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Apparent digestibility of differently processed grain legumes, cow pea and mung bean in black tiger shrimp, *Penaeus monodon* Fabricius and associated histological anomalies in hepatopancreas and midgut

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Abstract

Experiments were conducted to test the effect different treatment process like dehulling, soaking, autoclaving, germination and germination in combination with autoclaving on proximate composition and antinutritional factors (ANFs) of legume seeds, cow pea and mung bean. An *in vivo* digestibility trial was conducted in black tiger shrimp *Penaeus monodon* to determine the coefficient of total tract apparent digestibility (CTTAD) of differently processed legume seeds. The CTTADs were determined by comparing the concentrations of digestibility marker (Cr_2O_3) in the feed and faeces of the juvenile shrimp (4 ± 0.5 g). Seeds processed by germination in combination with autoclaving were low in ANFs and higher in proximate composition with increased protein contents of 18.3 and 15.6% in cow

Abbreviations: ANF, antinutritional factors; ANOVA, analysis of variance; AOAC, Association of Official Analytical Chemists; B, F and R (cells), types of hepatopancreatic cells; Bb, brush border; CEC, columnar epithelia cells; CPA, autoclaved cow pea; CPD, dehulled cow pea; CPG, germinated cow pea; CPGA, autoclaved germinates of cow pea; CPR, raw cow pea; CPS, soaked cow pea; Cr_2O_3, chromic oxide; L, lumen; M Et, myo-epithelial layer; MBA, autoclaved mung bean; MBD, dehulled mung bean; MBG, germinated mung bean; MBGA, autoclaved germinates of mung bean; MBR, raw mung bean; MBS, soaked mung bean; PM, periotrophic membrane; RF, reference diet; RW, raw; SL CC, sloughed cellular contents; SPSS, Statistical Package for Social Sciences; TD, test diet; TI, test ingredient; Ver., version; W, wall

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pea and mung bean, respectively. Though trypsin inhibitor activity was significantly (P<0.05) high in germinated seeds, there was a significant reduction by 83.3 and 81.21% on germination followed by autoclaving. Due to unexpected mortality of shrimp, dietary treatments containing raw, soaked, and germinated cow pea and mung bean were removed from the trial. There was a significant difference (P<0.05) in the CTTAD values between the feedstuffs and various treatment processes made. Higher CTTAD dry matter (DM), crude protein (CP) and nitrogen free extract (NFE) were obtained with seeds processed with germination in combination with autoclaving and the trend is similar in both the seeds tested. CTTAD for DM, CP and crude lipid of the grain legumes ranged between 0.683–0.885, 0.684–0.834 and 0.704–1.302, respectively. Histological examinations on hepatopancreas and midgut of shrimp sampled at slaughter revealed some common anomalies. With the exception of the shrimp fed dehulled cowpea, histology was normal in all the shrimps sampled at the end of the digestibility trial.

Keywords: *Penaeus monodon*; Digestibility; Plant feedstuffs; Dehulling; Soaking; Autoclaving; Germination; ANFs; Histology

1. Introduction

Legume seeds are high energy, medium protein ingredients which include peas, grams and beans and other closely related species within the Fabaceae family. In shrimp feed, soybean meal is the most widely used among various available plant protein feedstuffs, owing to its nutritional quality, favorable cost and consistent availability (Lim and Dominy, 1990; Samocha et al., 2004). Only recently, researchers have begun to evaluate the suitability of grain legumes such as feed pea (Cruz-Suarez et al., 2001; Davis et al., 2002; Bautista-Teruel et al., 2003), lupin (Sudaryono et al., 1999), cow pea and rice bean (Eusebio, 1991) in shrimp feeds. India is a major producer of grain legumes globally which are frequently used in poultry and livestock diets (Singh, 2003). Grain legumes offer flexibility in feedstuff selection to the feed manufacturer as they have the potential to provide both good energy and moderate protein to the diet. However, the utilization of available protein and carbohydrates in legume feedstuffs are much less than that calculated from the chemical composition because of the presence of various antinutritional factors (ANFs) such as trypsin inhibitors (TI), phytates, saponins and polyphenolic compounds (Liener, 1989; Siddhuraju et al., 2002; Siddhuraju and Becker, 2005). To inactivate or reduce the above mentioned antinutrients, various conventional, simple processing methods have been used such as dehulling, dry heating, roasting, boiling, soaking (in water, alkali and acid), solvent extraction, germination and fermentation (Shastry and John, 1991; Elemo et al., 1998; Siddhuraju et al., 2002). However, none of these methods is able to remove completely all the detected antinutrients that are present in feed materials. Hence, a combination of processing methods is generally more effective than a single method (Siddhuraju and Becker, 2005).

Determination of digestibility can be used to select ingredients that optimize the nutritional value and cost of formulated feeds. Digestibility of a feedstuff by the animal depends not only on the animal digestive tract architecture, physiology and environmental conditions, but also on the physical and nutrient characteristics of the feedstuff (Lee and Lawrence,

1985; Akiyama et al., 1989). Previous studies demonstrated that cells and tissues of the midgut gland (Vogt et al., 1985) and digestive tract of shrimp are very sensitive to the different diets. Many researchers reported histological alterations with relation to ANFs in fishes (Ciereszko and Dabrowski, 2000; Salaro et al., 2000) and hence they can be used as a scale to measure the effect of ANFs on physiology of cultured organisms (Tacon, 1997). The current study reports a comparative assessment of the variations in proximate composition, ANFs and digestibility of differently processed two under utilized legumes such as cow pea and mung bean in *Penaeus monodon* and associated histological anomalies in hepatopancreas and midgut of post-fed shrimp.

