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# Albacore tuna spleen trypsin: Potential application as laundry detergent additive and in carotenoprotein extraction from Pacific white shrimp shells

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ABSTRACT

Partitioned trypsin from spleen of albacore tuna (*Thunnus alalunga*) by ATPS was evaluated for its potential applications in laundry detergents and in the recovery of carotenoprotein from Pacific white shrimp (*Litopenaeus vannamei*) shells. The partitioned trypsin was extremely stable toward various surfactants and bleach agents and showed excellent stability and compatibility with commercial liquid and solid detergents. Additionally, partitioned trypsin showed an efficient hydrolysis and recovery of carotenoprotein from Pacific white shrimp shells. The carotenoprotein recovery was maximized by the hydrolysis of shrimp shells using 0.8 trypsin units/g shrimp shells at 25 °C for 45 min and shrimp shells/buffer ratio of 1:2 (w/v). Carotenoprotein consisted of 72.4% protein, 18.8% lipid, 7.1% ash, 1.6% chitin, and 73.3 μg total astaxanthin/g sample. It was rich in essential amino acids. When the hydrolytic activities of albacore tuna and bovine trypsins used in the extraction of carotenoprotein in Pacific white shrimp shells were compared, the recovery efficacy of protein and pigment by albacore tuna trypsin was similar to that achieved by bovine trypsin. These results suggest that albacore tuna trypsin could be used as an ideal choice for application in detergent formulations and for extraction of carotenoproteins.

## 1. Introduction

Because of the limited biological resources and increased environmental concerns, the interest in full utilization of seafood processing wastes has been increasing. Fish viscera, one of the most important by-products of the fishery industries, have been recognized as a potential source of digestive enzymes, especially proteinases (Klomklao et al., 2009a). Trypsin is one of the most important digestive enzymes from fish and aquatic invertebrates viscera (Jellouli et al., 2009). Trypsin has been used increasingly for industrial applications because trypsins from marine animals tend to be more stable at alkaline pH. They are most active between pH 8.0 and 10.0 (Bougatef, 2013).

Proteinases with high activity and stability in a high alkaline range are interesting for bioengineering and biotechnological applications. One of these applications is in the detergent industry. In fact, the pH of laundry detergents is generally in the range of 9.0–12.0. Thus, proteolytic enzymes incorporated into detergent formulations must exhibit significant activity and stability at high pH and temperature, in addition to their stability with detergent compounds like surfactants and oxidising agents (Younes et al., 2015). Another application of proteolytic enzymes is the carotenoprotein extraction from shrimp shell waste considered as a severe burden on the environment. Carotenoprotein has been reported to have bioactivity, e.g. antioxidative, antimicrobial, etc. (Senphan et al., 2014; Laywisadkul et al., 2017). Studies have been carried out on the recovery of carotenoprotein from shrimp waste by different techniques such as solvent extraction, supercritical fluid extraction (Babu et al., 2008) and vegetable oil (Sowmya et al., 2011). However, there are many disadvantages to those methods such as low protein content, high ash and chitin levels and low stability of carotenoprotein. To increase the extraction efficiency, hydrolysis processes

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mediated by proteinases have been implemented.

Albacore tuna (*Thunnus alalunga*) has been an important species for canning in Thailand with a large volume of raw materials used. Approximately two-thirds of the whole fish are used and the remaining viscera become waste. Accordingly, those viscera, which are the essential sources of potential proteinases, can be recovered for further uses (Nalinanon et al., 2010). Based on our previous study, proteinases from albacore tuna spleen were partitioned using an aqueous two-phase system (ATPS), which were identified as trypsin-like serine proteinases (Poonsin et al., 2017). However, no information regarding the use of partitioned trypsin from albacore tuna spleen for detergent composition and as a food-processing aid, particularly to recover the bioactive components from fish/shellfish-processing wastes, has been reported. Therefore, this study aimed to investigate the stability of partitioned trypsin from albacore tuna spleen with commercial laundry detergents, oxidants and surfactants agents, as well as its efficacy in carotenoprotein extraction from Pacific white shrimp shells and to characterise the resulting carotenoproteins.

## 2. Materials and methods

### 2.1. Chemical

*Nα*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), Coomassie Brilliant Blue R-250, β-mercaptoethanol (βME), *N,N,N′,N′*-tetramethyl ethylene diamine (TEMED), sodium perborate and a wide range of molecular weight markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetone, anhydrous sodium sulphate, ethylenediamine tetraacetic acid (EDTA), petroleum ether, sodium dodecyl sulphate (SDS), Tris (hydroxymethyl) aminomethane, Triton X-100, Tween 20, Tween 80 and urea were obtained from Merck (Darmstadt, Germany) and all other reagents were of analytical grade.

### 2.2. Sample preparation

Internal organs from albacore tuna (*Thunnus alalunga*) obtained from Tropical Canning (Thailand) Public Co. Ltd., Hat Yai, Songkhla, Thailand, were packed in polyethylene bags, kept in ice and transported to the Department of Food Science and Technology, Thaksin University, Phatthalung within 2 h. Only the spleen was collected. The spleen from albacore tuna were defatted with acetone and used for trypsin extraction according to the method of Klomklao et al. (2009a) and Poonsin et al. (2018).

Pacific white shrimp shells (*Litopenaeus vannamei)* were obtained from Sea Wealth Frozen Food Co., Ltd., Songkhla, Thailand. The samples were then ground to obtain the particle size of 1.0–2.0 mm using a waring blender and were kept at −20 °C until needed for carotenoprotein extraction.

### 2.3. Partitioning of trypsin by aqueous two-phase system

Trypsin from the spleen of albacore tuna was partitioned by ATPS according to the method of Poonsin et al. (2017). Trypsin after ATPS separation was used for evaluation in laundry detergents and extraction of Pacific white shrimp carotenoprotein.

### 2.4. Trypsin activity assay

The activity of both partitioned albacore tuna trypsin and bovine pancreatic trypsin (EC 3.4.21.4) (Sigma, St. Louis, MO) were measured using BAPNA as a substrate according to the method of Poonsin et al. (2017). One unit of trypsin activity was defined as the amount of trypsin causing an increase of 1.0 at $A_{410}$ per minute.

