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Optimization of immobilized metal ion affinity chromatography for single-step purification of recombinant ovine growth hormone expressed in *Escherichia coli*

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Abstract

In the present investigation, Sepharose 6B gel with 1,4-butanediol diglycidyl ether as spacer arm, iminodiacetic acid as the ligand and Cu^{2+}, Ni^{2+} as metal ions were used to prepare an immobilized metal ion affinity (IMA) gel. The binding capacities of recombinant ovine growth hormone (roGH) onto IMA gels were maximized in the packed bed column. Parameters influencing the purification efficiencies such as pH, ionic strength and flow-rate were optimized to achieve improved separation. The roGH was purified from inclusion bodies with an overall yield of 73.5% and purity of 94.3% using a Cu^{2+}–iminodiacetic acid (IDA) column. However, the Ni^{2+}–IDA column was more successful in purifying the roGH from crude cell lysate in a single-step with a yield of 83% and purity of 92.5%.

Keywords: *Escherichia coli*; Immobilized metal ion affinity chromatography; Growth hormone; Proteins; Iminodiacetic acid

1. Introduction

Immobilized metal ion affinity chromatography (IMAC) is a powerful affinity method for purification, which takes advantage of the interaction between biomolecules and transition metals [1]. Optimization of a chromatographic process on a preparative scale using IMAC demands a thorough understanding of the fundamental factors governing the various interactions involved. Knowledge of these aspects can provide a rationale for selecting a suitable chelating ligand and establishing appropriate chromatographic conditions for purification [2,3]. The performance of an IMA gel is a complex function of a large number of variables. It not only depends upon the sorbent matrix, the chelating agent, method of immobilization and metal ion, but also depends on the solution conditions (solution buffer, salt concentration, pH, etc.) used for the study. Hence, it is desirable to study the adsorption capacity of these supports under varying solution conditions to be used during equilibration, washing and elution. Immobilized metal ion affinity chromatography facilitates single-step purification of recombinant proteins by incorporating a His tag in the recombi-

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nant plasmid vector, which has a high affinity towards some of the metal ions [4].

Growth hormone plays an important role in metabolism, protein synthesis and cell proliferation. Ovine growth hormone is synthesized from sheep anterior pituitary gland and is required for normal growth and lactation in mammals [5]. Ovine growth hormone, which has an isoelectric point at pH 6.3, is a monomer of M_r 22 000 [6], which encodes a single polypeptide chain of 191 amino acids containing two disulfide bridges [7]. Ovine growth hormone is a nonglycosylated protein and hence a prokaryotic expression system has been preferred over other available expression systems [8–10]. Recently, transgenic sheep expressing ovine growth hormone have been reported to exploit its utility for higher growth of farm animals [11].

Recombinant ovine growth hormone (roGH) is expressed in *E. coli* in the form of intracellular insoluble, biologically inactive inclusion bodies [8]. The roGH expression vector was constructed using plasmid pQE 30 as the starting material [8]. The vector also had a unique $(His)_6$ tag to facilitate single step purification using immobilized metal ion affinity chromatography. The roGH is then isolated, solubilized and purified from the same using chromatographic techniques. The methods published for the isolation and purification of roGH using Ni^{2+}–NTA (nitrilotriacetic acid) [8] and liquid chromatography [9] provide high purity (up to 95%) with a low yield, but the procedures are cumbersome. Thus, a new chromatographic matrix was used, which minimized the tedious inclusion body isolation steps and provided a single-step purification of recombinant ovine growth hormone to give a better yield with similar purity levels.

2. Experimental

2.1. Materials

Sepharose 6B was procured from Pharmacia-LKB Biotechnology (Uppsala, Sweden). Iminodiacetic acid, imidazole, trichloroacetic acid (TCA), ampicillin, kanamycin and 1,4-butanediol diglycidyl ether were from Sigma (St. Louis, MO, USA); copper sulfate and urea were from Merck (Darmstadt, Germany); Tris, deoxycholate and phenylmethyl sulfonyl fluoride (PMSF) were from Amresco (USA); and isopropyl-β-D-thiogalactopyranoside (IPTG) was from BioSynth (Sweden). Ethylenediaminetetraacetic acid disodium salt (EDTA), nickel chloride, and sodium hydroxide were locally purchased from Qualigens (India). Other chemicals are of analytical grade. All solutions and buffers were prepared in ultra pure water obtained from a Milli-Q unit (Millipore, Bedford, MA, USA). The recombinant plasmid vector (pAROGH), clone of ovine growth hormone was obtained from the National Institute of Immunology (NII) (New Delhi, India). Anti-oGH antibodies were from NIDDK (NIH, Bethesda, MD, USA). Horseradish peroxidase (HRPO) conjugate was from the reagent bank of the National Institute of Immunology.

