ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

EFFECT OF TRANSGLUTAMINASE ON EMULSIFYING PROPERTIES OF CASEINOMACROPEPTIDE

M.Sc. THESIS

Gülnaz ARTAN

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Gülnaz ARTAN
(506141545)

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Thesis Advisor: Prof. Dr. Meral KILIÇ AKYILMAZ

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TRANSGLUTAMİNAZ ENZİMİNİN KAZEİNOMAKROPEPTİDİN EMÜLSİYON ÖZELLİKLERİNE ETKİSİ

YÜKSEK LİSANS TEZİ

Gülnaz ARTAN
(506141545)

Gıda Mühendisliği Anabilim Dalı

Gıda Mühendisliği Programı

Tez Danışmanı: Prof. Dr. Meral KILIÇ AKYILMAZ

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Gülnaz Artan, a M.Sc. student of ITU Institute of Science student ID 506141545, successfully defended the thesis/dissertation entitled "EFFECT OF TRANSGLUTAMINASE ON EMULSIFYING PROPERTIES OF CASEINOMACROPEPTIDE", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Meral KILIÇ AKYILMAZ** Istanbul Technical University	.....
Jury Members :	**Prof. Dr. Gürbüz GÜNEŞ** Istanbul Technical University	.....
	Ass. Prof. Celale KIRKIN Özyeğin University	.....

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To Science ,

FOREWORD

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Gülnaz ARTAN

Food Engineer

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ABBREVIATIONS

CMP : Caseinomacropeptide
KMP : Kazeinomakropeptit
NaCS : Sodium-caseinate
WPI : Whey Protein Isolate
Tgase : Microbial Transglutaminase
Tgaz : Mikrobiyal Transglutaminaz
EAI : Emulsifying Activity Index
ESI : Emulsion Stability Index
SDS : Sodium Dodecyl Sulfate

SYMBOLS

°C	**:** degree Celsius
U	**:** enzyme unite
g	**:** gram
mL	**:** mililiter
mM	**:** milimolar
w	**:** weight
v	**:** volume
min	**:** minute
µL	**:** microliter
T	**:** turbidity
A	**:** absorbance at 500 nm,
F	**:** dilution factor
L	**:** path length of cuvette in meters.
ϕ	**:** oil volume fraction of the emulsion
C	**:** protein amount (g/mL) before emulsification
A(0)	**:** the absorbance at 0 time
A(15)	**:** the absorbance after 15 min.

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SUMMARY

Caseinomacropeptide (CMP) is a polypeptide with biological activity which is released from casein during cheese production. In recent years, CMP is investigated because of its physiological, nutritional and technological functions. In the present study, effect of transglutaminase (Tgase) on emulsifying properties of CMP was investigated. In addition, emulsifying properties of enzyme treated and untreated CMP were compared with those of Na-caseinate and whey protein isolate.

Firstly, influencing factors on emulsifying properties of CMP were studied. Emulsions were formed with sunflower oil and CMP solution at different concentration (0.25-5%), ionic strenght (0-500 mM NaCl) and pH (2.5-10). Emulsion activity index (EAI), emulsion stability index (ESI) and creaming index of emulsions were measured. No significant effect of concentration of CMP was observed on emulsion stability. Increase in CMP concentration caused decrease in emulsion activity and creaming index values. Change in ionic strength did not affect emulsion activity and creaming index values. But it increased emulsion stability at 10 mM NaCl, while higher salt concentrations negatively affected stability. Emulsion activity and stability of CMP were found to show a minimum around pH 4.0.

In the second part of the study, effect of transglutaminase enzyme on emulsifying properties of CMP was studied at concentration of 0.5%, ionic strength of 10 mM NaCl and pH 3.5, 4.5 and 6.5. CMP solutions were treated with 1, 5 and 20 U Tgase/g CMP and then heat treated at 80°C for enzyme inactivation. EAI of CMP was increased as the activity of the enzyme was incresed. ESI of CMP was highest at the highest enzyme concentration at pH of 3.5 and 4.5, while there was no effect of enzyme on ESI at pH 6.5. Creaming index of CMP decreased with enzyme treatment at pH 3.5 and 4.5.

To determine the effect of inactivation heat treatment on the emulsion properties of CMP, CMP emulsions were prepared with or without enzyme (20 U Tgase/g CMP) and heat treatments. Both enzyme and heat treatment were found to increase the emulsion activity and stability of CMP.

CMP was found to have similar emulsion activity and stability with Na-caseinate and whey protein isolate. Enzyme treatment of CMP with 20 U Tgase/g CMP significantly improved emulsion properties.

TRANSGLUTAMİNAZ ENZİMİNİN KAZEİNOMAKROPEPTİDİN EMÜLSİYON ÖZELLİKLERİNE ETKİSİ

ÖZET

Kazeinomakropeptid (KMP) peynir üretimi sırasında oluşan biyolojik aktiviteye sahip bir polipeptiddir ve gerek fizyolojik ve besinsel, gerekse teknolojik özellikleri bakımından son yıllarda araştırılmaktadır. Bu çalışmada, kazeinomakropeptidin emülsiyon özelliklerine transglutaminaz (Tgaz) enziminin etkisi incelenmiştir. Ayrıca enzimsiz ve enzimli KMP'nin emülsiyon özellikleri sodyum kazeinat ve peyniraltı suyu protein izolatının özellikleri ile karşılaştırılmıştır.

Çalışmanın ilk aşamasında, uygun emülsiyon koşulları belirlenmesi için denemeler yapılmıştır. Peptid konsantrasyonu %0,25-5,00, iyonik kuvvet (0-500 mM NaCl) ve pH'nın (2,0-10,0) emülsiyon aktivite ve stabilitesine etkileri belirlenmiştir. Emülsiyonlar hacimce %25 ayçiçek yağı ve %75 protein çözeltisi kullanılarak ve elde edilen karışım 15500 rpm'de 2 dk homojenize edilerek hazırlanmıştır.

Protein çözeltilerinin hazırlanması aşamasında gerekli miktarda proteinler tartıldıktan sonra deney dizaynına göre distile su veya 0,1 M fosfat buffer kullanılarak uygun derişime sahip protein çözeltileri hazırlanmıştır. Çözeltiler 15 dakika magnetik karıştırıcı yardımı ile orta hızda karıştırıldıktan sonra 15 dakika boyunca da ultrasonik karıştırıcı yardımıyla çözündürülmüştür. Ardından 4°C'de 24 saat depolanarak iyice hidrate olmaları sağlanmıştır.

Emülsiyon özelliklerinin belirlenmesi aşamasında kullanılan parametreler; emülsiyon aktivite indeksi (EAI), emülsiyon stabilite indeksi (ESI), kremalaşma indeksi ve emülsiyon kapasitesidir (EC). Emülsiyon aktivite ve stabilite indeksleri türbidimetrik olarak belirlenmiştir. 9 mL protein çözeltisi ve 3 mL yağ kullanılarak emülsiyon örnekleri hazırlanmıştır. Hazırlama işleminin hemen ardından emülsiyondan 50 µL örnek alınmıştır ve 20 mL %0,1 (kütle/hacim) SDS çözeltisi ile seyreltilmiştir. Seyreltme işleminin ardından spektrofotometrede 500 nm'de ABS değeri okunmuştur ve emülsiyon aktivite indeksi hesabı yapılmıştır. 15 dakika sonra tekrar aynı emülsiyon örneğine ait 50 µL örnek alınıp 20 mL %0,1 (kütle/hacim) SDS çözeltisi ile seyreltilmiştir. Seyreltme işleminin ardından 500 nm'de ABS değeri okunmuştur ve emülsiyon stabilite indeksi hesaplanmıştır. Emülsiyon kapasitenin belirlenmesi için %2 (kütle/hacim) protein çözeltisine ait örneklerden 4 mL alınarak farklı yağ fraksiyonlarında emülsiyonlar hazırlanmıştır. Emülsiyonun, su içinde yağ emülsiyonundan yağ içinde su emülsiyonuna döndüğü minimum yağ miktarı iletkenlikölçer ile tespit edilmiştir. Emülsiyonun iletkenliğinin ani bir düşüş gösterdiği durum faz değişiminin olduğu nokta olarak kabul edilmiştir. Faz dönüşümüne neden olan minimum yağ miktarı belirlendikten sonra hesaplama yapılmıştır. Kremalaşma indeksinin belirlenmesi için ise 9 mL protein çözeltisi ve 3 mL yağ kullanılarak hazırlanan emülsiyonlardan 0. dakika için TScan yardımı ile

ölçüm alınmıştır. Örnekler 4°C'de 24 saat bekletildikten sonra tekrar TScan ile ölçüm alınmıştır ve gerekli hesaplamalar yapılmıştır.

Peptid konsantrasyonunun emülsiyon özelliklerine etkisini belirlemek için peptid konsantrasyonu %0,25-5,0 oranlarında değişen, sabit iyonik güç (10mM NaCl) ve pH (pH 8,0) değerindeki emülsiyonlar hazırlanmıştır. Emülsiyon stabilite indeksi (ESI) konsantrasyon değişikliğinden etkilenmezken emülsiyon aktivite indeksi (EAI) ve faz ayrılma yüzdesi olarak da tanımlayabileceğimiz kremalaşma indeksi konsantrasyon artışıyla paralel olarak düşmüştür.

