Immune-enhancement effects of tuna cooking drip and its enzymatic hydrolysate in Balb/c mice

Min-Ji Kim¹ · Koth-Bong-Woo-Ri Kim¹ · Nak-Yun Sung² · Eui-Hong Byun² · Hee-Sup Nam³ · Dong-Hyun Ahn¹

Received: 29 November 2016 / Revised: 24 March 2017 / Accepted: 29 September 2017 / Published online: 16 December 2017
© The Korean Society of Food Science and Technology and Springer Science+Business Media B.V., part of Springer Nature 2017

Abstract Tuna cooking drip (TCD) is a protein rich by-product of canned tuna processing that is typically discarded. In this study, the immune-enhancing activities of TCD and its enzymatic hydrolysate (EH-TCD) were investigated by intraperitoneally administering Balb/c male mice with TCD and EH-TCD for 2 weeks. This administration resulted in an increase in the weight of the spleen and thymus (200–800 mg/kg) and enhanced the proliferation rates of splenocytes (200–800 mg/kg). TCD and EH-TCD significantly increased the production of immunostimulatory cytokines (interleukin-10 and interleukin-2). In addition, TCD and EH-TCD increased serum IgG1 and IgG2a levels in a concentration-dependent manner. Particularly, EH-TCD had a greater immune-enhancing effect than TCD. These results suggest that TCD and EH-TCD exert immune-enhancing effects through an IgG antibody response and T cell activation, and EH-TCD can be used as an immunostimulatory agent.

Keywords Enzymatic hydrolysate · Immune-enhancement · Immunoglobulin · Immunostimulatory cytokines · Tuna cooking drip

Introduction

In recent years, opportunistic or complex infections associated with immunosuppression are difficult to treat with antibiotics alone, have become challenging to clinicians [1]. In addition, most immunomodulatory pharmaceuticals are unsuitable for chronic or prophylactic use. Thus, new immunomodulators that can augment nonspecific host defense mechanisms must be identified [2].

Enzymatic hydrolysates of marine proteins have been reported as important resources of novel immune peptides with strong immunostimulating effects [1, 3]. Thus, evaluating the immunomodulatory activity of marine-derived peptides may yield interesting results.

The global tuna catch reached approximately 4.76 million tons in 2011, and nearly 2 million tons of tuna are destined for canning annually [4]. The tuna canning industry generates approximately 50% of raw material as solid wastes or by-products, like bones, heads, viscera, dark muscle, and cooking drip [4]. However, while some of these by-products are used to make other products, the rest are discarded as waste, resulting in environmental pollution [5].

Tuna cooking drip (TCD) is generated by a steaming process. Although this TCD contains high-quality proteins, lipids, micronutrients, and minerals, only some of the cooking drip is used as a food additive [6]. In addition, the cooking drip is prone to gelation because of its low denaturation temperature, making it difficult to be used in food processing [7]. The number of studies aimed at recovering the essential nutrients from fish by-products has recently increased.

Enzymatic hydrolysis of protein is an attractive approach for improving their nutritional and functional characteristics [8]. It has been reported that various

✉ Dong-Hyun Ahn
dhahn@pknu.ac.kr

¹ Department of Food Science and Technology/Institute of Food Science, Pukyong National University, Busan 48513, Republic of Korea

² Department of Food Science and Technology, Kongju University, Kongju 32588, Republic of Korea

³ Research and Development Center, Dongwon F&B, Seongnam-Si 13212, Republic of Korea

peptides or hydrolysates derived from fish proteins show better biological activities than those derived from crude protein [9]. Increased angiotensin-I-converting enzyme inhibitory activity and antioxidant activity of the enzymatic hydrolysate (EH-TCD) of TCD have been reported [7]. In a previous study, Kim et al. [10] isolated three tuna-derived peptides, TP-D, TP-I, TP-N, from desalinated TCD at 10 kDa, and reported that TP-D exhibited anti-obesity activity in 3T3-L1 cells. Additionally, the major active components in TCD and EH-TCD were suggested to have immunomodulatory activity, but the main active peptide has not been identified.

