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RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

Extraction of protein from fresh rainbow trout (*Onchorhynchus mykiss*) viscera and smoked trout trimmings using commercial enzymes

Ticari enzimler kullanılarak taze alabalık (*Onchorhynchus mykiss)* iç organlarından ve tütsülenmiş alabalık kırpıntılarından protein ekstraksiyonu

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Abstract: In the current investigation, fresh trout viscera and smoked trout trimmings were enzymatically extracted using papain, alcalase, protamex, and flavourzyme. Protein extraction was performed at different concentrations (0.5%, 1%, 1.5% and 2%) and times (30 minute, 1 hour and 4 hours). The moisture, crude protein, lipid and crude ash contents of trout viscera used as raw materials, in the study, were respectively found as 60.26±0.78%, 12.18±0.21%, 31.18±0.36% and 1.33±0.07%, while these values in smoked trout trimmings were determined as 54.53±0.93%, 18.39±0.13%, 17.71 ± 1.06% and 8.50±0.13%, respectively. Following the conclusion of the study, protein content (g protein/100 g waste) and Protein Recovery Rate (PRR, %) in liquid protein hydrolysate extracted from trout viscera and smoked trout trimmings were found to be significantly affected by enzyme type, enzyme concentration, and extraction time. The results showed that the flavourzyme, followed by the protamex, produced the highest protein content (g protein/100 g waste) and PRR (%) in the liquid protein hydrolysate extracted from trout viscera. Furthermore, the protamex, followed by the flavourzyme, was shown to have the highest protein content (g protein /100 g waste) and PRR (%) in the liquid protein hydrolysate extracted from smoked trout trimmings.

Keywords: Trout, viscera, protein extraction, smoked trimmings, commercial enzymes

Öz: Bu çalışmada, alabalık iç organları ve tütsülenmiş alabalık kırpıntıları papain, alkalaz, protameks ve flavourzyme kullanılarak enzimatik olarak ekstrakte edilmiştir. Protein ekstraksiyonu, farklı konsantrasyonlarda (%0,5, %1, %1,5 ve %2) ve sürede (30 dakika, 1 saat ve 4 saat) gerçekleştirilmiştir. Çalışmada, hammadde olarak kullanılan alabalık iç organlarının nem, ham protein, lipid ve ham kül içerikleri sırasıyla %60,26±0,78, %12,18±0,21, %31,18±0,36 ve %1,33±0,07 olarak bulunurken, bu değerler tütsülenmiş alabalık kırpıntılarında sırasıyla %54,53±0,93, %18,39±0,13, %17,71±1,06 ve %8,50±0,13 olarak belirlenmiştir. Çalışmanın sonuçlanmasının ardından, alabalık iç organlarından ve tütsülenmiş alabalık kırpıntılarından ekstrakte edilen sıvı protein hidrolizatlarının protein içeriği (g protein / 100 g atık) ve Protein Geri Kazanım Oranlarının (PRR, %), enzim tipi, enzim konsantrasyonu ve ekstraksiyon süresinden önemli ölçüde etkilendiği bulunmuştur. Sonuçlar, alabalık iç organlarından ekstarkte edilen sıvı protein hidrolizatlarda en yüksek protein içeriği (g protein/100 g atık) ve PRR' nin (%) flavourzyme ile elde edildiğini bunu protameksin takip ettiğini göstermiştir. Ayrıca, tütsülenmiş alabalık kırpıntılarından ekstakte edilen sıvı protein hidrolizatlarında, protameks, ardından flavourzyme ile en yüksek protein içeriğine (g protein / 100 g atık) ve PRR'ye (%) sahip olduğu gösterilmiştir.

Anahtar kelimeler: Alabalık, iç organlar, protein ekstraksiyonu, tütsülenmiş kırıntı, ticari enzimler

INTRODUCTION

Rainbow trout **(***Onchorhynchus mykiss***)**, which is originated in North America and is the most commonly grown variety of trout in our country, is marketed fresh in our nation as well as smoked and exported abroad. According to TEPGE (Republıc of Turkey Mınıstry of Agrıculture and Forestry Agrıcultural Economic and Policy Development Institute), the production of rainbow trout in Turkey is estimated to reach 113,678 tons (TEBGE, 2020). The majority of the trout produced in our country are shipped fresh or smoked in processing factories. In industries that process smoking of trout have two types of waste which are produced. These include visceral waste that happens during the cleaning of the fish prior to the smoked process, as well as smoked flesh trimmings, which consists of head, skin, bone, and flesh parts, and is created during the processing of smoked fillets after they have been smoked. The heads (15.3% of total weight), bones (6.9% of total weight), tails (2.3% of total weight) and intestines (8.8% of total weight) of the trout were removed from the fish before it was smoked as a fillet. Thus, around 33% of the body of the fish was considered waste to be discarded in the procedure (Kotzamanis et al., 2001). Smoked trout trimmings are also used to make lower-value items such as fish meal and fish feed after they have been smoked. Its portion is around 3–5 % depending on numerous processing

conditions such as the size of the fish and the kind of smoker (Tolasa et al., 2012). Most of the fish wastes in Turkey are used to make fish meal with low biological and economic value. The remainder is dumped into the environment as a pollutant without being assessed. These sources, on the other hand, may be transformed into protein products that are nutritionally valuable, functional, easily digested, and have a high economic value. Studies conducted that have previously been performed on fish waste have revealed a large amount of protein. Zamora-Sillero et al. (2018) states that the protein content in fish waste might be 10-20% (w/w) of the total protein in fish.

