Research Article

Biological Activity of Persian Sturgeon Recombinant Growth Hormone Molecules Trapped In Inclusion Bodies (IBs)

Nasr Ehsan[1*], Hovhannisyan Hrachya[2], Pourkazemi Mohammad[1]

[1] *Iranian Fisheries Research Organization, P.O. Box: 14155-6116 Tehran, Iran*
[2] *Scientific and Production Center "Armbiotechnology" NAS RA, Yerevan, Armenia*

Received 2 December 2016 Accepted 13 January 2017

Abstract

The biological activity of Persian sturgeon (PS) enfolded crude recombinant growth hormone (rGH) recovered from inclusion bodies (IB) estimated by administrating to fish fingerlings. The psrGH IBs expressed in *E. coli* were dissolved in cold guanidine HCl solution, immediately diluted ~1000 fold in cold sodium chloride 0.9% solution do not allow the rGH folding and administrated to fishes. It was revealed that intramuscularly injections of rGH at dosage of 0.5 µg/ g once a week for 8 weeks significantly accelerate the growth of fishes. Comparison of the mean growth rate and daily weight and length gain dates of Persian sturgeon fingerlings administrated by pure and crude grade rGH revealed the existence at list 2-5% potentially biologically active psrGH molecules in IBs. This fact enables to avoid the time and cost consuming processes of refolding and purification of rGHs.

***Keywords*:** Inclusion body, Persian sturgeon, Recombinant growth hormone, Biological activity

Introduction

The overexpression of recombinant proteins in industrially useful microorganisms showed, however, that recombinant protein did not form its native, soluble and bioactive conformation. Instead of native protein inactive aggregates (inclusion bodies) accumulated in the host cell (Rudolph and Lilie, 1996). Thus, the accumulated r-protein requires solubilization and folding steps prior to purification by chromatography, and the overall protein recovery is significantly affected by the efficiency of these pre-purification steps. Therefore, the accumulated proteins need to be solubilized using high concentrations of denaturants such as urea or guanidine hydrochloride (GnHCl), followed by removal of denaturants for protein refolding. Therefore, considerable efforts have been made to increase the efficiency of solubilization and refolding as a means of improving the overall recovery of biologically active r-protein from inclusion bodies.

However, expressing a protein in inclusion body form can be advantageous. Large amounts of highly enriched proteins can be expressed as inclusion bodies. Trapped in insoluble aggregates, these proteins are for the most part protected from proteolytic degradation (De Bernardez, 1998; Lilie et al., 1998; Misawa et al., 1999).

The major advantages associated with the formation of inclusion bodies are expression of a very high level of protein of the cellular, easy isolation of the inclusion bodies from cellular due to differences in their size, density and solubility as compared with cellular contaminants, lower degradation of the expressed protein, resistance to proteolytic attack by cellular protease and homogeneity of the protein of interest in inclusion bodies which help in reducing the number of purification step to obtain pure protein (Singh and Panda, 2005).

The general strategy used to recover active protein from inclusion bodies involves three steps: inclusion body isolation and washing; solubilization of the aggregated protein; and refolding of the solubilized protein. It is generally believed that recombinant therapeutic proteins to be properly folded and fully functional. However, recombinant proteins expressed in bacteria often are being made faster than they can fold into the native structure, accumulated in inclusion bodies (Marston, 1986; Schein, 1989). Although proteins trapped in insoluble inclusion bodies (IBs) are generally believed to be misfolded and inactive (Baneyx and Mujacic, 2004), some of current research no longer supports this assumption.A growing number of studies in the scientific literature describe IBs as

Corresponding authors E-mail:
* *Nasr_ehsan1357@yahoo.com*

entities formed by functional protein species with native secondary structure. Furthermore, the structural and functional diversity of the model proteins used in these studies leaves little room to speculate about these observations being artifacts or peculiarities of certain protein species (Garcia-Fruitos et al., 2007; Garcia-Fruitos et al., 2007
The aim of this study was evaluation of biological activity of psrGH from IB's by administration to fishes immediately after GnHCl solubilization without folding.

Materials and Methods

The recombinant *E. coli* DE3(rGH) was constructed earlier by our laboratory.

