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High-level expression of biologically active glycoprotein hormones in *Pichia pastoris* strains—selection of strain GS115, and not X-33, for the production of biologically active N-glycosylated ^{15}N-labeled phCG

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Abstract The methylotrophic yeast *Pichia pastoris* is widely used for the production of recombinant glycoproteins. With the aim to generate biologically active ^{15}N-labeled glycohormones for conformational studies focused on the unravelling of the NMR structures in solution, the *P. pastoris* strains GS115 and X-33 were explored for the expression of human chorionic gonadotropin (phCG) and human follicle-stimulating hormone (phFSH). In agreement with recent investigations on the N-glycosylation of phCG, produced in *P. pastoris* GS115, using ammonia/glycerol-methanol as nitrogen/carbon sources, the N-glycosylation pattern of phCG, synthesized using NH_4Cl/glucose–glycerol–methanol, comprised neutral and charged, phosphorylated high-mannose-type N-glycans ($Man_{8–15}GlcNAc_2$). However, the changed culturing protocol led to much higher amounts of glycoprotein material, which is of importance for an economical realistic approach of the aimed NMR research. In the context of these studies, attention was also paid to the site specific N-glycosylation in phCG produced in *P. pastoris* GS115. In contrast to the rather simple N-glycosylation pattern of phCG expressed in the GS115 strain, phCG and phFSH expressed in the X-33 strain revealed, besides neutral high-mannose-type N-glycans, also high concentrations of neutral hypermannose-type N-glycans ($Man_{up\text{-}to\text{-}30}GlcNAc_2$). The latter finding made the X-33 strain not very suitable for generating ^{15}N-labeled material. Therefore, ^{15}N-phCG was expressed in the GS115 strain using the new optimized protocol. The ^{15}N-enrichment was evaluated by ^{15}N-HSQC NMR spectroscopy and GLC-EI/MS. Circular dichroism studies indicated that ^{15}N-phCG/GS115 had the same folding as urinary hCG. Furthermore, ^{15}N-phCG/GS115 was found to be similar to the unlabeled protein in every respect as judged by radioimmunoassay, radioreceptor assays, and *in vitro* bioassays.

Keywords *Pichia pastoris* GS115 · *Pichia pastoris* X-33 · Human chorionic gonadotropin · Human follicle stimulating hormone · Glycosylation · Hypermannosylation · ^{15}N-Labeling

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Abbreviations

2AB	2-Aminobenzamide
CD	Circular dichroism
CHO	Chinese hamster ovary
DMEM	Dulbecco's minimum essential medium
DO	Dissolved oxygen
GLC-EI/MS	Gas-liquid chromatography–electron impact mass spectrometry
hCG	Human chorionic gonadotropin
hFSH	Human follicle stimulating hormone
HPLC	High-performance liquid chromatography
HSQC	Heteronuclear single quantum coherence
LH	Luteinizing hormone
MAbs	Monoclonal antibodies
MALDI-TOF-MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
NMR	Nuclear magnetic resonance
PCR	Polymerase chain reaction
phCG	Human chorionic gonadotropin expressed in *Pichia pastoris*
phFSH	Human follicle stimulating hormone expressed in *Pichia pastoris*
PNGase F	Peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase F
RIA	Radioimmunoassay
RRA	Radioreceptor assays
TIC	Total ion current
TPPI	Time proportional phase incrementation
wcw	Weight cell weight
YPD	Yeast peptone dextrose

Introduction

One of the popular host-cell systems for the expression of recombinant proteins derived from eukaryotic genomes [1] is the methylotrophic yeast *Pichia pastoris*. This species, which is able to perform post-translational modifications such as glycosylation, is easier to manipulate genetically and to culture than mammalian cells. The *P. pastoris* expression system uses only pure reagents, such as methanol, glucose or glycerol, biotin, trace salts, and water. The media are free of toxins, and in addition bacterial contaminations are prevented by the use of methanol, which suits perfectly with pharmaceutical requirements. Furthermore, *P. pastoris* cell cultures can be grown at high cell densities and protein purification is usually simple because of the low level of native yeast proteins secreted [2].

Biologically active hCG and hFSH have successfully been expressed in *P. pastoris* using the GS115 and X-33 strains, yielding phCG and phFSH, respectively [3, 4] (unpublished data for the X-33 strain). The wild-type *P. pastoris* X-33 strain has been created from the GS115 strain by PCR of the wild type HIS4 gene, and transforming GS115 to His+ by homologous recombination. The strains X-33 and GS115 differ only by one basepair in the HIS4 gene. Recently, we have investigated in high detail the glycosylation pattern of phCG expressed in *P. pastoris* strain GS115, demonstrating the occurrence of (phosphorylated) high-mannose-type structures [5].

The glycosylation studies on phCG, as mentioned above, were carried out in the context of a larger project focused on the unraveling of the NMR structures in solution of hCG and hFSH. So far, this research has led to the unraveling of the NMR structure in solution of the α-subunit of native, urinary hCG [6–9], a structure that shows some differences with the α-subunit in the crystal structure of hCG [10, 11]. Of special interest is that the solution structure of the α-subunit exhibits an increased structural disorder [8] compared to the α-subunit in the crystal structure of the α,β-dimer.

In order to generate biologically active ^{15}N-labeled glycohormones in *P. pastoris* for NMR studies, in the present paper an optimization of the earlier reported fermentation protocol [4] is presented. Furthermore, attention is paid to the influence of the GS115 and X-33 strains on the N-glycosylation of *P. pastoris*-expressed hCG and hFSH. Finally, the most optimal protocol and strain have been used to generate biologically active ^{15}N-labeled phCG.

Materials and methods

General

[^{125}I]NaI and [1,2,6,7,16,17-^{3}H]-testosterone were obtained from Perkin Elmer Life Sciences (Boston, MA) and $^{15}NH_4Cl$ was purchased from Isotec (St Louis, MO). *Pichia* expression vectors, yeast extract, peptone, and yeast nitrogen base without amino acids, required for growing *P. pastoris* cells, were obtained from Invitrogen Corp (Carlsbad, CA).

All the DNA manipulations were according to standard techniques [12]. For hCG expressions using the *P. pastoris* strain GS115 and the pPIC9k expression vector, full details have been reported [3, 4]. For expressions using the *P. pastoris* strain X-33, the pGAPZalpha vector was used to prepare phFSH/X-33 and phCG/X-33 (unpublished data).

Growth media

The preculturing of *P. pastoris* cells was carried out in YPD (1% yeast extract, 2% peptone, and 2% dextrose), containing 100 μg/ml geneticin. The 1-liter medium for the culturing in the fermenter vessel contained 42.9 g KH_2PO_4, 1 g $CaSO_4{\cdot}2H_2O$, 14.3 g K_2SO_4, and 11.7 g $MgSO_4{\cdot}7H_2O$. The

nitrogen and carbon sources needed were prepared separately. The medium used is an adaptation of the FM22 medium [13, 14], wherein $(NH_4)_2SO_4$ has been substituted by NH_4Cl. To this end, 55 g NH_4Cl were dissolved in 550 ml H_2O, and the solution was sterilized by filtration through a 0.22 μm filter. One hundred milliliter of this solution were pumped into the fermenter vessel every 24 h except on day 2, when 50 ml were pumped. A 200 ml solution of 50% (w/v) glucose, containing 1 ml of PTM1 trace salts, replaced the previously used glycerol [4] during the batch phase, and a 50% glycerol solution (2 g glycerol in total) was used during the fed-batch phase of 20 min. PTM1 consisted of 24 mM $CuSO_4$, 0.53 mM NaI, 19.87 mM $MnSO_4$, 0.83 mM Na_2MoO_4, 0.32 mM boric acid, 2.10 mM $CoCl_2$, 0.15 mM $ZnCl_2$, 0.23 mM $FeSO_4$, and 0.82 mM biotin.