Adding enzymes to 'waste' material from fish, along with other procedures like filtering and centrifugation, was discovered many years ago to be an efficient technique to separate and recover the proteins contained in the waste (Kristinsson, 2007). The proteins in fish processing waste can be separated from other compounds to which they are associated with hydrolysis by using proteases. Several different authors have studied and described the enzymatic proteolysis and solubilization of proteins from a variety of different fish materials (Aspmo et al., 2005; Vieira et al., 1995). Pigott and Tucker (1990) define fish protein hydrolysate as a liquid product prepared from fish with the addition of proteolytic enzymes to accelerate hydrolysis under controlled conditions, resulting in a protein mixture. Proteins extracted from fish muscle by using enzyme have been found to contain a variety of bioactive peptides with nutritional and functional properties (Benjakul and Morrisey, 1997; Theodore et al., 2008). Furthermore, a wide range of bioactivities, including antihypertensive, antithrombotic, immune-modulatory, and antioxidant characteristics, have been found in peptides generated from the enzymatic breakdown of proteins (Dong et al., 2005; Fitzgerald et al., 2005; Ghaly et al., 2013; Liaset and Espe, 2008; Underland et al., 2009).

Researchers have shown that by applying regulated enzymatic hydrolysis, a wide variety of high-quality protein components may be generated from low-value waste materials (Quaglia et al., 1987; Aspmo et al., 2005). The enzyme utilized in hydrolysis has a significant impact on the content and properties of the extracted proteins, as well as the amino acid sequence of the peptides generated. The functional characteristics of the generated extracted proteins, which are referred to as hydrolysate, are significantly influenced by protease species and protein substrate. Temperature, hydrolysis time, and enzyme concentration all have an impact on the speed and specificity of protein hydrolysate production. The quality of the recovered protein is most strongly influenced by the duration of the hydrolysis process. All parameters that can influence the structure of the product such as pH, hydrolysis duration, enzyme-substrate level and temperature, can influence enzymatic hydrolysis (Utomo et al., 2014; Ananey-Obiri et al., 2019).

There has been a lot of studies on the use of commercial proteases to convert fish processing waste and inadequate or low-value fish into protein hydrolysates (Quaglia and Orban, 1987; Uhlig, H., 1998; Wu et al., 2015). The choice of enzyme is critical in the extraction of proteins from fish byproducts and waste (Ramakrishnan et al., 2013). Alcalase, -chymotrypsin, Neutrate, papain, pepsin, trypsin, pancreatin, flavourzyme, bromelain, pronase E, protamex, orientase, thermolysin, validase, protease amano and protease N are some of the most often used proteolytic enzymes. Among the commercial enzymes employed as proteolytic agents, effective investigations have been achieved by using plant proteases such as papain, bromelain, and ficin, as well as bacterial prostheses such as alcalase, neutrate, protease N, and protamex (Ananey-Obiri et al., 2019).

Using various proteases, successful investigations have been carried out on extracting fish protein hydrolysate from fish internal organs, which are discarded by fish processing companies (Batista et al., 2010; Chalamaiah et al., 2012; Siddik et al., 2021). Many enzymes are employed in the commercial production of fish protein hydrolysate. The amount of protein obtained from these enzymes has been determined on the waste profile and the surrounding circumstances. In this investigation, trout viscera and smoked trout trimmings were extracted at varied rates (0.5%, 1%, 1.5%, and 2%) and times (30 minutes, 1 hour, and 4 hours) by utilizing commercial enzymes (protamex, flavourzyme, alcalase and papain), and extracted protein content (g protein/100 g waste) and Protein Recovery Rate (PRR, %) were measured in liquid protein hydrolysate.

MATERIAL AND METHODS

Material

Fresh trout viscera and smoked trout trimmings were received from the processing factory of of Kılıç Holding Bafa Su Ürünleri A.Ş., which processes fresh and smoked trout in Maraş, Turkey. The enzymes needed for protein hydrolysate production, including alcalase, protameks, and flavourzyme, were provided by Sigma-Aldrich, and papain enzymes, which were supplied by Novozymes A/S (Bagsvaerd, Denmark).

