
Concentrated pineapple extract and Na-Cid[®] facilitates the digestion of soybean meal and shrimp feed

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Bupphadong, F., Chirapongsatonkul, N., Kuepethkaew, S., Klomklao, S. and U-taynapun, K. (2019). Concentrated pineapple extract and Na-Cid[®] facilitates the digestion of soybean meal and shrimp feed. *International Journal of Agricultural Technology* 15(6):823-834.

Abstract Soybean meal has been used as an alternative plant protein source to replace fishmeal in shrimp feed for decades. However, the use of soybean meal is problematic owing to low digestibility in the digestive tract of shrimp and anti-nutritional factors that are found in plant derived protein. In addition, the residues of undigested feedstuff have been found to cause inflammation in the digestive tract of shrimp leading to white feces syndrome (WFS). This disease negatively affected the production of Pacific white shrimp (*Litopenaeus vannamei*) in Southeast Asia including Thailand. The study examined the ability of concentrated pineapple extract and Na-Cid[®] to increase the digestibility of soybean meal and shrimp feed via protease activity. The results clearly showed that Na-Cid[®] could facilitate the digestion of soybean meal and shrimp feed over a wide range of pH and temperature. The proteins released from Na-Cid[®]-treated soybean meal and shrimp feed were highest at a pH range between 5.0 and 8.5 while the lowest amounts were found at pH 4.0 and 9.0–10.0, respectively. Test temperatures in the range of 25–50 °C did not affect the ability of Na-Cid[®] to facilitate digestion. The results showed that Na-Cid[®] was able to increase the digestion of protein in soybean meal and shrimp feed by approximately 18–26% and 27–33%, respectively. It is demonstrated the capability of the concentrated pineapple extract to increase *in vitro* digestibility of soybean meal protein. Our finding may provide a potent pretreatment procedure to solve the problem regarding the digestibility of protein in feedstuff leading to reduce the undigested residues remaining in the digestive tract of shrimp and the subsequent occurrence of white feces syndrome (WFS).

Keywords: Concentrated pineapple extract, Digestion, Na-Cid[®], Pacific white shrimp (*Litopenaeus vannamei*)

Introduction

Aquaculture production has expanded rapidly since aquatic animals have been considered as high quality food for humans. Therefore, the demand for aquaculture feed has increased enormously. Fishmeal, which is considered a quality protein source promoting the growth of aquatic animals, has thus been

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required in increasing quantities. However, the supply of fishmeal has been insufficient because of a reduction in the natural fish stock leading to high prices and market volatility (Tantikitti, 2014). Several alternative protein sources have been searched for. There has been increasing interest in plant protein sources because of their low price, sufficient quantities, and relatively consistent nutrient composition (Tantikitti, 2014). Among plant protein sources used to replace fishmeal in aquatic animal feed, soybean meal and soybean products are suitable sources according to their protein levels and amino acid profile. Even though the advantages of soybean products are obvious, concerns about the low levels of methionine and essential fatty acids that do not meet the requirements of aquatic animals as well as anti-nutritional factors have been documented (Hardy, 1996; Francis *et al.*, 2001; Samocha *et al.*, 2004; Gatlin *et al.*, 2007). There have been proposals for pretreatment and processing technologies that are currently available to solve the mentioned disadvantages of soybean meal and make it a potential protein source for aquatic animals (Tantikitti, 2014). Pretreatments through alcohol-extraction, enzyme treatment processes, and biological/microbial fermentation have been shown to remove and/or inactivate certain anti-nutritional factors (Tibaldi *et al.*, 2006; Shao *et al.*, 2019b).

Pacific white shrimp (*Litopenaeus vannamei*) is widely used in commercial aquaculture but its production value has been threatened by rising disease frequency and severity (Xiong *et al.*, 2016). The emerging occurrence of a disease associated with the dysfunction or impairment of the shrimps' digestive system, white feces syndrome (WFS), has been reported. This disease has led to economic losses in China, India, Vietnam, Thailand, and other countries (Tangprasittipap *et al.*, 2013; Mastan, 2015; Tang *et al.*, 2016; Hou *et al.*, 2018). Certain species belonging to the genus *Vibrio* and aggregated, transformed, microvilli (ATM) resembling gregarines and the microsporidian *Enterocytozoon hepatopenaei* (EHP) have been reported to be associated with WFS in Thailand (Tangprasittipap *et al.*, 2013; Sriurairatana *et al.*, 2014). However, the white fecal strings, which are usually observed in the pond of diseased shrimp, might result from inflamed tissues shed from the digestive tract of the shrimp.