2. Materials methods

Legume feedstuffs like cow pea (*Vigna unguiculata*) and mung bean (*Phaseolus aureus*) processed by treatment methods like dehulling, soaking, autoclaving, germination and germination in combination with autoclaving were used for digestibility assessment.

2.1. Feedstuffs and processing

Whole grain legumes (food grade) were obtained from the local market and were cleaned by hand to remove foreign materials before processing. The seeds were divided in to six portions as required for the different treatment processes employed. Five parts were subjected to different individual treatment processes and one part served as raw (RW). The seeds were dehulled in rubber roll sheller and segregated from the undecorticated and broken grains. Soaking of whole grain legumes was undertaken for 24 h in distilled water. For autoclaving, the soaked seeds were placed in a conical flask. After adding tap water (2 ml/g dry seeds) the mixture was autoclaved at 121 °C for 30 min. Prior to germination, seeds were sterilized by soaking in ethanol for 1 min. Sterilized seeds were soaked in distilled water for 12 h at room temperature (25 °C). The soaked seeds were kept between thick layers of cotton cloth and allowed to germinate in the dark at room temperature for 2 days. The germinated seeds were rinsed with distilled water. The germinated seeds were autoclaved or mashed. All the wet processed feedstuffs (soaked, autoclaved, germinated, germinated + autoclaved) were dried at 50 °C overnight in an electric oven and ground to fine particles.

2.2. In vivo digestibility trial

2.2.1. Experimental diet preparation

The coefficient of total tract apparent digestibility dry matter (DM), protein (CP), lipid (CL) and nitrogen free extract (NFE) in processed legume seeds were determined *in vivo* using chromic oxide (Cr_2O_3) as an inert marker. The reference diet (RF) (Table 1) was formulated to satisfy the protein and lipid requirements of *P. monodon* (Smith and Tabrett, 2004). Twelve test diets composed of 0.50 reference diet (RD) and 0.50 test ingredient were prepared by following regrinding and re-extruding technique described by Smith and Tabrett (2004). Test diets contained the following test ingredients: cow pea-raw (CPR), dehulled (CPD), autoclaved (CPA), soaked (CPS), germinated (CPG) and autoclaved germinates

Table 1
Ingredient composition (g kg^{-1}) of reference and test diets

Ingredients	Reference diet	Test diet
Fish meal[a]	245	122.5
Wheat gluten	160	80
Squid meal[b]	80	40
Defatted soybean meal	120	60
Wheat flour	279	134.5
Soy lecithin	20	10
Di-calcium phosphate	20	10
Sardine body oil	30	15
Cholesterol	5	2.5
Spirulina	5	2.5
Cr_2O_3	5	5
Vitamin mixture[c]	10	5
Mineral mixture[d]	10	5
Sodium alginate	8	8
Test ingredient[e]	–	500

[a] Sardine meal obtained from Coastal Aquatic Proteins, Mangalore, India.
[b] Mantle powder prepared from fresh squid obtained from local fish market.
[c] Vitamin mix (10 g) provided the following levels of nutrients—vitamin A acetate, 87,912 IU; cholecalciferol (D3), 2200 IU; tocopheryl acetate (E), 550 IU; menadione, 22 mg; D-calcium pantothenate, 189 mg; pyridoxine HCl, 77.6 mg; riboflavin, 66 mg; niacin, 330 mg; folic acid, 22 mg; thiamin mononitrate, 73.9 mg; biotin, 2.2 mg; cyanocobalamin (B12), 0.1 mg; inositol, 220 mg; butylated hydroxytoluene, 22 mg.
[d] Mineral mix (10 g) provided the following levels of nutrients—Cu, 2.8 mg; Fe, 39.1 mg; Zn, 107.3 mg; Mn, 41.1 mg; K, 1674 mg; I, 10 mg; Co, 0.5 mg; Se, 0.4 mg.
[e] Either cow pea or mung bean processed by different processing methods.

(CPGA); mung bean-raw (MBR), dehulled (MBD), autoclaved (MBA), soaked (MBS), germinated (MBG) and autoclaved germinates (MBGA). Initially water was added to all the dry ingredients (except sodium alginate and vitamins mix) and thoroughly mixed until homogenous in a Hobart-type mixer and made in to dough like consistency. The dough was pressure pelletized using a Hobart meat grinder with 2 mm die and air dried. Then dried pellet was reground and passed through 200 μm sieve. Sieved feed was thoroughly hand mixed with fish oil and vitamin mix before adding with water containing sodium alginate binder (8 g kg^{-1} in 200 ml of water). The dough was cold pelletized again into a bath of 10% aqueous calcium chloride. The spaghetti like feed was left in the calcium chloride for 5 min before being removed and dried overnight at room temperature. Dried pellets were stored in 4 °C until use. Proximate composition of the reference diet was analyzed and the test diets were calculated using the respective analyzed proximal values of processed ingredients and reference diet (Table 2).