### 2.5. Evaluation of partitioned trypsin from albacore tuna spleen as a laundry detergent additive

#### 2.5.1. Effect of surfactants and oxidising agents

The effects of some surfactants and oxidising agents on partitioned trypsin stability were studied by incubating the enzymes with Tween 20, Tween 80, Triton X-100 (1% and 5% v/v), SDS (0.1%, 0.5%, 1% and 5% w/v), hydrogen peroxide (1%, 5%, 10% and 15% v/v) and sodium perborate (0.5%, 1%, 2% and 3% w/v) at 40 °C for 30 and 60 min. The residual activities were measured at pH 9.5 and 55 °C. Percentage of enzyme activity was estimated considering 100% the highest activity detected in this assay. The control was conducted in the same manner except that deionised water was used instead of surfactants and oxidising agents.

#### 2.5.2. Compatibility and stability with commercial laundry detergents

The compatibility and stability of the partitioned trypsin with commercial solid laundry detergents were investigated using Attack (Kao, Thailand), Omo (Uniliver, Thailand), Pao (Lion, Thailand) and Breeze (Uniliver, Thailand). Commercial detergents were diluted in tap water to give a final concentration of 7 mg/mL to simulate washing conditions. The compatibility of the partitioned enzyme with commercial liquid detergents was also studied using Attack (Kao, Thailand), Omo (Uniliver, Thailand), Pao (Lion, Thailand), Breeze (Uniliver, Thailand) and Hygiene (I.P., Thailand). The liquid detergents were diluted 100-fold in tap water (1/100) to simulate washing conditions.

The endogenous proteinases contained in these detergents were inactivated by heating the diluted detergents for 1 h at 80 °C prior to the addition of the enzyme preparation. The partitioned trypsin was incubated with different detergents at 30 °C, 40 °C, 50 °C and 60 °C for 30 and 60 min. The remaining activities were determined under standard assay conditions. The enzyme activity of the control, without detergent, incubated under the similar conditions, was taken as 100%.

### 2.6. Extraction of carotenoprotein from Pacific white shrimp shells using partitioned trypsin from albacore tuna spleen

#### 2.6.1. Effect of hydrolysis time

Carotenoprotein from the shells of Pacific white shrimp was extracted by the method of Klomklao et al. (2009b). The control study was performed in the same manner without partitioned trypsin. All samples were determined for protein recovery and total carotenoid content. The hydrolysis time rendering the highest total carotenoid and protein content was chosen for further study.

#### 2.6.2. Effect of enzyme concentration

Different amounts of partitioned trypsin (0, 0.05, 0.1, 0.2, 0.3, 0.5, 0.7, 0.8, 0.9, 1.0, 1.2 units/g sample) were added to the suspension of ground Pacific white shrimp shell in 0.5 M EDTA, pH 9.5 (1:3 ratio, w/v). The hydrolytic reaction was carried out at 25 °C for 45 min, and the extraction of carotenoprotein was performed as previously described. The partitioned albacore tuna trypsin concentration, which was able to hydrolyse Pacific white shrimp shell with the highest total carotenoid and protein content was selected for further steps.

#### 2.6.3. Effect of Pacific white shrimp shell and buffer ratio

Ground Pacific white shrimp shell was blended with 0.5 M EDTA (pH 9.5) at a ratio of 1:0.5, 1:1, 1:2, 1:3, 1:4 and 1:5 (w/v). Partitioned albacore tuna trypsin at a level of 0.8 units/g sample was added and the reaction was maintained at 25 °C for 45 min. The carotenoprotein was extracted in the same manner as previously described. The buffer ratio used for mixing the ground shrimp shells showing the highest total carotenoid and protein content was selected for further study.

#### 2.6.4. Measurement of protein recovery

Carotenoprotein samples (100 mg) were mixed with 10 mL of 0.5 M

NaOH, followed by incubating at 85 °C for 1 h. The mixture was centrifuged at 9000 × *g* for 10 min. The protein contents of supernatant were measured by the Biuret method (Robinson and Hogden, 1940) using bovine serum albumin as a standard.

#### 2.6.5. Determination of total carotenoid content

Total carotenoid content was determined according to the methods of Simpson and Haard (1985) and Klomklao et al. (2009b). The content (C) of carotenoid in carotenoprotein sample was calculated using the equation given by Saito and Regier (1971):

$$C\ (\mu g/g\ sample) = \frac{A_{468} \times \text{volume of extract} \times \text{dilution factor}}{0.2 \times \text{weight of sample used in grams}}$$

where 0.2 is the $A_{468}$ of 1 μg/mL standard astaxanthin.

### 2.7. Characterisation of carotenoprotein

Carotenoprotein recovered with and without partitioned albacore tuna trypsin under optimal conditions, yielding the highest protein recovery and carotenoid content, was subjected to analysis. The optimum conditions for carotenoprotein extraction by partitioned albacore tuna trypsin were 0.8 trypsin units/g shrimp shells, a hydrolysis time of 45 min at 25 °C and a shrimp shell/buffer ratio of 1:2 (w/v).

#### 2.7.1. Proximate analyses

Protein, ash and fat contents of shrimp shells and the carotenoprotein extracted with or without partitioned trypsin were determined according to the method of AOAC (2012). The contents were expressed on a dry weight basis.

#### 2.7.2. Determination of chitin content

Chitin of shrimp shells and the carotenoprotein extracted with or without partitioned trypsin were measured according to the method of Simpson and Haard (1985) as modified by Senphan and Benjakul (2014).

#### 2.7.3. Determination of amino acid composition

Amino acid composition of carotenoprotein extracted with partitioned albacore tuna trypsin was analysed according to the method of Ganno et al. (1985) with a slight modification. The sample was hydrolysed under reduced pressure in 4 M methanesulphonic acid in the presence of 0.2% (v/v) 3–2(2-aminoethyl) indole at 100 °C for 22 h. For analyzing the tryptophan content, the sample was hydrolysed by 1.5 M mercaptoethanesulphonic acid to avoid the decomposition of tryptophan (Penke et al., 1974). The hydrolysates were neutralised with 2.8 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.04 mL was applied to an amino acid analyser (MLC-703, Atto Co., Tokyo, Japan).

#### 2.7.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Carotenoprotein extracted with or without partitioned trypsin from albacore tuna spleen were determined for protein patterns using SDS-PAGE following the method of Laemmli (1970) as modified by Klomklao et al. (2009b).

### 2.8. Comparison of bovine and albacore tuna trypsin as extraction aids for carotenoprotein recovery from Pacific white shrimp shells

The recovery of carotenoprotein from Pacific white shrimp shells were compared using partitioned albacore tuna trypsin and trypsin from bovine pancreas. At the same level of the enzyme (0.8 units/g sample), trypsins from both sources were added to the suspension of ground Pacific white shrimp shell in 0.5 M EDTA (pH 9.5) at the ratio of 1:2 (w/v). The hydrolytic reaction was carried out at 25 °C for 45 min, and the extraction of carotenoprotein was performed as previously described. All samples were determined for protein recovery and total carotenoid content.

