REPORT

Biological activity in vitro *and* in vivo *of an* in silico *designed secretagogue peptide to be used in fish*

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ABSTRACT

In teleost fish, secretion of the growth hormone (GH) is regulated by several hypothalamic factors that are influenced by the physiological state of the animal. GH in fish is involved in many physiological processes that are not overtly growth-related such as: saltwater osmoregulation, antifreeze protein synthesis, and the regulation of sexual maturation and immune functions. This study was conducted to characterize a decapeptide A233 (GKFDLSPEHQ) designed by molecular modeling to evaluate its function as a GH secretagogue (GHS). In pituitary cell culture, the peptide A233 induces GH secretion and it is also able to increase superoxide production in tilapia head-kidney leukocyte cultures. This effect is blocked by preincubation with the GHS receptor antagonist [D-Lys3]-GHRP6. Immunoneutralization of GH by addition of anti-tilapia GH monoclonal antibody blocked the stimulatory effect of A233 on superoxide production. These experiments suggest a GH-mediated mechanism for the action of A233. The *in vivo* biological action of the decapeptide was also demonstrated for growth stimulation in goldfish and tilapia larvae ($p < 0.001$). Superoxide dismutase levels, antiprotease activity, and lectin titer were enhanced in tilapia larvae treated with this novel molecule. The decapeptide A233 is able to function as GHS in teleosts and enhance parameters of the innate immune system in the fish larvae. This study won the Annual Award of the Academy of Sciences of Cuba in 2012.

Keywords: growth hormone secretagogue, growth hormone, tilapia, teleost fish, GHRP-6, innate immune system, fish larvae

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RESUMEN

Demostración de la actividad biológica *in vitro* e *in vivo*, de un péptido secretagogo diseñado *in silico* para ser usado en peces. La secreción de la hormona de crecimiento (HC) en peces teleósteos es regulada por varios factores hipotalámicos que dependen de su estado fisiológico. Ella interviene en procesos fisiológicos asociados indirectamente con el crecimiento de estos animales, como la osmorregulación de agua salada, la síntesis de proteínas anticongelantes, y la regulación de la maduración sexual y de las funciones inmunológicas. En este estudio se caracterizó un decapéptido denominado A233 (GKFDLSPEHQ), diseñado por modelación molecular, y se evaluaron sus efectos como secretagogo de la HC (SHC). En cultivos de células pituitarias, este péptido indujo la secreción de la HC e incrementó la producción de superóxido dismutasa en cultivos de leucocitos de la cabeza del riñón: efecto bloqueado al preincubar las células con el antagonista de SHC [D-Lys3]-GHRP6. La inmunoneutralización de la HC por la adición de un anticuerpo anti-HC de tilapia bloqueó la producción de superóxido dismutasa estimulada por el péptido A233. Ello sugiere que la acción de este péptido está mediada por la HC. También se demostró su actividad biológica *in vivo*, mediante la estimulación del crecimiento en larvas de *goldfish* y de tilapia ($p < 0.001$). Los niveles de superóxido dismutasa, de actividad antiproteasa y los títulos de lectina aumentaron en larvas de tilapia tratadas con esta molécula. El decapéptido A233 funciona como SHC en peces teleósteos, y potencia los parámetros de la respuesta inmune innata en sus larvas. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba, en el año 2012.

Palabras clave: secretagogo de la hormona de crecimiento, hormona de crecimiento, tilapia, peces teleósteos, GHRP-6, sistema inmune innato, larva de pez

*I*ntroduction

Growth hormone (GH) has pleiotropic functions in all vertebrates. In addition to its essential role in the regulation of body growth and development, it can also influence reproduction, immunity, osmoregulation, and behavior [1]. In teleosts, secretion of GH is regulated by several hypothalamic factors that are influenced by the physiological state of the animal. Besides the pituitary gland, the GHgene is also expressed in other tissues of fish, especially in lymphoid organs and cells [2].

The synthetic GH secretagogues (GHSs) consist of a family of ligands, first described by Momany *et al*. [3]. GHS bind to a receptor inducing calcium mobilization, as identified by [4] in pigs and humans. GHS

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receptor (GHSR) has been identified in teleost fish and birds [5]. More than one receptor has been proposed for zebrafish, catfish, and goldfish [6]. Ghrelin is the endogenous ligand of this receptor. GHSR is expressed predominantly in the brain and pituitary, but it is also expressed in many peripheral organs including immune system cells. GRLN modulates the immune system [7] and it has been shown to stimulate phagocytosis in fish leukocytes, and this effect is mediated in part by GH secreted by leukocytes [8].

The A233 molecule was described by the molecular modeling of the human GRLN receptor using combined techniques of homology modeling, molecular dynamics, and exhaustive conformational search techniques. Then, a virtual library was built with several thousands of structures having such characteristics, to perform a conformational analysis. Afterwards, a massive docking experiment was performed against the receptor model. The aim of this study was to assess the biological activity of synthetic peptide A233 as a stimulator of growth and the innate immune system of teleost fish, through studies performed *in vitro* and *in vivo*.