It has been demonstrated that anti-nutritional factors and some residues of soybean meal contribute directly or indirectly to the inflammation of the shrimp intestine. The indirect determinant is probably related to an induced mucosal inflammation resulting from the transformation of the intestinal microbiota into an undesirable community (Zhou *et al.*, 2018). Therefore, the objective of this work was to examine the ability of the concentrated pineapple extract, Na-Cid[®], in increasing the digestibility of soybean meal and shrimp feed. Since pineapple contains a potent protease enzyme, bromelain, which is able to increase protein

digestibility, it has been used in commerce as a feed enzyme in animal feed (Pariza and Cook, 2010). The capability of Na-Cid[®] to digest soybean meal and shrimp feed was evaluated in various conditions in order to provide the optimum pH and temperature range for further application.

Materials and Methods

Soybean meal and shrimp feed, produced by the ThaiUnion Feedmill, was purchased from the commercial animal feed supplier in Nakhon Si Thammarat Province, Thailand. The soybean meal and shrimp feed was stored correctly in order to prevent contamination and nutrient losses. All materials used throughout this study were mixed thoroughly and kept in aliquots at 4 °C.

Na-Cid[®], the concentrated pineapple product, was obtained from Yeast Master Co., Ltd. All other chemicals used in this study were of analytical grade and obtained from Sigma (Sigma-Aldrich).

Protease assay

The proteolytic activity of Na-Cid[®] was determined through a non-specific protease activity assay following the method of Cupp-Enyard (2008) with slight modification. A substrate, 1% (w/v) casein in 0.5 M potassium phosphate buffer (pH 7.5), was mixed with 2% (v/v) of Na-Cid[®]. The reaction was initiated by incubating at 37 °C for 30 min and the reaction was stopped by adding trichloroacetic acid (TCA) at the final concentration of 10% (w/v). The reaction was centrifuged at 10,000 × g for 15 min at 4 °C, the supernatant was filtered through a 0.45 µm polyethersulfone syringe filter. The protein content in the clear flow-through was measured according to the Lowry method (Lowry *et al.*, 1951) using tyrosine as the standard. One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmole of tyrosine per min under the assayed condition. Protease activity was expressed in units/ml while specific enzyme activity was expressed as units/mg protein.

Experimental design

The experimental design for the evaluation of the suitable amount of Na-Cid[®] and optimum condition for the digestion ability of Na-Cid[®] was set up as a completely randomized design (CRD) with 6 replications.

Measurement of protein content

The protein content in Na-Cid[®] and the Na-Cid[®] treated soybean meal and Na-Cid[®] treated shrimp feed was determined according to the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as the standard.

Evaluation of the suitable amount of Na-Cid[®] for digestion

The appropriate amount of Na-Cid[®] to facilitate the digestion of soybean meal was verified. Soybean meal, 10 g per each reaction as substrate, was thoroughly mixed with Na-Cid[®] at 0% (control), 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, and 3.0% (v/v) in 0.1 M citrate buffer (pH 6.0). The mixtures in each reaction were incubated at 37 °C for 30 min followed by incubation at room temperature for 30 min. The reaction mixtures were incubated in the Stomacher[®] 400 Circulator (Seward) at 200 rpm. After 15 min, all reaction mixtures were centrifuged at 10,000 × g for 15 min at 4 °C, the supernatant was then collected for the determination of the protein amount by the Lowry method as described above. The released protein represented the digestion ability of Na-Cid[®].

Optimal pH analysis for the digestion ability of Na-Cid[®]

The digestion ability of Na-Cid[®] was determined in different pH conditions, pH 4.0–10.0, and using a buffer as follows: 0.1 M citrate buffer (pH 4.0, 4.5, 5.0, 5.5, and 6.0), 0.1 M sodium/potassium phosphate buffer (pH 6.5, 7.0, and 7.5), 0.1 M Tris-HCl buffer (pH 8.0 and 8.5), and 0.1 M sodium carbonate buffer (pH 9.0, 9.5, and 10.0). Soybean meal and shrimp feed, 10 g per each reaction as substrate, was thoroughly mixed with Na-Cid[®] at the desired concentration obtained from the above experiment in the aforementioned buffers. Sterile distilled water (0% Na-Cid[®]) was used in the control treatment. The digestion ability of Na-Cid[®] was represented by µg of the released protein into the reaction mixture. Moreover, the resultant released protein through the use of Na-Cid[®] was compared with the released protein in the control and present as the percentage increase of the released protein compared to the control.