İyonik gücün KMP emülsiyonu üzerindeki etkisini belirlemek için ise, %0,5 peptid konsantrasyonunda sabit pH 8,0'de iyonik kuvvet 0-500 mM NaCl aralığında değiştirilerek emülsiyonlar hazırlanmış ve emülsiyonların özellikleri incelenmiştir. Maksimum EAI değeri 10 mM NaCl varlığında görülmüş ve tuz miktarı arttıkça EAI değerinin düştüğü gözlenmiştir. ESI ve kremalaşma indeksi değerleri iyonik güç değişiminden önemli derecede etkilenmemişlerdir.

Emülsiyon özelliklerinin pH değişimi ile gösterdiği farklılıkları belirlemek için %0,5 KMP konsantrasyonunda iyonik güç 10 mM NaCl varlığı ile sabit tutularak 2,5-10,0 pH aralığında emülsiyonlar hazırlanmıştır. KMP'nin emülsiyon akvitesinin ve stabilitesinin pH 4,0 dolaylarında en düşük değerlere sahip oldukları bulunmuştur.

Bu çalışmalardan elde edilen sonuçlar neticesinde %0,5 protein konsantrasyondaki 10 mM NaCl ile sabit iyonik kuvvete sahip olan KMP çözeltilerinin emülsiyon hazırlanmasında kullanılmasına karar verilmiştir.

Çalışmanın ikinci aşamasında Tgaz enzimi uygulamasının kazeinomakropeptidin emülsiyon özellikleri üzerindeki etkisi incelenmiştir. 1, 5 ve 20 U Tgaz/g KMP aktivitede Tgaz ile 40°C'de 1 saat muamele edilmiş, enzim inaktivasyonu için 80°C'de 2 dakika ısıl işlem uygulanmış, KMP çözeltilerinin 3,5, 4,5 ve 6,5 pH'lardaki emülsiyonları oluşturulmuş ve bu çözeltilerden elde edilen emülsiyonların özellikleri incelenmiştir.

Yapılan çalışmaların sonucunda, 1 U Tgaz/g KMP kullanımının emülsiyon özelliklerinde değişikliğe neden olmadığı görülmüştür. 5 U Tgaz/g protein enzim kullanımı ise pH 4,5'da etkisini en fazla şekilde gösterirken diğer pH değerlerinde çok fazla değişikliğe neden olmamıştır. 20 U Tgaz/g KMP enzim kullanımı ise her üç pH değerinde de EAI ve ESI değerlerini önemli derecede arttırken pH 3,5'da kremalaşma indeksini önemli derecede düşürerek kazeinomakropeptidin emülsiyon özelliklerini önemli düzeyde iyileştirmiştir. Sonuç olarak, kazeinomakropeptidin emülsiyon özelliklerinin en fazla 20 U enzim kullanımı ile pH 3,5'da iyileştiği saptanmıştır.

Enzim kullanımının emülsiyon kapasitesi 1 U Tgaz/g KMP kullanımı ile artarken daha yüksek miktarlardaki enzim uygulamaları emülsiyon kapasitesini etkilememiştir.

Enzim inaktivasyonu için uygulanan ısıl işlemin emülsiyon özelliklerine etkisini belirlemek için ise, işlem uygulanmayan KMP çözeltisi, sadece ısıl işlem uygulanan KMP çözeltisi, sadece enzim uygulanan KMP çözeltisi ve enzim ve ısıl işlem uygulanan KMP çözeltisi kullanılarak oluşturulan emülsiyonların özellikleri incelenmiştir. İncelenen tüm pH değerlerinde 20 U Tgaz/g KMP enzim kullanılan ve inaktivasyon işlemi uygulanan emülsiyonların özelliklerinin diğer emülsiyonların özelliklerinden daha iyi olduğu saptanmıştır.

Sodyum kazeinat ve peyniraltı suyu protein izolatı ile karşılaştırıldığında KMP'nin emülsiyon kapasitesinin yüksek olmasına rağmen aktivite ve stabilite özelliklerinin farklı olmadığı saptanmıştır. 20 U Tgaz/g KMP enzim ile muamele edilen KMP emülsiyonu sodyum kazeinat ve peyniraltı suyu protein izolatı emülsiyonları ile kıyaslandığında ise EAI ve ESI değerlerinin en fazla olan örnek olduğu görülmüştür. Enzimli ve enzimsiz KMP'nin kremalaşma indeksi değerleri diğer emülgatörlerden farksız bulunmuştur. Sonuç olarak 20 U Tgaz/g KMP enzim kullanımı ile kazeinomakropeptidin emülsiyon özellikleri önemli ölçüde iyileştirilmiştir.

1. INTRODUCTION

1.1 Literature Review

1.1.1 Caseinomacropeptide

In recent years, the biological activity of milk components, especially found in whey, have been studied. Because of being most common and in largest supply, bovine milk components are main source materials for these studies. One of the bioactive components of whey is caseinomacropeptide (CMP) or glycomacropeptide (GMP). During the manufacture of cheese, rennet hydrolyzes Phe105- Met106 bond of κ-casein and two fractions are formed: para-κ-casein with amino acid residues from 1-105, and glycomacropeptide (GMP) from 106-169, which remains soluble in milk whey (Abd El-Salam et al., 1996). Glycosylated form of CMP is called GMP. Both forms are present in cheese whey.

György et al. (1954) first reported the presence of protein-bound sialic acid as a bifido-factor in human and cow milk not destroyed or altered even by autoclaving. Delfour et al. (1965) also reported that sialic acid bound protein is kappa-casein (κ-CN) and a sialic acid-rich peptide was formed after cheese manufacturing process by action of rennet.

Bioactivity of glycomacropeptide (GMP) has been studied by several researchers. Some physiological functions of GMP are listed below (Brody, 2000):

(1) Binding of cholera and *Escherichia coli* enterotoxins; GMP inhibits the binding of cholera toxins to their oligosaccharide receptors on cell walls (Kawasaki, et al., 1992; Oh et al., 2000) and protects cells from infection by influenza virus (Kawasaki et al., 1993).

(2) Inhibition of bacterial and viral adhesion; GMP also inhibits the adhesion of cariogenic bacteria such as *Streptococcus mutans, S. sanguis* and *S. sobrinus* to the oral cavity (Neeser et al., 1988; Neeser et al., 1994; Vacca Smith and Bowen, 2000) and modulates the composition of the dental plaque microbiota (Guggenheim et al., 1999; Schupbach et al., 1996).

(3) Suppression of gastric secretions; Stan and Chernikov (1979) showed that GMP inhibits gastric secretions and slows stomach contractions in dogs. In addition, in the following years Stan et al. (1983) reported that entering the blood to cause gastric acid inhibition is necessary for the GMP. Guilloteau et al. (1987) found that intravenous injection of GMP afforded no inhibition of gastric secretions or changes of digestive hormone blood plasma levels in preruminant calves. On the other hand, Yvon et al. (1994) reported that receptors on the intestinal mucosa were triggered by GMP.

(4) Promotion of bifidobacterial growth; Bezkorovainy et al. (1979) found that glycopeptide from a bovine milk casein chymotryptic digest to have one-tenth the growth-promoting activity of human milk solids.

Thomä-Worringer et al. (2006) reported the growth-promoting activity of CMP or several *Bifidobacteria* species in rich media.

Gilles Robitaille (2013) reinforced the position of CMP as an efficient additive for probiotic growth and reported that it is not necessary to fractionate CMP into aCMP and gCMP to be fully active.

(5) Modulation of immune system responses; CMP also exerts immunomodulating activities, as it inhibits mitogens from inducing the proliferation of lymphocytes, and can even induce apoptosis of certain lymphocytes (Matin & Otani, 2000; Monnai & Otani, 1997; Otani et al., 1996; Otani & Monnai, 1995).

In addition to the bioactivity of CMP, it exhibits some particular nutritional properties. The fact that CMP is rich in branched-chain amino acids and low in methionine makes it a useful diet ingredient for patients suffering from hepatic diseases (Abd El-Salam et al., 1996). Moreover, CMP has no phenylalanine in its amino acid composition, which makes it suitable for nutrition in cases of phenylketonuria. On the other hand, CMP can cause hyperthreoninemia because of its high content of threonine (Fanaro & Vigi, 2002; Rigo et al., 2001). Zinc absorption was also increased by CMP supplementation (Kelleher et al., 2003). Large amounts of sialic acid are found in the brain and in the central nervous system in the form of gangliosides and glycoproteins, which contribute to the functioning of cell membranes and membrane receptors and to normal brain development. An in vivo experiment with laboratory animals has shown that the exogenous

administration of sialic acid increased the production of ganglioside sialic acid in the brain, improving learning ability (Wang et al., 2001). This effect could also be achieved with dietary CMP (Wang et al., 2004).

Besides its biological and nutritional properties of CMP, it also has several structure-functional properties such as solubility at wide pH range, gelling, emulsifying and foaming abilities which are shown to be promising for applications in food and nutrition industry. Besides possible health-promoting benefits, the functional properties of CMP make it an interesting ingredient for use in the development of novel foods (Dickinson, 2003).

1.1.2 Emulsifying properties of CMP

For food and food-related industries, knowledge about emulsions is important for a number of reasons. Firstly, many natural and processed foods consist emulsions comparatively or completely such as cream, beverage, soups, cakes, sauces, ice cream etc. Secondly, emulsions have been used as a delivery system for functional food ingredients, such as vitamins, flavors, preservatives etc. (McClements, 2016).So emulsifying properties are important in many food applications of ingredient proteins and these commonly discussed in terms of emulsifying capacity (EC), emulsifying stability (ESI), and emulsifying activity (EAI).

