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Endo 1-3(4) Beta-glucanasesupplementation of Barley Based Diet and Its Effect on Some Hematological Parameters of Common Carp (*Cyprinus carpio*)

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Abstract: The aim of this study was to evaluate the effects of purified b-glucanase on someblood parameters of common carp (*Cyprinus carpio*) fed on barley based diet. Carbohydrates are cheap energy sources in animal nutrition. These materials are rich in anti-nutrient factors which affect the animal performance. Some main compositions are called non-starch polysaccharides (NSPs) which composed of hexose and pentoses (arabinoxylans and xylans). Beta-glucans are the main NSP in barley and oats. In this study, 90 fish in three treatments (30 fish/treatment) and each treatment has three replicates (each replicate contains 10 fish/ 50L glass aquarium) fed by enzyme treated diets in 3 levels of 0% as control, 0.1% and 0.5% for 8 weeks. Enzyme supplementation at the level of 0.1% increased blood parameters such as blood sugar, cholesterol and triglyceride but decreased total protein and uric acid ($P<0.05$). In term hematological parameters, best performance was evaluated for enzyme treatment at the level of 0.5%. All parameters such as W.B.Cs, R.B.Cs, hemoglobin and hematocrit in this level (0.5%) was higher than other treatments ($P<0.05$). Authors suggest that beta-glucanase addition in aqua-feeds based on barley can be useful for fish health and maybe as immunity stimulator.

Key words: Non-Starch Polysaccharides · Glucose · Cholesterol · Triglycerides · Hematocrit

INTRODUCTION

Common carp (*Cyprinus carpio*) is one of common cultural species in all around the world. Even though there are plenty of documents in carp nutrition but there should be some studies to improve this field. In some cases researchers replaced some diets with others or added some biological agent into diet to achieve better feed conversion ratio (FCR) and better growth either, but some of them succeed [1,2] and some did not [3,4]. Carbohydrates are cheap energy source for animal feed but these materials are composed of some anti-nutritional factors that influence the animal performance. The endosperm cell walls of wheat, barley, rye, oats and so many cereals have these anti-nutritive properties. One of the most important anti-nutrients exists in animal diets are non-starch polysaccharides (NSPs) which divided into two main groups: pentoses(arabinoxylans and xylans) and hexoses. It has been clearly demonstrated that the primary mechanism of the anti-nutritional effects of the soluble NSP activity is related to their viscous properties and hence the presence of soluble beta-glucans in barley is one of the major causes of growth depression and poor feed conversion ratio in monogastric animals [5].

Fishes, like other non-ruminant animals, do not produce digestive enzymes whichdegrade the cell wall; so they cannotstorage NSPs found in various concentrations of the plant materials used for animal feeding [6]. The NSPs and lignin are commonly referred to as dietary fiber (DF), because of its indigestibility in the small intestine and will be an index for the fraction of the plant material potentially available for fermentation in the large intestine. In large intestine a variable fraction of DF will be fermented into short-chain fatty acids and thereby provide energy for the host. In addition DF components can interfere with the digestion and absorption processes in the small intestine and the production of digestive enzymes [7]. The chemical composition of barley varies considerably with variety, growing conditions and year [8] which contains implications for the nutritive value [9].

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A single or elementary plant fiber is a single cell, typically of a length from 1 to 50 mm and about 10-50 mm in diameter. Plant fibers are like microscopic tubes, i.e., cell walls surrounding the central lumen. The lumen contributes to the water uptake behavior of plant fibers. The fiber consists of several cell walls. These cell walls are formed from oriented reinforcing semi-crystalline cellulose microfibrils embedded in a hemicellulose-lignin matrix of varying composition [10]. The main DF components of the cell wall of barley are cellulose, arabinoxylans and mixed-linked beta 1-3(4) D-glucan (beta- glucan) [11]. Beta-glucan is a linear homo-polymer of D-glucose residues which are linked through beta-1,3 glycosidic bonds in the main chain. This polysaccharide, comprising the highest percentage of the fungal cell walls, has a major role in providing the cell wall with rigidity and protection. This is generally achieved through the assistance of other cell wall components, such as chitin and different proteins [12].