Optimal temperature analysis for the digestion ability of Na-Cid[®]

The temperature for the digestion ability was analyzed in a range of 25–50 °C. Soybean meal and shrimp feed, 10 g per each reaction, was thoroughly mixed with 2% (v/v) Na-Cid[®] in buffers that gave the highest digestion ability and incubated at temperatures of 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, and 50 °C for 30 min. Sterile distilled water (0% Na-Cid[®]) was used as the control in all the test temperatures. Thereafter, the experimental analysis was performed as that described for the optimal pH measurement. The digestion ability of Na-Cid[®] was presented by the amount of released protein in the reaction mixture and percentage increase of the released protein compared to the control.

Statistical analysis

Statistical analysis was evaluated using SPSS 16.0 for Window (SPSS Inc.). To test whether there was a significant difference in the released protein in the control (without Na-Cid[®]) and with Na-Cid[®] under the same condition, a Student's *t* test was conducted. A *P* value of <0.05 was considered statistically significant. The digestibility of soybean meal and shrimp feed under different analysis conditions was examined by one-way ANOVA. Duncan's Multiple Range Test (DMRT) was used for the analysis of the significant differences. Significant differences were stated at $P<0.05$.

Results

Na-Cid[®] was examined to measure its protease activity and the result is shown in Table 1. The appropriate amount of Na-Cid[®] that gave the precise and desirable activity was determined. The concentration of Na-Cid[®] varied in a range of 0–3.0% for soybean meal digestion. The result showed that the activity of Na-Cid[®] was increased in a dose-dependent manner with significance from 0% to 2.0%. There was no statistical difference in the digestion ability when using Na-Cid[®] at a concentration greater than 2.0% (Figure 1). The concentration of 2% Na-Cid[®], the lowest amount that gave the highest digestion ability among all treatments, was chosen for further analyses.

Optimum pH for the digestion ability of Na-Cid[®]

Soybean meal and shrimp feed were treated with 2% Na-Cid[®] compared with the control or 0% Na-Cid[®] under pH 4.0–10.0. In all pH tests, significantly higher amount ($P<0.05$) of the resultant released protein was observed in the 2% Na-Cid[®] treatment for both soybean meal (Figure 2A) and shrimp feed (Figure 2B). The amount of released protein in the control treated in all buffers was similar (around 80–90 μg) while more than 100 μg of the released protein was detected in the 2% Na-Cid[®] treatment. In order to compare the digestion ability of Na-Cid[®] at different pH, the increase in released protein in the 2% Na-Cid[®] treatment was calculated by comparison with that of the control. The results showed that Na-Cid[®] was able to digest soybean meal highest at a pH between 5.0 and 8.5 which was significantly higher ($P<0.05$) than those detected at other pH (Table 2). A similar result was obtained for the digestion of shrimp feed. The maximum digestion capability of Na-Cid[®] was observed in a wide range of pH (pH 5.0–8.5). The lowest digestion ability of Na-Cid[®] was detected at pH 4.0 and 9.0–10.0 for soybean meal and shrimp feed, respectively (Table 2).

Table 1. Protease activity of Na-Cid[®]

Protease activity (Units/ml)	Protein (mg/ml)	Specific activity ($\times 10^{-3}$ Units/mg protein)
9.48 \pm 2.11	100.25 \pm 13.22	94.56 \pm 9.88

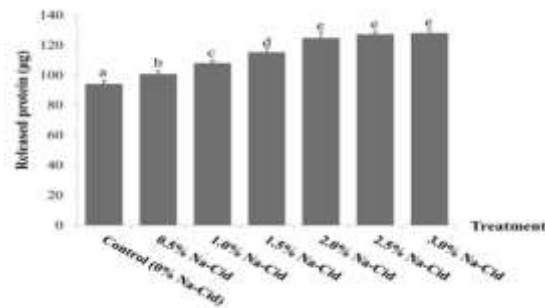


Figure 1. The digestion ability of Na-Cid[®] at the different concentrations using soybean meal as a substrate. The different letters indicate the significant difference among treatments