2.2. Preparation and characterization of IMA gel

The method published by Winzerling [12] was used to prepare the iminodiacetic acid (IDA)-Sepharose 6B gel. The IDA-chelated gel thus prepared was used to pack the column and the gel was characterized for metal loading capacities. All the experiments for metal loading were conducted at room temperature (25 °C). The column (5 ml packed bed volume) pre-equilibrated with 10 m*M* sodium acetate buffer of pH 4.0 was charged with metal ions by passing a solution of copper or nickel sulfate (20–50 m*M*). The loosely bound and excess metal ions in the column were removed by proper washing with the same buffer. The metal adsorption capacities of the IMA gel were measured by collecting the outlet samples of the column during the metal ion loading and washing step. The metal ion concentration in all the samples was measured using UV–Vis spectrophotometry and the amount of adsorbed metal ions was determined by applying a mass balance across the column (frontal analysis).

To measure copper and nickel concentration, the λ_{max} for copper and nickel were chosen as 778 nm and 395 nm, respectively. The column was then equilibrated with 6–7 column volumes of 20 m*M* sodium phosphate buffer followed by elution with 50 m*M* EDTA in the same buffer. After thorough washing with Milli-Q water, the column was ready for the next experiment. Each time, before starting

the experiment, the column was recharged with the metal ions to ensure the same efficiency of the column.

2.3. Expression and isolation of recombinant ovine growth hormone

Prokaryotic expression systems have been preferred to other available expression systems for the expression of roGH, since it is nonglycosylated [9,10]. The recombinant plasmid vector (pAROGH) was used to express roGH in *E. coli* M15 cells [8]. The expression vector has a unique $(His)_6$ affinity tag, which facilitates single-step purification of recombinant protein using metal ion affinity chromatography [13]. Due to its small size, the His tag is poorly immunogenic, therefore, it is not necessary to cleave it from the fusion protein after purification.

Transformed *E. coli* cells, harboring the recombinant plasmid were cultured at 37 °C in 5 ml of fresh Luria-Bertani medium containing 50 μg/ml of ampicillin and 25 μg/ml of kanamycin for 12–14 h. The freshly grown cultures were subcultured in larger volumes until an A_{600} value of 0.6–0.7 was obtained. The addition of IPTG to a final concentration of 1 m*M* induced the culture, which was continuously shaken at 37 °C for about 3.5 h. Recombinant ovine growth hormone was produced in the form of intracellular insoluble, biologically inactive inclusion bodies. The insoluble aggregates in the inclusion bodies turned out to be advantageous as they prevented the degradation of protein as has been described for other recombinant proteins and it also facilitates its purification. As the roGH is accumulated in the form of insoluble inclusion bodies, other soluble proteins of *E. coli* can be separated from the inclusion bodies by using different solubilizing steps following the protocol as described [14,15]. In this protocol *E. coli* cells expressing recombinant protein as inclusion bodies were solubilized in a suitable buffer in the presence of 8 *M* urea concentration. The protein solution was loaded onto a Ni^{2+}–NTA column and the bound proteins were eluted using a gradient concentration of imidazole in the elution buffer [15]. Fig. 2 represents the sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel scanned for inclusion body isolation steps.

2.4. Purification of roGH using IMAC

Purification of roGH using IMAC was carried out under denaturing conditions. Purified inclusion bodies or crude cell lysate was dissolved in the presence of 8 *M* urea in Tris buffer and the clear supernatant was used for roGH purification using IMAC. The optimization of different parameters such as salt concentration, pH and elution behavior was done initially for IMA gel. An Eppendorf tube was packed with 0.5 ml of Cu^{2+}–IDA gel. Protein lysates (pellet dissolved in 8 *M* urea) were loaded onto this matrix. Washing was done with Tris buffer (20 m*M*) and elution with different concentrations of imidazole. After the optimization of conditions, small syringe columns (10 cm×1.4 cm) were packed with 5 ml each of Cu^{2+}–IDA and Ni^{2+}–IDA gels and a thorough study of different parameters was performed.