Results and discussion

To evaluate the effect of the A233 peptide on GH secretion, we performed an *in vitro* culture of cells in the pituitary gland of tilapia (*Oreochromis* sp.), at a concentration of 10 nM stimulated GH secretion by these cells after an 8-h treatment. Owing to the importance of phagocytic cells in the immune response in fish, we evaluated the *in vitro* effect of A233 peptide in superoxide anion production in phagocytic leukocytes isolated from tilapia (*Oreochromis* sp.). The increase of superoxide anion production was statistically higher in cells stimulated with the highest dose of the peptide tested (10 nM). In this study, we demonstrated, for the first time, the effect of peptidic molecules having internal cycles and composed solely of L-amino acids that are capable of exerting, due to their chemical structure, similar functions to those attributed to GRLN, des-acyl GRLN and other peptidic GHS. Our results are alike to those obtained in tilapia (*Oreochromis mossambicus*), where the effect of GRLN on GH secretion *in vitro* was dependent on the concentration of the endogenous secretagogue used [9].

In teleosts, the morphology of the anterior kidney resembles the bone marrow of higher vertebrates and is a major hematopoietic organ where phagocytic cells are formed [10]. Phagocytic cells produce reactive oxygen species (ROS) such as superoxide anion, which help to eliminate many of the pathogens and parasites that infect these animals. The superoxide anion is a ROS produced by the NAPH oxidase complex and are well-known central components in the antimicrobial arsenal of activated phagocytes [11, 12].

Leukocytes isolated from tilapia anterior kidneys were treated with the specific antagonist secretagogue receptor [D-Lys3]-GHRP6 and subsequently stimulated with peptide A233. The stimulatory effect of peptide A233 on the increased production of superoxide anion was inhibited by pretreatment with the antagonist, suggesting that the A233 peptide action on tilapia leukocytes (*Oreochromis* sp.) is mediated by the GHSR1a secretagogue receptor. Previous studies showed that the effects of GHSs on immune cells may be mediated by the action of GH produced by these cells. The stimulatory effect of A233 on superoxide production was abolished by immunoneutralization with an anti-tiGH mAb mixture, suggesting the importance of GH secreted by leukocytes, as described by Yada *et al*. [8], indicating that the effect of these peptides is mediated through local production of GH.

The value of GHSs as useful growth enhancers is clear. These synthetic peptides are effective in stimulating production and release of endogenous GH as a physiological response, with no side effects on the pituitary or toxicity potential. Besides, their low molecular weight provides them enhanced biodistribution.

Considering the results mentioned above, it was also evaluated the biological function of the synthetic peptide A233 on somatic growth of tilapia (*Oreochromis* sp.) and goldfish larvae (*Carassius auratus*). Tilapia larvae showed a significant increase in growth at 20 and 30 days of treatment with peptide A233 (0.1 mg/L). All animals received the same commercial diet, so the increase in weight and height is due to the administration of peptide A233.

Its effects on innate immunity were additionally investigated. Innate immunity is the first line of defense against pathogens that infect fish [13], and fundamental in fish embryos and larvae, since they lack acquired immunity. In this sense, some parameters of the innate immune response in larvae treated with the peptide A233 were assessed, such as: title of lectins and antiprotease activity. Additionally, the enzymatic activity of SOD was determined in homogenates of larvae treated with peptide A233 as an indicator of antioxidant defense.

The treated larvae exhibited a better growth rate as well as an enhancement on some innate immune response parameters, improving the larvae quality. It could potentially give them a higher resistance to pathogens and better efficient adaptive response due to the cross talk between innate and acquired immune responses. Further experiments should be conducted to verify how the adaptive response is affected in fish with an enhanced innate immune response due to A233 administration. The parameters of the innate immune response are highly variable, and the application of an immunostimulant allows priming of the innate immune response in the larvae population. The antioxidant SOD activity was also increased in larvae after treatment.

Additionally, administration by immersion baths of recombinant tilapia neuropeptide Y in African catfish larvae (*Carassius gariepinus*) increased the concentration of reduced glutathione and SOD activity, without producing any effect on the activity of catalase [14]. Also, in larvae of tilapia treated with recombinant truncated tilapia GH, SOD and catalase activities were increased [15].

Such a rise in antioxidant defenses may neutralize deleterious byproducts of metabolism and counteract the oxidative stress associated with growth. The results obtained in this research associated with the parameters of the innate immune response and antioxidant defense of tilapia larvae indicate, for the

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first time, the role of the A233 peptide as a stimulator of the immune system in tilapia (*Oreochromis* sp.). Stimulation of growth directed to reduce the time of harvest and high mortality at fish larval stages and the use of immunostimulants to prepare them to cope with intensive farming are in line with goals of modern biotechnology.

Conclusions

The delivery of A233 by immersion baths to fish larvae stimulates growth, and due to the action of GH, it positively affects various parameters of the innate immunity, as evidence of the relationship between the immune and endocrine systems in fish.