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INVESTIGATING THE EFFECTS OF SALT, PHOSPHATE AND PH ON FUNCTIONAL PROPERTIES OF BEEF MYOFIBRILLAR PROTEINS

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ABSTRACT

This study aimed to investigate the effect of salt and phosphate on the functional properties of myofibrillar proteins (MPs) at pH 2, 4, 6, 8, and 10. The highest emulsion activity index (EAI) value (8.51 m^2/g protein) was obtained with the use of NaCl, and phosphate at pH 10. The highest emulsion stability index (ESI) result was determined (230.8 minutes) with the use of salt at pH 8. The addition of salt and phosphate significantly ($p<0.05$) improved the emulsifying properties of proteins. Considering the water absorption capacity (WAC), the highest value was obtained as 1.9 mL water/g protein at pH 6 and pH 8. The highest fat absorption capacity (FAC) value of 8.3 mL fat/g protein was found with the addition of phosphate at pH 6. The highest foam capacity (FC) and foam stability (FS) were obtained at pH 10 and 4, respectively.
Keywords: Myofibrillar proteins, functional properties, emulsion, foam

DANA ETİ MİYOFİBRİLER PROTEİNLERİNİN FONKSİYONEL ÖZELLİKLERİ ÜZERİNE TUZ, FOSFAT ve PH'NIN ETKİLERİNİN ARAŞTIRILMASI

ÖZ

Bu çalışmada, tuzun ve fosfatın miyofibriler proteinlerin fonksiyonel özellikleri üzerindeki etkisinin pH 2, 4, 6, 8, 10 değerlerinde araştırılması amaçlanmıştır. Emülsiyon aktivite indeksi (EAI), emülsiyon stabilite indeksi (ESI), köpük kapasitesi (FC), köpük stabilitesi (FS), su absorpsiyon kapasitesi (WAC) ve yağ tutma kapasitesi (FAC) incelenmiştir. pH 10'da tuz ve fosfat kullanımıyla, en yüksek EAI değeri (8,51 m^2/g protein) elde edilmiştir. pH 8'de, tuz kullanımıyla en yüksek ESI sonucu (230,8 dakika) belirlenmiş; tuz ve fosfat ilavesinin, proteinlerin emülsifiye edici özelliklerini önemli ölçüde iyileştirdiği ($p<0.05$) tespit edilmiştir. Fosfat ilavesiyle, pH 6 ve pH 8'de en yüksek WAC değeri (1,9

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mL su/g protein) elde edilmiştir. En yüksek FAC değeri (8.3 mL yağ/g protein) pH 6'da fosfat ilavesiyle elde edilmiştir. Köpürme özelliklerine bakıldığında, en yüksek FC değeri pH 10'da (%90) elde edilmiştir. Fosfat ve tuz kombinasyonunda, pH 4'te, en yüksek FS sonucu %100 olarak belirlenmiştir.
Anahtar kelimeler: Miyofibriler proteinler, fonksiyonel özellikler, emülsiyon, köpük

INTRODUCTION

Meat, which has high-quality proteins and is an ideal protein source, possesses a fundamental role as an essential food product for human consumption. Meat protein encompasses a great deal of essential amino acid residues and has a high digestibility (Pereira and Vicente, 2013). Although approximately 40% of the total protein consumption of humans is explained by animal protein sources, it is estimated that there will be a significant increase until 2050. The total meat consumption was predicted to increase as much as 102% between 2000 and 2050 (FAO, 2006).

The functional properties of proteins play a major role in the increase in the need for meat protein. In particular, the need for meat proteins is increasing in order to reduce the cost of meat products, improve the quality of existing products and produce different products (Jayasooriya et al., 2004). Myofibrillar proteins (MPs) such as myosin and actin have a significant role in terms of functional properties in meat. They are the biggest portion of proteins from muscle tissue and are partly soluble relative to stroma and sarcoplasmic proteins (Ramírez-Suárez and Xiong, 2003). Also, among muscle proteins, MPs are responsible for 97% of the water holding capacity and 75% of the emulsifying capacity of meat (Chang et al., 2012; Forrest et al., 2001; Zhang and Barbut, 2005). Thus, MPs have a significant influence on meat quality (Smith, 1988).

Functional characteristics of meat proteins include all physicochemical features that influence the behaviour of the proteins during meat products production process (Colmenero and Borderias, 1983). These properties, which also include solubility, viscosity, and gelation are needed to guarantee satisfying shape, texture, mouthfeel, and good cutting properties (Kinsella and Phillips, 1989). These characteristics are influenced by intrinsic factors such as molecular structure and composition, and extrinsic factors including temperature, pH, and non-meat ingredients (e.g. phosphate and salt) (Damodaran, 1996; Santhi et al., 2017).