2.2.2. *Digestibility trial*

Hatchery produced (Best India shrimp hatchery, Marakkanam, Chennai, India) post-larvae of *P. monodon* were grown up to juvenile shrimp in concrete tanks with a commercial shrimp feed (Waterbase Private Limited, India) labeled to have 35 g kg^{-1} crude protein. Juvenile shrimp (4 ± 0.5 g) grown in concrete tanks were randomly stocked in 27 circular plastic troughs (100-l). Each trough housed six shrimp with three replicate troughs per

Table 2
Analyzed proximate composition (g kg^{-1}) of reference and test diets[a]

Diets	Crude protein	Crude fat	Crude fibre	Ash	NFE[a]
Reference diet	450 ± 1.6	98 ± 1.9	23 ± 2.5	100 ± 5.1	329 ± 6.5
CPDH	356 ± 3.9	56 ± 0.4	30 ± 2.5	68 ± 0.0	489 ± 1.6
CPA	346 ± 1.3	55 ± 0.3	38 ± 0.1	68 ± 0.4	493 ± 1.3
CPG	372 ± 0.3	54 ± 0.1	44 ± 0.2	68 ± 0.1	463 ± 2.1
CPGA	371 ± 0.6	55 ± 0.2	43 ± 0.1	68 ± 0.3	464 ± 2.2
MBDH	362 ± 4.4	55 ± 0.1	26 ± 1.6	71 ± 0.1	486 ± 1.3
MBA	349 ± 0.7	55 ± 0.2	31 ± 0.05	71 ± 0.1	495 ± 0.5
MBG	366 ± 0.2	53 ± 0.1	30 ± 1.6	70 ± 0.4	481 ± 1.55
MBGA	367 ± 0.2	53 ± 0.2	26 ± 0.2	68 ± 0.2	486 ± 0.7

Values are means of triplicates ± standard deviation. Cow pea: CPDH, dehulled; CPA, autoclaved; CPG, germinate; CPGA, autoclaved germinates. Mung bean: MBDH, dehulled; MBA, autoclaved; MBG, germinate; MBGA, autoclaved germinates.

[a] Nitrogen free extract calculated by difference.

treatment. Filtered seawater was used with a daily water exchange of 80%. Temperature was maintained at 28 ± 2 °C. Photo exposure was provided as alternating 12 h light and dark periods. Salinity, pH, dissolved oxygen and ammonia–nitrogen concentrations in the water were measured once a week following the method of Strickland and Parsons (1972). Shrimp were acclimated to the digestibility trough for 1 week before the initiation of trial and adapted to consume each experimental diet containing Cr_2O_3. Shrimp were fed four meals per day (05:00, 11:00, 17:00 and 23:00 h) and allowed to feed 45 min after each feeding. The uneaten feed including a few faecal strands were siphoned from the bottom of the tanks after feeding time and discarded. Thereafter, the faecal matter was collected twice from the tank bottom before the next feeding (3 h after earlier feeding and 0.5 h before next feeding) for 20 days. The collected faeces was gently rinsed with distilled water to remove excess salt and dried in a hot air oven at 55 °C. The dried faecal material from each tank was pooled and stored frozen at −20 °C for analysis. CTTADs were determined according to Cruz-Suarez et al. (2001).

2.3. Chemical analysis

Experimental feedstuffs, reference diet and faecal samples were finely ground, sieved before analysis. Dry matter was calculated by gravimetric analysis following oven drying at 100 °C for 24 h. Ash content was determined gravimetrically by burning in Muffle furnace at 550 °C for 6 h. Kjeldahl method of AOAC (1990) was followed for estimation of crude protein content (AOAC 955.04). Crude fibre and crude lipid was analyzed according to Van Soest et al. (1991) and Folch et al. (1957). Nitrogen free extract was calculated by difference. The varying levels of ANFs such as trypsin inhibitors, phytic acid and tannins in feedstuffs were determined following Kakade et al. (1974), Wheeler and Ferrel (1971) and Burns (1971), respectively. The chromic oxide content of diets and faecal samples were analyzed following method of Furukawa and Tsukahara (1966). As a replacement for of conventional Kjeldhal flask, the samples were digested using rapid digestion microwave oven (Microwave 3000^{TM}).

2.4. Histological analysis

The shrimp fed raw, soaked and germinated feedstuffs died in the middle of the trial; hence the shrimp at this point were sampled and used for histological analysis. However, the remaining dietary treatments were sampled only at end of the trial. Shrimp from each tank were caught and injected with the Davidson's fixative directly in to the hepatopancreas first and then at several other points around the digestive tract. The cuticle was cut along the body length with a pair of scissors starting from the sixth abdominal segment and stabbed along the mid-side up to the cephalothorax at which point the cut was angled and turned in to rostrum just behind the eyestalk. Another deep cut was made directly down in to the muscle just behind the cephalothorax to facilitate the penetration of fixative. Fixed hepatopancreas and midgut were dehydrated in a graded ethanol series and embedded in paraffin. Tissues sections (3–4 μm) were stained with haematoxylin and eosin (Humason, 1972) and examined by light microscopy, following the structural illustrations and terminology of Vogt et al. (1985) and Bell and Lightner (1988).

2.5. Statistical analysis

Data on proximate composition, ANFs and CTTADs of grain legumes in *P. monodon* were subjected to one-way ANOVA to determine significant differences among the treatments. Duncan's multiple range test (Duncan, 1955) was applied to ascertain any significant differences between treatment means. All the above mentioned statistical analyses were performed using SPSS statistical software (Ver. 10 for Windows, SPSS, Chicago, IL, USA). Limits of significance for all critical ranges were set at $P<0.05$.