Methods

Enzymatic extraction of protein

The enzymatic extraction of protein from trout viscera and smoked trout trimmings was produced by the enzymatic method according to Ramakrishnan et al. (2013) and He et al. (2013) with slight modifications. The wastes were vacuum-packed into 5-kilogram packages, placed in ice-filled foam boxes, and transported to the Protein Research Laboratory at the Faculty of Fisheries, Department of Fishing and Processing Technologies, where they were held at -18°C until protein hydrolysate was produced. After being thawed at room temperature, frozen wastes were minced by using a meat grinder. The wastes were then heated for 20 minutes at 90°C to assure that endogenous enzymes were inactivated (Nasri et al., 2013). Each sample of minced viscera and smoked trout trimmings, whose enzymes were inactivated,

was cooled and homogenized by adding distilled water at a ratio of 1:1. The optimal hydrolysis temperatures, pH values, inactivation times, and temperatures of four different enzymes (papain, alcalase, protamex, and flavourzyme) used in the production of fish protein hydrolysate (FPH) were conducted in accordance with the manufacturer's recommendations. With the use of 2 N NaOH, the pH values at which these enzymes displayed optimal activity were adjusted to 8.0 for alkaline protease, 7.0 for protamex and flavourzyme, and 7.0 for papain. The hydrolysis temperature was set in accordance with the manufacturer's recommendations, with alcalase at 55°C, flavourzyme and protamex at 50°C, and papain at 40°C being selected as the best temperatures for each enzyme. During the hydrolysis stage, papain, alcalase, protamex, and flavourzyme were evaluated at three different hydrolysis times of 30 minutes, 1 hour, and 4 hours, as well as four different enzyme concentrations of 0.5%, 1%, 1.5%, and 2%. The inactivation time and temperature of each enzyme were applied according to the recommendation of the company from which the enzymes were obtanied, in order to terminate the hydrolysis in the samples that were applied for a certain time, temperature and enzyme-substrate concentration. Accordingly, it was applied for alcalase at 85°C for 10 minutes, for flavourzyme at 90°C for 5 minutes, for protamex at 85°C for 10 minutes and for papain for 30 minutes at 70°C and then 15 min cooled. The cooled extracted solution was then centrifuged at 3600 rpm for 20 minutes to separate into phases.

Figure 1 shows the flow chart to the production of extracted fish protein hydrolysate. The formation of four different phases was achieved in all as an oil phase in the top phase, a light oil phase in the second phase, a protein phase in the third phase, and an insoluble material phase in the fourth phase. The protein content in the hydrolyzing solution were collected from the liquid proteins in step 3 for the production of hydrolyzed fish protein extract (Figure 2).

Figure 1. The production of hydrolyzed fish protein extract flow chart

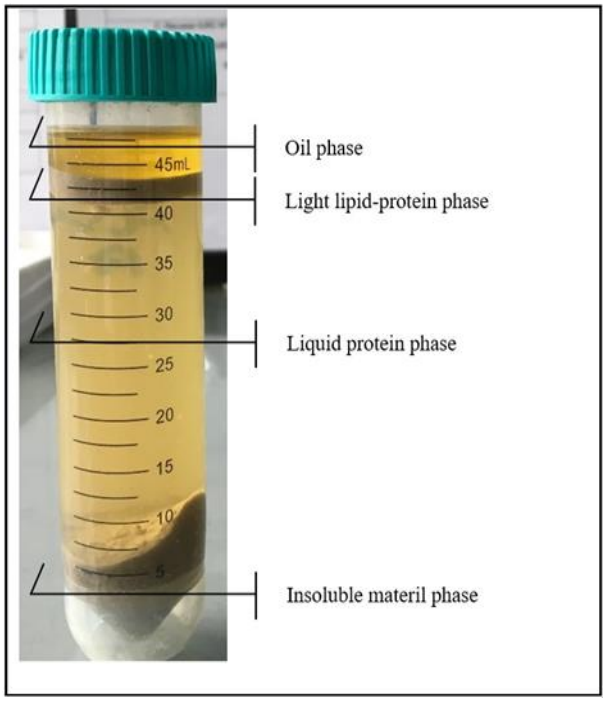

Figure 2. Fractions of soluble hydrolyzed fish protein extract derived from trout viscera and smoked trout trimmings

Chemical analysis

The proximate composition of the trout viscera and the smoked trout trimmings were determined in triplicate by using the following methods: lipid content by Bligh and Dyer (1959), moisture content by AOAC (1998), total crude protein by Kjeldahl technique (AOAC 981.10, 1998) and ash content by AOAC (1998). The quantity of protein in the extracted solution samples was measured by using the Lowry method (1951), using bovine serum albumin as a standard protein. Absorbance was measured at 660nm in a UV/vis spectrophotometer. Protein Recovery Rate (%) estimated by Ovissipour et al. (2009) using the formula below:

$$\text{Protein Recovery Rate (\%)} = \frac{\text{[the content of protein present in the hydrolysate]}}{\text{[the initial content of protein present in the extracted mixture]}} \times 100$$

Statistical analysis

Using the SPSS (SPSS 16.0 Inc. Chicago, IL) package program, one-way analysis of variance (ANOVA) and General Linear Model (GLM) was used in data obtained in the study, and Duncan multi-way analysis of variance was used to assess the differences between the means at the 0.05 significant level.

RESULTS AND DISCUSSION

Proximate composition of trout viscera and smoked trout trimmings

Crude protein, lipid, crude ash and moisture contents of trout viscera and smoked trout trimmings used in the production of protein hydrolysates are given in Figure 3.

