ID 14120_Ervia Yudiati

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IMMUNOSTIMULATORY EFFECTS OF ULVAN ON TRYPSIN-MEDIATED PROTEIN DIGESTION IN THE GUT OF PACIFIC WHITELEG SHRIMP *Litopenaeus vannamei*

ABSTRACT

Litopenaeus vannamei has emerged in the aquaculture industry. Production consistency, nutrition, and disease management play critical roles, particularly the digestive enzymes such as trypsin. This study assesses Ulvan, an immunostimulant from *Ulva lactuca*, on shrimp trypsin activity. Trypsin has been found to significantly enhance the activity of hemocyanin phenoloxidase, a crucial component of humoral immunity. This study aims to evaluate the potency of ulvan related to trypsin as an immunostimulant agent, extracted from *Ulva* sp. Ulvan, extracted using various methods (P-HWE, O-HWE, P-A-HWE, and O-A-HWE), was evaluated using different doses of (0 g kg^{-1} (Control), 0.75 g kg^{-1} (ULV-0.75), 1.50 g kg^{-1} (ULV-1.50), and 3.00 g kg^{-1} (ULV-3.00) of feed). The O-A-HWE exhibited the fastest and highest increase in trypsin activity on day 4, surpassing the control on days 2, 3, 7, and 8. The P-HWE, O-HWE, and P-A-HWE also showed significant changes in trypsin activity compared to the control on specific days. Meanwhile, trypsin activity in Ulvan-fed shrimp did not significantly differ from the control on days 0 and 1. The differences emerged on day 2 and 3, notably between ULV-1.50 g kg^{-1} and ULV-0.75 g kg^{-1}. The ULV-3.00 g kg^{-1} showed no significant difference from ULV-1.50 g kg^{-1}. O-A-HWE demonstrated significant differences in trypsin activity compared to other Ulvan extracts, suggesting its potential to enhance shrimp health.

KEYWORDS: immunostimulant; shrimp; trypsin activity; ulvan

ABSTRAK: Efek Imunostimulasi Ulvan dan Digesti Protein Tripsin pada Pencernaan Udang Vaname *Litopenaeus vannamei*

Litopenaeus vannamei *memiliki peran yang besar dalam industri akuakultur. Konsistensi dalam produksi, nutrisi, dan pengelolaan terhadap penyakit merupakan bagian yang sangat penting, terutama enzim pencernaan, di antaranya tripsin. Tripsin berfungsi untuk meningkatkan fenoloksidase hemosianin, yang peranannya penting untuk kekebalan. Studi ini mengevaluasi Ulvan, yaitu bahan yang bersifat imunostimulan dari* ***Ulva*** *sp., terhadap aktivitas tripsin udang. Ulvan, diekstraksi menggunakan berbagai metode (P-HWE, O-HWE, P-A-HWE, dan O-A-HWE), dievaluasi menggunakan perlakuan dosis yang berbeda (0 g kg^{-1} (Kontrol), 0,75 g kg^{-1} (ULV-0,75), 1,50 g kg^{-1} (ULV-1,50), dan 3,00 g kg^{-1} (ULV-3,00) pada pemeliharaan udang vaname selama 10 hari. O-A-HWE menunjukkan peningkatan aktivitas tripsin tercepat dan tertinggi pada hari ke-4, melebihi kontrol pada hari ke-2, 3, 7, dan 8. P-HWE, O-HWE, dan P-A-HWE juga menunjukkan perubahan signifikan dalam aktivitas tripsin dibandingkan dengan kontrol pada hari-hari tertentu. Sementara aktivitas tripsin dalam udang yang diberi Ulvan tidak berbeda secara signifikan dari kontrol pada hari ke-0 dan 1, perbedaan mulai terlihat pada hari ke-2 dan 3, terutama antara ULV-1,50 g kg^{-1} dan ULV-0,75 g kg^{-1}. ULV-3,00 g kg^{-1} tidak menunjukkan perbedaan yang signifikan dari ULV-1,50 g kg^{-1}. O-A-HWE menunjukkan perbedaan signifikan dalam aktivitas tripsin dibandingkan dengan ekstrak Ulvan lainnya, sehingga berpotensi dalam meningkatkan kesehatan udang.*

