Effect of protease and phytase supplementation on growth performance and nutrient digestibility of rainbow trout (Oncorhynchus mykiss, Walbaum) fed soybean meal-based diets

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ABSTRACT
An experiment was conducted to evaluate the effects of growth, body composition, and nutrient digestibility of addition protease and phytase to soybean meal-based diet in rainbow trout. Seven soybean meal-based diets were prepared by adding protease, phytase, and mixture of both enzymes at two different levels (1 and 2 g kg$^{-1}$) to a base diet without enzymes. Mixed enzymes (1, 2 g kg$^{-1}$) were prepared by adding protease and phytase at the same doses (0.5 + 0.5 and 1 + 1 g kg$^{-1}$). Diets consisted of 31% fish meal and 44% dehulled hexane extracted soybean meal. At the start of the experiment, 45 rainbow trout (initial mean body weight 88 g) were stocked into each of 21 fibre tanks. After 90 days, there were no significant differences on the growth, feed conversion ratio, digestibility of protein and lipid among the groups. Results of this study showed that the addition of protease or phytase as mix to soybean meal-based diet could not increase growth and nutrient digestibility in trout.

1. Introduction
Total aquaculture production in Turkey at 2015 has reached to 240,000 ton. One hundred and eight thousand tons of this production constitutes trout production (Anonymous 2016). Soybean meal is mainly used in rainbow trout feed produced in Turkey (Yildirim 2008; Korkut et al. 2016). However, it contains antinutritional factors such as trypsin inhibitors that reduce the digestibility of protein (Cheng & Hardy 2003). Previous studies indicated that protease is capable of decreasing the effect of trypsin inhibitors (Caine et al. 1998), improving protein digestibility (Drew et al. 2005; Dalsgaard et al. 2012) and feed efficiency (Drew et al. 2005).

The other important antinutrient present in soybean meal is phytate, a phosphorus (P) storage molecule. Phytate can combine with protein as insoluble complexes and can adversely affect digestibility (Liu et al. 1998; Sugiura et al. 2001; Cao et al. 2007). Phytate is largely indigestible by fish because they do not produce phytase enzyme which hydrolyses phytate (Ramseyer et al. 1999). There are studies on the addition of different enzymes to different feedstuff in fish diet. However, there are only a few studies on the addition of protease and phytase individually to soybean meal in fish diet. In addition, there is no study on the addition as a combination protease and phytase to feedstuff in fish.

This study was conducted to examine the effect on growth parameters, feed conversion ratio (FCR), nutrient digestibility and body composition of the addition protease or phytase as individually or in combination at two different levels (1, 2 g kg$^{-1}$) to 44% soybean meal-based diet in rainbow trout.

2. Materials and methods
2.1. Experimental diets and analysis
The basal diet was formulated to contain 40% crude protein and 17.35 MJ kg$^{-1}$ digestible energy. Experimental diets were formulated to fulfil the nutritional requirements of rainbow trout (NRC 2011). Seven diets were formulated by adding protease (RONOZYME ProAct$^{TM}$ 75,000 PROT g$^{-1}$) and phytase (RONOZYME NP$^{TM}$ 10,000 FYT g$^{-1}$) and a mixture of protease and phytase enzymes at two different levels (1, 2 g kg$^{-1}$) to control diets (without enzyme) (Table 1). Protease and phytase enzymes were supplied by DSM Corporation. Phytase (Ronozyme NP; 10,000 units g$^{-1}$) is a commercial enzyme produced by fermentation of Peniaphora lycii. Protease (Ronozyme ProAct, 75,000 units g$^{-1}$) is a commercial enzyme produced by fermentation of B. licheniformis. In the experiment, dehulled, toasted and solvent extracted soybean meal was used. The ingredients used in the experiment were obtained from the local market. All the ingredients were ground into fine powder and sieved through a 320 μm mesh, enzymes at predetermined levels were added to ingredients. All ingredients were mixed in a mixer, then fish oil and 20% distilled water were added, and pellets made with a laboratory pelletizer and dried for about 16 h at 20°C. The particle size of the diets was 5 mm and stored at +4°C until used.