There have been some studies about the factors affecting the functional characteristics of MPs from different sources. Accordingly, Hong et al. (2014) showed that the pH-shifting process and microbial transglutaminase together resulted in the highest gel strength of porcine MPs whereas the use of microbial transglutaminase could improve their emulsion stability (Hong et al., 2014). Also, the long-term exposure of dielectric barrier discharge plasma to MPs from the longissimus dorsi muscle significantly improved their water-holding capacity (Sharifian et al., 2019). In addition, the water holding capacity and gel strength of beef MPs were improved by increasing pH, which was enhanced by ultrasound duration and power (Amiri et al., 2018). Omana et al. (2010) showed that foam characteristics of MPs from chicken thigh meat are substantially enhanced using alkaline pH extraction (Omana et al., 2010). However, there has been no study to determine the combined effects of pH, salt, and phosphate on isolated MPs from beef *in vitro* so far. In this study, MPs were isolated from beef and were used for the investigation of the effects of salt and phosphate at various pH on their functional characteristics including water absorption capacity, fat absorption capacity, emulsifying properties, and foaming properties.

MATERIALS AND METHODS

Materials

In this study, minced beef meat from beef brisket was obtained from a local business in Tekirdağ, stored at -20°C, and then used as a source of MPs. Different amounts of salt and phosphate were used to investigate the functional properties of MPs.

Methods

Protein isolation

Proteins were isolated as previously described method by Malva et al. (2018) with some modifications. Briefly, 150 mL of ice-cold dH_2O and 10 g of ground beef were transferred to the blender, homogenized for 1 min, and transferred into 15 mL tubes. Then, they were centrifuged at 2500 rpm for 10 min. The pellets were transferred into the blender, using ice-cold 150 mL of 0.6 M KCl. After homogenization for 1 min, the mixture was transferred into 15 mL tubes and centrifuged at 2500 rpm for 10 min. Then, the supernatants were collected and passed through 0.45 µm filters (Millex filter). Soluble extract including MPs was dried at 30°C. Then, the dried powders were washed three times, each of which was followed by centrifugation. The powder form of purified proteins was obtained upon incubation at 30°C for 2 h. 10% SDS-PAGE gel electrophoresis was also performed to qualitatively evaluate the proteins after isolation (Laemmli, 1970).

Optimization of quantities of sodium chloride and phosphate

Optimization of quantities of sodium chloride and phosphate was performed using a range of 0.01-0.05 g of sodium chloride and 0.004-0.012 g of phosphate for 0.1 g of MPs in the concept of experimental research design. These ranges of sodium chloride and phosphate quantities were determined based on the calculations of their quantities used in the production of meat products. In these calculations, some assumptions were used. The assumptions used in this study are given as follows: Practically, in sausage production, 60 kg lean meat is used for 100 kg sausage dough. Since beef meat contains 20% protein, the amount of meat protein in this recipe is 12 kg. Approximately 50% of meat proteins are myofibrillar proteins and the amount of them in the recipe is about 6 kg. In 100 kg sausage production, 2 kg NaCl and 0.5 kg phosphate are used as additives. It can be assumed that this amount of additives interact with 6 kg MPs. For convenience in experimental studies, the average amount of additives used for each 0.1 g of MP was calculated. The results showed that 0.03 g of NaCl and 0.008 g of phosphate are used for each 0.1 g of MP. These values were accepted as average values and the range of sodium chloride (0.01-0.05 g) and phosphate (0.004-0.012 g) was used for the optimization of their quantities based on the functional properties of the proteins. Five different levels were used for NaCl (0.01, 0.02, 0.03, 0.04 and 0.05 g) and phosphate (0.004, 0.006, 0.008, 0.010 and 0.012 g) in preliminary experiments and only the best combination was used in further studies.

The investigation of the influence of salt, phosphate, and pH on the functional properties of MPs

Water absorption capacity (WAC) and fat absorption capacity (FAC)

The analyses of WAC and FAC were performed as the previously described method by Segura-Campos et al. (2013) and Lili et al. (2015) with some modifications. For this purpose, 0.1 g (W) sample and 5 mL of distilled water for WAC or corn oil for FAC were weighed and transferred into 15 mL centrifuge tubes. They were adjusted to pH 2, 4, 6, 8, and 10, using 0.1 M HCl or 0.1 M NaOH, and vortexed at room temperature for 1 min. These mixtures were incubated at 30°C for 30 min. The initial volume of the mixtures was recorded as V1. The tubes were centrifuged at 2500 RPM for 20 min and the supernatant volumes were recorded as V2. Equation 1 was used for the calculation of WAC and FAC. The results were given as sample g per mL water and as sample g per mL fat.

