparation. In our previous studies [7], the basic adsorption properties for the RC-based IMAMs have been extensively investigated by evaluating their efficiency on PGA purification process. In addition, IDA was selected as chelator owing to its highest cupper ion chelating capacity and protein adsorption capacity. According to literature [7] about IMMA, the coupled IDA density could be significantly influenced by various reaction conditions such as pH, temperature, coupling time, shaking rate, etc. The larger amount of IDA was coupled, the higher cupper ion capacity and protein adsorption capacity in IMAM were achieved. However, it was noted that the concentration of sodium hydroxide in the epoxidation process was observed to be a critical factor affecting the IMAM performance.

2. Experimental

2.1. Materials and equipments

The RC membranes, with a diameter of 47 mm, an average pore size of 0.45 μm, and a thickness of 160 μm, were purchased from Sartorius (Germany).

p-Dimethylaminobenzaldehyde (DAB), 6-APA were purchased from Sigma (USA); penicillin G from MDBio (Taiwan); ethylenediaminetetraacetic acid disodium salt anhydrate (EDTA) and EPI from Tedia (Fairfield, OH, USA); IDA from Acros Organics (Geel, Belgium). Other reagents were of analytical grade and from local suppliers.

2.2. Preparation of crude PGA extract

Fermentation broth from Escherichia coli cultivation was obtained. The cells were harvested by centrifuging 100 ml of crude broth at 14,000 × g for 20 min

(Centrifuge Z323K, Hermle, Germany). The pellet obtained was washed twice with DI water, then resuspended in lysis buffer (0.1M KH_2PO_4, pH7.5), and sonicated for 12 min. The solution was centrifugated at 18,000×g for 20 min and the supernatant was collected [5, 8].

2.3. Analysis procedures

2.3.1. Protein concentration

Protein concentration was measured by the Biorad Protein Assay using bovine serum albumin as standard [11].

2.3.2. Enzyme activity

PGA activity was determined using the colorimetric method proposed by Balasingham et al. [12], and one unit (IU) of enzyme activity was defined as the amount of enzyme required to produce 1 μmol 6-APA per min at 37 °C, pH 8.0.

2.3.3. Copper ion analysis

To quantify the immobilized copper density of membrane, the membrane was washed with 10 ml of 0.1 M EDTA for 1 h. The eluent was collected and the concentration of Cu^{2+} was determined by a spectrophotometer (Metertek SP-830, Metertech Inc., Taiwan) at 800 nm [13].

2.3.4. Determination of EPI and IDA densities on RC membrane

The EPI and IDA densities were determined by measuring the differences in oxygen and nitrogen contents, respectively, between the original RC membrane and the membrane from the intermediate reaction step using elemental analyzer (EA) (Heraeus F002, Hanau, Germany).

2.3.5. Mechanical Analysis

The analysis of the mechanical strength of the membranes was carried out by using a pulling force test machine (AI-7000M, Universal Testing Machine Company, Taiwan). The membrane was cut into 1 cm × 3 cm and operated under a 6 *N*/ min scan rate at room temperature.

2.3.6 The amount of epoxide groups on the membrane surface

According to reference 14, the analysis of the amount of epoxide groups was carried out using a piece of modified membrane were aminated with 25% ammonium hydroxide solution, washed, and assayed with acid orange II reagent (0.5 m*M* acid orange II in HCl, pH 3). For the determination of blank value of non-aminated RC membrane was used. After 24 h at room temperature, acid orange solution was removed and the modified membrane were washed with HCl (pH 3). Acid orange II content was measured spectrophotometrically by

treating the membrane with NaOH solution (pH 12) for 15 min and comparing the absorbance of supernatant at 490 nm with that of NaOH solution with acid orange II at various concentrations.