As known, NSPsare the most anti-nutritive components that found in the cell wall of cereals. Monogastric animals like pigs, poultry and fishes have no digestive enzyme to reduce these anti-nutrients but ruminants break them by their microbial colonies in their intestine. There are some ways to reduce these materials and improve diet's nutritional value such as pelleting [13], extrusion [14], soaking [15], Gamma irradiation [16] and enzyme treatment, [17]. Enzymes are biological products that catalyze the biochemical reactions involved in celllife. Enzymes are proteins of high molecular weight (between 10,000 and 500,000 Daltons), precipitated by alcohol, acetone and ammonium sulphate. Like all proteins, they are sensitive to the physicochemical environment, variations in which may modify their activity [18]. With enzyme supplementation the effect of varying beta-glucan level is minimal. A series of experiments were conducted to study the effects of enzyme supplementation of diets containing high levels of a local variety of barley on the performance of broiler chickens. There are some differences between resulted data in some cases that would be due to variation in soluble beta-glucan content of different barley cultivars grown under different environmental conditions [19]. The purpose of the present study was to study the effects of enzyme addition to diets based on locally grown barley and fed to common carp (*Cyprinus carpio*) in the form of pellets.

MATERIALS AND METHODS

Fishes and Experimental Conditions: This experiment carried out at a 3×3 factorial as a complete randomized design in the fisheries research center of Gorgan University of Agriculture Sciences andNatural Resource, Gorgan, Iran. Fishes brought from Institute of Aquaculture of the Marjani and transferred to research center and exposed in 2 ppt salt bath before introducing to aquaria.90 individuals of common carp (*Cyprinus carpio*) with average weight of 13.46±0.17 g distributed into 9 glass aquaria groups containing 10 fish per group which divided into 3 subgroups. Aquariafilled up to 50 liter, temperature adjusted to 25±1°C and water aerated as well as possible. Diets prepared and fishes fed by 3.5% of wet body weight twice a day at 8AM and 8PM. The method for feed preparation and enzyme addition are described in the next sections. Initial diet (experimental diet without enzyme) used for first week offishes adaptation to new experimental situation and after that, fishes fed by experimental diets (Table 1) for 8 weeks of trial. No fish died during experiment. Fish were weighed every 2 weeks and theamount of diet fed was adjusted accordingly.

Enzyme Preparation: Beta- glucanase (Endo beta 1-3(4) D-glucanase, EC 3.2.1.6) is in 16th glucanohydrolase family which degrades the carbohydrate polymers into its component residues by breaking the beta-glycosidic bonds [12]. Enzyme added to diets by dissolving in buffer sullotion (Citrate,-Phosphate buffer, pH=4.8). For making a Citrate-Phosphate buffer (pH=4.8), 252 ml of 0.1N citric acid (Merck, Germany) and 248 ml of 0.2N dibasic sodium phosphate were mixed (Merck, Germany) in 1000 ml distilled water according to manual ,[20]. As resulted in some researches, optimum conditions of this enzyme related to pH, temperature and even some other factors such as wetness [21]. 9 gbeta- glucanase (Sigma, USA) dissolved in 20 ml Citrate-Phosphate buffer (pH=4.8) and sprayed the solution on dried food by water sprayer.

Feeding Trial: Feed stuffs assayed for protein and gross energy contentbefore mixing, thus formulated and stabilized by winfeed nutrition software (version 2.8, Cambridge University, UK). Although nutrient requirements of carp are well documented, we preferred to use the most reliable reference [22]. The feed formulation is given in table 1.

