The World Biotech Report 1985

Volume 1: Europe

The World Biotech Report 1985
Volume 1: Europe

Proceedings of Biotech '85 Europe,
Geneva, May 1985

Contents

ORIGINAL PAGE IS
OF POOR QUALITY

Healthcare product development

1 The science & the commerce

2 Opportunities for genetic technologies

New plant genetics

Strategy & finance

Advances in sensors

3 Environmental pollution control

4 Immobilized systems

5 Enzymes, engineering & industry

Poster presentations

Human growth hormone: microbial expression & purification

Tony Atkinson, Director
Jeremy R Court & Roger F Sherwood
Microbial Technology Laboratory

George W Jack
Therapeutic Products Laboratory
CAMR
UK

Growth hormone (GH), prolactin and chorionic somatomammotropin (CS) are a family of polypeptides related by general function, immunochemistry and structure (1); this despite their specific biological activities and synthesis of the latter in the placenta and the two former hormones in the pituatory. All 3 hormones commonly possess lactogenic and growth promoting properties and have a similar size (190 to 199 amino acids dependent on specie) and protein structure. Recent rDNA work, extending previous studies, has shown the existence of multiple genes for human GH (hGH) and for human CS (hCS), has demonstrated that hCS is a variant of hGH and indicates a variable rate of evolution in this gene family.

Presented at Biotech '85 (Europe): Online Publications, Pinner, UK, 1985.

Rat GH was the first of the growth hormone gene family to be cloned and sequenced (2), thus yielding the amino acid structure of the mature- and pre- hormone and was later expressed as a fusion protein with -lactamase in E.coli (3). Subsequently hGH was cloned (4) and expressed (5,6) in E.coli; as now also have bovine GH (7-10) and porcine GH (9).

The conditions necessary for high level expression of hGH, bGH and pGH have been thoroughly investigated. In one example (9) both bGH and pGH were constructed following similar principles to Goeddel et al (5) for the construction of hGH. In these cases a substantial N-terminal portion of the hormone gene (in contrast to the pre-hormone possessing a signal peptide) is made synthetically and fused in phase to the remainder of the gene, to produce an ATG initiation codon in front of the triplet codon for the first amino acid (phe in all cases). Synthetic met-hGH has thus been expressed at high level (2 to 15% soluble protein) employing 2 tandem lac promoters in the plasmid pHGH 107 (5) or a tandem trp-lac promoter construction (11). High level expression (20 to 30% soluble protein) of met-bGH and met-pGH have been obtained employing trp promoter vehicles. Somewhat surprisingly it was shown that reduction in the usually critical distance between the Shine-Dalgarno ribosomal binding site and the ATG codon in the original pHGH 107 met-hGH construct from the latter's 11 base pairs to 7 base pairs (the natural spacing of the lac promoter) actually reduced expression of the gene by about 40%. In an entirely separate construction Schoner et al (10) showed that a met-bGH gene produced by direct fusion of the ATG codon onto the first triplet codon (for phe) of bGH yielded a system expressing poorly with either the E.coli trp or lpp promoters in a thermoinducible runaway replication vehicle. However, expression levels of up to 30% soluble cell protein could be obtained by introducing additional codons 3' to the ATG initiation codon. High level expression of met-bGH, without introducing extra codons, was obtained by converting the coding sequence to a two-cistron expression system (10) to obviate the low efficiency of mRNA secondary structures, overcome fortuitously in the Seeburg et al (9) construction by the use of synthetic and different codons in the N-terminal construction.

Growth hormones constructed to be intracellular, as above, are produced, as are many other over-expressed foreign proteins, as dense cytoplasmic inclusion bodies (12) in which many of the aggregated proteins' thiols are in the reduced rather than the oxidised form. The exception however is met-hGH which appears to be freely soluble and biologically active in the E.coli cell (13) although the formation of granules of met-hGH in some instances has been noted (12). Active, soluble and "natural" hGH can be produced in bacteria. For instance, while P.aeruginosa transformed with a plasmid containing a gene encoding the mature form of hGH, preceeded by an ATG codon, expresses met-hGH in its cytoplasm; the hGH protein of cells transformed with a plasmid containing a gene for the natural hGH precursor is transported across the inner membrane and has the N-terminal amino acid sequence of authentic mature hGH of Phe-Pro-Thr-Ile (14).