KATA KUNCI: aktivitas tripsin; imunostimulan; udang; Ulvan

INTRODUCTION

The aquaculture industry has experienced remarkable growth, particularly in the production of whiteleg shrimp, *Litopenaeus vannamei*. This species has emerged as one of the significant contributors to aquaculture both in production volume and economic value. It is not surprising that whiteleg shrimp shows a remarkable surge of production from 154.5 thousand tonnes in 2000 to 5812.2 thousand tonnes in 2020, with yearly average production of 1678.4 (2005), 2648.5 (2010), and 3803.6 (2015) thousand tonnes (Food and Agriculture Organization, 2022). Unfortunately, in Indonesia, the production was decresed. In 2015, Indonesia procuded 786,654 tonnes, but was then declined to 265,000 tonnes (2016) (Febriani *et al.*, 2018).

In order to sustain such production consistency, nutrition and disease management play critical roles in whiteleg shrimp farming. A stable feed supply with sufficient nutrients will ensure optimal growth of farmed shrimp. Nutrition itself encompasses the intake, breakdown, absorption, and utilization of nutrients. Digestive enzymes, such as trypsin, play a vital role in breaking down dietary proteins to acquire these nutrients. Trypsin, along with chymotrypsin, elastase, and thrombin, is crucial for protein digestion, particularly in crustaceans like penaeid shrimp (Aguiñaga-Cruz *et al.*, 2019; Hernández *et al.*, 2023). Trypsin, a serine protease found across various organisms, primarily cleaves peptide chains at the carboxyl side of lysine or arginine amino acids. Besides its digestive role, trypsin has been implicated in various functions within the immune system. Recent research suggests its involvement in diverse roles in invertebrate innate immunity. For instance, trypsin treatment in shrimp has been found to significantly enhance the activity of hemocyanin phenoloxidase, a crucial component of humoral immunity (Li *et al.*, 2018). Our study demonstrates that immunostimulants like Ulvan can increase phenoloxidase activity.

Ulvan, a sulfated polysaccharide present in the cell walls of green seaweed belonging to the *Ulvale* genus, is closely associated with algal proliferation in coastal waters and lagoons

experiencing eutrophication (Fletcher, 1996). Green seaweed typically contains 9-36% w/v sulfated polysaccharide in the form of Ulvan, a heteropolysaccharide with anionic properties and a molecular weight ranging between 150-2000 kDa. This polysaccharide comprises monosaccharides like rhamnose (45%), glucuronic acid (22.5%), xylose (9.6%), and iduronic acid (5%) (Lahaye & Robic, 2007). Furthermore, Ulvan is characterized by a chemical composition that includes Ulvanobiuronic acid and Ulvanobiose as recurring disaccharides, unique to Ulvan (Cindana Mo'o *et al.*, 2020; Kidgell *et al.*, 2019; Mohan *et al.*, 2019). The research aimed to investigate the impact of the immunostimulant Ulvan on protein digestion activity, such as trypsin, while specifically examining any potential side effects or synergistic effects in enhancing immune function.

MATERIALS AND METHODS

Collection of *Ulva lactuca*

Ulva lactuca samples, the primary source of Ulvan sulfated polysaccharides, were obtained from Ngrehan Beach, Gunungkidul Regency, Special Region of Yogyakarta, during low tide. The seaweed was hand-collected and directly placed in plastic net bags to allow the seawater to drip. After collection, the samples were meticulously stored in plastic bags for transportation to the laboratory. A thorough cleaning process was conducted to eliminate impurities and epiphytes, succeeded by a wash with fresh water. Post-washing, the samples were air-dried and finely powdered using a blender and sieved with a flour filter sieve.

Extraction of Ulvan from *Ulva lactuca*

The primary extraction methods used were polysaccharide-hot water extraction (P-HWE) and polysaccharide-acidic hot water extraction (P-A-HWE) with H_2O_2 and ascorbic acid. The P-HWE extract produced was then converted into oligosaccharide-hot water extraction (O-

HWE), and the P-A-HWE extract produced was then converted into oligosaccharide-acidic hot water extraction (O-A-HWE) using oven heating.

