Effects of enzymatic hydrolysate from seahorse Hippocampus abdominalis on testosterone secretion from TM3 Leydig cells and in male mice

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Abstract Protein hydrolysates, the so-called bioactive peptides, are specific protein fragments that have positive effects on several body functions and may improve human health. Marine organism-derived protein hydrolysates and bioactive peptides have shown to possess many physiological functions. Seahorses, used in traditional medicine, are generally ground to powder form either for direct application or for application following dissolution in warm water. In this present study, we purified, hydrolyzed, and characterized two bioactive peptides (ALC and PEP) obtained from seahorse (Hippocampus abdominalis). The hydrolysates derived from seahorse significantly upregulated the expression of cyclin D and cyclin E and increased cell proliferation and testosterone level in the TM3 mouse Leydig cell line. These findings suggest that the hydrolysates stimulate the proliferation of TM3 cells via the AKT, ERK, and JNK pathways. The decline in the circulating testosterone levels in older men is associated with various adverse health effects. Following daily intake of hydrolysates for 12 weeks, the circulating level of testosterone and the sperm count in mice were measured. We found increased sperm motility (sperm count) as well as an increase in the testosterone level in male mice following a 12-week intake of hydrolysates derived from H. abdominalis. Hence, it can be suggested that seahorse-derived hydrolysates play an important role in improving male health by improving the serum testosterone level.

Keywords Bioactive peptides · Hydrolysates · Hippocampus abdominalis · Leydig cells · Testosterone

Introduction

The seahorse is a marine teleost fish well known for its unusual characteristic feature (male pregnancy) and its unique medicinal properties. Medicinal properties of many animals in the Syngnathidae family, including seahorses, are well documented in all versions of China Pharmacopoeia including those from the Liang dynasty (A.D. 502–557). Several recent studies have shown that seahorse derivatives have multiple biological activities, including appetite stimulation, anti-tumor, anti-inflammatory, anti-aging, anti-fatigue, and anti-platelet activities (Zhang et al. 2003; Himaya et al. 2012). Hippocampus abdominalis (Lesson 1827) is one of the most commonly available seahorse species used in traditional medicine (Vincent 1995; Vincent et al. 2011). Previous studies on the seahorse bioactive components mainly focused on analysis of trace elements, amino acids, and organic solvent-soluble components (Vincent 1995; Vincent et al. 2011).
Marine organisms are an excellent source of bioactive compounds. Many studies have shown that marine bioactive peptides have positive effect on health (Ryan et al. 2011; Ibáñez et al. 2012). These peptides were derived from marine organisms by one of the three methods such as solvent extraction, enzymatic hydrolysis, and microbial fermentation (Kim 2013). The enzymatic hydrolysis of various proteins may act as potential modulators of metabolism during digestion (Rutherford-Markwick and Moughan 2005). In enzymatic protein hydrolysis, the specific type of the enzyme is important because it determines the cleavage pattern of the specific peptide bonds (Kim 2013). There are many enzymes, both natural (trypsin, pepsin, and proteinase K) and commercial (alcalase and flavourzyme) protease (Ketnawa et al. 2014). Bioactive peptides generally contain 3–20 amino acid residues per molecule, and their activity depends on their respective amino acid composition and sequence. Previous other studies reported food protein as a source of peptides with a diverse range of bioactivities, like antioxidant, anticancer, and cholesterol-lowering effects (Erdmann et al. 2008; Shahidi and Zhong 2008). Currently, there is a continuous search for marine-derived hydrolysates for its role as functional food ingredient and in prevention of diseases (Lordan et al. 2011).