$$WAC/_{FAC} = \frac{V1-V2}{W} \quad (1)$$

Emulsifying properties

The emulsion activity index (EAI) and emulsion stability index (ESI) of the samples were determined as the previously described method by Kudre et al. (2018) with some modification. Corn oil (4 mL) and 1% myofibrillar protein solution (10 mL) were homogenized with a homogenizer (T25 Ultra-Turrax, IKA-Werke GmbH, Staufen, Germany) for 2 minutes. 1 mL emulsion was diluted by 99 mL of 0.1% SDS. The samples were adjusted to pH 2, 4, 6, 8, and 10. The mixtures were vortexed for 20 s and then the initial absorption (A0) of the mixtures and absorption of mixtures at minute 10 (A10) were

measured at 500 nm by spectrophotometer (Optizen POP UVVis, Mecasys Co. Ltd., Daejeon, Kore). Those values were used for the calculation of EAI and ESI:

$$EAI\ \left(m^2/g\right) = \frac{(2\times2{,}303\times A\times DF)}{(l\times\emptyset\times C)} \quad (2)$$

$$ESI\ (\text{min}) = \frac{A0}{(A0-A10)} \times \Delta t \quad (3)$$

where DF is the dilution factor of the emulsion (100), l is the length of the spectrophotometer cuvette (m), Φ is a fraction of oil volume, and C is the protein concentration in the aqueous phase (g/m^3) and Δt is 10 minutes.

Foaming properties

Foaming capacity (FC) and foaming stability (FS) were determined as the previously described method by Segura-Campos et al. (2013) and Kudre et al. (2018) with some modifications. 20 mL solution containing 1% sample and distilled water was mixed at 10000 rpm for 2 minutes by the homogenizer. The pH value of the solutions was adjusted to pH 2, 4, 6, 8, and 10 and the analysis was performed for every pH value. FC was calculated using foaming volume in 30 s. FS values were calculated using foaming volumes at 30 min. Equations 4 and 5 were used for calculation:

$$FC\ (\%) = \frac{Vf}{V0} \times 100 \quad (4)$$

$$FS\ (\%) = \frac{Vt}{V0} \times 100 \quad (5)$$

Statistical analysis

All analyses were performed in duplicate, and the mean values were presented. Analysis of variance (One-way-ANOVA) was applied to determine whether there was a significant difference among the means. The significance of the differences among the means was tested at the $p<0.05$ level. Significant sources of variation were subjected to the Duncan test, which is a multiple comparison test. Statistical analyses were performed using SPSS 22.0 (SPSS Inc., IL, USA).

RESULTS AND DISCUSSION

Isolation of MPs

Proteins were isolated from minced beef meat and qualitatively checked by 10% SDS-PAGE gel electrophoresis analysis. The results confirmed that soluble extract and the powder form of the final product including MPs with several protein bands distributed in a range of 25-250 kDa (Figure 1).

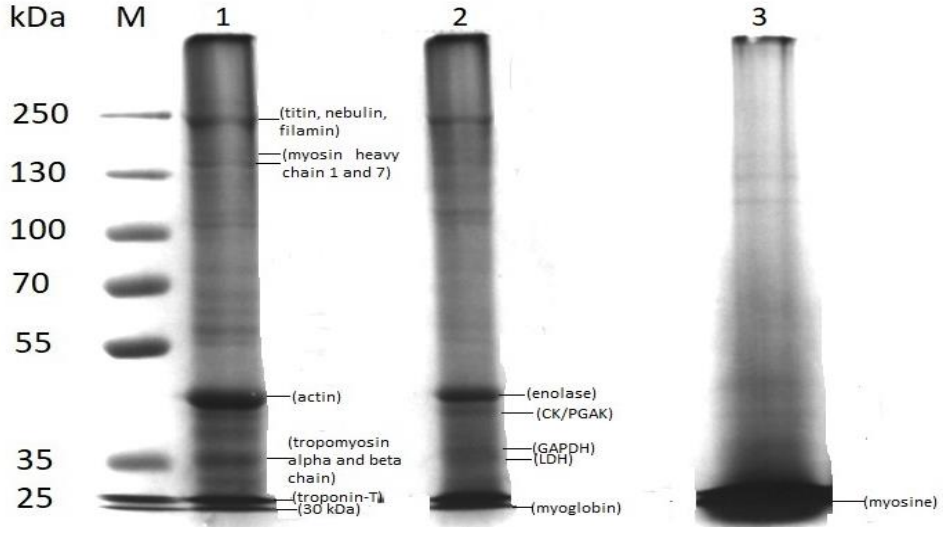

Figure 1. SDS-PAGE display of the purified MPs (1), sarcoplasmic (2) and myosine (3) proteins. M indicates protein marker bands. Abbreviations are shown in brackets; (PGAK: phosphoglycerate kinase, CK: creatine kinase, GAPDH: glyceraldehyde phosphate dehydrogenase, LDH: lactate dehydrogenase).