3. Results

3.1. Effect processing techniques on proximate composition of legume seeds

Changes in proximate compositions of cow pea and mung bean as a function of different treatment processes (Table 3) were significant ($P<0.05$). Among the treatment process, germination improved the protein content by 18.3 and 15.6% in cow pea and mung bean, respectively. While dehulling significantly ($P<0.05$) reduced the fibre content in both the seeds, soaking and autoclaving did not have any substantial changes in proximate composition. Though protein content was slightly decreased on autoclaving, there was no significant difference ($P>0.05$) in proximate composition between germinates and autoclaved germinates.

3.2. Effect processing techniques on ANFs

Significant differences ($P<0.05$) in antinutritional contents were found between legume seeds subjected to different processing methods. Reductions of ANFs were observed to various extents depending on different treatment processes employed, except germination which substantially ($P<0.05$) increased the trypsin inhibitor activity (Table 4). There noticed

Table 3
Changes in proximate composition of cow pea and mung bean as a function of different treatment processes (g kg^{-1} dry matter)

Component	Raw	Dehulled	Autoclaved	Soaked	Germinated	Autoclaved germinates
Cow pea	247 ± 1.2 a	264 ± 2.6 b	242 ± 1.2 a	248 ± 1.5 a	294 ± 1.5 c	293 ± 2.5
Crude protein						
Crude lipid	16 ± 0.6 c	15 ± 0.5 bc	13 ± 0.6 b	14 ± 0.3 c	11 ± 0.6 a	11 ± 1.2 a
Crude fibre	52 ± 0.5 b	38 ± 0.05 a	53 ± 0.8 b	53 ± 0.6 b	64 ± 1.8 c	63 ± 0.8 c
Crude ash	38 ± 0.6 b	36 ± 0.5 a	36 ± 0.5 a	37 ± 0.8 a	35 ± 0.4 a	35 ± 1.0 a
NFE[a]	646 ± 1.6 b	649 ± 3.1 b	657 ± 2.6 b	649 ± 2.0 b	596 ± 4.2 a	598 ± 4.5 a
Mung bean	245 ± 2.5 a	275 ± 1.8 bc	249 ± 0.6 a	253 ± 1.0 a	283 ± 0.1 c	284 ± 1.5 c
Crude protein						
Crude lipid	13 ± 0.5 b	11 ± 0.9 b	11 ± 0.04 b	13 ± 0.7 b	7.8 ± 0.3 a	8.3 ± 0.6 a
Crude fibre	42 ± 0.5 c	29 ± 0.05 a	39 ± 0.3 b	35 ± 2.4 b	37 ± 2.5 b	29 ± 0.7 a
Crude ash	49 ± 0.3 c	42 ± 0.6 b	41 ± 0.4 b	41 ± 1.0 b	39 ± 0.4 a	37 ± 1.7 a
NFE[a]	656 ± 3.4	643 ± 2.5	660 ± 1.0	658 ± 1.0	63.28 ± 0.31	642 ± 1.4

Values are means of triplicates ± standard deviation. Means in the same row sharing different letters are significantly different (P<0.05).

[a] Nitrogen free extract calculated by difference.

a similar trend in reduction or increase in ANFs for both grain legumes. Though trypsin inhibitor activity was significantly (P<0.05) high in germinated seeds, a significant reduction was observed by a level of 83.3 and 81.21% on germination followed by autoclaving. Autoclaving of raw/and germinated grain legumes showed significant (P<0.05) reduction in all the three ANFs. Reductions of tannin and phytic acid contents on germination ranged between 55–60% and 70–81%, respectively and it further reduced upon subsequent auto-

Table 4
Effect of processing on potential antinutritional factors (ANF) in cow pea and mung bean

Antinutritional factor (dry weight basis)	Treatment processes					
	Raw	Dehulled	Autoclaved	Soaked	Germinated	Autoclaved germinates
Cow pea						
Trypsin inhibitors (mg g^{-1})	13.7 ± 0.5 c	12.3 ± 1.2 c	2.5 ± 0.2 a	12.9 ± 1.0 c	35.7 ± 0.6 d	2.3 ± 1.2 b
Phytic acid (mg g^{-1})	12.8 ± 0.2 c	11.4 ± 0.3 c	7.6 ± 0.2 b	9.5 ± 0.3 bc	3.9 ± 0.4 a	1.1 ± 0.3 a
Tannins (mg g^{-1})	9.7 ± 0.4 d	1.6 ± 0.0 a	6.5 ± 0.4 c	7.8 ± 0.3 c	4.3 ± 0.0 b	1.3 ± 0.0 a
Mung bean						
Trypsin inhibitors (mg g^{-1})	14.9 ± 0.8 c	11.3 ± 1.5 c	2.1 ± 1.5 a	13.3 ± 2.5 c	44.7 ± 3.5 d	2.8 ± 0.3 b
Phytic acid (mg g^{-1})	13.7 ± 0.4 c	12.8 ± 0.3 c	8.6 ± 0.1 b	11.5 ± 0.5 bc	2.8 ± 0.1 a	1.4 ± 0.1 a
Tannins (mg g^{-1})	7.8 ± 0.2 c	1.4 ± 0.1 a	4.1 ± 0.2 b	6.5 ± 0.3 c	3.1 ± 0.2 ba	2.2 ± 0.2 a

Values are means of triplicates ± standard deviation. Means in the same row sharing different letters are significantly different (P<0.05).

claving. Dehulling reduced the tannin content significantly ($P<0.05$) in both cow pea and mung beans.