2.5. SDS–PAGE and immunoblot analysis

The qualitative measurement of the protein was done using 12% SDS–PAGE gel, run according to the method of Laemmli [16]. Total cell lysates and purified proteins were resolved on SDS–PAGE and stained with Coomassie blue. The loading volume in SDS–PAGE was always 20 μl. For immunoblot analysis another 12% SDS–PAGE gel, without staining, was electrophoretically transferred onto a nitrocellulose membrane at 30 V for 12 h. The nonspecific sites of the membrane were blocked with non-fat milk and washed thoroughly with phosphate-buffered saline (PBS) containing Tween 20. After blocking, the nitrocellulose membrane was incubated with oGH polyclonal antibodies. Anti rb IgG conjugated to HRPO was used as second antibody. The antigen–antibody complexes were detected upon developing with diaminobenzidine as substrate.

2.6. Protein estimation for yield and purity of roGH

Total protein concentration in the sample fractions collected was measured at 280 nm using a UV spectrophotometer after TCA precipitation. Imidazole interferes in the protein estimation and hence it

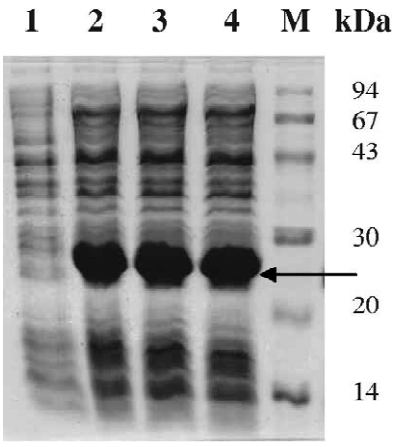

Fig. 1. Expression of ovine growth hormone in *E. coli*. Lanes 1, uninduced *E. coli* M15 cells transformed with recombinant plasmid vector; 2, 3 and 4, *E. coli* cells induced with 1 m*M* IPTG. *M*, molecular mass standards; kDa, kilodaltons.

was removed using TCA precipitation. The precipitated protein was redissolved in 8 *M* urea.

To calculate the recombinant oGH content in each well of the SDS–PAGE gel, Bio-Rad software for intensity measurement was used. One standard SDS–PAGE gel with different concentrations of pure bovine serum albumin (BSA) was run and intensity was measured for each band having different protein content. Total protein balance and roGH balance was done for each experiment to calculate the yield and purity of recombinant ovine growth hormone.

3. Results and discussion

3.1. Characterization of the IMA gel capacity for Cu^{2+} and Ni^{2+} ions

The pre-equilibrated column was loaded with metal ions by passing metal ion solution under desired conditions. The loosely bound and excess metal ions in the column were removed by thorough washing with the same buffer. The loading capacity of IDA gel with copper ions was found to be 26 ± 1 μmol/ml of gel and with nickel ions 21 ± 1 μmol/ml of gel, respectively, with sodium acetate–acetic acid buffer using frontal analysis. The metal loading capacity was found to remain the same even after repeated use of the gel. Each time before starting the experiment the column was recharged with the metal ions to ensure the same efficiency of the column.

3.2. Expression and isolation of roGH

E. coli cells harboring recombinant plasmid vector were induced with 1 m*M* IPTG after the A_{600} showed a value of 0.6–0.7. After induction, the expression of roGH was analyzed on SDS–PAGE gel and stained with Coomassie blue. A predominant band corresponding to the expected size of oGH (M_r 22 000) standard was observed in the total cell extract of induced *E. coli* cells (Fig. 1). A new protein band indicated by an arrow can be seen in

