

Single Step Purification of Recombinant Collagen from *Escherichia coli* using Weak Cation Exchange Chromatography

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Collagen is expressed by gene-engineering for significant medical and biotechnological importance. In purification of collagen, ion exchange, widely used to purify and separate collagens, is a high capacity, cheap, efficient and rapid method for purification of collagen. Here we develop a single step system for purification of recombinant collagen which improves purification of collagen. In this experiment, the effects of parameters such as pH, salt type and salt concentration on the chromatographic behavior of recombinant collagen from *E.coli* on the cation-exchanger were evaluated with the aim of optimizing the ion-exchange purification of collagen. The collagen yield was also taken consideration in purification by the resulted lyophilized collagen. We describe the purity of recombinant collagen using SDS-PAGE gel electrophoresis and size-exclusion chromatography. All in all, the results demonstrate that single step purification may achieve high purity collagen and reduce the cost. And purified collagen can be used as the study of structure and preparation of biomaterials.

Key words: Purification; recombinant protein; ion-exchange chromatography

Chromatography is a major unit operation in biological sample purification processes¹⁻⁴. Sometimes, the needed purity of biological sample is achieved through multiple chromatography steps combined which are typically expensive and time-consuming, and result in high losses of the resulted protein⁵. So the effective methods of purification of recombinant proteins from biological mixtures at all levels from laboratory to large scale is regarded as a major challenge in the biotechnology industry, despite the availability of a wide range of innovative approaches to the problems. However, a rapid, economic and precision method for purification of recombinant protein is limited. Purification, more than almost any other activity, defines the profitability of a

bioprocess and represents about 70% of the operating cost of the entire bioprocessing.

The most recombinant proteins involve a peptide "tag" with six contiguous histidine residues to the N- or C- terminus. Protein with His-tag is purified by metal affinity chromatography⁶. But this method is expensive on its own yields highly pure protein with His-tags remaining attached⁷. Unfortunately, metal matrix needs to be chelated at fixed periods. It is difficult to keep using constantly for purification of protein, and making buffer systems have toxic reagents such as imidazole. So ion-exchange chromatography (IEC) is generally used as recombinant protein purification processes⁸. However, many parameters may be involved such mobile phase (salt type, salt concentration, pH⁹), stationary phase (type of ion-exchange groups, ion exchanger capacity, base matrix property) and operating other variables (flow rate, sample concentration, loading, elution condition, additives)¹⁰. Therefore, IEC is chosen

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by many variables condition changed, optimization of which is labor-intensive and time-consuming. In consideration of large scale purification, organic extraction¹¹, three phase portioning^{12, 13} and metal ion-precipitation¹⁴ were not fit for purification of recombinant collagen. The reason is that these methods have a negative effect of collagen stability and yields of the resulted collagen are reduced¹⁵.

In this study, we explore weak cation-exchange chromatography processes for purification of recombinant collagen by using a single-step developed. Starting buffer conditions were carried out in order to improve the impurity protein adsorption and the target collagens were obtained from flow through peak. The starting buffer conditions (pH, buffer concentration, and salt concentration, etc) and elution were optimized. Other investigated parameters included flow rate, inlet collagen concentration. The effects of these parameters for purification of collagen were analyzed in order to provide the understanding of performance of weak cation-exchange system. The peak shape, the recovery and the purity of collected peaks were examined in order to verify the purification method for recombinant collagen. This study indicated the efficient purification of recombinant collagen and could maintain a high stability of collagen by using single-step chromatography, which reduced the loss of recombinant collagen and also is beneficial to the large scale for biotechnology industry.

MATERIALS AND METHODS

Chromatography media and column

HiScale CM Sepharose FF column (carboxymethyl group, weak acid type, particle diameter 100 μ m, column size 16mm \times 20cm i.d., total

bed volume 40mL, GE Healthcare Lifesciences, England) was used as a weak cation-exchange chromatography column and media.