3.3. Apparent digestibility

The shrimp fed raw, soaked, and germinated cow pea and mung bean started to die 1 week into the trial. Hence those dietary treatments were removed. Other dietary treatments continued without interruption or disease problem. The water quality parameters across experiments were: salinity, 28–30.1‰; temperature, 25.5–28.5 °C; dissolved oxygen, >5.7 mg l^{-1}; total ammonia–nitrogen, 0.04–0.08 mg l^{-1}; nitrite–nitrogen, 0.08–0.10 mg l^{-1}; and pH 7.9–8.2.

CTTAD dry matter, crude protein, crude lipid and NFE (ANFED) of test diets and test feed stuffs (Tables 5 and 6, respectively) were significantly ($P<0.05$) affected by the processing methods handled on both the grain legumes. CTTAD of test ingredients were slightly higher than the test diets except for NFE and no trend in CTTADs related to ingredient composition (*i.e.* levels of crude protein, crude lipid, or NFE) was apparent. Though the shrimp fed germinated seed died in the middle of the trial, the CTTAD were significantly ($P<0.05$) higher in shrimp fed autoclaved germinates and followed by autoclaved and dehulled seeds with a similar trend in both the seeds tested. CTTAD for DM, CP and CL of the grain legumes ranged between 0.683–0.885, 0.684–0.834 and 0.705–1.302, respectively.

3.4. Histological anomalies

Figs. 1A–D and 2 show the transverse and longitudinal sections of the tubules through the medial region of hepatopancreas. Hepatopancreas from a shrimp fed RD and test diets CPA, CPGA, MBA and MBGA (Fig. 1A) exhibited normal star-shaped lumina (L). The cell types (B, F and R) described by Bell and Lightner (1988) are well preserved. The tubules are surrounded by a basal lamina separating the glandular cells from the haemal sinuses between the tubules. A brush border (Bb) is evident on the luminal surface of the cells. A thin myo-epithelial layer (M Et) surrounds the tubules. Between tubules haemal sinuses (Sin) are seen.

Hepatopancreas of shrimp fed CPR, CPS, CPG, MBR, MBS, and MBG revealed some common abnormalities among the dietary treatments (Fig. 1B–D). It includes unusual enlargement of vacuoles of B cells (Fig. 1B and D), loss of the acinar structure of the organ (Fig. 1C and D), and occurrence of basophilic granules (Fig. 2) in the basal lamina of glandular cells. Cell vacuolization became more prominent, the cells taking on a foamy appearance (Fig. 1B). Acinar structure typical of healthy animals was disorganized because of the sloughing off of the cell lining (Fig. 1C). The connective tissue constituting the capsule of the organ and that surrounding the acini was moderately well conserved. The tubular lumina lost their star like outline. The sloughed cellular content (SL CC) was dispersed within the lumina and among the acini (Fig. 1C). From the shrimp sampled at the end of the digestibility trial, shrimp fed CPD only showed vacuolization in B cells.

Fig. 3A–D shows the longitudinal sections of the midgut from shrimp fed experimental diets. In shrimp fed RD and test diets CPA, CPGA, MBA and MBGA, midgut wall (W) is uniformly lined with tall, quite narrow columnar epithelial cells (CEC). The columnar

Table 5
Coefficient of total tract apparent digestibility coefficient (CTTAD) in test diets containing differently processed cow pea and mung bean consumed by black tiger shrimp *Penaeus monodon*

Diets	RD
Dry matter	0.74 ± 0.002
Crude protein	0.88 ± 0.001
Crude lipid	0.79 ± 0.014
NFE	0.74 ± 0.005

Diets	Cow pea	Mung bean	Cow pea	Mung bean	Cow pea	Mung bean	Cow pea	Mung bean
Dehulled	0.71 ± 0.003 a	0.76 ± 0.001 a	0.75 ± 0.004 a	0.81 ± 0.004 a	0.78 ± 0.001 b	0.81 ± 0.008 b	0.73 ± 0.002 a	0.68 ± 0.004 a
Autoclaved	0.75 ± 0.007 b	0.77 ± 0.001 b	0.84 ± 0.004	0.79 ± 0.004 a	0.72 ± 0.001 c	0.77 ± 0.007 a	0.76 ± 0.006 b	0.72 ± 0.004 b
Autoclaved Germinates	0.75 ± 0.003 b	0.81 ± 0.008 c	0.86 ± 0.008	0.86 ± 0.001 b	0.80 ± 0.004 a	0.83 ± 0.006 b	0.79 ± 0.002 c	0.75 ± 0.003 c

RD, reference diet; NFE, nitrogen free extract. Values are means of triplicates ± standard deviation. Means in the columns sharing same letters are not significantly different ($P<0.05$).

