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## **Advanced purification strategy for CueR, a cysteine containing copper(I) and DNA binding protein**

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## Abstract

Metal ion regulation is essential for living organisms. In prokaryotes metal ion dependent transcriptional factors, the so-called metalloregulatory proteins play a fundamental role in controlling the concentration of metal ions. These proteins recognize metal ions with an outstanding selectivity. A detailed understanding of their function may be exploited in potential health, environmental and analytical applications. Members of the MerR protein family sense a broad range of mostly late transition and heavy metal ions through their cysteine thiolates. The air sensitivity of latter groups makes the expression and purification of such proteins challenging. Here we describe a method for the purification of the copper-regulatory CueR protein under optimized conditions. In order to avoid protein precipitation and/or eventual aggregation and to get rid of the co-purifying *Escherichia coli* elongation factor, our procedure consisted of four steps supplemented by DNA digestion. Subsequent anion exchange on Sepharose FF Q 16/10, affinity chromatography on Heparin FF 16/10, second anion exchange on Source 30 Q 16/13 and gel filtration on Superdex 75 26/60 resulted in large amounts of pure CueR protein without any affinity tag. Structure and functionality tests performed by mass spectrometry, circular dichroism spectroscopy and electrophoretic gel mobility shift assays approved the success of the purification procedure.

**Keywords:** Metalloregulatory proteins, cysteine, copper(I), DNA binding, four step purification

## Introduction

Metal ions bound to proteins play important roles in biochemical processes either by the stabilization of the structure of these proteins or by participating in enzymatic reactions. For instance, transition metal ions are often necessary for the optimal function of transcription factors, however, the increased amounts of the otherwise essential metal ions may cause toxic effects in living organisms [1,2]. Accordingly, the concentration of these metal ions must be under a strict metal ion sensitive and selective control in the cell [2–9]. Understanding the details of bacterial metal ion regulatory mechanisms may forward the design of molecules that selectively bind specific metal ions.

Metalloregulatory proteins represent a sub-class of transcriptional regulators that respond to the change of metal ion concentration or availability by balancing the expression of cellular metal uptake and efflux/detoxification systems [6,7]. MerR proteins are one of the ten metal ion regulatory protein families that are distinguished in bacteria [7]. Representative examples are e.g. the Hg<sup>II</sup>-ion binding MerR (the name of the family originates from this protein) and the Cu<sup>I</sup>-ion regulatory CueR proteins [9]. A characteristic of the MerR family members is the similarity observed in the first ~100 amino acids of their sequences [10]. The N-terminal DNA binding domain contains a helix-turn-helix- $\beta$ -hairpin motif followed by a long dimerization helix forming an antiparallel coiled-coil structure [11]. Significant differences are, however, found in the C-terminal effector (metal ion) binding domains (Fig.1) allowing dimeric MerR proteins to distinguish between various metal ions.

The dimer of MerR apo-protein binds to a 19 – 20 bp (base pair) segment of the promoter region of DNA between the –35 and –10 sites. The regulatory mechanism is based on the conformational change of the protein upon metal ion binding, which influences the DNA structure and initiates the RNA polymerase action. As a consequence, a series of proteins are expressed, which participate in the removal of the unwanted metal ions from the cell.

Crystal structures of CueR with Cu<sup>I</sup>-, Ag<sup>I</sup>- and Au<sup>I</sup>-ions reflect that all of these ions are bound in the effector binding domain close to the C-terminus [11]. A loop is formed around the metal centers via the coordination of two cysteine thiolates, which restricts the metal ion into a linear coordination geometry. According to *in vitro* experiments, CueR gives a transcriptional-activation response to single-charged, but not to double-charged metal ions [11]. Recently, the crystal structures of the metal ion free modified protein and the Ag<sup>I</sup>-bound

form, both cocrystallized with DNA, have been published providing more insight into the influence of metal ion binding on the structure of the DNA [12]. Based on the combination of experimental studies with CueR model peptides and quantum chemical calculations we have proposed the participation of a protonated Cys thiol in the metal ion binding domain of CueR and the operation of a protonation switch in the mechanism of the protein [13]. However, the potential role of the protonation/deprotonation of Cys112 in the function of CueR has not been proved, yet. It is also unexplored how CueR so successfully rejects soft divalent metal ions. A further open question is whether the CCHH fragment close to the C-terminus, which has otherwise no direct influence on the transcriptional activity of CueR [14], plays any role in the operation of the protein. In order to better understand the mechanism of the selective metal ion recognition and regulation of CueR we aim at expressing and purifying the wild type CueR from *E. coli* for subsequent structural and activity investigations. Although a published method for the purification of CueR is available in the literature [15], based on the protocol applied for ZntR, a related MerR homologue [16], we have faced difficulties to adapt it. Therefore, in this paper we describe an alternative procedure for the purification of CueR eliminating the precipitation step of the protein, which may provide general guidelines for working with air-sensitive DNA binding proteins.

**Fig. 1** – near here

## **Materials and methods**

### *Strains and media*

*E. coli* DH10B F<sup>+</sup> *endA1 recA1 galU galK deoR nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ<sup>-</sup>* [11,17] was applied as cloning host for recombinant DNA work and *E. coli* BL21(DE3) F<sup>+</sup> *ompT gal [dcm] [lon] hsdS<sub>B</sub>* [18] for the overexpression of CueR protein. Bacteria were grown in LB medium [19] containing ampicillin (100 μg/ml) at 37 °C.

### *Plasmid construction*

The gene of the wild type CueR in a pET24a (Kan<sup>R</sup>) plasmid was kindly provided by prof. Alfonso Mondragon (Department of Molecular Biosciences, Northwestern University, Evanston, Illinois, USA). The DNA segment, encoding CueR, was recloned into a pET21a

(Amp<sup>R</sup>) plasmid (Novagen). The gene was amplified by PCR using T7 sequencing primers (T7 forward primer: 5'-TAATACGACTCACTATAGGG-3' and T7 reverse primer: 5'-GCTAGTTATTGCTCAGCGG-3') then cloned between NdeI and BamHI sites to create pET21a-CueR. A stop codon prior to the BamHI cleavage site assured the expression of the protein without any additional amino acids encoded by the plasmid.

### *Protein purification*

*E. coli* BL21 (DE3) bacteria expressing the wild type CueR from the pET21a-CueR expression vector were first grown in 50 ml LB/Amp<sup>+</sup> medium (including 0.1 mg/ml ampicillin at final concentration) at 37 °C for ~ 4 hours until OD<sub>600</sub> = 0.6 – 1.0 was reached. This pre-culture was sedimented by centrifugation at 4 °C and 18000 × g for 10 min. The cells were re-suspended in 50 ml fresh LB/Amp<sup>+</sup> medium, and 6.5 ml of this culture was used to inoculate 650 ml LB/Amp<sup>+</sup> medium. When OD<sub>600</sub> of 0.4 – 0.6 was attained, the expression of CueR was induced by the addition of IPTG to a final concentration of 0.1 mM. The cultures were incubated overnight at 20 °C to avoid aggregation otherwise observed at 37 °C. The cells were harvested by centrifugation at 4 °C and then suspended in 20 mM Tris/HCl buffer, pH 7.5 to a total volume of 40 mL. Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> was added to the sample before and after cell lysis to a final concentration of 2 mM. The cells were lysed by sonication and the extract was centrifuged at 4 °C and 18000 × g for 20 min. Nucleic acids in the supernatant were digested at RT for 1.5 hours in the presence of DNase I (25 µg/mL) and MgCl<sub>2</sub> (2 mM). The sample was diluted with 20 mM Tris/HCl, pH 7.5 to a conductivity of 2.3 mS/cm and filtered through a 0.45 µm GHP Acrodisc<sup>®</sup> GF 25 mm Syringe Filter (Life Sciences).

After the preparatory procedures, the CueR protein was purified in four chromatographic steps (Fig. 2) in the order of anion exchange, affinity chromatography, a second anion exchange and a finally gel filtration. Between each purification step the pooled fractions were ultrafiltrated three times in a Millipore 5124 Amicon Stirred Cell Model 8400, 400 mL (N<sub>2</sub> gas, PLBC 3000 membrane) with the binding buffer used during the following purification step.