In our previous study, desalinated TCD was found to be a potential immunomodulator [11] and increased cytokine production by RAW 264.7 cells and splenic cells in vitro. Therefore, we conducted animal experiments to evaluate the immune-enhancement effects of TCD and EH-TCD intraperitoneally administered to Balb/c, and to determine their effects on serum immunoglobulin levels and cytokine production from spleen cells.

Materials and methods

Preparation of desalted TCD and EH-TCD

TCD obtained in Korea in 2015 was centrifuged to remove any suspended solids that may have interfered with the desalting step. The desalted TCD was membrane-filtered (membrane 2319/size 200 Da) to obtain TCD of 30 Brix and 1% salinity. The obtained TCD was sterilized at 110 °C for 10 s. The TCD was introduced into the reactor (Integrated Jacketed Reactor, DooYoung Hi-Tech Co., Ltd., Seoul, Korea) and heated for 15 min at 75 °C followed by cooling at 50 °C. The commercial enzymes used were enzyme A (protease from *Bacillus licheniformis* and *Bacillus subtilis* + protease from *Aspergillus oryzae*) and enzyme B (protease from *Aspergillus oryzae*). Enzyme A and enzyme B were added at a ratio of 38.34:61.66, until a solids concentration of 1.05% by the weight ratio of TCD. The reaction was carried out for 24.5 h at 20 rpm agitation. To inactivate the enzymes, the samples were heated for 15 min at 90 °C. The EH-TCD was spray-dried.

Determination of molecular weight

The molecular weight distribution profile of the sample was determined using a Waters GPC system (Milford, MA, USA) consisting of a Waters 515 pump and Waters 410 differential refractometer. A TSK gel 2000SWXL (7.8 × 300 mm) column (Tosoh Corp., Tokyo, Japan) was used with 0.2 M NaNO₃ + 0.01 M NaH₂PO₄, pH 7, as the mobile phase. The calibration curve was obtained using

polyethylene glycol and polyethylene oxide. The results were obtained and processed using Empower software (Waters).

Animal experiments

Balb/c male mice were purchased from Orient Bio Inc. (Sunngnam, Korea). The mice were 6 weeks old and maintained in a temperature-controlled environment (20 ± 2 °C, humidity: 50 ± 10%) with a 12 h light-12 h dark cycle. Mice were given free access to water and food throughout the experiment. Any aspect of the work involving experimental animals and their care was approved by the Animal Ethics Committee of Pukyong National University (2016-11). After 7 days of acclimation, mice were randomly divided into four groups: control group (sterile saline solution) and TCD or EH-TCD group (200, 400, and 800 mg/kg body weight). All mice were administered TCD or EH-TCD (200, 400, and 800 mg/kg body weight) intraperitoneally once daily for 2 weeks. The mice were weighed at the beginning and end of the experiments.

Assay of spleen and thymus indices

Twenty hours after the last intraperitoneal administration, the body weight of mice was measured and the mice were sacrificed. The spleen and thymus were then excised and weighed immediately. Organ indices were calculated according to the following formula: W_1/W_0 , where W_1 corresponds to the weight of the spleen or thymus and W_0 corresponds to the weight of the whole body.

Assay of splenic lymphocyte proliferation

Splenocyte proliferation was measured by an MTT assay of Park et al. [12] with some modifications. The mice spleens were collected aseptically and gently ground in RPMI 1640 medium. The splenocytes were centrifuged at 1800 rpm for 5 min at 4 °C and the red blood cells were removed using lysis buffer. Next, the splenocytes were re-suspended in RPMI 1640 complete medium and adjusted to a concentration of 1×10^7 cells/mL. The cell suspension was seeded into 96-well plates and stimulated with 3 µg/mL concanavalin A (ConA) and 1 µg/mL lipopolysaccharide (LPS), or cultured without ConA and LPS. The well plates were incubated at 37 °C in 5% CO₂. After 70 h of incubation, 30 µL of MTT solution (5 mg/mL) was added and incubated for another 2 h. Finally, 100 µL of solubilization solution (dimethyl sulfoxide) was added to completely dissolve the purple formazan crystals. Absorbance was measured with a microplate reader at 540 nm. The proliferation effect of splenocytes was evaluated according to the

following formula: Proliferation index (%) = [Abs sample/ Abs control] × 100.

