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Divalent metal cation chelators enhance chromatographic separation of structurally similar macromolecules: separation of human growth hormone isoforms

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Abstract

Human GH isoforms were separated by anion-exchange chromatography using a linear NaCl gradient in the presence and absence of EDTA and EGTA. SDS–PAGE showed that glycosylated 24-kDa hGH did not appreciably separate from other hGH variants in the absence of metal chelators. However, in the presence of metal chelators, glycosylated 24-kDa hGH separated from the bulk of the hGH isoforms. Human GH isoforms were also separated by size-exclusion chromatography in the presence and absence of metal chelators. Glycosylated 24-kDa hGH eluted with the bulk of the hGH isoforms in both separations. The inclusion of metal chelators in chromatographic buffers to alter the charge and/or size of proteins by stripping their metals may be a generally useful strategy in their fractionation.

Keywords: Human growth hormone; Metal chelators

1. Introduction

A collection of structurally and functionally similar proteins taken together constitute what is known as hGH [1–9]. There are two hGH genes, hGH-N and hGH-V, that code for hormones with different amino acid sequences. Structural variants of hGH are also produced from alternative splicing of hGH mRNAs. Post-translational modifications such as deamidation, phosphorylation, glycosylation, acetylation, aggregation and enzymatic cleavage can also contribute to the structural heterogeneity of hGH. The identification of hGH variants raises questions about the contribution of each isoform in the regulation of the many physiological actions of hGH. A given isoform of hGH may possess biological activities that differ from those of other hGH isoforms.

Reports have shown that some isoforms of hGH have enhanced biological activities and others are completely bioinactive or are limited in the range of metabolic, anatomic and physiological processes that they can regulate [1–8].

Although many of the isoforms of hGH have been identified, they have not all been isolated and characterized. Isoforms of hGH are proteins of molecular masses of 5, 17, 20, 22, 24, 36 and 45 kDa which are also heterogeneous in charge as deter-

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mined by Western blots of pituitary extracts separated by one-dimensional SDS–PAGE and two-dimensional isoelectric focusing SDS–PAGE [7–9]. The most abundant isoform of pituitary hGH is a 22-kDa hGH-N gene product. A glycosylated 24-kDa hGH was recently identified and isolated in a denatured state by preparative SDS–PAGE [9]. However, to determine the biological activity of glycosylated 24-kDa hGH, isolation of the native protein from other isoforms is required.

Chromatographic separation of individual hGH isoforms has been a difficult task, as evidenced by the unfinished isolation of each variant. The structural similarities among the members of this family make their separation difficult. The differences in their physicochemical properties are too slight to be easily separated by the chromatographic methods currently available. However, altering the physicochemical properties of the hGH isoforms may provide a way to separate them.

In this study, metal chelators were incorporated into the chromatographic buffers to strip hGH-associated divalent metal cations. If divalent metal cations are bound to the hGH isoforms, then stripping them off of the proteins will alter their physicochemical properties. Specifically, the net molecular charge on a particular hGH isoform will change after stripping bound divalent cations. Similarly, the net molecular size of a particular hGH isoform will change after stripping bound divalent cations if the cations serve as a bridge that holds subunits of an oligomer together. Isoforms of hGH were incubated with divalent metal cation chelators. The effect of this treatment on the charge and size of hGH isoforms was assessed by comparing the separation of glycosylated 24-kDa hGH from other hGH variants in both anion-exchange chromatography and size-exclusion chromatography.

2. Experimental

2.1. Anion-exchange chromatography

A 30×1-cm Bio-Rad Econo-column was packed to a height of 20 cm with anion exchange adsorbent (DEAE-TSK Toyopearl SP-650 M, Supelco, Bellefonte, PA, USA). The column was attached to a medium pressure liquid chromatography system (Bio-Logic Workstation, Bio-Rad, Hercules, CA, USA) and separations carried out at a flow-rate of 1.0 ml/min at 25°C.