First, the filtered solution was loaded onto a HiLoad Sepharose Fast Flow Q 16/10 column, which had been equilibrated with 5 column volume (CV) of 20 mM Tris/HCl, pH 7.5 (Buffer A). The bound proteins were eluted with a linear gradient of 20 mM Tris/HCl, 1M NaCl, pH 7.5 (Buffer B) from 15 % to 60 % in 6 CV. The CueR containing fractions were collected and diluted with 20 mM Tris/HCl, pH 7.5 to a conductivity of 7 mS/cm.

In the second step the sample was filtered and loaded onto a HiPerp Heparin FF 16/10 column, preequilibrated with a 20 mM Tris/HCl, 50 mM NaCl, pH 7.5 buffer. The bound protein was eluted with a linear gradient of 20 mM Tris/HCl, 1 M NaCl, pH 7.5 from 0 % to 50 % in 10 CV.

**Fig. 2** – near here

Following affinity chromatography, the fractions containing CueR were pooled and diluted 3 times with 20 mM Tris/HCl, pH 7.5. The ultrafiltered sample was loaded onto a Source 30 Q 16/13 column, preequilibrated with 5 CV of 20 mM Tris/HCl, pH 7.5. The bound protein was eluted with linear gradient of 20 mM Tris/HCl, 1M NaCl, pH 7.0 from 0 % to 60 % in 6 CV. The CueR fractions were collected and filtered again.

Finally, the sample was loaded onto a HiLoad Superdex 75 26/60 column, preequilibrated with a buffer required for the subsequent application (Tris or HEPES containing also 1 mM dithiothreitol (DTT) as reducing agents). Proteins were eluted with isocratic elution and then concentrated by ultrafiltration. Collection of CueR is not recommended in NH<sub>4</sub>Ac buffers (pH 7.0) since aggregation of the protein was observed in this buffer.

Protein samples obtained during the purification were analyzed by standard SDS-PAGE gel electrophoresis [19] using *Any kD™ Mini-PROTEAN®TGX™* (Bio-Rad) gels and Coomassie staining.

#### *Protein identification*

Proteins were identified by peptide mass fingerprint analysis (see details in the Supplementary Material), carried out with a MALDI-TOF mass spectrometer (Bruker, Reflex III) Protein bands were cut out from the SDS-PAGE gel, and sliced into small pieces. The samples were reduced with DTT, alkylated with iodoacetamide and digested with trypsin at 37 °C for 4 hours. Extracted tryptic peptides were spotted onto the MALDI target plate using 2,5-Dihydroxybenzoic acid (DHB) as a matrix and measured in positive reflectron mode. Detected peptide masses were subjected to database search against the Swissprot protein database on our in-house Mascot (Version: 2.2.07, Matrix Science) search engine.

#### *Analysis of the purified protein*

Intact protein analysis were performed on an LTQ-Orbitrap Elite (Thermo) mass spectrometer coupled with a TriVersa NanoMate (Advion) chip-based electrospray ion source. All the masses were measured in the Orbitrap in positive ion mode with the highest resolution ( $R = 240\,000$  at  $400\text{ m/z}$ ). For the top-down analysis ion-trap CID fragmentation was carried out in order to prevent multiple fragmentations and allow for the detection of the possible disulphide bridges (see Scheme 1).

**Scheme 1** – near here

#### *Circular Dichroism spectroscopy*

Circular dichroism spectra were recorded on a Jasco J-815 spectropolarimeter. Camphor-sulfonic acid served as a calibration material for the instrument. All spectra were recorded with 1 nm steps and a dwell time of 2 s per step, using a 0.2 mm quartz cell (SUPRASIL, Hellma GmbH, Germany), in the wavelength range of 180-260 nm. The protein was dissolved in 5 mM HEPES, pH = 7.7 buffer yielding  $c_{\text{protein}} = 2.3 \times 10^{-5}$  M concentration. The raw spectra were baseline-corrected with the water spectrum.

#### *Electrophoretic gel mobility shift assay*

The DNA binding capability of the CueR was evidenced by electrophoretic gel mobility shift assay (EMSA). The volume of the protein-DNA reaction mixture was 5  $\mu\text{l}$  by mixing 1  $\mu\text{l}$  50  $\mu\text{M}$  DNA solution (in 20 mM Tris/HClO<sub>4</sub>, 0.1 mM NaClO<sub>4</sub>, pH 8.0 buffer) with 4  $\mu\text{l}$  100  $\mu\text{M}$  Protein solution (in 20 mM Tris/HCl, 1 mM DTT, pH 7.5 buffer). After 4 hours incubation at 37 °C this solution was mixed with 1  $\mu\text{l}$  6  $\times$  Loading Dye (10 mM Tris-HCl, pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA - Thermo Fischer Scientific) to load it onto 2% (2.0 g/100mL) agarose gel and then run for 30 minutes applying 100 V. After the electrophoresis, the gel was submerged into 1  $\mu\text{g/ml}$  ethidium bromide solution for 20 min, and washed two times with water for 10 min. A 35 bp long PcopA promoter sequence was applied as a specific dsDNA: 5'-AAAGGTAAACCTTCCAGCAAGGGGAAGGTCAAGA-3', while the sequence of the 35 bp non-specific DNA was 5'-GCTGTACATATCGGTAGATTTTCGATCGGTAGATGA-3'.

## **Results and discussion**



### *Difficulties encountered during the purification process of CueR and their solutions*

Our aim was to obtain the wild-type CueR without any affinity tag, which is indispensable for further experiments on the metal ion binding selectivity of the protein. CueR is made up of 135 amino acids. The average molecular weight of the apo-protein is 15235.1 Da, and the theoretical pI is 5.72. This would allow for applying cation exchange at pH 3.0 being a great advantage in the purification process of an air-sensitive protein. The  $pK_a$  of cysteine thiols is ca. 8.4 [21]. A lower pH ensuring the protonated state of cysteines should decelerate the oxidation leading to the formation of intra- and/or intermolecular disulfide bonds and thus, to the potential formation of polymeric aggregates. However, it turned out that CueR in its highly positively charged form under acidic condition binds tightly to the negatively charged DNA being another reason for precipitation. Streptomycin sulfate, a water soluble antibiotic binding to the bacterial ribosome, is a widely used agent to precipitate nucleic acids. In our experiment streptomycin sulfate caused not only the removal of nucleic acids, but most of the CueR protein disappeared from the solution, too (data not shown). This necessitated a change of the purification strategy.

In order to perform anion exchange the increase of the pH was required. Taking into account the acid-base properties of the cysteine thiol groups, pH = 7.5 was chosen for this procedure.  $Na_2S_2O_4$  was added to the samples before and after the lysis of cells to prevent the oxidation of CueR. Anion exchange alone was not enough to obtain an adequately purified CueR (see later in Fig. 6, lane 2). Therefore, the samples were loaded on a gel filtration column, preceded by a concentration step. However, precipitation was observed during the ultrafiltration step (data not shown). This experiment was repeated in the presence of DTT as a reducing agent and argon-flushed buffers but the precipitation could not be avoided. The precipitated proteins were analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showing two clear bands on the gel: a ~ 15 kDa size protein expected to be CueR, and an unknown protein with a size of ~ 45 kDa (Fig. 3). The latter protein appeared in all fractions of the previous purification steps (even in gel filtration experiment separating the molecules based on their size) together with the target CueR molecule.

**Fig. 3** – near here

The two bands were cut from the gel and analyzed with peptide mass fingerprinting. As a result of this procedure the 15 and 45 kDa proteins were identified as the CueR

(CUER\_ECOLI) and an Elongation factor of *E. coli* (EFTU1\_ECOLI), respectively. The most intense peaks of the mass spectra obtained from the bottom and upper protein band matched to CueR with 44 %, and to the Elongation factor with 58 % sequence coverage, respectively. CueR co-precipitated with another nucleic acid binding protein, an elongation factor, suggesting that CueR interacted with this elongation factor through DNA. Therefore, we expected that CueR precipitation might have been prevented by digesting DNA with DNaseI enzyme before the first chromatographic step. Indeed, CueR precipitation was not observed in further downstream anion exchange and gel filtration purification steps after digesting DNA with DNaseI. However, the purity of the CueR containing fractions collected from the gel filtration column was found to be insufficient (data not shown).