The psrGH butch fermentation in *E. coli*:

An aliquot (1 ml) of overnight Persian sturgeon recombinant Growth hormone (psrGH) *E. coli* DE3(rGH) overnight culture was inoculate in 10 ml of LB-broth containing 50 µg/ml ampicillin and grow at 37 °C by intensive aeration. After 2 hours of growth 100 µl IPTG was added to culture to induce rGH expression and incubation continued for 4 hours.

Inclusion body separation and rGH recovery

The cells harvested, suspended in 20 ml of 20 mM phosphate buffer (pH 7.2), and lysed by lyzocim. The resulting homogenate was centrifuged at 8000 rpm for 40 min at 4°C, and the pellet was washed with 20 m1 of 1 m sucrose and then with 20 ml of 4% Triton X-100, 20mM phosphate buffer, and 1 ml EDTA (pH 7.2) to remove soluble components, bacterial cell wall and cell membrane, and lipid components. The IBs were dissolved in cold guanidine HCl solution, diluted immediately ~1000 fold in cold sodium chloride 0.9% solution to minimize solvent concentration and do not allow the rGH refold and store in cold.

Fish rearing and rpsGH administration:

The fingerlings held indoors in pools (120 × 60 × 65 cm) filled with 400 l fresh water. Fresh water from a common reservoir supplied circularly, after filtration, at a running speed of 500-600 ml/min. Fish kept on a natural photoperiod at 23 - 26°C for 2 weeks before the study began. Each group of fish fed with commercial fish food pellets an amount of food equal to 3% of their total body weight twice daily. The IB suspension and purified hormone were intramuscularly injected to fishes once a week for 8 weeks.

The correlation index (CI)

CI was calculated by formula CI= weight/length

Statistical analyses:

Duncan's new multiple-range test (randomized block design). The 95% confidence level ($P < 0.05$) was used unless otherwise stated. Growth rates were compared using a one-way analysis of variance ANOVA (SPSS).

Results

The psrGH IBs dissolved in guanidine HCl solution and immediately diluted 1000 time in sodium chloride 0.9% solution to minimize Gnd HCl impact and not allow the rGH fold. A total of 60 PS fingerlings were randomly divided into four groups, with averages of 20±0.15 g for body weight and 17±0.2 cm for fork length and reared. One of the groups was control one next group was made intramuscularly injections of solubilized IB crude grade PS GH by dosage of 0.5 µg/g and the rest two groups were administrated of purified native rGH 0.01 and 0.05 µg/g dosages every week for 8 weeks. The morphometric characteristic (body weight and length) of fishes were recorded at the start of trial then every fourth week over 8-week period. The mean weight and length of fishes are presented in table 1.

Table1. Body weight and length of PS fingerlings injected by putre refolded rGH and crude grade solvents from IBs, over 8 weeks cultivation. (W- mean weight (g) and L - length (cm) of fishes. rGH IB – rGH from inclusive bodies, prGH – purified native rGH)

Cultivation period	Recombinant growth hormone dosage (*µg*/g)							
	Control		rGH IB 0.5		pnrGH 0.01		pnrGH 0.05	
	W	L	W	L	W	L	W	L
At the start	20.0	17.4	20.0	17.2	20.1	17.0	20.4	17.3
First 4 weeks	28.9	19.4	38.9	21.0	36.6	20.9	40.9	21.3
Second 4 weeks	35.1	20.7	44.7	22.5	42.2	22.3	49.0	23.0