Chromatography apparatus

Most experiments were performed on a liquid chromatography system AKTA purifier 100 (GE Healthcare Life sciences), which was fully automated. The AKTA system had two 900 pumps, a 900 UV-Vis variable wavelength detector, IV-908 sample injector, and the automated controller designed for Windows 2000.

Chemicals and reagents

All buffer ingredients, obtained from readily available commercial sources, were of analytical grade in these studies. All buffer ingredients characteristics are given in Table 1. All mobile phases, prepared in ultrapure water, were filtered through a 0.22 μ m cellulose-nitrate filter (Thermo Fisher, USA). The filter system was from Millipore, USA.

Pretreatment of recombinant collagen

The recombinant collagen (isoelectric point (pI) 5.1, Mr 97KDa) used in this study were produced in situ autoclaved fermentor to achieve high-level expression of target protein from our laboratory^{16, 17}.

The cultured cells were harvested by centrifugation and were washed with distilled water; cell suspensions were disrupted via high press cracker (Rannie 15.5, Denmark APV) at 700 bar. The homogenate was precipitated with sodium chloride (NaCl) and then with hydrochloric acid until a pH of 2 was obtained for the removal of the non-target protein; the supernatant was then desalted by an ultrafiltration filter (30 kDa cut-off, Millipore). And recombinant collagens were lyophilized for storage.

Table 1. Characteristics of buffer ingredients used.

Buffer agents	pKa	Suggested Concentration (mmol/L)	Mr	Counterion
Sodium hydrogen phosphate	7.2	50	358.14	Na ⁺
Citric acid	3.13	20	192.14	Na ⁺
Acetic acid	4.76	50	60.05	Na ⁺

Chromatography conditions experiments used for purification of recombinant collagen

Based on ion-exchange can quickly carry on the preliminary purification and be suited for

large scale production, CM Sepharose FF resin column was used to purify the recombinant collagen. The column was washed with the elution buffer until the OD₂₈₀ of the flow through was

below 0.01, and then further washed with starting buffer until OD_{280} was below 0.003. After the column balanced, recombinant collagen samples were injected into the AKTA system, collecting peaks according to peak time.

In the chromatography conditions experiments, starting buffer solutions, phosphate buffer, were prepared from stock buffer (20mM-50mM, pH 3.8) and contained from 0.15 to 0.5 of NaCl. Elution buffer (phosphate buffer) conditions (20-50mM, pH 3.8), considered in purifying collagen, mainly were prepared different concentrations. Dissolved collagen in starting buffer was prepared directly before use and was not filtered. And the conditions of collagen solutions were in agreement with that of starting buffer. Changes of pH played a significant role in purification of collagen, so it was also considered as one of chromatography conditions.

Sodium dodecyl sulfate–polyacrylamide (SDS-PAGE) gel electrophoresis analysis

SDS-PAGE was conducted using a 12% acrylamide separating gel and a 5% acrylamide stacking gel¹⁶. The fractions samples were prepared for gel loading by adding sample dissolved buffer, containing 1% SDS, 5% β -mercaptoethanol, 25 mM Tris–HCl pH 7.5, 1% glycerol and 0.05% bromophenol blue, and then boiled at 100 °C for 5 min prior to gel loading. Map sample was loaded (20 μ g/lane) gels were stained with Coomassie Brilliant Blue G-350 or silver for detection. The sample was separated by SDS–PAGE, from which approximate molecular weight determinations and collagen purity were estimated.

Size-exclusion chromatography analysis

Analytical size-exclusion chromatography was run using a Hiload 26/60 superdex 200pg (GE Healthcare Lifesciences, England). The mobile phase was 50mM phosphate buffer solution (pH 7.2) containing 0.15M NaCl. Flow rate was 0.5 ml/min. Sample volume loading was 0.5ml and collagen concentration was 1mg/ml.