Assay of serum IgG1 and IgG2a, IL-10, and IL-2

To determine the serum levels of IgG1 and IgG2a, we used the sandwich enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with 50 µL of anti-mouse IgG1 or IgG2a (BD Pharmingen, Franklin Lakes, NJ, USA) in coating buffer overnight at 4 °C. After washing, the plates were blocked with 2% bovine serum albumin for 1 h at room temperature (RT). Serial dilutions of standard IgG1 and IgG2a (BD Pharmingen) and dilutions of serum samples were added and incubated for 2 h at RT. A horseradish peroxidase-conjugated secondary antibody (BD Pharmingen) was then added, followed by incubation with o-phenylenediamine solution for 30 min at RT. Finally, 2 N H₂SO₄ was added to stop reaction and the absorbance in each well was measured using an ELISA reader at 490 nm. Interleukin (IL)-10 and IL-2 levels were measured using a mouse ELISA kit (mouse ELISA set; BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis

The data were analyzed using one-way analysis of variance with Duncan's multiple range test. Experimental values are presented as the mean ± standard deviations (S.D.). Probability values (*p*) less than 0.05 were considered statistically significant.

Results and discussion

Molecular weight distribution of EH-TCD

The molecular weight (MW) distribution of EH-TCD was analyzed by gel permeation chromatography using an HPLC system. The chromatographic data (Fig. 1) showed that EH-TCD was enriched in peptides with molecular weights (MWs) lower than 5 kDa. The MW distribution profiles were composed of peptides of > 10 kDa (0.33%), 5–10 kDa (2.04%), and < 5 kDa (97.66%). Numerous studies have reported that low-MW peptides are potent bioactive peptides [1, 12]. The ability of peptides to modulate the host immune response indicates that peptides stimulate T-helper (Th) cells and enhance cytokine production [1, 13]. The high proportion of peptides with low-MW suggests that these peptides have immunomodulatory properties.

Spleen and thymus weight

To investigate the immunomodulatory effect of TCD and EH-TCD, we first evaluated its protection against degenerative changes in the spleen and thymus. As shown in Table 1, all doses of TCD and EH-TCD significantly increased the spleen and thymus indices in mice. Compared to the control group, in the high-dose group (800 mg/kg) of TCD and EH-TCD, the spleen indices were approximately 1.24- and 1.34-fold higher, respectively, and the thymus indices were approximately 1.26- and 1.09-fold higher, respectively. These results indicate that TCD and EH-TCD markedly increased the spleen and thymus indices without any side-effects on the body weight of healthy Balb/c mice.

ConA and LPS-induced lymphocyte proliferation of TCD and EH-TCD

The effects of TCD and EH-TCD on splenic lymphocyte proliferation were evaluated, as shown in Fig. 2. Compared to the control group, TCD significantly promoted the proliferation of mitogen-free splenocytes and LPS- and ConA-stimulated splenocytes in the 800 mg/kg treatment group. However, although TCD stimulated the proliferation of splenocytes, proliferation was much lower compared to the ConA- and LPS-stimulated splenocytes. Similar results were obtained for EH-TCD, and the highest splenocyte proliferation rate was observed in the 800 mg/kg treatment group. Unlike TCD administration, the proliferation rate of mitogen-free splenocytes was increased or similar compared to that of ConA- and LPS-stimulated splenocytes in the 200 and 400 mg/kg treatment groups. Therefore, EH-TCD showed a greater mitogenic effect than TCD. The positive effects of TCD and EH-TCD on splenic lymphocyte proliferation indicate that they can enhance cellular immunity [14].

IL-2 and IL-10 production

The major Th1 cytokine, IL-2 activates cellular immune responses of dendritic cells and monocytes [15]. IL-2 promotes T cell proliferation as a T cell growth factor [16] and enhances immunoglobulin J-chain transcription in B cells [17]. IL-10 was previously grouped as a Th-2-type cytokine, but was recently reported to be produced by regulatory T-cells that can suppress effector T-cells [18]. To investigate whether TCD and EH-TCD increase the production of IL-2 and IL-10, IL-2 and IL-10 production in splenocytes were measured using an ELISA assay (Fig. 3). Our results demonstrated that T cells from mice that received the highest dose of TCD secreted more IL-10 than the control group. High doses of TCD may induce regulatory immune responses. Moreover, IL-2 and IL-10 were

