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PRETREATMENT STRATEGIES FOR IMPROVED ASTAXANTHIN RECOVERY FROM CRAB CARAPACE WASTE: ENZYMATIC VS. STEAM-EXPLOSION METHODS

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ABSTRACT

Crab meat pasteurization industries produce carapace waste containing astaxanthin. The carapace composition includes minerals, proteins, and chitin, which can hinder the astaxanthin extraction. This study investigated the effects of alcalase and steam-explosion pretreatments on the astaxanthin yield and antioxidant activity of astaxanthin extracted from the carapace powder. The alcalase pretreatment was determined using OVAT (One-Variable-At-A-Time) in a Completely Randomized Design, including particle sizes (20–100 mesh), carapace-to-alcalase ratios (1:1 up to 1:5 w/v), and hydrolysis times (10–180 min). The steam-explosion pretreatment was carried out by heating carapace powder in a steam-explosion at 130–140 ºC for 3 min. Subsequently, astaxanthin was extracted using a sonicator with acetone 1:7 (w/v) at 50 ºC for 20 min. The best alcalase pretreatment results were obtained with a particle size of 80–100 mesh, carapace-to-alcalase ratio of 1:2 (w/v), and a hydrolysis time of 40 min, which increased the relative astaxanthin yield by 23.69% and had no significantly effect on IC_{50} compared to the control (unpretreated carapace). The steam-explosion pretreatment decreased the relative astaxanthin yield by 63.89%, but increased on IC_{50} by a factor of 2 compared with the control (indicating increased antioxidant activity). HPLC chromatograms showed that the main component of all extracts was trans-astaxanthin. The results suggest that alcalase pretreatment is a potential to increase the yield, whereas steam-explosion pretreatment is a potential to increase the antioxidant activity of astaxanthin extracted. The development of astaxanthin recovery technology and the utilization of carapace waste can support a sustainable blue economy.

Keywords: Alcalase; HPLC; Hydrolysis time; IC_{50}; Steam-explosion

INTRODUCTION

The blue swimming crab (*Portunus pelagicus*) is a highly valuable species found abundantly in the western Pacific and eastern Indian Oceans, including Indonesia, where the major fishery commodity is mainly exported as pasteurized crab meat. Indonesia is a major contributor to the global crab meat market, accounting for more than 40% of the total contribution from 74 countries that export pasteurized crab meat (Setioko *et al*., 2024). Based on data from the Ministry of Maritime Affairs and Fisheries of Indonesia (MMAF), the production volume of crab in 2023 was 127,040.68 tonnes (MMAF, 2025). Crab processing produces up to 80% waste consisting of viscera and crab shells, including shoulder, leg, tip, claw, and carapace (Hossain & Shahidi, 2024), posing an environmental problem. The persistent underutilization of crab carapace presents a substantial opportunity to advance its use toward broader applications, such as protein hydrolysate, astaxanthin, chitin, and chitosan production for biomedical, food, cosmetic, feed, and nutraceutical industries, thereby transforming waste into high-value products and mitigating ecological impact.

Crab carapace contains pigments in addition to chitin, protein, and calcium carbonate. Stachowiak & Szulc (2021) and Su *et al*. (2018) describe astaxanthin as the primary pigment found in the main layer of the exoskeleton and in subepidermal chromatophores of the crustacean carapace, which is removed during chitin processing because it imparts an orange-red colour. Meanwhile, chitin and chitosan must be colorless to be used in various products. Shah *et al*. (2016) explained that astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is a red carotenoid from the xanthophyll group with the chemical formula of $C_{40}H_{52}O_4$ and molecular weight of 596,84 g/mol. The antioxidant activity of astaxanthin is 10 times higher than that of β-carotene, canthaxanthin, zeaxanthin, and lutein, 65 times stronger than vitamin C, and 100 times more effective than α-tokoferol. Therefore, an appropriate biorefinery method is needed to utilize raw materials sustainably and produce value-added products. Aneesh *et al*. (2023) extracted chitin and chitosan using the residue after protein and astaxanthin extraction from shrimp (*Solenocera choprai*) shells.