The growth hormone source for these experiments was partially purified human pituitary extract that had been fractionated by Sephadex G-100 in ammonium acetate followed by Sephadex G-100 chromatography in acetic acid [9]. In the control fractionation, 1.0 mg of partially purified human pituitary extract containing hGH isoforms was solubilized in 1.0 ml of loading buffer A (12.5 m*M* Bis–Tris at pH 7.5). After applying the extract to the anion-exchange column that was previously equilibrated with ten column volumes of loading buffer A, unbound proteins were washed out with loading buffer A. The bound proteins were eluted with an increasing salt gradient of NaCl in loading buffer A to a final concentration of 0.25 *M* NaCl. During the fractionation, samples (3.0 ml) were collected using a Bio-Rad fraction collector (model 2128). The protein elution profile was monitored by light absorbance at 280 nm. The column fractions were analysed by SDS–PAGE (13.5% T, 2.7% C_{Bis}) by the method of Laemmli [10]. A silver staining technique using potassium permanganate was used for visualization of the proteins in the polyacrylamide gels [11]. The molecular masses of the markers for SDS–polyacrylamide gels (Dalton Mark VII-L, Sigma) were 14 200 (α-lactalbumin), 20 100 (trypsin inhibitor), 24 000 (trypsinogen), 29 000 (carbonic anhydrase), 36 000 (glyceraldehyde-3-phosphate dehydrogenase), 45 000 (egg albumin), and 66 000 (bovine albumin).

In the experimental fractionation, EDTA and EGTA were incorporated into the chromatographic buffers. Partially purified human pituitary extract containing hGH isoforms (1 mg) was solubilized in 1 ml of loading buffer A containing 0.5 m*M* EGTA and 0.5 m*M* EDTA. After applying the extract to the anion-exchange column previously equilibrated with 10 column volumes of loading buffer A containing divalent metal cation chelators, unbound proteins were washed out with additional buffer. The bound proteins were eluted with an increasing salt gradient of NaCl in loading buffer A containing divalent metal cation chelators to a final concentration of 0.25 *M* NaCl. The protein elution profile was monitored

by light absorbance at 280 nm. The column fractions were analysed by SDS–PAGE and silver staining [10,11].

2.2. Western blot analysis

Selected fractions from the DEAE anion exchange fractionations, with and without chelator, were separated by SDS–PAGE on a 13.5% T, 2.7% C_{Bis} SDS–polyacrylamide gel [10]. The resolved proteins were blotted onto nitrocellulose membranes according to Towbin [12]. The blotted nitrocellulose membranes were probed with a 1:1000 dilution of rabbit anti-hGH primary antibody (NIDDK-anti-synth hGH-IC-4, developed in rabbits, AFP-1613102481), in blocker, for 16 h. The Western blot was visualized by using a biotinylated goat anti-rabbit secondary antibody in conjunction and an ABC alkaline phosphatase visualization kit (Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer's instructions.

2.3. Size-exclusion chromatography

In the control fractionation, proteins from the human pituitary extract were separated using a HiPrep Sephacryl S-100 High Resolution size-exclusion column (Pharmacia Biotech, Torrance, CA, USA), which contained 47 μm beads. The column had an inner diameter of 26 mm and gel bed height of 60 cm. The column was attached to a Bio-Logic Workstation. Chromatographic separation was carried out at 25°C. Two milligrams of partially purified human pituitary extract containing hGH isoforms were solubilized in 2.0 ml of loading buffer B (50 m*M* NaH_2PO_4, 150 m*M* NaCl, pH 7.0) and the extract was then applied. The proteins were chromatographed with a buffer flow-rate of 1.3 ml/min and 3.0-ml fractions were collected. The proteins in the column fractions were analysed by SDS–PAGE and silver staining [10,11]. The size-exclusion column was calibrated with proteins from the Mol-Ranger molecular mass marker kit (Pierce) with molecular masses of 12 500 (cytochrome C), 25 000 (chymotrypsinogen A), 45 000 (hen egg albumin), and 67 000 (bovine serum albumin).

In the experimental fractionation, EDTA and EGTA were incorporated into the chromatographic buffer. Two milligrams of partially purified human pituitary extract containing hGH isoforms were solubilized in 2.0 ml of loading buffer B containing 1.0 m*M* EDTA and 1.0 m*M* EGTA. The size-exclusion column was equilibrated and the extract was then applied. The proteins were chromatographed with a buffer flow-rate of 1.3 ml/min and 3.0 ml fractions were collected. The proteins in the column fractions were analysed by SDS–PAGE and silver staining [10,11].

