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One-step isolation of adenosine triphosphate from crude fermentation broth of *Saccharomyces cerevisiae* by anion-exchange chromatography using supermacroporous cryogel

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Abstract

Adenosine triphosphate (ATP) is an important high-energy compound widely used in biological and therapeutic fields. It can be produced by phosphorylation of adenosine monophosphate (AMP) with microbial cells in industrial scale and the effective isolation of ATP from microbial fermentation broth is a challenging work. In this work, we develop a novel one-step method to directly separate ATP from fermentation broth of *Saccharomyces cerevisiae* by anion-exchange chromatography using supermacroporous cryogel. The cryogel bed with tertiary amine groups was prepared by grafting *N*,*N*-dimethylaminoethyl methacrylate (DMAEMA) monomer chains onto the matrix of a polyacrylamide-based cryogel in a glass column and its properties of liquid dispersion, water permeability, porosity as well as the ligand density were measured. Chromatographic separation of ATP from the fermentation broth by the cryogel was carried out using deionised water and 0.01 M HCl as running buffer, respectively. The breakthrough characteristics and elution performance in the cryogel bed were revealed and analyzed. The purities of the obtained ATP were analyzed quantitatively by high performance liquid chromatography (HPLC). The maximal purity of ATP by the one-step separation method was 95.5% using 0.01 M HCl as running buffer in this work. The corresponding chromatographic behaviors were investigated and analyzed.

Keywords: Cryogel; Anion-exchange; Adenosine triphosphate; Purification; Adsorption; Elution

1. Introduction

ATP is an important phosphate compound in living cells and it plays a significant role in various metabolic circles and biological processes as a high-energy resource. Its disodium salt is widely used as a therapeutic agent to the brain and cardiovascular system diseases. ATP was prepared formerly by extraction from animal muscles or by chemical synthesis. Due to the high costs of starting materials or the difficult additional separations, these methods were seldom applied in industrial scale. In the recent decades biosynthesis means using enzymes (polyphosphate kinase, acetate kinase, adenylate kinase, pyruvate kinase and polyphosphate-AMP phosphotransferase) coupled with special substrates [1–4] or using microbial cells (*Saccharomyces cerevisiae*, *Candida boidinii*, *Brevibacterium ammoniagenes*, *Escherichia coli*) with the required enzymatic system [1,5–9] has been applied to prepare ATP. Among them phosphorylation of AMP by yeast cells to produce ATP is an economical and easy way in industrial processes and employed in many production plants. Generally, the isolation processing of ATP from crude fermentation broth of yeast cells involves multiple steps, i.e., filtration or centrifugation, charcoal adsorption, alcohol precipitation, adsorption using anion-exchange resin and the related elution and concentration. The isolation process requires long time (several days to weeks) and the product obtained by resin adsorption still contains large amounts of contaminations, such as AMP and adenosine diphosphate (ADP). In the case of high temperature (the environmental temperature above 30–35 °C) degeneration of the feedstock within the resin adsorption column occurs easily, which always results in the high contaminations in

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the final product. Therefore, it is of great significance to promote the traditional purification process or develop new separation methods.

Supermacroporous cryogel is a novel chromatographic media for bioseparation, which attracts the increasing interests for direct isolation of target biomolecules from fermentation broths or unclarified feedstocks with cells or cell debris in downstream processes [10–13]. The cryogel can be prepared under solvent freezing conditions [10–29]. Supermacroporous cryogels always have interconnected pores with pore size of several microns to several hundreds of microns, which permit the free-passage of microbial cells or cell debris without blockage. The mass transfer resistance of the target molecules from the feedstock bulk to the binding sites on the pore surface is low and the adsorption equilibrium is established quickly. Therefore, high throughput of crude feedstocks with effective purification can be performed in cryogel beds. This method has been demonstrated to be efficient in capturing target biomolecules, such as antibody fragments, plasmid DNA, bacteriocins, biotinylated retrovirus, recombinant His-tagged lactate dehydrogenase, urokinase, bacterial endotoxins, inclusion bodies, bovine serum albumin (BSA) and lysozyme [11,13–25].

In this work, we present a new one-step method for chromatographic isolation of high-purity ATP directly from crude fermentation broth of *S. cerevisiae* using anion-exchange cryogel. The breakthrough characteristics and elution performance of ATP from the crude feedstock, as well as the purity of the ATP obtained under different conditions in the cryogel column will also be investigated and analyzed.