P-HWE Extraction Method and O-HWE Production

The P-HWE method was conducted according to Tabarsa *et al.* (2018) with slight adjustments. Dried *U. lactuca* powder (20 g) underwent lipid, pigment, and low molecular weight compound removal using ethanol (80% EtOH, 200 mL, Merck, Germany) under constant stirring overnight. After centrifugation at 8,000 rpm for 10 minutes, the supernatant was discarded, and the depigmented *U. lactuca* powder was air-dried. Distilled water (400 mL) was added to the depigmented powder (20 g), and extraction was carried out at 65°C with stirring for 2 hours. The supernatant collected after centrifugation at 10,000 rpm for 10 minutes underwent polysaccharide precipitation using EtOH (99%) to achieve a final EtOH concentration of 70%. The mixture was stored at 4°C overnight, and the precipitate was obtained after centrifugation at 10,000 rpm for 10 minutes. The polysaccharide precipitate was dried at room temperature. The polysaccharide yield was calculated from the depigmented powder obtained after EtOH precipitation. Subsequently, the dried polysaccharide (P-HWE) underwent a heating process in an oven at 145°C for 4.5 hours to obtain oligosaccharide extract (O-HWE) following Yudiati *et al.* (2018).

P-A-HWE Extraction Method and O-A-HWE Production

The P-A-HWE method, a modification, included a depigmentation step involving the addition of hydrogen peroxide (H_2O_2) and ascorbic acid (1:1 ratio, concentration 17.26 mM) (Chen *et al.*, 2016a). Subsequent procedures, including depigmentation, extraction, and precipitation, mirrored those outlined in the P-HWE extraction method (Tabarsa *et al.*, 2018). The polysaccharide yield was determined from the depigmented powder obtained after ethanol

precipitation. Following this, the dried acidic polysaccharide (P-A-HWE) underwent a heating process in an oven at 145°C for 4.5 hours to yield the hot acidic oligosaccharide extract (O-A-HWE) (Yudiati *et al.*, 2018).

Feeding Trial and Experimental Design

Ulvan was dried and supplemented into the shrimp feed at a dosage of 0.15% (1500 mg kg^{-1} of feed) (SGH® commercial pellets, PT Suri Tani Pemuka, Indonesia). Each of the four extract types (P-HWE, O-HWE, P-A-HWE, and O-A-HWE) was prepared with 1500 mg feed supplementation. This involved 30:1 (w/v) dissolving the extract in sterile water and evenly spraying it onto the feed, which was then dried at room temperature. The feed with the extract was weighed, prepared as a stock, and administered according to the daily feeding schedule for each treatment. The SGH® commercial pellets, equivalent to 5% of the average body weight per day, were fed four times daily (04:00, 10:00, 16:00, and 22:00). The proximate analysis of SGH® feed revealed: a water content of 11%; protein 32-36%; lipid 6.5-7.0%; crude fiber 3%; ash 12%, and energy 16.5-17.0 MJ kg^{-1}.

The experimental setup utilized a completely randomized design. Three hundred and seventy-five healthy *L. vannamei* (5.45-6.22 g) were obtained from the Brackish Water Aquaculture Development Center, Ministry of Marine Affairs and Fisheries, Jepara. The shrimp were stocked in rectangular plastic tanks (145 L) filled with 100 L of seawater at a density of 25 shrimp per tank. The tanks were aerated, and approximately 10% of water renewal occurred daily (Azhar & Yudiati, 2023; Yudiati *et al.*, 2019). Water parameters were monitored for pH (7.99-8.40), dissolved oxygen (5.22-5.51 $mg.L^{-1}$), salinity (30 ppt), temperature (29.33-31.09ºC), with nitrate, nitrite, and ammonia levels undetected using Merck colorimetric kits. Water parameters were observed daily at 08:00 and 16:00. A 3-day acclimatization period was observed until the shrimp displayed normal behavior and consumed the provided feed. The

survival rate (%) was evaluated at the end of the experiment. Three individual shrimp samples from each replicate were collected daily for immune parameter analysis for ten days. The shrimps were still alive for hemolymph sampling and brought back to the tank. After reaching maximum trypsin activity, further experiments were conducted using Ulvan to determine the fastest time it could reach maximum trypsin activity. Different concentrations were used, with Ulvan added to shrimp feed at concentrations of 0 g kg^{-1} (Control), 0.75 g kg^{-1} (ULV-0.75), 1.50 g kg^{-1} (ULV-1.50), and 3.00 g kg^{-1} (ULV-3.00) of feed.

