Simplified purification of equine chorionic gonadotropin (eCG)–an example of the use of magnetic microsorbents for the isolation of glycoproteins from serum

Christine Müller, Kerstin Wagner, Katrin Frankenfeld, Matthias Franzreb

Section: Bioprocessing and Biological Engineering

Simplified purification of equine chorionic gonadotropin (eCG) – an example of the use of magnetic microsorbents for the isolation of glycoproteins from serum

Christine Müller, Kerstin Wagner, Katrin Frankenfeld, Matthias Franzreb*

C. Müller, M. Franzreb

Karlsruhe Institute of Technology, Institute of Functional Interfaces, Hermann von Helmholtz Platz 1, 76344 Eggenstein Leopoldshafen, Germany

e-mail: Matthias.franzreb@kit.edu

Phone: +49 7247/823595

Fax: +49 7247/823478

K.Wagner

INNOVENT e. V., Biomaterials Department, Prüssingstrasse 27b, 07745 Jena, Germany

K. Frankenfeld

fzmb GmbH Institute for Medical Technology and Biotechnology, Geranienweg 7, 99947 Bad Langensalza, Germany

Abstract

Classical purification of the glycoprotein equine chorionic gonadotropin (eCG) from serum includes pH fractionation with metaphosphoric acid, two ethanol precipitation steps as well as dialysis followed by fixed-bed chromatography. A simplified process requiring only 1/3 of the solvent and improving the yield from 53 to 65% has been developed. The process comprises an ultra-/diafiltration step after the first ethanol precipitation, directly followed by an

adsorption/desorption procedure based on magnetic microadsorbents with N,N-diethyl-ammonium functionalization. The process reaches an overall purification factor of eCG of more than 1800 and an average product activity of 1300 IU_{ELISA}/mg. After adapting the parameters of the fractionation and the type of magnetic microadsorbents, the new concept is likely to be transferable to other serum proteins.

Keywords

Equine chorionic gonadotropin, *glycoprotein*, *magnetic adsorbent*, *serum*,

Introduction

Equine chorionic gonadotropin (eCG) is a 60 kDa glycoprotein hormone composed of two non-covalently bound subunits. The total carbohydrate content of eCG is 45%, of which 10% correspond to sialic acid. This contributes to the very low isoelectric point (pI) of 1.8 of the hormone, a unique property which can be used for purification. The average molecular weights of the α and β subunits are 16.96 kDa and 43.72 kDa, respectively (Christakos & Bahl 1979). Equine chorionic gonadotropin can be detected in mare sera between days 40 and 130 of pregnancy (Aggarwal et al. 1980; Cole & Hart 1930; Murphy & Martinuk 1991). Early in this period it attains its highest levels of about 60-80 IU/ml in a bioassay based on rats (Allen 1969). eCG is applied in veterinary medicine as well as in animal breeding. The classical purification scheme for eCG can be divided into two main steps. First, the generation of an intermediate product by precipitation and filtration steps removing the main contaminants like albumin. And second, the generation of a final extract by chromatographic steps. To obtain the intermediate, fractionation with metaphosphoric acid and two-step ethanol precipitations with 50 and 75% (v/v) at 4°C (Aggarwal et al. 1980; Gospodarowicz & Papkoff 1967) are applied. Subsequently, the pellet is dissolved and desalted, resulting in a solution suitable for chromatography.

In this paper, we suggest a purification of eCG which differs in both sections. First, the initial generation of the intermediate is reduced by two steps decreasing the amount of ethanol necessary by 66% and omitting the corresponding solid-liquid separations. Second, the final purification of the resulting eCG intermediate is accomplished by the use of magnetic microsorbents instead of conventional chromatography. The possibility of isolating biomolecules directly from crude suspensions by using magnetic microsorbents has been known for some time in different areas of biotechnology. Small-scale bioseparation experiments applying magnetic microsorbents have been reported for e.g. whey proteins (Meyer et al. 2007; Heeboll-Nielsen et al. 2004), cell homogenates (Hubbuch et al. 2001), or plant extracts (Heeboll-Nielsen et al. 2004). In addition, a one-step enrichment of glycoproteins from serum, using magnetic beads with attached lectins as ligand was described recently for bioanalytical use by Loo et al. (2010). For the first time, however, we describe the integration of a magnetic separation step into a

fully scalable downstream processing scheme for the purification of a commercially used glycoprotein from serum.