### 2.9. Statistical analysis

A completely randomised design was used throughout the study. Experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS for windows, SPSS Inc., Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Evaluation of partitioned trypsin from albacore tuna spleen as a laundry detergent additive

#### 3.1.1. Stability of partitioned trypsin in the presence of surfactants and oxidising agents

In order to be effective during washing, a good detergent proteinase must be stable and compatible with all commonly used detergent compounds such as oxidising agents, surfactants and other additives, which might be present in the formulation (El Hadj Ali et al., 2009). As shown in Table 1, partitioned trypsin from albacore tuna spleen was highly stable in the presence of non-ionic surfactants like Tween 20, Tween 80 and Triton X-100, retaining 96.6–98.4% of the activity at a concentration of 1% (v/v) after incubation at 40 °C for 30 and 60 min and retaining 95.4–96.5% of the activity at a concentration of 5% (v/v) after incubation at 40 °C for 30 and 60 min. The stability of partitioned trypsin against a non-ionic surfactant was higher than proteinase from *Nocardiopsis* sp. which retained 76% of its initial activity in the presence of 5% (v/v) Tween 20 and Tween 80 after incubation at 40 °C for 60 min (Moreira et al., 2002), and alkaline proteinases from *Serranus scriba* viscera which retained 73.7% of its activity after incubation at 30 °C for 60 min in the presence of 5% (v/v) Triton X-100 (Nasri et al., 2015).

**Table 1**
Stability of partitioned trypsin from albacore tuna spleen in the presence of various surfactants and oxidising agents.

| Surfactants/ oxidising agents | Concentration | Relative activity (%) 30 min* | Relative activity (%) 60 min* |
|---|---|---|---|
| Control | 0 | $100.00^{m} \pm 0.10$ | $100.00^{n} \pm 0.99$ |
| Tween 20 | 1% (v/v) | $98.06^{k} \pm 0.55$ | $97.60^{kl} \pm 0.41$ |
| | 5% (v/v) | $96.27^{i} \pm 0.12$ | $95.68^{gh} \pm 0.55$ |
| Tween 80 | 1% (v/v) | $97.23^{j} \pm 0.26$ | $96.61^{ij} \pm 0.45$ |
| | 5% (v/v) | $96.34^{i} \pm 0.30$ | $95.38^{g} \pm 0.25$ |
| Triton X-100 | 1% (v/v) | $98.39^{kl} \pm 0.51$ | $98.26^{lm} \pm 0.50$ |
| | 5% (v/v) | $96.54^{i} \pm 0.17$ | $96.28^{hi} \pm 0.11$ |
| SDS | 0.1% (w/v) | $98.99^{l} \pm 0.12$ | $98.46^{m} \pm 0.57$ |
| | 0.5% (w/v) | $98.42^{kl} \pm 0.36$ | $98.16^{lm} \pm 0.06$ |
| | 1% (w/v) | $85.87^{c} \pm 0.38$ | $85.54^{c} \pm 0.10$ |
| | 5% (w/v) | $19.56^{a} \pm 0.11$ | $19.39^{a} \pm 0.06$ |
| $H_2O_2$ | 1% (v/v) | $92.44^{f} \pm 0.55$ | $91.58^{e} \pm 0.52$ |
| | 5% (v/v) | $89.70^{e} \pm 0.20$ | $87.75^{d} \pm 0.92$ |
| | 10% (v/v) | $87.85^{d} \pm 0.11$ | $85.51^{c} \pm 0.46$ |
| | 15% (v/v) | $84.81^{b} \pm 0.11$ | $82.99^{b} \pm 0.06$ |
| Sodium perborate | 0.5% (w/v) | $97.80^{jk} \pm 0.21$ | $97.14^{jk} \pm 0.36$ |
| | 1% (w/v) | $96.05^{i} \pm 0.71$ | $95.19^{g} \pm 0.45$ |
| | 2% (w/v) | $94.69^{h} \pm 0.06$ | $93.34^{f} \pm 0.15$ |
| | 3% (w/v) | $93.37^{g} \pm 1.05$ | $91.65^{e} \pm 0.70$ |

The enzyme was incubated with different surfactants and oxidising agents at 40 °C for 30 and 60 min. The remaining activity was measured at pH 9.5 and 50 °C.
The different letters in the same column denote the significant differences ($P < 0.05$).
* Values are mean ± standard deviation ($n = 3$).

Interestingly, the partitioned albacore tuna trypsin was highly stable against the strong anionic surfactant (SDS) and retained 85.5–99.0% of its activity in the presence of 0.1%, 0.5% and 1% (w/v) SDS after incubation at 40 °C for 30 and 60 min. The stability of partitioned albacore tuna trypsin was different from those trypsins from other fish species which are not very stable in the presence of SDS (Younes et al., 2015; Nasri et al., 2015; Ktari et al., 2012; El Hadj Ali et al., 2009). Nevertheless, at higher concentrations, partitioned albacore tuna trypsin was less stable against SDS and retained 19.6% and 19.4% of its activity in the presence of 5% (w/v) SDS after incubation at 40 °C for 30 and 60 min, respectively. This might be due to a higher concentration of SDS led to the denaturation of the enzyme (Bhuyan, 2010).

Stability of the enzymes toward oxidising agents is a very important characteristic for their eventual use in detergent formulations. The partitioned trypsin is stable even at high concentrations of hydrogen peroxide (15% v/v). It retained 84.8% and 83.0% of the initial activity after incubation at 40 °C for 30 and 60 min, respectively. The stability of partitioned trypsin was higher than trypsin from grey triggerfish (*Balistes capriscus*) which retained 39% of its initial activity in the presence of 5% (v/v) hydrogen peroxide after incubation at 40 °C for 60 min (Jellouli et al., 2009), and alkaline proteinases from *S. scriba* which retained about 73.7% of its activity after incubation at 30 °C for 60 min in the presence of 5% (v/v) hydrogen peroxide (Nasri et al., 2015). In addition, the enzyme was found to be highly stable towards sodium perborate, retaining 91.7–97.8% of its initial activity in the presence of 0.5–3% (w/v) sodium perborate after incubation at 40 °C for 30 and 60 min. The result was similar to zebra blenny (*Salaria basilisca*) trypsin (Ktari et al., 2012) and was higher than trypsin from striped seabream (*Lithognathus mormyrus*) which retained 40% of its initial activity in the presence of 1% (w/v) sodium perborate after incubation at 30 °C for 60 min (El Hadj Ali et al., 2009). These results suggested that the enzyme stability in the presence of surfactants and oxidising agents was dependent on source and structure of enzyme. A difference in the structure of enzyme may exist in different marine fish trypsins (El Hadj Ali et al., 2009).