Fig. 1 Molecular weight distribution profile of enzymatic hydrolysate of tuna cooking juice

Table 1 Effect of tuna cooking drip and enzymatic hydrolysate of tuna cooking drip on spleen and thymus index of mice

Group	Dose (mg/kg BW)	Spleen index (mg/g)	Rate (group/normal)	Thymus index (mg/g)	Rate (group/normal)
Normal	0	3.76 ± 0.16 ^c	1.00	1.54 ± 0.08 ^c	1.00
TCD	200	4.15 ± 0.02 ^b	1.10	1.69 ± 0.06 ^b	1.10
	400	4.27 ± 0.23 ^b	1.14	1.60 ± 0.07 ^{bc}	1.04
	800	4.65 ± 0.14 ^a	1.24	1.95 ± 0.02 ^a	1.26
Normal	0	3.76 ± 0.16 ^c	1.00	1.54 ± 0.08 ^b	1.00
EH-TCD	200	4.58 ± 0.06 ^b	1.22	1.73 ± 0.06 ^a	1.12
	400	4.86 ± 0.26 ^a	1.29	1.78 ± 0.07 ^a	1.16
	800	5.03 ± 0.14 ^a	1.34	1.68 ± 0.02 ^a	1.09

All values are represented as Mean ± S.D. n = 6. Means with different letters (a–c) in the same column are significantly different ($p < 0.05$) Spleen index and thymus index = spleen or thymus weight/body weight

also produced by splenocytes cultured with ConA and LPS. TCD enhanced IL-2 production in mitogen-free splenocytes and LPS- and ConA-stimulated splenocytes. As a result, significant increases were observed in the 400 and 800 mg/kg treatment groups. Compared to TCD, IL-2 secretion in mitogen-free splenocytes and ConA- and LPS-stimulated splenocytes was greater after EH-TCD administration, showing a concentration-dependent increase. At higher concentrations, TCD enhanced IL-10 production in mitogen-free and LPS-stimulated splenocytes, but not in ConA-stimulated splenocytes. In contrast, EH-TCD enhanced IL-10 production in mitogen-free and ConA- and LPS-stimulated splenocytes. In our results, the increase in cytokine production varied among mitogen free or mitogen treatment group. Similar results were also reported by Ni et al. [19]. Compared to TCD, a significant increase in

cytokine production was observed in the EH-TCD treatment groups. Endogenous and/or exogenous enzyme hydrolyzes fish protein and creates active peptides with various biological activities [20, 21]. Bioactive peptides in fish protein hydrolysates, which are obtained by enzymatic hydrolysis, have been isolated from fish tissues, such as sardine muscle, tuna muscle, and pacific hake. These peptides mainly exhibit angiotensin-I-converting enzyme inhibitory activity and anti-oxidant activity [22]. Some studies have reported that fish-derived peptides function as multifunctional peptides due to the activity enhancement of bradykinin that can stimulate macrophages, enhance lymphocyte migration, and increase lymphokine secretion [23–25]. Our results suggest that intraperitoneal administration of TCD and EH-TCD regulates the production of IL-2 and IL-10 in lymphocytes in vivo.

Fig. 2 Effect of tuna cooking drip (A) and enzymatic hydrolysate of tuna cooking drip (B) on proliferation in lipopolysaccharide (LPS) and concanavalin A (ConA)-activated lymphocytes. Cells were treated in the absence or presence of LPS (1 μ g/mL) or ConA

(3 μ g/mL) for 24 h. All values are represented as the mean $\pm$ S.D. $n = 6$. Means with different letters ($a-d$) above the bars are significantly different ($p < 0.05$)

Fig. 3 Effect of tuna cooking drip (A) and enzymatic hydrolysate of tuna cooking drip (B) on IL-2 and IL-10 production in LPS- and ConA-activated lymphocytes. Means with different letters ($a-d$) above the bars are significantly different ($p < 0.05$)

Fig. 4 Effect of tuna cooking drip (**A**) and enzymatic hydrolysate of tuna cooking drip (**B**) on IgG1 and IgG2a levels in serum. All values are represented as the mean $\pm$ S.D. $n = 6$. Means with different letters (*a–d*) above the bars are significantly different ($p < 0.05$)