Trypsin Extraction from Shrimp Gut

Trypsin extraction from shrimp gut procedure modified from Tang *et al.* (2022) and Khantaphant and Benjakul (2010). The 100 mg of shrimp gut was homogenized with 500 µL volumes of 20 mM Tris–HCl buffer (pH 8.0) containing 5 mM $CaCl_2$ for 3 min using a sterile plastic grinder. The homogenate was centrifuged at 8,000∗ *g* for 30 minutes at 4°C. The supernatant obtained was referred to as shrimp gut extract.

Evaluation of Trypsin Activity

The trypsin activity measurement followed the procedures developed by Senphan and Benjakul (2012) and Kumlu and Jones (1995) with slight modification, using BAPNA as the substrate. A shrimp gut extract (100 µL), appropriately diluted, was mixed with the addition of 200 µL of 10 mM substrate, followed by incubation at 37°C for 20 minutes. To halt the reaction, 200 µL of cold 10% (w/v) trichloroacetic acid was introduced. Absorbance was then measured at 410 nm using a UV-vis spectrophotometer (R-Biopharm Well Reader; Germany). A blank solution was prepared similarly, omitting the enzyme addition before introducing the trichloroacetic acid. Each sample underwent triplicate runs. Trypsin activity (U) was calculated as the enzyme quantity needed to convert 1 µmol of substrate per minute.

Ethical Statement

The use of animals in this study follows the ethic protocol of animal welfare for research at Diponegoro University and SNI 8037.1: 2014 (SNI, 2014).

Data Analysis

The data underwent analysis of variance (ANOVA) followed by the least significant difference (LSD) test for further comparisons. Different letters assigned to data points at the same sampling time indicate significant differences ($p < 0.05$) in trypsin activity.

RESULTS AND DISCUSSION

Trypsin Activity in Four Types of Ulvan

Trypsin, a serine protease, assists in protein digestion and promotes the production of other digestive enzymes (Lemos *et al.*, 2000). In *Litopenaeus vannamei*, its trypsin-like protease plays a crucial role in breaking down myofibrillar and collagen proteins, leading to muscle softening (Peng *et al*., 2019). This study demonstrates a positive correlation between the immunostimulant Ulvan and trypsin production. Based on Figure 1, the fastest and highest increase in trypsin activity significantly differed from the control, which occurred on day 4 for O-A-HWE. On other days, trypsin activity significantly exceeded the control in O-A-HWE on days 2, 3, 7, and 8, while it was significantly lower than the control on day 6. Another extract, P-HWE, showed significantly higher trypsin activity compared to the control on days 3, 4, 6, 7, 8, and 9. Similarly, another extract, O-HWE, exhibited significantly higher trypsin activity compared to the control on days 2, 3, 8, and 9 and significantly lower activity on day 4. Another extract, P-A-HWE, displayed significantly higher trypsin activity compared to the control on days 4, 7, and 8, while it was significantly lower on day 9.

Furthermore, our study highlights the broader implications of immunostimulants in aquaculture. While immunostimulants like Ulvan may not directly increase digestive protein, they are crucial in supporting immune system biosynthesis, indirectly influencing protein digestion efficiency. The study demonstrated the effects of replacing dietary fish meal with Antarctic krill (*Euphausia superba*) meal on increasing the growth performance, immunity, and muscle quality of white shrimp *L. vannamei* (Wei *et al.*, 2022). In the hepatopancreas of shrimp fed diets containing 100 g kg^{-1} fish meal and protease-complex at 175 mg kg^{-1}, the activities of digestive enzymes such as trypsin, lipase, and amylase were observed to be higher compared to those fed diets with only 100 g kg^{-1} fish meal, but lower than those fed diets containing 200 g kg^{-1} fish meal (Song *et al.*, 2017). Another study examined the impact of tributyrin supplementation, alone or in combination with fructooligosaccharides, in high soybean meal diets on the growth performance, innate immunity, and intestinal morphology of *Litopenaeus vannamei* shrimp. The addition of tributyrin to the diet notably boosted trypsin activity in the hepatopancreas, hemolymph ACP and AKP activities and also improved mucosal thickness (Liu *et al.*, 2021).