3. Results

The chromatogram of the anion-exchange fractionation of hGH isoforms in the absence of divalent metal cation chelators is shown in Fig. 1. The absorbance profile in the top panel shows one main absorbance maxima centered at fraction 39 with a few minor peaks and shoulders before and after the main absorbance peak. The presence of one main absorbance peak indicates a low degree of resolution of hGH isoforms. SDS–PAGE analysis of the chromatographic fractions is shown in the bottom panel of Fig. 1. Human GH isoforms were observed in fractions 37–48. The 24-kDa hGH variant was prominent in fractions 43–45; however, slight traces were observed in fractions 42 and 46–48. The stained polyacrylamide gels demonstrate that the glycosylated 24-kDa hGH isoform did not significantly separate from the other hGH isoforms. The insert in the chromatogram of Fig. 1 is a Western blot of DEAE fractions that was probed with an anti-hGH primary antibody. The fractions were selected from different areas of the chromatographic separation. Lane A is representative of fractions 35 and 36. Lane B is representative of fractions 39–41. Lane C is representative of fractions 42–47. Lane D is representative of fractions 49–53. Lane E is representative of fractions 54–58. Lane F is representative of fractions 59–62. The Western blot indicates that the 22- and 24-kDa proteins in the fractions from different parts of the chromatogram are hGH isoforms.

The chromatogram of the anion-exchange fractionation of hGH isoforms in the presence of divalent metal cation chelators is shown in Fig. 2. The absorbance profile in the top panel displays two

