The Effect of Abalone Embryo Digested Protein Fractions (AEDPFs) on the Viability of Murine Fibroblast Cell Line

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Abstract

Nowadays abalones are used as the supplements for skin care products and dietarys supplement for anti-skin aging, but the effect of abalone extract on skin is still unclear. This study aimed to investigate the effect of abalone embryo digested protein fractions (AEDPFs) on viability of murine fibroblast cell lines (NIH 3T3) as a cellular model for skin fibroblast. First, abalone embryos were digested by pepsin and trypsin which are major proteinases of the human's digestive system. The digested samples were fractionated into four sizes of < 3, 3-10, 10-30, and > 30 kDa. The NIH 3T3 cell line was treated with the abalone embryo undigested crude (AEUC) and AEDPFs at different concentrations (100, 200, and 400 µg/ml). The NIH 3T3 cell viability was performed using MTT assay. Results revealed that viability of the fibroblasts treated with AEDPFs and AEUC was not significantly different (p>0.05). All abalone embryo fractions did not lose ability to support cell growth or show low cytotoxic effects after being digested. More than 90% of AEDPFs were small peptides (less than 10 kDa). This suggested that the abalone embryos could be potential source of active peptides after being digested by the digestive enzymes and are potentially safe to use as components in cosmetic and food supplementary products.

Keywords: *Abalone embryo, in vitro digestion, hydrolysate, murine fibroblast, cell viability, cell growth*

บทคัดย่อ

ในปัจจุบันมีการนำผลิตภัณฑ์จากชิ้นส่วนและตัวอ่อนของหอยเป๋าฮื้อมาใช้เป็นอาหารเ สริมหรือผลิตภัณฑ์บำรุงผิวต่าง ๆ ที่ช่วยต่อต้านริ้วรอยของผิว อย่างไรก็ตามยังไม่มีรายงานการศึกษาเพื่อยืนยันประสิทธิภาพของอาหารจากสารสกัดจากหอยเป๋าฮื้อภายหลังการย่อยในระบบทางเดินอาหาร การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาเปรียบเทียบผลกระทบต่อการรอดชีวิตของเซลล์ไฟโบรบลาสต์เพาะเลี้ยง (NIH 3T3) เมื่อได้รับสารจากตัวอ่อนหอยเป๋าฮื้อก่อนและหลังกระบวนการย่อยในหลอดทดลอง โดยทำการย่อยตัวอ่อนหอยเป๋าฮื้อด้วยเอนไซม์เปปซินและทริปซิน ซึ่งเป็นเอนไซม์ย่อยโปรตีนในระบบทางเดินอาหารของมนุษย์ แยกกลุ่มสารตามขนาดโมเลกุลออกเป็น 4 กลุ่ม คือ ขนาดเล็กกว่า 3 กิโลดาลตัน จนถึงใหญ่กว่า 30 กิโลดาลตัน แล้วนำไปทดสอบกับเซลล์ไฟโบรบลาสต์ของหนูเทียบกับสารจากตัวอ่อนหอยเป๋าฮื้อที่ไม่ผ่านการย่อยที่ระดับความเข้มข้น 100, 200 และ 400 ไมโครกรัม/มิลลิลิตร และตรวจสอบอัตราการรอดชีวิตของเซลล์ด้วย MTT assay ผลการทดลองพบว่าการเจริญและการอยู่รอดของเซลล์นั้นไม่แตกต่างอย่างมีนัยสำคัญทางสถิติที่ระดับ p>0.05 อนุมานได้ว่าที่สารที่ผ่านการย่อยแล้วไม่ได้สูญเสียความสามารถในการสนับสนุนการเจริญและมีความเป็นพิษต่อเซลล์ต่ำมาก สารที่สกัดได้ประกอบด้วยเปปไทด์ที่มีโมเลกุลขนาดเล็ก (เล็กกว่า 10 กิโลดาลตัน) มีปริมาณมากกว่า 90% ทำให้ตัวอ่อนหอยเป๋าฮื้อเป็นแหล่งของเปปไทด์ที่มีศักยภาพสูง และมีแนวโน้มความปลอดภัยที่จะนำไปพัฒนาเป็นส่วนผสมของผลิตภัณฑ์เสริมอาหารและความงามต่อไป

คำสำคัญ: *ตัวอ่อนหอยเป๋าฮื้อ การย่อยในหลอดทดลอง สารที่ผ่านการย่อย ไฟโบรบลาสต์ของหนู การอยู่รอดของเซลล์ การเจริญของเซลล์*

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1. Introduction

Abalone, a common name for marine gastropod mollusks, is herbivorous and resides in constant salinity zone along the rocks and reefs. Abalones distribute worldwide along coastal waters in tropical and temperate areas (Geiger, 1999). The abalones are commonly found in the temperate zone of the northwestern Pacific Ocean and Indonesian waters (Bali) and New Caledonia. Abalone shells from these last two sites differ somewhat from the holotype by being slightly more elongate and having a dark sepia color (Geiger, 1998).