Production of phCG in *P. pastoris* GS115

The production of phCG/GS115 was performed using a BioFlo 3000 fermenter (New Brunswick Scientific, Edison, NJ), equipped with a 3-liter bioreactor constantly maintained at 28°C.

The *P. pastoris* cells were revived by inoculation from a frozen glycerol stock of a single colony into 5 ml YPD, containing 100 μg/ml geneticin (preculturing). After 2 days of incubation at 30°C, 1 ml of the culture was transferred into 100 ml of the same medium, and incubated for 3 days at 30°C. Then, the culture was spun down at 5,000 rpm, and the cells were resuspended in 10 ml of the supernatant.

After autoclaving the fermentation medium (1 l) in the fermenter vessel, 100 ml of the NH_4Cl solution, 50 ml of the glucose solution (see above), and 6 ml of trace salts were added in the vessel before inoculation of the cells. The pH was automatically maintained at 5.0 by addition of 5.0 M KOH. The agitation was set to 600 rpm, and O_2 was automatically mixed with air to maintain the dissolved oxygen (DO) level at the set point of 30%. The console automatically adjusted the gas flow, varying between one and two vessel volume. The cell density was estimated every 24 h by measuring the wet cell weight and the absorbance at 600 nm. Foaming was prevented by the addition of 100 μl Antifoam 289. The culture used the glucose present in the vessel in about 3.5 h, and then the glucose feeding was gradually adjusted according to the DO reading at 6 ml/h. The cells started to utilize O_2 while they were actively growing, the agitation was set to 750 rpm, and towards the end of the batch phase they were utilizing 90% of pure O_2. In total, 100 g of glucose was used during this batch growth phase, which lasted for about 20 h. Then, when all the glucose was consumed, an increase in both DO and pH was observed. The glycerol fed-batch phase was initiated by feeding the culture with the glycerol solution and lasted 20 min. The cells were starved for 30 min before starting the induction phase. Then, the culture was fed with a 50% (v/v) methanol solution (containing 6 ml/l of trace salts) at a rate of 6 ml/h. Once the culture was adapted to grow on methanol, the rate was increased and the feeding solution was replaced by a 90% (v/v) methanol solution (containing 6 ml/l of trace salts). DO spikes were regularly monitored to ensure the viability of the culture; the induction phase lasted 96 h.

At the end of the fermentation, the pH of the medium was adjusted to 7.4, EDTA and NaN_3 were added to a concentration of 10 and 1 mM, respectively, and the cells were separated from the 3-liter of supernatant by centrifugation at 5,000 rpm with a Beckman centrifuge for 20 min at 20°C.

phCG/GS115 was purified using an earlier described protocol using sequential Phenyl Sepharose (Amersham Biosciences, Piscataway, NJ) and SP-Sepharose Fast Flow (Amersham Biosciences) column chromatography [4], yielding about 25 mg/l full biological active glycohormone.

Production of phCG and phFSH in *P. pastoris* X-33

The production of phCG/X-33 and phFSH/X-33 was carried out in a 10-liter fermentor vessel using ammonia/glycerol–methanol as nitrogen/carbon sources (unpublished data of R.R. Dighe), yielding about 17 and 22 mg/l biological active glycohormone, respectively.

Release and isolation of N-glycans of phCG and phFSH with PNGase F or Endo H

Glycoprotein samples (phCG or phFSH) were reduced and *S*-carboxymethylated according to standard procedures [5, 15], except that the reducing glycoprotein solution was boiled for 3 min instead of being incubated for 2 h at 37°C.

Reduced and carboxymethylated glycoprotein or glycopeptide (derived from phCG) samples were dissolved (2 mg/ml) in 20 mM NaH_2PO_4/Na_2HPO_4, pH 7.2, containing 10 mM EDTA, and digested with peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase F, EC 3.5.1.52; Roche Applied Science, IN) (0.5 U/mg) for 24 h at 37°C. Alternatively, samples were dissolved (2 mg/ml) in 20 mM NaH_2PO_4/Na_2HPO_4, pH 5.5, containing 10 mM EDTA, and digested with endo-β-*N*-acetylglucosaminidase H (Endo H, EC 3.2.1.96; Roche Applied Science, IN) (1 mU/100 μg).

For the N-glycan profiling of phCG/GS115, the PNGase F digest was separated on a Superdex G75 column (60× 2.6 cm; Pharmacia, Uppsala, Sweden) using 50 mM NH_4HCO_3, pH 7.0, as eluent at a flow rate of 1 ml/min, monitored at 214 nm (Uvicord, LKB). In the case of phCG/X-33 and phFSH/X-33, the PNGase F digest was fractionated on a Bio-Gel P-10 column (75×2 cm, BioRad) using 50 mM NH_4HCO_3, pH 7.0, as eluent at a flow rate of

13 ml/h, monitored by refractive index detection (Bischoff 8100 RI detector). The N-deglycosylated glycoproteins and the carbohydrates were isolated and lyophilized four times.

N-glycans released from glycopeptides were directly labeled with 2-aminobenzamide (2AB) without prior separation from the peptide moiety.

Preparation, isolation, and fractionation of phCG/GS115 glycopeptides

Reduced and carboxymethylated phCG/GS115 (15 mg), dissolved in 7.5 ml 20 mM NaH_2PO_4/Na_2HPO_4, pH 8.0, was digested with 100 μg trypsin (EC 3.4.21.4; Roche Applied Science, IN) for 2 h at 37°C. The digest was stored for 20 min at −80°C to deactivate the enzyme, then lyophilized. The tryptic digest was dissolved in buffer A (20 mM Tris/HCl, pH 7.5, containing 0.3 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.02% NaN_3) and applied to a 2-ml ConA-Sepharose (Sigma-Aldrich, St Louis, MO) column, equilibrated in the same buffer. After washing with buffer A, the column was eluted with 100 mM methyl α-D-mannopyranoside in buffer A, and the glycopeptide-containing fraction obtained was lyophilized.

HPLC fractionations were carried out on a Waters 600 HPLC system. The glycopeptide fraction was separated on a Vydac 214TP510 C_4 column (4.6×250 mm; Grace Vydac, Hesperia, CA). Elutions were performed with a gradient of acetonitrile in 0.1 M triethylamine phosphate, pH 6.5 (built up from buffers B and C), at a flow rate of 1.5 ml/min, monitored at 214 nm. Buffer B consisted of 5% acetonitrile in 0.1 M triethylamine phosphate, pH 6.5; buffer C of 60% acetonitrile in 0.1 M triethylamine phosphate, pH 6.5. For gradient details, see relevant figure captions. Isolated glycopeptides were desalted on a HiTrap column (Pharmacia), then lyophilized three times. One of the Vydac fractions was further fractionated on a Lichrosorb-NH_2 10 μm column (25× 0.46 cm, Alltech, Breda, The Netherlands), equipped with a LiChrospher Amino 5 μm guard column (7.5×4.6 mm). Elutions were performed with a gradient of 30 mM K_2HPO_4/KH_2PO_4, pH 6.8, in acetonitrile, at a flow rate of 1.5 ml/min, monitored at 214 nm. Relevant fractions were concentrated under a N_2 stream, and lyophilized.