RESULTS AND DISCUSSION

The viability of target collagen for recombinant collagen purification was carried out using a CM FF column, which was validated for binding non-target. To determine whether target collagen could be purified from recombinant collagen, buffer was PBS buffer with additional NaCl and others conditions strategies were taken considered in: (1) increase flow rate, (2) increase NaCl concentration in buffer, (3) reduce sample loading to avoid the excess of loading and so on. **Effect of buffers' concentrations (starting buffer and elution buffer)**

Buffer concentrations affect the binding between target collagen and non-target collagen on the CM FF column. Buffers' concentrations were listed in the material method parts. Experiments around this buffer with different concentrations (20-50mM) containing 0.15M NaCl were processed in CM FF column.

A results analysis using electrophoresis lanes for fractions of recombinant collagen is shown in Fig.1. CM FF chromatography of recombinant collagen showed that the major bulk of collagen did not bind with the column material

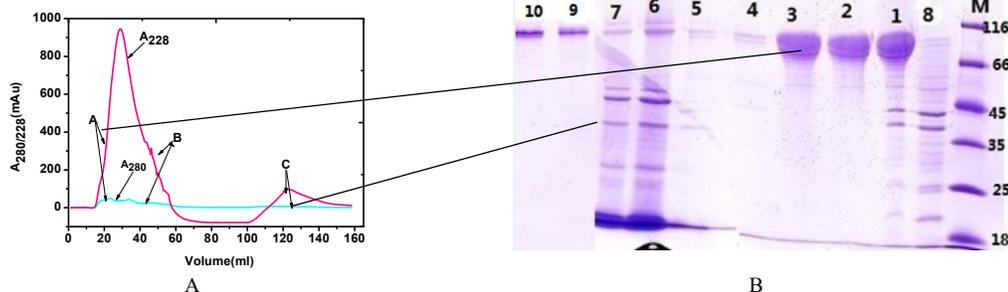


Fig. 1. CM FF chromatography and SDS-PAGE analysis of recombinant collagen. (A) Chromatogram of the ion-exchange profile. (B) SDS-PAGE electrophoresis gel of the fractions shown in (A); M: Protein Marker; lane 1, 2, and 3: end of the washing phase B peaks; lane 4 and 5: flow through A peaks; lane 6, 7, and 8: elution fraction C peaks. And A, B and C peaks were marked in Fig.1.A

(Fig. 1A). SDS-PAGE analysis (A to B fractions in Fig. 1A; lane 1, 2 and 3 were from buffer of different concentrations) revealed that most of the constituents are target collagens. Elution with different NaCl resulted in a wide peak which contained many other proteins in the C fractions (C peak in Fig. 1A, lane 6, 7 and 8 respectively). The unbound collagen species of approximately 98 kDa, which could not be identified in the course of this work, was washing together with collagen. This macromolecule is readily visible in the initial map by gel analysis (Fig. 1B, lane 1, 2 and 3). It is quite similar that this collagen is a part of A peak, as it is known to be existed in the used starting recombinant collagen sample, and evidence could be obtained in the further experiment.

Another well known procedure of recombinant collagen purification is the use of 50mM buffer with 0.15M NaCl, pH 4.0. The resulting gel map is shown in lane 9 and 10 of Fig. 1B. A molecular weight of the target collagen was about 98 kDa, as seen by Coomassie staining. Lane 4 and 5 indicated that target collagen did not purify and separated when PBS buffer concentration was low 50mM. The similar collagen was flowed through lane 1, 2 and 3, and little other protein was adsorbed on CM FF column.

In this study, we observed that the target collagen failed to bind to the CM FF column and non-target collagen binds but was subsequently adsorbed during column wash operations. Many other proteins of recombinant collagen were adsorbed on the column. This provides the foundations for us to achieve the pure collagen.