Table 6
Coefficient of total tract apparent digestibility (CTTAD) in differently processed cow pea and mung bean consumed by black tiger shrimp *Penaeus monodon*

Diets	IADMD		IACPD		IACLD		IANFED	
	Cow pea	Mung bean	Cow pea	Mung bean	Cow pea	Mung bean	Cow pea	Mung bean
Dehulled	0.68 ± 0.008 a	0.78 ± 0.004 a	0.60 ± 0.008 a	0.65 ± 0.007 a	0.79 ± 0.060 b	1.18 ± 0.138 a	0.68 ± 0.004 a	0.65 ± 0.009 a
Autoclaved	0.77 ± 0.015 b	0.81 ± 0.004 b	0.77 ± 0.014 b	0.70 ± 0.001 b	0.77 ± 0.005 a	0.70 ± 0.047 a	0.73 ± 0.008 b	0.72 ± 0.007 b
Autoclaved germinates	0.76 ± 0.006 b	0.88 ± 0.017 c	0.83 ± 0.025 c	0.83 ± 0.002 c	1.06 ± 0.121 c	1.30 ± 0.086 b	0.77 ± 0.003 c	0.75 ± 0.002 c

NFE, nitrogen free extract. Values are means of triplicates ± standard deviation. Means in the columns sharing same letters are not significantly different (P<0.05).

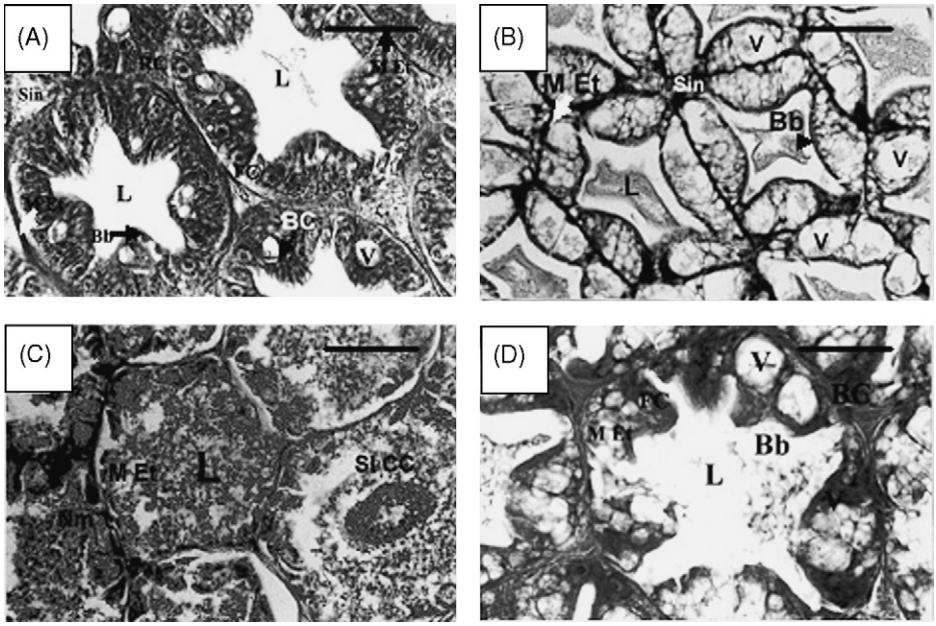

Fig. 1. Transverse section through hepatopancreas of shrimp fed test diets containing cow pea and mung bean: (A) hepatopancreas from shrimp fed reference diet showing normal tubules; (B) hepatopancreas showing sever vacuolization in B-cells of tubules; (C) hepatopancreatic tubules showing sloughing of myo-epithelial layer and sloughed cellular contents; (D) hepatopancreatic tubules showing disorganized acinar structure because of the sloughing off of the cell lining.

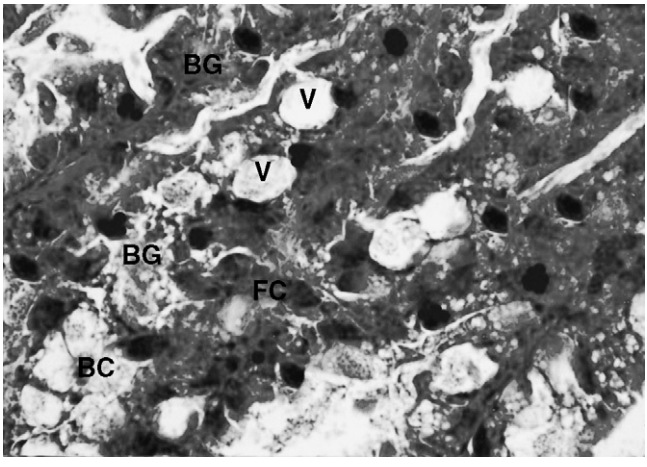

Fig. 2. Longitudinal section through hepatopancreas of shrimp fed test diet showing distinct basal granules.