An ideal detergent enzyme should be stable and active in the detergent solution for a long period of time (El Hadj Ali et al., 2009; Espósito et al., 2009). At the same oxidising agents and surfactants concentration, partitioned albacore tuna trypsin activity slightly decreased with increasing incubation time (Table 1). In the presence of an oxidising agent, the activity of proteinase from Tambaqui (*Colossoma macropomum*) decreased with increasing incubation time (Espósito et al., 2009). From the results, the high stability of the partitioned trypsin from albacore tuna spleen toward surfactants and oxidising agents for a long period of time is a very important characteristic for its eventual use in detergent formulation.

### *3.1.2. Compatibility and stability of partitioned trypsin in the presence of commercial laundry detergents*

The suitability of an enzyme preparation for use in commercial laundry detergents depends on its compatibility with the detergents over a wide temperature range. The enzyme should have adequate temperature stability to be effective in a wide range of washing temperatures (Banik and Prakash, 2004). Therefore, the compatibility and stability of partitioned trypsin from albacore tuna spleen was investigated. The enzymes were pre-incubated in the presence of various commercial detergents of different composition at 30–60 °C for 30 and 60 min. The data presented in Fig. 1a,b shows that the partitioned trypsin is highly stable in the presence of several commercial solid detergents. The enzymes retained 91.3%, 91.9%, 91.8% and 96.2% of their initial activity after 30 min incubation at 30 °C in the presence of Attack, Omo, Pao and Breeze, respectively (Fig. 1a). At 40–60 °C, the enzymes were found to be less stable than that at 30 °C. At high temperature, the enzyme most likely underwent denaturation and lost its activity (Klomklao et al., 2009a). However, the enzymes retained more

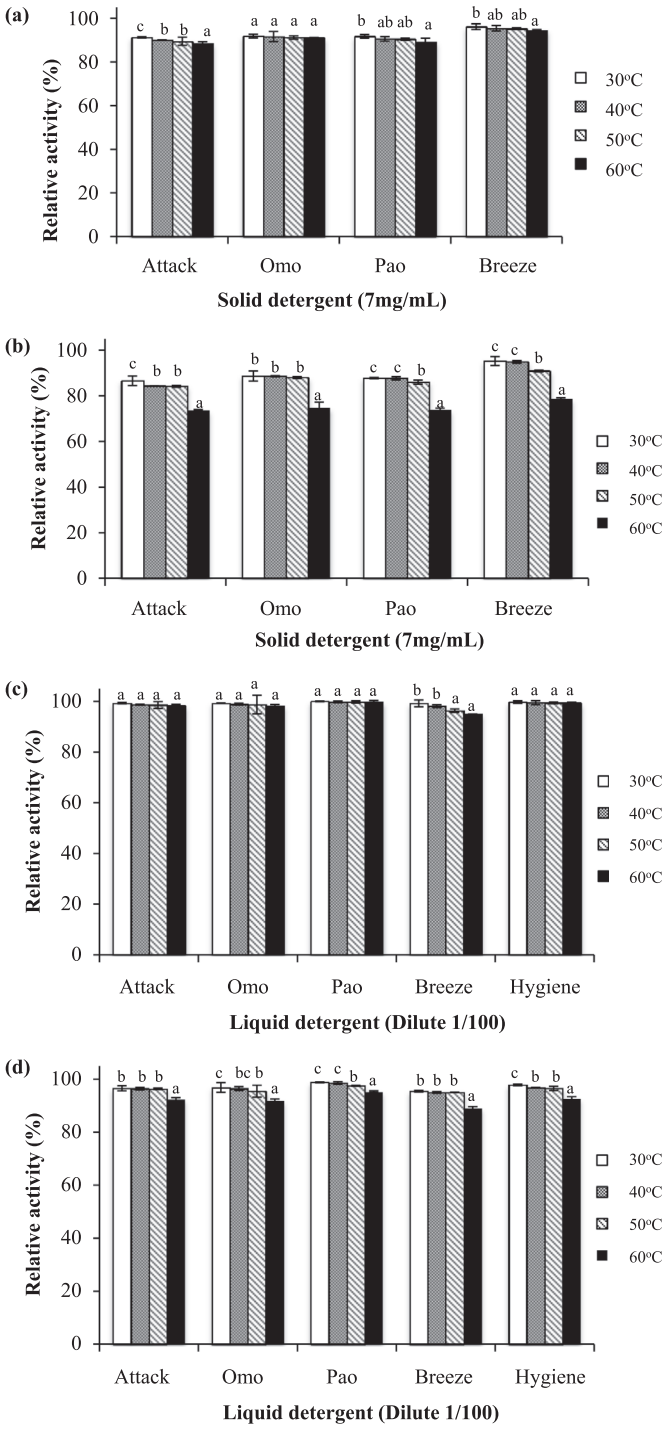


**Fig. 1.** Stability of partitioned trypsin from albacore tuna spleen in the presence of various commercial solid detergents for 30 min (a) and 60 min (b) and commercial liquid detergents for 30 min (c) and 60 min (d) at 30, 40, 50 and 60 °C. The enzyme was pre-incubated with commercial solid detergents at 7 mg/mL and the remaining activity was measured at pH 9.5 and 50 °C, using BAPNA as a substrate. Enzyme activity of control sample without any detergent, incubated under the similar conditions, was taken as 100%. Different letters indicate significant differences ($P < 0.05$). Bars represent the standard deviation from triplicate determinations.

than 88% of their activity after incubation at 40–60 °C for 30 min. Moreover, the partitioned albacore tuna trypsin also retained 73.7–95.2% of its activity when incubated with the detergent Attack, Omo, Pao and Breeze at 30–60 °C for 60 min (Fig. 1b). Either hand washing or machine-washing of cloths normally takes 60–90 min

(Banik and Prakash, 2004). Hence, the partitioned trypsin from albacore tuna spleen present in detergent should remain active during the washing period. The stability of the partitioned trypsin from albacore tuna spleen in the presence of various commercial solid detergents was similar to proteinase from red scorpionfish (*Scorpaena scrofa*) viscera which retained more than 83% of its activity in commercial solid detergent at a concentration of 7 mg/mL (Younes et al., 2015). Sila et al. (2012a) also reported that proteinases from goby (*Zosterisessor ophiocephalus*) retained 87% when incubated with commercial solid detergent at 30 °C for 1 h.

