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SHORT COMMUNICATION

Effects of dietary β-conglycinin and glycinin on digestive enzymes activities, intestinal histology and immune responses of juvenile turbot *Scophthalmus maximus*

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Turbot *Scophthalmus maximus* is a valuable commercial carnivorous fish in Europe and Asia because of its high quality flesh and rapid growth. In current turbot farming, fish meal is used as the main dietary protein source (Bonaldo, Parma, Mandrioli, Sirri, Fontanillas, Badiani & Gatta 2011). However, global aquaculture has continued to grow while the production of fish meal is static. Thus, the research on alternatives to fish meal is an international research priority (Hardy & Kissil 1997). Among the alternatives, soy product is one of the most promising because of the security of supply, price and good amino acid profile (Storebakken, Refstie & Ruyter 2000). However, soybean meal can be used in carnivorous fish diets only at relatively low levels. The main limitation is principally due to its reduced digestibility, palatability but more importantly the presence of anti-nutritional factors, such as protease inhibitors, tannins, lectins, saponins and allergens (reviewed by Francis, Makkar & Becker 2001).

The presence of allergenic components in soybeans greatly restricts their use in diets for livestock species. Beta-conglycinin (7S) and glycinin (11S), the two major storage protein components in soybean which account for 70–80% of protein composition, act as allergens to several animals and humans (Garcia, Torre, Marina & Laborda 1997). They can cause reduced growth and increased incidence of intestinal diseases, such as enteritis in piglets and calves (L'Hocine & Boye 2007). There is little published data about the effects of β-conglycinin and glycinin on fish. Rumsey, Siwicki, Anderson and Bowser (1994) reported that soy preparations with high levels of antigenic β-conglycinin and glycinin induced inflammation in rainbow trout. It was also found that β-conglycinin inclusion induced inflammation and oxidation, and caused dysfunction of intestinal digestion and absorption in Jian carp *Cyprinus carpio* var. Jian (Zhang, Guo, Feng, Jiang, Kuang, Liu, Hu, Jiang, Li, Tang & Zhou 2013). However, the role of dietary glycinin in animal gastrointestinal function has not been extensively studied. In food production, heat treatment is used to denature protein to reduce allergenicity. Demonte, Carlos, Lourenco and Dutra de Oliveira (1997) reported that reduction in immunogenicity occurred when glycinin was exposed to high temperatures in the presence of native anti-glycinin serum. However, there are different viewpoints regarding the effect of heat treatment. It is unlikely that heat alone will denature the protein sufficiently to reduce allergenicity because of the complex structure of

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the number epitopes present in β-conglycinin and glycinin (Wilson, Blaschek & deMejia 2005). Whether heating denaturation could efficiently reduce allergenicity of these two proteins in fish still needs to be clarified. Therefore, the objective of this study was to investigate the effects of purified β-conglycinin and glycinin with heat treatment or not on digestive enzymes activities, intestinal histology and immune responses of juvenile turbot.

Beta-conglycinin and glycinin were isolated according to the procedure of Wu, Murphy, Johnson, Fratzke and Reuber (1999). The purity of the isolated β-conglycinin and glycinin fractions were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970).

Part of the isolated fractions of β-conglycinin (7S) and glycinin (11S) after adjusted to pH 7.0 were heated to 75 and 90°C, respectively, for 3 h in water bath to complete denaturation (Deak, Murphy & Johnson 2007). After that, fractions were cooled at room temperature and then lyophilized.

The basal diet (diet control) was formulated to contain 48% crude protein and 12% crude lipid. The other four diets were supplemented with 60 g kg^{-1} of β-conglycinin, 60 g kg^{-1} of heat treated β-conglycinin, 60 g kg^{-1} of glycinin and 60 g kg^{-1} of heat treated glycinin respectively (Table 1). The five isonitrogeous and isolipidic experimental diets were named as control, 7S, H7S, 11S and H11S respectively. The β-conglycinin or glycinin concentration used in this study was approximately equal to that in 30% soybean meal supplement. The preparation of the test diets followed the previous study of Yun, Ai, Mai, Xu, Qi and Luo (2012).