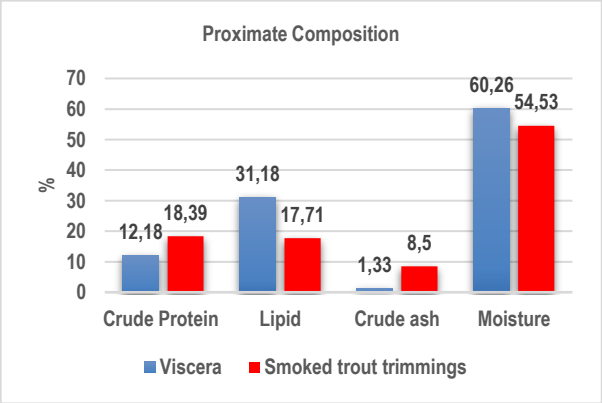

Figure 3. The Proximate composition of trout viscera and smoked trout trimmings

In the proximate composition analysis, trout viscera included 60.26±0.78% moisture, 12.18±0.21% crude protein, 31.18±0.36% lipid, and 1.33±0.07% ash, while smoked trout trimmings contained 54.53±0.93% moisture, 18.39±0.13% crude protein, 17.71±1.06% lipid, and 8.50±0.28% crude ash.

Taheri et al. (2013) observed that trout viscera had moisture content of 71.65%, fat content of 13%, protein content of 15% and ash content of 2.73%. Dong et al. (1993) found that minced salmon viscera contained 78.7% moisture, 12.1% protein, 18.1% lipid and 7.1% crude ash. Kotzamanis et al. (2001) determined the average crude protein, fat content, ash content and moisture of trout waste (head, tail, bone, and intestines) to be 14.5%, 11.1%, 3.3%, and 70.1%, respectively. In a previous study, Tokur (2007) reported that the moisture, crude protein, lipid, and crude ash content of smoked trout were 61.14%, 26.53%, 6.4%, and 1.71%, respectively, which was in contrast to our findings. In the study, written by Tosun and Özden (2014), the researchers found that the protein, fat, moisture, and ash contents of hot-smoked rainbow trout were 22.06%, 7.02%, 66.70%, and 3.50%, respectively. Those findings were in contrast to the data provided in this study, which included fish viscera and smoked trout trimmings. The proximate composition of fish as well as viscera varies according to the fish species, sex, age, nutritional status, season and health (Villamil et al., 2017; Korkmaz and Tokur, 2019). Lipid and protein content of cultured fish have been observed to depend mostly on the activity of fish muscle and feed (Thammapat et al., 2010). The current research findings, when compared to previous study findings for proximate compositions, are predicted to be dependent on feeding and moisture content of used materials (Kotzamanis et al., 2001; Kołakowska et al., 2006). After the

smoking process, previous studies showed that the proximate composition of the fish might alter based on a variety of parameters such as the brine concentration and time applied, the smoked temperature and duration, and the kind of fish (Bjørnevik et al., 2018; Fuentes et al., 2010; Jittinandana et al., 2002; Tosun and Özden, 2014). This might explain why differences in proximate composition were observed.

Papain

Table 1 shows the protein content (g protein/100 g waste) and Protein Recovery Rates (%) in the liquid protein hydrolysate extracted from trout viscera and smoked trout trimmings using four different rates of papain during three different time periods.

Table 1. Protein content (g protein/100 g waste) and Protein Recovery Rates (PRR %) in liquid protein hydrolysate derived from trout viscera and smoked trout trimmings using papain[1,2]

		Enzyme concentrations (%)							
	Extraction Time	0.5	1	1.5	2	0.5	1	1.5	2
		Viscera				Smoked trout trimmings			
Protein Content	30 min.	5.93^{a1} (0.01)	6.23^{a4} (0.01)	6.18^{a3} (0.01)	6.12^{a2} (0.02)	5.59^{a1} (0.01)	5.60^{a1} (0.01)	5.85^{a2} (0.01)	5.92^{a3} (0.01)
	1 h	6.15^{b1} (0.01)	6.33^{b4} (0.01)	6.31^{b3} (0.01)	6.18^{b2} (0.01)	5.87^{b1} (0.01)	6.33^{b2} (0.01)	6.36^{c3} (0.01)	6.40^{c4} (0.01)
	4 h	6.13^{b1} (0.01)	6.37^{c3} (0.01)	6.39^{c4} (0.01)	6.29^{c2} (0.01)	6.01^{c2} (0.01)	6.24^{c4} (0.01)	5.99^{b1} (0.01)	6.14^{b3} (0.00)
PRR (%)	30 min.	48.71^{a1} (0.06)	51.12^{a4} (0.08)	50.70^{a3} (0.04)	50.23^{a2} (0.14)	30.41^{a1} (0.06)	30.47^{a1} (0.03)	31.80^{a2} (0.06)	32.18^{a3} (0.06)
	1 h	50.46^{c1} (0.07)	51.98^{b4} (0.10)	51.81^{b3} (0.09)	50.76^{b2} (0.06)	31.92^{b1} (0.03)	34.42^{c2} (0.08)	34.56^{c3} (0.06)	34.81^{c4} (0.08)
	4 h	50.34^{b1} (0.06)	52.28^{c3} (0.11)	52.44^{c4} (0.12)	51.64^{c2} (0.11)	32.65^{c2} (0.05)	33.94^{b4} (0.04)	32.58^{b1} (0.07)	33.38^{b3} (0.02)