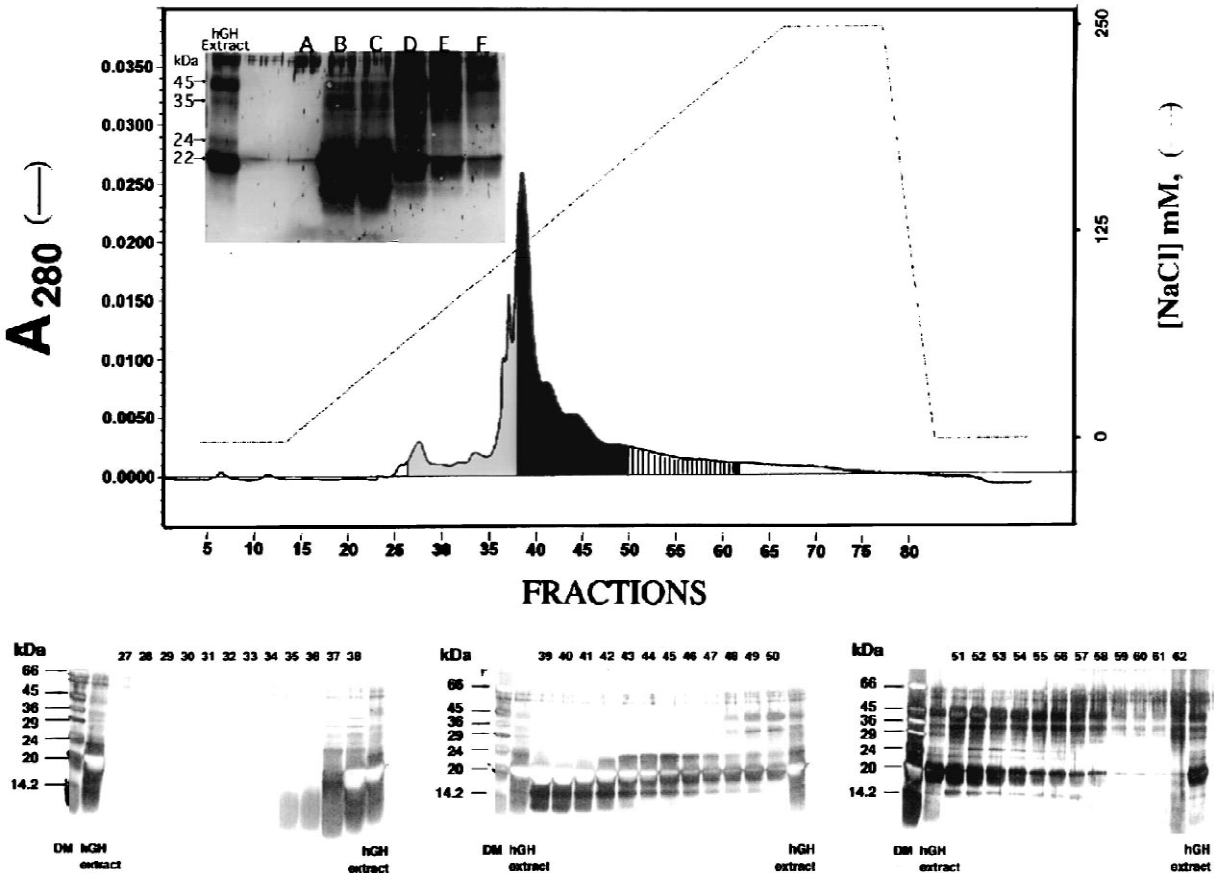

Fig. 1. Separation of hGH isoforms by anion-exchange chromatography in the absence of divalent metal cation chelators. The top panel shows the absorbance profile for the anion-exchange fractionation of hGH isoforms in the absence of metal chelators. Areas of the chromatographic profile shaded in grey, black or with vertical lines designate fractions that were analysed by SDS–PAGE. The bottom panel shows the silver-stained analytical SDS polyacrylamide gels (13.5% T, 2.7% C_{Bis}) of chromatographic fractions. Polyacrylamide gels containing fractions 27–38, 39–50 and 51–62 represent regions of the chromatographic profile shaded in grey, black or with vertical lines, respectively. The insert is a Western blot, probed with an anti-hGH primay antibody, of samples fractionated on the DEAE column. The fractions were selected from different areas of the chromatographic separation. Lane A is representative of fractions 35–36. B is representative of fractions 39–41. Lane C is representative of fractions 42–47. Lane D is representative of fractions 49–53. Lane E is representative of fractions 54–58. Lane F is representative of fractions 59–62.

major maxima at approximately fractions number 30 and 34, respectively. In addition, there are five prominent absorption maxima occurring before the major peaks and two absorption shoulders occurring after the major peaks that display extensive tailing as the salt gradient increases. Chromatographic fractions of the experimental anion-exchange fractionation were analysed by SDS–PAGE as shown in the bottom panel of Fig. 2. Human GH isoforms were observed in fractions 27–48. The glycosylated 24-kDa hGH isoform eluted from the anion exchange column in fractions 38–46 which correspond to fractions where NaCl concentrations range from 105 to 148 m*M*. The fractions containing glycosylated 24-kDa hGH were significantly separated from the bulk of the other hGH isoforms. The insert in the chromatogram of Fig. 2 is a Western blot of DEAE fractions that was probed with an anti-hGH primary antibody. The fractions were selected from different areas of the chromatographic separation. Lane A is representative of fractions 27–30. Lane B is representative of fractions 31–33. Lane C is representa-