Ingredient compositions and proximate analysis of the diets are given in Table 1. The moisture, crude protein, crude fibre and ash contents of the experimental diets, feces samples and body composition were determined according to standard Association of Official Analytical Chemists (AOAC) methods.
Spyridakis et al. (1989). Apparent digestibility coefficients were calculated by the indirect method of Jodoin et al. (1982). Apparent digestibility coefficients were calculated as follows:

\[
\text{ADC} = 100 - \left(\frac{\text{Cr}_2\text{O}_3 \text{ in diet} (\%)}{\text{Cr}_2\text{O}_3 \text{ in feces} (\%)}\right) \times \left(\frac{\text{nutrient in feces} (\%)}{\text{nutrient in diet} (\%)}\right)
\]

Moisture was analysed by oven drying at 105°C for 24 h. Crude ash was determined using a muffle furnace at 550°C for 24 h. Crude protein was determined by the Kjeldahl method and multiplying by a factor of 6.25. Crude ash was determined using a muffle furnace at 550°C for 24 h. Crude fibre was determined by sample digestion with H₂SO₄ and NaOH. Lipids were extracted from samples using the chloroform:methanol mixture according to Bligh and Dyer (1959). The homogenate was filtered through Whatman No. 1 filter paper and rinsed with chloroform. The filtrate was allowed to stand in a cold room for separation and the lower phase containing lipid was collected. The solvent was evaporated in an evaporator at 40°C. Total lipid content was calculated gravimetrically.

### 2.2. Experimental animals and culture conditions
Rainbow trout were obtained from Egirdir Fisheries Faculty, Turkey. Fish were acclimated to experimental conditions for 2 weeks. At the start of the experiment, 45 rainbow trout (initial mean body weight 88 g) were stocked into each of 21 fibre-glass tanks. Each tank was provided with running spring water. The fish were hand fed twice daily (09:00 and 16:00 h) at a ratio of 2.5% of their body weight. All experiments were done in triplicate. The feeding trial was conducted for 12 weeks. At the end of this period, five fish from each tank were sacrificed and frozen at −20°C for further tests. Water quality parameters such as dissolved oxygen level and temperatures were kept above 7.20 ± 0.5 mg L⁻¹ and 11 ± 1°C, respectively.

### 2.3. Digestibility trial
Apparent digestibility was calculated by the indirect method of Spyridakis et al. (1989). Apparent digestibility coefficients were measured with 0.5% chromic oxide as a marker. Feces of fish were collected during the feeding trial. Fecal material collection was carried out manually by siphoning. After feeding, the floors of tanks were siphoned to clean them. Feces were collected by siphon before other feeding. Feces of each treatment were kept at −20°C and then oven dried at 50°C for 48 h and analysed. Apparent digestibility coefficients were calculated as follows:

\[
\text{ADC} = 100 - \left(\frac{\text{Cr}_2\text{O}_3 \text{ in diet} (\%)}{\text{Cr}_2\text{O}_3 \text{ in feces} (\%)}\right) \times \left(\frac{\text{nutrient in feces} (\%)}{\text{nutrient in diet} (\%)}\right)
\]

Growth, FCR and nutrient digestibility were analysed by one way analysis of variance (ANOVA). All data were calculated by using SPSS computer program (SPSS 2000). The Duncan test was used to determine the differences among treatment means (P = .05).

### 3. Results
Growth performance of rainbow trout fry fed with the different enzyme diets is shown in Table 2. Final weights, weight gain (WG) and specific growth rate (SGR) of rainbow trout fry fed with phytase and protease addition to diet containing 44% soybean meal showed no significant differences with fish fed control diet (P > .05). The supplement of enzymes to diet had no significant effect on FCR of rainbow trout. In addition, protein and lipid digestibility were not improved by supplemented enzymes (P > .05) (Table 3). There were no differences among the groups in the body composition (Table 4) and the survival rate was 100% in all groups.

### 4. Discussion
#### 4.1. Phytase
In the present study, the addition of phytase to diets including soybean meal does not affect growth and FCR in rainbow trout.

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**Table 1. Formulation of experimental diets and proximate analysis (% dry matter).**

| Ingredients (g kg⁻¹) | Control | Phytase (1 g kg⁻¹) | Phytase (2 g kg⁻¹) | Protease (1 g kg⁻¹) | Protease (2 g kg⁻¹) | Phyt + Prot (1 g kg⁻¹) | Phyt + Prot (2 g kg⁻¹) |
|---------------------|---------|------------------|------------------|-------------------|-------------------|----------------------|----------------------|
| Fish meal           | 31.00   | 31.00            | 31.00            | 31.00             | 31.00             | 31.00                | 31.00                |
| Soybean meal        | 44.00   | 44.00            | 44.00            | 44.00             | 44.00             | 44.00                | 44.00                |
| Wheat flour         | 4.00    | 4.00             | 4.00             | 4.00              | 4.00              | 4.00                 | 4.00                 |
| Corn flour          | 8.00    | 8.00             | 8.00             | 8.00              | 8.00              | 8.00                 | 8.00                 |
| Soybean oil         | 11.70   | 11.70            | 11.70            | 11.70             | 11.70             | 11.70                | 11.70                |
| Vit-Min*            | 0.50    | 0.50             | 0.50             | 0.50              | 0.50              | 0.50                 | 0.50                 |
| Cr₂O₃               | 0.50    | 0.50             | 0.50             | 0.50              | 0.50              | 0.50                 | 0.50                 |
| Pellet binder       | 0.30    | 0.30             | 0.30             | 0.30              | 0.30              | 0.30                 | 0.30                 |