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Haliotis diversicolor is one of the most important animals in aquaculture industry. Abalone aquaculture in both natural marine water and artificial tanks requires large quantities of healthy embryos, so embryo production is a fundamental stage in abalone aquaculture (Li et al., 2006). The abalone aquaculture uses excess amount of eggs and sperms for external fertilization. However, space is limited, not all embryos have been reared to adults. The rest of the embryos have been used as components in skin care products or supplements that claims to help increasing skin strength and elasticity according to collagen activity (Levitan, 2006).

Fibroblasts are widely-distributed connective tissue cells found in all vertebrates. They are traditionally defined as cells of mesenchymal origin that produce interstitial collagen. The definition of the fibroblast is based on morphological characteristics that vary widely depending on a location and metabolic activity within individual cells of that organism, as well as the overall activity level of the organism itself. (Eghbali-Webb, 1994; Kanekar et al., 1998; Baudino et al., 2006; Baum and Duffy, 2011). The fibroblasts are often perceived as a kind of uniform, comparable to cell function, regardless of whether they come from skin, heart, or other tissues (Souders et al., 2009). The fibroblasts produce various extracellular matrix (ECM) components, primarily collagens type I and III, attached to different structural supports. This is a continuous process that new collagens are being deposited while older collagens are broken down (Petrov et al., 2002; Wynn, 2008).

Murine fibroblast cell line (NIH 3T3) is derived from the embryos of *Mus musculus f. domenstica* ("Swiss mouse"). The cell line has since become a standard fibroblast cell line. It has been used as a model system in a multitude of different studies (Todaro and Green 1963; Leibiger et al., 2013). For example, Chen et al., (2013) assessed an *in vitro* response of a mouse embryonic fibroblast cell lines to pearl nano-powder. The cytotoxicity of the filtrated pearls was tested with the mouse embryonic fibroblast cell line NIH-3T3. Results showed that the cultured cells in the medium containing the pearl powder at 24, and 72 h had cell viability greater than 90%.

2. Objectives

This study aimed to fractionate different molecular sizes of abalone embryo digested protein fractions (AEDPFs) and to determine effects of AEDPFs on viability of murine fibroblast cell line using MTT assay compared to abalone embryo undigested crude (AEUC).

3. Materials and methods

3.1 Preparation of AEDPFs and AEUC

The preparation process was modified from Kapsokefalou and Miller (1991) method and all steps were illustrated in Figure 1. Frozen-dried abalone embryos (kindly provided by Phuket Abalone Farm Co. Ltd., Thailand) were resuspended in ultrapure water to the concentration of 7.5 mg/ml. The abalone embryo suspension (2 mL) was adjusted to pH 2.0 for gastric enzyme digestion using 0.1 N HCl. Pepsin (Bio Basic, Canada) was added at the enzyme to substrate ratio of 1:100 (w/w) and placed on a shaker at 200 rpm at 37°C for 5 h. Then the pH was later adjusted to 6.5 using pH 7 buffer (prepared from KH_2PO_4 and NaOH) suitable for small intestinal enzyme digestion. Trypsin (Bio Basic, Canada) was added at the enzyme to substrate ratio of 1/100 (w/w), before incubation at 37°C on a shaker at 200 rpm for 4 h. The abalone embryos were then centrifuged at 15,000 *g* for 15 min to precipitate non-dissolved fractions and supernatants were collected and pooled. The supernatants were further fractionated using the size-excluded spin columns (Vivaspin®, UK) firstly with the filter size of 30-kDa, before separating with 10-kDa and 3-kDa filter columns. A control group (AEUC) was the abalone embryos that were not digested and fractionated. All AEDPFs and AEUC samples were lyophilized and kept at -25°C until use.