Materials and methods

Extraction and purification of eCG

Blood was collected from native Haflinger mares between 50 and 100 days of gestation. After separation of erythrocytes and fibrin from the whole blood, the serum was stored at -20°C without additional preservatives. The serum had an average of 200 mu/ml (mouse units) determined by using immature female mice[1]. In order to reduce the amount of bioassays necessary for routine measurements, they were replaced by a commercial immunoassay (ELISA) for eCG from the company of DRG Instruments GmbH, Marburg, Germany. The sera used of nominal 200 mu/ml resulted in activities of the immunoassay between 39 and 57 IU_{ELISA}/ml. The reason for the variation can be twofold: First, the activity of the serum donated by a pregnant horse is monitored using bioassays on a weekly basis only. However, depending on the state of pregnancy and other factors a certain day to day variation exists. Second, bio- and immunoassays use different epitops of eCG. Therefore, an alteration of the molecule structure during treatment may influence one type of assay while the other shows no deviation. Nevertheless, in an extensive study Lecompte showed that both types of assays are in good correlation as long as the sialic acid content of the eCG is not changed and a suitable standard is used for the calibration (Lecompte et al. 1998).

To simulate the classical purification scheme, the serum was treated according to a process suggested by Gospodarowicz and Papkoff (1967). In the beginning a fractionation was carried out with batches of 500 ml or 1 l. In the first step the pH of the serum was adjusted to 3.0 by addition of a fresh solution of 0.5 M metaphosphoric acid, followed by centrifugation at 9000 g and the discarding of the resulting precipitate. The pH of the supernatant was then adjusted to 4.5 with 1 M NaOH before adding one volume of cold (-20°C) ethanol. After 2 hours at 4°C, the precipitate was removed by centrifugation and another volume of cold (-20°C) ethanol was added to the supernatant, giving a final ethanol fraction of 75% (v/v). The resulting precipitate was collected after 20 hours at 4°C, dialyzed or diafiltrated, and lyophilized.

In contrast to this, the simplified scheme for the production of an eCG intermediate was carried out as follows. After the precipitation with 0.5 M metaphosphoric acid and the first ethanol precipitation, the crude eCG obtained was concentrated and desalted directly by means of diafiltration, the second ethanol precipitation and following centrifugation step were skipped. The resulting product was not lyophilized, but directly used for further purification.

[1] A fixed correlation exists between the biological activities determined in mice or rats: 3.8 mu/ml = 1 IU/ml. Therefore the used serum corresponded to an average of 53 IU/ml.

In both cases final purification was done on the 20 ml scale using magnetic anion exchanger particles with N,N-diethyl-ammonium group (DEAP) as functional group. The DEAP-functionalized magnetic particles were synthesized by activation of carboxylated magnetic polyvinyl alcohol beads (M-PVA) with EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) followed by further reaction with N,N-diethyl-1,3-propane diamine. After functionalization the particles were thoroughly washed with water and stored at 4°C until use. Further details about the use of magnetic beads and the principle of ligand coupling using EDC chemistry can be found in Hermanson et al. (1992).

Before application the particle suspension was washed twice with 0.01 M sodium acetate buffer pH 4 as the binding buffer. For effective binding, the conductivity of the eCG intermediate must not exceed 0.8-1.2 mS/cm. 3-5 g magnetic particles/l were incubated for 20 min with the solutions of the eCG intermediate at pH 4. Subsequently, the supernatant was discarded and the particles were washed three times for 5 min with a 0.01 M sodium acetate buffer of pH 4. For elution, the particles were incubated twice in a 0.01 M phosphate buffer of pH 8 with 0.3 M NaCl for 20 min. During the incubation of the eCG intermediate or the elution step, the particles were stirred with a glass rod at intervals of 5 min. After the elution step, the particles were regenerated with a 1 M NaCl for 20 min. The described protein purification using magnetic beads equals the recommended scheme of the so-called High Gradient Magnetic Fishing, a unit operation combining rapid batch adsorption in a stirred tank followed by capture of the loaded beads by pumping the solution through a magnetic separator (Franzreb et al. 2006).