Martin-Diana et al. (2005) studied emulsifying properties of whey protein concentrate (WPC) and CMP. They reported that when compared with WPC, the emulsifying activity index of CMP was lower (36 m^2/g for CMP and 185 m^2/g for WPC).They found that, emulsifying stability index of WPC changed significantly due to pH and ionic strength whereas, CMP had a more stable emulsifying activity index with respect to pH than WPC. The authors suggested that CMP can be preferred in industrial processes involving pH changes.

It is well known that the solubility of proteins often is influenced by pH, thereby affecting the emulsifying properties. CMP is a hydrophilic water soluble molecule with its net negative charge even at low pH values (Haertle´ & Chobert, 1999). Chobert et al. (1989) studied solubility and emulsifying properties of κ-casein and its caseinomacropeptide. According to their results, while the solubility of CMP did not change within the range of pH 1-10, the emulsifying properties of peptide was effected by pH. In that study, although minimum solubility of CMP was between pH

1 to 5, maximum solubility was between pH 5-10 and solubility was not affected by heat treatment. Moreover, emulsion activity of CMP showed a wavy distribution due to pH changes. These results showed that the emulsifying properties of the peptide did not relate to its solubility.

The glycosylation also affects the functional properties of CMP. Glycan moiety significantly affects the surface activity, so emulsification properties of the glycosylated and non-glycosylated CMP fractions are different from each other. Kreuß et al. (2009) studied the effect of glycosylation on the interfacial properties of bovine caseinomacropeptide and reported that non-glycosylated one showed a higher emulsifying activity index of 150.7 m^2/g while the glycosylated one exhibited a lower value of 98.5 m^2/g. In addition, the stability of emulsions of CMP was 1.4 times higher than GMP. These authors reported that the pH of the emulsion influenced both the formation and stability of the emulsions formed by CMP. At the oil–water interface, above the p*I* there is an average stabilized adsorption of peptide with negative repulsion between individual oil droplets. At the p*I*, due to lack of repulsive forces, the oil droplets coalesce. Below the p*I*, there is repulsive positive charge which stabilizes the oil droplets.

Emulsifying properties of CMP is also affected by the hydrophilic nature of CMP which reduces the emulsifying activity as compared to native amphipathic κ-casein. The emulsification activity of GMP can be modified by conjugating it with other molecules such as lactose (Moreno et al. 2002) and fatty acids (Wong et al. 2006). According to the study of Moreno et al. (2002), the conjugation of lactose with GMP using Maillard reaction chemistry increased the emulsifying activity while the peptide solubility was not decreased significantly. Wong et al. (2006) studied functional and biological activities of casein glycomacropeptide influenced by lipophilization with medium and long chain fatty acid. They conjugated long-chain fatty acids to GMP using lipophilization reaction and reported that the modified GMP had improved functionality as a surfactant with enhanced antibacterial activity towards Gram-negative bacteria.

1.1.3 Emulsifying activity of milk proteins

Caseins and whey proteins are two main classes of milk proteins. Caseins are fractionated into four distinct proteins; α_{s1}-, α_{s2}-, β- and κ-caseins and all of these

proteins are phosphoproteins (Fox, 2009). Subfractions of caseins are amphiphilic; they have one side consisting of hydrophilic amino acids and the opposite side consisting of hydrophobic (or lipophilic) ones. The distribution of the many of the charged residues, the hydrophobic residues and especially the phosphoserine residues are not uniform along the polypeptide chain. Moreover, not only the caseins tend to associate with themselves and with each other but also all of them are able to bind calcium. The most calcium sensitive caseins are α_{s1}-Casein, α_{s2}-casein and β-casein.

On the other hand, κ-casein is insensitive to calcium. κ-Casein is capable of stabilizing other case ins against calcium-induced precipitation and allows the formation of colloidal sized aggregates (Horne, 1998, 2003). β-lactoglobulin, α-lactalbumin, immunoglobulins and bovine serum albumin are subfractions of whey proteins. Approximately 50% of the total whey protein in bovine milk is β-Lactoglobulin that contains two disulphide bonds and a single free thiol group. High levels of secondary, tertiary and quaternary structures are present in whey proteins, unlike caseins (Edwards et al., 2009; Kinsella & Whitehead, 1989).

Protein products possess important functional properties such as water binding, emulsification, foaming, whipping and gelation (Singh, 2010). Emulsification by caseinates and whey proteins are important applications in the food industry. They are used for production of emulsion-type products, e.g. coffee whiteners, whipped toppings, cream liqueurs, dietary formulations, liquid nutritional products and medical foods etc. (McClements, 2016). The amphiphilicity of primary structure of milk proteins is the basis for their emulsifying ability. Being amphiphilic determines their ability to adsorb to the oil-water interface (Dickinson, 2008; McClements, 2016; Singh & Ye, 2009). Na-caseinate, whey protein isolates and milk protein concentrates are emulsifying ingredients commonly used in the food industry. Na-caseinate is produced from acid-precipitated casein by dissolving in NaOH and being spray-dried subsequently (Walstra, 1999). Whey protein isolate is produced from whey by using ultrafiltration techniques or complex formations (Miller, 2006). Milk protein concentrate is manufactured by filtration processes (microfiltration, ultrafiltration and diafiltration) which removes lactose and soluble mineral while retaining milk proteins, followed by spray-drying (Martin,2010).

α_{s1}-casein and β-caseins possess amphiphilic structure and have strong tendencies to adsorb at oil-water interfaces and stabilise oil-in-water emulsions. β-casein has an

extensive hydrophobic region anchored directly at the surface to adsorb and a hydrophilic region (40-50 residues at the N-terminus) protruding extensively into the aqueous phase. On the other hand, α_{s1}-casein has a loop-like structure. It does not have such a pronounced inequality in the distribution of hydrophobic and hydrophilic residues in its primary structure (Dickinson, 1992). It has been suggested that α_{s1}-casein adsorbs to the oil-water interface via peptides towards the middle of its sequence, although β-casein has hydrophobic region at the end of its conformation and this difference may cause the protein to form a thinner adsorbed layer than does β-casein (Dalgleish, 1996; Dickinson, 1992). Because of its relatively higher surface activity, β-casein can displace α_{s1}-casein from the oil-water interface (Dickinson et al., 1988a; Dickinson & Stainsby, 1988b). However, Hunt and Dalgleish (1994) observed that there was no preference for α_{s1}-casein or β-casein in sodium-caseinate-stabilised emulsions. Euston et al. (1996) and Srinivasan et al. (1996) reported that the preferential adsorption of β-casein in sodium caseinate was dependent on the protein concentration used for making the emulsions. When the amount of a_s-casein was higher than β-casein, α_{s1}-casein was present at the interface at high protein concentrations. On the other hand, at low protein concentration, β-casein was adsorbed in preference to a_s-casein. High amounts of κ-casein at the surface were observed by Srinivasan et al. (2000). Consequently, the amount and structure of the adsorbed caseins on the surface is not clear yet (Ye, 2008).

The state of the protein particles in the milk protein concentrates (MPC) influenced the emulsifying properties of MPC and the stability of the emulsions formed with MPC (Ye, 2011). Ye (2011) also reported that when casein micelles in the MPCs were dissociated by reduction of the calcium content, the emulsifying ability was improved through the fine emulsion with a smaller droplet size at a lower protein concentration. The surface protein composition and the surface protein concentration were also altered in the emulsions formed by these MPCs with different casein states(Ye, 2011). The size and the amount of unadsorbed casein in the aqueous phase were changed by these alterations in the surface protein concentration and composition due to alteration in the aggregation of casein in MPC. Thus, the stability of an emulsion may be decreased by the depletion flocculation induced by the unadsorbed protein at high protein concentration. It is suggested that while a protein emulsifying agent is chosen, depletion flocculation may need to be considered(Ye,

2011). For the best emulsion stability, the properties of the protein products should be taken into account (Ye, 2011).

Ye and Singh (2001) reported that before homogenization, $CaCl_2$ addition higher than the critical concentration to Na-caseinate solution increases the surface protein coverage and alters the casein composition of the adsorbed layer. Although β-casein adsorption is hardly affected, α_{s1}-casein adsorption at the droplet surface is enhanced. Protein concentration is another parameter for emulsion stability. Ye and Singh (2001) also reported that the protein concentration above 6.0 wt% results in very high depletion flocculation and the flocculation leads to creaming because of strong emulsion droplet network. Depletion flocculation has not been observed in whey-protein-based emulsions. It appears that depletion flocculation in Na-caseinate emulsions formed because of the presence of casein aggregates formed from the self-assembly of Na-caseinate in the aqueous phase of the emulsion at concentrations above 2 wt% (Ye & Singh, 2001). The addition of moderate amounts of $CaCl_2$ to emulsions containing excess sodium caseinate has been shown to eliminate depletion flocculation and to improve the creaming stability (Ye & Singh, 2001). From another point of view, bridging flocculation destabilized the emulsion at low protein content, because of low surface protein coverage (McClements, 2016). Although the emulsions are stable against flocculation, coalescence and creaming for several weeks at about 2.0 wt% caseinate concentration, when the protein content is increased to above 3.0 wt%, unadsorbed caseinate gives rise to depletion flocculation (Dickinson & Golding, 1997; Euston & Hirst, 1999).

Dalgleish (1996) reported that β-lactoglobulin formed denser and rather thinner (2-3 nm at neutral pH) adsorbed layer than caseins. Because of its amphiphilic nature, β-lactoglobulin readily adsorbs at the oil-water interface, where it partially unfolds. Additionally, the partial unfolding of the globular whey-protein structure following adsorption causes exposure of the reactive sulphydryl group, leading to slow polymerization of the adsorbed protein in the aged adsorbed layer via sulphydryl-disulphide interchange (McClements, Monahan & Kinsella, 1993). Dickenson et al. (1989), Euston et al., (1996) and Hunt & Dalgleish (1994) reported no preferential adsorption between β-lactoglobulin (β-lg) and a-lactalbumin (α-la). However, Closs et al. (1993) found that β-lg was adsorbed in preference to α-la in emulsions formed with whey proteins, or with mixtures of β-lg and α-la. As a result, more information

about the individual proteins in mixed films containing several different proteins is needed.