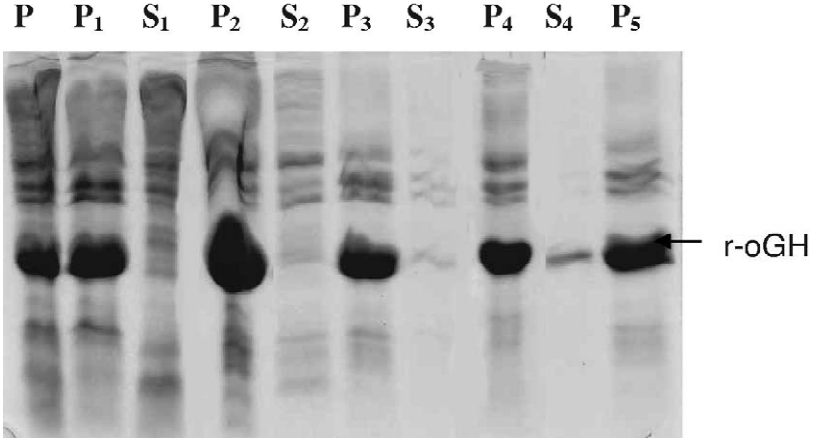

Fig. 2. Isolation of inclusion bodies from total cell lysates. Pellet P is crude cell lysates and pellet P_5 is the inclusion body of rOGH with very few contaminants. P_1, S_1, P_2, S_2 ... are the pellet and supernatant samples in the different washing steps of inclusion body isolation.

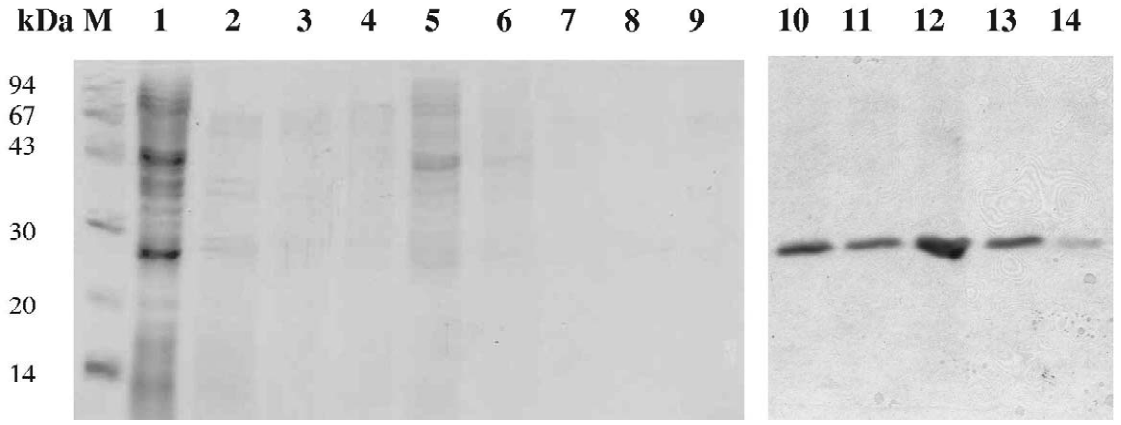

Fig. 3. Purification of roGH after inclusion body isolation using the Cu^{2+}–IDA column. *M*, molecular mass standard. Lanes 1, protein lysate loaded onto the column; 2, flow through; 3, washing; 4, washing with 10 m*M* imidazole; 5, first fraction of 50 m*M* imidazole elution; 6, second fraction of 50 m*M* imidazole elution; 7, third fraction of 50 m*M* imidazole elution; 8, first fraction of 100 m*M* imidazole elution; 9, second fraction of 100 m*M* imidazole elution; 10, first fraction of 200 m*M* imidazole elution; 11, second fraction of 200 m*M* imidazole elution; 12, first fraction of 400 m*M* imidazole elution; 13, second fraction of 400 m*M* imidazole elution; 14, first fraction of 800 m*M* imidazole elution.

Fig. 1 (lane 3), which does not appear in the uninduced cells (lane 1), indicating an efficient expression of recombinant ovine growth hormone in the form of insoluble and inactive inclusion bodies. The recombinant protein with the His tag runs slower on SDS gel and appears to have an M_r a few thousands higher than expected. This is in accordance with the retarded mobility on SDS–PAGE shown by the histidine-tagged fusion proteins [14,16,17].

Inclusion bodies were isolated from soluble proteins of *E. coli* cells by different solubilizing and washing steps. The initial cell pellet was first suspended in Tris buffer to isolate the easily soluble *E. coli* proteins then the pellet was suspended in a detergent-like 2% deoxycholate two times to isolate the hardly soluble native membrane proteins except the inclusion body. In each step, samples of pellet and supernatant were taken and after suspending in the loading buffer, total cellular proteins were separated by 12% SDS–PAGE. The final pellet (P_5) containing the roGH was found to have far less contaminant than the original cell pellet (Fig. 2).