Process analyses

The identity and purity of the eCG were controlled by the following analytical methods:

Total protein: The protein content was determined by the method of Bradford using bovine serum albumin as the standard.

eCG concentration: eCG concentrations were measured with an ELISA kit for eCG (DRG Instruments GmbH, Marburg, Germany). Procedures were performed according to the manufacturer's protocol.

SDS-PAGE: Purity of the eCG after the final precipitation was analyzed by SDS-PAGE on a 12% Tris/glycine polyacrylamide gel. 12 µg with purified eCG per well, along with standard marker proteins and a commercial eCG (Calbiochem-Merck Biosciences, Darmstadt, Germany) with a biological activity of 10000 IU/ml. 70 IU of the commercial eCG were loaded per well. Each sample was denatured by boiling for 3 min at 95°C and cooled immediately prior to loading. Electrophoresis was carried out at 90 V for 10 min, followed by 120 V until tracking dye reached the end of the gel. Proteins in the gel were stained with Coomassie Brilliant Blue R250.

Western blot: For Western blot analyses, the proteins were also separated on 12% Tris/glycine polyacrylamide gel. The proteins obtained were transferred to a PVDF membrane which was incubated for 3 h at room temperature with 7.5% (v/v) skim milk in phosphate-buffered saline with 0.1% Tween (TBST buffer). Then, the blocking solution

was discarded and the membrane was washed with TBST buffer for 5 times, 5 min each. Afterwards, the diluted primary antibody in the blocking solution was incubated for 1 h at room temperature (dilution ratio 1:2000) (Acris Antibodies GmbH, Herford, Germany). The primary antibody was poured out and the membrane was washed with TBST for another 5 times. Then, the secondary antibody was added in the blocking solution (dilution ratio 1:5000) (Acris Antibodies GmbH, Herford, Germany) and incubated for 1 hour at room temperature. Afterwards, the membrane was washed with TBST for another 5 times. Finally, the horseradish peroxidase (HRP)-conjugated secondary antibody was detected by ECL reagent luminescence (Amersham Biosciences/GE Healthcare). Chemoluminescence was detected with a Lumi-Imager F1 (Boehringer, Mannheim, Germany).

MALDI-ToF MS analysis: The proteins of interest were cut out of the gel after staining with Coomassie and washed in distilled water for 10 min. This was removed and the gel slices were washed with 10 mM NH_4HCO_3 for 10 min and afterwards, destained with acetonitrile and 10 mM NH_4HCO_3 (1/1) for 10 min. The gel pieces were then reduced with 10 mM NH_4HCO_3, including 10 mM dithiothreitol (DTT) for 15 min at 60 °C. The DTT solution was allowed to cool, and then it was removed. The gel slices were then alkylated with 55 mM iodoacetamide for 15 min in the dark. This solution was removed and the gel pieces were washed alternately with pure NH_4HCO_3 and acetonitrile/ NH_4HCO_3solution for 10 min until the gel pieces were destained. The gel then was dried, and covered with trypsin solution. 20 µl NH_4HCO_3solution were added to prevent the gel from drying out and incubated overnight at room temperature. MALDI-ToF mass spectra were recorded by a 4800 Plus Analyzer time-of-flight mass spectrometer (Applied Biosystems, Carlsbad, USA) operated in the reflector mode. 2600 laser pulses were focused on the target. For measurement in the positive ion mode, 0.5 µl sample were mixed with 0.5 µl α-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in acetonitrile/trifluoroacetic acid, 1:1 v/v).