*Provided the following per kg diet; 4,000,000 IU vitamin A, vitamin D₃ 480,000 IU, 2400 mg vitamin E, 2400 mg vitamin K₃, 4000 mg vitamin B₁, 6000 mg vitamin B₂, 4000 mg Niacin, 10,000 mg Cal.D. Pantothenate, 4000 vitamin B₆, 10 mg vitamin B₁₂, 100 mg d-Biotin, 1200 mg folic acid, 40,000 mg vitamin C, 60,000 mg inositol, 23,750 mg manganese, 75,000 mg zinc, copper 5000 mg, cobalt 2000 mg, iodine 2750 mg, selenium 100 mg, magnesium 200,000 mg.

#Dig. En. (MJ kg⁻¹)b | 17.35 | 17.35 | 17.35 | 17.35 | 17.35 | 17.35 | 17.35 | 17.35 |

7bDig. En.: digestible energy value was calculated from published values for the diet ingredients (NRC 2011).
Table 2. Growth parameters of rainbow trout fed with protease and phytase-supplemented diets.

| Growth parameters | Initial weight (g) | Final weight (g) | Weight Gain (g) | SGR (% day$^{-1}$) | FCR |
|-------------------|-------------------|-----------------|-----------------|-------------------|-----|
| Control           | 88.00 ± 2.17      | 232.20 ± 2.16   | 144.19 ± 3.99$^b$ | 1.08 ± 0.04$^b$ | 1.62 ± 0.01 |
| Phytase 1 (1 g kg$^{-1}$) | 87.22 ± 1.12 | 236.06 ± 2.54 | 148.84 ± 1.49$^b$ | 1.11 ± 0.00$^b$ | 1.61 ± 0.03 |
| Phytase 2 (2 g kg$^{-1}$) | 87.96 ± 0.56 | 228.76 ± 3.56 | 140.80 ± 3.06$^b$ | 1.06 ± 0.01$^b$ | 1.62 ± 0.06 |
| Protease 1 (1 g kg$^{-1}$) | 89.86 ± 0.24 | 226.87 ± 5.27 | 137.00 ± 5.21$^b$ | 1.03 ± 0.03$^b$ | 1.73 ± 0.07 |
| Protease 2 (2 g kg$^{-1}$) | 87.93 ± 0.42 | 229.87 ± 1.16 | 141.94 ± 1.52$^b$ | 1.07 ± 0.01$^b$ | 1.67 ± 0.03 |
| Phyt + Prot 1 (1 g kg$^{-1}$) | 86.36 ± 2.41 | 224.67 ± 3.26 | 138.31 ± 3.55$^b$ | 1.06 ± 0.03$^b$ | 1.62 ± 0.05 |
| Phyt + Prot 2 (2 g kg$^{-1}$) | 89.53 ± 1.31 | 224.16 ± 5.43 | 134.62 ± 5.56$^b$ | 1.02 ± 0.03$^b$ | 1.70 ± 0.08 |

Notes: Values in the column having the same superscript are not significantly different (P > .05). Weight gain (WG) = (final body weight - initial body weight). Specific growth rate (SGR) (% day$^{-1}$) = [(ln final body weight - ln initial body weight)/days] × 100. Feed conversion ratio (FCR) = feed consumed (g)/weight gain (g).

Table 3. Mean percent apparent dry matter, crude protein and total lipid digestibility of rainbow trout fed with protease and phytase supplemented soybean meal-based diets (%).

| Groups            | Protein | Lipid |
|-------------------|---------|-------|
| Control           | 90.38 ± 0.64 | 89.90 ± 0.41 |
| Phytase 1 (1 g kg$^{-1}$) | 89.87 ± 0.56 | 88.64 ± 0.63 |
| Phytase 2 (2 g kg$^{-1}$) | 90.51 ± 0.26 | 88.05 ± 0.25 |
| Protease 1 (1 g kg$^{-1}$) | 87.80 ± 0.72 | 89.43 ± 0.46 |
| Protease 2 (2 g kg$^{-1}$) | 89.79 ± 0.22 | 89.43 ± 0.46 |
| Phyt + Prot 1 (1 g kg$^{-1}$) | 86.76 ± 0.84 | 89.43 ± 0.46 |
| Phyt + Prot 2 (2 g kg$^{-1}$) | 89.42 ± 0.31 | 88.43 ± 0.46 |