IgG1 and IgG2a levels

Serum immunoglobulin levels were examined at the end of the experiments (Fig. 4). The serum levels of IgG1 and IgG2a increased with increasing TCD concentration, which was not observed in the control group. Moreover, for EH-TCD, the serum IgG1 and IgG2a levels increased in a dose-dependent manner, resulting in a significant increase in IgG2a levels. Both IgG1 and IgG2a are thought to regulate specific adaptive immune responses. It has been reported that Th2-type cytokines, such as IL-4, regulate immunoglobulin class-switching and result in increased IgG1 production [26]. In addition, IgG2a production has been correlated with the activity of Th1 cells [27]. As shown in Fig. 3, comparison of the increased production of IL-2 and IL-10 related to Th1 and Th2 cells revealed that IL-2 production was markedly increased in the TCD and EH-TCD groups. Therefore, the significant increase in

serum IgG2a indicates that EH-TCD enhances Th1 activity.

In conclusion, our results demonstrated that intraperitoneal administration of TCD and EH-TCD promotes antibody and cytokine production by B and T lymphocytes. TCD and EH-TCD significantly affected immune enhancement-related cytokine production (IL-2 and IL-10) and blood serum levels (IgG1 and IgG2a) in the 400 and 800 mg/kg treatment groups. The EH-TCD treatment group showed a greater immune enhancement effect than the TCD group. Therefore, an increase in the secretion of IL-2, IL-10, IgG2a, and IgG1 verified the immune-enhancement effects of TCD and EH-TCD through the regulation of T cells, and may serve as a valuable source for clinical use as a natural immunomodulator. Further studies examining the immunomodulating effect of orally administered EH-TCD are underway to determine the mechanisms of cytokine production, natural killer cell activity, and complete blood count test.

Acknowledgements This study was a part of the project entitled ‘Functional materials and foods using fisheries by-products’, funded by the Ministry of Oceans and Fisheries, Korea (20130279).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