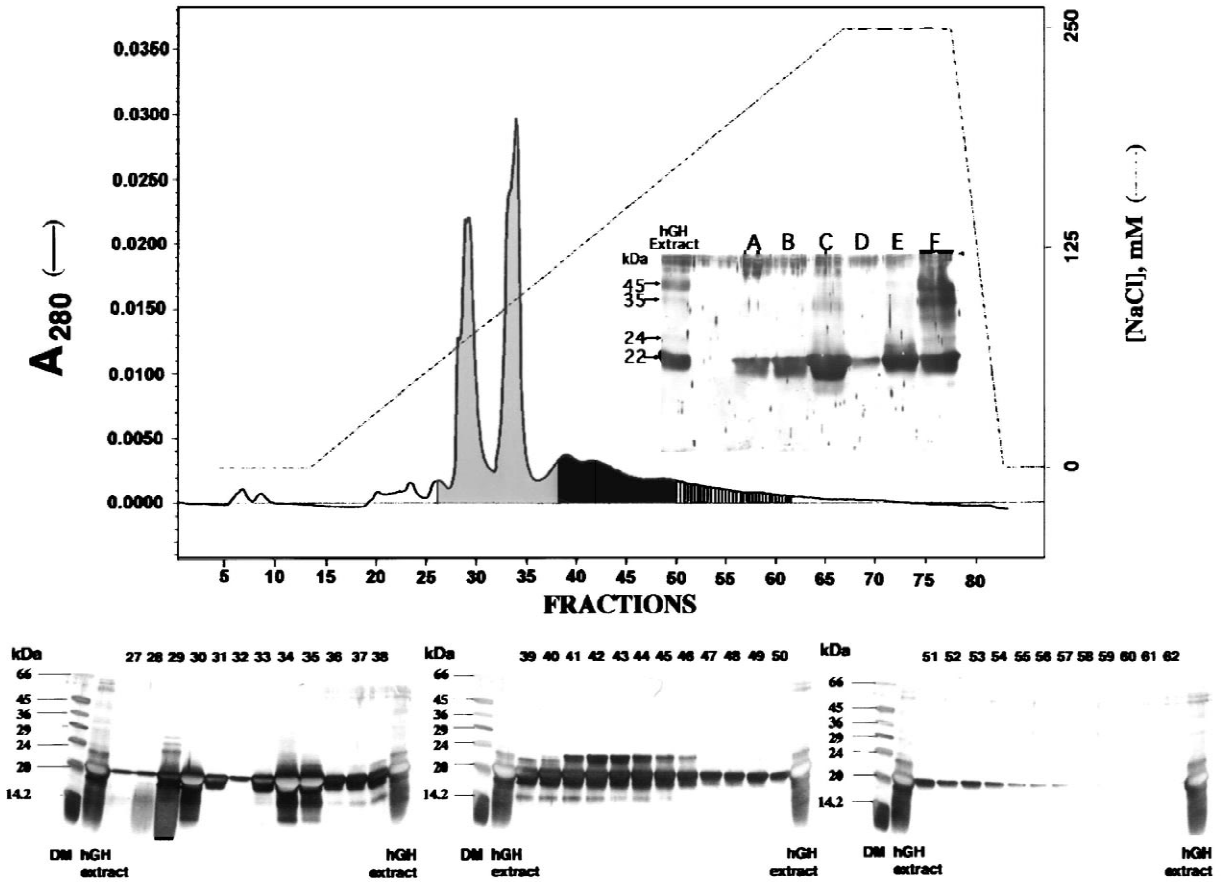

Fig. 2. Separation of hGH Isoforms by anion-exchange chromatography in the presence of divalent metal cation chelators. The top panel shows the absorbance profile for the anion-exchange fractionation of hGH isoforms in the presence of metal chelators. Areas of the chromatographic profile shaded in grey, black or with vertical lines designate fractions that were analysed by SDS–PAGE. The bottom panel shows the silver stained analytical SDS polyacrylamide gels (13.5% T, 2.7% C_{Bis}) of chromatographic fractions. Polyacrylamide gels containing fractions 27–38, 39–50 and 51–62 represent regions of the chromatographic profile shaded in grey, black or with vertical lines, respectively. The insert is a Western blot of samples fractionated on the DEAE column that was probed with a rabbit anti-hGH primary antibody. The fractions were selected from different areas of the chromatographic separation. Lane A is representative of fractions 27–30. Lane B is representative of fractions 31–33. Lane C is representative of fractions 34 and 35. Lane D is representative of fractions 36–38. Lane E is representative of fractions 41–46. Lane F is representative of fractions 48–53.

tive of fractions 34 and 35. Lane D is representative of fractions 36–38. Lane E is representative of fractions 41–46. Lane F is representative of fractions 48–53. The Western blot indicates that the 22- and 24-kDa proteins in the fractions from different parts of the chromatogram are hGH isoforms.

The chromatogram displaying the size-exclusion fractionation of hGH isoforms in the absence of divalent metal cation chelators is shown in Fig. 3. The absorbance profile in the top panel exhibits one minor absorption maximum at fraction 45 and a major absorbance maxima at fraction 55 followed by small absorption shoulders. Chromatographic fractions were analysed by SDS–PAGE as shown in the bottom panel of Fig. 3. The stained polyacrylamide gels demonstrate that glycosylated 24-kDa hGH did not significantly separate from the other hGH variants and was localized in the absorbance peak centered at fraction 55.