The emulsifying ability of whey protein and sodium caseinate is much higher than that of "aggregated" milk protein products, such as MPC and calcium caseinate (Euston & Hirst, 1999; Singh & Ye, 2008; Ye, Srinivasan, & Singh, 2000). Both sodium caseinate and whey protein products (WPC and WPI) show excellent emulsifying ability, and it is possible to make stable emulsions at a relatively low protein-to-oil ratio (about 1:60) (Ye & Singh, 2001). Conversely, much higher concentrations of MPC or calcium caseinate are required to make a stable emulsion and larger droplets are formed in these protein-stabilized emulsions under similar homogenization conditions. The relatively low emulsifying ability of MPCs has limited their applications in some food formulations (Ye, 2011). In addition, Hunt and Dalgleish (1994) reported that at low concentration, there was no preferential adsorption between caseinate and whey protein, but the amount of caseinate at the surface was much greater than the amount of whey protein at high concentrations.

Aiqian Ye (2008) studied interfacial composition and stability of emulsions made with mixtures of commercial sodium caseinate and whey protein concentrate. He reported that the interfacial composition and the stability of emulsions were dependent on the protein concentration used to form the emulsion. Although the protein concentration was lower than 3%, the surface contained higher proportions of whey proteins than caseins; caseins were adsorbed in preference to whey proteins at higher protein concentrations (Ye, 2008). The different amounts of adsorbed individual proteins at the surface could be attributed mainly to different states of protein molecular structure at the surface, which was related to the concentration at the surface (Ye, 2008). Ye (2008) also reported that an increase in the creaming stability of emulsions with an increase in the protein concentration was observed at low concentrations. However, the creaming stability decreased markedly as the total protein concentration of the system was increased above 2% (sodium caseinate >1%) (Ye, 2008). This was attributed to depletion flocculation, caused by sodium caseinate occurring in these emulsions. The whey proteins in the system did not retard this instability in emulsions made with mixtures of sodium caseinate and WPC (Ye, 2008).

1.1.4 Transglutaminase (Tgase)

Proteins can be modified by chemical, enzymatic and physical methods for the improvement and development of new functional properties. Transglutaminase (Tgase) is an enzyme that can modify proteins by means of amine incorporation, crosslinking and deamidation (Motoki et al, 1998).

As shown in Figure 1, transglutaminase can catalyze 3 types of reactions.Tgase catalyzes acyl transfer reactions between primary amines (acyl acceptor) including the ε-amino group of lysine residues and the γ-carboxyamide group of peptide or protein-bound glutamine (acyl donor) and thus covalent cross-links are introduced in proteinaceous systems. If the acyl acceptor is the ε-amino group of protein-bound lysine, intramolecular and/or intermolecular cross-links (isopeptide bonds) are formed. It is results of protein polimerization. When primary amines are not existed, water can react as acyl acceptor. It leads to the deamidation of the glutamine residue under formation of glutamic acid and ammonia (Jaros et al., 2006).

Amino acids or peptides can be introduced into a protein by the acyl transfer reaction (Ikura et al. 1981). The methionine and lysine content of casein or soybean proteins can be enhanced by enzymatic attachment of lysylpeptides and lysylmethionine (Seguro et al. 1996; Yokoyama et al. 2004). Tgase is a good alterative for improving the nutritive value of foods because of the incorporated amino acids that act like amino acids in native protein (Motoki & Kumazawa 2000). In addition, functional properties of proteins such as emulsifying properties, solubility, foaming and gelation properties can be modified by the the cross-linking reaction (Faergemand et al 1997). Functionality may also be enhanced by the deamidation reaction (Hamada, 1994).

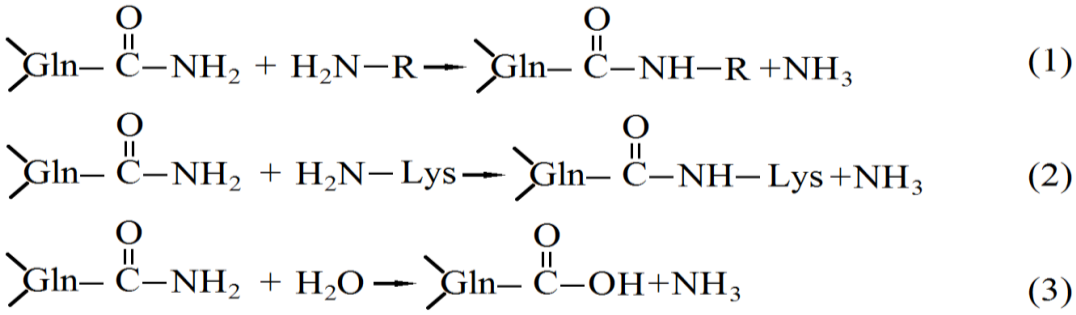


Figure 1.1: Reaction Catalyzed by Transglutaminase. (1)Acyl transfer reaction. (2)Cross-linking reaction. (3)Deamidation. (Jaros et al., 2006).

Molecular weight of Tgase is smaller than that of other known enzymes and it is noticable because of its calcium independence (Motoki et al., 1998). To expand the application of proteins, Tgase crosslinking has been used to improve their stability and functional properties (Motoki et al., 1998).In addition to the functional properties, Tgase affects solubility and hence gelation, emulsification, foaming, viscosity and water-holding capacity, which all depend on protein solubility (Gaspar et al., 2015). There is greater number of studies about using Tgase at food products such as meat products, dairy products, soybean products, wheat products, and its good effects on functional properties of final products (Motoki et al., 1998). Using Tgase for the production of dairy products is suggested because of good results such as improving gel strength, surface viscosity, water-holding capacity, stability, rennetability and mechanical properties and decreasing permeability (Özrenk, 2006).

1.1.5 Effects of Tgase on emulsifying activity of milk proteins

Gelation, rheological, emulsifying or renneting properties and solubility, hydration and heat stability of proteins can be modified by Tgase cross-linking applications (Lorenzen, 2000). The macromolecular structure of a protein influences the susceptibility of a protein to Tgase-induced cross-linking (Traore´ et al. 1992). Caseins, the main proteins in milk, are particularly good substrate for Tgase (Lorenzen, 2000). Low degree of tertiary structure, flexible, random-coil arrangement and the absence of any disulphide bonds in the α_{s1}- and β-caseins may leave the reactive groups exposed to the enzyme (Færgemand et al. 1998; O'Connell and Kruif, 2003). On the other hand, because of their globular structures, whey proteins are less efficient to cross-link (Sharma et al. 2002).

Flanagan et al. (2003) studied the effects of cross-linking on heat stability, solubility and emulsifying and foaming properties of Na-caseinate by incubating an aqueous solution at a level of 4% (w/v) with Tgase ([E:S] of 1:50 and 1:20) for 185 min at 20°C, followed by an inactivation step (80°C for 1 min). The solubility of Na-caseinate was improved by cross-linking, especially at acidic conditions (pH 2 and 3). Solubility was almost 100%, despite of being 57 and 74% for the unmodified samples, respectively (Flanagan et al., 2003). On the other hand, incubation of Na-caseinate with Tgase alone is of limited benefit for improvement in the emulsifying and foaming properties of Na-caseinate (Flanagan et al., 2003). Flanagan et al.

(2003) reported that Tgase may be used to modify the heat stability properties of Na-caseinate but have limited effect to improve emulsifying properties. However, Tang et al. (2005) incubated aqueous Na-caseinate solutions (1 or 2% [w/v], pH 7.5) with rather high concentrations of mTgase (15 U/g proteins, incubated at 37^{o}C for varying time) and the reaction was stopped chemically by adding N-ethyl-maleoyl-amide. The results indicated that increasing incubation time has a positive effect on emulsifying activity index (EAI) and emulsion stability of mTgase-treated Na-caseinate emulsions (Tang et al., 2005). As a result, these authors concluded that process conditions were more effective on emulsion properties of proteins.

Hinz et al. (2007) also studied influence of enzymatic cross-linking on milk fat globules and emulsifying properties of milk proteins. In this study, raw whole milk and recombined milk (untreated or Tgase-treated skimmed milk by adding butterfat) were treated with 0.05 g/L Tgase at 30°C. The crosslinking of whole and skim milk caseins increased with incubation time. Whereas the extensive clustering of fat globules in cross-linked raw whole milk occurred, less clustering of fat globules in recombined milk comprised of Tgase-treated skim milk was observed. Fat globule size of raw whole milk decreased with increasing homogenization pressure but Tgase treatment followed by homogenization caused a rise in particle size with incubation time (0h<6h<24h). On the other hand, while at 180 bar pressure increasing incubation time decreased the particle size, at 800 bar pressure particle size increased with Tgase incubation time of skim milk which the recombined milk was prepared. As a result, emulsifying properties of milk proteins are affected by Tgase-induced crosslinking.

Færgemand et al. (1998) studied emulsifying properties of milk proteins (sodium caseinate and β-lactoglobulin) crosslinked with 10 U/g microbial transglutaminase for 2h at 40°C. The degree of crosslinking changed due to emulsion preparation and determined via SDS-page and droplet size measurements analyses. They found that high extent of cross-linking milk proteins reduced the droplet size stability of oil droplets in o-w emulsions. However, limited cross-linking was beneficial. Hence, enzymatic cross-linking seems an interesting tool for controlling the stability or instability of milk protein stabilized oil-in-water emulsions.