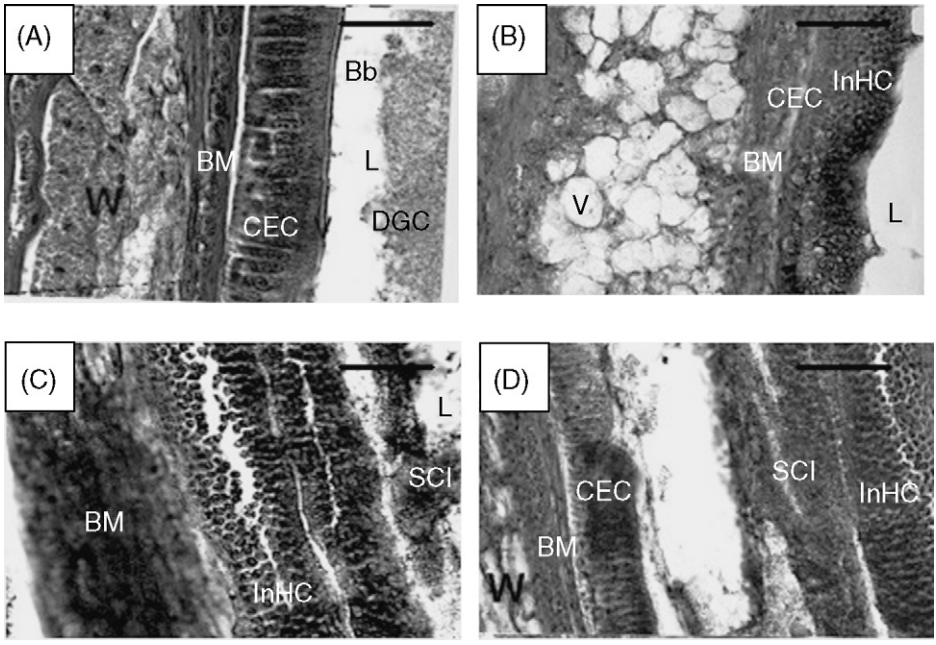

Fig. 3. Longitudinal section through midgut of shrimp fed diets containing cow pea and mung bean: (A) midgut of shrimp fed reference diet showing normal tissue; (B) midgut of shrimp showing abnormal vacuolation in midgut wall; (C) midgut of shrimp showing infiltration of inflammatory haemocytes in to the lumen of the gut and degenerating gut wall; (D) midgut of shrimp showing occurrence of inflammatory haemocytes and sloughing of columnar epithelial cells.

epithelial cells rest on a basement membrane above the gut wall. Tubular lumen (L) is dispersed with digested remains of feed material. The cell types and structure described by Bell and Lightner (1988) are well preserved. The free border of the CEC is conspicuously striated with a brush border (Bb). Goblet cells which secretes mucous are interspersed among the CEC. A thin chitinous periotrophic membrane (PM) is evident on luminal surface of the Bb (Fig. 3A).

Midgut of shrimp fed CPR, CPS, CPG, MBR, MBS, and MBG revealed abnormalities like detachment of columnar epithelial, vacuolation and swelling in gut wall (Fig. 3B), necrosis of epithelial cells of mucousal lining (Fig. 3D) and occurrence of inflammatory haemocytes (Fig. 3C and D), While vacuolation (Fig. 3) and swelling of gut was a common abnormality, inflammatory haemocytes is prominent only in shrimp fed raw cow pea (CPR).

4. Discussion

In the present study cow pea and mung bean processed with germination in combination with autoclaving obtained higher protein content, lower ANFs and maximum CTTADs. The significant increase in crude protein content of the cow pea and mung bean on germination,

can be attributed to production of growth enzymes (Sunday et al., 2001) and consumption of other seed components during the germination process. Similar to these observations, a significant increase in protein content on germinations of seeds have been reported by Uwaegbute et al. (2000) and Rivas-Vega et al. (2006) in cow pea and Mubarak (2005) in mung bean. They also reported a significant influence on crude fibre and crud lipid content. However, in the present study only slight changes were noticed in those parameters which may be due to differences in the germination duration, which was restricted to 2 days in the present study. Otherwise the increase in duration of germination will leads to increase in crude fibre content of germinates (Oloyo, 2004). The observation made in the study on reduction of both phytic acid and tannin content of the seeds germination are in agreement with the earlier reports. Eskin and Wiebe (1983) reported that germination reduced phytic acid content in germinating seed, due to increased phytase activity. After 48 h of germination, tannin contents of mung beans (Barroga et al., 1985) and *Dolichos lablab* (Shastry and John, 1991) were reduced by 23–36 and 85%, respectively. Shastry and John (1991) reported that germination increased trypsin inhibitory activity in legumes, attributed to trypsin inhibition to the phenolic content in the seeds. The increase in the trypsin inhibitory activity in the seed of cow pea and mung bean during germination can be attributed to the increase in the phenolic content of the seed. As seed hulls are rich in fibre and tannin content (Liener, 1989) dehulling significantly reduced both crude fibre (Eusebio, 1991; Davis et al., 2002) and tannin content (Booth et al., 2001), and their removal leads to a slight increase in protein content. Phytic acid and trypsin inhibitors, however, are concentrated in the cotyledons, so that dehulling did not show any significant influence on ANFs. Though autoclaving made marginal changes in proximate composition of the seeds, ANFs were significantly reduced with a pronounced impact on TIA. Similarly, pressure cooking/autoclaving of raw seeds of various legumes significantly decreased the various antinutrients (Khalil and Mansour, 1995; Siddhuraju and Becker, 2005; Mubarak, 2005). The rate of reduction of phytic acid and tannin content in the present study are consistent with the findings of other workers who found such reductions on autoclaving of food legumes (Rehman and Shah, 2005). Hence, seeds processed by germination in combination with autoclaving were low in ANF and constructive in proximate composition as influenced by the germination process. Though soaking processes was less effective in reducing phytic acid and TIA, it significantly reduced tannin content of both cow pea and mung bean and it accords with earlier investigations in different varieties of legume seeds (Vijayakumari et al., 1997).