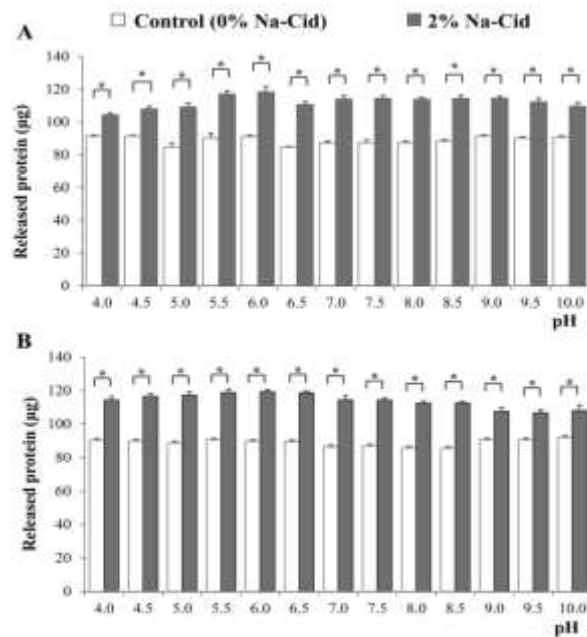


Figure 2. Influence of pH on the digestion ability of Na-Cid[®] represented by the protein released into the reaction mixture. (A) Soybean meal and (B) Shrimp feed was treated with 2% (v/v) of Na-Cid[®] while sterile distilled water was used to replace Na-Cid[®] as the control (0% Na-Cid[®]). The results were expressed as mean \pm SD (n=6). The asterisk located above the black line indicates the significance ($P < 0.05$) between the treatment (with or without Na-Cid[®]) of the same pH. The statistical analysis used is the Student's *t* test

Table 2. Percentage increase of the released protein of the soybean meal and shrimp feed treated with 2% (v/v) of Na-Cid[®] compared to the control (0% Na-Cid[®]) analyzed at different pH

pH	Percentage increase of the released protein compared to the control ¹	
	Soybean meal	Shrimp feed
4.0	14.15±2.09 ^a	26.67±2.27 ^b
4.5	18.17±3.22 ^b	29.61±3.41 ^{bc}
5.0	28.79±2.42 ^c	32.19±4.06 ^c
5.5	29.98±4.04 ^c	31.42±1.94 ^c
6.0	29.77±2.68 ^c	33.06±1.81 ^c
6.5	30.50±2.02 ^c	32.84±1.92 ^c
7.0	30.66±1.92 ^c	32.36±2.28 ^c
7.5	30.63±2.93 ^c	31.57±3.48 ^c
8.0	30.28±2.76 ^c	31.53±3.26 ^c
8.5	29.44±3.78 ^c	31.74±3.56 ^c
9.0	25.44±3.10 ^d	18.87±2.59 ^a
9.5	24.52±3.31 ^d	17.91±2.38 ^a
10.0	21.10±2.71 ^c	17.40±2.73 ^a

^{1/}: Mean ± SD (n=6) of the calculated value.

Mean values within a row with different superscript letters were significantly different ($P<0.05$).

Optimum temperature for the digestion ability of Na-Cid[®]

In all temperature tests, the treatment of soybean meal and shrimp feed with 2% Na-Cid[®] increased the released protein significantly higher ($P<0.05$) than that of the control (Figure 3). The released protein in the control treated at all incubation temperatures was around 80–90 µg for soybean meal and 100 µg for shrimp feed which suggested that temperature in the test range did not promote the digestion.

Similarly to the pH experiment, to compare the effect of temperature on the action of Na-Cid[®], the increase in the released protein in the 2% Na-Cid[®] treatment was calculated by comparison with that of the control. The results showed that Na-Cid[®] could increase the protein digestion of soybean meal and shrimp feed by approximately 18–26% and 27–33%, respectively (Table 3). However, for the soybean meal digestion, a lower temperature seemed to be more suitable for the action of Na-Cid[®]. The statistically higher activity of Na-Cid[®] was observed at 25–40 °C for soybean meal ($P<0.05$). Differently, 30–45 °C was the appropriate temperature of Na-Cid[®] to digest the shrimp feed (Table 3).

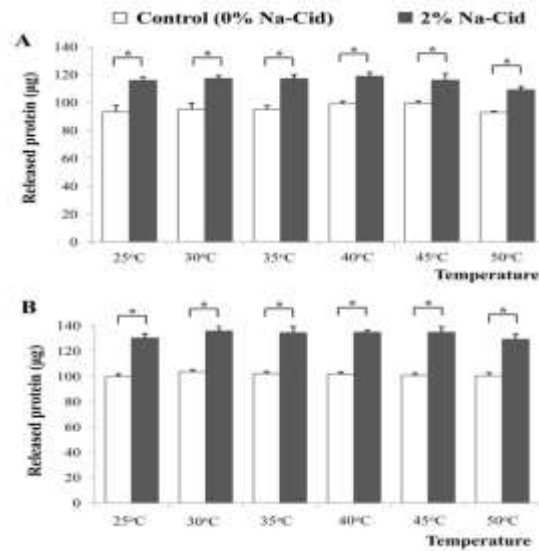


Figure 3. Influence of incubation temperature on the digestion ability of Na-Cid[®] represented by the protein released into the reaction mixture. (A) Soybean meal and (B) Shrimp feed was treated with 2% (v/v) of Na-Cid[®] while sterile distilled water was used to replace Na-Cid[®] as control (0% Na-Cid[®]). The results were expressed as mean \pm SD (n=6). The asterisk located above the black line indicates significance ($P < 0.05$) between the treatment (with or without Na-Cid[®]) at the same temperature. The statistical analysis used is the Student's *t* test