In order to achieve better separation, we started a new purification procedure by loading the DNaseI digested mixture directly onto an anion exchange column. After this step affinity chromatography purification by a Heparin column and a subsequent anion exchange were introduced. The glycosaminoglycan heparin mimics the polyanionic structure of nucleic acids. Consequently, nucleic acid binding proteins, such as CueR show a strong affinity to heparin, too. One may notice that heparin carries negative charges, and thus it can also work as a cation exchange column. However, in our system the positively charged proteins were removed in the flow-through fraction of the preceding anion exchange step. Anion exchange was introduced as the third chromatographic step in the protocol. This was necessary because a ~ 31 kDa protein contamination appeared in the collected fractions, in addition to CueR, when gel filtration was applied directly after affinity chromatography (Fig. 4).

**Fig. 4** – near here

The separation of CueR from the ~ 31 kDa protein was successful in the purification process complemented with this second anion exchange, introduced between the affinity purification and gel filtration steps. A Source 30 Q 16/13 column was selected, with a higher resolution as compared to that of Sepharose FF. The anion exchange at this step was performed at pH = 7.0 (Fig. 5C). The final gel filtration produced a single well separated peak (Fig. 5D).

In summary, pure CueR fractions were obtained in the four-step chromatographic procedure (Fig. 5), preceded by the digestion of the DNA, as it was reflected by the SDS-PAGE analysis (Table 1, Fig. 6). The average yield was 4 mg protein per 1 L culture.

**Table 1** – near here

**Fig. 5** – near here

**Fig. 6** – near here

#### *Analysis of the integrity of the purified protein*

The monoisotopic molecular mass of the singly charged ( $MH^+$ ) intact CueR was determined as 15225.5 Da by deconvolution of the mass spectrum. This value is in a good agreement with the theoretically calculated protonated monoisotopic mass of 15226.6 Da. In order to investigate the oxidation status of the cysteine residues, a collision-induced dissociation (CID) was performed. The ion at 897  $m/z$  ( $z = 17$ ) was chosen for fragmentation in the ion trap. The most abundant fragment peak at 789.66  $m/z$  ( $z = 3$ ) was further fragmented and identified as the C-terminal PGDDSADCPIIENLSGCCHHRAG part of the protein (Fig. 7). Analyzing the  $MS^3$  spectrum showed that the majority of the peaks corresponding to sequences containing various number of Cys residues e.g. the  $y_{10}$  [LSGCCHHRAG] $^+$ ,  $y_7$ : [CCHHRAG] $^+$  and  $y_6$ : [CHHRAG] $^+$  display masses expected from the reduced forms. Signals of fragments with a disulfide bridge typically between Cys129 and Cys130 represent only a small fraction of the CueR molecules. The fact that during ion-trap CID experiment the protein was broken between Cys112 and Pro113 indicates that Cys112 is not linked to the other Cys residue (Cys120) of the metal ion binding loop through an intramolecular disulfide bridge. All in all, from the  $MS^3$  experiment we have a clear evidence that all of the four Cys residues can be kept in their reduced form in the large majority of the protein molecules.

**Fig. 7** – near here

#### *Characterization of the purified CueR*

The solution structure of the protein was tested by CD spectroscopy. The recorded spectrum (Fig. 8) is characteristic for proteins that are rich in  $\alpha$ -helices, in agreement with the crystal structures of the metal-bound forms of CueR (PDB id: 1Q05, 1Q06, 1Q07 [11]). The evaluation of the CD spectra by BeStSel program yielded  $\sim 43\%$   $\alpha$ -helical content [22].

**Fig. 8** – near here

The electrophoretic mobility shift assay (EMSA) clearly shows that the specific 35 bp long DNA fragment (the *PcopA* promoter region of the regulated *copA* gene) is shifted in the gel in the presence of the protein (Fig. 9). Similar effect was not observed with the non-specific DNA. This undoubtedly verifies the proper folding of the CueR, which functions as a specific DNA binding protein recognizing the promoter sequence.

**Fig. 9** – near here

## Conclusion

In this work, we described an alternative purification strategy for a cysteine containing nucleic acid binding protein, the copper-efflux regulator CueR. In a previously published method, based on the purification protocol of a related MerR family member protein ZntR, the proteins were precipitated before the chromatographic purification. Such steps often lead to a significant loss of proteins, and the re-folding may also be problematic. Consequently, instead of the precipitation step, we removed the nucleic acids by enzymatic digestion followed by a multi-step chromatographic procedure to achieve a proper protein separation.

This four-step purification method resulted in CueR protein samples of high purity (Table 1) with an the average yield of 4 mg protein from 1 L culture. It is worth mentioning that the yield can be increased and the process can be shortened by the elimination of the second anion exchange step, if the very high purity of the protein is not crucial.

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**Table 1:** Purity of CueR after the consecutive steps of the purification as estimated from SDS-PAGE analysis.

<b>Step</b>	<b>Purity (%)</b>
Crude extract	1
Nucleic acid digestion	1
Sepharose column (pooled peak)	20
Heparin column (pooled peak)	80
Source column (pooled peak)	95
Sephadex column (pooled peak)	99

## Caption of Scheme and Figures

**Scheme 1:** During the CID experiments, the peptides are fragmented at the amide bonds along the backbone, generating b- and y-type fragment ions [20].

**Fig. 1:** The crystal structure and the amino acid sequence of the Cu<sup>I</sup>-binding CueR (PDB id: 1Q05 [11]). Gray-scale coding applied for the various domains: the DNA-binding domains are marked with white, dimerization helices with light grey, metal-binding domains with dark grey and Cu<sup>I</sup>-ions with black spheres. (The sequence of a fragment with unresolved structure is italicized.)

**Fig. 2:** Flow chart of the four-step chromatographic purification protocol.

**Fig. 3:** SDS-PAGE image of the precipitated proteins with the molecular weight standard given in kDa.

**Fig. 4:** Chromatogram of gel filtration and SDS-PAGE analysis of the combined and concentrated CueR containing fractions obtained in a procedure without performing a second anion exchange between the affinity chromatography and gel filtration steps.

**Fig. 5:** Representative chromatograms of the various purification steps (dotted lines denote the collected fractions). The dashed lines show the percentage of the applied, high ionic strength buffer during the elution. **A:** first anion exchange on a Sepharose FF 16/10 column; **B:** affinity purification on a Heparin FF 16/10; **C:** second anion exchange on Source 30 Q 16/13; **D:** gel filtration on HiLoad Superdex 75 26/600 with isocratic elution.

**Fig. 6:** SDS-PAGE analysis performed at the various stages of the purification.

M: molecular weight standard given in kDa; 1: supernatant, 2: sample after the first anion exchange; 3: sample after affinity purification; 4: sample after the second anion exchange; 5: sample after gel filtration; 6: final concentrated sample.



**Fig. 7:** MS<sup>3</sup> CID spectrum of CueR showing fragments of [PGDDSADCPIIENLSGCCHHRAG]<sup>3+</sup> from the C-terminal part of the protein. The expected *m/z* values calculated by Protein Prospector program are shown in the table. Identified fragments from the spectrum are highlighted in bold and italic.

**Fig. 8:** CD spectrum of CueR in 5 mM HEPES buffer recorded in a 0.2 mm quartz cell.

**Fig. 9:** Gel mobility shift assay with CueR and 35 bp specific and non-specific DNA fragments. Lane 1 and 6 contain the 100 bp DNA ladder as marker. The free specific and non-specific DNA samples are in lanes 2 and 3 respectively. Lane 4 shows the effect of CueR on the specific DNA while lane 5 represents the effect of CueR on the non-specific DNA.