2. Experimental

2.1. Materials

N,N′-methylene-bis-acrylamide (MBAAm, 99%) and DMAEMA (98%) were bought from Sigma-Aldrich (Steinheim, Germany). Acrylamide (AAm, 99.9%) was from Biobasic (Toronto, Canada). *N,N,N′,N′*-tetramethylethylenediamine (TEMED, 99%) was purchased from Amresco (Ohio, USA). ATP (98.6%), ammonium persulfate (APS, 98%) and other chemicals used (analytical grade) were obtained from local sources. All reagents were used as received.

The fermentation broth suspension of *S. cerevisiae* used in the cryogel chromatography contained 3.3 g wet cells per liter. The total concentration of ATP was 7.7 g/L. The fractions of solute contents in the culture compounds were 47.1% for ATP, 27.9% for ADP, 16.1% for AMP and 8.9% for other nucleic acid or protein derivative contaminations, respectively. The OD value at 600 nm of the suspension was 0.2.

2.2. Preparation of anion-exchange cryogel

The polyacrylamide-based cryogel was prepared by free radical cryo-copolymerization of AAm and MBAAm initiated by TEMED and APS in a glass column (I.D. 16 mm, length of 200 mm) under freezing-temperature variation condition, as reported previously [24–28]. The weights of AAm and MBAAm to the total mass of reactive solution were 5.7% and 1.3%, and the weights of APS and TEMED to the total monomers were 1.2% and 0.5%, respectively. The reaction mixture was frozen under freezing-route B described in [26]. The anion-exchange cryogel (length of 79 mm) was prepared in a similar manner as those in [22,29]. Briefly, the initiator mixture of 5 mL 1 M NaOH and 15 mL 0.05 M $K_5[Cu(HIO_6)_2]$ was passed through the matrix cryogel by a peristaltic pump at the flow velocity of 1.5 cm/min and kept at a constant temperature of 321 K for 30 min. Then 15 mL 1.5 M DMAEMA monomer solution was pumped into the column and maintained 2 h at a constant temperature of 321 K for grafting polymerization. After that the reaction was terminated and washed with 200 mL 0.1 M HCl followed by enough deionised water to remove the residual monomers. Theoretical plate, water permeability and porosity of the cryogel were measured as those previously [24–28]. The ligand density of the anion-exchange groups grafted on the cryogel was determined by the dried cryogel weights before and after graft polymerization and the monomer molecular weight [22].

2.3. Chromatography of ATP by anion-exchange cryogel

The chromatographic isolation of ATP from the fermentation broth suspension of *S. cerevisiae* using the anion-exchange cryogel was carried out at a constant flow velocity of 2 cm/min. The fermentation broth was diluted threefold with deionised water as feedstock. The column was equilibrated with deionised water. The feedstock was loaded and the column was washed at the same liquid velocity with water. The elution was carried out using 0.03 M NaCl followed by 1 M NaCl. The column effluent was recorded over time and collected fractionally for further analysis. Column cleaning was performed with 0.5 M NaOH, 1 M NaCl and deionised water.

The chromatographic experiment was also performed using 0.01 M HCl as running buffer. The fermentation broth was diluted threefold with 0.01 M HCl. The column was equilibrated with 0.01 M HCl and the feedstock was loaded at the same flow velocity as above. The column was washed with 0.01 M HCl. The elution was carried out using 0.03 M NaCl in 0.01 M HCl followed by 1 M NaCl in 0.01 M HCl. The column effluent was collected fractionally and analyzed. The chromatography processes were monitored using a flow-through UV spectrometer at 254 nm.

2.4. Analysis

Quantitative analysis of the purity of ATP was performed by HPLC in a Waters Alliance liquid chromatograph system (2695) equipped with a photodiode array detector (2996) and a SymmetryShield RP C_{18} column (5 μm, 4.6 mm × 250 mm) with 50 mM potassium phosphate buffer (pH 6.5) as mobile phase. The flow rate of 1 mL/min, injection volume of 20 μL and column temperature of 30 °C were applied and the detection by adsorbance was at 259 nm.

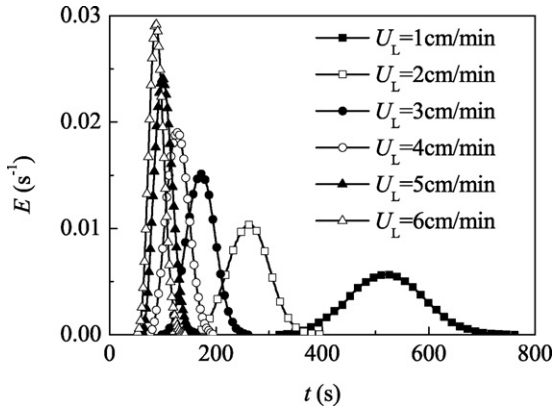

Fig. 1. Residence time distributions at various liquid velocities in the anion-exchange cryogel bed (16 mm diameter, 79 mm length).