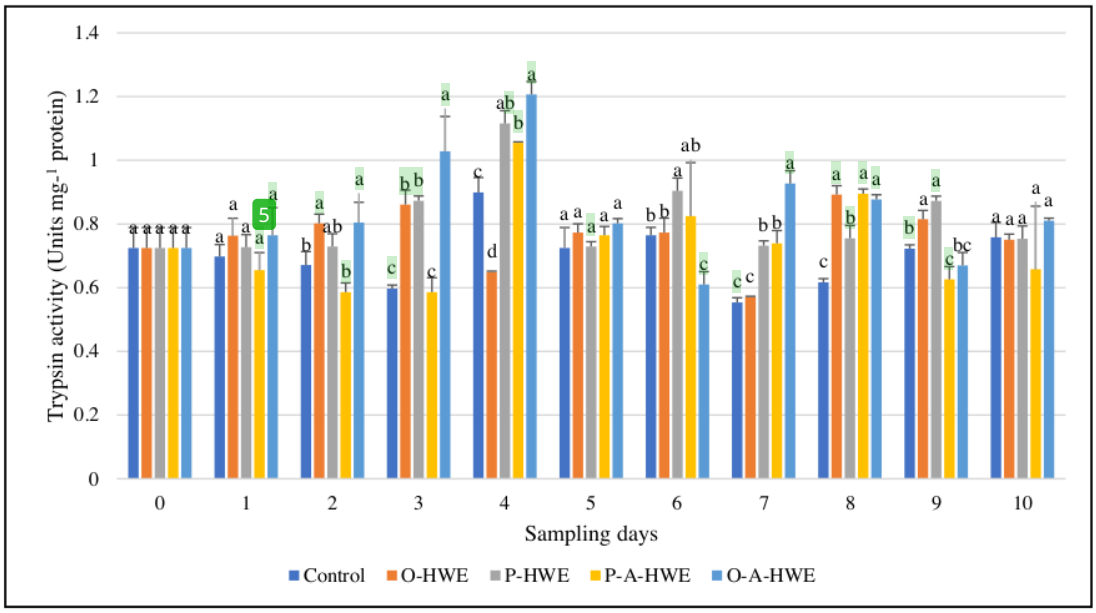

Figure 1. The trypsin activity in *Litopenaeus vannamei* before, every day and 10th day of rearing with oral supplementation of P-HWE, O-HWE, P-A-HWE, and O-A-HWE in feed. Letters above the bars indicate significant differences ($p < 0.05$, LSD test)

Trypsin Activity Variation over Four Days with Three Different Ulvan Doses (O-A-HWE)

Pattern recognition serves as the initial phase in innate immunity. Immunostimulants, like any infection, are identified in the hemocoel of shrimp through the recognition of various PAMPs present in the immunostimulants. Unlike antigen-specific responses, the introduction of immunostimulants triggers a broad response that expedites the detection and elimination of a wide range of infectious pathogens (Secombes, 1994). Furthermore, the immune system of *L. vannamei* can be enhanced through oral administration of polysaccharide supplements from seaweed, utilizing recognition pathways such as the Lipopolysaccharide and β-1,3-Glucan Binding Protein (Azhar & Yudiati, 2023; Chen *et al.*, 2016b; Yudiati *et al.*, 2016;). Our previous study showed that the fastest increase in immune phenoloxidase activity occurred in O-H-HWE.

Based on Figure 2, the trypsin activity of white shrimp *L. vannamei* fed with diets supplemented with different doses of Ulvan showed no significant difference compared to the control. Based on Figure 3, the trypsin activity of white shrimp *L. vannamei* fed with diets supplemented with different doses of Ulvan showed no significant difference compared to the control on day 1.

Based on Figure 4, the trypsin activity of white shrimp *L. vannamei* fed with diets supplemented with different doses of Ulvan showed a significant difference compared to the control on day 2. However, no significant difference was observed between the doses themselves. Based on Figure 5, the trypsin activity of white shrimp *L. vannamei* fed with diets supplemented with different doses of Ulvan showed a significant difference compared to the control on day 3. Specifically, ULV-1.50 g kg^{-1} exhibited a significant difference compared to ULV-0.75 g kg^{-1}, while no significant difference was observed compared to ULV-3.00 g kg^{-1}.