[1]Parentheses indicate the standard deviation

[2] Different letters in the same column and numbers in the same row indicate differences at a significance level of 0.05 ($p<0.05$)

In the extracted of trout viscera with papain enzyme, there was a particularly notable increase in the content of protein with respect to extraction time in all papain concentrations studied, except for 0.5% which shown an increase after 1 hour of extraction and no significant differences between the time intervals ($p<0.05$) of 1 hour and 4 hour extraction. The highest protein content after 30 minutes, 1 hour and 4 hours of extraction were determined to be 1% enzyme concentration with 6.23g protein/100g waste, 1% enzyme rate with 6.33g protein/100g waste and 1.5% enzyme rate with 6.39g protein/100g waste, respectively ($p<0.05$). As result of 4 hour extraction, the PRR (%) of trout viscera treated with 0.5%, 1%, 1.5% and 2% papain contcentrations significantly increased from 48.71% to 50.34%, from 51.12% to 52.28%, from 50.70% to 52.44% and from 50.23% to 51.64%, respectively ($p<0.05$). Under consideration of all extraction periods and papain concentrations, it was shown that 1 and 4 hour of extraction, as well as 1% and 1.5% papain concentrations, resulted in increased protein content and PRR (%) in liquid protein hydrolysate extracted from trout viscera ($p<0.05$).

After extracted of smoked trout trimmings for 30 minutes, 1 hour, and 4 hours, the highest protein content was found in samples extracted with 2% papain concentration with 5.92g of waste, 2% papain concentration with 6.40g of waste, and 1% papain concentration with 6.24g of waste, respectively ($p<0.05$) (Table 1). When the enzyme concentration was raised from 0.5% to 2%, the PPR (%) for liquid protein hydrolysate from smoked trout trimmings increased from 30.41% to 32.18% after 30 minutes of extraction, from 31.91% to 34.81% after 1 hour of extraction, and from 32.65% to 33.38% after 4 hours of extraction ($p<0.05$).Using smoked trout trimmings treated with papain, the results revealed that all extraction periods had a statistically significant impact on protein content and PRR (%), with 1 hour having the highest protein content and PRR (%) achievable with the use of 1%, 1.5%, and 2% ($p<0.05$).

Papain (EC 3.4.22.2), a plant cysteine protease endolytic enzyme, is obtained by cutting the skin of an unripe papaya (*Carica papaya* L.) and then collecting and drying the latex that flows from the cut (Mombaya, 2012; Hoyle and Merritt, 1994; Shahidi et al., 1995). According to Uhlig (1998), papain has wide proteolytic activity against proteins, short chain peptides, amino acid esters, and amide linkages and has been commonly used in the food and pharmaceutical industries. Utoma et al. (2014) used papain enzyme to hydrolyze catfish fillet wastes for 6, 12, 24, 36, and 48 hours and found that the content of protein in the liquid protein hydrolysate increased significantly with time. Similarly, it was found in this study that as the extraction time increased, so did the content of protein in the liquid hydrolysate solution extracted from viscera and smoked trout trimmings. Fan et al (2018) discovered that the hydrolysis duration in the hydrolysis of trout bone proteins caused an increase in the hydrolysate grade, which was similar to the values found in this study. Adler-Nissen (1986) stated that the enzyme

substrate ratio has an important influence on peptide bonding of the protein substrate as well as on the pH and temperature. According to Noman et al. (2018), the enzyme/substrate ratio over 3% (w/w) in the hydrolysis of Chinese sturgeon (*Acipenser sinensis*) by using papain had no effect on the degree of hydrolysis. They noted that their findings were most likely due to enzyme aggregation, which causes an increase in substrate diffusion inhibition, resulting in reaction rate saturation. Similar findings from this study were also disclosed in that, when the impact of the ratio was studied on the protein contents of liquid protein hydrolysate derived from trout viscera, it was shown that higher papain concentration greater than 1.5 % resulted in reduced protein contents in all periods. This impact, on the other hand, was not observed in smoked trout trimmings. This finding indicates that not only the type of enzyme, enzyme concentrations, and duration of the hydrolysis, but also the type of waste used, have a significant impact on protein content and recovery during enzymatic extraction.

Alcalase

Table 2 shows the protein content (g protein/100 g waste) and Protein Recovery Rates (%) in the liquid protein hydrolysate extracted from trout viscera and smoked trout trimmings using four different rates of alcalase during three different extration periods.