Testosterone is the principal hormone secreted by the interstitial cells of Leydig of the testis and plays an important role in maintaining male health, by promoting spermatogenesis and improving muscle mass and physical strength in a dose-dependent manner (Bhasin et al. 2001; Ibebunjo et al. 2011; Sattler et al. 2011; Schwartz and Holtorf 2011; Horstman et al. 2012; Nigro and Christ-Crain 2012). Serum testosterone levels decline progressively with aging resulting in certain pathophysiological changes. The decline in testosterone with aging is related to decreases in both hypothalamic and testicular function, and in the number of testicular Leydig cells (Matsumoto 2002). Recent studies have shown that replacement of testosterone improves the symptoms of andropause in aging males such as decrease in lean muscle mass and osteopenia (Steidle et al. 2003; Barqawi and Crawford 2006). Testosterone is the major androgen and the Leydig cells of the testes are known to be the primary source of androgens (Barqawi and Crawford 2006; Steidle et al. 2003). The number of testicular Leydig cells decreases with age, resulting in decreased testosterone production (Griswold and Behringer 2009; O’Shaughnessy and Fowler 2011).

The objective of the present study was to investigate the effects of ALC and PEP, hydrolysates derived from seahorse by enzymatic hydrolysis, on mouse Leydig (TM3) cells and male mice. In addition, cellular- and molecular-regulating mechanisms of ALC and PEP hydrolysates in enhancement of the testosterone production were investigated.

**Materials and methods**

**Cell line**

Mouse Leydig cells (TM3 cells) were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). TM3 cells were cultured in Dulbecco’s Modified Eagle Medium (Corning Cellgro, Manassas, VA, USA) supplemented with 10% Fetal Bovine Serum (heat inactivated FBS; Corning Cellgro, Manassas, VA, USA) in a 95% humidified chamber with 5% CO₂ at 37 °C. The culture medium was changed at an interval of 2 days.

**Preparation of H. abdominalis protease enzymatic hydrolysates**

The dried seahorse powder samples (5 gm) were mixed with a volume of 100 ml of distilled water and it was then hydrolyzed with pepsin and alcalase (substrate: enzyme ratios of about. 100:1) at 37 °C for 24 h. At the end of 24 h, the hydrolysates were adjusted to pH 7.0 and the enzymes were inactivated at 100 °C for 10 min in a water bath. Then the hydrolysates were then centrifuged for 20 min at 10,000 rpm and the resulting soluble and insoluble fractions were separated. The soluble fraction was dried using a freeze dryer and stored at −20 °C for further use.

**MTT viability assay**

In order to determine the toxicity of hydrolysates, we performed the MTT assay. Cells were treated with different concentrations of the hydrolysates at 0.05, 0.2, 0.5 and 1 mg/mL and were incubated for 24 h. To check antioxidant activity, cells were treated with seahorse-derived hydrolysates and H₂O₂ for 2 h with 20 µL of MTT (5 g/L in PBS) reagent added to each well, and the plates were incubated for 4 h. One hundred and fifty µL DMSO was added to each well, which was followed by shaking for 20 min. The optical density of supernatant was measured at 570 nm (Bui et al. 2014; Kwon et al. 2016).

**Western blot analysis**

Cellular protein extracts were prepared. TM3 cells were washed with cold, sterile PBS, and lysed in RIPA lysis buffer (with 1 mM PMSF, Biosesang, Seongnam, Gyeonggi, Korea), sonicated briefly, and centrifuged for 20 min at 13,000 rpm at 4 °C. A BCA assay (Pierce Biotechnology, Rockford, IL, USA) was used to measure the protein concentration of the cell lysate. Lysates containing equivalent amounts of protein were analyzed by 10–12% sodium dodecyl sulfate polyacrylamide
electrophoresis. Proteins separated by SDS-PAGE were transferred to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Billerica, MA, USA). The transfer occurred over 1–2 h at 100 V. The membranes were blocked with 5% nonfat dry milk in TTBS for 1–2 h at room temperature or overnight at 4 °C. Next, primary and secondary antibodies were applied. The following primary antibodies were used: anti-cyclin D, anti-cyclin E, anti-p38MAPK, anti-pERK1/2, anti-JNK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-pJNK, anti-pP38MAPK, anti-GAPDH, anti-AKT, anti-pAKT, and anti-ERK1/2 (Cell Signaling, Danvers, MA, USA). The membranes were washed with TTBS and incubated with secondary antibodies at room temperature for 30 min. Blots were developed using an enhanced chemiluminescence ECL kit (LPS solution, Daejeon, South Korea).