Characterization of ^{15}N-phCG/GS115

SDS-PAGE

The purity of ^{15}N-phCG/GS115 was checked by SDS-PAGE under reducing conditions. The gel was composed of a 4% stacking gel at pH 6.8 and a 12% running gel at pH 8.8. The bands were visualized with Coomassie Blue.

Radioimmunoassay (RIA)

The hCG activity of the purified ^{15}N-phCG/GS115 material was determined by RIA using various polyclonal and monoclonal antibody-based RIAs as described earlier [3, 16]. Predetermined quantities of urinary hCG or purified ^{15}N-phCG/GS115 material were diluted in RIA buffer (0.5 M sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 50 mM EDTA), then incubated with appropriate dilutions of either hCG polyclonal antiserum or monoclonal antibodies against hCG and [^{125}I]hCG (100,000 cpm/tube) overnight at room temperature. The antigen-antibody complexes were precipitated by adding an appropriate dilution of normal rabbit/ mouse serum, goat anti-rabbit/ anti-mouse IgG and 2.5% polyethylene glycol. The tubes were centrifugated at 5,000×*g*, the supernatant was discarded, and the radioactivity in the pellet was counted in a gamma counter.

Radioreceptor assays (RRA)

The ability of ^{15}N-phCG/GS115 to bind to luteinizing hormone (LH) receptors was assayed by RRA, using a crude rat testicular homogenate as a source of LH receptors [3, 17]. The receptor preparation was incubated with varying concentrations of ^{15}N-phCG/GS115 or urinary hCG in the presence of [^{125}I]hCG (200,000 cpm/tube) as the tracer. The non-specific binding was determined by evaluating the binding of [^{125}I]hCG to the receptor in the presence of a large excess of unlabeled hCG (400 ng/ml). The samples were incubated at room temperature for 2–3 h, followed by addition of 1 ml RRA buffer consisting of 0.05 M phosphate buffer, pH 7.4, containing 5 mM $MgCl_2$ and 1 mg/ml BSA. The samples were centrifugated for 15 min at 4,000×*g* at 4°C. The supernatant was discarded and the pellet was counted using a Perkin Elmer auto-gamma counter.

Mouse Leydig cell in vitro bioassay

The ability of ^{15}N-phCG/GS115 to stimulate testosterone production in mouse Leydig cells, obtained from adult male Swiss mice (2–3 months old) was assayed as described [17]. Testes were decapsulated, suspended in Dulbecco's minimum essential medium (DMEM), pH 7.4, containing 0.05 M Hepes and 0.1% bovine serum albumin, and stirred in an ice bath on a magnetic stirrer for 15 min. The cell suspension per testis was filtered through a nylon cloth, oxygenated, and incubated for 1 h at 34°C (shaking water bath). After centrifugation for 10 min at 750×*g*, the cells were washed twice with

DMEM, and then resuspended in 5 ml medium. Aliquots of the cell suspension (0.4 ml/tube) were incubated with different dilutions of ^{15}N-phCG/GS115 or urinary hCG for 4 h at 34°C (shaking water bath) following oxygenation. Testosterone, secreted upon stimulation with ^{15}N-phCG/GS115 or urinary hCG, was determined by a testosterone specific RIA, as described earlier [17], using specific testosterone antiserum (at 1:50,000 dilution) and [1,2,6,7,16,17-^{3}H]-testosterone (6,000 cpm/tube) in a total volume of 400 µl. Binding reactions were carried out for 1 h at room temperature followed by 1 h at 4°C. At the end of the incubation period, free testosterone was removed by adding an equal volume of dextran-coated charcoal (1 g charcoal and 0.1 g dextran/ml). Subsequently, the tubes were centrifugated at 5,000×*g* for 15 min at 4°C. 700 µl of each supernatant were thoroughly mixed with 1 ml scintillation cocktail (2.5 g 2,5-diphenyloxazole, 200 mg *p*-bis[2-(5-phenyloxazolyl)]-benzene, 10.5 ml methanol in 500 ml toluene). The solutions were incubated at room temperature in the dark for at least 4 h, and radioactivity was counted in a Pharmacia Liquid Scintillation counter.

Analysis methodologies

Monosaccharide analysis

Desalted glycoprotein samples were subjected to methanolysis, followed by re-*N*-acetylation and trimethylsilylation, and the generated mixtures of trimethylsilylated methyl glycosides were analyzed by GLC-EI/MS, as described [5, 18].

Methylation analysis

The hypermannose-type chain fraction derived from phFSH/X-33 (1 mg) was permethylated using methyl iodide and solid sodium hydroxide in dimethyl sulfoxide. After hydrolysis, the mixture of partially methylated monosaccharides was converted into a mixture of partially methylated alditol acetates, which was analyzed by GLC-EI/MS, as described [19, 20].

2AB-labeling of N-glycans and HPLC profiling

Enzymatically released glycoprotein N-glycans were derivatized with 2-aminobenzamide, as described [5, 21]. Neutral N-glycan profiling on normal phase TSKgel Amide-80 and charged N-glycan profiling on weak anion-exchange Vydac 301 VHP5410 were carried out as reported previously [5].

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

MALDI-TOF mass spectra were recorded on a Voyager-DE PRO mass spectrometer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands) with implemented delayed extraction technique, equipped with a N_2 laser (337 nm, 3 ns pulse width). Spectra (positive-ion mode) were recorded in the linear mode at an accelerating voltage of 25 kV using an extraction delay of 600 ns. Peptide samples (0.5 µl), when necessary desalted with Omix tips C18 (Varian, Lake Forest, CA), were mixed in a 1:1 ratio on the target with α-cyano-4-hydroxycinnaminic acid (10 mg/ml) dissolved in acetonitrile—0.3% aqueous trifluoroacetic acid (50:50, v/v). ^{15}N-phCG/GS115 (2 mg/ml) was dissolved in 0.2% trifluoroacetic acid and boiled for 3 min. Then, 0.5-µl samples were mixed in a 1:1 ratio on the target with 3,5-dimethoxy-4-hydroxycinnaminic acid (sinapinic acid, 10 mg/ml) dissolved in acetonitrile—0.3% aqueous trifluoroacetic acid (50:50, v/v).

Amino acid analysis

^{15}N-phCG/GS115 (400 µg) was hydrolyzed in 6 M HCl at 110°C for 22 h. After trimethylsilylation, the derivatized amino acids were analyzed by GLC-EI/MS on a Fisons Instruments GC 8060/MD 800 system (Interscience), equipped with an AT-1 column (30 m×0.25 mm, Alltech, Breda, The Netherlands). The temperature was maintained for 2 min at 80°C, and then increased at 4°C/min until 180°C, which was kept for 5 min.

Circular dichroism (CD)

Samples were prepared by dissolving 0.5 mg ^{15}N-phCG/GS115 in 300 µl 50 mM sodium phosphate buffer, pH 5.6. CD measurements were carried out between 260 and 190 nm at room temperature on a Jasco J-810 spectropolarimeter, using a 1-mm path length cell, 1-nm bandwidth, 1-s response time, and a scan speed of 50 nm/min.