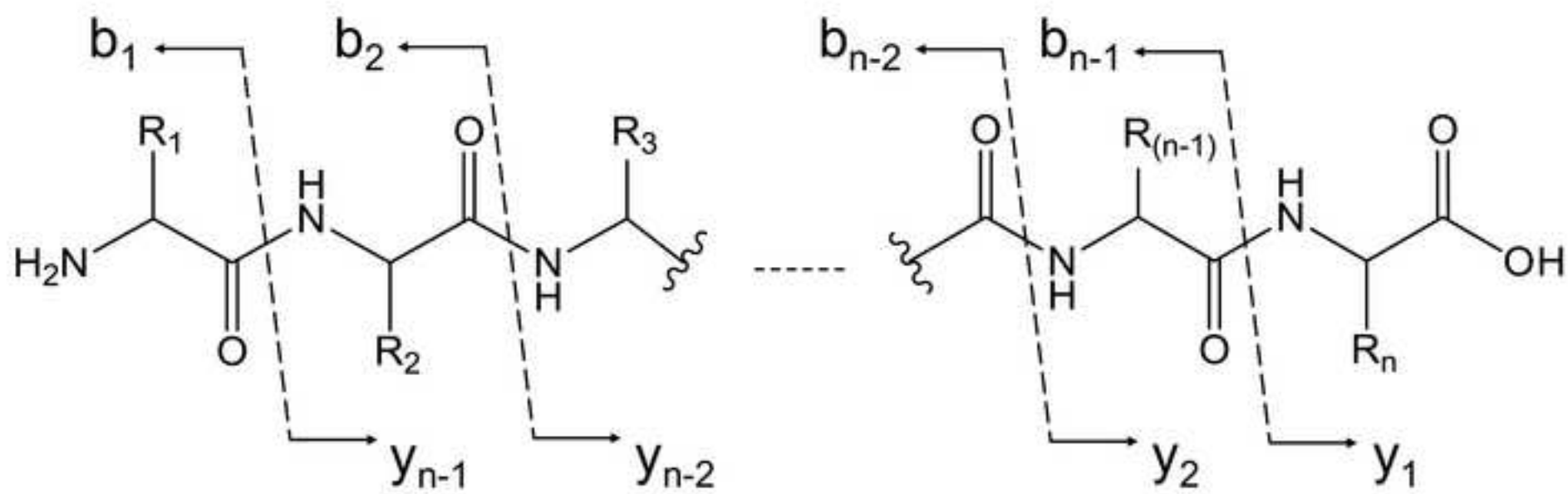
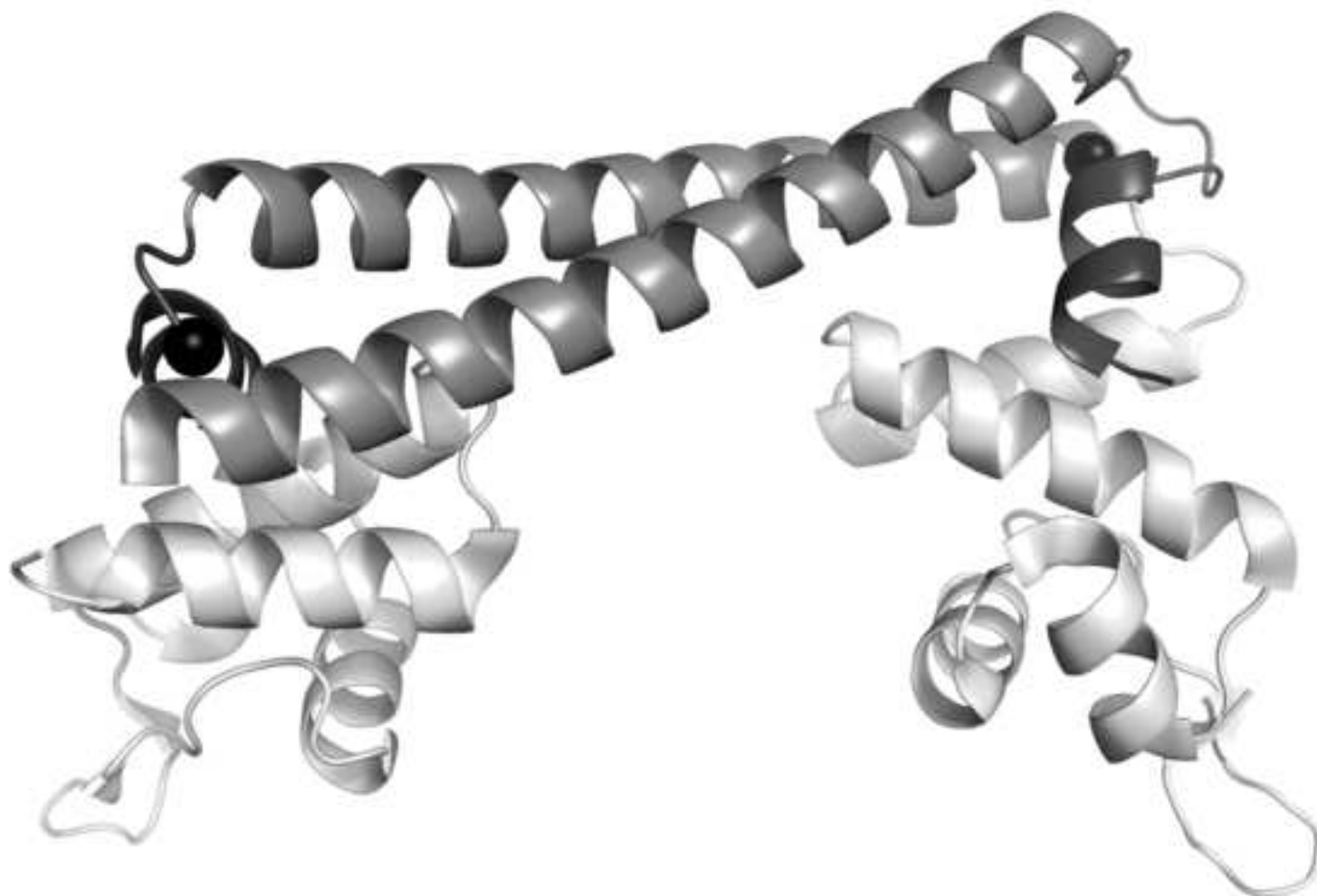


Fig. 1 - single column  
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MNISDVAKITGLTSKAIRFYEEKGLVTPPMRSENG  
YRTYTQQHLNELTLLRQARQVGFNLEESGELVNL  
FNDPQRHSADVKKRRTLEKVAEIERHIEELQSMRD  
QLLALANACPGDDSADCPIENLSGCCHHRAG

**Fig. 2 - 1.5 column**

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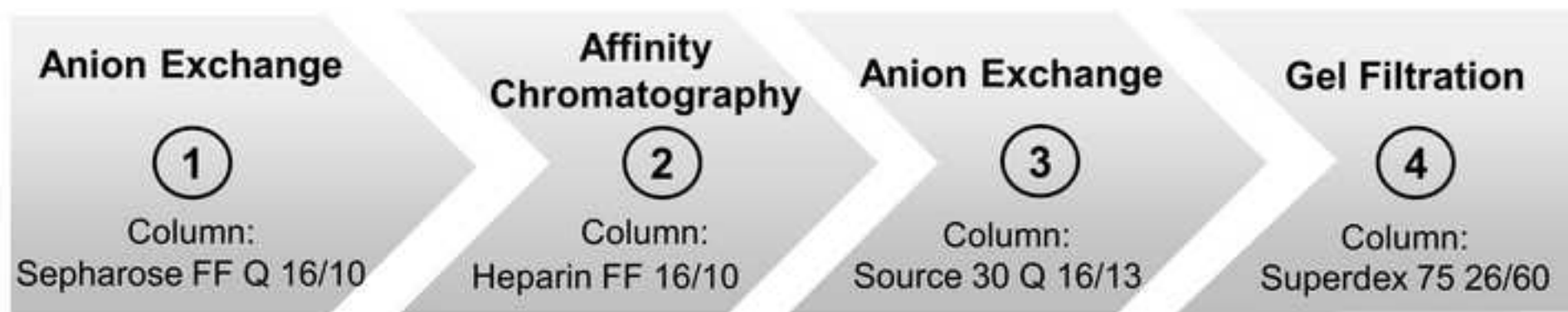