Research Article

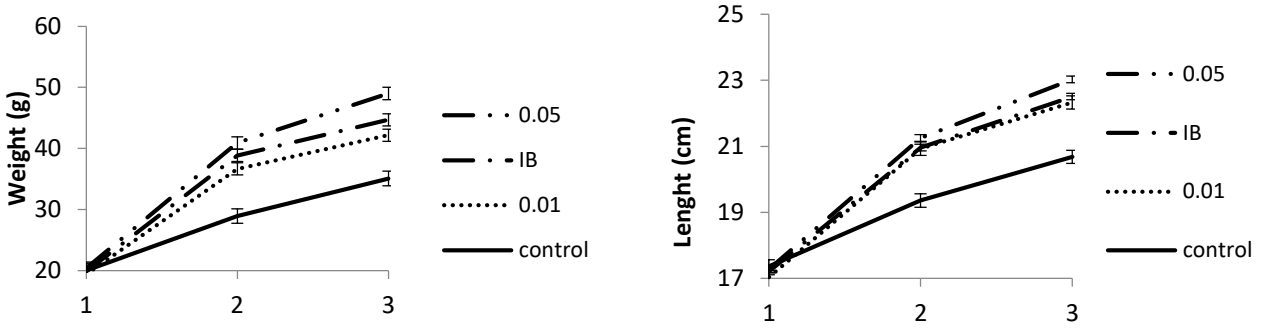

Figure 1. The influence of PS rGH administration on weight and length growth of fishes

At the end of 8th week, fish from groups received rGH and control group showed significant differences ($P < 0.01$) in weight and length (Table. 2). It was notable that growth enhancement of this treated groups were actually evident at the eight week Thus, after 8 weeks of rGH IB receiving group, the mean weight and length were 27.35% and 8.65% more than control group. Furthermore, the mean weight of groups 0.05 µg/g and 0.01 µg/g of pnrGH were respectively, 39.7% and 20.3% more than control group and the mean length of group groups 0.05 µg/g and 0.01 µg/g were respectively, 11.4% and 8.0% more than control group. At the end of experiment, the survivability of all fishes was 94 %. The daily weight increase of control fishes for 4 weeks was 144% while the increase weight of fishes administrated with dose 0.5 µg/g rGH IB, 0.01, 0.05µg/g pnrGH were 194.5%,188 % and 200% respectively but for the next 4 weeks were 115%, 115%, 120% and control was 121%. Length increase of control group for 4 weeks was 111% while the increase weight of groups administrated were 122%, 122%, 123% respectively but for the next 4 weeks were 107%, 107%, 108%, respectively, in groups administrated and control 106.7 %. Significant acceleration of fishes growth was observed for the first 4 weeks GH administration while the following administrations influence was no significant. It was revealed that once a week intramuscularly administrations of dissolved IB suspensions, were effective for first four weeks, after that the daily gains of treated and untreated fishes are not significantly differ (Fig. 1).

IB curve located between the curves of purified GH doses of 0.01 and 0.05 µg/g. As shown in Fig. 1 and 2 the first GH administration significantly increase growth rate of fishes for first 4 weeks but the 5th and following administrations were not show any influence on growth rate of fishes for the next 4 weeks comparing in control group.

Statistically analysis confirmed the trustworthiness of differences between the mean weight and length among group administrated with control group. Thus, one can therefore conclude that, unfolded IB solution accelerate the weight and length growth of Persian sturgeon. Due to the lack of appropriate standards in this bioassay, the activity of the psrGH IBs was quantified by extrapolation of the dates from growth curves of rGH administration.

The comparative studies of growth curves revealed that that the 0.5 µg/g dose of crude unfolded rGH solution possesses biological activity between 0.1 and 0.3 doses of purified and refolded hormone (Fig, 1).

Table 2. One-way analysis of variance ANOVA of the trial dates of weight and length.

ANOVA		Sum of Squares	Df	Mean Square	F	Sig.
Weight	Between Groups	22268.577	2	11134.288	291.861	.000
	Within Groups	7782.447	204	38.149		
	Total	30051.024	206			
Length	Between Groups	495.008	2	247.504	150.216	.000
	Within Groups	336.122	204	1.648		
	Total	831.130	206			

*Df- degrees of freedom; F- frequencies; Sig- signify

Thus IBs contain about 2-5% biologically active psrGH molecules or they are folded in fish body fluid. This finding enables to avoid the time and cost depending process of refolding and purification of rGHs from inclusion bodies.

Discussion:

Solubilization and folding of inclusion body proteins into bioactive forms is cumbersome, results in poor recovery and accounts for the major cost in production of recombinant proteins from prokaryotic organisms. Our results and other authors date shows that the yield GH is about 30% of total protein synthases in *E. coli*. During solubilization of IB, dialyse and purification a huge amount of recombinant molecules are lost. Because of, the previous purification GH is the main factor making its use more expensive and prohibitive to be used in aquaculture.