NMR spectroscopy

NMR spectra were recorded on a 900-MHz NMR spectrometer (Bruker Avance DRX). Lyophilized ^{15}N-phCG/GS115 (15 mg) was dissolved in 500 µl 90% (v/v) H_2O, containing 10 mM EDTA and 10% $^{2}H_2O$ (99.9%; Cambridge Isotope Laboratories Inc., MA). The ^{15}N-HSQC spectrum was recorded at a probe temperature of 57°C and pH 4.4, using Echo/Antiecho-TPPI gradient selection with

decoupling during acquisition [22–24]. The experiments were obtained with 128×1024 points, 128 scans using a spectral width of 2737 Hz in the ^{15}N dimension and 9920 Hz in the ^{1}H direction. NMR data sets were processed using in house developed software with zero filling, π/2 shifted sine bell window function for *t1*, π/2 shifted squared sine bell window function for *t2*, and automatic spline baseline correction in both dimensions.

Results and discussion

Optimization of the production of recombinant phCG in *P. pastoris* GS115

In general, *P. pastoris* can provide high expression levels of foreign proteins [3, 13], being strongly dependent on the methanol-regulated promoter of the alcohol oxidase 1 gene (AOX1) [25, 26]. In order to produce ^{15}N-labeled glycohormones in an economical way, an optimization of earlier protocols [4, 14, 27] was investigated. Due to the high costs of ^{15}N-labeled ammonia, the use of ammonia was not explored. Although both $^{15}NH_4Cl$ and $(^{15}NH_4)_2SO_4$ are equally priced, the use of $(NH_4)_2SO_4$ results in a precipitation of K_2SO_4, which can hamper cell growth. As an exchange of medium would be required during the fermentation to remove K_2SO_4 [27], $^{15}NH_4Cl$ was selected instead. Looking for a reduction of the costs of a potential ^{13}C labeling, introductory experiments showed that partial replacement of glycerol [14, 28] by glucose turned out to be an excellent option. Therefore, in the present work the nitrogen/carbon sources NH_4Cl/glucose-glycerol-methanol were explored to replace the earlier used [4] ammonia/glycerol-methanol. The finally developed production process, yielding the highest cell densities and yields (about 25 mg/l phCG/GS115) so far, has been detailed in "Materials and methods."

For protein expression in *P. pastoris*, it is crucial to supply an optimal amount of NH_4Cl. However, continuous diluted NH_4Cl feeding was not explored. Previous protocols [14] recommended to feed the culture every 24 h with 10 g/l of NH_4Cl. Following this strategy, however, we found that on the second day of the fermentation, 5 g/l were enough to ensure acidification of the medium by the cells because there was no change in the DO. The short glycerol fed-batch phase of 30 min was long enough to derepress the AOX1 promoter [14]. At the end of the batch phase (48 h), just before methanol induction, the wet cell weight (wcw) reached 109 g/l (Fig. 1), and 48 h after the beginning of the methanol induction, the wcw reached 233 g/l, then decreased to 198 g/l because of the dilution effect. It should be noted that this is the first time that such high cell densities are reported for recombinant glycoprotein hormones in *P. pastoris*. This is probably due to the use of NH_4Cl instead of ammonia [4] or $(NH_4)_2SO_4$ for which in both cases the highest wcw never exceeded 62 g/l (OD 150) (unpublished data). During the adaptation phase of the methanol induction, 50% aqueous methanol was supplied at a flow rate of 6 ml/h for 8 h. The choice of diluted methanol was to avoid a toxic methanol accumulation, which would provoke cell death. Methanol feeding was progressively increased in order to maintain a good O_2 drop, and after 30 h, a solution of 90% aqueous methanol was used as carbon source.

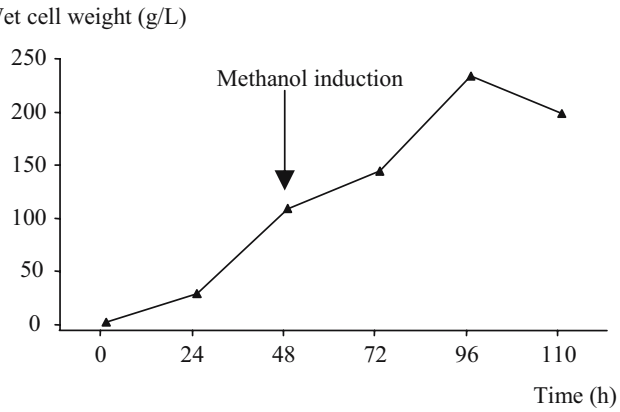


Fig. 1 Growth curve of *Pichia pastoris* cells during the fermentation with NH_4Cl/glucose–glycerol–methanol as nitrogen/carbon sources

Summarising, a highly efficient protocol using the nitrogen/carbon sources NH_4Cl/glucose-glycerol-methanol for the production of phCG in *P. pastoris* GS115 could be established.

Comparison of the N-glycan profiles of phCG/GS115, phCG/X-33, and phFSH/X-33

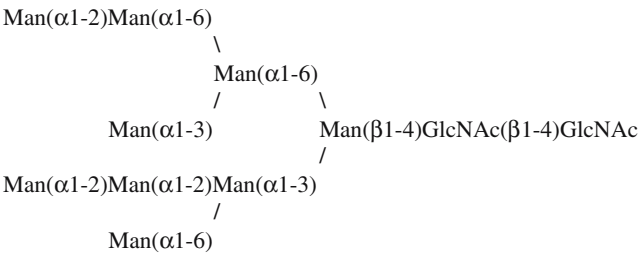
Recently, we have investigated the glycosylation pattern of phCG/GS115 expressed in *P. pastoris* strain GS115 using ammonia/glycerol-methanol as nitrogen/carbon sources [5]. The N-glycans consisted of neutral (80%) and charged, phosphate-containing (20%) high-mannose-type structures. Mannosyl O-glycans were not detected. Neutral oligosaccharides were composed of $Man_8GlcNAc_2$ (11%), $Man_9GlcNAc_2$ (47%), $Man_{10}GlcNAc_2$ (28%), $Man_{11}GlcNAc_2$ (10%), $Man_{12}GlcNAc_2$ (4%), and traces of $Man_{13–15}GlcNAc_2$. Mono- and di-phosphate-containing oligosaccharides were present, whereby the mono-phosphate compounds ranged from $Man_9PGlcNAc_2$ to $Man_{13}PGlcNAc_2$. For the detailed isomer composition of the various $Man_{8–11}GlcNAc_2$ ensembles, see [5].

In order to search for differences in N-glycosylation when glycoprotein hormones are expressed in *P. pastoris* strain GS115 or X-33, of importance for future NMR studies, N-glycan screenings were carried out on the phCG/GS115 (new conditions), phCG/X-33, and phFSH/X-33

batches. Monosaccharide analysis performed on phCG/GS115 revealed a carbohydrate content of 30% (by mass), and the presence of Man and GlcNAc in the molar ratio of 9:2, comparable with phCG/GS115 when using ammonia/glycerol-methanol as nitrogen/carbon sources [5]. However, monosaccharide analysis of phCG/X-33 and phFSH/X-33 revealed a carbohydrate content of 37 and 39% (by mass), respectively, and Man and GlcNAc were present in the molar ratio of 33:2 and 36:2, respectively. In view of the relatively high amounts of mannose found, the possible presence of free mannose in the samples was excluded via an additional purification by size-exclusion chromatography (Hitrap). As no differences were found between the first and the second monosaccharide analysis of each probe, these data indicate the presence of hypermannosylation in both phCG/X-33 and phFSH/X-33.

After reduction and carboxymethylation, the three samples were digested with PNGase F, and the completeness of the N-deglycosylation was checked by SDS-PAGE.