3.3. Optimization of different parameters

The Cu^{2+}–IDA and Ni^{2+}–IDA columns were used for the purification of roGH and different parameters were optimized to compare the efficiency of the matrices. The solution and environment conditions were optimized to give the maximum yield and purity of recombinant ovine growth hormone. All experiments were done on a column volume of 5 ml. All the samples collected from the column were run on 12% SDS–PAGE gel and the total protein concentration was measured at 280 nm after TCA precipitation. Intensity of the roGH band in the SDS–PAGE gel was measured to calculate the amount of roGH in each sample collected. The elution profile of roGH was drawn and the yield and purity of recombinant ovine growth hormone were determined.

3.3.1. Effect of pH

The different functional groups present in proteins change their charge characteristic when the pH of the system varies. Protons affect the immobilized metal ions as well as the surface properties of the proteins in the sample [18]. Thus, when the pH is varied, the adsorption of different proteins onto IMA gel also varies. In our study, the solution buffer (Tris–HCl) with different pH was used to find the optimum pH for maximum binding of roGH onto the Cu^{2+}–IDA gel. No salt was used in the protein load for pH

optimization and the elution was done with a step gradient of imidazole. The roGH binding onto metal affinity columns was found to be maximum at pH 8.3.

3.3.2. Effect of imidazole on roGH elution

After loading the crude protein lysate onto the affinity column, few proteins other than roGH also bind to the column as some of the native *E. coli* proteins also have histidine at their surface. The binding of these proteins to the affinity matrix may also be due to other surface accessible amino acids like cysteine, tryptophan etc., or due to ionic interactions. The elution of bound protein was optimized to give the maximum purity of recombinant ovine growth hormone in the eluted fractions. Different concentrations of imidazole of different volumes were used and it was seen that bound proteins other than roGH eluted out with a low concentration of imidazole (50 m*M*) in the washing buffer (Tris, pH 8.3). The major part of the bound roGH was eluted with the 200 m*M* and 400 m*M* imidazole concentrations only (Fig. 3).

3.3.3. Effect of salt concentration

The influence of the ionic strength on the adsorption of proteins onto Cu^{2+}–IDA and Ni^{2+}–IDA gels was investigated. The increase in the protein adsorption with increasing ionic strength indicates the suppression of electrostatic interactions and involvement of surface histidine towards protein adsorption [19,20]. Different concentrations of salt (NaCl) like 0.5 *M*, 1 *M* and 2 *M* were used to study the effect of salt concentration. In the presence of very high salt concentration (>2 *M* NaCl) the protein becomes aggregated and hence much higher concentrations of salt were not used for the study.

A comparative study of yield and the purity of roGH at different salt concentrations for both Cu^{2+}–IDA and Ni^{2+}–IDA columns is shown in Table 1. In the experiments using protein load with no salt concentration, the yield and the purity of roGH were found to be very low for both columns due to nonspecific binding. At 2 *M* salt concentration the eluted fractions were found to have very few contaminating proteins resulting in a high yield and purity of roGH for both columns.

The binding of proteins to the matrix through ionic interactions was suppressed by increasing the salt concentration, which helped in eliminating many contaminating bands from elutes. The yield and the purity of roGH were found to be maximum at 2 *M* salt concentration in the protein load for the Ni^{2+}–IDA column. This may be because two histidine residues are required for protein binding with Ni^{2+} ions while Cu^{2+} ions are able to recognize even a single histidine residue.

3.3.4. Effect of flow rate

The flow-rate influences the binding of roGH onto the affinity columns as shown in Table 2. Proper binding of the protein to the metal ions requires sufficient equilibration time. At comparatively high flow-rate the protein load does not get enough equilibration time for binding and also there is a high pressure of the solution buffer across the column.