The chromatogram displaying the size-exclusion fractionation of hGH isoforms in the presence of metal chelators is shown in Fig. 4. The absorbance

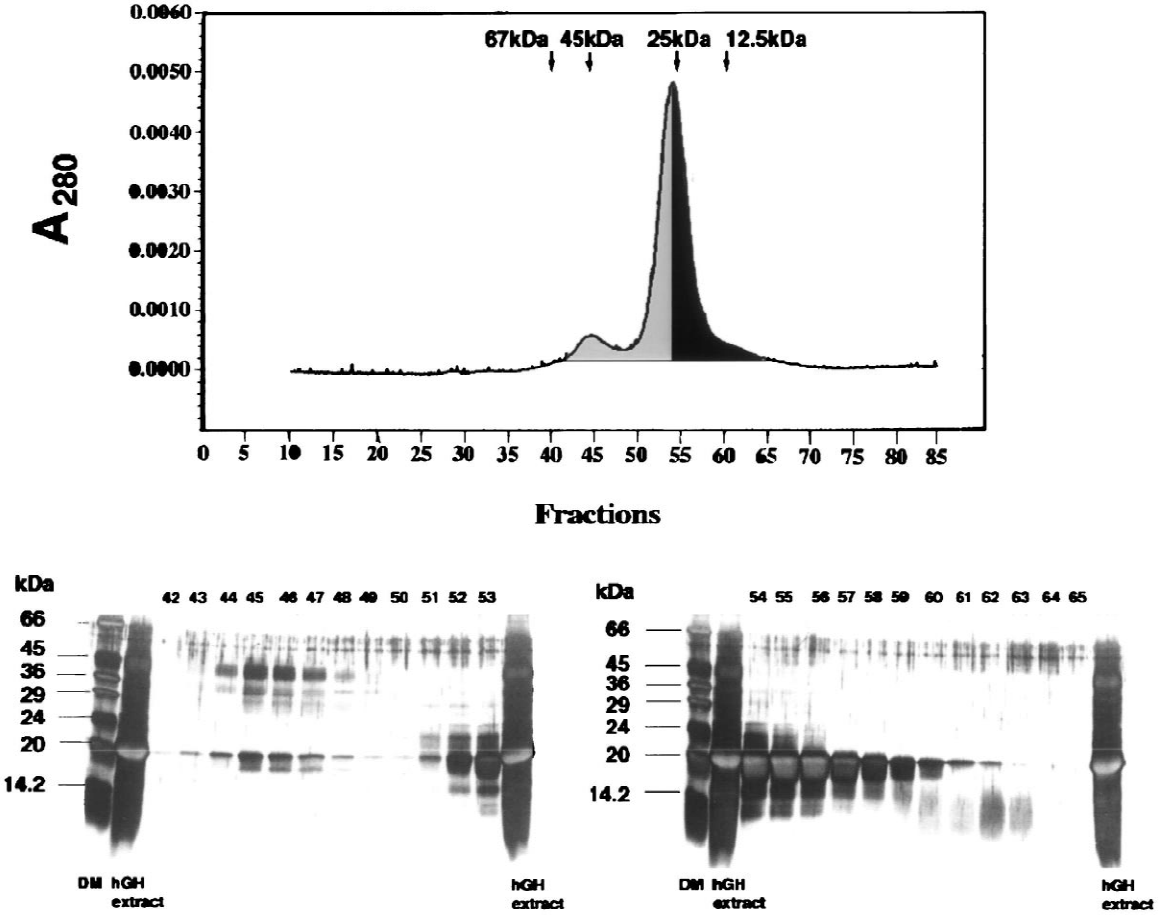

Fig. 3. Separation of hGH isoforms by size-exclusion chromatography in the absence of divalent metal cation chelators. The top panel shows the absorbance profile for the size-exclusion fractionation of hGH isoforms in the absence of metal chelators. Areas of the chromatographic profile shaded in grey and black designate fractions that were analysed by SDS–PAGE. The bottom panel shows the silver stained analytical SDS polyacrylamide gels (13.5% T, 2.7% C_{Bis}) of chromatographic fractions. Polyacrylamide gels containing fractions 42–53 and 54–65 represent regions of the chromatographic profile shaded in grey and black, respectively.