Juvenile turbot were obtained from fish farm (Jiaonan, Shandong, China) and transferred to an indoor flow-through water system in the National Research Center for Marine Science and Technology (Qingdao, China). Fish were acclimated to the system and fed control diet for 2 weeks. After that, turbot with initial body weight of 6.80 ± 0.02 g (mean ± SEM) were randomly distributed to 5 groups, and each group had 3 replicates. Thirty fish in a 300 l tank was used as a replicate. Fish were fed with the experimental diets to apparent satiation twice daily at 07:00 and 18:00 hours. During the 4-week feeding trial, water temperature was 14–16°C, pH 7.8–8.2 and salinity 28–30‰.

At the end of the feeding trial, fish were randomly chosen and killed by a sharp blow on the head. The ventral belly surface of the fish was opened to expose the abdominal cavity. Samples for enzyme analysis were taken at 12 h postprandial from the following gastrointestinal (GI) sections: central part of the pyloric caeca (PC), mid gut (MG, ½ the distance between the pyloric caeca and the intestinal constriction) and hind gut (HG, from the constriction to the anus). Each section was rinsed with ice-cold distilled water to remove the eventual remaining gut contents. Tissue samples of 5 fish per tank were pooled and frozen in liquid nitrogen. After that, they were stored at −80°C until enzyme assay. For histological analyses, MG and HG from four individual samples per tank were collected and fixed in 10% neutral buffered formalin (4% formaldehyde, pH 7.0) for 24 h, then transferred to 70% ethanol and stored at 4°C until further processing.

Contents of dry matter, crude protein, crude lipid and ash in feed ingredients and the experimental diets were analysed by the standard method (AOAC 1995).

To obtain an adequate crude enzyme extract solution, the samples were thawed and homogenized (1:20) in ice-cold Tris (2 mM)-mannitol (50 mM) buffer (pH 7.1). After centrifugation (9000 *g*, 30 min, 4°C), the supernatants were collected and kept at 4°C for analysis.

Amylase activity was measured by a colorimetric method based on the detection of residual starch after their reaction with iodine solution as described by Pimstone (1964). Incubations were performed at 37°C. The data acquisition system was run at 660 nm with a microplate reader (Model Multiskan spectrum, Thermo, Waltham, MA, USA). One unit (U) of amylase activity was defined as the amount of enzyme that degraded 10 mg substrate (starch) per 30 min. Specific activity was expressed as amylase units/g protein.

Lipase activity was measured by the colorimetric method as described by Winartasaputra, Mallet, Kuan and Guilbault (1980) and Hasan, Shah and Hameed (2009). The test supernatant was incubated substrate buffer solution of triglyceride. Incubations were performed at 37°C. Then, the product of the reaction was immediately measured at 420 nm with an Ultraviolet Visible Spectrophotometer (UV-2401PC, Shimadzu Corp., Kyoto, Japan). One unit (U) of lipase activity was defined as the amount of enzyme that degraded 1 μmol

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Table 1 Ingredients and compositions of the experimental diets (dry-matter basis)

	Experimental diets				
	Control	**7S**	**H7S**	**11S**	**H11S**
Ingredients (g kg^{-1})					
Fish meal*	380	300	300	300	300
Wheat meal	251	261	261	261	261
Fish oil	70	80	80	80	80
Soybean lecithin	20	20	20	20	20
Mineral and vitamin premix†	20	20	20	20	20
Choline chloride	2.5	2.5	2.5	2.5	2.5
$Ca(HPO_4)\cdot 2H_2O$	3	3	3	3	3
Attractant‡	3	3	3	3	3
Ethoxyquin	0.5	0.5	0.5	0.5	0.5
Amino acid mix§	170	170	170	170	170
Cellulose	80	80	80	80	80
Beta-conglycinin (7S)		60			
Heated β-conglycinin (H7S)			60		
Glycinin (11S)				60	
Heated glycinin (H11S)					60
Proximate composition (%)					
Dry matter	95.63	95.12	95.12	95.45	95.11
Crude protein	48.45	48.17	48.12	48.44	48.53
Crude lipid	12.02	12.65	12.33	12.66	12.44
Ash	8.59	8.96	9.01	8.77	8.69

Control, a basal diet; 7S, addition of 60 g kg^{-1} β-conglycinin to the basal diet; H7S, addition of 60 g kg^{-1} heat-treated β-conglycinin to the basal diet; 11S, addition of 60 g kg^{-1} glycinin to the basal diet; H11S, addition of 60 g kg^{-1} heat-treated glycinin to the basal diet.