Table1: Barley based diet formulation

Feed stuff (%)	Quantity (%)
Barley flour	52
Fish meal	33
Fish oil	12
Vitamin and mineral supplementary*	2
Chromium oxide (Cr_2O_3)	1
Approximate composition of feed	
Dry matter (%)	90.07
Crude protein (%)	31.6
Gross energy (KCal/kg)	3369.12
Ash (%)	4.94
Lipid (%)	3.864
Crude fiber (%)	0.198
Lysine	2.11
Methionine	0.77

* Vitaminet water soluble multivitamin plus trace elements manufactured by DamloranPharma Co. Birjand, Iran.

Ingredients per Each Gram: Vitamin A: 10000 IU; Vitamin D3: 3000 IU; Vitamin E: 3 mg; Vitamin B1: 2 mg; Vitamin B2: 2 mg; Vitamin B6: 1 mg; Vitamin K3: 2 mg; Nicotinamide: 15 mg; Calcium pantothenate: 5 mg; Cu2+: 3 mg; Fe2+: 12 mg; Zn2+: 15 mg; Mn2+: 25 mg.

Blood Sampling and Assay: At the end of 8th week of trial, 9 fish per replicate selected and blood samples gathered from caudal vein by cutting caudal fins and collecting into 2ml tubes containing the anticoagulant K2EDTA (50 µl.ml^{-} blood) for CBC and 2ml tubes without anticoagulant agent employed for biochemical analyses. As in mammalian samples, glucose determinations are most useful when performed on serum separated from cellular elements shortly after sampling. If more than 30 minutes to an hour is required to get whole blood sample back to the laboratory and processed, fluoride anticoagulant needs to be used to slow glycolysis, but also other enzymatic processes in the serum and in test reagents used in automatic serum analyzer [23]. Blood samples were imediately centrifuged at 12850 g and the plasma was seperated [24] All blood samples were assayd by Elan auto-analyser (Ependorf, Germany) for biochemical analyse. Glucose assayed by glucose oxidase method, urea by urease assay, uric acid by Gochman and Schmitz's enzymatic method, cholestrol by cholestrol oxidase method, trygelycerids by gelycerophosphat dehydrogenase enzymatic method and total protein by Biuret method assayd in triplicate and CBC assay performed according to official methods [25].

Analytical Procedure: Data analyzed by SPSS 18 software. All data from experiments were subjected to one way ANOVA and Significant differences among the means were determined by using Duncan's multiple-range test at $p<0.05$.

RESULTS

As shown, enzyme application in diets affected the biochemical (Table 2) and blood hematological parameters (Table 3). It's obvious that enzyme application at the level of 0.1% affected the biochemical characteristics such as glucose, uric acid, triglycerides and total protein significantly ($P<0.05$). Total protein was higher in control group than enzyme levels but the difference between enzyme treatments was not significant ($P>0.05$). Urea had no significant difference. In biochemical parameters uric

Table 2: Blood biochemical characteristics of common carp

	Parameter					
Treatment	Sugar (mg.dl^{-1})	Uric acid (mg.dl^{-})	Cholesterol (mg.dl^{-})	Triglycerides (mg.dl^{-})	Total protein (g.dl^{-})	Urea (mg.dl^{-})
0%	82.33^{c}	0.525^{a}	162^{b}	305^{c}	3.5^{a}	7^{a}
0.1%	100^{a}	0.36^{c}	180^{a}	350^{a}	3.26^{c}	6.76^{a}
0.5%	91.66^{b}	0.4^{b}	169^{b}	338^{b}	3.36^{b}	7^{a}

Means in the same column with superscripts of different letters differ significantly at $p<0.05$

Table 3: Blood hematological characteristics of common carp

	Parameter			
Treatment	WBC (μl⁻)	RBC (mil.μl⁻)	Hemoglobin (g.dl⁻)	Hematocrit (%)
0%	6050[c]	1.39[b]	9.75[b]	29.28[c]
0.1%	6533[b]	1.41[b]	10.1[b]	30.13[b]
0.5%	6900[a]	1.43[a]	10.43[a]	31.3[a]

Means in the same column with superscripts of different letters differ significantly at p<0.05

acid in control group was higher than enzyme treatments and even in urea significantly but not in latest one (P<0.05). Cholesterol showed the same values between 0.5% enzyme treatment and control group but cholesterol level in 0.1% enzyme treatment was higher than other groups (P<0.05). Cholesterol and triglycerides were higher in 0.1% enzyme level (P<0.05) (Table 2).