Results and discussion

Due to the high salt (conductivity: 10-13 mS/cm) and protein (60 mg/ml) content of serum, it is not possible to directly apply ion exchange adsorbents. Consequently, several precipitation steps are needed in order to reduce the foreign protein content and the conductivity. The isoelectric point of the main serum protein equine serum albumin is in the range of 4.65-4.9. Therefore, a first precipitation step with metaphosphoric acid (pH 3) was effective and removed 70-80 % of the total serum protein. The resulting supernatant was further purified by a first ethanol precipitation adjusting a fraction of 50% (v/v) and holding it for 2 h at 4°C. The classical purification scheme reported by Gospodarowicz and Papkoff (1967) uses a second ethanol precipitation at 75% (v/v) ethanol, in the course of which the eCG precipitates. Afterwards, the precipitate is dissolved in water and dialyzed. At a later stage of our investigations, we replaced dialysis by diafiltration, because the latter is less time-consuming and guarantees that conductivities below 1 mS/cm necessary for a purification applying ion exchangers are reached. As the last step of the production of the eCG intermediate, the desalted solution was lyophilized in order to reduce the

volume and provide for long-term storage. The eCG intermediate was stable in the frozen or lyophilized form, while it lost its activity within days when stored in solution in a refrigerator. For analysis, the lyophilizate was dissolved in an amount of water just large enough to reach a clear solution, the aim being to adjust a maximum concentration for the following chromatographic or batch adsorption steps.

While removing the majority of serum proteins and salts, the procedure described consumes large amounts of solvents and also results in substantial activity losses. Over a period of two years, the classical purification scheme of Gospodarowicz and Papkoff (1967) was repeated three times at 500 ml in order to have reliable data, on the basis of which the simplified method suggested could be judged. Table 1 shows a summary of the recovery rates and purification factors of the resulting intermediate product achieved.

Table 1

The eCG content after the ethanol precipitation steps was not determined due to the negative influence of ethanol on the ELISA. The resulting intermediate product contained less than 0.3% of the original total protein, giving an average purification factor of 246. However, the recovery rate was in the range of 53% only because of the multiple and partly harsh purification steps. The calculated concentration factor strongly depended on the amount of water needed to dissolve the lyophilizate, explaining the comparatively large standard deviation of this value. In addition, it must be mentioned that the eCG content of the serum itself showed large variations from batch to batch reaching from 39 to 57 IU_{ELISA}/ml. In relation to their mass, the lyophilizates had an activity of 130 IU_{ELISA}/mg, which, according to our experience, roughly corresponds to a bioactivity of around 400 IU/mg measured in rats. Considering the bioactivity of at least 1000 IU/mg required for commercial eCG products, the intermediate requires further purification.

It was the aim of our project to develop a purification process for eCG which requires fewer steps, is much faster and reaches higher recovery rates. It was therefore studied at which point of the classical purification scheme a direct ultra-/diafiltration could be used to produce an alternative intermediate eCG product suitable for further purification with magnetic microadsorbents. In first trials, we tested ultra-/diafiltration directly after the precipitation step with metaphosphoric acid. However, the filtration process was laborious and the resulting concentrate had a total protein content which prevented effective binding of the eCG onto the magnetic adsorbents (data not shown). As shown in Table 2, execution of ultra-/diafiltration after the first ethanol precipitation step was much more effective.

Table 2

About 0.25% of the original total protein remains in the UF/DF concentrate, comparable to the 0.3% in the case of the classical scheme for the production of the eCG intermediate. However, the avoidance of the second ethanol precipitation step resulted in a smaller activity loss and therefore in a higher purification factor and recovery.

A comparison of the first two lines of (Table 1 and Table 2) shows that, within the investigated range, the efficiency of the applied steps does not depend on the batch size. Experiments starting with 500 ml, 1667 ml serum showed similar losses of approx. 10% after the metaphosphoric precipitation.

Tables 1 and 2 show the sum parameters describing the overall performance of the purification schemes. In order to obtain a clearer impression of the similarities and differences of the intermediate products resulting from the two schemes, several more sophisticated analytical techniques were applied. Hereinafter, the intermediate products generated by the application of the classical scheme and the simplified scheme will be referred to as c-eCG-i, and s-eCG-i, respectively.