Table 2. Protein content (g protein/100 g waste) and Protein Recovery Rates (%) in the liquid protein hydrolysate from viscera and smoked trout trimmings utilizing alcalase[1,2]

		Enzyme concentrations (%)							
	Extraction Time	**0.5**	**1**	**1.5**	**2**	**0.5**	**1**	**1.5**	**2**
		Viscera				**Smoked trout trimmings**			
Protein Content	**30 min.**	6.22^{b2} (0.02)	6.21^{b2} (0.02)	6.15^{a1} (0.02)	6.47^{b3} (0.01)	5.95^{b1} (0.01)	6.13^{a2} (0.02)	6.09^{a2} (0.06)	6.11^{b2} (0.07)
	1 h	6.14^{a1} (0.01)	6.15^{a1} (0.02)	6.14^{a1} (0.02)	6.18^{a1} (0.06)	6.16^{c2} (0.01)	6.20^{b2} (0.06)	6.25^{c3} (0.01)	5.92^{a1} (0.04)
	4 h	6.14^{a1} (0.07)	6.15^{a12} (0.01)	6.15^{a12} (0.01)	6.16^{a2} (0.01)	5.38^{a1} (0.07)	6.15^{a2} (0.01)	6.19^{b2} (0.01)	6.19^{c2} (0.00)
PRR (%)	**30 min.**	51.10^{b2} (0.13)	50.97^{b2} (0.17)	50.46^{a1} (0.13)	53.13^{b3} (0.11)	32.37^{b1} (0.07)	33.31^{a2} (0.13)	33.11^{a2} (0.32)	33.24^{b2} (0.38)
	1 h	50.40^{a1} (0.06)	50.53^{a1} (0.08)	50.39^{a1} (0.05)	50.73^{a1} (0.50)	33.49^{c2} (0.04)	34.01^{c3} (0.46)	34.95^{c4} (0.10)	32.17^{a1} (0.22)
	4 h	50.39^{a1} (0.05)	50.51^{a2} (0.09)	50.48^{a12} (0.05)	50.56^{a2} (0.09)	$29.^{24a1}$ (0.36)	33.46^{b3} (0.05)	34.02^{b4} (0.06)	33.65^{c2} (0.02)

[1]Parentheses indicate the standard deviation

[2] Different letters in the same column and numbers in the same row indicate differences at a significance level of 0.05 ($p < 0.05$)

The highest protein content in liquid protein hydrolysate from trout viscera was obtained after 30 minutes of extraction at all alcase concentrations, and it then significantly decreased ($p<0.05$), except for samples treated with 1.5 % alcalase, which had no significant influence on the protein content throughout the extraction period ($p>0.05$). By increasing the alcalase concentration from 0.5 to 2%, the PRR/%) of liquid protein hydrolyzate generated from trout viscera increased after 30 minutes and 4 hours of extraction. However, no significant change in PRR (%) was seen after 1 hour of extraction at all concentarions. In the extraction of trout viscera with alcalase, the results revealed that the highest PRR (%) and protein content were obtained after 30 minutes of extraction and at 2% alcalase concentration ($p<0.05$).

The highest protein content and PRR (%) in protein liquid protein hydrolysate derived from the smoked trout trimmings treated with the alcalase was found after 1 hour extraction and 1.5% alcalase concentration. Findings from the current study reveal that alcalase extraction of smoking trout trimmings had a significant impact on protein content and PRR (%) depending on extraction time and alcalase concentrations ($p<0.05$). (Table 2).

Many researchers have found Alcalase® 2.4L, an alkaline enzyme produced from *Bacillus licheniformis* and developed by Novozymes for the detergent industry, to be one of the most effective enzymes for solubilizing proteins among the numerous proteases tested (Diniz and Martin, 1997; Aspmo et al., 2005). Because of its high degree of hydrolysis (DH), which can be attained in a relatively short time compared to moderately neutral or acidic enzymes, Alcalase® 2.4L (*Bacillus licheniformis*) is frequently preferred for fish extraction (Lee, 2007).

Protein Recovery Rate (%) refers to the percentage of total proteins which are soluble in the raw material, as well as the percentage of protein from the extracted materials in the protein phase and nitrogen recovery reflects the yield of proteins that can be recovered during the extraction process (Benjakul and Morrisey,1997). Benjakul and Morrisey (1997) revealed that enzyme concentration, reaction time, and waste/buffer ratio all have a significant effect on extraction and nitrogen recovery (NR) in protein hydrolysates produced from pacific whiting solid wastes using alcalase. The same researchers found that the enzyme concentration between 0 and 34 AU/kg significantly increased nitrogen recovery (NR), but when the enzyme concentration was higher (57 AU/kg),

there was no significant effect on NR. In this study, it was revealed that, in addition to extraction duration and enzyme concentrations, the materials used had a significant impact on the content of protein and PRR (%) after alcalase extraction. Shahidi et al. (1995) used alcalase, neutralase, and papain to extract protein hydrolysate from capelin (*Mallotus villosus*). The extraction solution was also subjected to autolytic hydrolysis. The results showed that the protein recoveries using commercial enzymes achieved 22,9%, 51,6% and 70% compared with the efficiency of autolytic hydrolysis. Similarly, they also observed that while considerable soluble protein was generated at the beginning of the hydrolysate, adding more enzymes throughout the stationary phase of hydrolysis had little influence on the dissolution of the hydrolysate. The presence of a high concentration of soluble peptide in the reaction mixes appears to limit the rate of hydrolysis. Ovissipour et al. (2009) produced protein hydrolysate from the viscera of Persian sturgeon (*Acipenser persicus*) using commercially available Alcalase, and they observed that protein recovery ranged from 34.97% to 61.96% depending on the hydrolysis progressed (30-205 min) protein source. It was reported by them that enzyme absorption onto insoluble protein particles is rapid, cleaving the polypeptide chains that are only weakly linked to the surface of the particles. The more compacted the core proteins are, the longer it takes for them to be hydrolyzed to be broken down (Klomklao and Benjakul, 2017).