Fig. 3 - single column  
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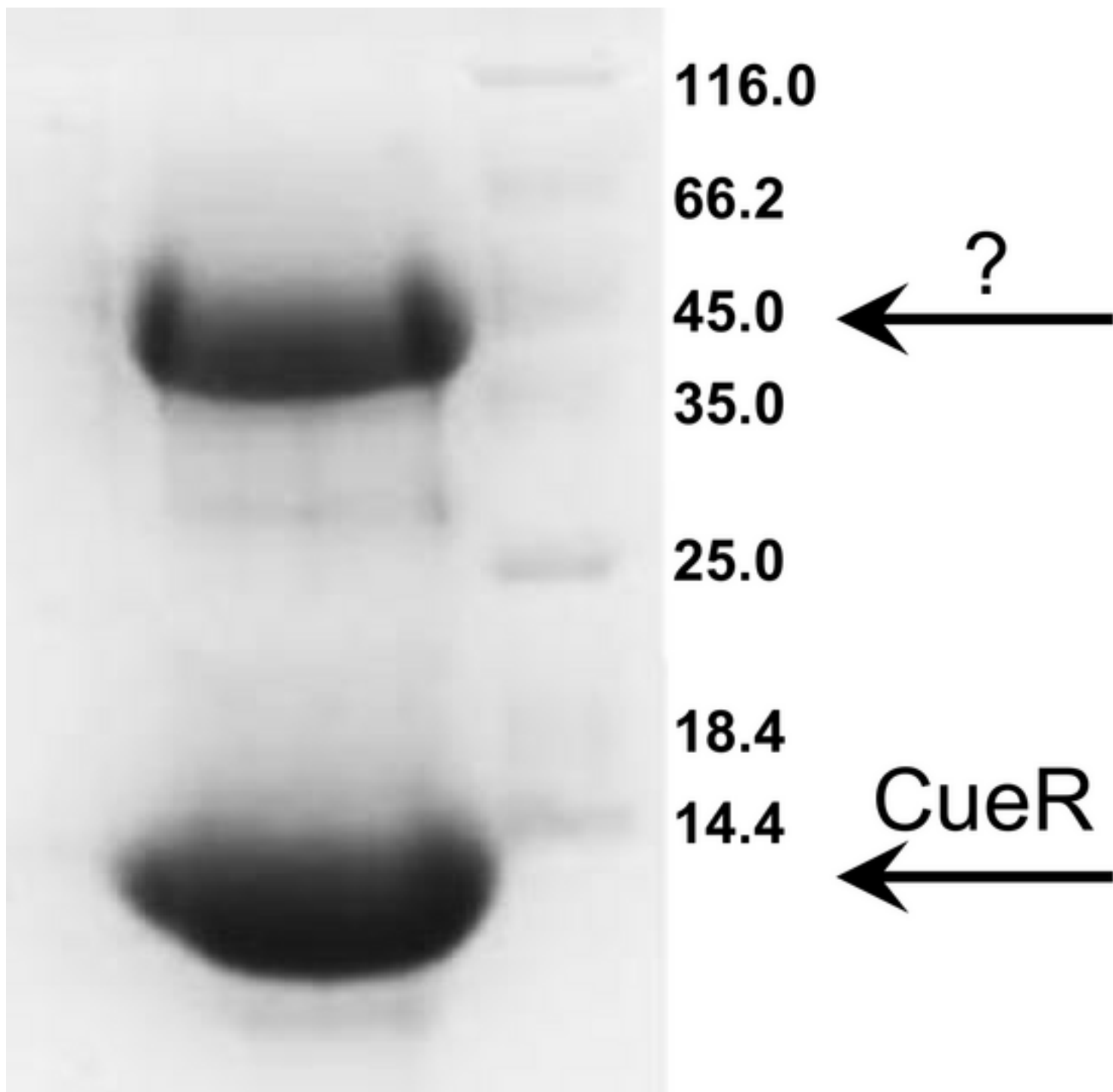


Fig. 4 - 1.5 column  
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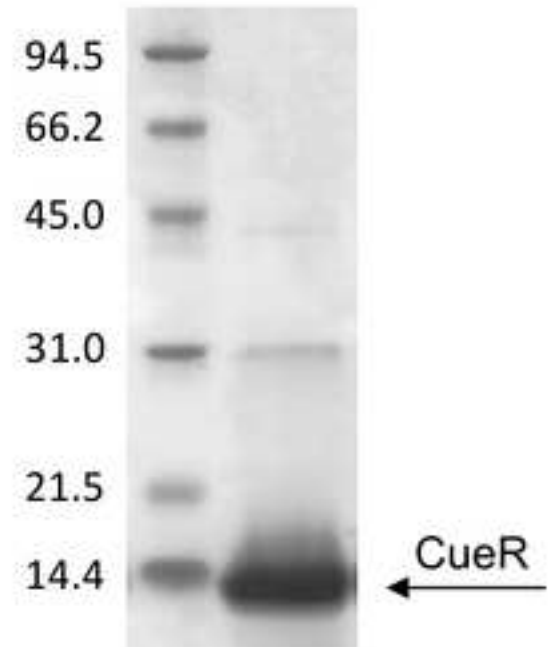
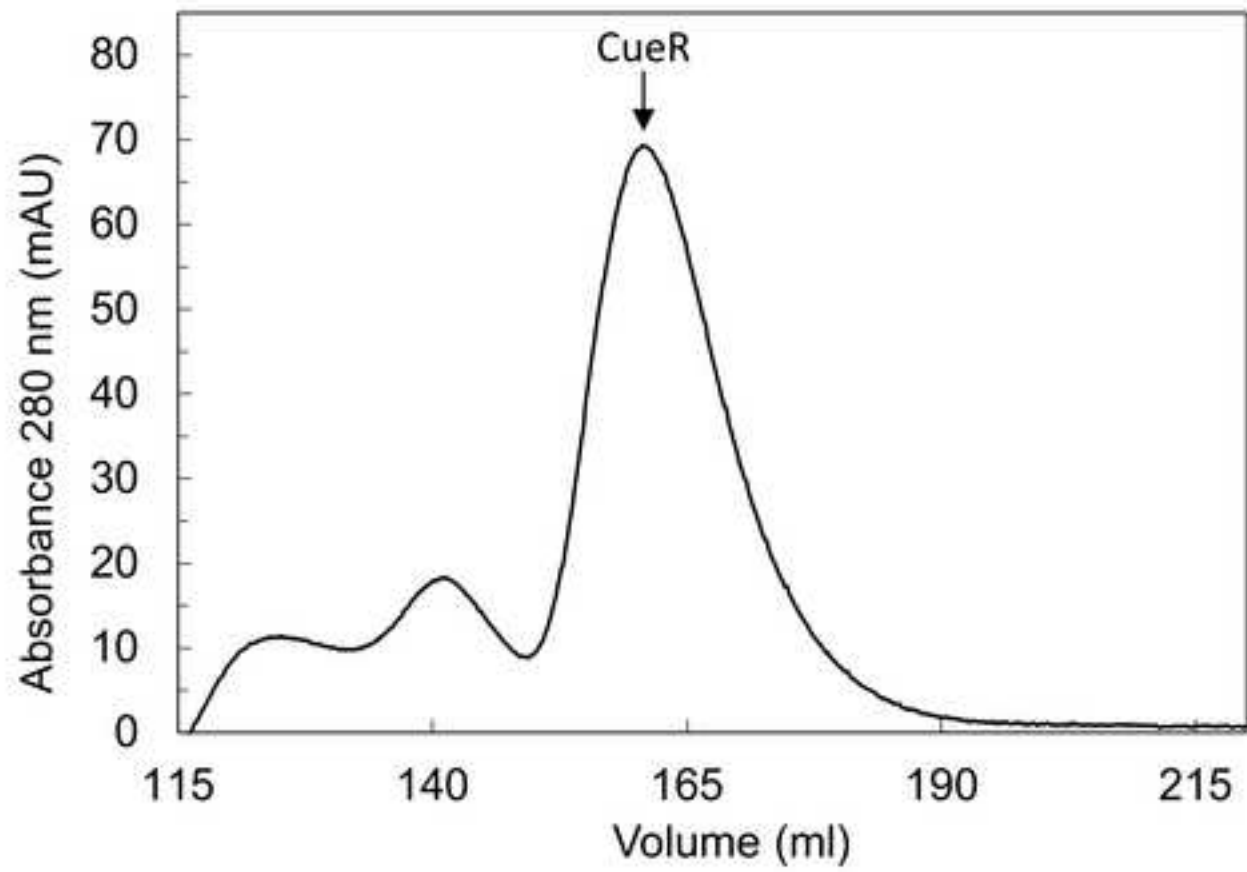