Effect of sodium chloride

In the experiments, different concentrations of NaCl were performed by ion-exchange chromatography. The results are shown in Fig. 2, the column was equilibrated with 50mM PBS buffer containing different concentrations of NaCl, pH 4.0 at a flow rate 2ml/min, 5 ml of recombinant collagen samples was then loaded onto the column. At a flow rate of 2 ml/min, the column was first washed with the equilibrium buffer, then with 50mM PBS buffer containing NaCl concentrations (0.3, 0.4 and 0.5M) to elute bound non-target collagen. The resulting data (presented as a percentage of the initial amount of the applied collagen, calculated via the protein yield assay) demonstrated that higher recovery of target collagen was obtained for a NaCl concentration of 0.15M.

As shown in Fig. 2A, increasing NaCl concentration reduced non-target collagen to bind to CM FF column. The results indicated that adsorption was a combination of ionic-strength, hydrogen bond interaction, and isoelectric point of collagen. Therefore, increasing ionic strength weakened interaction of electrostatic or hydrophobic.

In Fig. 2B, SDS-PAGE analysis also confirmed unbound collagen and bound non-target collagen. Lane 1, 5, 7 and 9, in PBS buffer containing high NaCl concentrations, nearly did not bind to column, and lane 2, 8 was in PBS buffer containing lower NaCl concentration. And elution fractions were lane 3 and 4, lane 3 was bound little many other collagens when NaCl concentration was high and lane 4 was bound a lot many other

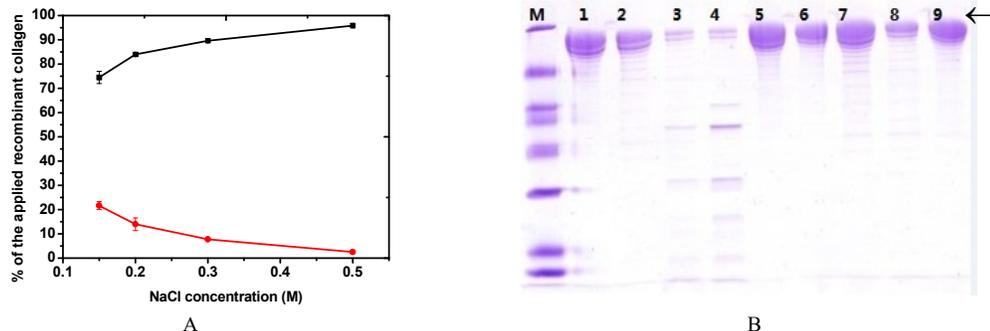


Fig. 2. Ion-exchange chromatography at different NaCl concentrations. A: The CM FF column was equilibrated with 50mM PBS buffer (pH 4.0) at a flow rate 2ml/min. After 5ml of collagen solution was injected into the column. Non-adsorbed collagen (■, flow through of collagen unbound), and adsorbed collagen (●, dissociation of collagen bound). All data are shown as percentages of the amounts of the applied collagen. B: SDS-PAGE analysis of collecting fractions; lane 1, 2, 5, 6, 7, 8 and 9 were unbound collagen; lane 3 and 4 were bound collagen

collagens as NaCl concentration was low. Therefore, NaCl concentration played a role in purification of recombinant collagen, as adsorption was concerned with ionic-strength, the optimum of which was beneficial to bind non-target collagens.

Effect of flow rate

The study was tested in a 50mM PBS starting buffer containing 0.15M sodium chloride and 50mM PBS elution buffer containing 0.3M sodium chloride. The interaction from hydrophobic bond to binding between unbound and bound collagen are much stronger than those from electrostatic interaction¹⁷. As shown in Fig.3, a slight change was observed on the percentage of washing out and eluted collagen of different flow rate. This indicated that both unbound collagens and bound many other collagens displayed the effect of the flow rate on the adsorption. There was more time for many other collagens adsorbed on the CM FF column when low-flow rate was under 2.5ml/min, in contrast to high-flow rates, collagen had no enough time for adsorption and limited diffusion. Because of many amounts recombinant collagens loaded, flow rate was taken considered in purification of protein for enough time diffusion. So studying flow rate shows great hope as a viable method for purification of recombinant collagen.