Also, the partitioned albacore tuna trypsin is extremely stable in the presence of commercial liquid detergents (Fig. 1c,d). The enzyme retained 95% of its initial activity with Breeze and more than 98% with Attack, Omo, Pao and Hygiene even after 30 min incubation at 30–60 °C (Fig. 1c). In addition, the enzyme also retained 89.2–98.8% of its initial activity in the presence of Breeze, Attack, Omo, Pao and Hygiene after incubation at 30–60 °C for 60 min (Fig. 1d). These findings were similar to those trypsin from striped seabream (*L. mormyrus*), which retained 91.5% in commercial liquid detergent after incubation at 30 °C for 30 min (El Hadj Ali et al., 2009). Younes et al. (2015) found that proteinases from red scorpionfish (*S. scrofa*) were stable in the presence of several commercial liquid detergents. Interestingly, the partitioned albacore tuna trypsin was more stable than Purafect 2000E, a commercial alkaline proteinase used in detergent formulations, which retained about 70% of its activity in the presence of commercial liquid detergents after incubation at 40 °C for 60 min (Ktari et al., 2012). The obtained results clearly indicate that the performance of enzymes in detergents depends on a number of factors, including the detergent compounds. Considering its promising characteristics, albacore tuna spleen trypsin may be considered a potential candidate for future use in detergent processing industries.

### 3.2. Extraction of carotenoprotein from Pacific white shrimp shells using albacore tuna trypsin

#### 3.2.1. Effect of hydrolysis times on carotenoprotein extraction from shrimp shells

The effect of partitioned trypsin from albacore tuna spleen at various hydrolysis times on carotenoprotein recovery from Pacific white shrimp shells is depicted in Fig. 2. The recovered protein content of shrimp shells homogenate treated without (the control) or with partitioned albacore tuna trypsin at 0.2 and 0.8 units/g sample increased when increasing the hydrolysis time (Fig. 2a). At the same hydrolysis time, the recovered contents of shrimp shells hydrolysed with partitioned trypsin (0.2 and 0.8 units/g sample) were higher than that of the control ($P < 0.05$). This indicated that the addition of partitioned albacore tuna trypsin to the shrimp shells homogenate was effective in improving the protein recovery (Fig. 2a). Further, the autolysis caused by the endogenous proteinases in shrimp shells was noted to play a key role in the hydrolysis of the protein. From the results, the rapid hydrolysis of protein was observed within the first 45 min ($P < 0.05$). Thereafter, no marked changes in the recovered protein content were noticeable in shrimp shells homogenate with and without the addition of partitioned albacore tuna trypsin ($P > 0.05$). The results suggested that after 45 min the enzymatic reaction reached a steady-state phase. This typical curve was also reported by Sila et al. (2012b) when trypsin from Tunisian barbel (*Barbus callensis*) was used for carotenoprotein recovery from shrimp wastes. Generally, the enzyme absorbs rapidly onto the insoluble protein particles, and the polypeptide chains that are loosely bound to the surface are cleaved. The more compacted core proteins are hydrolysed more slowly. The rate of enzymic cleavage of peptide bond controls the overall rate of hydrolysis (Klomklao et al., 2009b). However, available substrate for hydrolysis decreases as time of reaction increases (Klomklao et al., 2009b). Trypsin was used to extract carotenoprotein from brown shrimp shell waste (Chakrabarti, 2002) and black tiger shrimp shells (Klomklao et al., 2009b). Protein

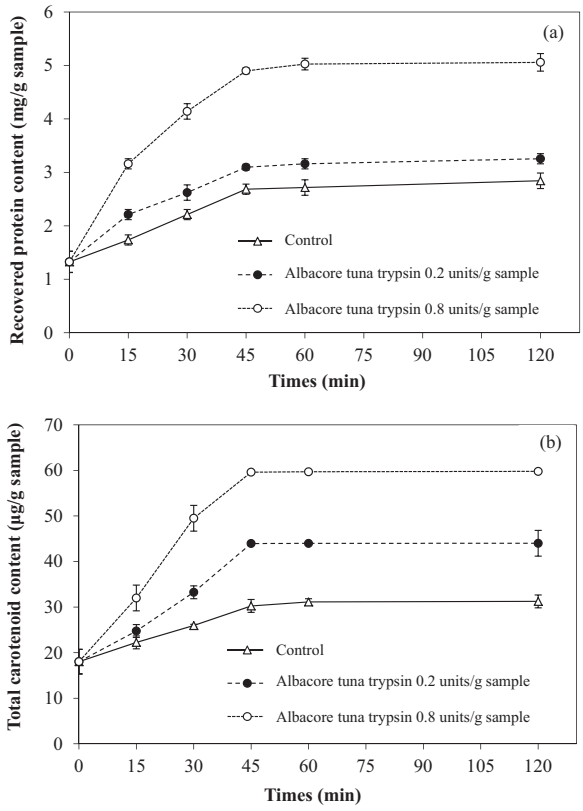


**Fig. 2.** Recovered protein (a) and total carotenoid (b) contents of Pacific white shrimp shells without and with the aid of trypsin from albacore tuna spleen at a level of 0.2 and 0.8 units/g sample for various times at 25 °C. Bars represent standard deviation from triplicate determinations.

recovery was used as an indicator for the cleavage of peptide bond, and the release of the carotenoprotein (Sila et al., 2012c).

When total carotenoid contents of carotenoprotein extracted from Pacific white shrimp shells with and without the aid of partitioned albacore tuna trypsin at different hydrolysis times were determined (Fig. 2b), similar trends were found to those of the recovery of carotenoprotein (Fig. 2a). No marked changes in carotenoid content were observed after 45 min of hydrolysis ($P > 0.05$). At the same hydrolysis time, the total carotenoid contents of shrimp shells added with partitioned trypsin was generally higher than that without trypsin treatment ($P < 0.05$) (Fig. 2b). Cano-Lopez et al. (1987) reported that proteolytic enzymes were used to disrupt the protein-carotenoid bond, thus increasing carotenoid recovery. Albacore tuna trypsin possibly had the relative narrow specificity for catalyzing hydrolysis of peptide bonds on the carboxyl side of lysine and arginine residues. This might facilitate the release of a stable carotenoprotein complex, leading to the increased recovery of carotenoprotein from shrimp shells (Klomklao et al., 2009b). From the results, the hydrolysis time of 45 min was found to be optimal for carotenoprotein production.