The protein patterns obtained by SDS-PAGE and coomassie staining of c-eCG-i, s-eCG-i, and commercial eCG are shown in Figure 1. The commercial product exhibits an astonishing plurality of protein bands, with the strongest band around 45 kDa. According to Christakos and Bahl (1979), the molecular weights of the α and β subunits of eCG are 16.96 kDa and 43.72 kDa, respectively. Hence, the most intense band correlates with the β-subunit, while the α-subunit seems to be shifted slightly to higher molecular weights. The eCG intermediates show closely related patterns.

Fig. 1

eCG is a highly glycosylated protein with a total carbohydrate content of 45% (González et al. 1998). Glycoproteins containing more than 10% carbohydrate bind less SDS per g than unglycosylated proteins (Moore et al. 1980). The result is a lower negative charge to mass ratio and a concomitant decrease in mobility in SDS gels and higher apparent molecular weights. This could explain the variance of the molecular weight of the α and β subunit.

In order to obtain a clearer idea, Western blotting was used to confirm the results of gel electrophoresis. A positive signal for the β subunit of eCG of approximately 45 kDa was detected (Fig. 2).

Fig. 2

To identify eCG completely in the SDS-PAGE of the intermediate eCG products produced, the protein bands for c-eCG-i and commercial eCG visible at the height of the corresponding molecular weight of the α and β subunits were cut out from the SDS-PAGE (Fig. 1). To control tryptic digest, bovine serum albumin (BSA) was used. Figures 3 and 4 show the spectra of the bands at approx. 45 and 20 kDa. For both molecular weights and, hence, for both subunits, the spectra of the commercial eCG and c-eCG-i showed nearly identical peptide masses. In the case of the 20 kDa band of the eCG intermediate, several peaks are distributed around the main peak at m/z 842, which indicates that there are several peptide variants which differ in their glycosilation. Besides the comparison with the commercial standard, the Mascot software was used to search the NCBInr database. However, no matching results were found, not even for the commercial product. This result was no surprise, because the spectra existing in the database consider peptides only, but not the carbohydrate residues of the protein.

Fig. 3

Fig. 4

To determine whether the simplified scheme for eCG intermediate production was suitable to produce a commercially valuable product with only one additional purification step, small-scale adsorption experiments applying magnetic anion exchange particles were conducted with the classical eCG intermediate as well as with the one resulting from the simplified scheme. Table 3 shows a comparison of the properties of the final eluates. The purifications starting from the classical and simplified eCG intermediates were repeated three times (two times 23 ml and once 500 ml scale) or three times (all 100 ml scale).

Table 3

The two experiments of classical eCG intermediate production on different scales show nearly identical yields (38% and 40%). The main differences in the experimental results are the total protein content and the corresponding purification factors. The experiment in the 500 ml scale was performed in an automated High-Gradient Magnetic Fishing set-up (Franzreb et al. 2006). In this case, the increase of the total protein remaining in the eluate probably results from the dead volume of the device and the fact that only three washing steps were performed. A reduction of the total protein in the eluate corresponding to an improvement of the purification factor could be achieved by further washing steps. However, in relation to the decisive parameter of the specific activity (IU/mg) of the lyophilisate, total protein contents below approx. 0.3 mg/ml play only a minor role, because the resulting mass of the lyophilisate is determined mainly by the salt included.

In two cases (Eluate c-eCG-i_{23ml} and Eluate s-eCG), the eluate finally was diafiltrated until a conductivity below 1 mS/cm, lyophilized, and weighed in order to check the specific activity. The eluates starting from c-eCG-i_{23ml} and s-eCG-i showed activities of 540 IU_{ELISA}/mg and 1300 IU_{ELISA}/mg, respectively. This shows that the specific activities finally achieved mainly depend on eCG concentration of the eluat. Obviously, purification with magnetic anion exchange adsorbents worked in both cases, but the higher recoveries of the simplified process for eCG intermediate generation eventually result in a product of higher specific activity.