Yang et al. (2016) study effects of microbial Tgase crosslinking on the functional properties of yak and cow caseins. Although being more soluble, having better

emulsifier properties, yak caseins whose solubility lower than cow caseins, have a better emulsifying activity. It is because of good hydrophobic-hydrophilic balance in yak caseins (Yang et al., 2016). After cross-linking, caseins solubility decreased, while the emulsifying properties increased due to casein polymerization. Although yak emulsifying properties was better at the beginning, cow caseins emulsion activity increased more. The results showed that the crosslinking degree of both caseins was different at same Tgase concentration, but changes in functional properties were same.

Liu and Damodaran (1999), studied the effect of Tgase-catalyzed polymerization of β-casein on its emulsifying properties. They found that although emulsifying index decreased, the storage stability of emulsions increased with increased polymerization with Tgase. Enhanced steric stabilization or stronger cohesive interaction may be the cause of the enhanced emulsion stability. Compared to casein, as described above, studies on the properties of Tgase-treated whey proteins are less in the literature.

Kester and Richardson (1983) reported several techniques such as chemical, physical and enzymatic methods to improve the functional properties of whey proteins. According to their results, proteolysis of whey proteins was limited by enzymatic modification. Due to the limited proteolysis, solubility of whey proteins improved and foaming and emulsification activity enhanced.

Sharma et al. (2002) studied the emulsifying properties of an industrial α-lactalbumin concentrate cross-linked before and after emulsification by a microbial transglutaminase. They reported that the emulsion properties of cross-linked α-lactalbumin were influenced by sequence of cross-linking. Maximum crosslinking was observed at 0.5 %(w/v) protein concentration, at 50°C, pH 5 and at 5h incubation time. While the emulsions of α-lactalbumin concentrate without crosslinking were generally unstable, cross-linking before emulsification decreased the stability further. On the other hand, when cross-linking was carried out after emulsification, the emulsion stability was improved.

In this study, Tgase was used to improve emulsifying properties of CMP. As CMP is a peptide, crosslinking with Tgase might enhance its emulsifying activity and stability. The results were compared with emulsifying properties of two commonly used commercial emulsifiers, Na-caseinate and whey protein isolate.

2. MATERIAL AND METHODS

2.1 Materials

Commercial caseinomacropeptide (CMP) (Lacprodan® CGMP-10) was kindly provided by Arla Food Ingredients (Viby J, Denmark). Composition of CMP is given in Table 2.1. Commercial Na-caseinate (NaCS) was obtained from Maysa Gida (İstanbul, Türkiye). Native Whey Protein Isolate Low Lactose (WPI) (Prolacta® 95LL Instant) was purchased from Lactalis Ingredienst (Bourgbarré, France). NaCS contained 91% protein, 4% ash and 1% fat. WPI contained 89% protein, 3% ash and 2% fat according to the manufacturer.

Table 2.1: Chemical Composition of CMP.

Component	Amount (%)
Dry matter	95
Protein	80
CGMP	64
β-lactoglobulin	9
α-lactalbumin	6
Sialic acid	6
Ash	5
Lactose	2

Microbial transglutaminase (Activa® MP Transglutaminase, Ajınomoto, Japan) was supplied from a local ingredient supplier. According to the producer company, this product contains maltodextrin and 1% enzyme (100 U/g solid). Refined sunflower oil was obtained from a local retail market.

Sodium dodecyl sulfate (SDS) and sodium chloride were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Sodium dihydrogen phosphate dihydrate was provided by Riedel-deHaen (Seelze, Germany) and di-sodium hydrogen orthophosphate (BDH Laboratory Supplies, England). Distilled and de-ionized water was used for the preparation of all solutions.

2.2 Methods

2.2.1 Emulsion preparation

Sodium dihydrogen phosphate dihydrate and di-sodium hydrogen orthophosphate were dissolved in deionized water to prepare a buffer solution (pH 8.0, 0.1 M). Solutions of CMP, Na-CS and WPI were prepared in water or in 0.1 M phosphate buffer at pH 8.0 by stirring on a magnetic stirrer for 15 min and on ultrasonic stirrer for 15 min. Solutions were stored at 4°C overnight for complete hydration.

For determining effect of ionic strength, the solutions of CMP were prepared at a concentration of 0.5% (w/v) in phosphate buffer. NaCl at concentrations ranging from 10 to 500 mM was added to adjust ionic strength. CMP concentrations were varied between 0.25 and 5% (w/v) in buffer and NaCl concentration was adjusted to 10 mM to assess the effect of protein concentration. Effect of pH was determined by adjusting pH of protein solutions to values in the range of 2.5-10.0 by using 4 N NaOH and HCl.

Oil-in-water emulsion was prepared by using 3 mL sunflower oil and 9 mL of protein solution at an oil volume fraction of 25% (v/v). Oil and protein solutions were blended with a probe homogenizer (Ultra Turrax T18, IKA Werke, Staufen, Germany) with a shear gap width of 0.5 mm at a rotation speed of 15500 rpm for 120s.

Table 2.2: Experimental desing of parameters.

Parameters	Peptide concentration % (w/v)	Medium	NaCl (mM)	pH
peptide concentration	0.25-5.0	phosphate buffer	10	8.0
ionic strength	0.5	phosphate buffer	0-500	8.0
pH	0.5	distilled water	10	2.5-10.0

2.2.2 Enzyme application

Solution of CMP was prepared in water at a concentration of 5% (w/v) because enzyme was inhibited in phosphate buffer. Transglutaminase was added at levels of 1, 5 and 20 U/g CMP to this solution and incubated at 40°C for 1 h with constant stirring. Enzyme was inactivated by heat treatment of the solution to 80°C for 2 min

in a water bath. The solution was cooled to room temperature immediately after inactivation. Solutions were used for analysis after required dilution. Emulsions were prepared from enzyme-treated CMP with or without inactivation for determining effect of heat treatment. Emulsions of WPI and NaCS were also prepared in the same way for comparison.

Table 2.3: Experimental desing of enzyme application.

Sample Name	Enzyme U/gCMP	incubation (40°C /60 min)	inactivation (80°C /2 min)
CMP	-	-	-
CMP-IHT	-	+	+
1 U/g CMP-IHT	1	+	+
5 U/g CMP-IHT	5	+	+
20 U/g CMP-IHT	20	+	+
20 U/g CMP	20	+	-

2.2.3 Determination of emulsion capacity

Emulsion capacity, the ability of an emulsifier to emulsify a given amount of oil, was determined by the method of Karaca et al. (2011). Protein solutions were prepared at a concentration of 2% (w/v) for determining emulsion capacity. Emulsions were prepared with 4 mL of protein solution and various volumes of sunflower oil as described above. Volume of oil was increased or decreased until phase inversion was observed. Phase inversion was detected with conductivity meter (Hanna Instruments, Inc., USA) at a point where conductivity value of emulsion was decreased drastically. Average volume (mL) of the oil used before and after phase inversion point was divided by mass (g) of the protein solution to calculate emulsion capacity.

$$\boldsymbol{Emulsion\ Capacity} = \frac{\textbf{volume of oil at inversion}}{\textbf{mass of protein in emulsion}} \quad (1)$$

2.2.4 Determination of emulsion activity index and emulsion stability index

Emulsifying activity index (EAI) and emulsion stability index (ESI) were determined by applying the turbidometric method of Pearce and Kinsella (1978) as modified by Cameron et al. (1991). After preparing the emulsions, an aliquot of 50 μL was dispersed in 20 mL of 0.1% SDS solution. The absorbance of the diluted emulsion was then measured by a spectrophotometer (Biospec 1601 UV-Vis Spectrophotometer, Shimadzu, Kyoto, Japan) at 500 nm in 1 cm glass cuvettes. Absorbance values were held below 0.4 by dilution with SDS solution as suggested

by Pearce and Kinsella (1978). Turbidity was calculated by using the following formula:

$$\boldsymbol{T = \frac{2{,}303.A.F}{L}} \quad (2)$$

where T is turbidity, A is absorbance at 500 nm, F is dilution factor and L: path length of cuvette in meters.

The EAI (m^2/g) was then defined as:

$$\boldsymbol{EAI = \frac{2.T}{C.(1-\phi)}} \quad (3)$$

where $\boldsymbol{\phi}$ is oil volume fraction of the emulsion and C is mass of protein per unit volume (g/mL) of the protein aqueous phase before emulsion formation.

The ESI (min) was calculated by using the absorbance value directly after preparing the emulsions and after a time of 15 min with the following formula:

$$\boldsymbol{ESI = \frac{A(0)\,.\,15}{[A(0) - A(15)]}} \quad (4)$$

where A(0) is the absorbance at 0 time and A(15) is the absorbance after 15 min.

2.2.5 Creaming Index

The emulsions naturally began to separate into a transparent layer at the bottom and an optically opaque cream layer at the top after a period of storage. The total height of the emulsions and the height of the transparent layer were measured from backscattering values measured by a turbidimeter (TurbiScan Classic MA 2000, Formulaction, l'Union, France) to determine the creaming index and creaming.