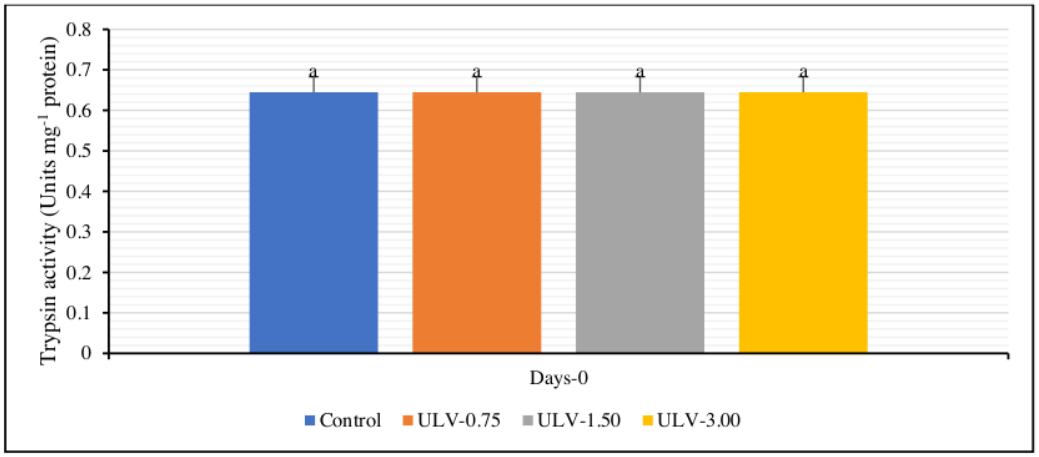

Figure 2. Trypsin activity (Units mg^{-1} protein) of white shrimp *Litopenaeus vannamei* fed diets supplemented with Ulvan at 0 g kg^{-1} (control), 1.50 g kg^{-1}, and 3.00 g kg^{-1} on days-0. Data points with different letters at the same sampling time indicate significant differences ($p < 0.05$)

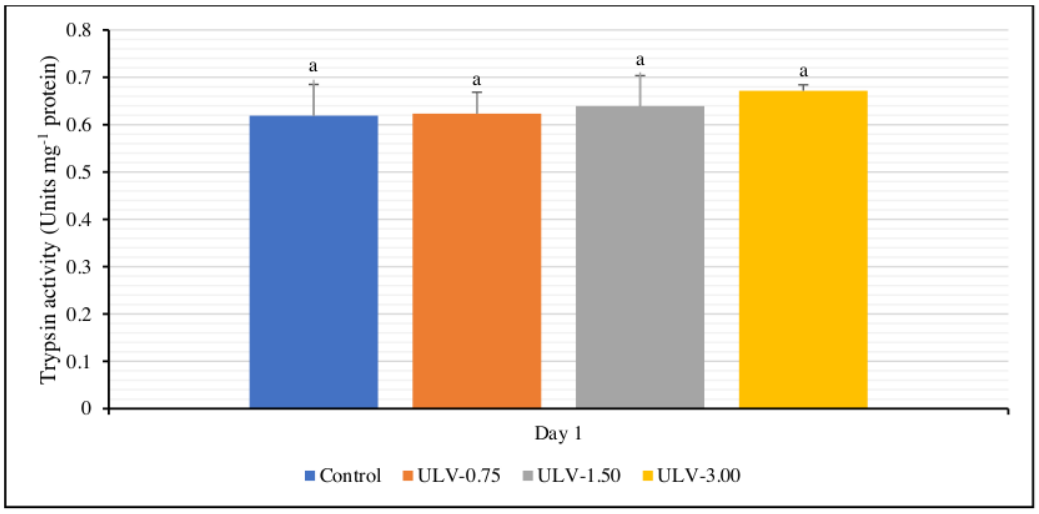

Figure 3. Trypsin activity (Units mg^{-1} protein) of white shrimp *Litopenaeus vannamei* fed diets supplemented with Ulvan at 0 g kg^{-1} (control), 0.75 g kg^{-1}, 1.50 g kg^{-1}, and 3.00 g kg^{-1} on day 1. Data points with different letters at the same sampling time indicate significant differences ($p < 0.05$)