Influence of salt, phosphate, and pH on WAC and FAC of MPs

Water Absorption Capacity (WAC) Results

WAC is explained as the capability of fresh meat to hold its water (Pearce et al., 2011). Poor WAC may influence the processed meat yield and quality (Savage et al., 1990). It is considered that water is held by about 85% through largely actin and myosin filaments of MPs in meat (Huff-Lonergan and Lonergan, 2005; Zeng et al., 2017). In this study, the WAC of beef MPs was determined using different quantities of sodium chloride and/or phosphate. The highest WAC value was determined in 0.1 g MP-0.04 g sodium chloride-0.004 g phosphate combination (data not shown). WAC of 0.1 g MP was then analyzed by the addition of 0.04 g sodium chloride and/or 0.004 g phosphate at different pH points (pH 2, 4, 6, 8, and 10), as well as the control sample using water. WAC analysis results showed that MPs with the addition of phosphate and/or salt generally had a higher WAC value than the control sample (0.8 g mL water/g protein) (Figure 2). Many studies have reported that salt and phosphate increase water absorption of meat products by adjusting their ionic strength (Choi et al., 2014; Cheng and Sun, 2008; Xiong et al., 2000; Whiting, 1984; Puolanne et al., 2001) and it has been shown that ionic strength influences the swelling degree of the MPs (Wilding et al., 1986). Besides, WAC analysis results revealed that the addition of phosphate or phosphate-salt caused the highest WAC value at relatively higher pH points (pH 6 - 10) compared to other tested pH points, and also gradually increased the WAC value of MPs towards alkaline pH conditions. It is clear that phosphate resulted in high WAC at basic pH conditions (Figure 2). Phosphate has a basic character and its addition to meat results in an increase in pH, which causes improvement in water absorption of beef and porcine muscle MPs as shown in some reports (Amiri et al., 2018; Bertram et al., 2004). Also, phosphate forms some complexes with magnesium- and calcium-bound protein, causing the improved solubilization of actin and myosin of MPs (Fernández-Martín et al., 2002). Thus, it was suggested that phosphate may improve the solubility of MPs, and cause an increase in pH, resulting in an increase in WAC.

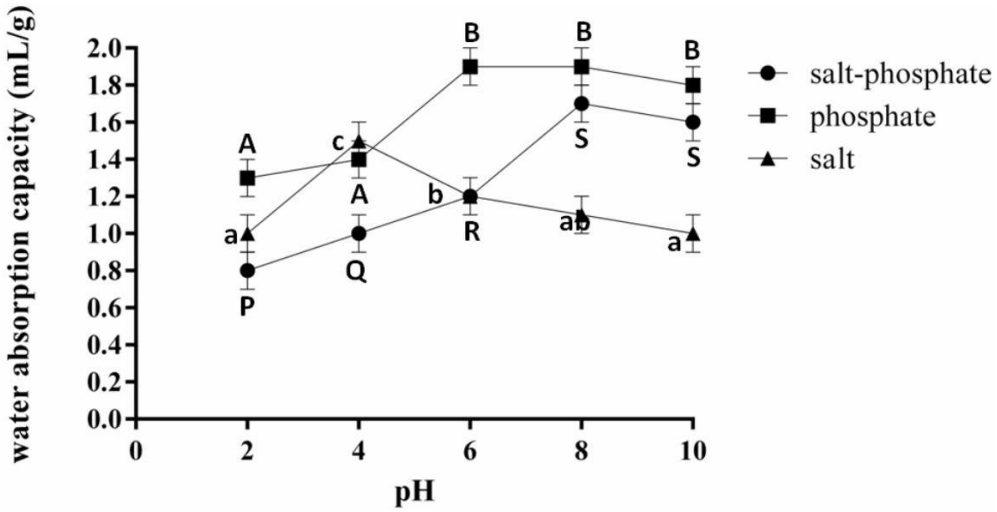

Figure 2. Water absorption capacity of combinations of 0.04 g NaCl-0.1 g protein, 0.004 g phosphate-0.1 g protein, and 0.04 g NaCl-0.004 g phosphate-0.1 g protein at different pH points.

a, b, c, d, e: Mean values corresponding to the usage of salt with proteins at different pH values not followed by a common letter differ significantly ($P < 0.05$).

A, B, C, D, E: Mean values corresponding to the usage of phosphate with proteins at different pH values not followed by a common letter differ significantly ($P < 0.05$).

P, Q, R, S: Mean values corresponding to the combined usage of salt and phosphate with proteins at different pH values not followed by a common letter differ significantly ($P < 0.05$).

Fat Absorption Capacity (FAC) Results

FAC is defined as the holding ability of fat by non-polar amino acid residues in proteins (Yongsawatdigul and Hemung, 2010). FAC is important for improving the flavor of comminuted meats (De Kanterewicz et al., 1987). In this study, the FAC of MPs was evaluated using different quantities of sodium chloride and/or phosphate. FAC analysis results indicated that the highest FAC value was obtained for 0.1 g MP combined with 0.04 g sodium chloride and 0.004 g phosphate, as in the WAC analysis result (data not shown). The FAC value of the control sample was determined as 5.4 mL fat/g protein.