In the case of phCG/GS115, the pool of released N-glycans was isolated via size-exclusion chromatography, then 2AB-labeled, and analyzed by HPLC. The molar ratio of $Man_8GlcNAc_2$:$Man_9GlcNAc_2$:$Man_{10}GlcNAc_2$:$Man_{11}GlcNAc_2$ was shown to be 19:38:27:16. Glycans larger than $Man_{11}GlcNAc_2$ were present in negligible amounts. Evidently, the neutral N-glycosylation patterns of the phCG/GS115 batches prepared using the nitrogen/carbon sources NH_4Cl/glucose–glycerol–methanol (this study) and ammonia/glycerol–methanol [5] contain the same components, although in slightly different molar ratios. The same holds for the charged N-glycans. Note that the $Man_9GlcNAc_2$ isomer mainly represents [5]:



In the case of phCG/X-33 and phFSH/X-33, the pools of N-glycans were fractionated by size-exclusion chromatography, yielding three fractions, denoted **P10.1**, **P10.2**, and **P10.3** (Fig. 2). Fraction **P10.1** contained the protein moiety. Monosaccharide analysis demonstrated the occurrence of carbohydrate in fractions **P10.2** (Man:GlcNAc=33:2 for phCG/X-33, and 36:2 for phFSH/X-33) and **P10.3** (Man:GlcNAc=10:2 for both samples); fraction **P10.2** contained small amounts of protein.

HPLC profiling of the 2AB-labeled **P10.3** fractions derived from phCG/X-33 and phFSH/X-33, showed in both

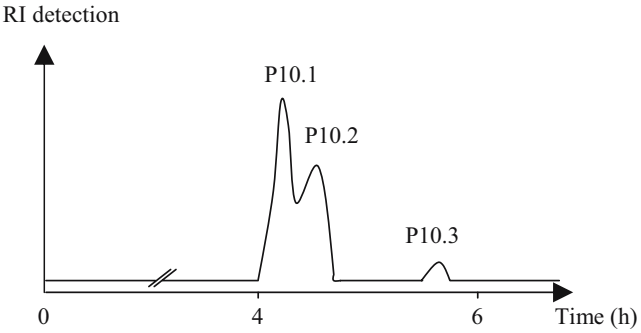


Fig. 2 Bio-Gel P-10 fractionation pattern of PNGase F-treated phFSH/X-33. Elutions were performed at a flow rate of 13 ml/h and monitored by refractive index detection. A similar profile was obtained with phCG/X-33. Peak 1, protein; peak 2, hypermannose-type N-glycans; peak 3, high-mannose-type N-glycans

cases a series of neutral oligomannose-type N-glycans, ranging from $Man_9GlcNAc_2$ to $Man_{11}GlcNAc_2$ (Fig. 3a and b); molar ratios of $Man_9GlcNAc_2$:$Man_{10}GlcNAc_2$:$Man_{11}GlcNAc_2$ are 43:39:17 for phCG/X-33, and 40:42:18 for phFSH/X-33. Comparing Fig. 3a and b, it is postulated that phCG/X-33 and phFSH/X-33 present slight differences in their $Man_{10}GlcNAc_2$ and $Man_{11}GlcNAc_2$ isomeric populations. In contrast to phCG/GS115 [5], phCG/X-33 and phFSH/X-33 did not contain phosphorylated N-glycans.

Fraction **P10.2** of phFSH/X-33 was subjected to methylation analysis, and it was found that the **P10.2** glycans are built up from terminal Man, 2-substituted Man, and 2,6-disubstituted Man in the molar ratio 1.2:2.3:1.0; very small amounts of 6- and 3,6-di-substituted Man were detected. It should be noted that, as expected, the hypermannose-type

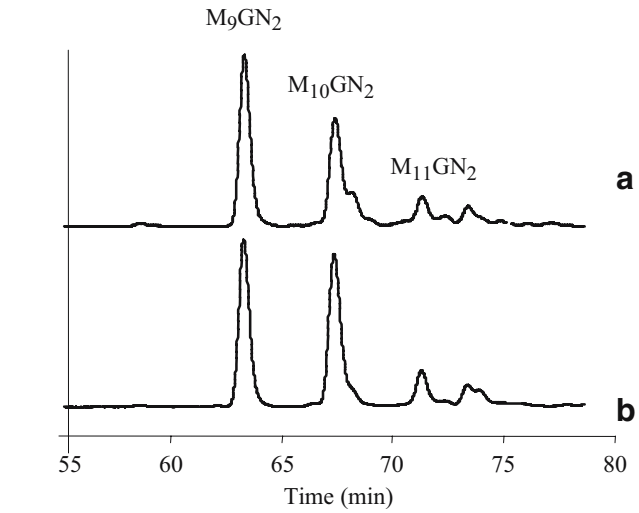


Fig. 3 HPLC profile of 2AB-labeled neutral oligosaccharides derived from glycoprotein samples expressed in strain X-33 on a normal-phase TSKgel Amide-80 column. Elutions were carried out with a gradient of ammonium formate, pH 4.4, in acetonitrile, at a flow rate of 0.8 ml/min [5]. **a** N-glycans of phCG/X-33; **b** N-glycans of phFSH/X-33. M = Man, GN = GlcNAc. Under the applied conditions, the acidic compounds elute in the void volume, not shown in the chromatograms

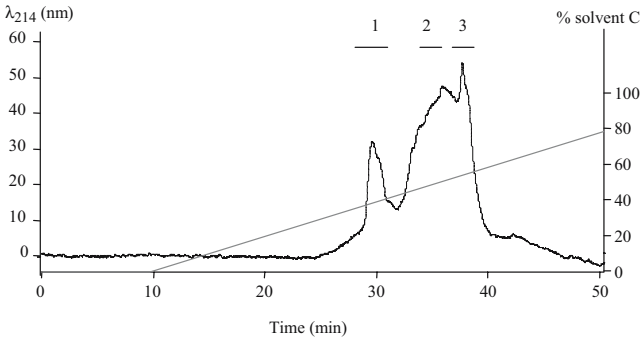


Fig. 4 HPLC elution profile at 214 nm of the phCG/GS115-derived tryptic glycopeptide fraction on a reversed-phase Vydac C_4 column. Elutions were carried out with a gradient of 0–80% (v/v) solvent C [60% acetonitrile in triethylamine phosphate] in solvent B [5% acetonitrile in triethylamine phosphate] at 1.5 ml/min

chains did not contain (α1–3)-linked Man residues in the outer part [29].

Based on the finding of hypermannosylation when using the X-33 strain, the GS115 strain, yielding a more simple N-glycosylation pattern, was selected for generating ^{15}N-labeled phCG for NMR studies.

This is the first glycosylation study on glycoproteins expressed in the *P. pastoris* wild type X-33 strain, a strain that has become commercially available recently. The found hypermannosylation confirms the recent findings of [30] for the glycosylated chicken cystatin expressed in the X-33 strain, whereby hypermannose-type N-glycans up to 50 Man residues were indicated. Probably, the difference of one basepair in the HIS4 gene of the X-33 strain causes a frame shift, and therefore induces translation modifications. *P. pastoris* strains are generally preferred to *Saccharomyces cerevisiae* strains, because they were known for their suitability to express recombinant glycoproteins with only high-mannose-type, and not hypermannose-type N-glycans. With the introduction of the *P. pastoris* wild type X-33 strain, one should really realize the differences in glycosylation machinery among *P. pastoris* strains.