It was therefore necessary to develop more cost effective production of functionally active therapeutic proteins preparations for parenteral administration in crude grade form without the need for the proteins renaturation and purification. Many researchers describe IBs by functional protein species with native secondary structure (Garcia-Fruitos *et al*., 2007; Garcia-Fruitos *et al*., 2007; Jevsevar *et al*., 2005). Recent reviews in this area have reported IBs containing properly folded proteins (Doglia *et al*., 2008; Ventura and Villaverde, 2006), casting doubt on the paradigm of considering recombinant protein solubility as equivalent to protein conformational quality (Baneyx and Mujacic, 2004; Barannikova, 1987). However, none of them evaluated the possible effects of GH IBs crude preparations.

In this study for the first time was evaluate the effect of intramuscular administration of solubilized IB crude grade GH molecules to PS fingerlings and found significant increase in weight and in length of young fishes. From our date IBs demonstrated 20-30 fold less active than folded and purified GH, indicating on existence of 3-5% bioactive rGH proteins in intact inclusion bodies. Estimation of daily gain of weight and length revealed that once of week intramuscularly administrations of IB are effective during first four weeks, after that the daily gains are not significantly differ from control fihes.

Conclusion

For the first time demonstrated that crud IB hydrolysates 0.5 µg/g b.w. intramuscular administration accelerates weight and length growth of PS fingerlings equal with effectiveness of ~0.02 µg/g b.w. of purified rGH, indicating on the existence of about 2-5% bioactive recombinant GH molecules in IB aggregates.

Acknowledgements

This study was supported by the International Sturgeon Research institute Rast, Iran.

References

1. Baneyx F. and Mujacic M. (2004) Recombinant protein folding and misfolding in Escherichia coli. Nature Biotechnology. 22: 1399 – 1408.
2. Barannikova, I. A. (1987) Review of sturgeon farming in the Soviet Union. Journal of Ichthyology, 27: 62–67.
3. De Bernardez C.E. (1998) Refolding of recombinant proteins. Current Opinion in Biotechnology, 9: 157–163.
4. Doglia S., Ami D., Natalello A., Gatti-Lafranconi P.and Lotti M. (2008) Fourier transform infrared spectroscopy analysis of the conformational quality of recombinant proteins within inclusion bodies. Biotechnology Journal,3: 193-201.
5. Garcia-Fruitos E., Aris A. and Villaverde A. (2007) Localization of functional polypeptides in bacterial inclusion bodies. Applied and Environmental Microbiology, 73: 289-294.
6. Garcia-Fruitos E. Martinez-Alonso M., Gonzalez-Montalban N., Valli M., Mattanovich D., and Villaverde A. (2007) Divergent genetic control of protein solubility and conformational quality in *Escherichia coli*. Journal of Molecular Biology, 374: 195-205.
7. Jevsevar S., Gaberc-Porekar V., Fonda I., Podobnik B., Grdadolnik J. and Menart V. (2005) Production of nonclassical inclusion bodies from which correctly folded protein can be extracted. Biotechnology Progress, 21: 632–639.
8. Lilie H., Schwarz E., and Rudolph R. (1998) Advances in refolding of proteins produced in *E. coli*. Curr. Opin. Biotechnology, 9: 497–501.
9. Marston F.A.O. (1986) The purification of eukaryotic polypeptides synthesized in *Escherichia coli*. Biochemical Journal, 240(1): 1-12.
10. Misawa S. and Kumagai I. (1999) Refolding of therapeutic proteins produced in *Escherichia coli* as inclusion bodies. Biopolymers, 51: 297-307.

11. Rudolph R. and Lilie H. (1996) In vitro folding of inclusion body proteins. The Journal of Federation of American Societies for Experimental Biology, 10: 49-56.
12. Schein C.H. (1989) Production of Soluble Recombinant Proteins in Bacteria. Nature Biotechnology, 7: 1141 – 1149.
13. Singh S.M. and Panda A.K (2005) Solubilization and refolding of bacterial inclusion body proteins. Journal of Bioscience and Bioengineering, 99: 303–310.
14. Ventura S. and Villaverde A. (2006) Protein quality in bacterial inclusion bodies.Trends Biotechnol. 24(4): 179-185.