However, the structure of the crab carapace is rigid due to the high mineral content and matrix composed of protein and chitin. Efficient environmentally friendly pretreatment is required before extraction to open the carapace matrix and separate astaxanthin from proteins and minerals, thereby increasing astaxanthin recovery while retaining other valuable compounds, such as chitin. Hopkins *et al*. (2025) reported that enzymatic pretreatment using alcalase offers high flexibility, low energy, and sustainable material utilization. Antunes-Valcareggi *et al*. (2017) significantly increased the extraction yield ($p<0.05$) by approximately 8-fold compared to the control (without pretreatment) by performing deproteinization and

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demineralization pretreatment. Deproteinization was carried out with alcalase, followed by demineralization with a 7% HCl solution, before astaxanthin extraction from crab (*Callinectes sapidus*) carapace using acetone. Furthermore, the astaxanthin extraction residue is used to recover chitin.

Liquid waste generated by the demineralization process using strong acids/bases is corrosive and can adversely affect the environment (Hu *et al*., 2020). This indicates a need for alternative pretreatment methods, such as mechanical demineralization, that can disrupt the carapace matrix structure. Montoya *et al*. (2021) applied demineralization before extracting astaxanthin from crab carapace and shrimp heads. Demineralization was performed in an autoclave, followed by astaxanthin extraction with various organic solvents.

Steam-explosion has recently been used for biomass decomposition, and Hu *et al*. (2020) developed a steam-explosion process to extract chitin from shrimp carapaces. The vapor permeability during steam-explosion heating can disrupt interactions among minerals, proteins, and chitin in the carapace, increasing the reactive surface area and accelerating the extraction. Bonfiglio *et al*. (2021) studied the extraction of xylitol and carotenoids from switchgrass and *Eucalyptus globulus* with pretreatment using steam-explosion. Based on the results, steam-explosion is expected to be an alternative pretreatment method prior to astaxanthin extraction.

Sufficient information is not available regarding pretreatment with alcalase and steam-explosion before astaxanthin extraction from dried crab (*P. pelagicus*) carapace. The influence and potential applications of different pretreatment methods for enhancing astaxanthin yield and the antioxidant activity of astaxanthin extracts will be discussed in this article. This study aimed to evaluate the effects of alcalase and steam-explosion pretreatments on the astaxanthin yield and the antioxidant activity of astaxanthin extracted from dried crab carapace.

RESEARCH METHODS

Time and Location

The research was conducted from January to July 2024 in the Faculty of Agricultural Technology laboratory, Universitas Gadjah Mada (Special Region of Yogyakarta, Indonesia).

Materials

The crab (*P. pelagicus*) carapace was obtained in December 2023 from the crab-peeling miniplant of UD Lautan Bening in Banyudono Village, Kaliori District, Rembang Regency, Central Java Province, Indonesia. In the crab-peeling miniplant, the crab was steamed for 30 min before being the meat was separated from the crab shells. The crabs processed by the miniplant have a carapace length of 10–15 cm. Furthermore, the carapace was kept in a styrofoam box filled with ice until the temperature fell below 4 ºC, then transported to Universitas Gadjah Mada for astaxanthin extraction.

Preparation of Crab Carapace Powder

The crab carapace was washed thoroughly, then dried in a food dehydrator at 50 ºC for 2 h (the moisture content aproximately 5%), and ground in a grinder (Getra IC-108, China) for 30 sec at 25,000 rpm. The carapace powder was filtered using a sieve shaker (Haver & Boecker 59302 Oelde, Germany) with sieves measuring 20, 40, 60, 80, and 100 mesh. The carapace powder was positioned in aluminium pouches and stored at -20 ºC until used for astaxanthin extraction.

Proximate Analysis of Carapace Powder

The proximate content of blue swimming crab carapace powder (80–100 mesh), unpretreated, was determined according to the standard methods of the Association of Official Analytical Chemists (AOAC, 2005). Moisture and ash content were determined using the gravimetric method, with an oven for moisture and a furnace for ash. Protein content was determined using the Kjeldahl method, and lipid content was analyzed using the Soxhlet method.

Pretreatment Methods

Before astaxanthin extraction, pretreatment was carried out using alcalase or steam-explosion. Enzymatic pretreatment was performed using Alcalase® Enzyme (*Bacillus licheniformis*, Merck, Denmark) solution with enzyme activity ≥0.75 Anson u/mL at 25 ºC and pH 7.5, and the optimum hydrolysis temperature and pH for alcalase were 50 ºC and 9, respectively. The effect of crab carapace powder particle size (20–40, 40–60, 60–80, and 80–100 mesh) during alcalase pretreatment on the astaxanthin content and yield was investigated by treating carapace powder with alcalase at a ratio of 1:1 (w/v) for 120 min. Subsequently, the optimal carapace-to-alcalase ratio (1:1, 1:2, 1:3, 1:4, and 1:5 w/v, expressed as carapace mass per alcalase solution volume, which corresponds to the enzyme-to-substrate ratio of 0.75, 1.50, 2.25, 3.00, and 3.75 u/g carapace) was determined based on the highest astaxanthin content and yield by hydrolyzing the powder with the selected particle size for 120 min. The powder with the selected particle size and the carapace-to-alcalase ratio was hydrolyzed for 10, 20, 30, 40, 50, 60, 90, 120, 150, and 180 min to investigate the effect of hydrolysis time on astaxanthin yield. Alcalase was inactivated at 75 ºC for 5 min.