**Cell proliferation assay with the electric cell-substrate impedance sensing (ECIS) system**

Electric cell-substrate impedance sensing (ECIS) is a real-time, impedance-based method for assessing the activities of TM3 cells grown in cell culture. TM3 cells were added to the electrode arrays, which were attached to the electrode sensor surface, which act as insulators and increase the impedance and resistance response. Growth curve data were plotted in the impedance versus time curve (Dudek et al. 2010). For ECIS experiments, cells were seeded on the electrode plates. After incubation for 24 h, cells were treated with 0.5 mg/mL concentration of hydrolysate and the electrode arrays were placed in plate holder plates in the incubator and connected to the ECIS system. TM3 cell proliferation analysis was performed using the ECIS system software (Applied Biophysics, Troy, NY, USA) (Szulcek et al. 2014).

**Animals**

Male ICR mice were purchased from Orient Bio (5 weeks of age, Orient Bio Inc., Seongnam, Gyeonggi, Korea). The animals were acclimatized for at least 1 week before beginning the experiments. The animals were housed at room temperature (23 ± 2 °C) with constant humidity (55 ± 15%) on a 12 h light/12 h dark cycle, with free access to food and water. After 7 days of adaptation, eligible animals were randomly divided into groups of 10 mice each, including negative (injected with PBS only) and positive (seahorse-derived hydrolysate) control groups. Twenty mice were orally administered 25 mg/kg hydrolysates (ALC and PEP) daily for 12 weeks as the experimental group. Another 10 mice were orally administered with PBS daily for 12 weeks as the control group (Pang et al. 2015).

**Measurement of the number of sperm**

Male mice were weighed and anesthetized with ether on the next day after final dosing. Numbers of sperm heads in the testis were counted by a slightly modified method by Toth et al. (1989) by collecting sperm containing fluid from the cauda, which was later cut into several pieces. The sperm fluid and pieces of cauda were resuspended in PBS buffer, and the sperm count was assessed by determining the percentage of motile sperm (World Health Organisation 1999).

**Blood collection and tissue removal**

Blood and tissue samples were collected between 9:00 and 12:00 a.m. Mice were anesthetized with ether and exsanguinated by closed cardiac puncture. Sera were collected and stored frozen at −70 °C until further use. The testes and epididymides were removed rapidly and weighed (Liu et al. 2016).

**Measurement of serum testosterone**

The amount of testosterone in the TM3 cell media or blood samples collected from male mouse were measured by an ELISA kit from Gentaur (YHB1275Mo, Brussels, Belgium) as per the manufacturer’s instruction. The results were expressed as nmol/L (concentration of testosterone) as read from standard curves.

**Testicular histology**

Immunohistochemistry was performed using a standard protocol. Paraffin-embedded 3-μM sections were processed and stained with hematoxylin and eosin (H&E). Tissues from the right testis were fixed in Bouin’s fluid and stained with H&E stain. The tissues were dehydrated using a graded ethanol series, washed in xylene, and embedded in paraffin wax for sectioning. Samples were examined under the microscope (Olympus B ×51 microscope, Tokyo, Japan). The sections from each animal were evaluated for structural changes (Akpanah et al. 2003; Mahabadi et al. 2013).

**Statistics**

The data are expressed as the mean ± SD. The data were entered into a spreadsheet (Excel, Microsoft, Redmond, WA, USA and GraphPad Prism version 6.0, GraphPad Software, La Jolla, CA, USA) and the differences between
the control and seahorse-derived hydrolysate groups were examined by independent Student’s t test or by ANOVA.

Results

Seahorse-derived hydrolysate enhanced survival of TM3 cells

To evaluate the effects of ALC and PEP, mouse Leydig cells (TM3 cell) were incubated with seahorse-derived hydrolysate. TM3 cells were treated with 0.2, 0.5 and 1 mg/mL hydrolysate for 24 h. Hydrolysate (ALC and PEP) treatment at these concentrations significantly increased cell viability (Fig. 1A).