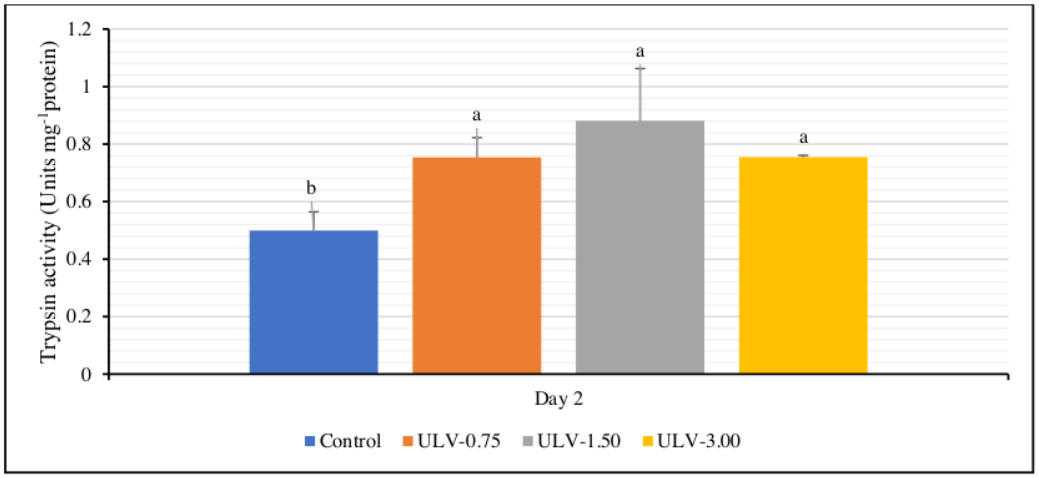

Figure 4. Trypsin activity (Units mg^{-1} protein) of white shrimp *Litopenaeus vannamei* fed diets supplemented with Ulvan at 0 g kg^{-1} (control), 0.75 g kg^{-1}, 1.50 g kg^{-1}, and 3.00 g kg^{-1} on day 2. Data points with different letters at the same sampling time indicate significant differences ($p < 0.05$)

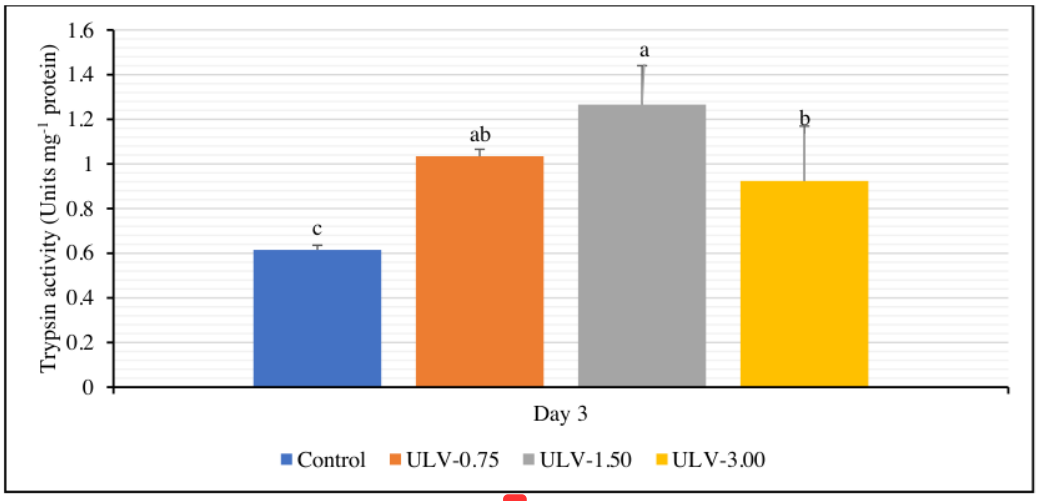

Figure 5. Trypsin activity (Units mg^{-1} Protein) of white shrimp *Litopenaeus vannamei* fed diets supplemented with Ulvan at 0 g kg^{-1} (control), 0.75 g kg^{-1}, 1.50 g kg^{-1}, and 3.00 g kg^{-1} on day 3. Data points with different letters at the same sampling time indicate significant differences ($p < 0.05$)

Based on Figure 6, the trypsin activity of white shrimp L. vannamei fed with diets supplemented with doses of ULV-1.50 g kg^{-1} and ULV-3.00 g kg^{-1} of Ulvan showed a significant difference compared to the control and ULV-0.75 g kg^{-1} on day 3. However, ULV-0.75 g kg^{-1} did not exhibit a significant difference compared to the control on day 3.