FAC of 0.1 g MP was then investigated at different pH points (pH 2, 4, 6, 8, and 10) in the presence of 0.04 g sodium chloride and 0.004 g phosphate. A higher FAC value than the control sample was obtained at each experimental point except two (0.1 g MP-0.04 g sodium chloride at pH 2 and 4) (Figure 3). It has been reported that there is an association between the function and the structure of meat proteins and emphasized the significance of solubility, hydrophobicity, and SH-group content of salt-soluble meat proteins, regarding fat binding and emulsifying properties (Li-Chan et al., 1985). A positive correlation was also reported between surface hydrophobicity and the fat absorption capacity of rockfish protein by Voutsinas et al. (1983). This study suggested that salt and/or phosphate may have increased the surface hydrophobicity of MPs.

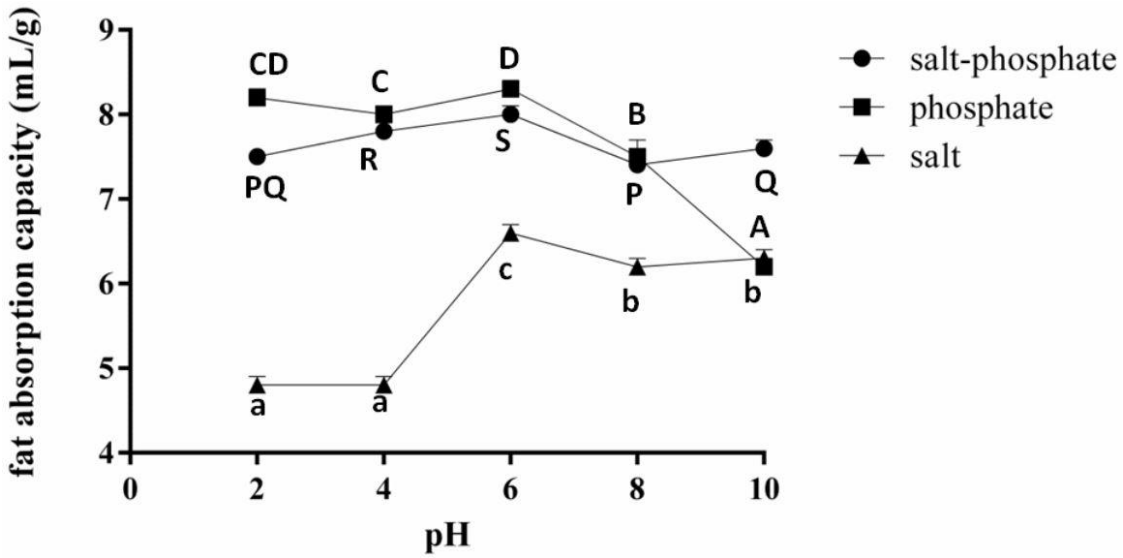

Figure 3. The fat absorption capacity of combinations of 0.04 g NaCl-0.1 g protein, 0.004 g phosphate-0.1 g protein, and 0.04 g NaCl-0.004 g phosphate -0.1 g protein at different pH points.

a, b, c, d, e: Mean values corresponding to the usage of salt with proteins at different pH values not followed by a common letter differ significantly (P < 0.05).

A, B, C, D, E: Mean values corresponding to the usage of phosphate with proteins at different pH values not followed by a common letter differ significantly (P < 0.05).

P, Q, R, S: Mean values corresponding to the combined usage of salt and phosphate with proteins at different pH values not followed by a common letter differ significantly (P < 0.05).

The use of MPs is suitable for food products needed for fat retention (Chen et al., 2017). Accordingly, Elizalde et al. (1988) have investigated the FAC value of different proteins including MPs. They have shown that the FAC of MPs has the highest value among other protein sources (WPC, BSA, BPI, SC, and SPI) as 5.25 mL fat/g protein (Elizalde et al., 1988). The FAC value of the control sample (5.4 mL fat/g protein) is compatible with the results of the study by Elizalde et al. (1988). Also, water-soluble MPs have higher fat absorption, relative to SPI and

WPI. It is ascribed that MPs may physically and structurally have bigger porosity which favors more fat entrapment and higher hydrophobic properties (Chen et al., 2017).

Emulsifying properties of MPs

Salt-soluble proteins including largely MPs are the major promotors of the emulsifying properties in comminuted meat (Hamm, 1986; Xiong, 1997). Emulsifying properties, *i.e.,* EAI and ESI, were investigated for MPs used together with different amounts of sodium chloride and/or phosphate. The highest EAI and ESI values were obtained by the addition of 0.05 g sodium chloride and 0.008 g phosphate on 0.1 g MPs (data not shown). EAI and ESI values were then investigated for 0.1 g MPs by supplementation of 0.05 g sodium chloride and/or 0.008 g phosphate, at different pH points (pH 2, 4, 6, 8, and 10), as well as the control group using water. The EAI of the control sample was determined as 4.98 m^2/g protein. The EAI values of the sample groups were given in Figure 4a. These results showed that the addition of salt and/or phosphate on MPs resulted in various EAI patterns at different pH points. Salt and salt-phosphate supplementation gradually increased EAI towards high pH values (Figure 4a). In line with this, high pH provided favorable emulsifying properties, as well as gel-forming properties (Chan et al., 2011).