Met-hGH has been expressed at high level in E.coli and purified to homogeneity on the large scale (13). Employing a derivative of the plasmid pHGH 107 (5) in which a trp promoter was inserted after the two tandem lac promoters (11), fermentation of E.coli RV308 containing the derivative plasmid under low tryptophan fermentation conditions gives constitutive production of met-hGH. Plasmid copy number, generation time of the E.coli cell, expression of the met-hGH gene and overall yield are unaffected by replacement of tetracycline (1 to 5 mg/l) in the culture with ampicillin (20 to 40 mg/l). All large scale cultures were routinely grown in the presence of 1 mg/l tetracycline and the data from several such cultures is summarised in Table 1.

Table 1

500 l Culture Parameters of the Production of met-hGH in E.coli

Generation time of E.coli cell	50 to 70 minutes
Average 500 l culture time	6.5 to 9.0 hours
Cell yield	20 to 30g cell paste/l
Overall yield	10 to 15 Kg cell paste
Molecules of met-hGH per cell	9×10^5 to 1.5×10^6
Average % soluble cell protein	8 to 15%
mg met-hGH/l culture	250-320 mg/l
Overall yield	125-160g met-hGH

Although met-hGH yields tended to decline variably at the end of log phase growth, this problem could be obviated by rapid (5 minute) cooling of the culture from 37°C to less than 10°C.

Accurate monitoring of met-hGH yields in culture required the development of a rapid reliable assay system free of the time constraints imposed by RIA or Rocket immunoelectrophoresis. A turbidometric assay was therefore developed employing sheep anti-hGH serum to precipitate authentic standard hGH or met-hGH in crude E.coli lysates in the presence of a non-ionic detergent such as Triton X-100. Monitoring the increase in A_{600} over 30 minutes at ambient temperature provided a rapid assay of hGH concentration in the range 0.1 to 16 mg/l. Data from this assay system, although consistently 20 to 30% lower than RIA or Rocket systems over the range 50 to 350 mg/l met-hGH was sufficiently rapid, sensitive and reliable to use in monitoring cultures and the initial extraction stages.

Early regulations required the killing of cells in the fermenter. In the case of met-hGH this could be accomplished, without loss or damage of this protein, by rapidly raising the pH from pH 7.0 (initial) to pH 11.5 to 12.0 with 10M NaOH. After 5 minutes exposure to this pH the count had fallen from 1.5×10^{10} viable cells/ml to about 6 viable cells/ml. It was noticeable however that at pH values below 11 the survival of E. coli was several logs higher and that at pH 11.5 E.coli survival was dramatically enhanced if cultures had previously been controlled in growth at pH 7.0 by the automatic addition of KOH or NaOH rather than, as normally, the addition of NH_3. Treatment at this pH resulted in lysis and release of met-hGH into the culture supernatent. Reduction of the pH to pH 7 to 8 caused precipitation of denatured proteins and a loss of 30 to 50% of the met-hGH which bound to the precipitated material. Lowering the pH to pH 8.5 reduced the loss but did not eliminate it and caused less precipitation of denatured protein. Complete recovery of met-hGH could be achieved by inclusion of non-ionic detergents, such as Triton X-100 or some of the Tween series, to final concentration above 0.1% in the alkaline lysis, followed by removal of denatured protein at pH 6.0 to 8.0

Met-hGH could be purified to homogeneity in a 4 step process from either a concentrated alkali whole culture lysate as above, or from pressure disrupted viable E.coli cells. Ammonium sulphate precipitation (20 to 50% sat^n.), followed by NaCl gradient elution on DEAE-sepharose at pH 8.5, gel filtration on Sephadex G-100 or AcA 44 at pH 8.0 and hydrophobic chromatography on phenyl-sepharose accomplished the purification. In the latter step the material was loaded (4 mg met-hGH per ml bed) at pH 8.0 in 1M NaCl and when unbound material had been removed met-hGH was eluted with water. The purification protocol is summarised in Table 2.