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Refolding of recombinant porcine growth hormone in a reducing environment limits in vitro aggregate formation

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Recombinant porcine growth hormone (rPGH) solubilized from bacterial inclusion bodies (IBs) using a cationic surfactant was oxidized to form disulphide bonds in a simple buffer solution containing 2-mercaptoethanol within an empirically derived optimal molar ratio of 2-mercaptoethanol:protein. A final yield of 55% monomeric rPGH was achieved at protein concentrations of up to 5 mg/ml without the need for removal of the 2-mercaptoethanol or the use of chaotrophic agents. In the absence of 2-mercaptoethanol only 15% monomeric rPGH was obtained, with the majority forming higher molecular weight aggregates. Using the procedure derived for porcine growth hormone, it may be possible to obtain high yields of native protein and overcome the need for using low protein concentrations and chaotrophic agents during in vitro refolding of other disulphide bonded recombinant proteins.

Aggregation; Growth hormone; Mercaptoethanol; Recombinant; Refolding

1. INTRODUCTION

Problems of aggregation and/or insolubility commonly encountered due to aberrant (non-native) disulphide bonding during the in vitro refolding procedures used to oxidize intra-disulphide bonded proteins have meant that yields of correctly refolded protein are generally low [1–4]. Various refolding protocols of differing complexity have been attempted to overcome these difficulties [3,5–7]. However, there appear to be no simple generally applicable means of obtaining high yields of recombinant biologically active disulphide bonded proteins. The degree of aggregation of recombinant proteins during in vitro refolding is generally controlled by using denaturants (3–5 M Urea or 1–2 M GnHCl) and by reoxidizing at very low protein concentrations, of the order of 1–100 μg/ml [3,5,8]. These constrains often pose serious problems, particularly during industrial scale production of relatively low value, high volume products such as animal growth hormones. The results reported here use 2-mercaptoethanol as a simple means of creating an optimal in vitro environment to form native disulphide bonds by air oxidation. Reoxidation of disulphide bonded recombinant proteins within an empirically optimized, nett reducing environment, may provide a relatively simple means of controlling the significant problem of aggregation during in vitro refolding.

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2. EXPERIMENTAL

2.1. *Solubilization of rPGH*

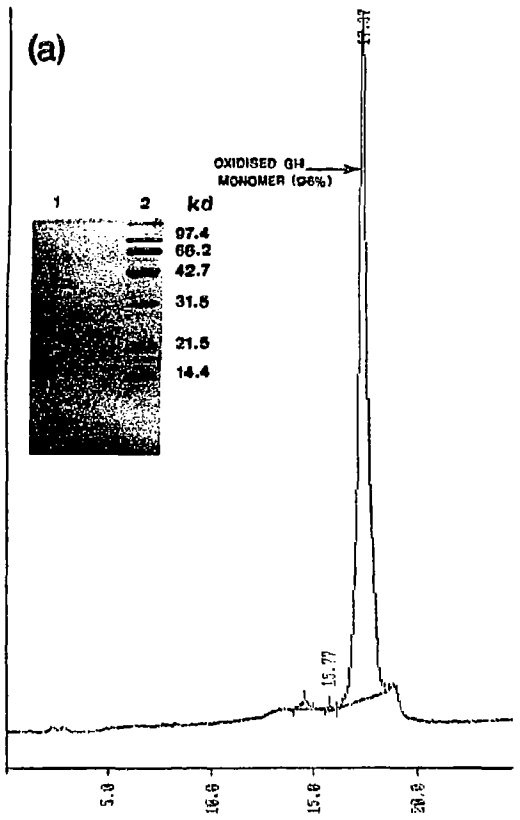
Methionyl rPGH derived from plasmid pMG93 was expressed in *E. coli* as described in UK Patent No. 8701848. The resultant IBs were solubilized using the cationic surfactant cetyltimethylammonium chloride (CTAC; ICI Australia Pty). Briefly, IBs were isolated by cell disruption, harvested by differential centrifugation and washed with 0.1 M citric acid, 0.2 M disodium phosphate pH 4.0 (2 ×) and distilled H_2O (2 ×) prior to use. IBs were used immediately or stored at pH 5 to 6.0 in a nitrogen-purged atmosphere. Approximately 50 mg of IBs (117 mg/ml dry weight) was solubilised at 10 mg/ml in a solution of 0.1 M Tris-HCl, pH 10.0, containing 2% mercaptoethanol (v/v) and 5% (w/v) CTAC for 1 h at 55°C. The solubilised inclusion bodies were clarified by centrifugation (10 000 ×*g*, 5 min) and the supernatant fraction immediately mixed with 8 bed volumes of Dowex 50W × 4 (100–150) mesh) ion exchange resin (Dow Chemical Corporation, USA) equilibrated in 0.1 M Glycine-HCl and 5 M urea, pH 10.0 (See Australian Patent Applications 11 412 and 15 010) to obtain surfactant free soluble rPGH.