Table 3. Percentage increasing in the released protein of the soybean meal and shrimp feed treated with 2% (v/v) of Na-Cid[®] compared to the control (0% Na-Cid[®]) analyzed at different temperatures

Temperature	Percentage increase of the released protein compared to the control	
	Soybean meal	Shrimp feed
25 °C	26.42 \pm 5.98 ^c	30.15 \pm 3.63 ^{ab}
30 °C	24.89 \pm 3.62 ^{bc}	32.40 \pm 3.95 ^b
35 °C	24.68 \pm 3.78 ^{bc}	33.50 \pm 3.58 ^b
40 °C	21.44 \pm 2.47 ^{ab}	33.53 \pm 2.41 ^b
45 °C	18.90 \pm 2.79 ^a	33.67 \pm 3.50 ^b
50 °C	18.61 \pm 1.88 ^a	27.19 \pm 3.97 ^a

^{1/}: Mean \pm SD (n=6) of the calculated value.

Mean values within a row with different superscript letters were significantly different ($P < 0.05$).

Discussion

Soybean meal and soybean products have been considered as the most suitable sources for fishmeal replacement in aquatic feed because of their protein levels and amino acid component that match the animals' requirements (Lim *et al.*, 1998; Samocha *et al.*, 2004; Tantikitti, 2014). However, the use of soybean meal to replace fishmeal is limited. A higher proportion causes negative effects, for example, disrupting the absorption and utilization of nutrients in the diet and suppressing the growth of aquatic animals. These adverse results may be due to several anti-nutritional factors such as a trypsin inhibitor, phytic acid, and saponin (Francis *et al.*, 2001). Therefore pretreatment of soybean meal has been highlighted for increasing the digestibility and reducing the anti-nutritional factors existing in plant protein sources. Many researchers have reported that fermented soybean meal is a good alternative protein source to replace fishmeal when using 20–30% replacement. The advantages of fermented soybean meal include increased growth performance of *L. vannamei* (Shao *et al.*, 2019a), *Macrobrachium nipponense* (Ding *et al.*, 2015), and *Fenneropenaeus indicus* (Sharawy *et al.*, 2016). These evidences reveal the potential of processed soybean meal in feed production for aquatic animals.

This study evaluated the ability of the concentrated pineapple extract, Na-Cid[®], to increase the digestibility of soybean meal as well as shrimp feed that contains soybean meal as the protein source. The results obtained clearly demonstrate the potent effect of the concentrated pineapple extract, Na-Cid[®], on the digestion of soybean meal and shrimp feed. This may be due to the protease activity of Na-Cid[®]. Moreover, according to the product data sheet, Na-Cid[®] contains organic acids, malic acid, and citric acid. These organic acids could facilitate digestion resulting in the increase of released protein from Na-Cid[®]-treated soybean meal and shrimp feed. Lückstädt (2008) has reviewed the contribution of organic acids in the enhancement of the digestibility of dietary components. It has been reported that feed supplemented with various organic acids enhances the growth performance of Atlantic salmon (Baeverfjord and Krogdahl, 1996), white shrimp (da Silva *et al.*, 2014), and the nutrient digestibility in Nile tilapia (Ng *et al.*, 2009) and yellow catfish (Zhu *et al.*, 2014).

The results in the present work also suggested that Na-Cid[®] was able to digest the test materials in a wide range of pH (pH 5.0–8.5) and temperature (25–40°C). These conditions are most likely to exist in the environmental conditions of the digestive tract of most aquatic animals including Pacific white shrimp. This product indeed facilitates and promotes the digestion of soybean meal and shrimp feed. The increased digestibility of feedstuff may help aquatic

animals to gain more nutrition from the feed. In addition, the risk of disease associated with the dysfunction of the digestive tract is consequently reduced. However, to achieve more insight into the effect of Na-Cid[®] in improving the quality of soybean meal, further studies should investigate the ability of Na-Cid[®] to degrade the proteins that act as an anti-nutritional factor.

Acknowledgement

This work was supported by Research and Research Industries, RRI (grant number MSD62I0040).

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(Received: 30 July 2019, accepted: 31 October 2019)