In order to confirm these results, we performed the proliferation assay with the ECIS system using 0.5 mg/mL concentrations of hydrolysate. The results were plotted in the impedance versus time curve. As expected, ALC and PEP significantly enhanced the proliferation of TM3 cells after 24 h compared with the control (Fig. 1B). This means that the area of electrode occupied by cells was increased but it does not state us whether the increase is caused by cell proliferation or cell size expansion.

To check if the increase is due to cell proliferation, we examined cell cycle-related proteins. Two cell cycle kinase
complexes, Cyclin D/cdk4-6 and Cyclin E/cdk2, are activated during the G1 phase of cell cycle (Bertoli et al. 2013). The expression levels of cyclin D and cyclin E were measured by western blot analysis using specific antibodies (Fig. 1C, D). The TM3 cell cycle response was determined following treatment with ALC and PEP at various concentrations (0, 0.2, and 0.5 mg/mL). The protein expression levels of cyclin D and cyclin E increased following treatment with ALC and PEP for 24 h (Fig. 1C). These results suggest that seahorse-derived hydrolysate promotes TM3 cell proliferation by modulating the cell cycle-related proteins.

At the level of the testes, oxidative stress is capable of disrupting the sperm-producing capacity (spermatogenesis) of Leydig cells as well as the capacity of the germinal epithelium to differentiate between normal and abnormal spermatozoa (Naughton et al. 2001; HALES et al. 2005). Many attempts have been made to improve male fertility and many experiments using natural antioxidant from plant extract have been performed to elucidate the beneficial effects of some natural products on semen quality and on the function of reproductive system. We investigated the antioxidant effects of hydrolysate (ALC and PEP) on H$_2$O$_2$-induced oxidative stress in cultured mouse TM3
Leydig cells (Fig. 2). In order to check the protective effects of hydrolysate against H$_2$O$_2$-induced cell damage, the TM3 cells were pre-treated with hydrolysate (0.05, 0.1, 0.2, 0.5, and 1 mg/mL) for 2 h and then exposed to H$_2$O$_2$ (1 mM) for an additional 24 h. The results revealed that pre-treatment with ALC and PEP protected the TM3 cells from subsequent H$_2$O$_2$-induced damage in a concentration-dependent manner (Fig. 2). These results showed the ability of the hydrolysate maintaining the state of TM3 cells healthy against oxidative stress.

**Effect of seahorse-derived hydrolysate on cell signaling pathways**

The regulation of cell growth and proliferation through multiple signaling pathways is primarily regulated by external signals provided by the surrounding cells. Mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) pathway is reported to be associated with the cell proliferation (Zhang and Liu 2002). In addition, the MEK/ERK pathway affects the proliferation and survival of cells in primary cultures of Leydig cells (Mendoza et al. 2011). Another key pathway responsible for controlling cell survival, division, and metabolism is the PI3 K-mTOR-AKT pathway.
Therefore, we tested whether peptide treatment affected the expression and phosphorylation of ERK, JNK, p38MAPK, and AKT. The hydrolysate induced activation ERK and AKT, but did not induce JNK and p38MAPK. Figure 3A shows that peptide treatment induced ERK and AKT phosphorylation. We tested whether the cell growth effects of hydrolysate was mediated via ERK and AKT. Cell proliferation assays were performed using cells pre-treated with ERK and AKT specific inhibitors. As shown in Fig. 3B, C, hydrolysate-induced cell growth was significantly inhibited by SB203580 and Ly294002 (Fig. 3B, C). These results indicated that the seahorse-derived hydrolysate treatment increased cell proliferation through activation of ERK and AKT pathways.

### Hydrolysate induced increase of testosterone synthesis in TM3 Cells

Testosterone reduction and associated symptoms occur in aging men. It has been variously referred to as andropause and testosterone level decreases by approximately 1% per year after age 30 (Matsumoto 2002). The Leydig cell is the major cellular source of testosterone in male mammals, and we next examined the testosterone synthesis in TM3 Leydig cells in the presence or absence of seahorse-derived hydrolysate. The TM3 cells were treated with different concentrations of hydrolysate (ALC and PEP) as mentioned in Fig. 4, and the media was collected and the amount of testosterone was measured by ELISA. Significant increases in testosterone were found in TM3 cells treated with hydrolysate in a dose-dependent manner (Fig. 4).