The steam-explosion pretreatment was carried out by heating carapace powder (80–100 mesh) in a steam-explosion for 3 min. This study used a simple steam-explosion with non-adjustable temperature and pressure. The steam-explosion consisted of a reactor vessel containing the sample and water, an oil-bath generating the temperature and pressure inside the reactor vessel, and an expansion chamber to explode the sample. The reactor vessel has a capacity of 200 mL and is equipped with a thermometer and a manometer. Based on temperature and pressure monitoring, it was found that during the heating process at 130 ºC for 3 minutes, there was a natural increase in temperature to 140 ºC and an increase in pressure from 2.5 to 3.5 kg/cm^2. Subsequently, the samples that had undergone pretreatment were dried using a freeze-dryer (Labconco, USA) for 48 h.

Extraction of Astaxanthin

Pretreated crab carapace powder was extracted for astaxanthin according to Antunes-Valcareggi *et al*. (2017) with a slight modification to the carapace-to-acetone ratio, 1:7 (w/v), respectively. The mixture was sonicated at 40 kHz and 50 ºC for 20 min using a bath sonicator (Baku BK-2000, China), and the astaxanthin extract was separated on Whatman #42 filter paper. The extraction process was repeated three times, and the filtrates

were combined; the acetone was then removed using a rotary evaporator (Ika RV10, China) at 50 ºC under vacuum. Astaxanthin extract was redissolved in methanol to a volume of 10 mL and stored at -20 ºC in an amber bottle until analysis. In this study, astaxanthin was also extracted from crab carapace powder (80–100 mesh) without pretreatment as a control.

Determination of the Astaxanthin Content of the Extract Solution and the Astaxanthin Yield

The astaxanthin content in the extract solutions from the control, alcalase pretreatment, and steam-explosion pretreatment was determined using a UV-vis spectrophotometer (Shimadzu UV-1280, Japan) at of 477 nm (Prayitno *et al*., 2022). This was calculated from the linear correlation equation of the *trans*-astaxanthin standard (Sigma-Aldrich SML0982, USA) in the methanol-soluble extract. The calculated content in the extract solution (mg/L) was considered as total carotenoids, while astaxanthin yield was estimated using the following equation:

$$\text{Astaxanthin yield } (\mu g/g \text{ dry weight}) = \text{Ast} \times V \times D \times 1000/w \quad \ldots\ldots(1)$$

where: Ast is astaxanthin content in the extract solution (mg/L), V represents volume of extract solution (L), D is dilution factor, and w is carapace mass (g dry weight)

Antioxidant Activity Analysis

The antioxidant activity of astaxanthin extract from control, alcalase pretreatment, and steam-explosion pretreatment was evaluated using the radical scavenging assay of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Chintong *et al*., 2019). The astaxanthin extract in methanol at various concentrations was mixed with 0.1 mM DPPH solution at a 1:1 (v/v) ratio and incubated for 30 min at room temperature in the dark. The samples were measured for absorbance at 517 nm using a spectrophotometer. The scavenging of DPPH radicals by astaxanthin extract of various concentrations was plotted on a curve to estimate the IC_{50} value. IC_{50} is known as the concentration of astaxanthin required to reduce DPPH radicals by 50%. The *trans*-astaxanthin standard was used as a reference, and the radical scavenging activity (RSA) of DPPH was determined based on the following equation:

$$\%RSA = [A_{DPPH} - (A_{sample} - A_{blank})]/A_{DPPH} \times 100\% \quad \ldots\ldots..(2)$$

where: A_{DPPH} is the absorbance of 0.1 mM DPPH solution in methanol, A_{sample} represents the absorbance of the astaxanthin extract solution with the addition of 0.1 mM DPPH solution, and A_{blank} is the astaxanthin extract solution without the addition of DPPH solution.