Mass mortality of shrimp observed with shrimp fed the test diet containing 500 g raw, soaked and germinated legume seeds kg^{-1} in the digestibility trial clearly indicate that legume seeds should be at least heat treated (autoclaving) or dehulled before inclusion in diets of juvenile *P. monodon*. Several reports have indicated that feed peas as potential ingredients for shrimp feeds (Smith et al., 1999; Cruz-Suarez et al., 2001; Davis et al., 2002; Bautista-Teruel et al., 2003). CTTAD for DM (0.73) and CP (0.92) values reported by Bautista-Teruel et al. (2003) with tiger shrimp fed field pea agree with present results. Bautista-Teruel et al. (2003) also obtained better survival and growth in shrimp fed feed pea meal; in the study, feed was prepared by extrusion cooking, which was found to denature most of the ANFs.

CTTADs of grain legumes were significantly different between treatment process. The difference in digestibility may be attributed to the denaturation of complex non-nutritive

molecules to simple nutritive ones or destruction of the ANFs of raw feedstuffs (Mansour et al., 1993; Cruz-Suarez et al., 2001) employed. CTTAD for DM and CP were significantly lower with shrimp fed dehulled seeds and higher in those fed seeds processed by germination in combination autoclaving. Lower digestibility of seed on dehulling might be attributed to level of ANFs present in the seeds. Among ANFs, trypsin inhibitor has received the most attention and has been reported to cause growth depression, poor feed efficiency and survival of fish (Wilson and Poe, 1985). ANFs that inhibit enzyme digestion in shrimp will decrease their growth rate (Garcia-Carreno, 1996). At the same time, significantly higher CTTAD for DM and CP in shrimp fed seeds processed with germination in combination with autoclaving might be due to relatively complete reduction in all ANFs (including crude fibre) and a significant improvement in crude protein content of geminated seeds. The improvement in digestibility, after autoclaving treatments, might be attributed to some other factors, such as disruption of protein structures and cell wall-encapsulated starch, starch gelatinisation, and physical disintegration of the legume seeds. Also, autoclaving is found to be most effective among the different processing methods for the reduction of various antinutrients in legume seeds (Rehman and Shah, 2005; Siddhuraju and Becker, 2005).

Eusebio (1991) reported that digestibility of protein content was higher in dehulled rice beans than raw seeds. Davis et al. (2002) and Cruz-Suarez et al. (2001) did not obtained a significant improvement in growth of shrimp fed dehulled feed pea. Though ANFs were significantly reduced by autoclaving, digestibility was inferior when compared to seeds processed with germination in combination with autoclaving and superior to seeds processed by dehulling. This fact might be attributed to the relatively higher crude fibre and ANFs of the autoclaved seeds than the seeds processed in combination with germination. The mortality observed with shrimp fed raw, soaked and germinated feedstuffs may be due to higher level ANFs in those feedstuffs. This is evidenced by the histological aberrations that observed in hepatopancreas and midgut of the shrimp sampled at the edge of death, which fed unprocessed feedstuffs. Many researchers reported histological alterations with relation to ANFs in fishes (Salaro et al., 2000; Ciereszko and Dabrowski, 2000) and shrimp (Vogt, 1990), and hence they can be used as a scale to measure the effect of ANFs on physiology of cultured organisms (Tacon, 1997). Midgut gland is the largest organ by volume in decapod crustaceans and has many biological functions, which include synthesis and secretion of digestive enzymes, absorption of digested dietary products, maintenance of reserves and organic substances, lipid and carbohydrate metabolism, distribution of stored reserves during the intermolt cycle, and catabolism of some organic compounds. A change in the histology of the midgut gland is observed prior to a growth depression response in shrimp (Vogt et al., 1985; Catacutan and De La Cruz, 1989). Numerous lipid vacuoles were observed in B-cells of the midgut gland cells of juvenile *P. monodon* fed a diet deficient in folic acid, riboflavin and ascorbic acid (Catacutan and De La Cruz, 1989). Marked histological alterations were evident within the midgut gland cells of post-larvae fed mimosine-containing diets, including the progressive destruction of the mid-gland epithelial cells (Vogt, 1990). The histological aberrations observed in the present study are in agreement with the aberrations on ingestion of toxic cyanobacteria by prawns. Cyanobacteria ingestion has been reported to induce haemocytic enteritis, a disease in which the epithelial lining of the midgut is damaged and the healthy mucosal lining is replaced by necrotic cells and layers of inflam-

matory haemocytes (Lightner, 1978). A similar condition is observed in the present study in shrimp fed raw plant feedstuffs.

Most of the antinutrients, at levels present as a result of incorporating alternate protein sources in shrimp feeds, do not lead to mortality, but could produce adverse effects and decrease productivity. In the present investigation, even the processed feedstuffs contained some little quantity of ANFs. The test diets in the present study was prepared to contain reference diet and test ingredient in the ratio of 50:50 as recommended by Smith et al. (1999). Hence at this given proportion, the shrimp could ingested more amount of ANFs when compared to their level in practical feed formulation. This shows the comparative tolerance of shrimp to ANFs (Tacon, 1997).

5. Conclusion

The results of the present study showed that higher proportions of unprocessed legume feedstuffs at the level of 500 g kg^{-1} test diets is fatal to shrimp. Histological examinations of the shrimp tissues were also confirmed the toxic effects of ANFs and further they could be used as valid tool to evaluate effect of ANFs in shrimp. The results with autoclaved seeds revealed that, the above problem could be overcome by at least heat processing the feedstuffs. *In vivo* digestibility trial revealed that CTTAD of feedstuffs could be enhanced significantly by processing the seeds with germination in combination with autoclaving.

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