Protamex

Protein content (g protein/100 g waste) and Protein Recovery Rates (%) in liquid protein hydrolysate extracted from viscera and smoked trout trimmings using protamex is shown in Table 3.

Table 3. Protein content (g protein/100 g waste) and Protein Recovery Rates (%) in liquid protein hydrolysate exctracted from viscera and smoked trout trimming using protamex [1,2]

		Enzyme concentrations (%)							
	Extraction Time	0.5	1	1.5	2	0.5	1	1.5	2
		Viscera				Smoked trout trimmings			
Protein Content	30 min.	6.51^{a1} (0.01)	6.54^{a2} (0.02)	6.62^{a3} (0.02)	6.70^{b4} (0.02)	6.54^{a1} (0.03)	6.69^{b3} (0.03)	6.72^{b3} (0.02)	6.58^{a2} (0.02)
	1 h	6.65^{b1} (0.02)	6.75^{c2} (0.01)	6.76^{c2} (0.01)	6.74^{b2} (0.01)	6.61^{b2} (0.04)	6.24^{a1} (0.34)	6.72^{b2} (0.01)	6.67^{b2} (0.02)
	4 h	6.65^{b12} (0.07)	6.62^{b12} (0.02)	6.70^{b2} (0.02)	6.37^{a1} (0.35)	6.75^{c2} (0.06)	6.65^{b1} (0.05)	6.64^{a1} (0.01	6.64^{ab1} 0.07)
PRR (%)	30 min.	53.47^{a1} (0.11)	53.69^{a2} (0.13)	54.32^{a3} (0.15)	55.04^{b4} (0.15)	35.57^{a1} (0.15)	36.38^{b3} (0.15)	36.56^{b3} (0.10)	35.79^{a2} (0.11)
	1 h	54.62^{b1} (0.13)	55.39^{c23} (0.08)	55.49^{c3} (0.11)	55.31^{b2} (0.10)	35.94^{a2} (0.20)	33.91^{a1} (1.87)	36.55^{b2} (0.06)	36.25^{b2} (0.12)
	4 h	54.61^{b12} (0.54)	54.31^{b12} (0.15)	55.03^{b2} (0.20)	52.34^{a1} (2.87)	36.72^{b2} (0.35)	36.19^{b1} (0.29)	36.08^{a1} (0.08)	36.12^{ab1} (0.39)

[1]Parentheses indicate the standard deviation
[2] Different letters in the same column and numbers in the same row indicate differences at a significance level of 0.05 ($p < 0.05$)

The content of protein and PRR (%) in the liquid protein hydrolysate of trout viscera extracted with protamex for 30 min. increased significantly as the enzyme concentration increased ($p<0.05$) and the highest protein content and PRR (%) were found in samples treated with 2% protamex ($p<0.05$). However, after 1 hour of extraction, the same effectiveness was not observed, with this increase seen only in increasing enzyme concentration from 0.5% to 1% ($p<0.05$). Furthermore, the enzyme concentration did not have a significant effect on the increase of protein content and PRR (%) after 4 hours extraction.

Protein content and PRR (%) in liquid protein hydrolysate produced from smoked trout trimmings treated with protamex for 30 minutes was highest at 1% and 1.5% enzyme concentrations; however, after the 4 hour extraction, the highest protein content was found at 0.5 % enzyme concentration. The increase in enzyme concentration in 1 and 4 hour extraction did not substantially contribute to the increase in protein content and PRR (%).

Protamex is a protease complex for *Bacillus* designed for food protein degradation. It has been demonstrated to exhibit non-bitter protein hydrolysates, unlike other endoproteases (Lee, 2007). Soufi-Kechaou et al. (2012) investigated the effect of extraction time on protein recovery rate in hydrolysates produced from cuttlefish (*Sepia officinalis*) viscera. Researchers have found that the rate of soluble nitrogen increases during hydrolysis. They noted that this was an indication that the proteins were solubilized under the influence of commercial enzymes used during hydrolysis and move from the substrate to the soluble phase. In the study, the total content of nitrogen in the soluble fraction increased rapidly in the first two hours and then the content of protein in the substrate decreased while reaching a stationary phase for the remainder of the hydrolysis reaction. The researchers obtained the maximum yield for protamex after approximately 120 minutes of extraction reaction. In this study, the highest content of protein was obtained in 1 hour extraction of visceral waste and 4 hour extraction for smoked trout trimmings. This shows that the material used in protein recovery is effective.

Molla and Hovannisyan (2011) used protamex to optimize the enzymatic hydrolysis of beluga (*Huso huso*) visceral waste proteins. They observed that increasing the temperature, time, and enzyme activity in the hydrolysis of beluga protein resulted in an increase in hydrolysis rate up to a certain point, but thereafter hydrolysis rate considerably decreases. They indicated that a decrease in hydrolysis rate with increasing enzyme activity levels, temperatures, and time might be attributed to a decrease in enzyme activity.

Flavourzyme

Table 4 shows the protein content (g protein/100 g waste) and Protein Recovery Rates (%) in the liquid protein hydrolysate extracted from trout viscera and smoked trout trimmings using four different rates of flavourzyme during three different time periods.