*Fish meal: steam dried fish meal (COPENCA Group, Lima, Peru).

†Vitamin premix supplied the diet with (mg kg^{-1} diet) the following compounds: retinyl acetate, 32; vitamin D_3, 5; DL-α-tocopherol acetate, 240; vitamin K_3, 10; thiamin, 25; riboflavin (80%), 45; pyridoxine hydrochloride, 20; vitamin B_{12} (1%), 10; Lascorbyl-2-monophosphate-Na (35%), 2000; calcium pantothenate, 60; nicotinic acid, 200; inositol, 800; biotin (2%), 60; folic acid, 20; choline chloride (50%), 2500; cellulose, 2473. Mineral premix consisted of (mg kg^{-1} diet) the following ingredients: $FeSO_4\cdot H_2O$, 80; $ZnSO_4\cdot H_2O$, 50; $CuSO_4\cdot 5H_2O$, 10; $MnSO_4\cdot H_2O$, 45; KI, 60; $CoCl_2\cdot 6H_2O$ (1%), 50; Na_2SeO_3 (1%), 20; $MgSO_4\cdot 7H_2O$, 1200; calcium propionate, 1000; zoelite, 2485.

‡Attractant: glycine and betaine (1:2).

§Amino acid mix (g kg^{-1} diet): aspartic acid 15, threonine 7.5, serine 7.5, L-glutamic acid 19.3, glycine 26, L-alanine 17, cysteine 0.6, L-valine 9, DL-methionine 6.8, L-isoleucine 6, L-leucine 7.3, Tyrosine 7.2, L-phenylalanine 6.4, histidine 7.5, L-lysine 11.8, L-arginine 15.1.

substrate (triglyceride) per minute. Specific activity was expressed as lipase units/g protein.

Maltase activity was analysed using maltose as substrate (Dahlqvist 1970). Incubations were performed at 37°C. The data acquisition system was run at 505 nm with the microplate reader. One unit (U) of maltase activity was defined as the amount of enzyme that degraded 1 mmol substrate (maltose) per minute. Specific activity was expressed as maltase units/mg protein.

Alkaline phosphatase and acid phosphatase activities in tissues were spectrophotometrically determined at 520 nm. Detection kits for Alkaline phosphatase (ALP) and acid phosphatase (ACP) activities were used (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Incubations were performed at 37°C. One unit of ALP activity was defined as the amount of enzyme that reacted with the matrix on alkaline condition and produced 1 mg phenol in 30 min. One unit of ACP activity was defined as the amount of enzyme that reacted with the matrix on acid condition and produced 1 mg phenol in 15 min. Specific activity was expressed as ALP or ACP units/g protein.

Lysozyme activity was measured using a commercial kit (Nanjing Jiancheng Bioengineering

Institute). Incubations were performed at 37°C. It was based on a turbidimetric assay to monitor the degradation of *Micrococcus lysodeikticus* cell. The activity was determined spectrophotometrically at 530 nm. Results were expressed as specific activity in the unit of lysozyme activity units per milligram protein.

Intestinal samples were routinely dehydrated in ethanol, equilibrated in xylene, embedded in paraffin and sectioned (5 μm). Tissues were stained with haematoxylin and eosin (H&E). Mucosal fold height (MFH) or mucosal fold fusion (MFF) was scored using a continuous scale from 1 to 10, representative for the MFH or degree of MFF from low to high. Blinded histological examination was performed using a light microscope.

All data were analysed by the software SPSS 11.0 for windows. Data were subjected to the one-way analysis of variance (ANOVA). Significant differences were indicated by Tukey's test. The level of significance was $P < 0.05$.

Results showed that protein concentrations of the isolated β-conglycinin and glycinin were 90.3 ± 1.5% and 94.1 ± 1.0% (mean ± SEM, $n = 4$) respectively. Figure 1 shows the SDS-PAGE gel loaded with β-conglycinin and glycinin fractions. The purity of β-conglycinin (Fig. 1, lane 2) and glycinin (Fig. 1, lane 3) were 75.87 ± 1.3% and 90.5 ± 2.0% (mean ± SEM, $n = 4$) respectively.