profile for separation of pituitary proteins in the presence of metal chelators matches the absorbance profile of pituitary proteins in the absence of metal chelators as shown in the top panel of Fig. 4. The minor absorbance peaks and major absorbance peaks of the two profiles, corresponding to fractions 45 and 55, respectively, coincide, as do the small absorption shoulders. Chromatographic fractions were analysed by SDS–PAGE as shown in the bottom panel of Fig. 4. The stained polyacrylamide gels demonstrate that glycosylated 24-kDa hGH did not significantly separate from the other hGH variants and that it was localized in the absorbance peak centered at fraction 55, as in the fractionation in the absence of divalent metal cation chelators.

4. Discussion

Proteins can bind divalent metal cations such as Fe^{2+}, Ca^{2+} and Mg^{2+} through their aspartic acid and glutamic acid residues. The macromolecular charge of an apoprotein will therefore vary, depending on number of divalent metal cations that are bound to it. A divalent metal cation can also serve as a bridge in

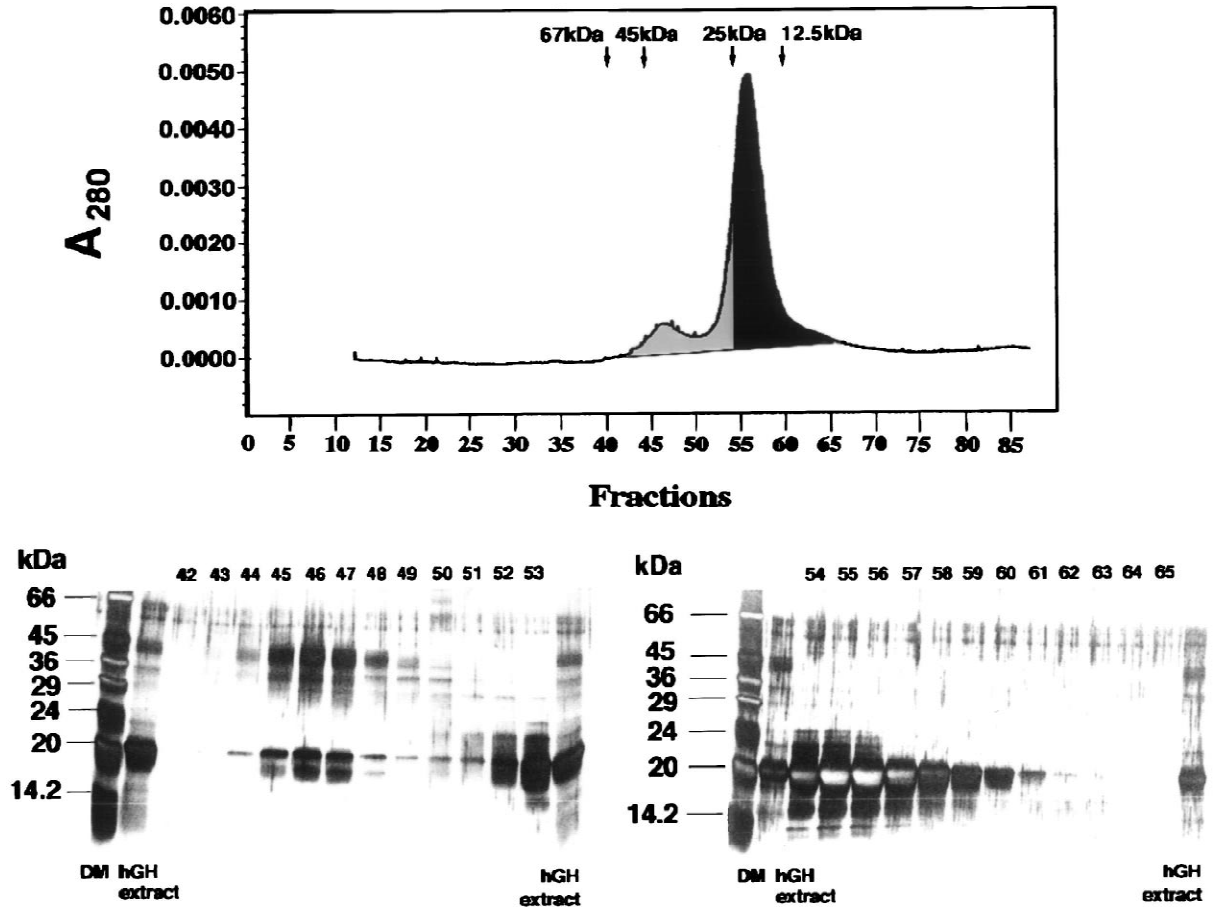

Fig. 4. Separation of hGH isoforms by size-exclusion chromatography in the presence of metal chelators. The top panel shows the absorbance profile for the size exclusion fractionation of hGH isoforms in the presence of metal chelators. Areas of the chromatographic profile shaded in grey and black designate fractions that were analysed by SDS–PAGE. The bottom panel shows the silver stained analytical SDS polyacrylamide gels (13.5% T, 2.7% C_{Bis}) of chromatographic fractions. Polyacrylamide gels containing fractions 42–53 and 54–65 represent regions of the chromatographic profile shaded in grey and black, respectively.

the formation of protein oligomers, and thereby alter a protein's size and shape.

EGTA and EDTA are chelating agents used in biochemical research to bind divalent metal cations. These metal chelators possess four carboxyl groups which are responsible for binding divalent metal cations. The chelators can therefore compete with the aspartic acid and glutamic acid residues of proteins and strip the divalent metal cations from them. A protein stripped of its divalent cations will therefore have an altered net macromolecular charge and/or shape.

Ion-exchange chromatography and size-exclusion chromatography are widely used methods in protein purification that separate macromolecules according to differences in their charge and size, respectively [13–15]. The strength of the binding of the protein to the ion-exchange absorbent depends on the net charge of the protein as well as on the surface distribution of the charge. In weak anion-exchange chromatography using DEAE, the absorbent has diethylaminoethyl functional groups that are positively charged and interact with the aspartic acid and glutamic acid residues of the proteins. In size-exclusion chromatography, proteins are separated on the basis of differences in their sizes and shapes.