### Sperm and serum testosterone level

We further studied the effects of alcalase and pepsin hydrolysate on mice model. 25 mg/kg of ALC and PEP was feed to mice every day for 12 weeks. The mean values of body weight and food intake are shown in Supplements 1 and 2. In case of PEP treatment, the average of food intake and body weight was increased though not significantly. The mean values of testicular weight are shown in Table 1. Although both hydrolysate-administered groups show the increments in testis weight, only the animals receiving ALC showed significant increase in testicular weights in comparison to the control group (Table 1).

To evaluate the effects of seahorse-derived hydrolysates on spermatogenesis, the number and motility of sperm was determined in control- and hydrolysate-treated mice. As shown in Table 1, when ALC and PEP were administered to mice for 12 weeks, averages in sperm counts in testis and sperm motility were increased when they were compared with the control group. However, these increments between the hydrolysate- and control-administered groups were not significant. Testosterone is the main secretory product of the testis and plays an important role during spermatogenesis (Weinbauer et al. 2010). Mice treated with ALC showed significant difference in serum testosterone and LH level compared with control and PEP group (Fig. 5A).

To determine whether the hydrolysates were associated with increase in testicular mass, we applied histomorphometry and assessed critical parameters of testicular architecture. Testes from both the hydrolysate-treated groups appeared normal and showed increase in cross-section diameter (Fig. 5D). Figure 5E shows the representative photomicrographs of testicular sections showing the Leydig cells in intertubular space from all the experimental groups. The section from hydrolysate-treated groups showed that the number of Leydig cells increased. This result suggests that the increase of the testicular weight and serum testosterone level occurs because of increased testicular size and Leydig cell accumulations in the interstitial tissue.

### Discussion

In Asian countries, like China and Vietnam, seahorses are used in traditional medicine. In several studies, the peptide hydrolysate obtained following enzymatic hydrolysis of the seahorse *Hippocampus* was found to have anti-inflammatory and neuroprotective effects, inhibits invasive migration, and also acts a ROS scavenger (Vincent et al. 2011). In this experiment, the hydrolysate (ALC and PEP) obtained from seahorse *Hippocampus abdominalis* was found to increase circulating testosterone level and Leydig

| Table 1 Enhanced spermatogenesis and sperm activity by seahorse-derived hydrolysate |
|---------------------------------|-----------------|-----------------|
| **Treatment**                  | **Testis weight (g)** | **Sperm number (n)** | **Sperm motility (%)** |
| Control                        | 0.096 ± 0.009     | 30.67 ± 6.037    | 90 ± 0.07             |
| Alcalase                       | 0.102 ± 0.009     | 37.33 ± 7.424    | 90 ± 8.66             |
| Pepsin                         | 0.099 ± 0.006     | 39.56 ± 4.592    | 92.22 ± 8.33          |

The 10 mice were orally administrated with PBS daily for 12 weeks, as the control group. Another 10 mice were orally administrated with alcalase and pepsin hydrolysates daily for 12 weeks, as the experimental group. All results are presented as the mean ± SD. *P < 0.05, **P < 0.001, compared to the control group.
Fig. 5 Effect of serum levels of testosterone and LH (Luteinizing hormone) after administration of seahorse-derived hydrolysate for 12 weeks. (A) Serum-free levels of testosterone at the end of the feeding period (12 weeks). Mean serum testosterone levels for hydrolysate-treated mice compared to those of the controls. Data represent the mean ± SD (n = 10/group). The serum testosterone levels after administration of ALC and PEP were compared to those of the controls. (B) Serum-free levels of LH (luteinizing hormone) at the end of the feeding period (12 weeks). LH level in the serum represents the mean ± SD (n = 10/group). The serum testosterone levels before and after administration of ALC and PEP were compared to those of the controls. (C–F) Histological analyses of mice testis treated with control or hydrolysate. Sections were stained with hematoxylin and eosin. Histomorphometrical assessment of circular seminiferous tubule (ST) cross-sectional diameters (C–D) and Leydig cell area (E–F). The magnification was approximately ×200 (C) or ×400 (E). Graph represents the changes in the diameter of the seminiferous tubules. Data are presented as the mean ± SD (n = 10/group). *P < 0.05, **P < 0.001, compared to the control group.
cell number in mouse-derived TM3 Leydig cell line and male mice.