In hematological parameters, enzyme addition in 0.5% level almost affected all parameters significantly (P<0.05) and then 0.1% level was in second place in balance with control group (Table 3).

DISCUSSION

Enzyme addition in monogastric animals like fishes would be very useful due to improving nutrient digestibility in at least 2 ways: (1) by supplying enzymes that animal cannot produce in sufficient quantity by itself, or (2) animal may produce enzyme itself but this exogenous enzyme would reduce the secretion of endogenous enzyme ,[26]. According to deficiency of studies focused on enzyme applying in fishes, this study is being compared with other monogastric animals like pigs and poultry. There is no difference between these animals except the energy content of diets that is high in terrestrial animals because of keeping blood warm and consequence reactions that need more energy to perform; For example energy requirement for chicks are 5 time than fishes and shrimps, [27]. Results are discussed as below:

Glucose: Glucose is probably the most studied of the non-enzymatic and non-protein components of fish serum. Glucose values tend to increase with increased age in fish, [23]. The inclusion of NSPs in the basic diet of monogastric animals including fish has been reported to delay the intestinal absorption of glucose, [28]. Beta- glucans basically consist of glucose residues joined by beta (1-3) and beta (1-4) linkages. Beta-glucanaseacts on these linkages and glucose will be the final product of these reactions, [29]. According to results, enzyme supplementation at level of 0.1% showed the higher value between other treatments (Table 2). These results were incompatible with, Og□uz and Göncüog□lu [30] who reported that enzyme application did not affect glucose but were compatible by Yuan's [31] results who reported that enzyme inclusion in levels of 180 and 360 mg/kg significantly (P<0.05) increased the amount of blood sugar due to breaking the NSPs to small residues of glucose. Ao *et al*. [32] reported that glucose increased according to Endopower (contain 300 unit/g xylanase activity and 220 unit/g beta-glucanase activity) addition at the level of 0.1% and NSPase (7 unit/g of α-1,6-beta-galactosidase and 22 unit/g of beta-1,4-mannanase) addition at the level of 0.2%. Resulted data from this experiment was compatible with Ao *et al*. [32] too.

Cholesteroland Triglycerides: Cholesterol levels can indicate disorders of lipid and lipoprotein metabolism and liver function which contribute to the decreased cholesterol levels, [33]. Increasing the NSP content in the diet of monogastric animals has been reported to decrease the utilization of lipids. Increase in digesta viscosity caused by intake of an NSP-containing diet has been shown to affect emulsification negatively and to reduce lipolysis [28]. One of the best reasons for high amount of cholesterol is due to high performance of enzyme at the level of 0.1% and improvement of emulsification. Present results was compatible with the studies of Mancini and Parillo [34] and Hajati [26] who reported that enzyme application increased the cholesterol level and was incompatible with Kermanshahi [35], Og□uz and Göncüoglu. [30] who reported that enzyme application did not affect the cholesterol level. Oguz and Göncüoglu [30] reported that enzyme application decreased the triglycerides. Kermanshahi [35,36] reported that enzyme applying itself in laying hens diet couldn't affect the triglyceride but the interaction between enzyme and dried barberryfruit and turmeric rhizome powder affected both hematocrit value and triglyceride significantly. Enzyme addition couldn't affect total cholesterol either.