Site-specific N-glycosylation of phCG/GS115

phCG/GS115 (N-glycosylation sites at αAsn52, αAsn78, βAsn13, and βAsn30) was reduced, carboxymethylated, and digested with trypsin. The resulting mixture of peptides and glycopeptides was subjected to ConA-Sepharose chromatography, and the glycopeptide-containing fraction, eluted with 100 mM methyl α-D-mannopyranoside, was subjected to HPLC analysis, yielding 3 subfractions denoted **1**, **2**, and **3** (Fig. 4). Each subfraction was digested with either PNGase F or Endo H, and analyzed by positive-ion mode MALDI-TOF-MS. Under these conditions, being specific for peptides, released carbohydrates are not detected as they do not crystallize with the matrix and/or fail to protonate. Due to the limited amount of material available, attention was paid only to the neutral N-glycans, representing 80% of the total N-glycan pool. Fraction **1**, digested with PNGase F, presented $[M+H]^+$ signals at *m/z* 1373.2 and 2070.9, corresponding to the peptides β9–20 and α76–92, respectively [31, 32]. Amino acid analysis performed on fraction **1** revealed the presence of about 50% β9–20 and 50% α76–92. Subfractionation of fraction **1** on Lichrosorb-NH_2 yielded 2 fractions, denoted **1-1** and **1-2**. MALDI-TOF-MS analysis of these fractions, digested with

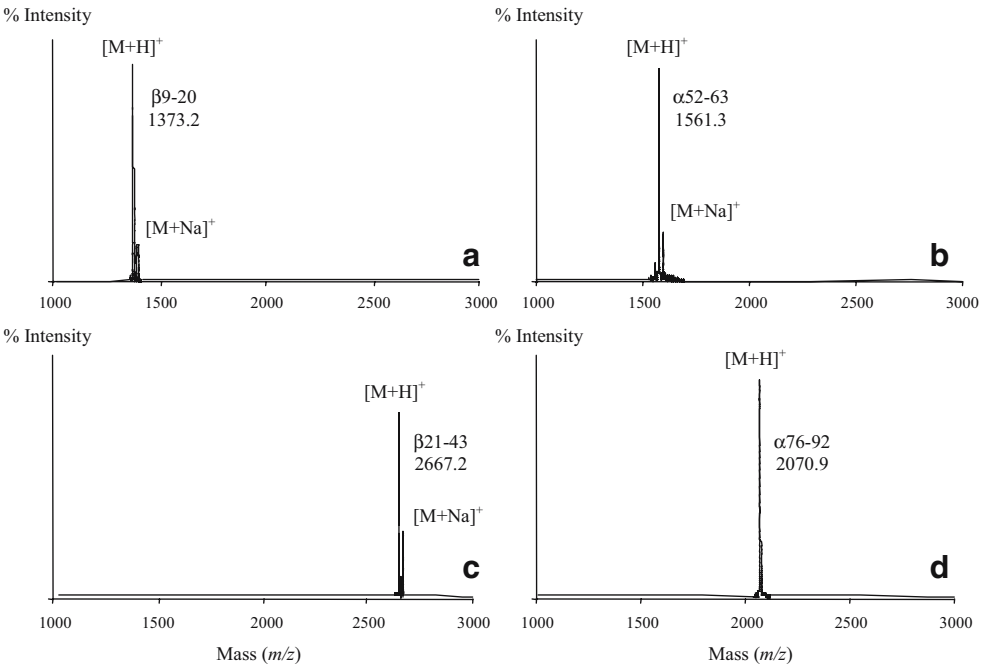


Fig. 5 MALDI-TOF mass spectra of deglycosylated tryptic glycopeptides derived from phCG/GS115, recorded in the positive-ion mode: **a** glycopeptide β9–20 (PNGase F); **b** glycopeptide α52–63 (endo H); **c** glycopeptide α21–43 (PNGase F); **d** glycopeptide α76–92 (PNGase F)

Table 1 Glycan chains occurring at the αAsn52, αAsn78, βAsn13, and βAsn30 sites in phCG/GS115. The quantifications are based on peak areas of the 2AB-labeled N-glycans in the HPLC chromatograms

Ensemble	α52–63	α76–92	β9–20	β21–43	Average	phCG
$Man_8GlcNAc_2$	9	9	20	36	19	19
$Man_9GlcNAc_2$	41	36	28	51	39	38
$Man_{10}GlcNAc_2$	37	30	27	11	26	27
$Man_{11}GlcNAc_2$	13	25	25	2	16	16

PNGase F, showed that fraction **1-1** corresponded to β9–20 (Fig. 5a) and fraction **1-2** to α76–92 (Fig. 5d) [31, 32]. Fraction **2** was resistant to PNGase F, but, digested with Endo H, it gave rise to a single $[M+H]^+$ peak at *m/z* 1561.3, corresponding to peptide α52–63 with one GlcNAc residue attached at αAsn52 (Fig. 5b). It should be noted that PNGase F does not release N-glycans from Asn residues that occur as N- or C-terminus in a peptide [33]. MALDI-TOF-MS analysis of fraction **3**, digested with PNGase F, demonstrated the presence of a single $[M+H]^+$ peak at *m/z* 2667.2, corresponding to peptide β21–43 (Fig. 5c) [31, 32]. Although the HPLC profile suggests a poor separation between fractions **2** and **3** (Fig. 4), MALDI-TOF-MS analysis of these fractions showed pure α52–63 and β21–43, respectively. Finally, the released 2AB-derivatized N-glycans of the fractions **1-1**, **1-2**, **2**, and **3** were subjected to HPLC analysis on TSKgel amide-80 (normal phase) and Vydac 301 VHP5410 (weak-anion exchange), and the results are presented in Table 1.

As is evident from Table 1, the N-glycosylation profiles at αAsn52 and αAsn78 are highly similar with dominating $Man_9GlcNAc_2$ and $Man_{10}GlcNAc_2$ structures. The N-glycosylation profile of βAsn13 presents similar amounts of $Man_{8-11}GlcNAc_2$, whereas for βAsn30 the $Man_9GlcNAc_2$ structure strongly dominates. Interestingly, compared with the other glycosylation sites, βAsn30 contains a very low amount of $Man_{11}GlcNAc_2$. It seems that the N-glycans in the α-subunit are more processed than those in the β-subunit. Within the β-subunit the N-glycans at βAsn13 are more processed than those at βAsn30. It should be noted that the N-glycan at αAsn52 in urinary hCG, located at the subunit interface [10, 11], is involved in receptor binding and in steroidogenic activity [34–36]. As phCG/GS115 is biologically active, the high-mannose-type N-glycans at αAsn52 seem to replace efficiently the sialylated glycan at αAsn52 in urinary hCG.

The difference in processing between βAsn13 and βAsn30 could be explained in terms of space availability. Crystallographic studies have shown that the N-glycans at βAsn13 and βAsn30 are located less than 7 Å from each other on the outward faces of the two adjacent β-strands [11]. Due to this proximity, it is postulated that there is a competition between the mannosyltransferase-directed growing of the more bulky high-mannose-type chains, as compared with the complex-type structures in urinary hCG, at both sites, whereby the faster growing bulky glycan at βAsn13 might prevent any further processing at βAsn30.