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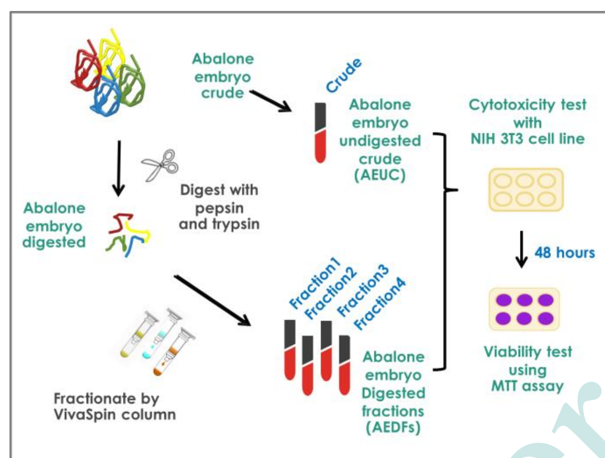

Figure 1 Experimental workflow for AEDPFs and AEUC preparation.

3.2 Viability Test

Murine fibroblast cell line (NIH 3T3) was kindly provided by Dr. Fahsai Kantawong, Chiang Mai University, Chiang Mai, Thailand, and seeded at 2×10^5 cells/plate in 2 mL Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic-antimycotic agent (Gibco, Invitrogen Taiwan Ltd., MD) . The cells were incubated at 37°C under 5% CO_2 with 95% air for two days. The AEDPFs (<3, 3-10, and >30 kDa) and AEUC were added into the serum-free DMEM at 100, 200, and 400 µg/ml. Control experiments were the serum-free DMEM medium without AEDF supplements. Two-days after the cultivation, 1000 µl of 50 µg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich, St. Louis, MO) was added to each well and continued incubation for 2 h at 37°C. The formazan crystals were then solubilized in dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm and 630 nm by using visible-light spectrophotometer. Percentage of cell viability was calculated by using the following formula:

$$\% cellviability = \frac{Abs_{570(treatment)} - Abs_{630(treatment)}}{Abs_{570(control)} - Abs630_{(control)}} X100$$

3.3 Statistical analysis

Mean and standard deviation of the percentage of cell viability were calculated across three replicates. The means of percent cell viability were compared according to the fractions and concentrations by two-way ANOVA followed by multiple comparisons of the means by Students' *t*-test and Tukey's honest significant difference (TukeyHSD) test using R program version 3.3.2 (R Development Core Team, 2008). Significant levels were determined at *p*-values less than 0.05 and 0.01.

4. Results

4.1 Distribution of AEDPFs

The abalone embryo suspension was turbid, blue-green, and non-viscous. After frozen- dried, the powder was fine grayish-green and water soluble. The embryos were enzymatically digested by pepsin and trypsin, and fractionated into four fractions by different molecular sizes (<3, 3-10, 10-30, >30 kDa). Dried weight ratio of the digested fraction was calculated to see the distribution of molecular size content (Table 1). More than 90% of the digested fraction was small-size peptides (less than 10 kDa). The AEDPFs contained proteins of molecular sizes <3 kDa, 3-10, 10-30, and >30 kDa accounting for 60%, 32%, 7% and 1% respectively (Figure 1).

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Table 1 Distribution on dry weight fractions of AEDPFs after lyophilized.

Molecular size	Dry weight (%)
< 3 kDa	107.59 mg (60)
3 – 10 kDa	56.89 mg (32)
10 – 30 kDa	1.52 mg (7)
> 30 kDa	12.03 mg (1)