1. Yang R., Zhang Z, Pei X, Han X, Wang J, Wang L, Long Z, Shen X, Li Y. Immunomodulatory effects of marine oligopeptide preparation from Chum Salmon (*Oncorhynchus keta*) in mice. Food Chem. 113: 464–470 (2009)
2. Yang MP, Eoum HY, Na KJ, Araki S, El-Abasy M, Motobu M, Hirota Y. Enhanced phagocytic activity of neutrophils caused by administration of egg white derivatives (EWD) in cats injected with cyclophosphamide (CPA). J. Vet. Med. Sci. 63: 269–274 (2001)
3. Gildberg A, Bøgwald J, Johansen A, Stenberg E. Isolation of acid peptide fractions from a fish protein hydrolysate with strong stimulatory effect on Atlantic salmon (*Salmo salar*) head kidney leucocytes. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 114B: 97–101 (1996)
4. Food and agriculture organization of the united nations. The state of world fisheries and aquaculture (2014)
5. Chalamaiiah M, Narsing Rao G, Rao DG, Jyothirmayi T. Protein hydrolysates from meriga (*Cirrhinus mrigala*) egg and evaluation of their functional properties. Food Chem. 120: 652–657 (2010)
6. Kim SK, Byun HG, Jeon YJ. Desalination of tuna boiled extract by electrodialysis. Korean J. Fish. Aquat. Sci. 32: 68–74 (1999)
7. Oh HS, Kim JS, Kim HS, Jee SJ, Lee JH, Chung IK, Kang KT, Heu MS. Improvement on the quality and functionality of skipjack tuna cooking drip using commercial enzymes. J. Korean Soc. Food Sci. Nutr. 36: 881–888 (2007)
8. In MJ, Chae HJ, Oh NS. Process development for heme-enriched peptide by enzymatic hydrolysis of hemoglobin. Bioresour. Technol. 84: 63–68 (2002)
9. Liceaga-Gesualdo AM, Li-Chan EY. Functional properties of fish protein hydrolysate from herring (*Clupea harengus*). J. Food Sci. 64: 1000–1004 (1999)
10. Kim YM, Kim EY, Kim IH, Nam TJ. Peptide derived from desalinated boiled tuna extract inhibits adipogenesis through the downregulation of C/EBP- α and PPAR- γ in 3T3-L1 adipocytes. Int. J. Mol. Med. 35: 1362–1368 (2015)
11. Kang BK, Kim KBWR, Ahn NK, Choi YU, Kim MJ, Bark SW, Pak WM, Kim BR, Park JH, Bae NY, Ahn DH. Immuno-stimulating activities of skipjack tuna *Katsuwonus pelamis* cooking juice concentrates on mouse macrophages and spleen cells. Korean J. Fish. Aquat. Sci. 47: 776–784 (2014)
12. Park YM, Won JH, Yun KJ, Ryu JH, Han YN, Choi SK, Lee KT. Preventive effect of ginkgo biloba extract (GBB) on the lipopolysaccharide-induced expressions of inducible nitric oxide synthase and cyclooxygenase-2 via suppression of nuclear factor- κ B in RAW 264.7 cells. Biol. Pharm. Bull. 29: 985–990 (2006)
13. Duarte J, Vinderola G, Ritz B, Perdígón G, Matar C. Immunomodulating capacity of commercial fish protein hydrolysate for diet supplementation. Immunobiology 211: 341–350 (2006)
14. Yu ZP, Xu DD, Lu LF, Zheng XD, Chen W. Immunomodulatory effect of a formula developed from American ginseng and Chinese jujube extracts in mice. J. Zhejiang Univ. Sci. 17: 147–157 (2016)
15. Huang WC, Kuo ML, Li ML, Yang RC, Liou CJ, Shen JJ. Extract of *Gynostemma pentaphyllum* enhanced the production of antibodies and cytokines in mice. Yakugaku Zasshi 127: 889–896 (2007)
16. Smith KA. Interleukin-2: inception, impact, and implications. Science 240: 1169–1176 (1988)
17. Johansen FE, Brandtzaeg P. Transcriptional regulation of the mucosal IgA system. Trends Immunol. 25: 150–157 (2004)
18. Bluesotne JA, Abbas AK. Natural versus adaptive regulatory T cells. Nat. Rev. Immunol. 3: 253–257 (2003)
19. Ni WY, Wu MF, Liao NC, Yeh MY, Lu HF, Hsueh SC, Liu JY, Huang YP, Chang CH, Chung JG. Extract of medicinal mushroom *Agaricus blazei* Murill enhances the non-specific and adaptive immune activities in Balb/c mice. In Vivo 27: 779–786 (2013)
20. Hsu KC. Purification of antioxidative peptides prepared from enzymatic hydrolysates of tuna dark muscle by-product. Food Chem. 122: 42–48 (2010)
21. Sarmadi BH, Ismail A. Antioxidative peptides from food proteins: a review. Peptides 31: 1949–1956 (2010)
22. Samaranayaka AG, Kitts DD, Li-Chan EC. Antioxidative and angiotensin-I-converting enzyme inhibitory potential of a Pacific Hake (*Merluccius productus*) fish protein hydrolysate subjected to simulated gastrointestinal digestion and Caco-2 cell permeation. J. Agric. Food Chem. 58: 1535–1542 (2010)
23. Balti R, Bougatef A, El-Hadj Ali N, Zekri D, Barkia A, Nasri N. Influence of degree of hydrolysis on functional properties and angiotensin I-converting enzyme-inhibitory activity of protein hydrolysates from cuttlefish (*Sepia officinalis*) by-products. J. Sci. Food Agric. 90: 2006–2014 (2010)
24. Cook MT, Hayball PJ, Birdseye L, Bagley C, Nowake BF, Hayball JC. Isolation and partial characterization of a pentraxin-like protein with complement-fixing activity from snapper (*Pagrus auratus*, Sparidae) serum. Dev. Comp. Immunol. 27: 579–588 (2003)
25. De Leo F, Panarese S, Gallerani R, Ceci LR. Angiotensin converting enzyme (ACE) inhibitory peptides: production and implementation of functional food. Curr. Pharm. Des. 15: 3622–3643 (2009)
26. Glimcher LH, Murphy KM. Lineage commitment in the immune system: the T helper lymphocyte grows up. Genes Dev. 14: 1693–1711 (2000)
27. Ravetch JV, Bolland S. IgG Fc receptors. Annu. Rev. Immunol. 19: 275–290 (2001)