After the feeding trial, the survival of turbot ranged from 98% to 100% ($P > 0.05$). The digestive enzyme activities in PC, MG and HG sections of gastrointestinal tract are presented in Table 2. The amylase activities in PC and MG of fish fed dietary 7S were significantly lower than those in fish fed the other diets ($P < 0.05$). In HG, no significant difference was observed in this enzyme activity ($P > 0.05$). The lipase activity in MG and HG of fish fed dietary 7S was significantly lower than those fed control, 11S and H11S diets ($P < 0.05$). The similar trend was observed in PC. The brush border enzymes (maltase and ALP) also showed the similar changes compared with the above digestive enzymes. In HG, the maltase and ALP activities in 7S treatment were significantly lower than those in the other treatments ($P < 0.05$). In MG, significant reduction was observed in ALP activity of fish fed 7S ($P < 0.05$), but not in maltase activity ($P > 0.05$). As shown in Table 2, fish fed 7S diet had significantly higher ACP activity in HG compared with those fed the other diets ($P < 0.05$). No significant difference in this activity was observed in MG ($P > 0.05$). There was a similar trend in lysozyme activity in HG. Fish fed 7S diet showed significantly higher activity compared with other treatments. No other difference was observed in this enzyme activity among the five treatments ($P > 0.05$). In this study, changed mucosal fold structure was observed in fish fed the 7S diet (Fig. 2). Fish fed 7S diet showed significantly reduced mucosal fold height in HG and increased mucosal fold fusion in MG and HG compared with the other treatments (Fig. 3, $P < 0.05$). No other significant difference was observed among the treatments.

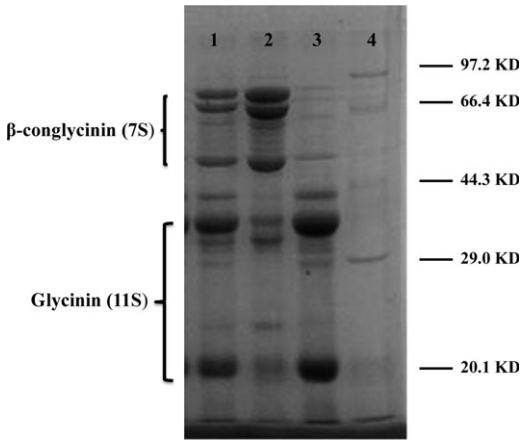

Figure 1 SDS-PAGE patterns of the isolated β-conglycinin and glycinin fractions. Lane 1: total soybean seed protein; lane 2: β-conglycinin fraction; lane 3: glycinin fraction; lane 4: standard.

In this study, dietary inclusion of β-conglycinin disordered intestinal function of turbot, including decreasing digestive enzymes activities, damaging intestinal histology and activating intestinal immune response. The gastrointestinal tract enzyme profile is an indicator of nutrient digestibility and absorption. In this study, activities of amylase, lipase and brush border enzymes (maltase and ALP) were significantly decreased by dietary β-conglycinin. The negative effects of dietary β-conglycinin on digestive and absorptive enzyme activities were also reported in Jian carp (Zhang *et al.* 2013). The explanation for these reductions in enzyme activities can only be speculative as current knowledge about β-conglycinin is weak. However, adverse effects of β-conglycinin on intestinal histology could be one of the reasons for brush border enzymes activities decreasing. As an

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Table 2 Activities of amylase, lipase, maltase, alkaline phosphatase, acid phosphatase and lysozyme in different sections of the gastrointestinal tract of turbot