Identification of Astaxanthin Extract Using HPLC

The astaxanthin extract from the control, alcalase pretreatment, and steam-explosion pretreatment was identified by HPLC (Hu *et al*., 2019), with slight modifications to the column temperature and astaxanthin solvent. First, the astaxanthin extract in methanol was passed through a 0.45 µm filter membrane before being injected into the HPLC (Shimadzu Prominence, Japan). The extract was passed through a C18 chromatography column, 4.6 mm x 150 mm, 5 µm (column Shimadzu®, Shimadzu, Japan) at a flow rate of 1 mL/min, isocratic pressure of 85 psi, wavelength of 474 nm, column temperature of 30 ºC, and injection volume of 20 µL. The mobile phase consisted of acetonitrile/methanol/dichloromethane (80:15:5, v/v/v) (Merck, Germany).

Statistical analysis

The data are shown as the mean of three replicates ± standard deviation. In the alcalase pretreatment screening stage, the experiment was conducted using the OVAT (One-Variable-At-A-Time) method in a Completely Randomized Design (CRD). The best results from the alcalase pretreatment screening were compared with those from the steam-explosion pretreatment and the control (unpretreated carapace) in a CRD. Astaxanthin content, astaxanthin yield, and IC_{50} were analyzed using analysis of variance (ANOVA). Duncan's test was used to determine significant differences between treatments ($p<0.05$). Statistical analysis was performed with SPSS® version 16 (IBM Co., USA).

RESULT AND DISCUSSION

Proximate Composition of Crab Carapace Powder

The results showed that minerals were the main component of the carapace of *P. pelagicus* (Table 1), followed by protein. Similar results were reported by Antunes-Valcareggi *et al*. (2017) and Pok *et al*. (2023), indicating that the main components of the carapace were minerals and proteins, in addition to water. Suryawanshi *et al*. (2020) reported that chitin fiber, in addition to minerals and proteins, made the carapace denser and more complex. Chitin fibre interacts with proteins through covalent bonds in the crustacean carapace, and the matrix is filled with minerals, specifically calcium carbonate. Therefore, pretreatment is required before astaxanthin extraction, namely a process to release the calcium-protein complex. The open carapace matrix facilitate contact between astaxanthin and the solvent.

Table 1. Proximate Composition of Crab Carapace

Crab Species	Raw Material	Moisture (%)	Protein (%db)	Lipid (%db)	Ash (%db)
Portunus pelagicus (present study)	carapaces powder	5.85±0.46	11.73±0.72	0.02±0.01	74.28±0.21
Callinectes sapidus (Antunes-Valcareggi *et al*., 2017)	carapaces and legs flour	35.80±0.10	33.00±1.00	1.93±0.07	56.2±1.20
Dilocarcinus pagei (Pok *et al*., 2023)	male whole carapaces	65.40±1.00	18.20±1.60	10.40±0.30	45.70±2.90
Dilocarcinus pagei (Pok *et al*., 2023)	female whole carapaces	72.50±3.10	17.40±1.50	11.10±1.50	40.30±2.20
Portunus segnis (Hamdi *et al*., 2020)	whole carapaces	55.60±0.80	12.90±0.80	4.40±0.40	53.80±0.50

Note: Literature values are for comparison only.

Table 1 shows that the proximate composition of each crab species differs. Factors that influence the biochemical composition of fish include feed source, biological variations (species, size, sex, and age), and environmental conditions (temperature, salinity, seasonal changes, and pH) (Fallah *et al*., 2013; Herawati *et al*., 2019; Nanda *et al*., 2021). The biochemical composition and color of crabs are influenced by their diet, with microalgae as the primary natural feed. Therefore, species, biochemical composition, and density of microalgae in the water significantly influence the color and biochemical composition. The presence of microalgae in the water can increase dissolved oxygen (DO) levels through their photosynthetic activity, and high DO levels improve crab quality (Kong *et al*., 2012).

Effect of Carapace Particle Size on the Astaxanthin Content of the Extract Solution and Astaxanthin Yield

Based on Table 2, the smaller the particle size of crab carapace, the higher the astaxanthin content and yield, so that more astaxanthin can be extracted from crab carapace by the solvent. Nwabanne (2012) explained that a smaller particle size of the material led to more damaged cell membranes during grinding, making the compounds in the material easier for the solvent to extract. Additionally, a smaller particle diameter means a shorter diffusion distance the solvent must travel within the particle to reach the surface and appear in the free extract phase. The process of extracting compounds using solvents starts with the solvent penetrating the solid matrix, then the target compound dissolves in the solvent, diffuses out of the solid matrix, and can then be collected (Smith, 2003).