Conclusions

A new concept for the purification of the hormone eCG from pregnant mare serum has been developed and compared to the classical purification process described by Gospodarowicz and Papkoff (1967). The new concept is based on a simplified pre-treatment of the serum applying two precipitation and one UF/DF steps, followed by purification applying anion exchangers in the form of magnetic microadsorbents. The new concept requires only 1/3 of the ethanol amount needed by the classical pretreatment and increases the average recovery from around 53 to 79%. In addition, specific binding to and elution from magnetic microadsorbents in a batch reactor, combined with magnetic separation, is a simple and fast process in comparison to

conventional fixed-bed chromatography. The produced intermediate and final eCG products were characterized by different analytical techniques, confirming the similarity to a commercial eCG product. The measured activity of 1300 IU_{ELISA}/mg indicates that the purity of the product achieved is sufficient for commercial use. However, this needs to be verified after the scale-up of the process by bioassays. In the case of a successful implementation of the new concept for the purification eCG, the same concept may be used for other serum proteins by adapting the parameters of fractionation and the type of magnetic microadsorbents used.

Acknowledgments The authors would like to thank their industrial partners (ABBIS, INLAB GmbH) of the joint project for the fruitful discussion. We also thank chemagen Biopolymer-Technologie AG in Baesweiler for the preparation of the carboxylated magnetic polyvinyl alcohol beads. Funding by the Federal Ministry of Economics and Technology (BMWi) is acknowledged.

References

Aggarwal BB, Farmer SW, Papkoff H, Stewart F Allen WR (1980) Purification and characterization of the gonadotropin secreted by cultured horse trophoblast cells. Endocrinology 106(6):1755-1759

Allen WR (1969) The immunological measurement of pregnant mare serum gonadotrophin. J Endocrinol 43(4):593-598

Christakos S & Bahl OP (1979) Pregnant mare serum gonadotropin. Purification and physicochemical, biological, and immunological characterization. J Biol Chem 254(10):4253-4261

Cole HH & Hart GH (1930) The potency of blood serum of mares in progressive stages of pregnancy in effecting the sexual maturity of the immature rat. Am J Physiol 93(1):57-68

Franzreb M, Siemann-Herzberg M, Hobley TJ, Thomas ORT (2006) Protein purification using magnetic adsorbent particles. Appl Microbiol Biotechnol 70: 505-516

González G, Castro B & Massaldi H (1998) Extraction of equine chorionic gonadotrophin from pregnant mare plasma by direct adsorption on chromatographic media. Biotechnol and Bioeng 57(1):22-25

Gospodarowicz D & Papkoff H (1967) A simple method for the isolation of pregnant mare serum gonadotropin. Endocrinology 80:699-702

Heeboll-Nielsen A, Dalkiaer M, Hubbuch JJ, Thomas ORT (2004) Superparamagnetic adsorbents for high-gradient magnetic fishing of lectins out of legume extracts. Biotechnol Bioeng 87(3):311-323

Heeboll-Nielsen A, Justesen SFL, Thomas ORT (2004) Fractionation of whey proteins with high-capacity superparamagnetic ion-exchangers. J Biotechnol 113(1-3):247-262

Hermanson GT, Mallia AK, Smith PK (1992) Immobilized affinity ligand techniques. Academic Press, San Diego

Hubbuch JJ, Matthiesen DB, Hobley TJ, Thomas ORT (2001) High gradient magnetic separation versus expanded bed adsorption: A first principle comparison. Bioseparation 10:99-112

Lecompte F, Roy F, Combarnous Y (1998) International collaborative calibration of a preparation of equine chorionic gonadotrophin (eCG NZY-01) proposed as a new standard. J Reprod Fert 113: 145-150

Loo D, Jones A, Hill MM (2010) Lectin magnetic bead array for biomarker discovery.J Proteome Res 9(10): 5496–5500

Meyer A, Berensmeier S, Franzreb F (2007) Direct capture of lactoferrin from whey using magnetic micro-ion exchangers in combination with high-gradient magnetic separation. Reactive and functional polymers 67:1577-1588

Moore W Jr, Ward D (1980) Pregnant mare serum gonadotropin. Rapid chromatographic procedures for the purification of intact hormone and isolation of subunits. J. Biol. Chem. 255(14):6923-6929

Murphy BD & Martinuk SD (1991) Equine chorionic gonadotropin. Endocr Rev 12(1):27-44

Table 1 Preparation of an eCG intermediate according to the purification scheme of Gospodarowicz and Papkoff (1967).