Fig. 5 - double column  
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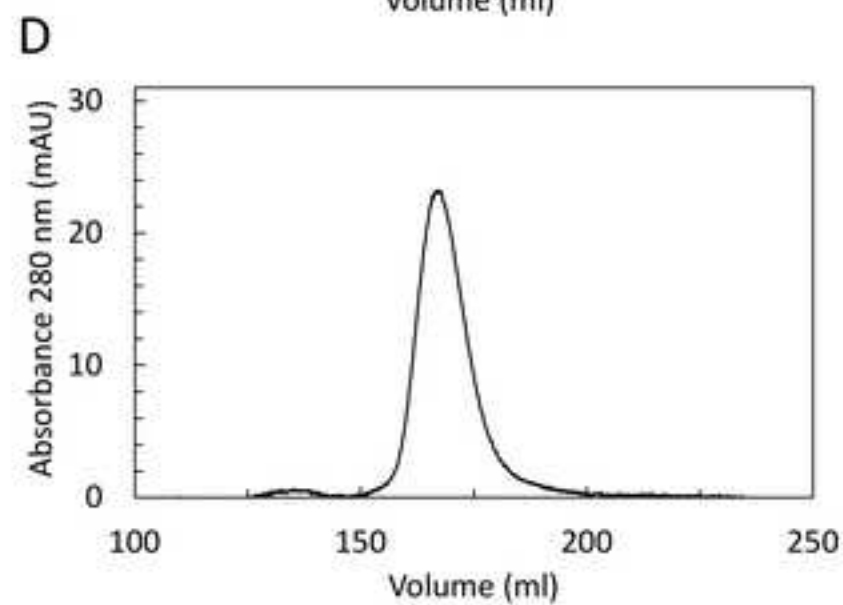
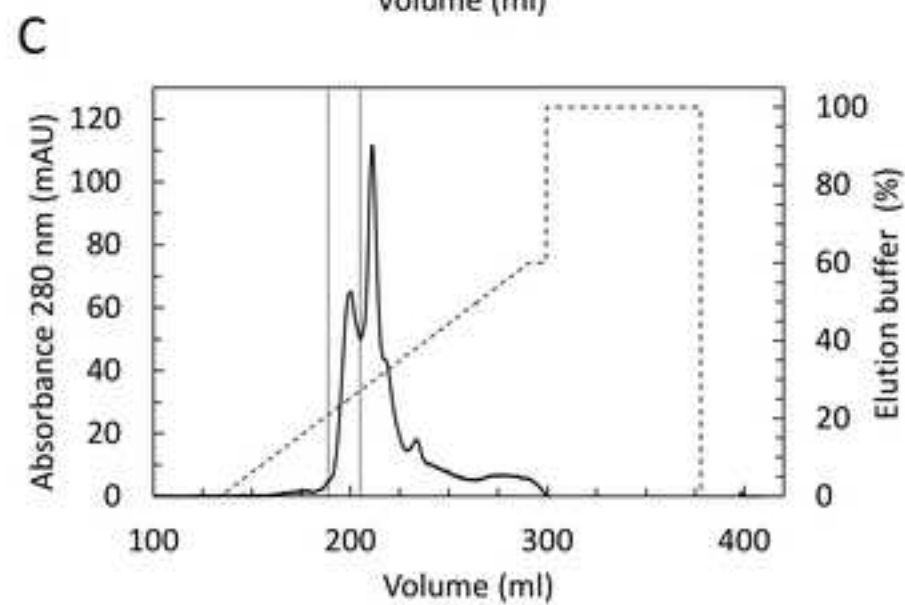
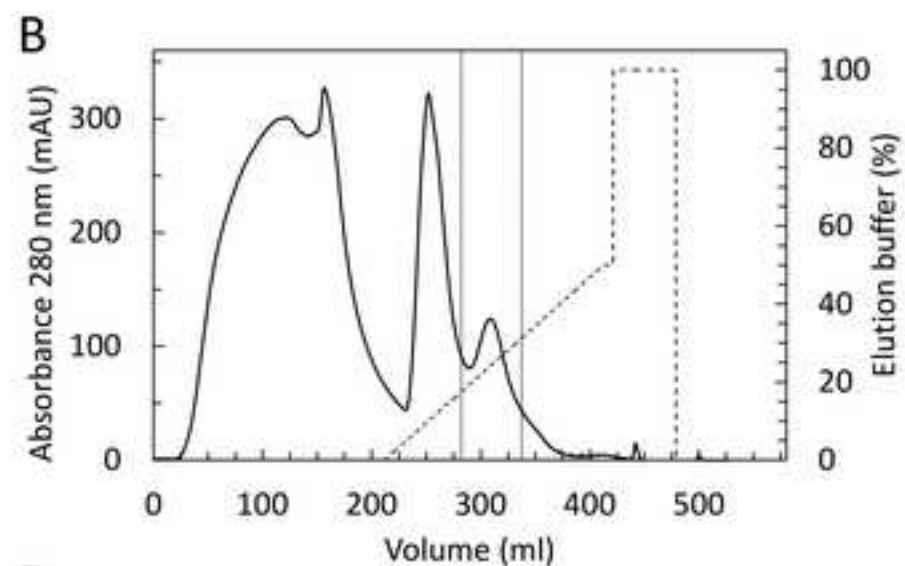
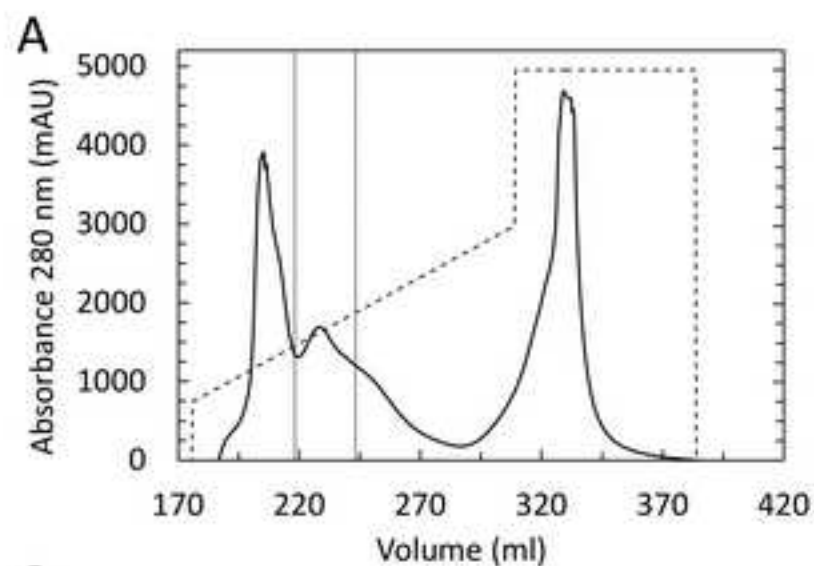