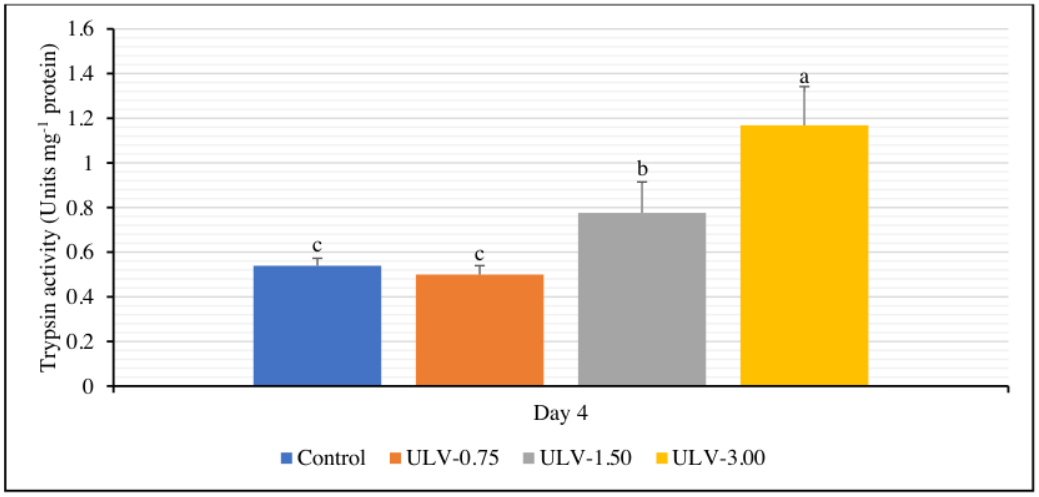

Figure 6. Trypsin activity (Units mg^{-1} protein) of white shrimp *Litopenaeus vannamei* fed diets supplemented with Ulvan at 0 g kg^{-1} (control), 0.75 g kg^{-1}, 1.50 g kg^{-1}, and 3.00 g kg^{-1} on day 4. Data points with different letters at the same sampling time indicate significant differences ($p < 0.05$)

Qiao *et al.* (2011) concluded that vitamin C and Chinese herbs served as effective immunostimulants for shrimp *L. vannamei*, with seven modified proteins, including trypsin, potentially contributing to enhanced immune responses. The trypsin activities in the hepatopancreas were assessed in both the 5 g kg^{-1} multi-species probiotic and 5 g kg^{-1} multi-enzyme preparation groups. Specifically, the combined use of complex probiotics and enzymes demonstrated overall positive effects, significantly enhancing growth performance, immunity, and disease resistance capacity (Zhang *et al.*, 2021).

Several studies (Gäde & Goldsworthy, 2003; Li *et al.*, 2018; Patel, 2017) have highlighted the crucial roles of trypsin, a protease, in the immune system. In line with our present study, Li *et al.* (2018) observed that the increase in trypsin levels correlates with the generation of hemocyanin-derived peptides in *L. vannamei*. Trypsin treatment has been shown to significantly enhance hemocyanin phenoloxidase, a key component of humoral immunity (Kim *et al.*, 2011). Additionally, trypsin purified from another organism, *Steinernema carpocapsae*, has been found to impact host hemocytes and actin filaments and regulate hemolymph melanization (Balasubramanian *et al.*, 2010). Additionally, investigating the long-term impacts of Ulvan supplementation on shrimp health and productivity, along with its potential interactions with other dietary elements, is essential. Further research is necessary to determine the most effective dosage and administration methods of Ulvan to optimize its beneficial effects on shrimp health and immune response. Moreover, conducting comparative studies between various immunostimulants and their effects on protein digestion and immune function could offer valuable insights into the most efficient strategies for improving shrimp health in aquaculture.

CONCLUSION

O-A-HWE had the highest increase in trypsin activity on day 4, surpassing the control on days 2, 3, 7, and 8. Other methods (P-HWE, O-HWE, and P-A-HWE) also affected trypsin activity significantly on specific days. While trypsin activity did not differ from the control on days 0 and 1, differences emerged by days 2 and 3, notably between ULV-1.50 g kg^{-1} and ULV-0.75 g kg^{-1}. The ULV-3.00 g kg^{-1} showed no significant difference from ULV-1.50 g kg^{-1}. O-A-HWE displayed significant differences in trypsin activity compared to other Ulvan extracts, suggesting its potential to enhance shrimp health. Further research is necessary to understand the long-term effects and optimal dosage of Ulvan and its future comparative studies with other immunostimulants in aquaculture.

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