Fraction	Protein content [mg/ml]	eCG [IU_{ELISA}/ml]	Volume [ml]	Recovery [%]	Purification factor	Concentration factor
Serum	59±2.5	39±12	500	100	1	1
HPO_3 ppt	15±4.8	29±11	613	90±18	3±1.5	0.73±0.3
Lyophilizate	1.1±0.2	185±109	72	53±6	246±110	5±3

Table 2 Preparation of an eCG intermediate using the proposed simplified purification scheme, including ultra-/diafiltration.

Fraction	Protein content [mg/ml]	eCG [IU_{ELISA}/ml]	Volume [ml]	Recovery [%]	Purification factor	Concentration factor
Serum	66±6.1	49±8.6	1667	100	1	1
HPO_3 ppt	8.5±2.1	41±14	1943	91±9.7	6.4±0.8	0.8±0.2
UF/DF Concen.	0.8±0.5	184±14	341	79±9.6	359±217	3.7±0.8

Table 3 Eluates resulting from final eCG purification applying magnetic anion exchange beads. The high-gradient magnetic fishing process started from the classical eCG intermediate (c-eCG-i) or the simplified eCG intermediate (s-eCG-i).

Fraction	Protein content [mg/ml]	eCG [IU_{ELISA}/ml]	Volume [ml]	Recovery [%]	Purification factor	Concentration factor
Eluate (c-eCG-i_{23ml})	0.06±0.02	310±14	15	38±1.7	7184±3074	7.95±0.3
Eluate (c-eCG-i_{500ml})	0.31	211	150	40	975	5.4
Eluate (s-eCG-i)	0.6±0.08	774±48	15	59±9.2	1818±138	16±0.9