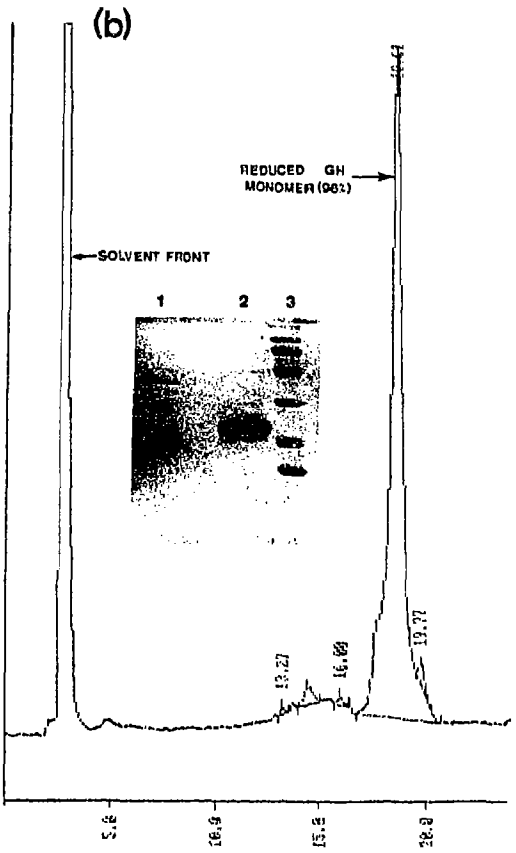
2.2. *Refolding of rPGH*

Solubilised surfactant-free rPGH (1.5 mg/ml, based on dry weight of IBs) was dialysed against 20 mM ethanolamine-HCl, pH 10.0, for 24–36 h in order to initiate oxidation of disulphides (refolding). For refolding in 2-mercaptoethanol, rPGH was exchanged using G-25 sephadex gel filtration (PD-10, 'desalting' columns; Pharmacia-LKB) into a solution of 20 mM ethanolamine-HCl, pH10.0, containing final concentrations of 5, 45, 55, 75 and 100 mM 2-mercaptoethanol. Refolding was for 24—48 h with shaking in an aerated environment at 4°C.

2.3. *HPLC Analysis*

RP-HPLC was performed using Cl alkyl-bonded silica columns (TSK-TMS 250, Toyo Soda Manufacturing Co., Tokyo, Japan; obtained through Pharmacia – LKB (Australia) Pty. Ltd.). Elution was performed at a flow rate of 0.5 ml/min at room temperature with water/acetonitrile mixtures containing 0.1 % (v/v) trifluoroacetic acid (TFA) as modifier. A stepwise linear gradient was constructed as follows: 100% buffer A (0.1% TFA in dH_2O) to 40% buffer B (100%