Emulsifying property analysis results showed that the MPs had the highest ESI value with the addition of salt at pH 8 among the other pH points (Figure 4a). This result is consistent with the studies reporting that the high pH values support the emulsion stability in meat combinations (Richardson and Jones, 1987; Young et al., 2005). Accordingly, Romero et al. (2011) have shown that crayfish proteins had more favorable emulsion stability at pH 8, relative to pH 2 (Romero et al., 2011). On the other hand, liver proteins from turkey included favorable emulsion stability at low and high pH values (Zouari et al., 2011), whereas salt soluble proteins obtained from meat having pH 6.5 included greater emulsifying properties (Kijowski and Niewiarowicz, 1978).

Emulsifying property analysis results showed MP-salt-phosphate had the highest EAI value at pH 10 and it was found as 8.51 m^2/g protein, whereas the MP-salt combination had the maximum value of ESI at pH 8 as 230.8 min (Figure 4a, 4b). Similarly, Ramachandran et al. (2009) reported that the EAI index of emulsifying properties of proteins isolated from various fishes was found between 2.193 m^2/g and 7.247 m^2/g whereas ESI values were determined between 53.33-128.33 min (Ramachandran et al., 2009). In addition, Mohan et al. (2006) showed that EAI values of MPs isolated from Rohu fish (*Labeo rohita*) were found as 6,25 m^2/g for 5 mg/mL protein and 11,09 m^2/g for 2.5 mg/mL protein. ESI results were found as 364 min for 5 mg/mL protein and 87 min for 2.5 mg/mL protein.

Foaming capacity (FC) and foaming stability (FS) results

FC and FS were investigated for MPs with the addition of different amounts of sodium chloride and/or phosphate. The highest FC and FS values were found in a combination of 0.1 g MP-0.03 g sodium chloride-0.01 g phosphate (data not shown). FC and FS with each combination of 0.03 g sodium chloride and 0.01 g phosphate at different pH points (pH 2, 4, 6, 8, and 10) were given in Fig 4a and 4b.

The FC of the control sample was determined as 40%. The addition of salt and/or phosphate to MP resulted in the greatest FC at alkaline pH, especially pH 10 (Figure 5a). Accordingly, Rocha-Estrada et al. (2010) have shown that myosin heavy chain (MHC) and paramyosin (PM) of mantle and fin of the jumbo squid *Dosidicus gigas* had great solubility and high foaming capacity at basic pH values. In line with this, foaming capacity was proportional to solubility. Also, Thawornchinsombut and Park (2005) showed that soluble fish proteins including MHC from pacific whiting mince had higher solubility at pH 7 and 10, relative to pH 4, when increasing the salt concentration. Thus, the present study suggested that MP solubility may be increased at alkaline pH conditions in line with the FC.