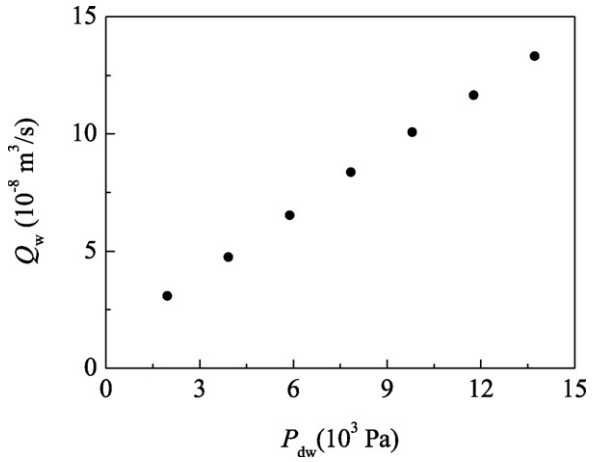

Fig. 3. Relationship of pressure drop vs. water flow rate in the anion-exchange cryogel column.

3. Results and discussion

3.1. Properties of anion-exchange cryogel

The polyacrylamide-based cryogel matrix prepared under the present condition has well-interconnected supermacropores as those reported previously [24,26]. The ligand density of the anion-exchange groups grafted on the cryogel is 94.6 μmol/mL-cryogel bed, which is expected to be available. This value is slightly lower than those reported in [22,29]. Residence time distributions (RTDs) at various superficial liquid flow velocities (U_L) obtained by tracer pulse method (3% acetone as tracer) are shown in Fig. 1 and the calculated values of height equivalent to theoretical plate (HETP) of the cryogel column are in the range of 0.12–0.23 cm for U_L from 1 to 6 cm/min (Fig. 2). These values are slightly higher than those cryogels reported in refs. [24,26]. This is due to the changes of pore spaces or porous channels within the cryogel induced by the graft polymerization, as pointed in [24]. The porosity of this cryogel is 84.8% measured by the content of free water and the cryogel volume, which is close to those obtained previously [26,27]. From the experimental results of pressure drop P_{dw} vs. flow rate Q_w shown in Fig. 3, the water permeability of the cryogel bed is 4.02×10^{-12} m^2, indicating that the flow resistances within the cryogel bed is low.

3.2. Capture of ATP by anion-exchange cryogel with deionised water as running buffer

Fig. 4 shows the chromatographic performance of ATP from the fermentation broth suspension of *S. cerevisiae* diluted three-fold with deionised water by the anion-exchange cryogel bed. The vertical axis represents A_{260} of the effluent samples diluted 60 times using 0.01 M HCl. Two-stage elution was conducted, i.e., with 0.03 M NaCl (elution 1) followed by 1 M NaCl (elution 2), and the bound ATP was eluted effectively. During the chromatography process, most of the yeast cells passed through the cryogel bed freely and only very small part of biomass was observed to be adsorbed by the cryogel at the inlet column section (about 5–10 mm height of the total bed height of 79 mm). This might be caused by the weak ionic interactions between the negative charged surfaces of biomass with the positive charged surface of the cryogel pore walls. These biomass were not des-

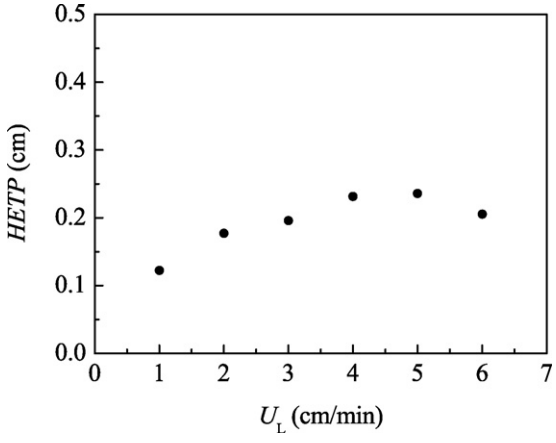

Fig. 2. Variation of HETP with liquid velocity in the anion-exchange cryogel bed.