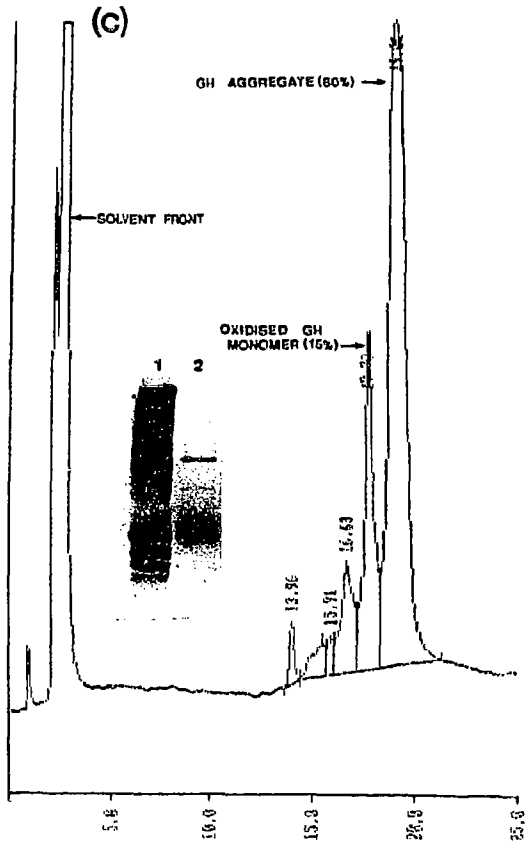


Fig. 1. RP-HPLC and 15% SDS-PAGE analyses comparing various purified 'standard' rPGH preparations and rPGH refolded without 2-mercaptoethanol. (a) RP-HPLC and SDS-PAGE analysis of a purified monomeric rPGH 'standard'. The rPGH is 96% pure by HPLC and has an M_r of 21.5K by SDS-PAGE. (b) RP-HPLC and SDS-PAGE analysis of reduced rPGH 'standard' prepared by treating the sample in (a) with 2% (v/v) 2-mercaptoethanol for 1 h in 3.0 M urea. (SDS-Gel: lane 1, purified rPGH; lane 2, reduced rPGH standard; lane 3, 'mol.wt.' markers.) Note that the correctly disulphide bonded (monomeric) and the reduced rPGH preparations are clearly resolvable by their respective retention times on RP-HPLC (typically retention times differ by 1 min) and by mobility on SDS-PAGE. (c) Solubilized rPGH refolded in the absence of 2-mercaptoethanol and analysed by RP-HPLC and SDS-PAGE. The species corresponding to oxidized, momomeric rPGH can be clearly identified as a peak (15% of protein) eluting with the same retention time as the purified rPGH 'standard' (cf. Fig. 1a) and with the same mobility on SDS-PAGE (cf. Fig. 1a, lane 1). The other major eluting protein species (80% of protein) with a peak retention time typically 1.5–2.0 min – greater than monomer, corresponds to a polydisperse 'aggregated', that is, inter-molecular disulphide bonded rPGH of varying molecular weight on SDS-PAGE (lane 1, refolded rPST; lane 2, purified 'standard' rPGH).

acetonitrile, 0.1% TFA) in 10 min; 40% buffer B to 70% buffer B in 15 min; and to 100% buffer B in 5 min. The 100% acetonitrile, 0.1% TFA eluent was maintained for a further 0.5 min before re-equilibration, prior to the next injection.

3. RESULTS AND DISCUSSION

3.1. *Refolding of rPGH in aqueous solution*

rPGH refolded against 20 mM ethanolamine-HCl, pH 10.0, was analysed by RP-HPLC to quantitate the

proportion of correctly disulphide bonded (i.e. monomeric, 21.5K rPGH) as a percentage of the total monomer and 'aggregated' forms. From the results shown in Fig. 1c approximately 15% of the rPGH was judged as monomeric after refolding. Approximately 80% existed as a polydisperse 'aggregate' species as judged by RP-HPLC and SDS-PAGE. No reduced rPGH was detected.

3.2. *Refolding of rPGH in the presence of 2-mercaptoethanol*

Samples of rPGH refolded in various concentrations of 2-mercaptoethanol were analysed by RP-HPLC. The yields of monomeric rPGH as a percentage of total peak area were respectively: 12% in the presence of 5 mM-mercaptoethanol; 25% in the presence of 45 mM 2-mercaptoethanol; 28% in the presence of 75 mM 2-mercaptoethanol, and 24% in the presence of 100 mM 2-mercaptoethanol (results not shown). The other major forms of rPGH present after refolding in 45–65 mM 2-mercaptoethanol comprised a major reduced species and a lesser proportion of the 'aggregated' species. The presence of these forms of rPGH was confirmed by SDS-PAGE.