In this present study, we investigated whether seahorse hydrolysate promotes Leydig TM3 cell proliferation and cell cycle progression through the Cyclin E and D, and AKT-ERK pathway. This signaling cascade is a key mediator of cell growth factor, androgen, follicle-stimulating hormone-dependent cell survival, proliferation, and differentiation (Lucas et al. 2014; Carrell et al. 2016). Therefore, this result may indicate that the signals were associated with an increased cell proliferation and testosterone production in TM3 cells after treatment with seahorse hydrolysates.

Recently great attention has been paid to study the bioactivity of natural products and their potential pharmacological utilization. In addition, many attempts have been made to improve male fertility, and many experiments using natural antioxidant from plant extract have been performed to elucidate the beneficial effects of some natural products on semen quality and on reproductive function. To investigate the antioxidant effects of seahorse hydrolysates, H$_2$O$_2$-mediated oxidative stress was induced in cultured mouse TM3 Leydig cells. Cellular mechanism to protect against oxidative stress in Leydig cells has not been routinely investigated, even though it plays an important role in male infertility (Lopes et al. 1998; Noh et al. 2012; Saleh and HCLD 2002). The fertilizing ability of human spermatozoa is inversely proportional to the oxidative stress (Sukcharoen and Keith 1996). In another study, the Leydig cells treated with low-dose testosterone showed cytoprotection by decreasing cellular ROS production (Hwang et al. 2011). Similarly, Kilinc et al. identified that deprivation of androgen increased oxidative stress in the testes (Kilinc et al. 2004). Our results showed the ALC and PEP protect Leydig TM3 cells against H$_2$O$_2$-induced cytotoxic effects.

Spermatogenesis is the process that occurs in the testicular seminiferous tubules in testes, and testosterone deficiency has a negative effect on sperm production (Saito et al. 2000). Leydig cells of the testes provide a primary source of testosterone in males that naturally decline during aging (Saito et al. 2000). The Leydig cells are activated by circulating levels of luteinizing hormone (LH) and testosterone from the Leydig cells. Maintaining a particular serum LH level is very important for initiating and supporting spermatogenesis (Haider 2004; Pourahid et al. 2014). Several reports showed that the extract containing garlic compound, ginger extract, yacon, and mistletoe has a positive effect on increasing testicular weight and serum testosterone level (Kasuga et al.
Citropsis articulata extracts of demonstrated that the androgenic effects of the aqueous leaf increased testicular weight, spermatogenesis, and Leydig cell proliferation by increasing serum testosterone and LH levels (Gakunga et al. 2014). We also observed an increase in the level of serum testosterone and LH level (Fig. 5A, B). Notably, a more profound increase in testicular weight and seminiferous tubules was found during hydrolysate administration (Table 1; Fig. 5C). This result came from proliferation of the interstitial Leydig cells, the important source of androgenic steroids (Zirkin and Chen 2000; Butler et al. 2008). The data from present study suggested the seahorse hydrolysate affects body weight and testicular weight, serum testosterone levels, and testicular integrity.

In this study, the ALC and PEP of natural hydrolysate showed significantly increased testosterone synthesis both in vivo and in vitro. Andropause syndrome is caused by the decreases in testosterone levels due to decreased production in the testicular Leydig cell. Consequences include reduction in muscle mass, decreased Leydig cell count, decreased lean body mass, decreased locomotive capacity, depression, increased fat accumulation, and inactivation of sperm (Vermeulen and Kaufman 1995; Trivason et al. 2011). The collective data support the suggestion that the increase in testosterone in the serum of hydrolysate-treated mice might be due to the increase in Leydig cells. Administration of hydrolysates derived from seahorse in the ICR mice led to increase in sperm count and sperm motility.

Therefore, seahorse-derived hydrolysate supplementation might improve the steroidogenic capacity of Leydig cells in older males.

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