SDS-PAGE and Size-exclusion chromatography analysis

To confirm collagen fractions of the optimized conditions of purifying, we used the combination of SDS-PAGE gel electrophoresis and size-exclusion chromatography and analyzed collagen fractions, the results were shown in Fig.4. Fig.4A was the chromatogram of target collagen,

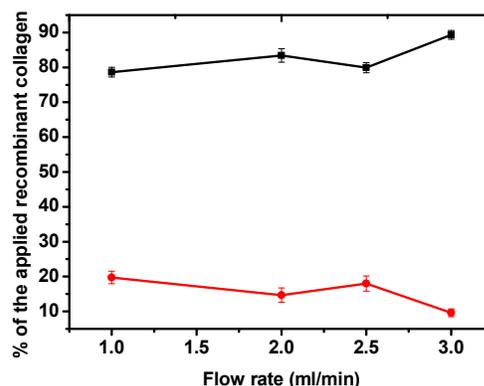


Fig. 3. Effect of flow rate on the binding of the collagen. For basic experimental conditions same as in Fig.2A. Non-adsorbed collagen (■, flow through of collagen unbound), and adsorbed collagen (●, dissociation of collagen bound). All data are shown as percentages of the amounts of the applied collagen. Flow rates for washing out of unbound and elution of bound collagen: 1.0, 1.5, 2.0, 2.5 and 3.0ml/min accordingly.

which was corresponding to lane 1 of gel electrophoresis by Coomassie staining in Fig.4C. It was a single peak, which had a good symmetry in Fig.4A, and lane 1 was also a single band in Fig.4C shown.

Fig.4B indicated that there were multiple peaks for the similar target collagen, which were corresponding to lane 2 in Fig.4C. So Fig.4 demonstrated that recombinant collagen was purified and separated under the optimal chromatographic conditions.

In summary, we have provided a single step for rapid, mild purification of recombinant collagen for industrial production. Both molecular and purity were analyzed by the combination of SDS-PAGE and size-exclusion chromatography.

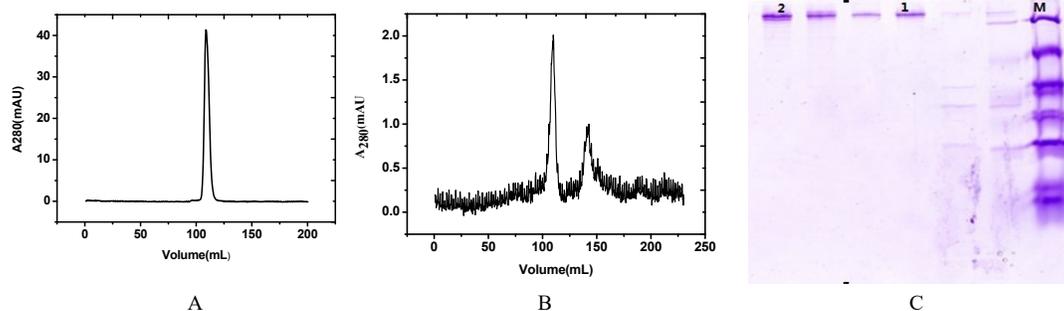


Fig. 4. SDS-PAGE and Size-exclusion chromatography analysis. A: lane 1 in Fig.4C corresponds to a single peak; B: lane 2 in Fig.4C corresponds to multiple peaks

We could show high purity collagen using CM FF column resulting in the discovery of different collagens of recombinant collagen. This includes that many other proteins were adsorbed on the column and separated. In addition, our experiment indicate that the optimal chromatographic conditions are vital for purification of recombinant collagen, and a single step for purification, having advantages to be applied as industrial enlargement, has the potential to improve the yield, processing time and cost.

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