Representative results comprising RP-HPLC and SDS-PAGE analysis of rPGH refolded in the optimal 2-mercaptoethanol concentration, 55 mM, are shown in Fig. 2. In addition to 28% oxidized monomeric rPGH, note the significant proportion of reduced (33%) rPGH in contrast to the results shown in Fig. 1c where the majority (80%) of the rPGH existed as a polydisperse aggregate population.

3.3. *Effect of protein concentration on yield of monomer during refolding in 2-mercaptoethanol*

rPST at protein concentrations of 1.5–7.5 mg/ml was refolded in 55 mM 2-mercaptoethanol and yield of monomeric rPGH estimated by RP-HPLC. Yields of 28%, 38% and 27% respectively for rPGH refolded at 1.5, 3.5 and 7.5 mg/ml were obtained (results not shown). As observed previously, the residual non-monomeric rPGH existed mainly as a reduced and lesser 'aggregated species'. Clearly, to maximise yield, both the respective concentrations of protein and 2-mercaptoethanol are required to be controlled during refolding, although surprisingly, higher yields of monomer were obtained at elevated prorein concentrations.

3.4. *Secondary oxidation/refolding of rPGH*

The results described above demonstrated the need for a critical ratio of [protein]:[2-mercaptoethanol] during refolding in order to increase yield of monomer at the expense of undesirable 'aggregated' forms. However, the presence of significant residual reduced rPGH (33%, cf. Fig. 2) even after 48 h of refolding in 55 mM 2-mercaptoethanol (irrespective of protein concentration) suggested that an additional oxidation step in the

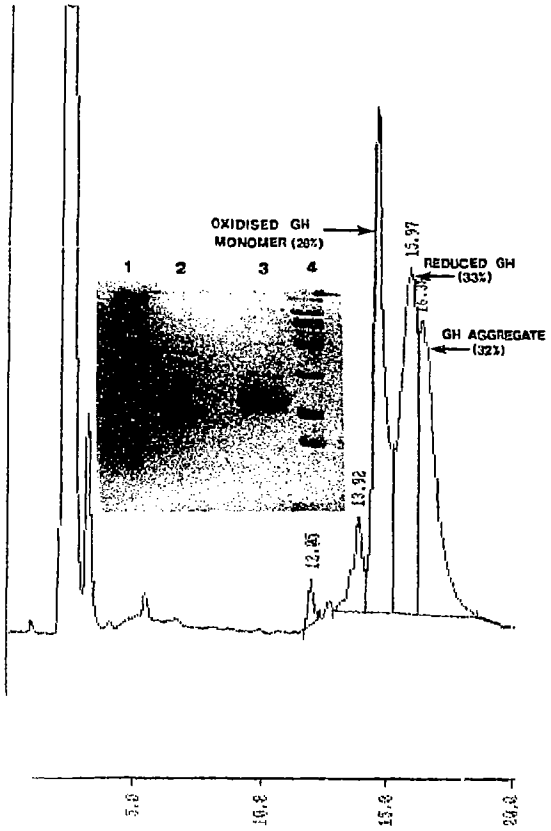


Fig. 2. Results of RP-HPLC and 15% SDS-PAGE analysis of rPGH at 1.5 mg/ml refolded in 55 mM 2-mercaptoethanol. The identity of the oxidized monomeric (28% of protein), reduced (33%) and 'aggregated' species of rPST was established from Fig. 1 and confirmed by SDS-PAGE (lane 1, rPGH refolded in 55 mM 2-mercaptoethanol; lane 2, purified 'standard' rPST; lane 3, reduced rPGH; lane 4, mol.wt. markers).

absence of 2-mercaptoethanol might increase the final yield of monomeric rPGH above 38%. Final yields of approximately 55% monomeric rPGH were obtained after secondary oxidation as judged by RP-HPLC (Fig. 3). The remaining rPGH (40%) was present as polydisperse aggregates as confirmed by SDS-PAGE (Fig. 3, lane 1).