Table 2. Astaxanthin Content of The Extract Solution and Astaxanthin Yield with Differences in Carapace Particle Size

Sample	Astaxanthin Content of the Extract Solution (mg/L)	Astaxanthin Yield (µg/g dry weight)
A	0.97±0.02^{a}	3.39±0.10^{a}
B	1.91±0.10^{b}	6.61±0.35^{b}
C	2.35±0.14^{c}	8.12±0.51^{c}
D	4.32±0.09^{d}	14.93±0.32^{d}

Note: Data with different superscript letters in the same column show significant differences ($p<0.05$). The sample is astaxanthin extract from crab carapace powder A (20–40 mesh), B (40–60 mesh), C (60–80 mesh), and D (80–100 mesh).

Crab carapace powder with a particle size of 80–100 mesh (149–177 µm) produces an extract with the highest astaxanthin content and yield ($p<0,05$) (Table 2), so this carapace powder was selected for the next stage of this study. Reducing particle diameter by grinding the material can increase extraction efficiency and decrease extraction time (Parjikolaei *et al*., 2015). In this study, crab carapace with a size of >100 mesh (<149 µm) was not used because the solids and extract were difficult to separate, corresponding with the statement of Zwingelstein *et al*. (2020) that the particle size of a material should be small to allow solvent penetration. However, extremely small particles can make it difficult to separate solids from the solution containing the target compound.

Effect of Carapace and Alcalase Ratio on the Astaxanthin Content of the Extract Solution and Astaxanthin Yield

Antunes-Valcareggi *et al*. (2017) reported that alcalase among the proteases that can hydrolyze crab carapace proteins, thereby facilitating and maximizing astaxanthin extraction. The amount of alcalase significantly affected astaxanthin content and yield ($p<0.05$) (Table 3). The carapace-to-alcalase ratio of 1:1 (w/v) produced an extract with low astaxanthin content and yield and did not differ significantly from the carapace-to-alcalase ratio of 1:5 (w/v) ($p>0.05$). The carapace-to-alcalase ratio of 1:2 (w/v) optimized the extraction process, yielding the highest astaxanthin content and yield ($p<0.05$), so that this ratio was selected for the next step of this study.

Table 3. Astaxanthin Content of The Extract Solution and Astaxanthin Yield with Different Ratios of Carapace and Alcalase

Ratio of Carapace and Alcalase (w/v)	Astaxanthin Content of the Extract Solution (mg/L)	Astaxanthin Yield (µg/g dry weight)
1:1	4.52±0.33^{a}	15.64±1.15^{a}
1:2	5.38±0.51^{b}	18.56±1.72^{b}
1:3	5.44±0.41^{b}	18.81±1.42^{b}
1:4	5.39±0.20^{b}	18.48±0.70^{b}
1:5	4.50±0.34^{a}	15.44±1.17^{a}

Note: Data with different superscript letters in the same column represent significant differences ($p<0.05$).

Deng *et al*. (2020) explained that increasing protease activity can hydrolyze proteins in the carapace more extensively. However, excessive protease did not significantly increase the amount of hydrolyzed protein. The amount of hydrolyzed protein allows the crab carapace matrix to become more open, generating more astaxanthin extract. However, the carapace-to-alcalase ratio of 1:5 (w/v) decreased astaxanthin content in the extract solution and astaxanthin yield (Table 3). This result was thought to be caused by increased damage to the carapace matrix due to excessive use of alcalase, allowing greater contact between astaxanthin and heat, light, and oxygen during hydrolysis and astaxanthin extraction. Carail *et al*. (2015) stated that released carotenoids become more susceptible to degradation caused by environmental conditions. The presence of water and oxygen can also promote the degradation of carotenoids

On the other hand, small amounts of alcalase (carapace-to-alcalase ratio of 1:1 w/v) also result in low astaxanthin content and yield. This is thought to be due to the small amount of alcalase used, resulting in only a small amount of protein being hydrolyzed, thus making it difficult to separate astaxanthin from the crab carapace. Yazdi *et al*. (2019) explained that low enzyme concentrations result in less than optimal protein hydrolysis. Therefore, enzymatic pretreatment cannot facilitate the release of bioactive compounds from the material.