2.4. IMAM preparation

The RC membranes were modified via a series of chemical reactions [7, 10, 13] to couple EPI, IDA and copper ions on the membrane surface. All reactions were carried out in a 200 ml glass bottle. A piece of RC membrane was immersed in a mixture of 10 ml DI water, 10 ml of various NaOH concentrations and 5 ml EPI, and shaken at 24 °C, 150 rpm for 12 h. The membrane was rinsed twice with DI water, and then immersed in various concentrations of IDA solution (dissolved in 1 M sodium carbonate), and shaken at 24 °C for 12 h. For coupling with Cu^{2+}, the membrane was reacted with 25 ml of 25 m*M* $CuSO_4$ for 1 h and then rinsed twice with the loading buffer (0.5 M NaCl, 10 m*M* sodium phosphate buffer, pH 7) to remove the unbound copper ions. The prepared IMAM membrane was preserved in phosphate buffer (PB: 50 m*M* phosphate buffer, pH 8) at 4 °C until use. The reaction scheme was presented in our previous study [7].

2.5. Batch adsorption experiments

For batch adsorption experiments, one piece of IMAM was put into a glass bottle and 10 ml of crude enzyme extract in 10 mM phosphate buffer was loaded. Then, elution was conducted by using the elution buffer. Different adsorption and elution conditions were tested.

2.6. Flow adsorption experiments

For flow experiments, 10 pieces of IMAM (under the optimal preparation conditions, metal ion capacity: 75.5±0.3 μmol Cu^{+2}/disc) were stacked in the cartridge. A 100 ml volume of crude PGA extract was loaded at 1.0 ml/min to the cartridge already equilibrated with loading buffer (0.5 *M* NaCl, 10 m*M* phosphate buffer, pH 8.5). Unbound protein was washed out with 90 ml of washing buffer (0.02 *M* NH_4Cl, 0.5 M NaCl, 10 m*M* phosphate buffer, pH 6.8). Then, bound enzyme was eluted with 90 ml of elution buffer (1.5 *M* NH_4Cl, 0.5 *M* NaCl, 10 m*M* phosphate buffer, pH 6.8). Every 3 ml of fraction was collected by the fraction collector and the enzyme activity and protein concentration of the sample were analyzed.

3. Results and discussion

3.1 Epoxidation process of RC membrane

The effect of various NaOH

concentrations in the range of 1.0 M to 2.0 M was investigated and the results are shown in Table1. It is well known that the pulling force of the membrane respects mechanical strength index, which could influence the performance of flow adsorption experiments. The lower pulling force means that the membrane is becoming fragile and would be easily damaged. In this experiments, it was found that the membrane became too fragile and could be easily distorted when the pulling forces ratio below twenty percentages. In this study, a pretest was conducted and the reaction time of epoxidation under different NaOH concentration was determined to prevent the pulling force ratio not to be lower than 20%. The results shown in Table1 exhibits that copper adsorption capacity reached a maximum value of 86.32 μmol Cu^{+2} / disc when using 1.6 *M* NaOH, accounting for a 33% increase compared to that with 1.0 *M* NaOH. However, the ratio of pulling force was obviously declined with the increase of NaOH concentrations from 1.6 *M* to 2.0 *M*. Therefore, it is critical to select proper EPI coupling reaction conditions with higher copper adsorption capacity but without giving up its configuration.

3.2 Pressure drop effect

It was observed that NaOH concentration caused not only lowering of mechanical resistance but also the pore size shrink of the membrane leading to high-pressure drop when packed in the column. In fact, pressure drop in the column would bring about uneven flow distribution. Pressure drop would become a more serious issue when a larger amount of membrane layers were packed in the operation column. It was necessary to investigate the pressure drop in flow adsorption experiments after the membrane being modified. To measure the pressure drop, ten pieces of IMAM prepared under various NaOH concentrations were stacked in the cartridge in the flow experiments. The results shown in Fig.1 indicated that the pressure drop had little difference when NaOH concentration was in the range of 1.0 *M* to 1.4 *M*. However, obvious pressure drop was observed when NaOH concentration was equal to or higher than 1.6 *M*. This demonstrated that the IMAM prepared under higher NaOH concentration would deteriate the membrane property and configuration though it would possess higher copper adsorption capacity.