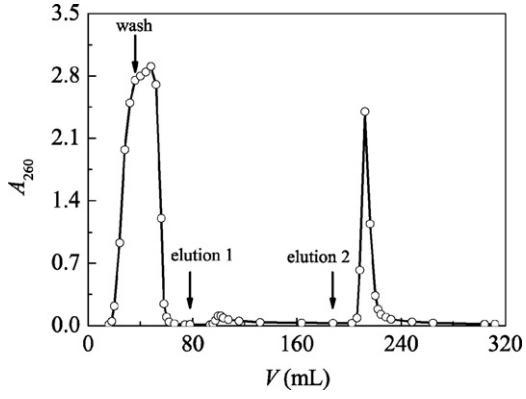

Fig. 4. Chromatographic behaviors of ATP from the fermentation broth by the anion-exchange cryogel bed. The column was washed with deionised water, eluted with 0.03 M NaCl (elution 1) followed by 1 M NaCl (elution 2). The liquid velocity was maintained at 2 cm/min in the chromatography process.

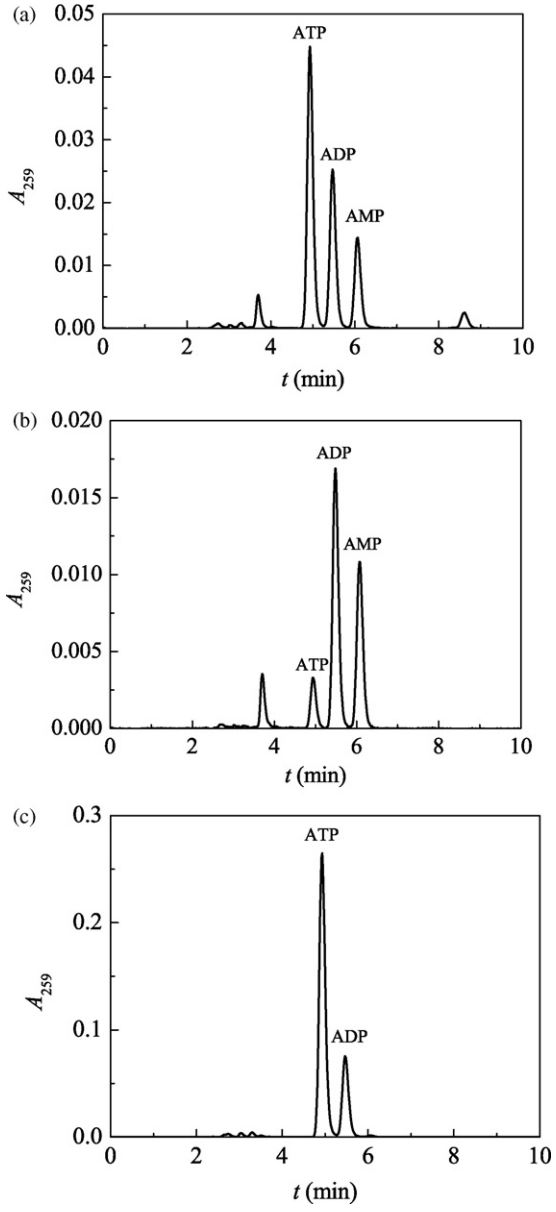

Fig. 5. HPLC results of ATP and other contamination contents in the crude fermentation broth (a) and the effluents for the loaded volume of 0.03 M NaCl from 40 to 59 mL (b) and 1 M NaCl from 12 to 38 mL (c), respectively.

orbed in elution steps 1 and 2, but were removed fully in the finally cleaning step.

Fig. 5 shows the HPLC profiles of the crude fermentation broth (a) and the effluent samples (b) in the elution 1 and (c) in the elution 2, respectively. These samples were diluted 500, 5, and 15 times using the mobile phase buffer in the HPLC measurements. The adsorbance values detected at 259 in the analysis were different due to the different solute concentrations in the samples. In order to show the relative fractions of ATP, ADP AMP and other impurities in each sample clearly, different scales of the vertical axis were used in these figures. Other HPLC profiles are not shown here. The relative contents of AMP, ADP, ATP and other impurities in different effluent fractions are summarized in Table 1.