The astaxanthin yield in this study was higher than that of other studies. Hooshmand *et al*. (2017) reported extraction of astaxanthin (carotenoid) from the carapace of fresh crab (*P. pelagicus*) with a carapace-to-acetone ratio of 1:4 (w/v), produced astaxanthin yield of 9.777 µg/g. Meanwhile, the

extraction of astaxanthin using sunflower oil 1:5 (w/v) at 78 ºC for 95 min produced a much lower yield, namely 0.372 µg/g.

Effect of Hydrolysis Time on the Astaxanthin Yield

Carapace protein was hydrolyzed with different periods and the carapace-to-alcalase ratio of 1:2 (w/v) (Table 3) to determine the effect of hydrolysis time and the optimal hydrolysis time. The result showed that astaxanthin yield increased with rising alcalase hydrolysis time up to 40 min. However, the yield decreased with hydrolysis time exceeding 40 min ($p<0.05$) (Fig. 1). This is because the longer the hydrolysis time by alcalase, the more astaxanthin was exposed to heat during the process. According to Lee *et al*. (2014), astaxanthin is easily decomposed even at room temperature, and more than 90% of astaxanthin decomposes when heated to 100 ºC for 5 sec. The total extractable carotenoids increased with increasing protein hydrolysis time until reaching a maximum (Hamdi *et al*., 2020). Sowmya & Sachindra (2012) explained that protein hydrolysis could release carotenoids bound to proteins, thereby increasing susceptibility to oxidation.

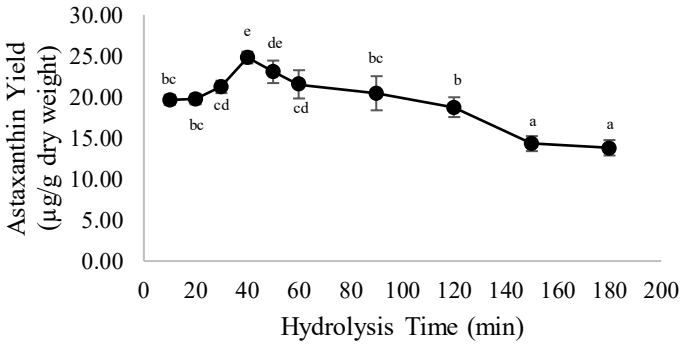

Figure 1. Astaxanthin Yield with Different Hydrolysis Times

The carapace-to-alcalase ratio of 1:2 (w/v), along with a hydrolysis time of 40 min, was the best alcalase pretreatment in this study. The astaxanthin yield (24.80±0.72 µg/g dry weight) from crab carapace powder was higher than previous results. Abd El-Ghany *et al*. (2023) reported that the astaxanthin yield obtained from *P. pelagicus* carapace powder through chemical extraction using acetone/hexane (1:1 v/v) was 19.46 µg/g dry weight, while 35.26 µg/g dry weight was from *C. sapidus*.

Effect of Pretreatment on the Astaxanthin Content of the Extract Solution, Astaxanthin Yield, Antioxidant Activity, and Compounds Contained

The astaxanthin extract from the best alcalase pretreatment results was obtained with a particle size of 80–100 mesh, carapace-to-alcalase ratio of 1:2 (w/v), and a hydrolysis time of 40 min compared to control (unpretreated carapace) and steam-explosion pretreatment. The astaxanthin content of the extract solution and the yield obtained with alcalase pretreatment were greater than those obtained with steam-explosion pretreatment and control ($p<0.05$) (Table 4). Deng *et al*. (2020) explained that only a small amount of astaxanthin could be extracted from the shrimp carapace control. Enzymatic pretreatment with protease and chitinase can facilitate astaxanthin extraction by reducing proteins and chitin reduction in the carapace, thereby preserving astaxanthin in its natural state.

Meanwhile, steam-explosion pretreatment reduced the astaxanthin content of the extract solution and the yield compared to control ($p<0.05$) (Table 4) because most *trans*-astaxanthin was degraded due to high temperatures during the heating process. The astaxanthin extract produced is yellow, compared to the red-orange extract obtained from alcalase pretreatment and control. This is consistent with the study by Jia *et al*. (2021) which shows that astaxanthin can undergo thermal degradation, leading to colour loss. The astaxanthin concentration decreases with increasing temperature and heating time. Tachaprutinun *et al*. (2009) also reported that the majority of astaxanthin in ethanol degraded when heated at 70 ºC for 2 h, during which *trans*-astaxanthin was isomerized to *cis*-astaxanthin.