#### 3.2.2. Effect of albacore tuna trypsin concentration on carotenoprotein extraction from shrimp shells

Fig. 3 shows the effect of partitioned trypsin from albacore tuna spleen at different levels on the recovery of carotenoprotein from Pacific white shrimp shells. The recovered protein and total carotenoid contents increased with increasing partitioned trypsin concentrations ($P < 0.05$) (Fig. 3a,b). However, no significant increases in both recovered protein and total carotenoid contents were found with treatment of partitioned albacore tuna trypsin at levels above 0.8 units/g sample ($P > 0.05$). Recovered protein content has been used as an indicator for the cleavage of peptide bond, whereas total carotenoid

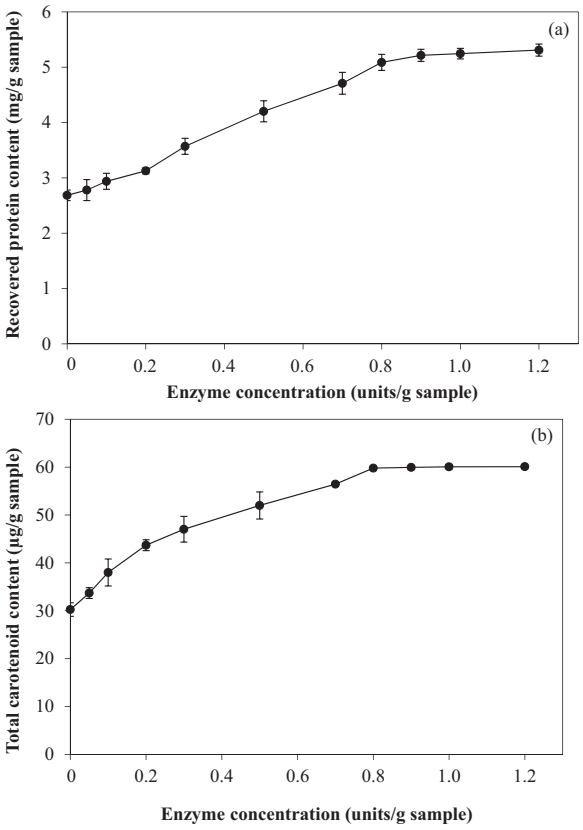


**Fig. 3.** Recovered protein (a) and total carotenoid (b) contents of Pacific white shrimp shells without and with the aid of trypsin from albacore tuna spleen at different levels. The hydrolytic reaction was conducted at 25 °C for 45 min. Bars represent standard deviation from triplicate determinations.

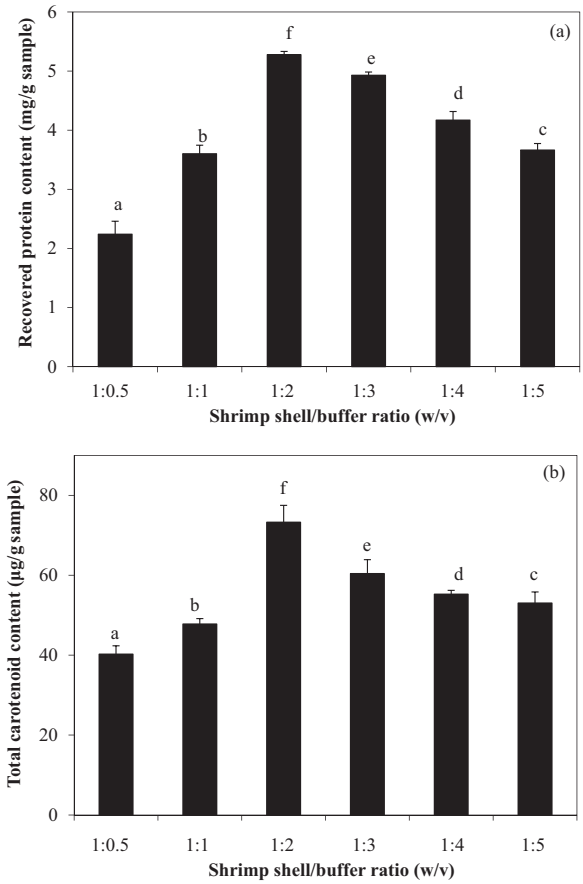


**Fig. 4.** Effect of shrimp shells/buffer ratio on recovered protein (a) and total carotenoid (b) contents for Pacific white shrimp shells treated with albacore tuna trypsin at a level of 0.8 units/g sample. The hydrolytic reaction was conducted at 25 °C for 45 min. Different letters indicate significant differences ($P < 0.05$). Bars represent the standard deviation from triplicate determinations.

content reflects the pigment content that can be recovered from the hydrolysis process (Klomklao et al., 2009b). Sila et al. (2012b) reported that the increase in Tunisian barbel (*B. callensis*) trypsin concentration (0–1 units/g shrimp shell) resulted in an increase in recovered protein and total carotenoid contents. In the present study, the increase in enzyme concentration caused an increase in recovered proteins and total carotenoid contents only in the concentration range of 0–0.8 units/g sample. The limited amount of protein substrate for hydrolysis reaction by proteinase most likely contributed to the plateau observed when partitioned albacore tuna trypsin above 0.8 units/g sample was used. Hence, the optimum level of trypsin from albacore tuna spleen for carotenoprotein extraction was 0.8 units/g sample.

### 3.2.3. Effect of substrate/buffer ratio on carotenoprotein extraction

In general, an increase in the Pacific white shrimp shell/buffer ratio resulted in an increase in the recovered protein as well as total carotenoid contents (Fig. 4a.b). The increase in ratio up to 1:2 significantly increased both protein and carotenoid contents ($P < 0.05$). Nevertheless, a ratio above 1:2 resulted in a significant decrease in protein and carotenoid contents ($P < 0.05$). Sufficient buffer provided buffering capacity for the reaction, and ensured the uniform dispersion. A Pacific white shrimp shell/buffer ratio of 1:2 was sufficient for enzymatic reaction. Increasing water or adding a buffer to the substrate enhanced enzyme homogeneity, promoted tissue swelling, and reduced the localized concentration of hydrolysis products (Benjakul and Morrissey, 1997). Sowmya et al. (2011) used proteinases to recover carotenoprotein from shrimp head and found that an increase in the ratio of added buffer resulted in an increase in carotenoid contents.

From the results, the optimum conditions for carotenoprotein extraction using partitioned albacore tuna trypsin were 0.8 units/g sample at 25 °C for 45 min and a Pacific white shrimp shell/buffer ratio of 1:2 (w/v).