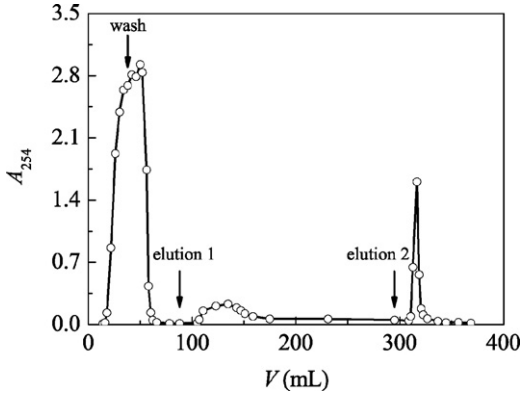

Fig. 6. Chromatographic behaviors of ATP from the fermentation broth by the cryogel bed. The column was washed with 0.01 M HCl, eluted with 0.03 M NaCl in 0.01 M HCl (elution 1) followed by 1 M NaCl in 0.01 M HCl (elution 2). The liquid velocity was maintained at 2 cm/min in the chromatography process.

It is seen that the crude culture contains ADP, AMP and at least five other contamination compounds. The effluents in the early stage of elution 1 with 0.03 M NaCl contained large amount of ADP, AMP and other impurities, indicating that these impurities were desorbed from the cryogel. The elution 2 was performed after about 110 mL of 0.03 M NaCl was loaded. In elution 2, 1 M NaCl was employed to elute the bound ATP from the cryogel column completely. As can be seen from Table 1, the ATP fraction in outflow by 1 M NaCl reached 72.5–74.4% and still contained 22.5–23.2% of ADP, indicating that further purification is still needed. The bound ATP and impurities were eluted completely after 115 mL 1 M NaCl was loaded.

3.3. Separation of ATP by anion-exchange cryogel with 0.01 M HCl as running buffer

In order to enhance the purity of ATP from the fermentation broth suspension, chromatographic experiments were also carried out with 0.01 M HCl as running buffer. Fig. 6 shows the chromatographic profile of ATP from the crude culture diluted

Table 1
HPLC results of compound contents in different elution fractions (deionised water as running buffer in the isolation process)

Elution step	Liquid fractions of elution 1 or 2 (mL)	ATP (%)	ADP (%)	AMP (%)	Other impurities (%)	Diluted times in HPLC
1	40–59	9.2	49.9	33.4	7.5	5
	74–102	10.8	73.0	13.0	3.2	1
2	12–38	74.4	22.5	0.5	2.6	15
	45–65	72.5	23.2	1.3	3.0	2
	115–147	0.0	0.0	0.0	0.0	5

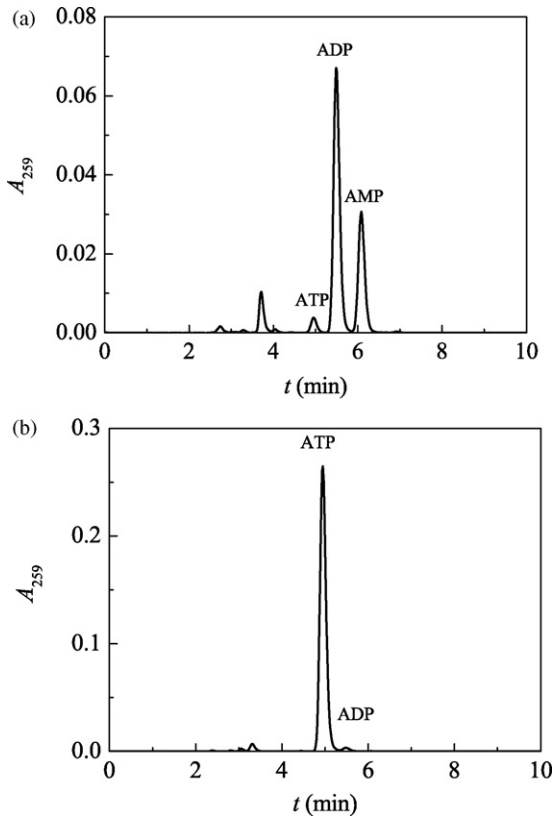

Fig. 7. HPLC results of ATP and other contamination contents in the effluents for the loaded volume of 0.03 M NaCl in 0.01 M HCl from 14 to 30 mL (a) and 1 M NaCl in 0.01 M HCl from 14 to 74 mL.

and washed using 0.01 M HCl and eluted with 0.03 M NaCl in 0.01 M HCl (elution 1) followed by 1 M NaCl in 0.01 M HCl (elution 2). In the measurements, each sample was also diluted 60 times in 0.01 M HCl, as same as those in Fig. 4. In this chromatography process, the yeast cells passed through the cryogel bed and nearly no biomass was observed to be adsorbed by the cryogel. The reason is that in the acid condition the ionic interactions between the negative charged surfaces of yeast cells with the cryogel pore walls were very weak.

