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Short Communications

Bacterial Lipopolysaccharides and Glutathione Mixed Disulfides as Possible Contaminants of Human Growth Hormone Produced with the Use of *E. coli* K12*

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In clinical trials of biosynthetically produced Human Growth Hormone (HGH) antibody production has been detected in some of the patients. Normal criteria of protein purity showed the protein to be homogeneous. Obviously, some component(s) in a very low concentration is/are attached to HGH and thereby make(s) some HGH-molecules antigenic. In this primary investigation two compounds were tested.

1. Lipopolysaccharides (LPS) from the outer membrane are known to be very "sticky" and also extremely antigenic when bound to a variety of biological molecules.[1]
2. Glutathione (GSH), known to be present in almost all living cells is to some extent "stored" as a mixed disulfide with different proteins in both eucaryotic and procaryotic cells. This for *E. coli* "excess-useless" bulk of protein (HGH) could be perhaps act as a storage protein for bacterial GSH, provided that the disulfide bonds of HGH are accessible for the mixed disulfide forming enzymes in the bacteria.

The amount by which these compounds remained after purification was analyzed in two different, but similar, sets of experiments. In the first case ^{14}C-labelled LPS were added to freshly prepared, HGH-containing, bacterial lysate and a purification was performed as a small scale copy of the industrial production. The lowest detectable amount of LPS in this system is 2–3 pmol (liquid scintillation counting). No radioactivity significantly higher than background was found, which after comparing with the amount of protein tested (20 nmol) shows that the highest possible concentration of LPS that could remain after purification is 120 ppm. Using alternative methods for radioactivity measurements where more sample could be used, and another criterion for "significantly higher than background" (twice the background was used here) would most likely lower this value. In the literature a corresponding value of 17 ppm is found to be antigenic in mice.[1] To what extent this value is transferable to humans, it is not possible to tell. A more sophisticated investigation needs to be done to be absolutely sure to eliminate LPS although the present investigation indicates that LPS are not the most likely compounds causing this antibody formation.

In the second case, mixed disulfide of HGH and ^{14}C-labelled GSH was synthesized and added to freshly prepared lysate as above. Two different mixed disulfide fractions were prepared and added in separate experiments. One with all four available SH-groups reacted and the other with only a fraction of the disulfides, preferably the C-terminal reacted. The purpose was to make one fraction "mixed disulfide saturated" and the other fraction similar to what could be produced *in vivo*. Details of the synthesis are described elsewhere.[2]

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Table 1. Distribution of radioactivity and HGH-activity after purification of HGH from lysate with HGH−GSH mixed disulfide (all four SH-groups reacted).

Radioactivity (cpm)[a]	
Added to lysate	320 000
Unretarded by first column	323 000
HGH-activity (IU)[b]	
Lysate	55
Added mixed disulfide	11
Total	66
Unretarded by first column	13
Eluted from first column	52
Total	65

[a] Radioactivity was measured by liquid scintillation counting. [b] HGH-activity was assayed using a Beckman Auto ICS rate nephelometer calibrated with the M33 gain card supplied with the instrument. The antibodies were raised in rabbits at Dakopatt A/S, Denmark using pituitary HGH (Crescormon, KabiVitrum) as antigen.

Table 2. Distribution of radioactivity among the different cysteine-containing peptides after reduction and labelling (^{14}C GSH) of HGH under *in vivo* conditions.[a] The sample was trypsin digested and the peptides separated on an RPC-column in an HPLC-system.[b]

Eluted peptide	Percentage radioactivity
42−64	13.5
159−167	10.8
179−183	27.6
184−191	26.8
Not retarded by column	14.7

[a] *In vivo* conditions in this investigation relate to the intracellular thiol concentration which for most cells is in the range of 1−10 mM. In these experiments reduction was performed with a dithiothreitol concentration of 3 mM at a pH of 7.5 and an HGH concentration equal to 0.4 mM. After removal of dithiothreitol with gelfiltration, labelling was performed by the addition of ^{14}C labelled glutathione disulfide, the excess of which was removed by another gelfiltration. [b] Trypsin digestion of the sample was performed essientally as described in (6). Peptide separation was done using a Waters μBondapac C18 column according to the manufacturers recommendation with a gradient made up from methanol and trifluoroacetic acid.

Table 1 shows the results from the addition to the lysate of HGH−GSH mixed disulfide, with all four SH-groups reacted, they indicate that all the "mixed disulfide saturated" ^{14}C-activity goes unretarded through the first column. At the same time, corresponding measurements of HGH-activity (Table 1) show that only 13 out of a total of 65 IU go unretarded through that column.

With the *in vivo* prepared mixed disulfide, the highest amount that could remain after purification was estimated to be 600 ppm(10 nmoles of protein tested, detection limit for GSH is 6 ppm). Whether HGH−GSH mixed disulfides can actually cause antibody production is not investigated at all and the relevance of the 600 ppm is therefore hard to evaluate. There are some investigations, however, concerning the biological activity when

the disulfide bonds of HGH are modified. If HGH is totally reduced and carbamidomethylated all the biological activity is retained.[3] Carboxymethylation instead of carbamidomethylation, on the other hand, gives no remaining activity.[4] Partial reduction and carboxymethylation of only the more readily reactive C-terminal disulfide does not interfere with the biological activity.[5]

Support for the finding of different reactivity of GSH towards the two disulfide bonds of HGH comes from the tryptic peptide separation of the *in vivo* prepared mixed disulfides. Table 2 demonstrates that the peptides originating from the C-terminal have incorporated twice the amount of radioactivity relative to the other disulfides, indicating that this is the most likely situation to occur *in vivo*. The different results depending on biological activity could be indicative for the behaviour of the immune system towards these molecules. The more substituted the HGH-molecules *i.e.* the larger the amounts of split disulfides and bound charged molecules, the greater the chance of forming biologically inactive as well as antigenic HGH-molecules. The possibility of bacteria creating "scrambled" HGH-molecules with mispaired intramolecular disulfide bonds, which could lead to formation of antigenic sites, is perhaps not very great but it can not altogether be ruled out.

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