4. CONCLUSION

The results reported in this study show yields of up to 55% monomeric rPGH at protein concentrations of 3–5 mg/ml in the absence of denaturants such as urea or GnHCl. These results are comparable to those claimed for rPGH refolded at low concentrations in urea [9] and represent, at least for rPGH, several significant departures from the current general dogma's for refolding recombinant proteins [1,5]: (i) high yield recovery of native recombinant growth hormones and most other recombinant proteins has almost invariably necessitated using appropriate concentrations of chaotrophic agents during refolding, and (ii) refolding of recombinant proteins at concentrations of 1 mg/ml or

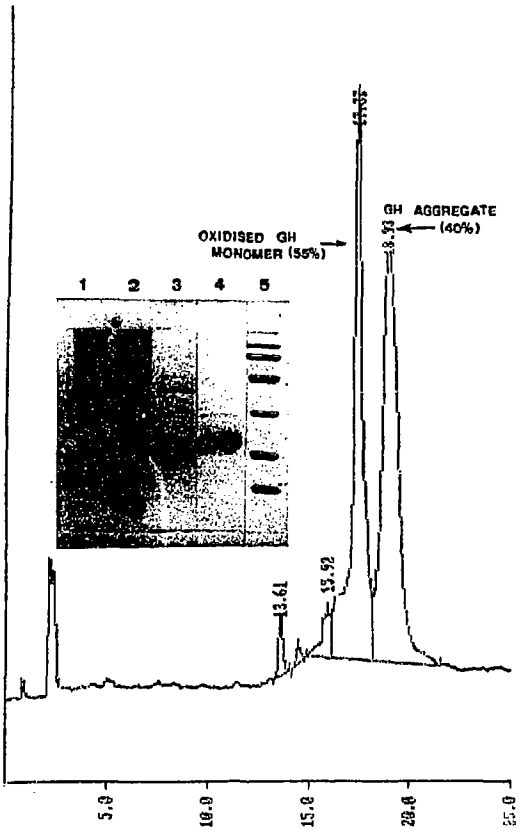


Fig. 3. Results of RP-HPLC and SDS-PAGE analysis of rPGH following secondary oxidation (refolding). The rPGH at 3.5 mg/ml was refolded for 24 h to obtain a yield of 38% monomer and approximately 30% residual reduced species, and subsequently exchanged via dialysis into 20 mM ethanolamine-HCl containing 50 mM $CuCl_2$ for 24 h with aeration. Final yield of rPGH monomer was 55% by HPLC. SDS-PAGE gel (lane 1, rPGH after secondary refolding; lane 2, rPGH after primary refolding in 55 mM 2-mercaptoethanol; lane 3, purified 'standard' rPGH; lane 4, reduced 'standard' rPGH and lane 5, mol. wt. markers). Note the absence of reduced rPGH in lane 1.

less (commonly 1–100 μg/ml) has been necessary to maximize yields of native protein. Surprisingly, using 2-mercaptoethanol, we observed an inverse relationship between yield of monomer and concentration of protein during refolding, up to a value of 5 mg/ml.

Clearly, the maintenance of a critical ratio of 2-mercaptoethanol:protein during disulphide bond formation provides an optimized in vitro environment that significantly lessens rPGH aggregation via otherwise undesirable intermolecular disulphide bonding. Moreover, the formation of the 'correct' disulphide bonds via air oxidation, occured without the necessity of removal of the 2-mercaptoethanol, where previously in the literature formation of protein disulphide bonds from the reduced state has involved substantially complete removal of reducing agent. It is interesting to consider whether the use of a nett reducing environment via a critical 2-mercaptoethanol:protein ratio during refolding will be generally applicable to the simplified and high yield recovery of the native forms of other disulphide bonded recombinant proteins.

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