Fig. 6 - single column  
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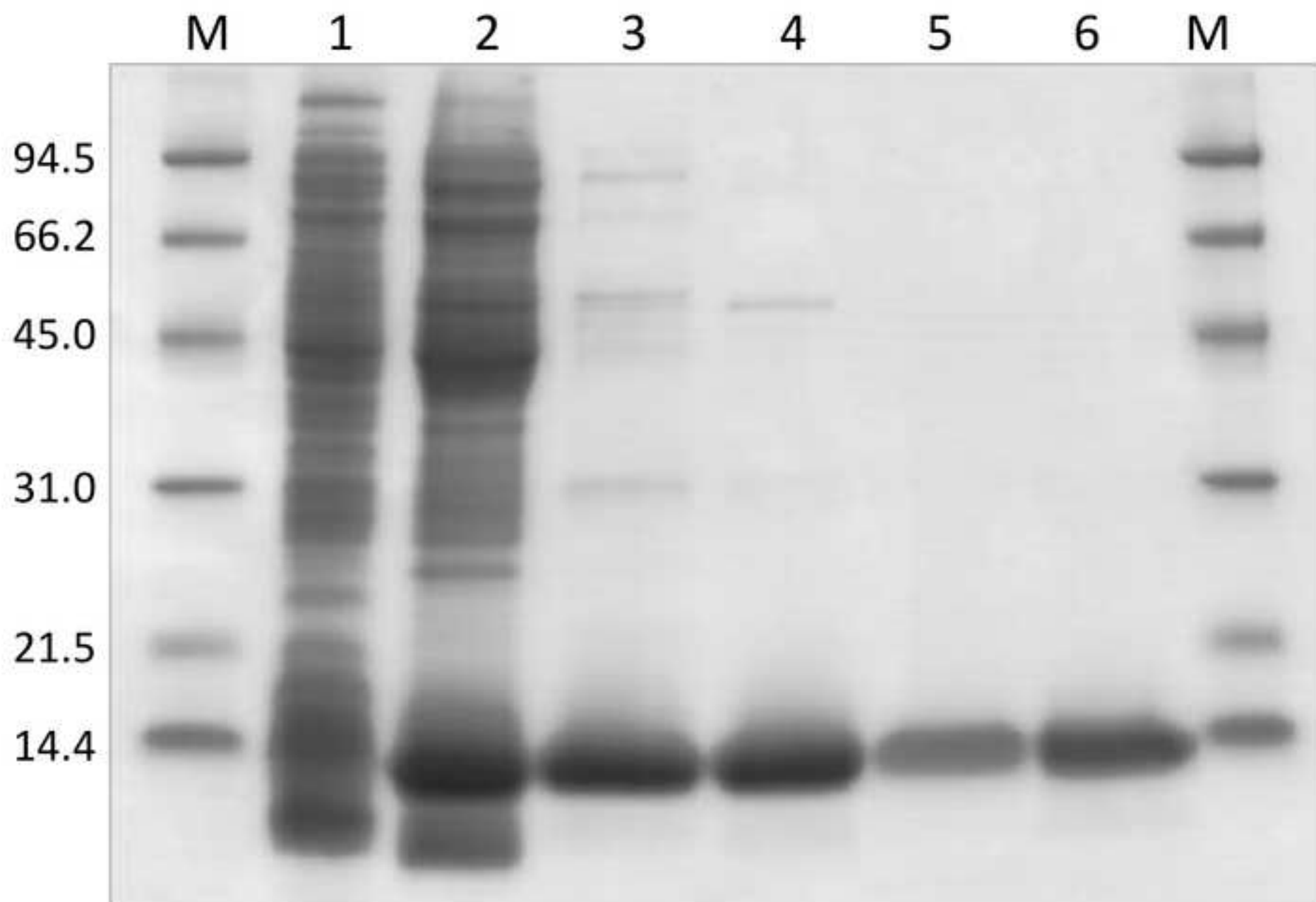
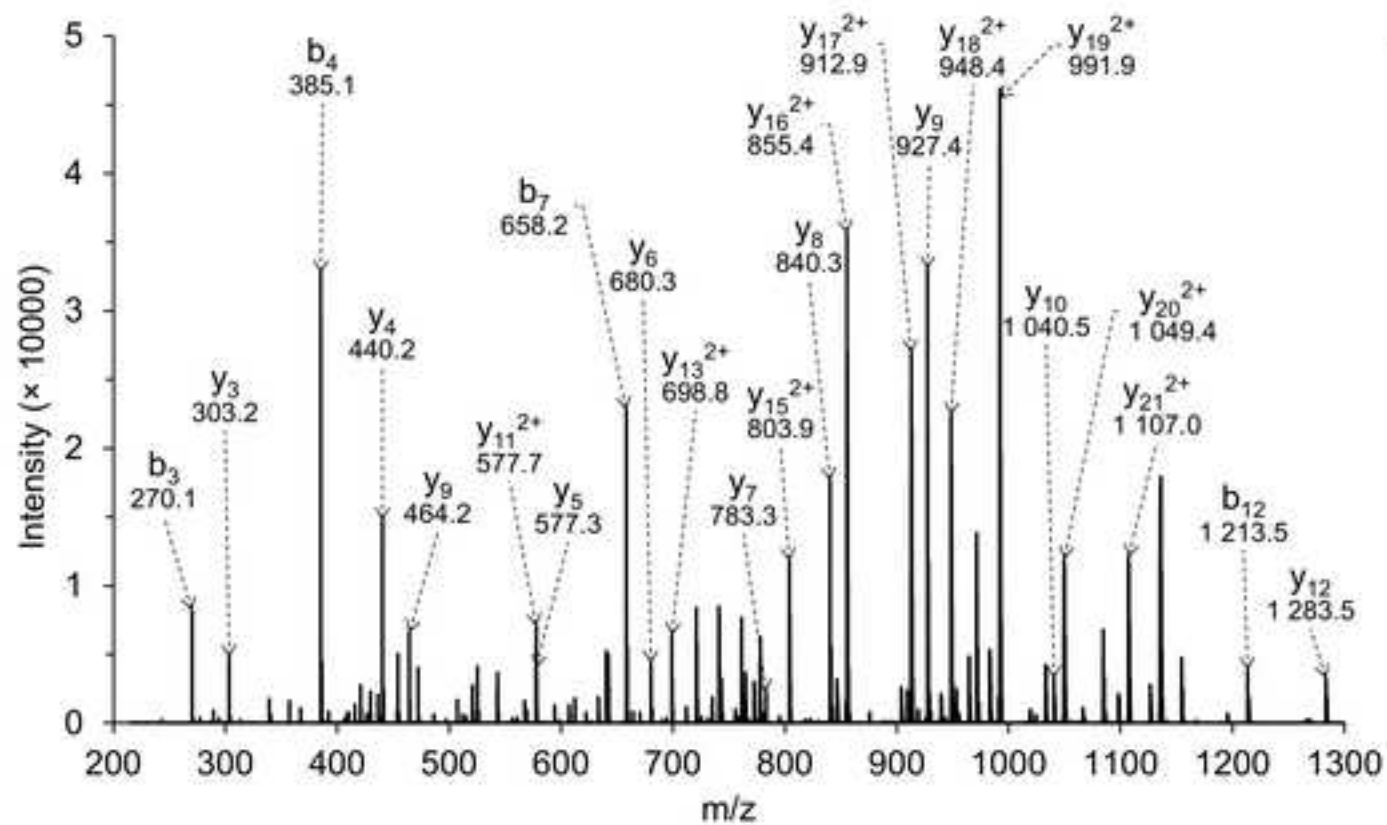




Fig. 7 - double column  
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<i>b</i> <sup>1+</sup>	<i>b</i> <sup>2+</sup>			<i>y</i> <sup>1+</sup>	<i>y</i> <sup>2+</sup>
—	—	1	P	23	—
155.1	—	2	G	22	2269.9
<b>270.1</b>	—	3	D	21	2212.9
<b>385.1</b>	—	4	D	20	2097.9
<b>472.2</b>	—	5	S	19	1982.9
<b>543.2</b>	—	6	A	18	1895.8
<b>658.2</b>	—	7	D	17	1824.8
<b>761.2</b>	—	8	C	16	1709.8
858.3	—	9	P	15	1606.8
<b>971.4</b>	—	10	I	14	1509.7
<b>1084.5</b>	—	11	I	13	1396.6
<b>1213.5</b>	—	12	E	12	<b>1283.5</b>
1327.5	—	13	N	11	1154.5
1440.6	—	14	L	10	<b>1040.5</b>
1527.7	—	15	S	9	<b>927.4</b>
1584.7	—	16	G	8	<b>840.3</b>
1687.7	—	17	C	7	<b>783.3</b>
1790.7	—	18	C	6	<b>680.3</b>
1927.8	<b>964.4</b>	19	H	5	<b>577.3</b>
2064.8	1032.9	20	H	4	<b>440.2</b>
2220.9	1111.0	21	R	3	<b>303.2</b>
2292.0	1146.5	22	A	2	147.1
—	—	23	G	1	76.0