Directly after preparing the emulsions, a 12 mL sample was filled in a glass tube with a length of 100 mm and a diameter of 15 mm. Backscattering value of the samples was measured at first minute and after 24 h of storage at 4ºC. Creaming index and formation of cream layer in emulsion was detected by the change in backscattering value. Creaming index was calculated by using the equation below (Anton et al., 2000):

$$\text{Creaming Index (\%)} = \left[\frac{\text{height of transparent phase}}{\text{total height of emulsion}}.100\right] \quad (5)$$

2.2.7 Statistical analysis

The trials and measurements were carried out at least in duplicate. Effect of treatments on measured variables was determined by ANOVA (IBM SPSS Statistics 23, U.S.A.). The differences between the means were determined at a significance level of 0.05 with the Tukey test.

3. RESULTS AND DISCUSSION

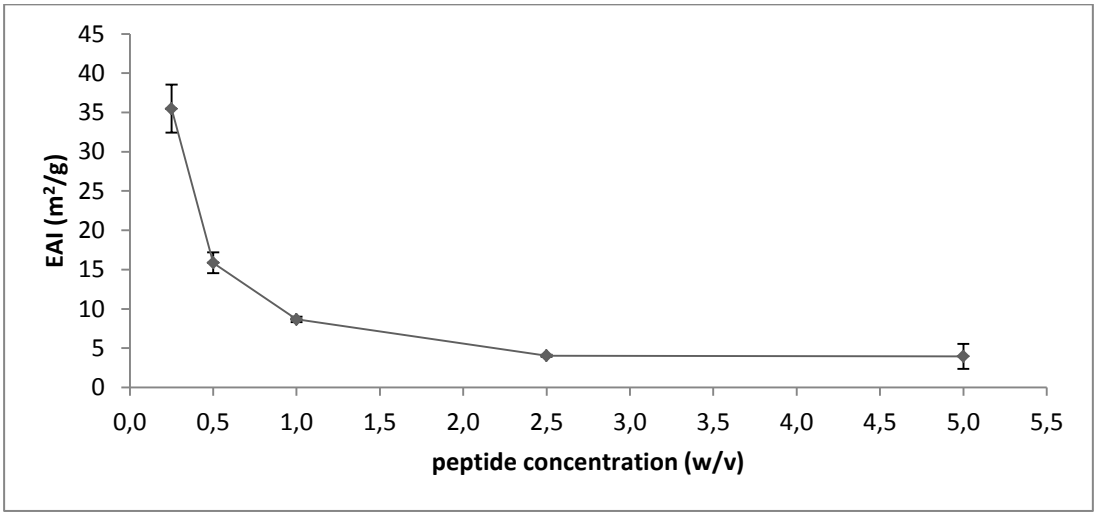
3.1 Emulsifying Properties of Caseinomacropeptide

The main aim of this part of the study is determining the behaviour of CMP in emulsions under different conditions. Because the structure and aggregation state of proteins can be changed by pH, ions and processing treatments before or after emulsification. The adsorptive behavior of milk proteins at the interface of oil-water can be effected irreversibly by this changes.

3.1.1 Effect of peptide concentration

The effect of peptide concentration on emulsifying properties of CMP is shown in Figure 3.1. Whereas peptide concentration has no statistically significant effect on the ESI values of CMP, EAI decreased with increasing peptide concentration (Figure 3.1.a,b). Increased protein concentration significantly reduces the EAI values. Proteins have good emulsifying properties as long as there was no protein aggregation (Smulders, 2000). Incease in concentration may lead to aggregation of CMP and worsen emulsification properties. It has been found that the emulsions made with low protein concentration have high coalescence stability that was determined by the surface excess of the droplets (Smulders, 2000). Similar results were found for sweet potato protein, almond, soy protein and wheat gluten (Agyarea et al., 2009, Liu et al., 1999b, Sze-Tao et al., 2000, Guo et al., 2011) and this trend was explained by better diffusion and adsoroption of protein on oil droplets which lead to formation of new droplets at low protein concentration. At higher protein concentration, protein adsorption reduced by limited diffusion so EAI decreased (Dagorn-Scaviner et al., 1987). There may not be no relationship between emulsion activity and stability (Chobert et al, 1989). A protein/peptide which have good emulsion activity can have a good or poor emulsion stability (Chobert et al., 1989).

(a)



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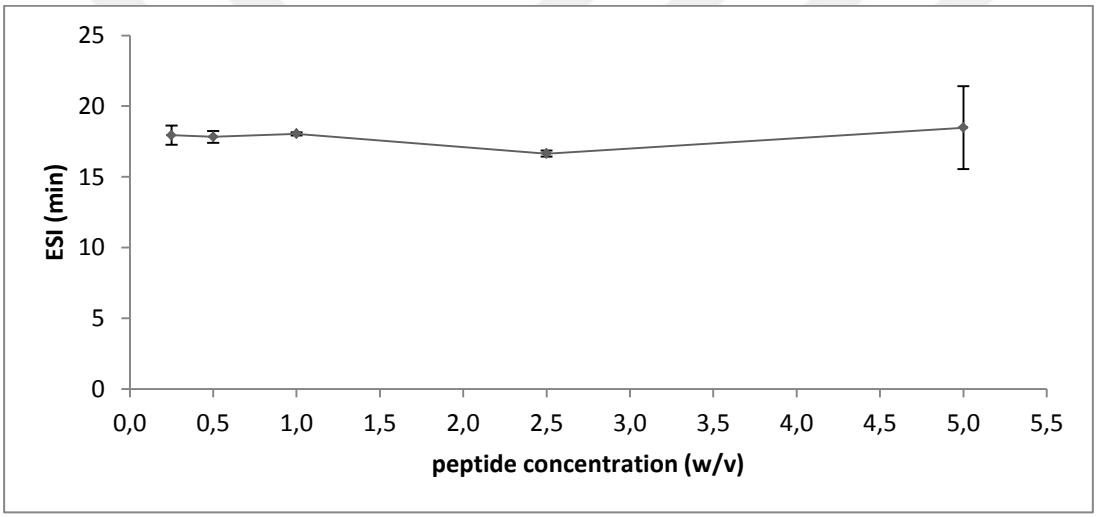


(c)

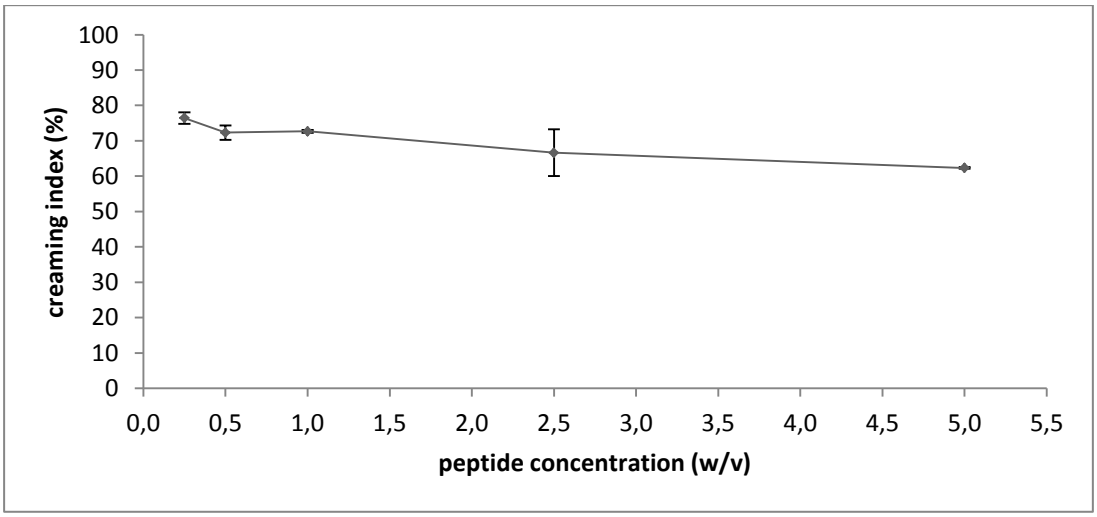


Figure 3. 1: Effect of peptide concentration on EAI (a), ESI (b) values and the creaming index after 24h (c) of CMP in pH 8.0 phosphate buffer, 10 mM NaCl concentration.

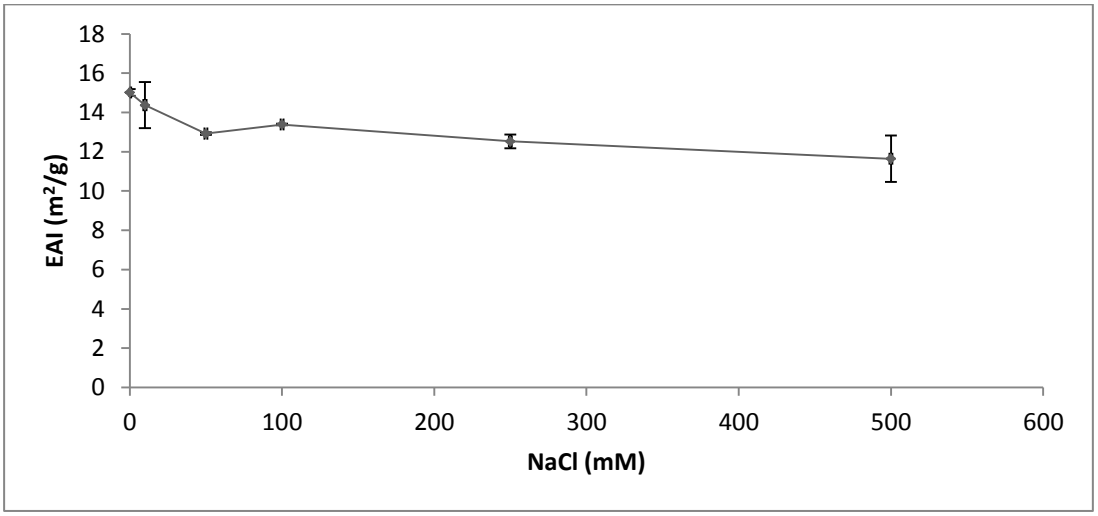
Martin-Diana et al. (2005) studied emulsifying properties of CMP. They analyzed the effects of protein concentration of 5-20%. They reported that protein concentration have a linear positive effect on EAI of CMP and linear and quadratic effect on ESI of CMP values. Protein concentaration used in this study was lower than those used by Martin-Diana et al. (2005). Dickinson & Golding, (1997) reported that high concentration NaCS have low stability and because of non-absorbed NaCS in aquase phase, deplation flocculation is occured. Sun and Gunasekaran (2009) reported that increasing WPI concentration caused decrease in droplet size and increse in negative charges on droplet surface which contibuted to the enhancement of emulsion stability due to steric stabilization and electrostatic repulsion. Ye (2011) also reported MPC and NaCS emulsion with low protein concentration demonstrated a high stability.