Increasing the enzyme concentration at all times in the extraction of trout viscera with the flavourzyme did not have a significant effect on the increase in the content of protein and PRR (%) in the liquid protein hydrolysate (Table 1). However, considering all extraction time for all enzyme concentarions, extraction times of 30 minutes and 4 hours resulted in the highest protein content and PRR (%) ($p<0.05$).

In the smoked trout trimmings, the highest protein content and PRR (%) in the liquid protein hydrolysate was recovered at 0.5% enzyme concentrations for 30 minutes, followed by 2% enzyme concentration for 4-hour extraction ($p<0.05$).

Table 4. Protein content (g protein/100 g waste) and Protein Recovery Rates (%) in the liquid protein hydrolysate extracted from viscera and smoked trout trimmings utilizing flavourzyme [1,2]

		Enzyme concentrations (%)							
	Extraction Time	0.5	1	1.5	2	0.5	1	1.5	2
		Viscera				Smoked trout trimmings			
Protein Content	30 min.	7.02^{b12} (0.00)	7.03^{c2} (0.02)	7.00^{b12} (0.01)	6.99^{c1} (0.01)	7.01^{c4} (0.01)	6.69^{b2} (0.02)	6.64^{b1} (0.03)	6.88^{b3} (0.02)
	1 h	6.99^{b3} (0.02)	6.51^{a1} (0.01)	6.94^{a2} (0.02)	6.97^{b3} (0.01)	6.00^{a1} (0.00)	5.99^{a1} (0.02)	6.00^{a1} (0.01)	6.03^{a2} (0.01)
	4 h	6.94^{a2} (0.02)	6.98^{b1} (0.01)	6.98^{ab2} (0.05)	6.90^{a2} (0.02)	6.05^{b1} (0.03)	6.31^{a1} (0.39)	6.90^{c2} (0.04)	6.96^{c2} (0.01)
PRR (%)	30 min.	57.62^{c23} (0.10)	57.68^{c3} (0.14)	57.45^{b12} (0.11)	57.43^{c1} (0.10)	38.10^{c4} (0.06)	36.39^{b2} (0.10)	36.12^{b1} (0.18)	37.41^{b3} (0.11)
	1 h	57.40^{a3} (0.19)	53.44^{a1} (0.09)	56.97^{a2} (0.17)	57.21^{b3} (0.11)	32.60^{a1} (0.03)	32.60^{a1} (0.10)	32.65^{a1} (0.05)	32.77^{a2} (0.05)
	4 h	57.33^{b2} (0.27)	57.33^{b2} (0.11)	57.32^{ab2} (0.39)	56.62^{a1} (0.11)	32.89^{b1} (0.18)	34.29^{a1} (2.14)	37.54^{c2} (0.20)	37.84^{c2} (0.06)

[1]Parentheses indicate the standard deviation

[2] Different letters in the same column and numbers in the same row indicate differences at a significance level of 0.05 ($p < 0.05$)

Flavourzyme is generated by a strain of *Aspergillus oryzae* and also is composed of a number of enzymes, including endoproteases and exopeptidases, each with varied activity and optimal pH values. Exopeptidase activities cause the removal of terminal amino acids that can cause bitter taste (Lee, 2007). It has been noted that hydrolysis of proteases such as flavourzyme, which can degrade bitter peptides (from Novozymes), contributes to eliminating the problem of bitter hydrolysates (Guerard, 2007). In the study of Nemati et al. (2012) with wastes belonging to shad *(Alosa caspia*) species, the protein recoveries of using flavourzyme throughout 1 hour increased significantly with time and their protein recovery obtained by flavourzyme during 60 minutes was 47.66%. Although it was shown in this investigation that visceral and smoked trout trimmings did not produce a significant increase in time on protein recovery in hydrolysates obtained with flavourzyme, protein recovery was assessed to be greater in trout viscera than that observed by Nemati et al (2012). Their also found that major peptide cleavage happened within the first 15 minutes of hydrolysis. Mohr (1980) stated that the proteins in the sarcoplasmic fraction may denature and precipitate during heating to the hydrolysis temperature, and the denatured proteins would be highly resistant to enzymatic degradation. Moreover, it was noted that there was no statistically significant difference found between the yield of proteins following enzymatic hydrolysis and hydrophobic interactions between peptides or self-assembly of larger peptides, meaning that precipitation would likely occur, reducing the yield of proteins (Mutilangi et al., 1996).

The flavourzyme, followed by protamex, produced the highest protein content and PRR (%) in liquid protein hydrolysate extracted from trout viscera when all times and rates were taken into consideration ($p<0.05$). Protamex, on the other hand, was found to be the enzyme responsible for the highest protein content and PRR (%) in smoked trout trimmings, followed by flavourzyme ($p<0.05$). Additionally, it was demonstrated that the effects of papain and alcalases were not comparable ($p>0.05$).

CONCLUSION

Based on the findings of the investigation, it was found that the content of and the protein recovery rate protein in the liquid protein hydrolysate were significantly affected by the type of waste, the amount of enzyme utilized, and the extraction time.

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