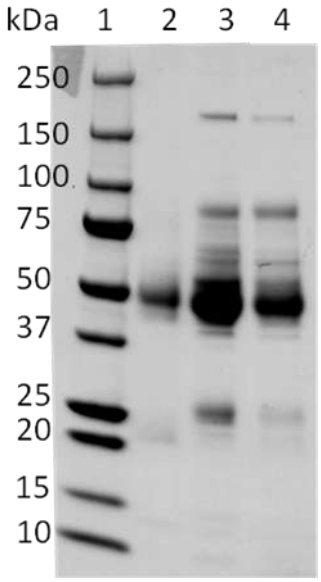

Fig 1

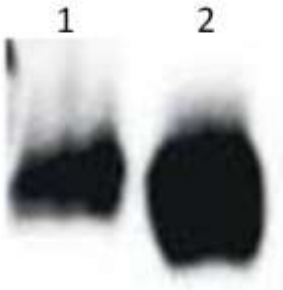

Fig 2

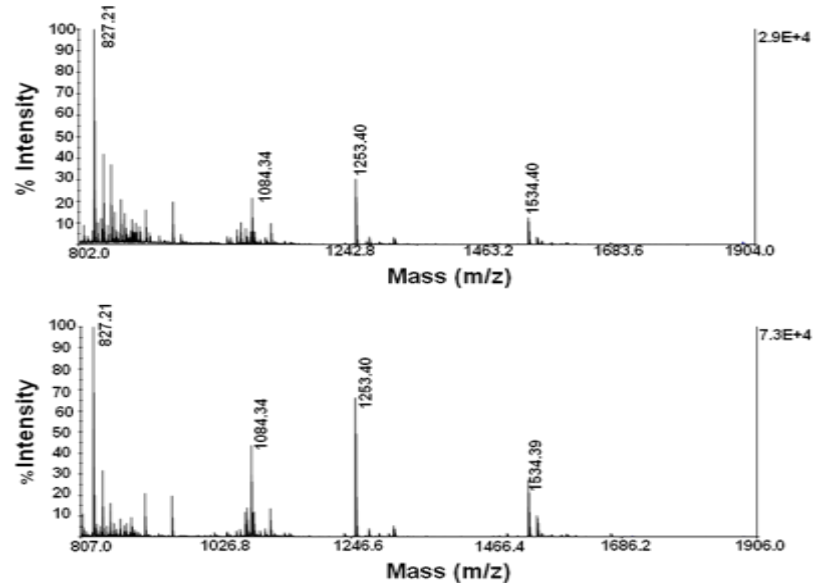
% Intensity
827.21
1084.34
1253.40
1534.40
2.9E+4
802.0
1242.8
1463.2
1683.6
1904.0
Mass (m/z)
% Intensity
827.21
1084.34
1253.40
1534.39
7.3E+4
807.0
1026.8
1246.6
1466.4
1686.2
1906.0
Mass (m/z)

Fig 3

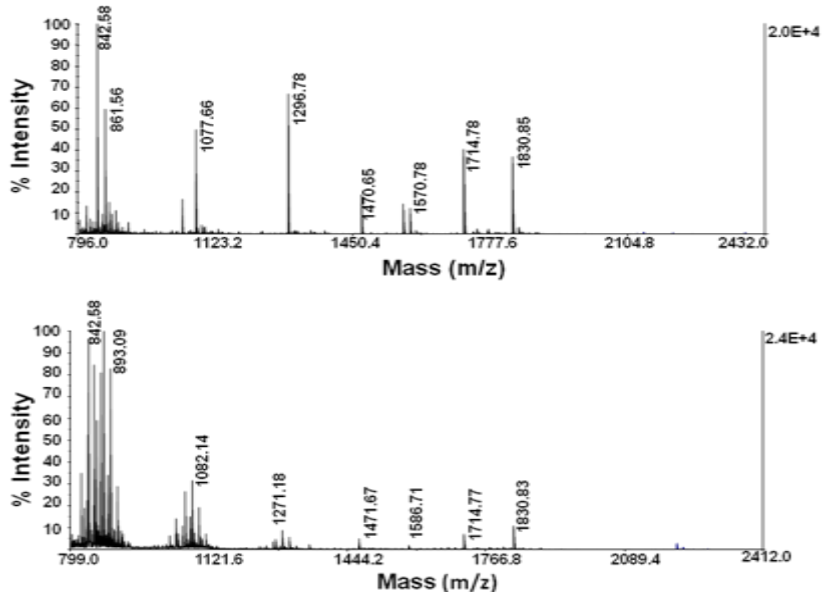
% Intensity
Mass (m/z)
842.58
861.56
1077.66
1296.78
1470.65
1570.78
1714.78
1830.85
2.0E+4
796.0
1123.2
1450.4
1777.6
2104.8
2432.0
% Intensity
Mass (m/z)
842.58
893.09
1082.14
1271.18
1471.67
1586.71
1714.77
1830.83
2.4E+4
799.0
1121.6
1444.2
1766.8
2089.4
2412.0

Fig 4

Fig. 1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of c-eCG-i, s-eCG-i, and commercial eCG. The proteins were stained with coomassie blue

Lane 1: Standard molecular weight marker, lane 2: commercial eCG, lane 3: c-eCG-i, lane 4: s-eCG-i

Fig. 2 Identification of the eCG β subunit by the application of a Western blot for the SDS gel, including the commercial eCG [lane 1], and c-eCG-i [lane 2]

Fig. 3 MALDI-ToF analysis of the bands around 45kDa of commercial eCG and c-eCG-i. The spectrum at the top shows the results of the commercial eCG

Fig. 4 MALDI-ToF analysis of the bands around 20kDa of commercial eCG and c-eCG-i. The spectrum at the top shows the results of the commercial eCG