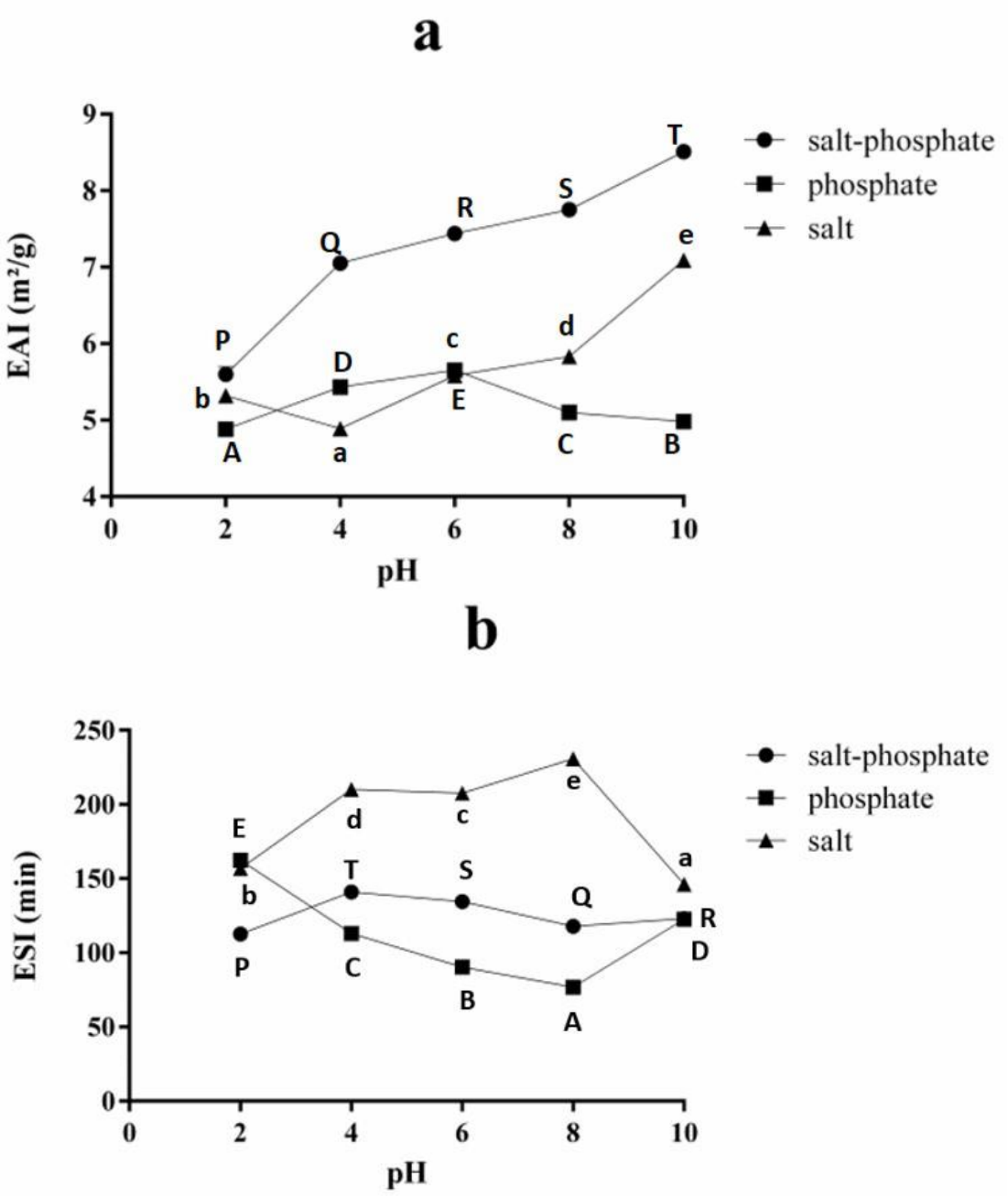

Figure 4. Emulsion activity index (EAI) (a) and emulsion stability index (ESI) (b) of combinations of 0.05 g NaCl-0.1 g protein, 0,008 g phosphate-0.1 g protein, and 0.05 g NaCl-0,008 g phosphate-0.1 g protein against different pH points

a, b, c, d, e: Mean values corresponding to the usage of salt with proteins at different pH values not followed by a common letter differ significantly (P < 0.05).

A, B, C, D, E: Mean values corresponding to the usage of phosphate with proteins at different pH values not followed by a common letter differ significantly (P < 0.05).

P, Q, R, S, T: Mean values corresponding to the combined usage of salt and phosphate with proteins at different pH values not followed by a common letter differ significantly (P < 0.05).