Table 1
Comparative study of yield and purity at different salt concentrations. Set conditions: pH 8.3; column flow-rate: loading, 0.6 ml/min; washing, 1.2 ml/min

Salt concentration	Yield of roGH (%)	Purity of roGH (%)
Cu^{2+}–IDA column		
(1) No salt	68.0	65.5
(2) 0.5 *M* NaCl	70.1	76.3
(3) 1.0 *M* NaCl	74.3	85.7
(4) 2.0 *M* NaCl	79.4	91.8
Ni^{2+}–IDA column		
(1) No salt	70.3	68.9
(2) 0.5 *M* NaCl	76.5	71.8
(3) 1.0 *M* NaCl	77.4	89.1
(4) 2.0 *M* NaCl	83.0	92.5

Table 2
Comparative study of yield and purity at different flow-rates. Set conditions: pH 8.3; 2 *M* NaCl in protein load, Cu^{2+}–IDA column (volume=5.0 ml)

Flow rate (ml/min)	Yield of roGH (%)	Purity of roGH (%)
(1) Loading 0.3 Elution 0.6	80.1	90.5
(2) Loading 0.6 Elution 1.2	79.4	91.8
(3) Loading 1.0 Elution 2.0	68.6	88.4

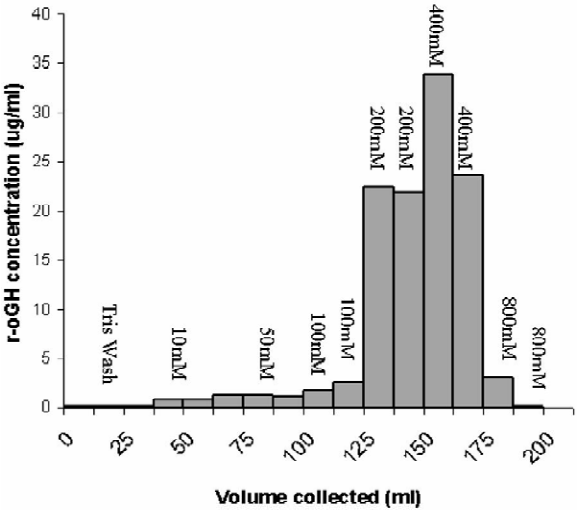

Fig. 4. Elution profile of roGH with different concentrations of imidazole from Cu^{2+}–IDA column using inclusion bodies as protein load.

Therefore, the yield of the roGH was found to be comparatively low due to loose binding of the protein. At comparatively moderate flow rate the proteins are allowed to bind strongly with the metal affinity column and a lower amount of roGH was lost in the washing step resulting in a good yield. The optimum flow-rate was found to be 0.5–0.6 ml/min for protein loading and 1.0–1.2 ml/min for protein elution in the IMA gel column used for the study.

3.4. Purification of roGH from inclusion bodies using the Cu^{2+}–IDA column

Although the yield of recombinant ovine growth hormone in the form of inclusion bodies was more than 85% after the inclusion body isolation steps, it contained some other native proteins of *E. coli*, which required metal ion affinity chromatography to get recombinant protein with high purity levels. Firstly, the crude cell lysates were processed for inclusion body isolation using different washing steps. Then, the cell pellet containing inclusion bodies of roGH (pellet P_5, Fig. 2) was solubilized completely using 8 *M* urea (prepared in 50 m*M* Tris buffer, pH 8.3) and loaded onto the Cu^{2+}–IDA column. The eluted samples with 200 m*M* concentration of imidazole and above contained very few contaminants as shown in the SDS–PAGE gel (Fig. 3). The overall yield and purity of roGH were calculated and found to be 73.5% and 94.3%, respectively, from the elution fractions (Fig. 4).

3.5. Single-step purification of roGH from crude cell lysate using the Ni^{2+}–IDA column

Isolation of inclusion bodies is the first step of purification before applying metal ion affinity chro-