Table 4. Body composition of rainbow trout fed with protease and phytase supplemented soybean meal-based diets (%).

| Groups            | Dry matter | Crude protein | Total lipid | Crude ash |
|-------------------|------------|---------------|-------------|-----------|
| Control           | 66.42 ± 3.73 | 20.71 ± 1.35 | 4.59 ± 0.31 | 2.00 ± 0.20 |
| Phytase 1 (1 g kg$^{-1}$) | 69.55 ± 2.23 | 19.48 ± 0.28 | 5.66 ± 0.07 | 1.77 ± 0.15 |
| Phytase 2 (2 g kg$^{-1}$) | 68.55 ± 3.86 | 19.81 ± 0.66 | 4.11 ± 0.27 | 1.90 ± 0.10 |
| Protease 1 (1 g kg$^{-1}$) | 69.03 ± 2.18 | 20.18 ± 0.53 | 5.32 ± 0.38 | 1.87 ± 0.12 |
| Protease 2 (2 g kg$^{-1}$) | 67.91 ± 3.84 | 19.76 ± 0.69 | 5.84 ± 0.71 | 1.70 ± 0.21 |
| Phyt + Prot 1 (1 g kg$^{-1}$) | 60.75 ± 2.82 | 19.98 ± 1.40 | 4.88 ± 0.50 | 1.95 ± 0.05 |
| Phyt + Prot 2 (2 g kg$^{-1}$) | 63.91 ± 3.83 | 20.74 ± 3.41 | 4.97 ± 0.43 | 1.80 ± 0.20 |

(P > .05). Similarly, Ayhan et al. (2008) reported that the addition of phytase to diet containing soybean meal in seabream had no effect on the growth parameter. Sajjadi and Carter (2004) also stated that phytase supplement to canola meal observed no effect on growth and feed efficiency of Atlantic salmon. Forster et al. (1999) informed that the addition of phytase to diet containing canola protein concentrate in rainbow trout showed no differences on growth parameters. In contrast, Ng and Chong (2002) showed that enzyme supplementing to tilapia diet containing 40% palm kern meal significantly improved growth and feed efficiency. But, this beneficial effect was not observed in fish-fed enzyme supplement to diets at the 20% inclusion level.

In this study, protein and dry matter digestibility of rainbow trout fed with phytase enzyme supplemented to soybean meal-based diets showed no significant difference among the treatment groups (P > .05). Similarly, Sajjadi and Carter, (2004), Forster et al. (1999), Teskeredzic et al. (1995) in salmonids and Papatryphon et al. (1999) in striped bass reported that supplementation of phytase enzyme to the diet did not show an increase in protein digestibility. In contrast, Vielma et al. (2004) and Sugiuia et al. (2001) showed increased protein digestibility on phytase addition to salmonid diet. The effects of phytase enzyme in a different study may depend on a variety of dietary factors such as the concentration of phytate in the diet, concentration and sources of protein in the diet and sources of enzymes. Enzymes may originate from different microorganisms. For maximal enzymatic activity, enzymes need different temperatures and pH levels.

4.2. Protease

In the present study, the addition of protease to diets in trout had no significant effect on growth, FCR, protein and dry matter digestibility. There are a few studies about the effects on the growth parameters of protease supplementation to soybean meal-based diet in trout. Similarly with our study, Dalsgaard et al. (2012) found that there were no differences in growth parameters and FCR with the addition of protease to soybean meal in rainbow trout diet. But, apparent digestibility of protein moderately improved by supplementing protease to soybean meal. Ayhan et al. (2008) also reported that the addition of protease to soybean meal diets in seabream had no effect on growth and protein digestibility. Drew et al. (2005) showed that the use of protease to Flex:Pea product in trout had no effect on nutrient digestibility. However, in the same study, protease was shown to increase nutrient digestibility of Canola:Pea product.

4.3. Mix enzyme (phytase + protease)

In the present study, the use of a combination of protease and phytase enzyme in basal diet did not show positive effect on the growth parameters, FCR and nutrient digestibility in rainbow trout. Ogunkoya et al. (2006) who also used an enzyme cocktail (Superzyme CS: xylanase, amylase, cellulase, protease and β-glucanase) in diet containing soybean meal for rainbow trout did not find a noticeable effect on growth performance.

As a result of this study, the addition of protease or phytase as individual or a combination to 44% soybean meal was not effective on growth, nutrient digestibility and body composition in rainbow trout.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was supported by the Türkiye Bilimsel ve Teknolojik Araştırma Kurumu (TUBITAK, Scientific and Technical Research Council of Turkey) [project no. 1100414].
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