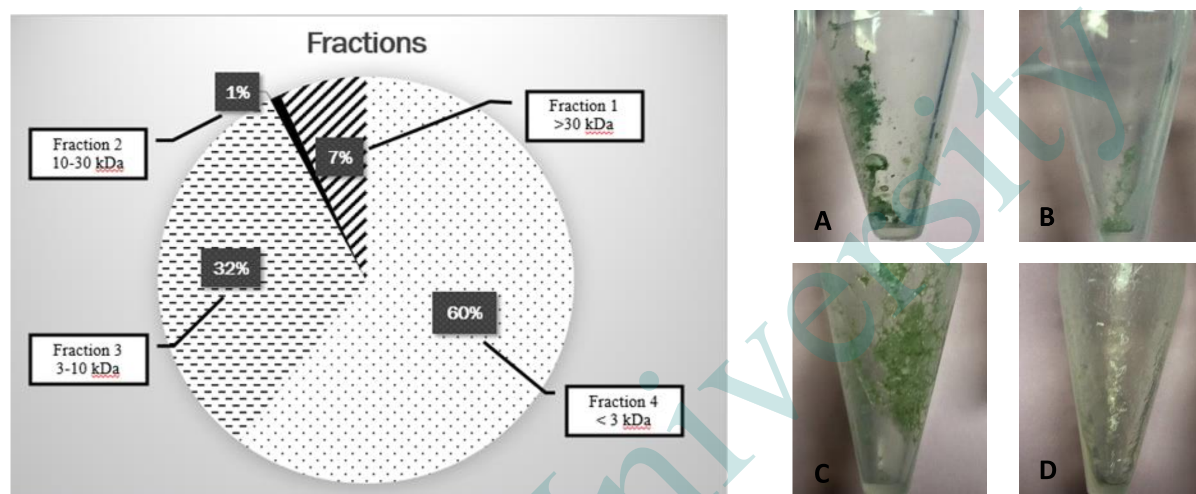

Figure 2 Percentage component of abalone embryo digested fractions (AEDPFs); (A) Fraction 1: > 30 kDa, (B) Fraction 2: 10-30 kDa, (C) Fraction 3: 3-10 kDa, and (D) Fraction 4: < 3 kDa

4.2 Cell viability analysis using MTT assay

Colorimetric detection of the tetrazolium reagents could estimate the number of viable cells based on the metabolic activity and indirectly indicate proliferation rate of the murine cells. The quantity of formazan (directly proportional to the number of viable cells) was measured by recording changes in absorbance at 570 nm. The absorbance at 630 nm was used as a reference to negate the effect of precipitated proteins or cellular debris that may interfere with the absorbance reading. Two-way ANOVA and multiple comparisons of the MTT results showed significantly different between the concentrations of the forth fraction of the AEDPFs at 200 and 400 µg/ml (p<0.01), while no difference between the fractions was observed (Table 2 and Figure 3).

The percentages of fibroblast cell viability treated with AEDPFs were not significantly different compared to the control sample. This result indicated that all *in vitro*-digested abalone extracts did not lose ability to support viability of the murine fibroblast cells. The viability of the cultured cells in the media containing AEDPFs at 400 µg/ml was greater than 100% meaning that the cell numbers were higher than the control. This represented the cell growth supportive effect of the abalone embryo supplement after being digested.

Table 2 Calculated probability (p-values) to measure effects of concentrations and fractions on the cell viability.

Factors	p-values
Concentration	0.00432 *
Treatment	0.74833
Concentration: treatment	0.57861

* indicated significant level at the p-value <0.01.

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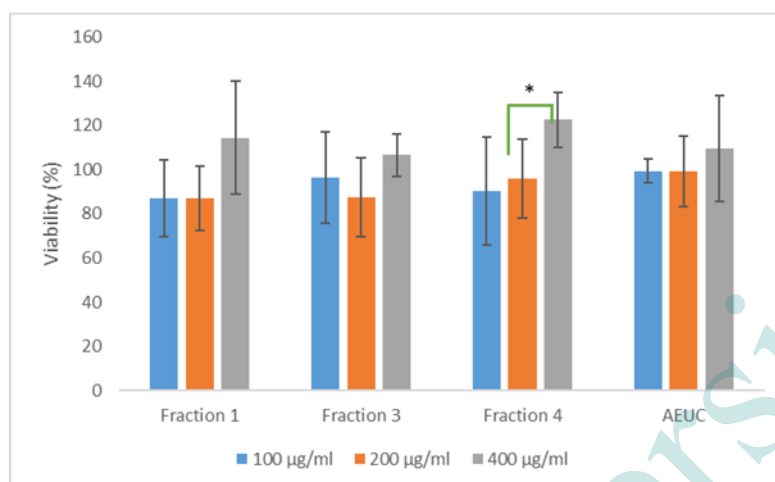

Figure 3 Percent viability of murine fibroblast cells after treated with different fractions of AEDPFs; fraction 1(> 30 kDa), fraction 3 (3-10 kDa), fraction 4 (< 3 kDa) and AEUC at 100, 200, and 400 µg/ml.

However, the result of the second faction (10-30 kDa) was not shown in Figure 3 due to the limitation of total weight of the fraction powder, not enough for minimum amount required for the MTT assay.