	Control	7S	H7S	11S	H11S	SEM*	*P*-value
Amylase activity (U g^{-1} pro)							
Pyloric caeca (PC)	96.2^{b}	69.6^{a}	85.4^{b}	86.3^{b}	92.3^{b}	2.9	<0.001
Mid gut (MG)	44.5^{b}	29.3^{a}	41.1^{b}	42.8^{b}	41.6^{b}	2.3	0.006
Hind gut (HG)	29.0	19.3	31.6	25.8	27.3	2.7	0.071
Lipase activity (U g^{-1} pro)							
Pyloric caeca (PC)	24.4	16.4	20.4	24.2	24.6	1.8	0.049
Mid gut (MG)	43.3^{b}	29.2^{a}	38.5^{ab}	44.1^{b}	46.1^{b}	2.8	0.011
Hind gut (HG)	28.8^{b}	16.8^{a}	20.5^{ab}	29.1^{b}	28.5^{b}	2.8	0.036
Maltase activity (U mg^{-1} pro)							
Mid gut (MG)	10.9	10.3	11.5	13.4	11.8	1.0	0.321
Hind gut (HG)	4.7^{b}	2.6^{a}	4.0^{b}	4.7^{b}	5.0^{b}	0.2	<0.001
Alkaline phosphatase (U g^{-1} pro)							
Mid gut (MG)	199^{b}	113^{a}	193^{b}	225^{b}	236^{b}	11	<0.001
Hind gut (HG)	136^{b}	108^{a}	129^{b}	127^{b}	139^{b}	4	0.002
Acid phosphatase (U g^{-1} pro)							
Mid gut (MG)	178	176	178	188	191	5	0.096
Hind gut (HG)	125^{a}	158^{b}	120^{a}	121^{a}	127^{a}	7	0.012
Lysozyme (U mg^{-1} pro)							
Hind gut (HG)	24.0^{a}	33.2^{b}	23.8^{a}	24.6^{a}	21.8^{a}	1.7	0.006

Means in the same line with different superscript letters are significantly different ($P < 0.05$) as determined by Tukey's test.
*Pooled standard error of the mean.

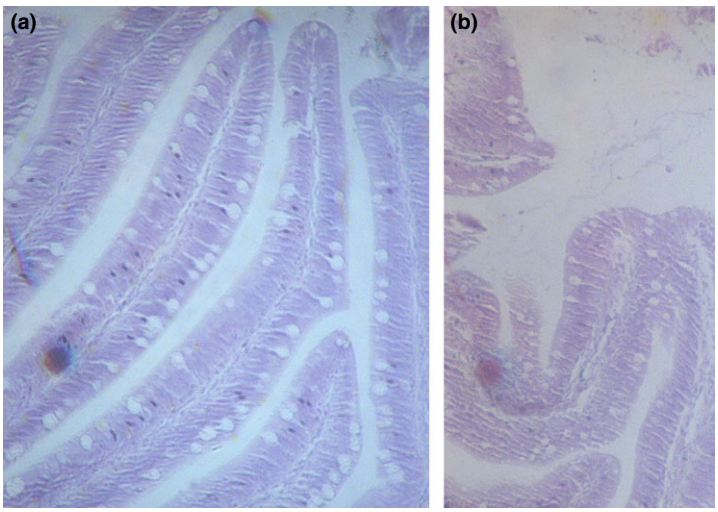

Figure 2 Histological sections of hind gut in turbot (H&E ×200). (a) Apparent normal structure of mucosal fold (in control treatment). (b) Changed structure of mucosal fold, displaying decreased height and increased fusion degree (in 7S treatment).

allergenic antigen contained in soybean meal (SBM), β-conglycinin is reported to cause inflamed intestinal lining and damage the microvilli, therefore reducing the production and secretion of digestive enzymes in piglets and calves (L'Hocine & Boye 2007). Chen, Hao, Piao, Ma, Wu, Qiao, Li and Wang (2010) reported that β-conglycinin directly induces intestinal damage by depressing intestinal epithelial-cell growth, damaging the cytoskeleton, and causing apoptosis in piglet

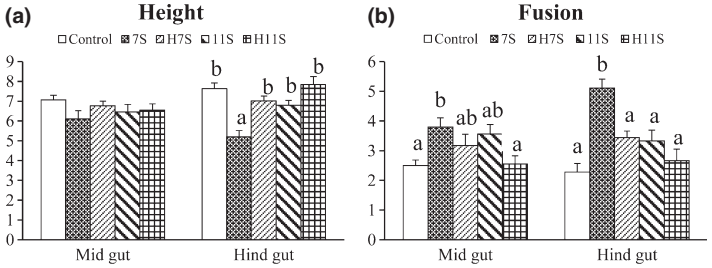

Figure 3 Details of evaluation of intestinal histology of turbot fed the experimental diets. (a) Mucosal fold height. (b) Mucosal fold fusion. Mucosal fold height (MFH) or mucosal fold fusion (MFF) was scored using a continuous scale from 1 to 10, representative for MFH or the degree of MFF from low to high.

intestine. In this study, the intestinal mucosal fold height was significantly shorter, and the degree of mucosal fold fusion was significantly higher in 7S treatment compared with the other treatments. These histomorphological changes induced by β-conglycinin inclusion could be due to its negative effects on intestinal epithelial cells, and thus depress production and secretion of digestive enzymes.