Creaming index of CMP emulsions decreased with increase in peptide concentration from 0.25 to 5% (Figure 3.4c). This means that as the peptide concentration was increased, the emulsion stability also increased. Kreuβ et al. (2009) reported that creaming index decreased at CMP concentration above 2%. Kreuβ et al. (2009) also reported that mean oil droplet size of CMP emulsions decreased with an increase in the peptide concentration (0.25-5%(w/w)). They explained this stabilizing effect by formation of more protein film and increase in viscosity with increasing protein concentration. In this study, even though ESI values did not change significantly, creaming values decreased with increasing protein concentration. As a conclusion; although higher peptide concentration has negative effect on EAI values, it affects emulsion stability of CMP and WPI stabilised emulsions positively.

3.1.2 Effect of ionic strength

Changes in EAI, ESI and creaming index of CMP by ionic strength are shown in Figure 3.2a-c. The EAI of emulsions without added NaCl and with 10 mM NaCl were significantly higher than the ones with more NaCl. ESI value without NaCl was found similar to that obtained with 10 mM NaCl. Further addition of NaCl up to 250 mM caused reduction in ESI but it increased again at 500 mM NaCl.

(a)



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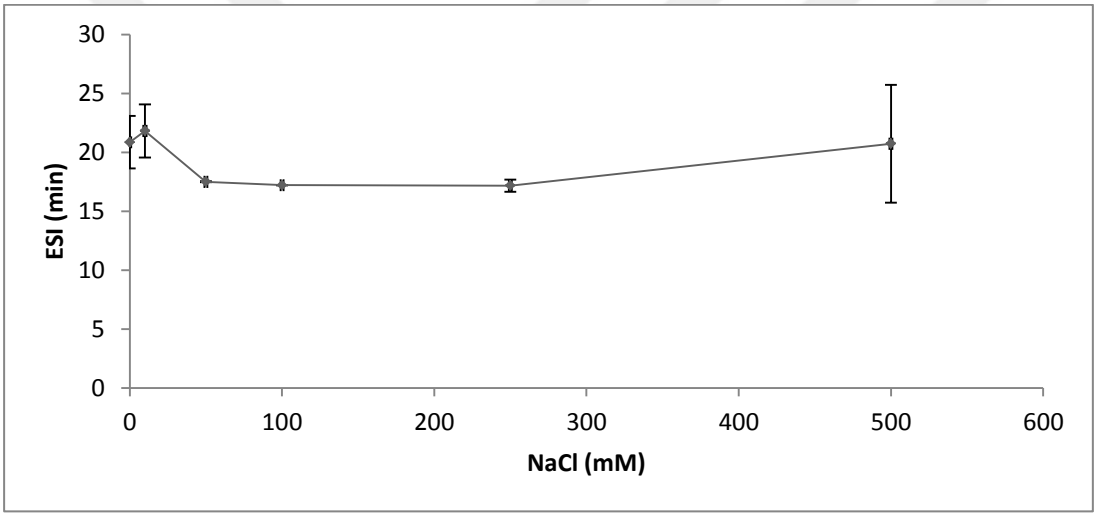


(c)

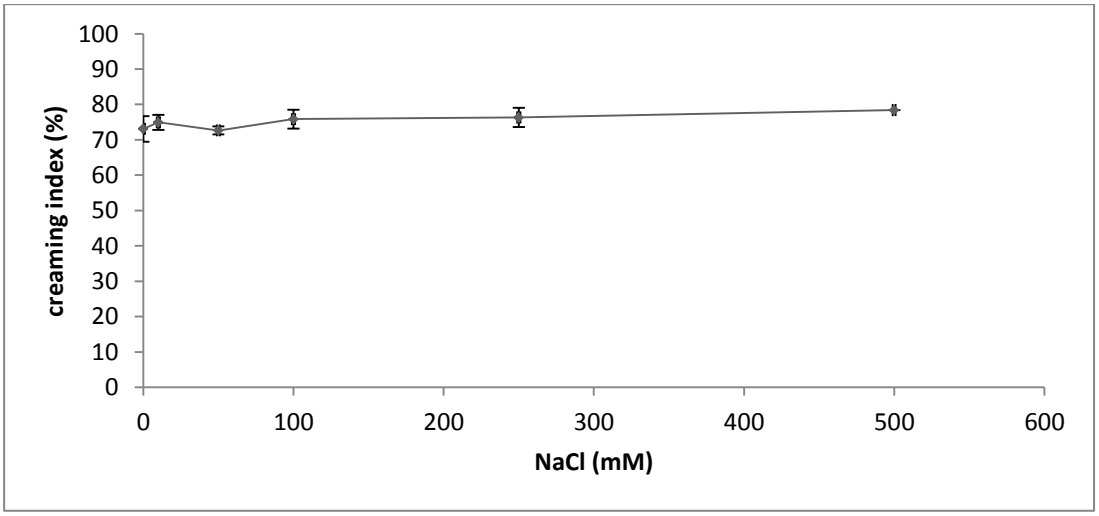


Figure 3. 2: Effect of ionic strength on EAI (a), ESI (b) values and creaming index after 24h (c) of CMP in pH 8.0 phosphate buffer at 0.5% (w/v) concentration.

Martin-Diana et al. (2005) reported no significant effect of added NaCl upto 700 mM on the EAI but a significant quadratic effect on ESI values of CMP. The surface properties of protein or peptides are severely affected by ionic strength because of changes in surface charge (Kreuβ et al. 2009). The net surface charge is decreased by increasing ionic strength and therefore the repulsive forces between single molecules and oil droplets decrease as well (Kreuβ et al. 2009). Reduction in surface charge might lead to more protein-protein interactions and less protein-oil interactions that could effect ESI. On the other hand, aggregation of proteins at high level of NaCl might stabilize the emulsion by increasing viscosity.

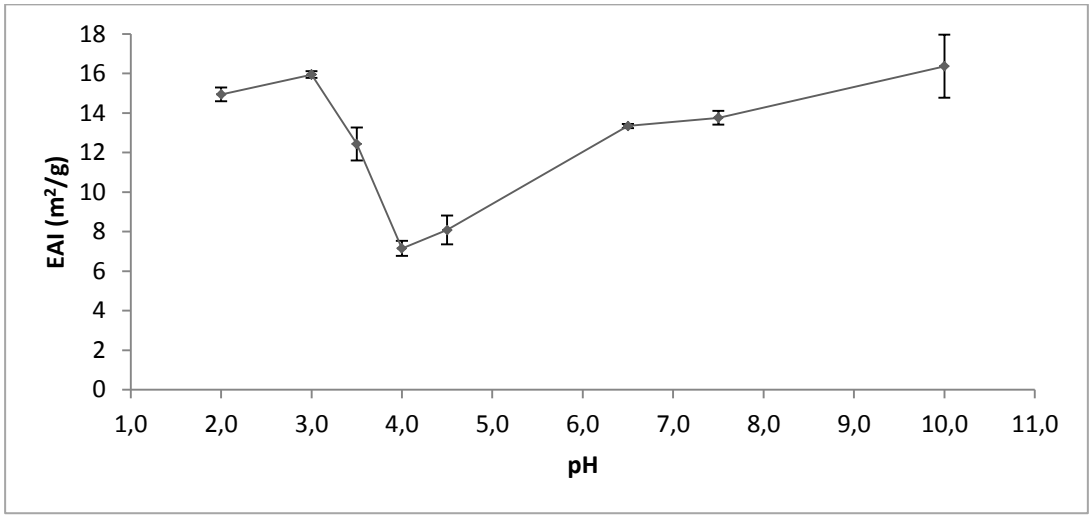
Ionic strenght between 0-500 mM NaCl had no remarkable effect on creaming index of CMP emulsions. Kreuβ et al. (2009) also reported that creaming index of gCMP was not affected by ionic strength but the emulsions of aCMP showed an increased creaming index up to 300 mM NaCl. This was explained by the loss of electrostatic repulsion in the case of gCMP. They interpreted the results according to presence or absence of glycan side chain.