Production and characterization of ^{15}N-phCG/GS115

Performing the culturing protocol using $^{15}NH_4Cl$/glucose-glycerol-methanol as nitrogen/carbon sources for the preparation of phCG in *P. pastoris* strain GS115, it was possible to isolate from 3 l culture supernatant about 25 mg of ^{15}N-phCG/GS115.

The purified protein showed two bands on SDS-PAGE with apparent molecular masses of 25 kDa and 35 kDa, corresponding to N-glycosylated α- and β-^{15}N-phCG/GS115, respectively (Fig. 6, lane 2). Theoretical masses of α- and β-^{15}N-phCG/GS115 (15.9 kDa and 21.3 kDa, respectively) were calculated considering that a $Man_9GlcNAc_2$ high-mannose-type structure was present at each N-glycosylation site. Apparent molecular masses are larger than theoretical masses, presumably because of partial shielding of the protein moiety, hindering the binding of SDS to the protein. The ^{15}N incorporation in the labeled phCG/GS115 was estimated by GLC-EI/MS of a trimethylsilylated protein hydrolysate. Six amino acids (valine, leucine, glycine, serine, threonine, and proline) could be clearly identified in the TIC chromatogram by comparing

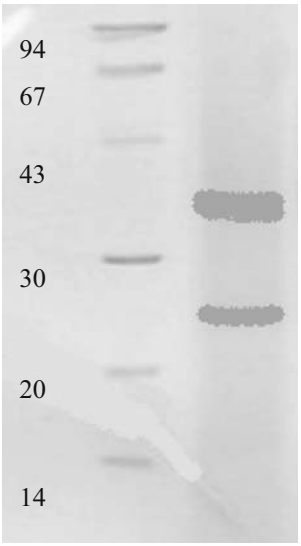


Fig. 6 12% SDS-PAGE gel of purified ^{15}N-phCG/GS115 stained with Coomassie Blue. Lane 1 indicates the protein molecular markers (phosphorylase B, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa). Lane 2 shows ^{15}N-phCG/GS115 (α-, β-subunits)

their mass spectra (data not shown) with reference data [37]. Calculation of the ratio of ^{15}N-enriched / non-enriched fragments using the mass spectra of the mentioned amino acids demonstrated that phCG/GS115 was ^{15}N-labeled for about 70%. The non-completed labeling of the hCG is due to the fact that the preculturing was performed with $^{14}NH_4Cl$.

As reported previously for glycoprotein hormones [38–40], characterization of intact hCG is quite difficult by MALDI-TOF-MS. Very broad peaks are obtained due to glycan heterogeneity and also here to the ^{15}N-incorporation of 70%. A typical mass spectrum of ^{15}N-phCG/GS115 contained peaks corresponding to the α- and β-subunits at 15.9 and 21.3 kDa, respectively (Fig. 7). Unfortunately, the ^{15}N-enrichment of phCG/GS115 could not be detected by this technique.

The secondary structure of ^{15}N-phCG/GS115 was studied by circular dichroism (CD), and compared with urinary hCG. Previous studies carried out on urinary hCG have suggested that covalent binding of carbohydrate results in an increase of the optical activity at 207 nm [41]. As is evident from Fig. 8, the CD spectra of both compounds, recorded under the same conditions, are superimposable, indicating that the structure of ^{15}N-phCG/GS115 is similar to that of urinary hCG. It can be inferred that ^{15}N-phCG/GS115 possesses the proper assembly of subunits. The high-mannose-type N-glycans of ^{15}N-phCG/GS115 [5], being partially phosphorylated, seem to have no influence on the secondary structure of the protein.

The reconstituted glycohormone showed similar biological activities as urinary hCG in terms of receptor binding, cAMP stimulation and steroidogenesis. The overall conformation of ^{15}N-phCG/GS115 was judged by radioimmunoassay using polyclonal antiserum against hCG [17]. All the

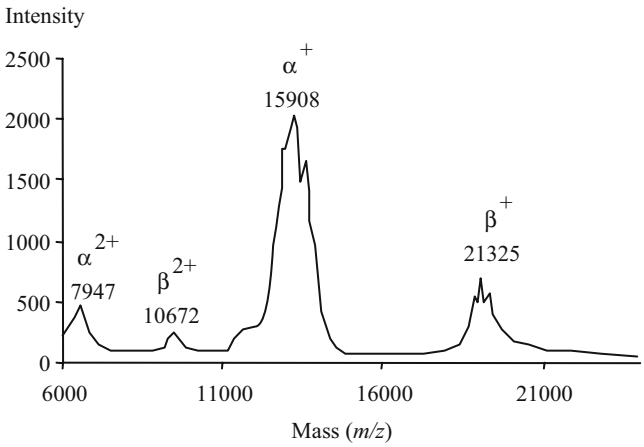


Fig. 7 MALDI-TOF mass spectrum of purified ^{15}N-phCG/GS115. The matrix consisted of 3,5-dimethoxy-4-hydroxycinnaminic acid (sinapinic acid, 10 mg/ml) dissolved in acetonitrile-0.3% aqueous trifluoroacetic acid (50:50, v/v). Before analysis, the sample was dissolved in 0.2% aqueous trifluoroacetic acid, and boiled for 3 min

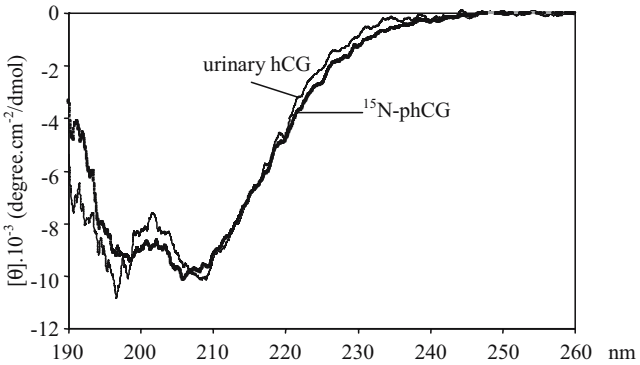


Fig. 8 CD spectra of urinary hCG (*thin line*) and ^{15}N-phCG/GS115 (*thick line*), recorded at room temperature. Samples (0.5 mg) were dissolved in 300 µl 50 mM sodium phosphate buffer, pH 5.6

RIA curves were analyzed using Graphpad prism software (version 3.03). As shown in Fig. 9a, the RIA displacement curve obtained with ^{15}N-phCG/GS115 is parallel to that observed with an authentic urinary hCG preparation, suggesting that the overall conformation in ^{15}N-hCG/GS115 is comparable to that of the natural hormone. To demonstrate that ^{15}N-phCG/GS115 is biologically active, its ability to bind to the LH receptor and elicit a biological response was next determined. As shown in Fig. 9b, ^{15}N-phCG/GS115 was able to inhibit completely the binding of ^{125}I-hCG to the receptor. Furthermore, the biological activity was demonstrated by incubating the mouse Leydig cells with equivalent receptor activities of hCG, ^{15}N-phCG/GS115 and phCG/GS115 for 4 h, and testosterone secreted into the medium was determined. Typical dose response curves are shown in Fig. 9c, where ^{15}N-phCG/GS115, phCG/GS115, and urinary hCG showed almost identical activities. ^{15}N-phCG/GS115 has therefore full biological activity as judged by RIA, RRA, and *in vitro* bioassays.