Uric Acid: In most monogastric animals, we should take care of nucleic acid supplies. For example Hajati [26] reported that enzyme addition in broiler chicks decreased the uric acids due to nutrient digestibility improvement. Uric acid formed by fish from exogenous and endogenous purine nucleotides and by catabolism of protein via purines. It is converted in the liver and to lesser extent in the kidney, to urea for excretion by the gills, [23]. Carbohydrateexcess concentration leads to uric acid production which deplete to urinary system, [37]. As seen in this study enzyme applying in 0.1% level could decrease the uric acid significantly than other treatments ($p<0.05$); the main reason for this phenomenon is due to blood sugar production. Hajati [26] reported that uric acid in young chickens decreased on 22^{th} and 44^{th} of experiment which is not compatible by this study.

Urea : Inclusion of NSPs in monogastric animal feeds lead in amino acid metabolismreduction. As NSPs take part in animal nutrition, the viscosity of digesta increases and proteins digestion occurs by some deficiencies and N secretion happens that reveals as excess in urea secretion, [28]. Most urea in fishes is produced by the liver, but urea passes rapidly though most internal membranes and is consequently found in all fish tissues. It's excreted in small quantity in relation to total nitrogen excretion, primarily by the gills, [23]. Borg *et al.* [38] mentioned that blood urea can reflect the state of protein metabolism and amino acid balance and said that when blood urea is low, the balance of amino acids balance is good, [31]. Even though the blood urea is lowest than others but this difference is not analytically significant ($P>0.05$). Another reason to describe the blood decrease refers to protein sparing role of carbohydrates. When carbohydrate is well utilized, animal body uses the carbohydrate for construction of some parts such as nervous system and prevents to amino acid catabolism. These amino acids will be employed for development of other parts of animal body, [37].

Yuan *et al.* [32] found that enzyme at the levels of 0 mg/kg (control) and 720 mg/kg was so high and in 360 mg/kg was lower values ($p<0.05$). This is because of best feed utilization in 360mg/kg treatment. Ao *et al*. [33] reported that inclusion of multi enzyme (Endopower) containing 300 unit/g xylanase activity and 220 unit/g beta-glucanase activity in 0.1% level increased the amount of blood urea in piglets. Results of this study were compatible with the results of Yuan et al. [32] and were in compatible with Ao *et al.* [33].

Total Protein: Alternate cause of low total proteins include decreased intake through starvation, decreased synthesis due to hepatic dysfunction, increased capillary permeability for plasma proteins, or degradation of protein by proteolytic enzymes released from endothelial cells destroyed by viruses or bacteria , [23]. As mentioned above, carbohydrates play an important role in protein sparing. Decrease in plasma protein probably reflects the accumulation of amino acids and dissolved proteins in animal tissues.

Hematological Parameters: As we know, CBC parameters dependent on age of fish, [23], but we saw in this study that enzyme could affect the hematocrit value due to WBCs and RBCs, hemoglobin, hematocrit values due to metabolism improvement that occurred by enzyme addition. One of most powerful hypothesis about RBCs increase is due to beta- glucan existence. As revealed by Brattgjerd [39] on Atlantic salmon (*Salmo salar*), Selvaraj *et al*. [40] on common carp (*Cyprinus carpio*) and Sung *et al*. [41] on tiger shrimp (,*Penaeus monodon*), beta-glucan (specially beta 1-3 glucan) is an immunity stimulant for animals, [28]. So glucan residues would cause the WBCs and maybe RBCs production in this experiment.

CONCLUSION AND PROPOSAL IDEAS

At the end, authors suggest that beta-glucanase addition in aqua-feed based on barley can be useful for fish health and maybe as immunity stimulator and supplemental tool for drug addiction. Enzyme addition would be more useful in seasons when animal need more energy for mobility or reproduction. There are a few works on enzyme addition for animals but there are fewer studies about fishes. Even study on effects of enzyme addition on reproduction tissues would be interesting.

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