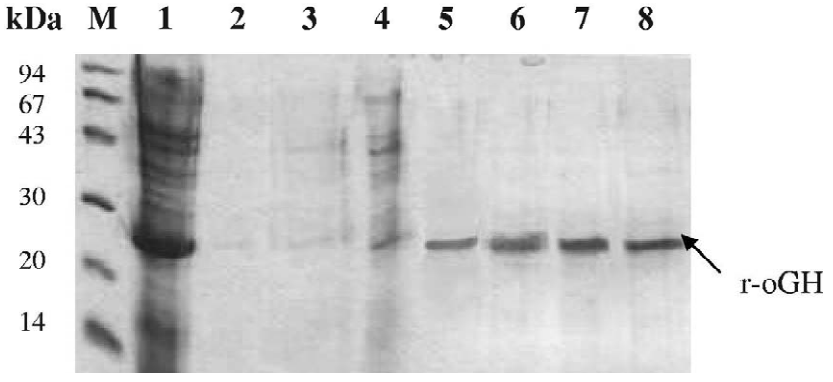

Fig. 5. Binding of roGH onto Ni^{2+}–IDA column with 2.0 *M* NaCl in the protein load. *M*, molecular mass standard. Lanes 1, protein lysate loaded onto the column; 2, flow through; 3, washing; 4, washing with 50 m*M* imidazole; 5, elution with 100 m*M* imidazole; 6, first fraction of 200 m*M* imidazole elution; 7, second fraction with 200 m*M* imidazole elution; 8, first fraction of 400 m*M* imidazole elution.

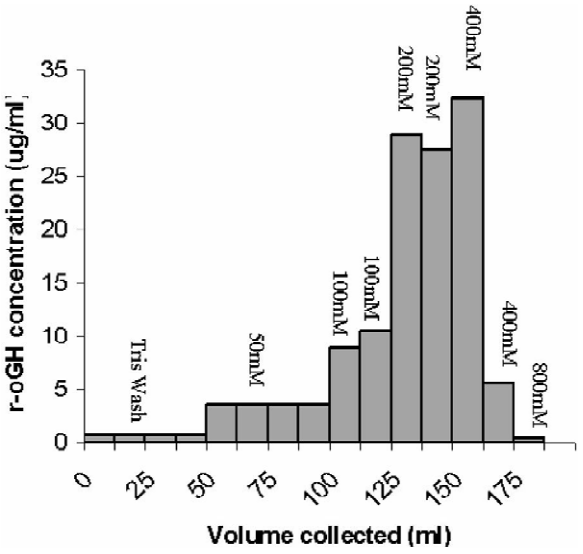

Fig. 6. Elution profile of roGH with different concentrations of imidazole from the Ni^{2+}–IDA column using crude cell lysates as protein load containing 2.0 *M* NaCl.

matography but the procedure is, however, cumbersome. So the emphasis was to avoid the inclusion body isolation step and directly load the crude cell lysate onto the affinity column so as to provide single-step purification. The total cell pellet was lysed by sonication. Unlike washing with detergents to purify the inclusion body pellet, the roGH was solubilized completely in 8 *M* urea (50 m*M* Tris buffer, pH 8.3). This crude protein lysate was directly loaded onto the metal ion affinity column under the optimized conditions and the bound protein was eluted with high concentrations of imidazole (Fig. 5). After the quantitative estimation of total protein and roGH in all sample fractions collected, the yield and purity of roGH was calculated and found to be 83% and 92.5%, respectively (Fig. 6).

An identical gel as in Fig. 5 was run on 12% SDS–PAGE gel and then transferred onto nitrocellulose membrane electrophoretically. The authenticity of the protein was verified by immunoblotting with rabbit anti-oGH antiserum. The roGH band (corresponds to about M_r 22 000) could be seen clearly on the nitrocellulose membrane. The other band (corresponds to about M_r 43 000) was of the dimer of recombinant protein (Fig. 7). The existence of such dimers of recombinant human growth hormone and bovine growth hormone has been reported recently [21,22]. Thus using IMAC, roGH could be purified in a single step from crude lysate.

4. Conclusions

Metal loading capacities of the self-prepared IDA-Sepharose gel were determined and found comparable to the commercially available gels. Also, the loading capacities remained high even after repetitive use of the gel. The maximum flow-rate for loading which could be used without affecting the yield was 0.6 ml/min. Use of a salt such as NaCl at a