The ^{15}N-HSQC NMR spectrum of ^{15}N-phCG/GS115 (Fig. 10) presents a broad chemical shift dispersion of backbone amide protons from 9.7 to 7.2 ppm, being characteristic of structured proteins. ^{15}N-phCG/GS115 (with 30 Pro residues and 2 N-termini) contains 233 amino acid residues that could potentially be observed in a ^{15}N-HSQC spectrum. In fact, approximately 220 cross-peaks are observed in the spectrum of Fig. 10, which indicates that the large hCG (molecular mass 37.2 kDa, including the oligosaccharide chains and the ^{15}N incorporation) is not aggregating at the NMR conditions, and that it has relatively uniform hydrodynamic properties. The cross-peaks in the ^{15}N-HSQC spectrum show a wide distribution in peak intensity. Although this could be due to sample heterogeneity of ^{15}N-phCG/GS115 due to different glycosylation patterns, or partially due to anisotropic tumbling, it appears more likely that this reflects differences in dynamics. Approximately 40 NH cross-peaks, that are more intense than most other signals, are all located in a

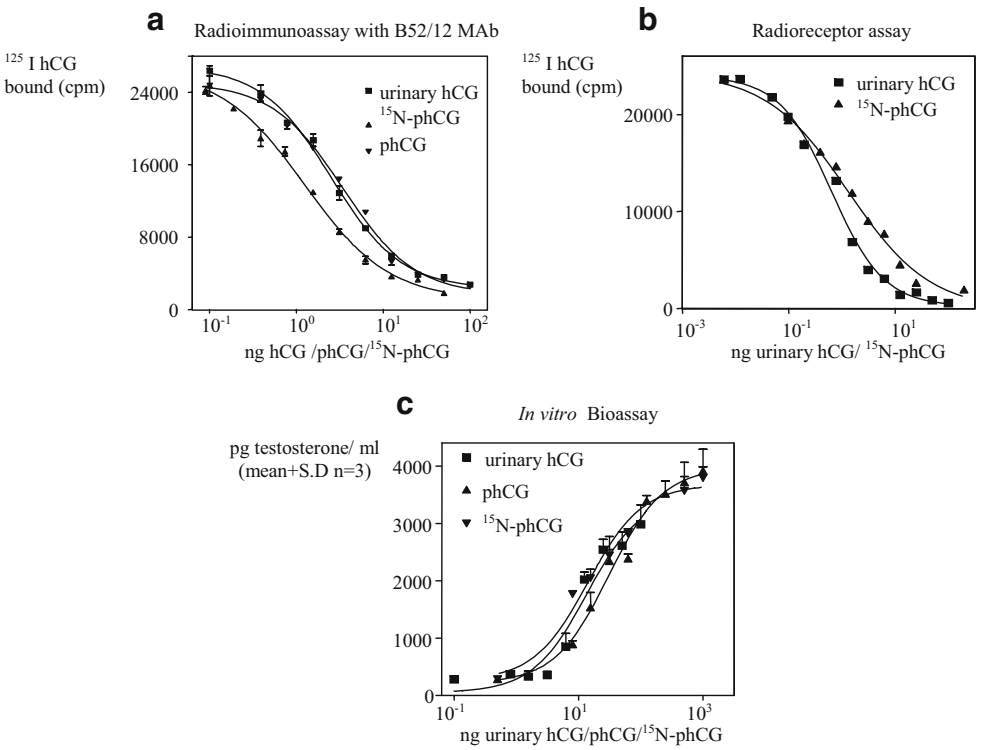


Fig. 9 Characterization of the hCG activity of purified ^{15}N-phCG/GS115. **a** Radioimmunoassay with B52/12 MAb. **b** Receptor binding activity of ^{15}N-phCG/GS115. Incubation with a rat LH receptor preparation along with [^{125}I]hCG; radioactivity bound to the receptor was determined. **c** Biological activity of ^{15}N-phCG/GS115. Incubation of urinary hCG, phCG/GS115 and ^{15}N-phCG/GS115 with mouse Leydig cells; testosterone secreted in the medium was measured by RIA

narrow region of the spectrum (F2=8.5–7.8 ppm/F1=125–130 ppm), and thus show limited chemical shift dispersion. These residues are likely located in an unstructured highly mobile part of ^{15}N-phCG/GS115.

The crystallographic structure of dimeric hCG [11] showed a disordered region with no electron density for the carboxylic terminal extension of β-hCG (βAsp111-βGln145). An intense cross-peak can be observed at 8.43/112.5 ppm, the random coil frequencies for Gly, which can then be assigned to βGly136. Therefore, we ascribe most of the intense signals to this part of the protein, which then demonstrates that the disorder in the X-ray structure is due to high mobility of hCG.

Final remarks

Until now, the production of labeled proteins for NMR purposes has been carried out mainly in bacterial systems, such as *E. coli.* Previously, also α- and β-hCG have been expressed in these bacteria [42, 43], and 6–10 mg/l of

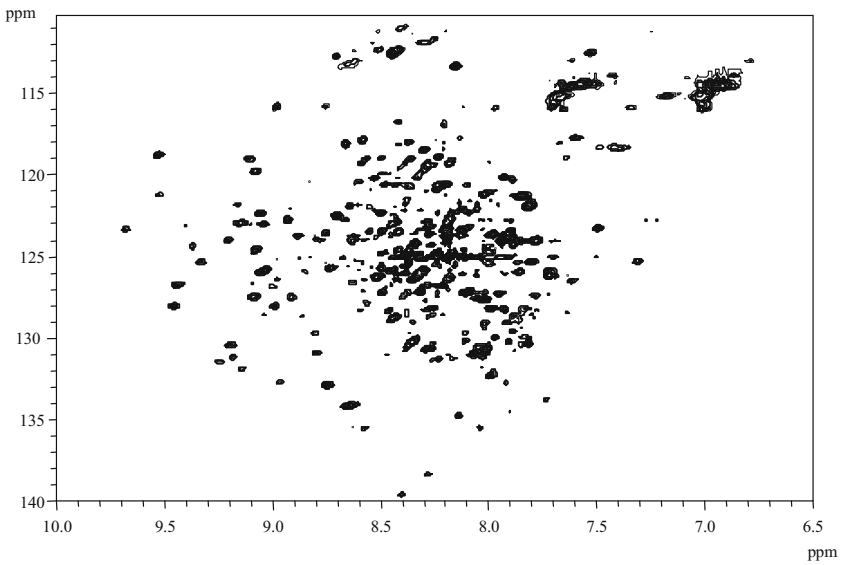


Fig. 10 ^{1}H-^{15}N HSQC spectrum of ^{15}N-phCG/GS115, recorded at a probe temperature of 57°C and pH 4.4

unfolded proteins could be produced. Refolding of each subunit followed by dimer assembly is then necessary to obtain an hCG heterodimer. However, non-glycosylated systems are obtained, usually not fully biologically active [42], which are not useful in case of studying native glycoproteins. Also eukaryotic systems were investigated for hCG expression, *e.g.* insect cells (α-hCG [44], β-hCG [45], and hCG [46]) as well as *Dictyostelium discoideum* (hCG [47]). hCG has also been expressed in Chinese hamster ovary (CHO) cells, and its glycosylation pattern was established [48]. This recombinant hCG is used for *in vitro* fertilization treatments [49, 50]. CHO cells [51, 52] were also used to produce uniformly ^{13}C- and ^{15}N-labeled hCG, but NMR structures have not been solved. *P. pastoris* was selected because this yeast can perform post-translational modifications, is easier to grow than CHO cells, and requires media of a simpler formulation. The present investigation has shown that this choice can generate very efficiently ^{15}N-labeled hCG to be used in NMR studies.

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