In a scenario where a protein is stripped of its divalent metal cations, a protein would have an altered net charge and consequently an altered affinity for a DEAE absorbent. Altering the charge of a protein in this manner provides a method of altering the affinity of proteins for an anion-exchanger and could enhance the separation of similarly charged proteins or isoforms of a given protein family. In our studies, the desorption of hGH isoforms from the DEAE absorbent, in the presence and absence of divalent metal cation chelators, differed. These data infer that some hGH isoforms bind divalent metal cations which, once stripped from the isoforms, leave them with an altered net charge. This concept is supported by other work which shows that hGH binds divalent metal cations [16–24].

In a similar scenario, a protein oligomer or aggregate that is stripped of its divalent metal cations would have an altered Stokes' radius and consequently an increased elution volume in a size-exclusion separation, if the integrity of the aggregate, or a portion thereof, is dependent on divalent metal cations serving as bridges between subunits or aggregates. In our studies, the separation of hGH isoforms by size-exclusion chromatography, in the presence and absence of metal chelators, did not differ. The absence of significant differences in the separation of hGH isoforms by size-exclusion chromatography, in the presence and absence of divalent metal cation chelators, argues against the idea that divalent metal cations form bridges between hGH isoforms to form protein aggregates. If hGH isoforms used metal cations as bridges in the formation of hGH oligomers, then stripping the metal cations would have altered the sizes of the oligomers and hence increased their elution volume.

An understanding of the physiological role of hGH will remain incomplete until purified preparations of each of the isoforms are available. In these experiments, the separation of glycosylated 24-kDa hGH from other hGH isoforms was enhanced by stripping divalent metal cations from the proteins and then separating the isoforms by anion-exchange chromatography. This strategy will aid in the eventual chromatographic isolation of this hGH isoform.

We have shown that the addition of metal chelators to chromatographic buffers can enhance the separation of structurally similar proteins. Stripping divalent metals from proteins to alter their charge and/or size is a simple and effective strategy that can easily be applied to the separation of other biological macromolecules by chromatographic methods that are sensitive to alterations in charge and/or shape of native proteins.

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