Table 4. Astaxanthin Content of The Extract Solution, Astaxanthin Yield, and Ic_{50} with Different Pretreatments

Pretreatment	Astaxanthin Content of the Extract Solution (mg/L)	Astaxanthin Yield (µg/g dry weight)	IC_{50} (ppm)
Control	6.03±0.79^{b}	20.05±2.64^{b}	3.30±0.37^{b}
Alcalase enzyme	7.05±0.25^{c}	24.80±0.72^{c}	3.70±0.33^{b}
Steam-explosion	2.17±0.27^{a}	7.24±0.90^{a}	1.56±0.19^{a}
Astaxanthin standard	-	-	4.19±0.31

Note: Data with different superscript letters in the same column represent significant differences ($p<0.05$). The IC_{50} value of astaxanthin standard is a comparison value.

Despite the lower astaxanthin content and yield, steam-explosion pretreatment produces an extract with lower IC_{50} values ($p<0.05$) (Table 4). The lower the IC_{50}, the stronger the antioxidant activity. Astaxanthin extract from steam-explosion pretreatment exhibited stronger antioxidant activity than that from alcalase pretreatment and the control. This is likely due to the greater amount of monoester astaxanthin that could be extracted, and to free (*trans*) astaxanthin being largely isomerized to *cis*-astaxanthin during the heating process in the steam-explosion. Astaxanthin extract with a higher content of monoesters-astaxanthin shows greater antioxidant activity than astaxanthin extract with a higher content of diesters-astaxanthin (Hwang *et al*., 2020). Astaxanthin isomers exhibit higher antioxidant activity than *trans*-astaxanthin, particularly 9-*cis* astaxanthin, which is 4-fold more potent than *trans*-astaxanthin (Liu & Osawa, 2007). This may also explain why the astaxanthin standard has lower antioxidant activity than the astaxanthin extract from this study (Table 4), as the standard used was all-*trans* astaxanthin (Fig. 2a).

All astaxanthin extracts from this study exhibited very strong antioxidant activity, with IC_{50} values ranging from 1.56 to 3.70 ppm (Table 4). IC_{50} values less than 10 ppm were considered very strong antioxidant activity, while IC_{50} values between 10–50 ppm were strong antioxidant activity, and those with IC_{50} values between 50–100 ppm were moderate

antioxidant activity. Meanwhile, IC_{50} values >250 ppm indicate an inactive antioxidant (Kalemba *et al*., 2024).

Steam-explosion, also known as autohydrolysis, involves hot steam penetrating the crustacean carapace, causing rapid decompression and thereby disrupting the interactions among chitin, minerals, and proteins. This matrix change can increase the reactive area between the internal compounds and the solvent (Hu *et al*., 2020). Therefore, astaxanthin in the esterified form (monoesters and diester-astaxanthin), which is strongly bound in the matrix, can be extracted from the carapace. Brotosudarmo *et al*. (2020) explained that astaxanthin could be esterified at one or both hydroxyl groups with fatty acids, such as oleic acid, palmitic acid, or linoleic acid.

The IC_{50} results for free radical measurements showed that all astaxanthin extracts exhibited antioxidant activity (Table 4) via a hydrogen donor mechanism, thereby reducing DPPH free radicals to a more stable state (Craft *et al*., 2012). The different structures of the terminal groups and the number of methyl groups affect the antioxidant activity of carotenoids (Lee *et al*., 2014). Astaxanthin has a molecular structure consisting of hydrocarbons with conjugated double bonds known as polyenes. Additionally, the presence of hydroxyl (OH) and keto (C=O) groups on each ionone ring confers hydrogen-donating capability to astaxanthin (Kraboun *et al*., 2021).

Based on the chromatograms (Fig. 2), the dominant compound present in all of the astaxanthin extract appeared at a retention time of 3.4 min, corresponding with the retention time of *trans*-astaxanthin standard at 3.46 min. The presence of trans astaxanthin in the astaxanthin extract control, with alcalase pretreatment, and steam-explosion was 78.38, 81.21, and 38.31% of the total extract, respectively. Previous studies have reported astaxanthin as a major carotenoid pigment, accounting for approximately 45–98% of the total carotenoids extracted from shrimp carapaces (Chintong *et al*., 2019; Prameela *et al*., 2017; Sowmya & Sachindra, 2012).

Variations in pretreatment before extraction can produce different amounts of peaks after the *trans*-astaxanthin peak. This includes control, with alcalase pretreatment, and steam-explosion producing 5, 9, and 7 peaks, respectively (Fig. 2). Montoya *et al*. (2021) reported that astaxanthin extracted from the carapace of *Callinectes sapidus* and *Callinectes bellicosus* using acetone consisted of all-*trans* astaxanthin, esterified astaxanthin, 9-*cis* astaxanthin, and 13-*cis* astaxanthin.