## 3.3. Characterisation of carotenoproteins extracted from Pacific white shrimp shells

### 3.3.1. Compositions of carotenoprotein

Total carotenoid contents of lyophilised carotenoprotein extracted with or without partitioned albacore tuna trypsin and dried shrimp shells are shown in Table 2. The freeze-dried carotenoproteins showed higher carotenoid content than shrimp shells. The level of the carotenoids associated with protein recovered with the aid of albacore tuna trypsin was approximately 6.08- and 2.19-fold higher than those of the shrimp shells and the control (without albacore tuna trypsin), respectively. The results suggested that trypsin from albacore tuna could be used to improve the recovery of carotenoid associated with protein from Pacific white shrimp shells. Additionally, trypsin used might cleave the peptides of proteins associated with carotenoids more selectively and effectively than the endogenous proteinases. This resulted in the greater content of carotenoid in carotenoprotein recovered.

For chemical compositions of shrimp shells and carotenoprotein recovered with or without the treatment of partitioned albacore tuna trypsin, carotenoprotein extracted using partitioned trypsin showed the highest protein (72.4%) and fat (18.8%) contents, followed by carotenoprotein extracted without partitioned albacore tuna trypsin treatment and the shrimp shells, respectively (Table 2). On the other hand, carotenoproteins recovered with the aid of partitioned albacore

**Table 2**
Carotenoid content and chemical compositions of Pacific white shrimp shells and carotenoprotein recovered with or without albacore tuna trypsin.

| Compositions | Shrimp shell | Carotenoprotein* | |
|---|---|---|---|
| | | Control | Albacore tuna trypsin-aided |
| Total carotenoid content (μg astaxanthin/g sample) | 12.05[a] ± 3.78 | 33.50[b] ± 5.66 | 73.25[c] ± 4.24 |
| Protein (% dry wt) | 38.57[a] ± 1.18 | 60.22[b] ± 0.69 | 72.37[c] ± 0.64 |
| Fat (% dry wt) | 4.70[a] ± 0.40 | 15.98[b] ± 0.17 | 18.79[c] ± 0.14 |
| Ash (% dry wt) | 21.36[c] ± 0.72 | 18.09[b] ± 0.61 | 7.14[a] ± 0.06 |
| Chitin (% dry wt) | 35.20[c] ± 0.56 | 5.61[b] ± 0.24 | 1.61[a] ± 0.03 |

The different letters in the same row denote the significant differences ($P < 0.05$).

* Values are mean ± standard deviation from triplicate determinations.

tuna trypsin had the lower ash (7.1%) and chitin (1.6%) contents, compared with the carotenoprotein recovered without partitioned albacore tuna trypsin and shrimp shells. Shell has been reported to contain protein, chitin and mineral, mainly $CaCO_3$ (Senphan et al., 2014). From the results, the trypsin-aided extraction process facilitated the release of protein, and fat from shrimp shells, while minerals and chitin were still retained in the shells. The high protein and fat contents were a result of the solubilization of protein during hydrolysis and the removal of insoluble unhydrolysed nonprotein substances. Sila et al. (2012c) reported that carotenoprotein from deep-water pink shrimp shells extracted by barbel trypsin had high protein (71.09%) and fat contents (16.47%). Klomklao et al. (2009b) found that carotenoprotein recovered from black tiger shrimp shells with the aid of bluefish trypsin had higher protein content and had lower ash and chitin contents than shrimp waste. Thus, proteins in shrimp shells could be effectively extracted from the shell with trypsin from albacore tuna. However, fat or lipoproteins could be co-extracted. Carotenoprotein is a stable complex, in which carotenoid is bound to a high density lipoprotein (Senphan et al., 2014). On the basis of the composition, the carotenoprotein extracted with albacore tuna trypsin could be used as a cheap source of pigment, protein and fat for human supplementation and in diets of cultured salmonid species.

### 3.3.2. Protein patterns

Fig. 5 displays the protein patterns of carotenoprotein recovered with and without albacore tuna trypsin treatment. The carotenoprotein recovered without albacore tuna trypsin addition (autolysis process) contained a variety of proteins with different molecular weights (MWs). The apparent MW of the major band was estimated to be 45 and 14.5 kDa. Proteins with apparent MW of 95, 66, 55 and 18.5 kDa were also found in the sample. For carotenoprotein extracted with the aid of partitioned trypsin, protein with MW of 45 kDa, which was most likely actin, totally disappeared. The results suggested that actin was susceptible to hydrolysis by trypsin added. Moreover, other proteins were also hydrolysed to some degree as evidenced by the decrease in band intensity with a coincidental increase in band intensity at dye front. Klomklao et al. (2009b) reported that MW of carotenoprotein from black tiger shrimp shells recovered with bluefish trypsin had two major proteins with MW of 211 and 45 kDa. The MW of carotenoproteins of *Homarus gammarus* ranged from 48 to 90 kDa (Buchwald and Jencks, 1968). Carotenoprotein from *Procambarus clarkia* had the proteins with MW of 22.4 and 19.2 kDa as the major proteins (Buchwald and Jencks, 1968). The differences in the MW of the carotenoproteins may be probably due to the differences in exoskeleton material (Klomklao et al., 2009b). Those proteins might be associated with carotenoid, particularly astaxanthin, which was the major carotenoid in Pacific white shrimp (Senphan et al., 2014). Carotenoids were reported to bind with proteins with noncovalent bond (Cheesman et al., 1967). From the result, it was noted that not all protein bands represented the proteins associated with carotenoids. However, some proteins might be involved in binding with carotenoids in the form of carotenoproteins.

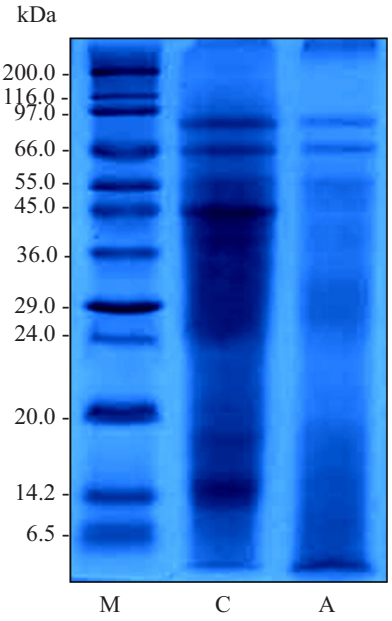


**Fig. 5.** Protein patterns of carotenoprotein recovered from Pacific white shrimp shells with and without albacore tuna trypsin. M, molecular weight standard; C: control (carotenoprotein recovered without trypsin); A: carotenoprotein recovered with albacore tuna trypsin.