3.3 Optimization in EPI coupling reaction

Based on previous results, it is necessary to find out the optimal EPI coupling conditions with higher PGA

purification performance and lower pressure drop characteristics. To optimize the EPI coupling condition, an objective function was defined as $f = \frac{\Delta PGA / PGA_0}{P / P_0}$, where PGA and P are the adsorbed PGA activity and the pressure drop in which the IMAM prepared with various NaOH concentrations (EPI coupling), respectively. PGA_0 and P_0 are the adsorbed PGA activity and the pressure drop with the IMAM prepared at 1.0 *M* NaOH. The objective function values were calculated and are shown in Fig.1. An optimal objective function value of f was obtained when using 1.4 *M* NaOH, where the IMAM prepared could adsorb PGA activity of PGA_0 with a relatively lower pressure drop of P_0. That is, the EPI coupling by using 1.4 *M* NaOH could reach higher PGA adsorption and lower pressure drop. The morphology of the membranes were further scanned with SEM and their images are shown in Fig.2**.** It was noted that the homogeneity of pore structure on IMAM with 1.4 *M* NaOH for EPI coupling could be retained (Fig.2b) in comparison to that of the untreated membrane (Fig.2a). However, a higher NaOH concentration obviously had a detrimental effect on the pore size of IMAM (Fig.2c, 2d). Therefore, 1.4 *M* NaOH for EPI coupling was selected for subsequent experiments. The effect of chelated Cu^{2+} density on the membrane was investigated and the results are shown in Fig.3. It was noted that the immobilized copper ion capacity increased while raising the time from 3h to 14h.. The maximum copper capacity were obtained when the time is 14h.

3.4 IDA concentration effect

The effect of IDA concentration on copper capacity and adsorbed PGA activity was studied and is shown in Fig.4. It was noted that copper ion and PGA activity adsorbed increased with an increase of IDA concentration. When using 1.0 *M* IDA, the maximum copper capacity and PGA activity were obtained, which were 75.5 μmol/disc Cu^{+2} and 1.8 IU/disc, respectively. Apparently, IDA concentration could influence the adsorption of copper ions and the chelation of PGA. Compared to the results with the literature [7], the adsorbed PGA activity was significantly enhanced accounted for a 133.16% increase. The high copper density on the modified membrane surface was beneficial to PGA adsorption.

The composition of O and N contents in the modified membrane was determined by EA (Elemental Analysis) and the results are shown in Table3. It was found that the use of 1.4 *M* NaOH leads to 37.5% of hydroxyl group in RC coupling with EPI,

which is higher than that reported in aqueous phase (30.8%) and alcohol phase (33.5%) with 1.0 *M* NaOH [10]. In addition, 47.9% epoxy group was coupled with IDA while using 1.0 *M* IDA, which is higher than that reported in aqueous phase (14.4%) and alcohol phase (14.0%) with 0.2 *M* IDA [10]. Moreover, there is 75.5 μmol (85.4% of the IDA coupled) copper ions chelated by the carboxyl groups in IDA. The main reason is that EPI is water-insoluble and it would not be evenly distributed in the aqueous solution. Without any external force, EPI drops can hardly touch the membrane surface since they were formed small droplets and precipitated in water. When NaOH concentration was raised, EPI drops would get more chances to contact the membrane surface and hence be able to react with the hydroxyl groups on the RC membrane. That is, the use of higher NaOH concentration would mainly improve the mass transfer rate of EPI drops from the aqueous solution to the membrane surface. High density of RC-EPI ligands effect on the improvement for the diffusion of IDA molecules or metal ions, the formation of high-density metal ions significantly enhances PGA affinity and thus increases the purification fold. As a result, the optimal conditions for forming RC-EPI-IDA ligands were found to be: one RC-EPI modified membrane immersed in 25ml, 1 *M* IDA solution (dissolved in 1 *M* Na_2CO_3), and shaken at 24℃, 120 rpm for12h.

3.5 Flow adsorption experiments

Fitton and Santarelli [6] reported that NH_4Cl was a more selective eluents with a higher fold purification than imidazole for purification of penicillin acylase in IMAM. Liu et al., [7], reported that NH_4Cl led to a similar result of recycling without the process of removing imidazole. Therefore, NH_4Cl was selected as washing and elution solution. The effect of NH_4Cl concentration on elution was studied and shown in Table2. When applying 0.2 *M* NH_4Cl as eluent, no PGA but only some impurity proteins was washed out. This meant that such concentration could be applied to wash impure protein. Meanwhile, when using 1.5 *M* NH_4Cl the PGA recovery was 98.9% and a purification fold of 10.23 could be reached. Consequently, 1.5 *M* NH_4Cl was selected for the subsequent flow through model operation.