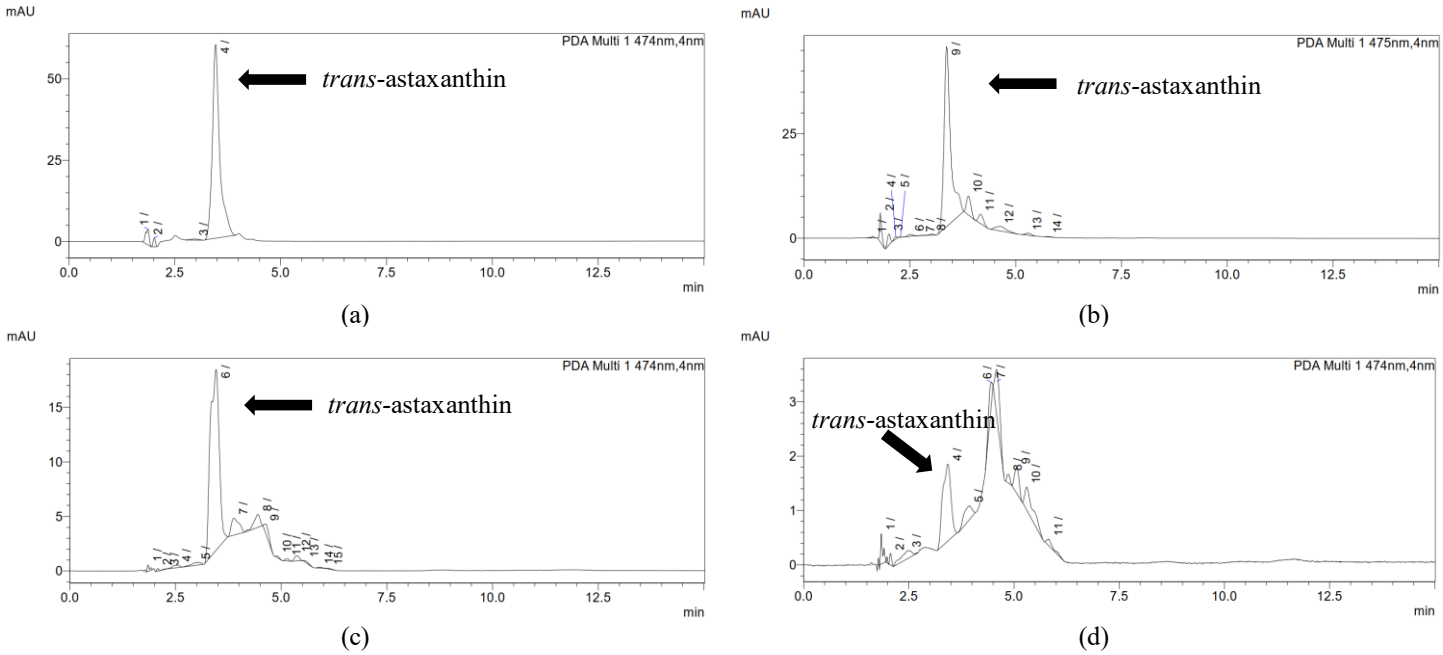

Figure 2. The Chromatograms of (a.) Astaxanthin Standard; (b.) Astaxanthin Extract Control; (c.) Astaxanthin Extract with Alcalase Pretreatment; and (d.) Astaxanthin Extract with Steam-Explosion Pretreatment

CONCLUSION

In conclusion, this study was an initial attempt to optimize astaxanthin extraction from dried crab carapace by introducing an alternative pretreatment method prior to extraction. The results showed that alcalase pretreatment with a carapace-to-alcalase ratio of 1:2 (w/v) and a hydrolysis time of 40 min could produce a higher astaxanthin yield compared to that obtained by steam-explosion. Alcalase pretreatment increased the relative astaxanthin yield by 23.69% compared with the control. The use of steam-explosion reduced the relative astaxanthin content and yield by approximately 65%, but the resulting extract had antioxidant activity that was two times stronger than the control. The amount of alcalase and hydrolysis time was found to affect the amount of astaxanthin extracted. In general, the results contribute to the development of astaxanthin recovery technology, namely alcalase and steamexplosion pretreatment, and the utilization of crab carapace waste to support a sustainable blue economy.

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