### 3.3.3. Amino acid compositions

The amino acid compositions of carotenoprotein recovered with the aid of albacore tuna trypsin are shown in Table 3. Carotenoprotein from Pacific white shrimp shells was rich in glutamic acid/glutamine (81.9 mg/g) and aspartic acid/asparagine (71.8 mg/g). Glycine and leucine were presented in high concentration in carotenoprotein. Armenta and Guerrero-Legarreta (2009) reported that carotenoproteins extracted from fermented Pacific white shrimp waste was rich in

**Table 3**
Amino acid compositions of carotenoprotein recovered with albacore tuna trypsin.

| Amino acid compositions (mg/g sample) | Carotenoprotein |
|---|---|
| Alanine | 38.58 |
| Arginine | 43.80 |
| Aspartic acid/Asparagine | 71.78 |
| Cysteine | 0.21 |
| Glutamic acid/Glutamine | 81.85 |
| Glycine | 54.10 |
| Histidine | 28.61 |
| Hydroxylysine | 0.66 |
| Isoleusine[A] | 32.19 |
| Leucine[A] | 52.60 |
| Lysine[A] | 39.88 |
| Methionine[A] | 16.47 |
| Phenylalanine[A] | 35.04 |
| Proline | 28.32 |
| Serine | 32.97 |
| Threonine[A] | 31.88 |
| Tryptophan[A] | 8.57 |
| Tyrosine | 29.04 |
| Valine[A] | 36.18 |
| Total amino acids | 662.73 |
| Total essential amino acids | 252.81 |
| Total non- essential amino acids | 409.92 |

[A] Essential amino acids in adults.

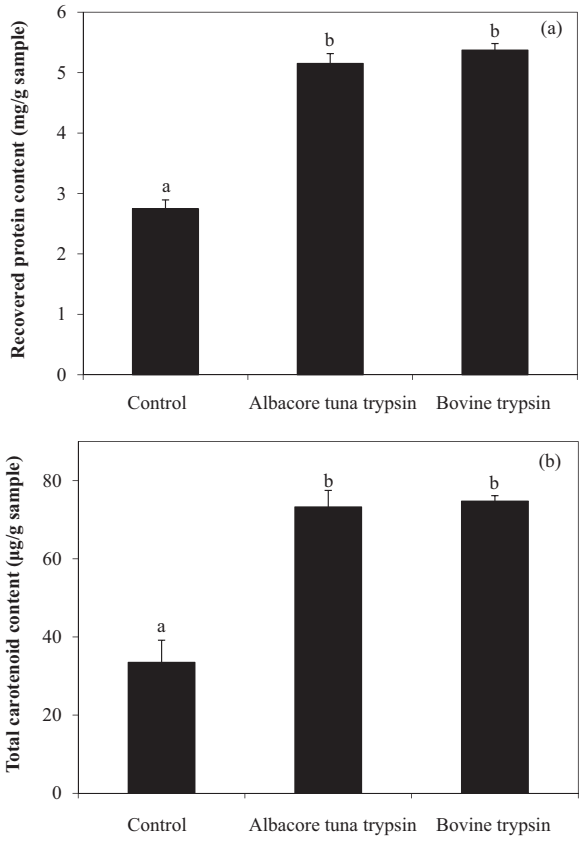


**Fig. 6.** Recovered protein (a) and total carotenoid (b) contents of Pacific white shrimp shells without and with the aid of albacore tuna trypsin and bovine trypsin at a level of 0.8 units/g sample. The hydrolytic reaction was conducted at 25 °C for 45 min. Different letters indicate significant differences ($P < 0.05$). Bars represent the standard deviation from triplicate determinations.

glutamic acid, aspartic acid, leucine and lysine (142.2, 126.7, 110.1 and 99.2 mg/g, respectively). Carotenoproteins derived from pink shrimp (*Parapenaeus longirostris*) contained high contents of glutamic acid/ glutamine (13.1 mol%), aspartic acid/ asparagines (10.92 mol%) and glycine (9.13 mol%) (Sila et al., 2012c). Glutamic acid and aspartic acid were the dominant amino acids in carotenoproteins isolated from shrimp wastes with the aid of bovine trypsin (Simpson and Haard, 1985). Furthermore, carotenoprotein extracted from Pacific white shrimp shells had high essential amino acid contents. Fish are unable to synthesize about half of the common amino acids, including arginine, histidine, isoleucine, leucine, lysine, cysteine, tyrosine, threonine, tryptophan and valine (Ya et al., 1991). For a diet of salmonid species, those essential amino acids should be supplemented to satisfy the requirements of these fish species. From the results, carotenoprotein from the shells of Pacific white shrimp recovered by albacore tuna trypsin contained essential amino acids required for solmonid species. However, low content of cysteine and hydroxylysine were found in carotenoprotein. Carotenoproteins from deep-water pink shrimp processing waste contained the low contents of cysteine (Sila et al., 2012c). Generally, low contents of cysteine and hydroxyproline were found in Pacific white shrimp shells and carotenoproteins (Senphan et al., 2014). Therefore, carotenoproteins extracted from Pacific white shrimp shells can be used as a promising source of feed for pen-reared salmoids together with the supplement of cysteine.

### *3.4. Comparison of bovine and albacore tuna trypsin as extraction aids for carotenoprotein recovery from Pacific white shrimp shells*

The recovery of proteins and carotenoids from Pacific white shrimp shells were compared using partitioned albacore tuna trypsin and trypsin from bovine pancreas. At the same concentration of the enzyme added (0.8 units/g sample), trypsins from both sources were noted to show more efficiency in the extraction of carotenoprotein compared to the control (without addition of enzyme) (Fig. 6) ($P < 0.05$). Albacore tuna trypsin showed similar recovery efficacy of protein or carotenoids, compared with bovine trypsin ($P > 0.05$) (Fig. 6a,b). Klomklao et al. (2009b) reported that the recovery efficacy of carotenoprotein by bluefish trypsin was similar to that of bovine trypsin. Babu et al. (2008) reported that trypsin showed higher recovery of carotenoprotein from shrimp head waste than did pepsin and papain. From the results, albacore tuna trypsin could be a potential application for the extraction of carotenoproteins from shrimp shells.

## 4. Conclusion

The partitioned trypsin from albacore tuna spleen exhibited a high stability in the presence of surfactants, oxidising agents and various laundry liquid and solid detergents. Additionally, partitioned albacore tuna trypsin showed an efficient hydrolysis and recovery of carotenoprotein from Pacific white shrimp shells. Carotenoprotein extracted with the enzymes had high protein, fat, pigment and essential amino acids contents. Thus, albacore tuna trypsin could be a potential novel enzyme for carotenoprotein extraction from shrimp shells. Extracted carotenoprotein could serve as a value-added nutritive food ingredient or as animal feed.

## Acknowledgments

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