Fig. 8 - single column  
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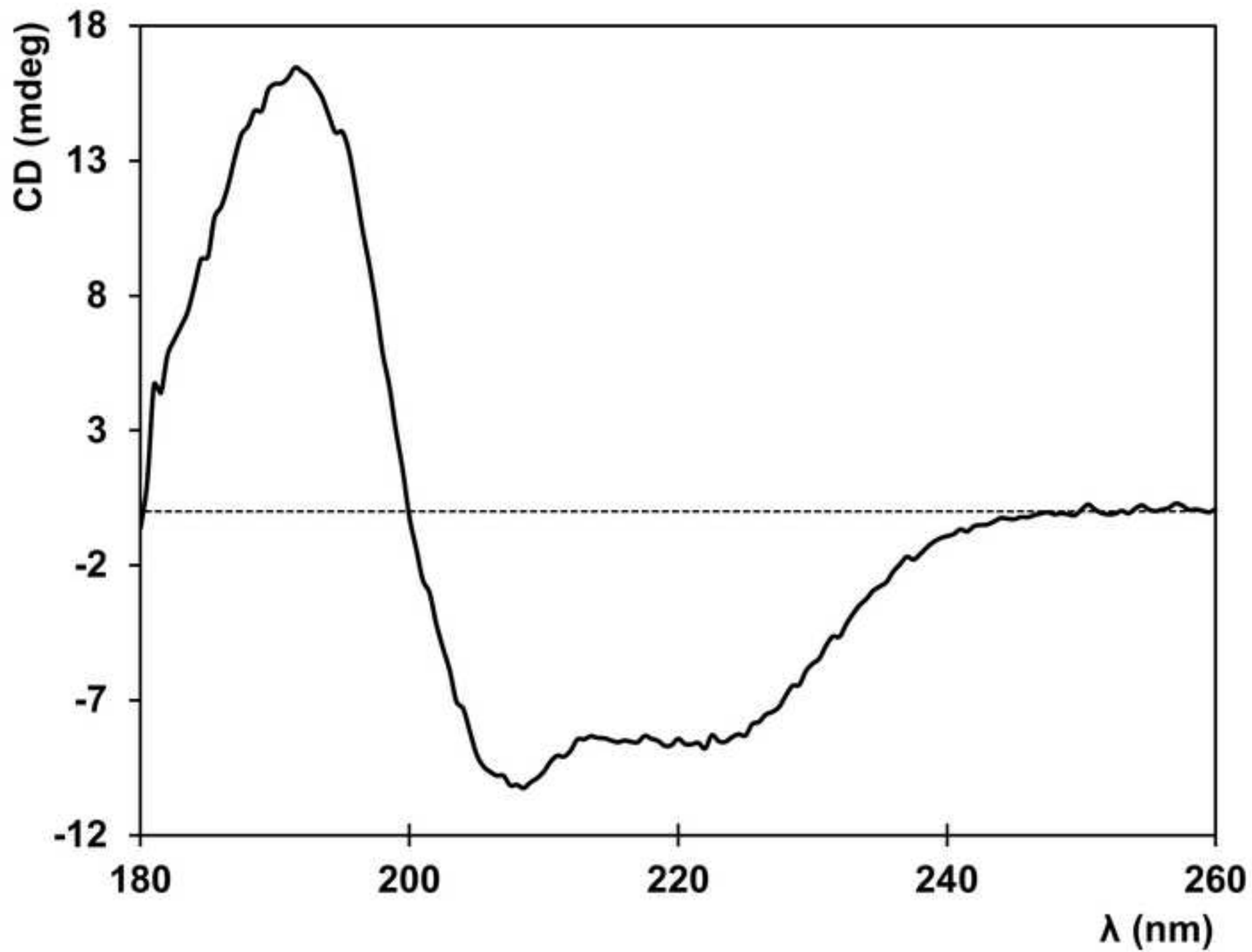
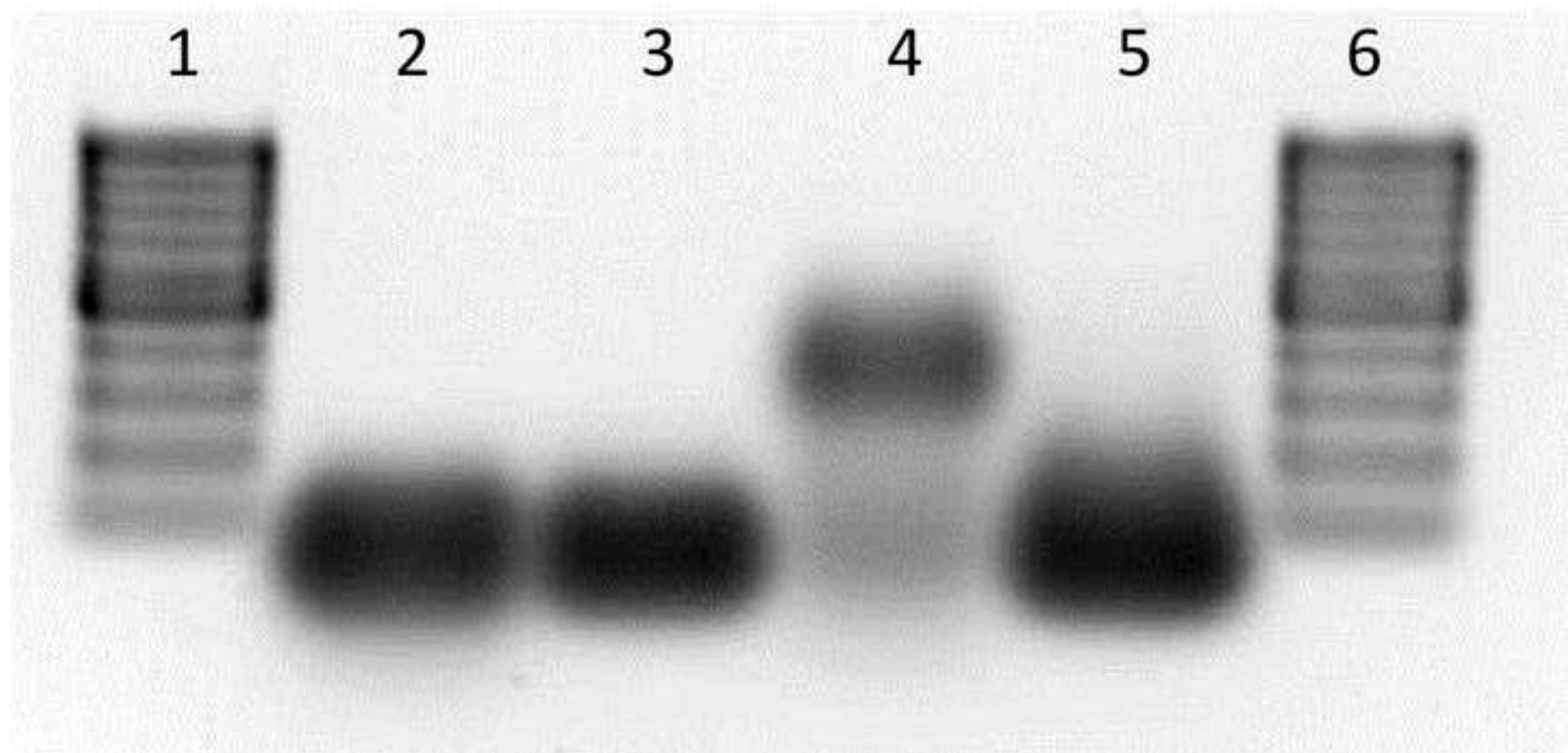


Fig. 9 - single column  
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# Graphical abstract: 5-step purification procedure of CueR protein

**DNA  
Digestion**

**1**

Enzyme:  
DNase I

**Anion  
Exchange**

**2**

Column:  
Sepharose FF Q

**Affinity  
Chromatography**

**3**

Column:  
Heparin FF

**Anion  
Exchange**

**4**

Column:  
Source 30 Q

**Gel  
Filtration**

**5**

Column:  
Superdex 75

**Supplemental Material**

[Click here to download Supplemental Material: Supplementary\\_PREP-15-707\\_R2.docx](#)