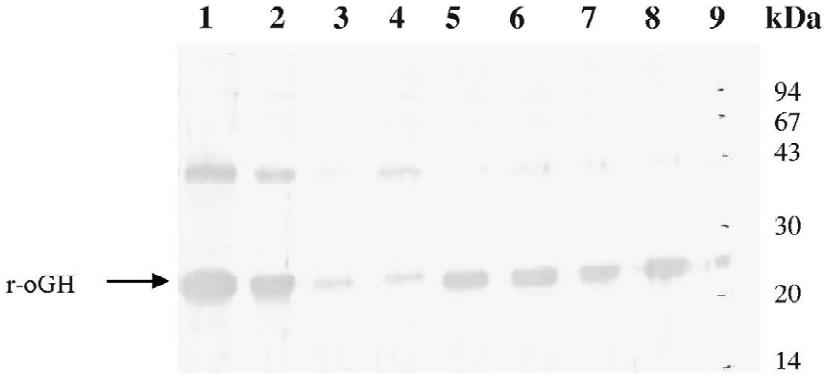

Fig. 7. Immunoblot analysis for the purification of roGH from crude protein lysates using the Ni^{2+}–IDA column. Lanes 1, protein lysate loaded onto the column; 2, flow through; 3, washing; 4, washing with 50 m*M* imidazole; 5, first fraction of 200 m*M* imidazole elution; 6, second fraction with 200 m*M* imidazole elution; 7, first fraction of 400 m*M* imidazole elution; 8, second fraction of 400 m*M* imidazole elution; 9, first fraction of 800 m*M* imidazole elution.

concentration of 2 *M* minimized non-specific interactions resulting in an improved recovery of roGH.

In our study, recombinant ovine growth hormone was purified from inclusion bodies with an overall yield of 73.5% and purity of 94.3% using Cu^{2+}–IDA affinity gel. Ni^{2+}–IDA affinity gel was used to purify roGH from crude cell lysate with an overall yield of 83% and purity of 92.5%, which is quite significant in comparison to the commercially available Ni^{2+}–NTA used to purify recombinant proteins from inclusion bodies [8]. It was concluded that Cu^{2+}–IDA and Ni^{2+}–IDA gels were successful in purifying roGH, containing a histidine tag at the amino-terminus, from inclusion bodies as well as from crude cell lysate.

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References

[1] J. Porath, J. Carlsson, J. Olsson, G. Belfrage, Nature 258 (1975) 598.
[2] M. Belew, T.T. Yip, L. Andersson, R. Ehrnstrom, Anal. Biochem. 164 (1987) 457.
[3] J. Porath, B. Olin, Biochemistry 22 (1983) 1621.
[4] M. Kashlev, E. Martin, A. Polyakov, K. Severino, V. Nikiforov, A. Goldfarb, Gene 130 (1993) 9.
[5] J.C. Juskevich, C.G. Guyer, Science 249 (1990) 875.
[6] J.M. Dellacha, M.A. Enero, J.A. Santome, A.C. Paladini, Eur. J. Biochem./FEBS 12 (1970) 289.
[7] C.H. Li, D. Gordon, J. Knorr, Ser. Biochem. Biophys. 156 (1973) 493.
[8] K.B.C. Appa Rao, L.C. Garg, A.K. Panda, S.M. Totey, Protein Expr. Purif. 11 (1997) 201.
[9] T.E. Adams, B. MacIntosh, M.R. Brandon, P. Wordsworth, N.K. Puri, Gene 122 (1992) 371.
[10] O.C. Wallis, M. Wallis, FEBS Lett. 2 (1989) 371.
[11] N.R. Adams, J.R. Briegel, K.A. Ward, J. Anim. Sci. 80 (2002) 2325.
[12] J.J. Winzerling, P. Berna, J. Porath, Methods 4 (1992) 4.
[13] E. Hochuli, H. Bobelli, A. Schacher, J. Chromatogr. 411 (1987) 177.
[14] R. Mukhija, P. Rupa, D. Pillai, L.C. Garg, Gene 165 (1995) 303.
[15] The QIAexpressionist, QIAGEN, Germany, 1992, p. 1.
[16] U.K. Laemmli, Nature 277 (1970) 680.
[17] M. Srivastava, J. Nayak, V. Mehrotra, R. Kaul, P. Sheela, S.K. Gupta, A.K. Panda, Process Biochem. 35 (1999) 451.
[18] S. Sharma, G.P. Agarwal, Anal. Biochem. 288 (2001) 126.
[19] W. Jiang, M.T.W. Hearn, Anal. Biochem. 242 (1996) 45.
[20] S. Sharma, G.P. Agarwal, J. Colloid Interface Sci. 243 (2001) 61.
[21] A.K. Patra, R. Mukhopadhyay, R. Mukhija, A. Krishnan, L.C. Garg, A.K. Panda, Protein Expr. Purif. 18 (2000) 182.
[22] T.J. Thamann, Anal. Biochem. 265 (1998) 202.