5. Discussion

This study found that above 90% of the AEDPFs was small peptides (less than 10 kDa). These small peptides were cleaved from their large protein precursors in the AEUC. The enzymatic digestion of the AEUC resembled to what could occur after the abalone embryos were consumed and digested *in vivo*. Nguyen et al., 2013 found that small abalone peptides effectively inhibited matrix metalloproteinases (MMPs) expression (i.e. MMP-2 and MMP-9) in HT1080 cells. These enzymes have important roles in skin aging by collagen degradation. This study had initially showed that the abalone embryo proteins (AUC) and peptides (AEDPFs) had very low cytotoxic effect on the murine fibroblasts. Some of the digested fractions also supported the fibroblast cell growth. The rationale for this could be that these fractions activated the production of growth-supportive extracellular matrix (ECM) molecules i.e. collagen types I and III, which encourage anti-skin aging (Petrov et al., 2002; Wynn, 2008). Therefore, the AEDPFs obtained from this study could be a potential source for several bioactive peptides.

6. Conclusion

The abalone embryo digested fractions (AEDPFs) were mostly small peptides that could be an alternative source for bioactive peptides. The AEDPFs were mildly toxic and growth supportive to the murine fibroblasts suggesting potential development as dietary supplements and skincare products.

7. Acknowledgements

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8. References

Baum, J., & Duffy, H. S. (2011). Fibroblasts and myofibroblasts: what are we talking about?. *Journal of Cardiovascular Pharmacology*, *57*(4), 376.

Baudino, T. A., Carver, W., Giles, W., & Borg, T. K. (2006). Cardiac fibroblasts: friend or foe?. *American Journal of Physiology-Heart and Circulatory Physiology*, *60*(3), H1015.

Chen, J. C., Kung, J. C., Hsieh, C. H., Hou, M. J., Shih, C. J., & Hung, C. C. (2013). Mineralization and osteoblast cells response of nanograde pearl powders. *Journal of Nanomaterials*, *2013*, 2.

Rangsit University

Eghbali-Webb M. (1994). Molecular Biology Intelligence Unit Molecular Biology of Collagen Matrix in the Heart. *Austin, TX: Landes*.

Geiger, D. L. (1998). Recent genera and species of the family Haliotidae Rafinesque, 1815 (Gastropoda: Vetigastropoda). *The Nautilus*, *111*(3), 85-116.

Geiger, D. L. (1999). Distribution and biogeography of the recent Haliotidae (Gastropoda: Vetigastropoda) world-wide. *Bollettino Malacologico*. *35*, 57-118.

Kanekar, S., Hirozanne, T., Terracio, L., & Borg, T. K. (1998). Cardiac fibroblasts: form and function. *Cardiovascular Pathology*, *7*(3), 127-133.

Kapsokefalou, M., & Miller, D. D. (1991). Effects of meat and selected food components on the valence of nonheme iron during in vitro digestion. *Journal of Food Science*, *56*(2), 352-355.

Li, H., Lin, W., Zhang, G., Cai, Z., Cai, G., Chang, Y., & Xing, K. (2006). Enhancement of larval settlement and metamorphosis through biological and chemical cues in the abalone *Haliotis diversicolor* supertexta. *Aquaculture*, *258*(1), 416-423.

Nguyen, V. T., Qian, Z. J., Ryu, B., Kim, K. N., Kim, D., Kim, Y. M., ... & Je, J. Y. (2013). Matrix metalloproteinases (MMPs) inhibitory effects of an octameric oligopeptide isolated from abalone *Haliotis discus hannai*. *Food Chemistry*, *141*(1), 503-509.

Levitan, D. R. (2006). The relationship between egg size and fertilization success in broadcast-spawning marine invertebrates. *Integrative and Comparative Biology*. *46*(3), 298-311.

Leibiger, C., Kosyakova, N., Mkrtchyan, H., Glei, M., Trifonov, V., & Liehr, T. (2013). First molecular cytogenetic high resolution characterization of the NIH 3T3 cell line by murine multicolor banding. *Journal of Histochemistry & Cytochemistry*, *61*(4), 306-312.

Petrov, V. V., Fagard, R. H., & Lijnen, P. J. (2002). Stimulation of collagen production by transforming growth factor-β1 during differentiation of cardiac fibroblasts to myofibroblasts. *Hypertension, 39*(2), 258-263.

R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Souders, C. A., Bowers, S. L., & Baudino, T. A. (2009). Cardiac fibroblast. *Circulation Research*, *105*(12), 1164-1176.

Todaro, G. J., & Green, H. (1963). Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *The Journal of Cell Biology*, *17*(2), 299-313.

Wynn, T. A. (2008). Cellular and molecular mechanisms of fibrosis. *The Journal of Pathology*, *214*(2), 199-210.

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