Flow experiments for PGA purification using a cartridge containing 10 pieces of IMAMs were performed and the results are displayed in Figure5. The PGA recovery of 97% and a purification fold of 21.31 could be obtained. The results were better than those obtained by using a packed column with Sepharose for Cu^{2+}

chelation [6] (97% recovery and 12.36-fold purification). Therefore, the IMAM for purification PGA was more suitable than packed column.

4. Conclusion

In this research, an immobilized metal affinity membrane with modified regeneration cellulose as matrix for purification of PGA was investigated. Factors such as EPI、IDA concentration and elution buffers were studied. Our investigation provided the optimal conditions for PGA purification using the IMAM process, where a 21.3 fold purification in specific activity in a single step with 97.1% recovery for PGA purification was achieved. From the economic viewpoint, the membrane process with the advantage of large surface area, short diffusion path, and low-pressure drop is easy to be scaled up industrially. This implied that IMAM provided other feasibility while the column process was difficult to meet the aim in large-scale purification process.

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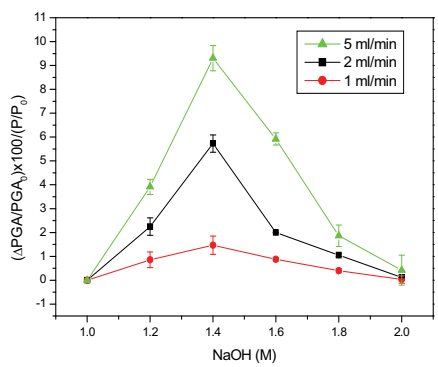

Fig.1 Effect of NaOH concentrations in EPI coupling reaction when NaOH concentration was in the range of 1.0M to 2.0M.

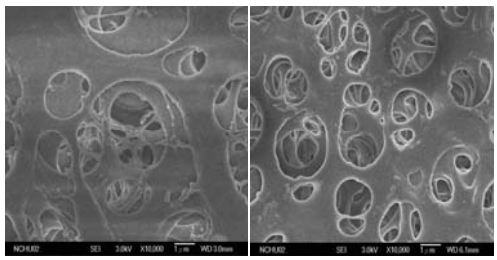

Fig.2a **Fig.2b**

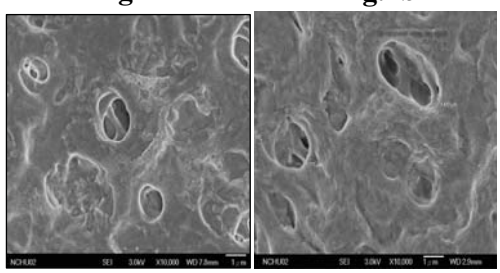

Fig.2c **Fig.2d**

Fig.2 Photographs of the original RC membrane (a)and the PGA immobilized membrane preparation by using 1.4M NaOH (b), 1.6M NaOH (c) and 2.0M NaOH(d)via the electron microscope (magnification times: 10,000×).

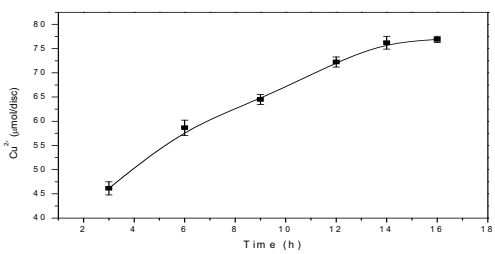

Fig.3 Effect of chelated Cu^{2+} density on the membrane by using 1.4M NaOH. Ranges denote the standard deviations of three tests.

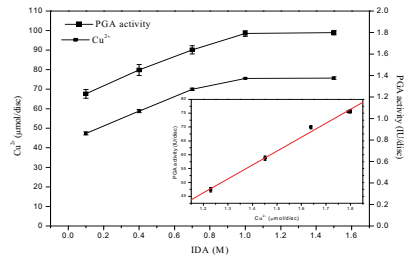

Fig.4 Effect of IDA conc. on adsorption PGA activity and chelating Cu^{+2} on the membrane; Loading buffer: 10mM phosphate, pH 6, 12h at 18 ℃, 100rpm. Loading protein: 25ml, 0.21mg/ml; activity: 0.121 IU/ml; Ranges denote the standard deviations of three tests.