The significantly higher ACP and lysozyme activities in hind gut of turbot suffered from β-conglycinin suggested that macrophage in hind gut may be activated by β-conglycinin. Activity of ACP was an indicator of cell-mediated immunity, and appeared to be a marker for macrophage infiltration in lamina propria and submucosa of the distal intestine as shown in the effects of SBM administration in Atlantic salmon (Bakke-McKellep, Sanden, Danieli, Acierno, Hemre, Maffia & Kroghdahl 2008). Increased activity of lysozyme was also reported in Atlantic salmon distal intestine with inflammation (Bakke-McKellep *et al.* 2008; Urán, Schrama, Rombout, Taverne-Thiele, Obach, Koppe & Verreth 2009). Therefore, the increases of ACP and lysozyme activities in fish fed β-conglycinin indicated that β-conglycinin could cause intestinal immune dysfunction. The negative effects of β-conglycinin on immune function were also reported in rats (Guo, Piao, Ou, Li & Hao 2007).

Previous studies have shown that fish meal can be replaced with soybean meal up to 20% level in diets for black sea turbot without negative effects on growth, nutrient utilization or nitrogen balance (Ergun, Yigit, Turker & Harmantepe 2008; Yigit, Ergün, Türker, Harmantepe & Erteken 2010). However, when the soybean meal inclusion level was up to 35%, significant decrease was observed in growth performance (Yigit *et al.* 2010). In this study, diets included β-conglycinin level corresponding to about 30% soybean meal inclusion level. It showed that the negative effects caused by β-conglycinin should not be ignored when using soybean meal as the alternative to fish meal.

Unlike β-conglycinin, untreated glycinin did not induce the intestinal dysfunction of turbot in the present study. Zhao, Qin, Sun, Zhang, Bao, Wang, Zhang, Zhang, Zhu and Sun (2008) reported that β-conglycinin appeared to be more resistant to digestion than glycinin in piglet because of their different protein structure. In the present study, the different effects of β-conglycinin and glycinin on intestinal function in turbot may be also caused by the different digestibility between these two proteins. Another possible reason could be that turbot is less sensitive to the immunogenicity of glycinin than β-conglycinin. This hypothesis was supported to some extent by the insignificant effects of glycinin on immune indices in the present study (Table 2). However, the present study was conducted for 4 weeks. Whether the glycinin would induce negative effects on the intestinal function of turbot in a longer period still needs to be clarified.

Heat treatment is widely used in food production. It can cause protein denaturation. Heat treated soybean meal could improve the nutritional value of SBM (Peres, Lim & Klesus 2003), the digestibility of nutrients (Haard, Dimes, Arndt & Dong 1996; Arndt, Hardy, Sugiura & Dong 1999), feed efficiency and growth of fish (Balogun & Ologhobo 1989). Wilson *et al.* (2005) attributed the improvement of SBM by heat to the anti-nutrients inactivating. In the present study, dramatic differences were shown in the effects of β-conglycinin on almost all the analysed parameters after heat treatment. Moreover, heat-treated β-conglycinin did not show any negative effect on intestinal

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function. It is known that the allergenicity of globular proteins depends on the tertiary structure of the antigen and on the integrity of the peptide sequence (Demonte *et al.* 1997). Thermal processing has been proved to be able to decrease the allergenicity of globular proteins by altering their structures (Sathe, Teuber & Roux 2005). In the present study, the negative impacts of β-conglycinin on intestinal function in turbot were alleviated after thermal processing. Possibly, this was because high temperature caused structural disorganization of β-conglycinin, which may contribute to the reduction of its immunogenicity. The present study suggested that heat application during the production of solvent-extracted soybean meal and during the extruded feed manufacturing will be helpful to use soybean meal as dietary protein sources. Further researches on effects of β-conglycinin with graded denatured levels on intestinal function of turbot are needed. It is good to get more theoretical basis for SBM using.

In conclusion, β-conglycinin induced disorders of intestinal function in turbot. Moreover, these negative effects could be alleviated by heat processing.

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