Fig. 7 shows two typical HPLC profiles of the effluent samples in elution 1 (a) and elution 2 (b) in the case that 0.01 M HCl was employed as running buffer in the chromatography. Both of these two samples were diluted 10 times before performing HPLC analysis and the obtained HPLC profiles were shown similarly as those above (Fig. 5). Variations of the relative contents of the target and impurities in different effluent fractions are summarized in Table 2.

It is seen that about 59.7% of ADP, 28.2% of AMP and 8.9% of other impurities were detected in the outflow for the loaded volume of 0.03 M NaCl in 0.01 M HCl from 14 to 30 mL. The ATP fraction in this sample was only 3.2%, indicating that the main impurities could be desorbed effectively using this elution buffer. With the increase of the loaded volume of 0.03 M NaCl in 0.01 M HCl, the fractions of ATP increased up to 88.2% and the total solute concentration was very low. Therefore, elution 2 was performed and the outflow of the peak was collected together and detected. As can be seen, the ATP fraction in the elution 2 was 95.5%, which was much higher than that obtained in the case of using water as running buffer. These results indicate that most of the impurities in the crude fermentation broth were removed by the anion-exchange cryogel chromatography.

The recovery efficiencies (defined as the ratio of the eluted pure ATP to that contained in the fermentation broth loaded onto the cryogel column) in elution 2 under the present two cases were estimated approximately from the quantitative analysis results and the obtained values were 51% and 58% in the case of using deionised water and 0.01 M HCl, respectively. These values mean that it is effective and thus preferred to get high-purity ATP using 0.01 M HCl as basic buffer in the chromatography process.

From the above experimental results, the buffer pH plays a crucial role to the adsorption and desorption of ATP, ADP and AMP onto the anion-exchange cryogel, thus to the corresponding purity of ATP in the isolation processing. In fact, ATP, ADP and AMP have negative charges in deionised water or in 0.01 M HCl due to the dissociation of phosphate groups. They were easily adsorbed onto the anion-exchange cryogel in the breakthrough process and eluted effectively by the salt buffer. However, the dissociation degree of phosphate groups in ATP was more complete than that in ADP or AMP, which resulted in the stronger binding of ATP to the cryogel than that of ADP and AMP. Thus, these two impurities were eluted firstly by the salt buffer of a low concentration. In the case of using 0.01 M HCl, the negative charges of ATP, ADP and AMP were less than those in the situation of using deionised water because the phosphate groups in these molecules were mainly in non-ionised form. Therefore, the main impurities (ADP and AMP) bound by the cryogel could be easily desorbed and removed in elution 1, which consequently resulted in the achievement of higher purity ATP than that using deionised water as running buffer.

Table 2
HPLC results of compound contents in different elution fractions (0.01 M HCl as running buffer in the isolation process)

Elution step	Liquid fractions of elution 1 or 2 (mL)	ATP (%)	ADP (%)	AMP (%)	Other impurities (%)	Diluted times in HPLC
1	14–30	3.2	59.7	28.2	8.9	10
	30–44	9.9	79.3	6.8	4.0	10
	44–60	27.0	66.0	2.8	4.2	10
	76–92	81.7	15.7	0.5	2.1	10
	140–220	88.2	11.8	0.0	1.0	10
2	14–74	95.5	1.4	0.0	3.1	10

4. Conclusions

Chromatography using the supermacroporous anion-exchange cryogel is a fast method for direct isolation of ATP from fermentation broth of yeast cells. The cryogel with tertiary amine groups is effective to isolate ATP from the culture suspension. In the present chromatography process, the bound contaminations (such as AMP and ADP) were found to be removed significantly in the elution process by 0.03 M NaCl in 0.01 M HCl and high-purity ATP was obtained in the followed elution process by 1 M NaCl in 0.01 M HCl. Therefore, the chromatography using anion-exchange cryogel is also a potential separation method to get high-purity ATP.

The running buffer is of importance to the purity of the obtained ATP and it is preferred to use 0.01 M HCl as running buffer compared with the deionised water in the chromatography process, indicating that the buffer pH is one of the most important factors to the adsorption and desorption of ATP, thus to the corresponding purities. However, the ATP capacity of the present cryogel is lower compared with the traditional anion-exchange resin and there is still a need to improve the recovery efficiency and the purity by further optimizing the chromatography conditions or by employing improved functional ligand and graft method.

Acknowledgments

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