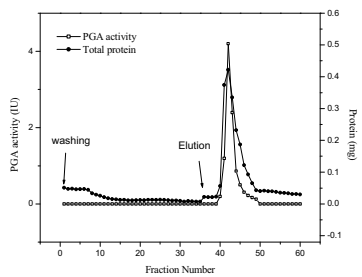

Fig. 5 Flow adsorption results of PAC using a membrane disc cartridge with 10 pieces of IMAM. Loading buffer: 0.5M NaCl, 10mM phosphate, pH 6, Loading protein: 250ml, 0.19mg/ml, activity 0.137IU/ml, specific activity 0.721IU/mg. Washing buffer: 0.02M NH_4Cl, 0.5M NaCl, 10mM Phosphate, pH6.5. Elution buffer: 1.5M NH_4Cl, 0.5M NaCl, 10mM phosphate, pH 6.5. Washing begins from fraction 1, elution from fraction 30

Table 1 Effect of NaOH concentrations on chelated copper ions capacity, PGA activity adsorbed, Transmembrane pressure(P) under flow rate of 2 ml/min

NaOH (M)	Time[a] (h)	Cu^{2+} (μmol disc)	F/F_0 / (%)[b]	PGA activity (U / disc)	P (psig)[c]
1.0	14	49.2±0.4	68.0±0.4	1.51±0.01	0.6±0.1
1.2	14	63.8±1.0	65.0±0.2	1.64±0.02	2.2±0.1
1.4	14	76.0±1.5	63.0±0.4	1.94±0.03	3.0±0.2
1.6	8	86.3±0.2	50.0±0.1	2.12±0.03	12.3±1.0
1.8	4	76.9±1.6	36.0±0.2	1.91±0.02	15.5±2.2
2.0	2	60.5±1.6	23.0±0.3	1.83±0.03	109.1±5.3

[a.] The time was RC membrane reacted with various NaOH solution.

Table 2 Effect of NH_4Cl concentration in elution buffer on PGA purification

NH_4Cl (M)	Enzyme (IU)	Protein (μg)	Specific activity (IU/mg)	Purification fold	Enzyme recovery (%)
0.01	0	38±8	0	0	0
0.02	0	70±7.0	0	0	0
0.04	0.09±0.01	76±11	1.182	1.64	4.7
0.06	0.12±0.01	83±15	1.448	2.01	6.4
0.08	0.15±0.01	84±10	1.779	2.47	7.9
0.1	0.22±0.01	99±19	2.228	3.09	11.6
0.3	0.43±0.01	106±21	4.076	5.65	22.6
0.5	0.93±0.03	154±20	6.047	8.39	49.7
0.7	1.30±0.02	194±15	6.697	9.29	68.8
1	1.62±0.03	229±18	7.062	9.79	84.4
1.5	1.89±0.03	256±26	7.377	10.23	98.9
2	1.88±0.03	270±24	6.958	9.65	98.9

Loading buffer:10mM phosphate, pH 6, 12h at 18℃, 100rpm
Loading protein:25ml, 0.19mg/ml, activity: 0.137IU/ml, specific activity 0.721IU/mg
Elution buffer:10mM phosphate, pH 6.5, shake for 1h at 24℃
Ranges denote the standard deviations of three tests.

Table 3 Capacity results in each reaction step of the construction of RC-EPI-IDA-M+2

	RC-EPI	RC-EPI-IDA	RC-EPI-IDA-M^{+2}
	Coupling capacity (μmol / disc)		
Aqueous phase[a]	151.76±0.65[*] (30.8%)	21.24±0.32 (14.0%)	2.72±0.05 (12.8%)
Alcohol phase[a]	164.98±0.39 (33.5%)	23.69±0.44 (14.4%)	2.75±0.02 (11.6%)
This study[b]	184.72±1.89 (37.5%)	88.39±2.25 (47.9%)	75.5±0.25 (85.4%)
	112.62±3.38[c]	88.39±2.25 (78.5%)	

[a] RC membrane with hydroxyl group of 493.07±0.73 μmole/disc, data adopted from Hu et al. [10].

[b] The amount of epoxy groups on RC-EPI membrane, was assayed according to Kroll *et al.* [14]

Ranges denote the standard deviations of three tests.