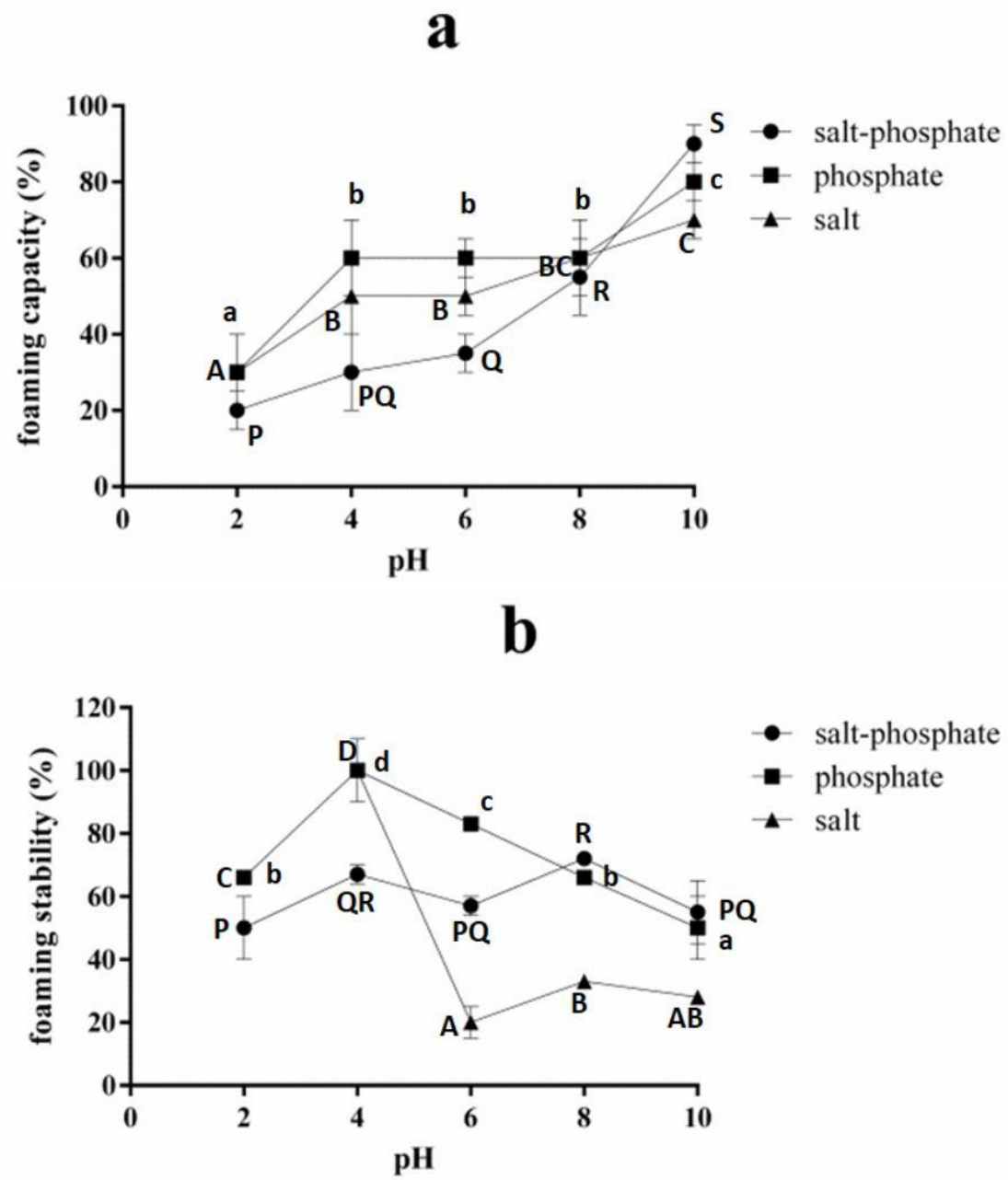

Figure 5. Foaming capacity (FC) (a) and foaming stability (FS) (b) of combinations of 0.03 g salt-0.1 g MP, 0.01 g phosphate-0.1 g MP, and 0.01 g phosphate-0.03 g salt-0.1 g MP against different pH points.
a, b, c, d, e: Mean values corresponding to the usage of phosphate with proteins at different pH values not followed by a common letter differ significantly (P < 0.05).
A, B, C, D, E: Mean values corresponding to the usage of salt with proteins at different pH values not followed by a common letter differ significantly (P < 0.05).
P, Q, R, S, T: Mean values corresponding to the combined usage of salt and phosphate with proteins at different pH values not followed by a common letter differ significantly (P < 0.05).

The FS of the control sample was determined as 20 % and the FS results were given in Figure 5b. Foaming property analysis results showed that MP-salt had the lowest FS at pH 6, compared to the other pH points; however, it was also decreased at pH 10. Similarly, FS of jumbo squid *D. gigas* was also minimum at pH 6 and it was decreased at extreme basic pH conditions (pH 12) in the presence of salt (Rocha-Estrada et al., 2010). The highest FC result was found to be 90% at pH 10 in the sample prepared with the addition of protein (0.1 g), NaCl (0.03 g), and phosphate (0.01 g), and the highest FS result was determined as 100% at pH 4 with the addition of NaCl or phosphate. The present study indicated that NaCl and phosphate addition to the proteins improved the foaming properties of the proteins. It is seen that both the foaming capacity and stability values were increased when compared with the control sample.

CONCLUSIONS

Due to various cultures and nutrition types in the world, research has been carried out on different products in different regions and studies are also carried out on improving the sensory properties of the products. In terms of both taste and health, the potential best recipe for the production stages of the products is sought. Studies on proteins, which are one of the most important components that contribute to the various properties of meat products, are also important in this respect. In this study, the effects of salt and phosphate used in almost every meat product on myofibrillar proteins were investigated. The amounts used in the study positively affected the functional properties of the proteins and higher results were obtained compared to the results obtained in the control sample.

There are various studies on myofibrillar proteins in the literature, but they are generally on white meat proteins, mostly fish and other marine animal proteins. Studies investigating the effect of the use of different food additives on the functional properties of red meat proteins in more detail will contribute to the meat products industry.

STATEMENT OF CONFLICT OF INTEREST

The authors have no conflict of interest with other persons and/or institutions.

AUTHOR CONTRIBUTIONS

The study was produced from Armin BJELAK's master's thesis, and Armin BJELAK took part in the formation of the study idea, the planning, and execution of the experiments, and created the text of the article. Banu SEZER took part in the execution of the experiments and created the text of the article. Yusuf SÜRMELİ took part in the creation of the experimental design, the evaluation of the data, and the editing of the manuscript. Hasan Murat VELİOĞLU took part in the formation of the idea of working as a supervisor, the creation of the experimental design, the evaluation of the data, and the control/editing of the draft of the manuscript. İsmail Hakkı BOYACI, as the project leader, took part in the creation of the experimental design, the evaluation of the data, and the control of the draft of the manuscript. The authors have read and approved the final version of the manuscript.

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