3.1.3 Effect of pH

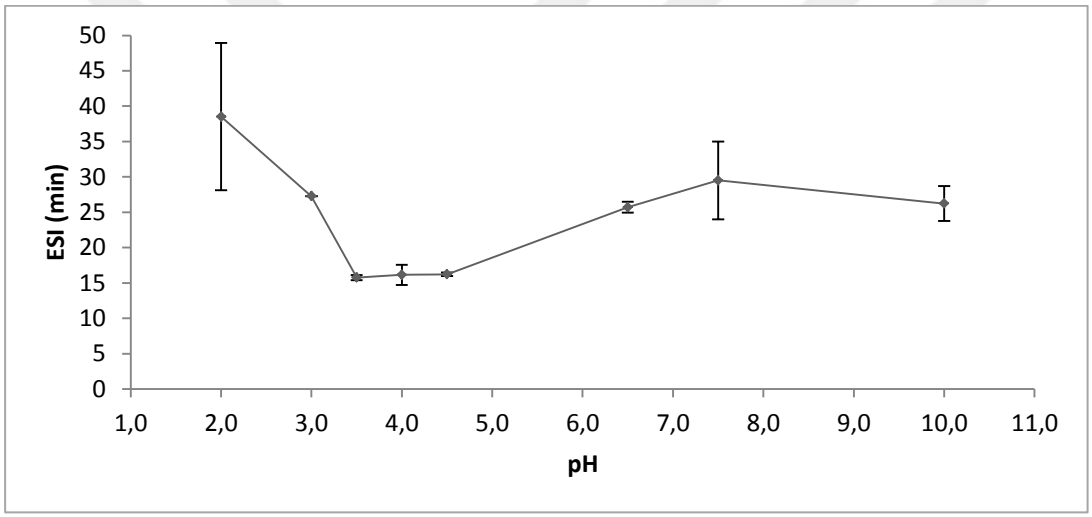
The pH has major influence on the surface and emulsifying properties of proteins/ peptides due to its effect on net surface charge (Kreuβ et al. 2009). The isoelectric point (pI) is the point along the pH scale at which a molecule carries no net charge. Around the pI, the electrostatic repulsive forces are decreased and the droplet surfaces are electrically charged at the pH away from pI of the protein. If the pH value is higher than pI, the molecule is positively charged but when pH is lower than pI, the molecule is negatively charged. Around the pI, van der Waals forces get to be dominant because of reducing electrostatic repulsive forces, it leads to increasing on droplet flocculation so reducing the emulsion stability. The emulsions are stable at pH values adequately above or below the pl, due to the possesing stronger electrostatic repulsion force (Hunt & Dalgleish, 1994).

CMP is an acidic peptide with pI between pH 3-4, higly soluble and heat stable (Thomä-Worringer et al. 2006). Because of having high amount of acidic amino acid side chains, its pI is in acidic range. In addition CMP does not have a single pI because of heterogeneity (Kreuβ et al., 2009).

(a)



(b)



(c)

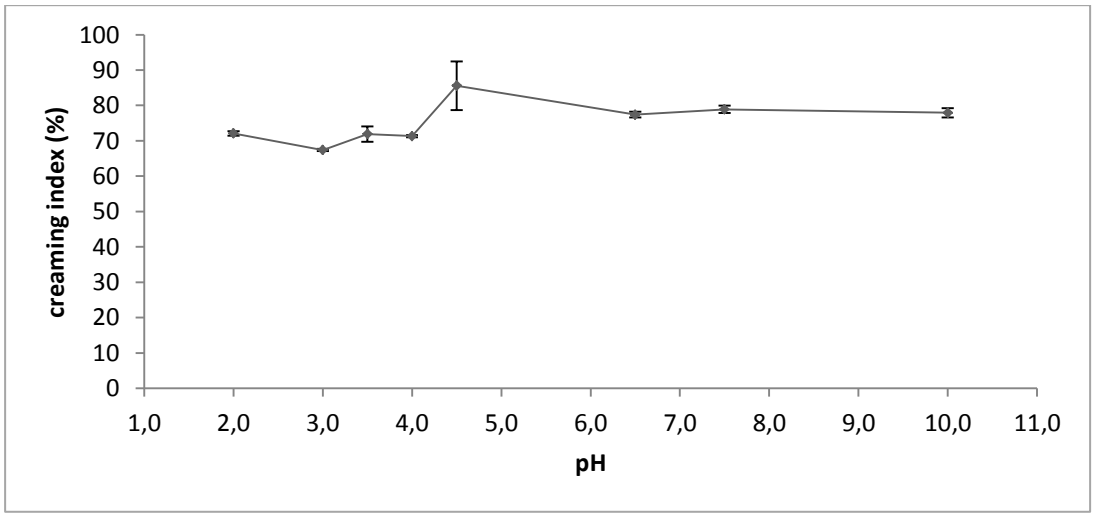


Figure 3. 3: Effect of pH on EAI (a), ESI (b) values and creaming index after 24h (c) of CMP. All emulsions were prepared with 0.5% (w/v) CMP concentration, 0.25 oil fraction and 10 mM NaCl.

EAI and ESI values showed a minimum and creaming index showed a maximum around pH 4.0 in the pH range of 2.5-10 (Figure 3.3a-c). pI value of CMP was reported to be pH 4 and this value is correspond to the value found by Thomä et al. (2006). The maximum values were observed at pH 10 and 2.5. These results are in agreement with those reported by Yang et al. (2016), Chobert et al. (1989) and Kreuβ et al. (2009). Tomczak et al. (2007) reported that CMP isoelectric point close to pH 3.8 and Rojas et al. (2013) reported it near to pH 3.6. Kreuβ et al. (2009) reported isoelectric points of 3.15 for gCMP and 4.15 for aCMP in bulk solution.

3.2 Influence of Tgase on Emulsifying Properties of CMP

Tgase enzyme is used generally in food applications for improving the technological properties of proteins by cross-linking between Gln (Glutamine) and Lys (Lysine) . (Jaros et al., 2006). In the present study, it was used for improving emulsifying properties of CMP. Emulsion were prepared at different pH and properties of emulsions were measured. The effects of the enzyme activity and inactivation application on the emulsion properties were determined.

3.2.1 Effect of Tgase activity on emulsifying properties of CMP

Tgase was added to CMP at activities of 1, 5 and 20 U/g CMP and enzyme was inactivated at 80°C for 2 min. Emulsions were prepared after inactivation and cooling of protein solutions. For elucidating heat application effect (CMP-IHT) on emulsifying properties of CMP, a control sample with no enzyme was prepared and inactivation heat treatment was applied.

As shown in Figure 3.4, using 20 U/g protein improved EAI values of CMP at all pH values. At acidic pH, values of EAI and ESI were greater than the values at higher pH. Among the Tgase-treated samples, only emulsions containing 20 U/g enzyme showed improvement at pH 6.5, while EAI values of samples containing 5 and 20 U/g increased at pH 3.5 and 4.5.

As shown in Figure 3.5, using 20 U/g protein improved ESI values of CMP at pH 3.5 and 4.5. This effect was observed the most at pH 3.5. The usage of the rate of 20 U/g CMP was found to enhance the emulsion stability. EAI and ESI values of emulsions generally showed minimum values at pH 4.5. Foegeding et al. (2006) reported that intermolecular interactions between adsorbed proteins affect the rheological

properties due to formed interfacial films. CMP and whey proteins, especially β-lactoglobulin, strongly interact in solution and form assembled structures due to electrostatic interactions (Martinez et al.,2009 and Martinez et al., 2010). Lam and Nickersen (2015) reported that EAI was found to increase when WPI aggregation was reduced and maximum aggregation was observed at pH 5.0 which is pI of β-lactoglobulin and near the pI of α-lactalbumin (pH 4.5). Based on this findings, pH effect on emulsions can be explained by aggregation of whey proteins because of being near to their pI.

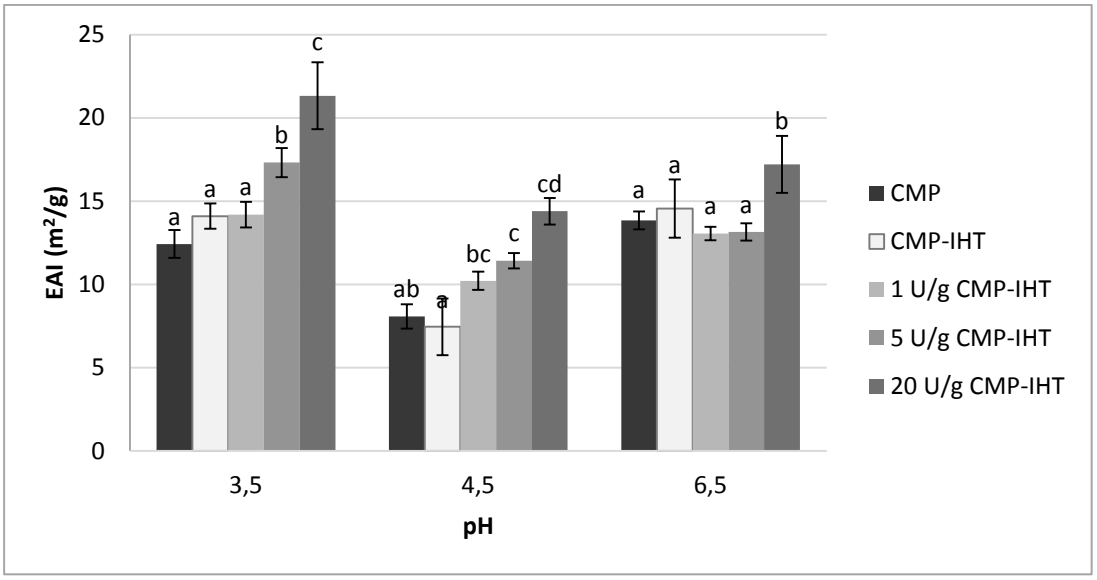


Figure 3. 4: Effect of Tgase enzyme application on EAI values of CMP. All emulsions were prepared with 0.5% (w/v) CMP concentration, 0.25 oil fraction and 10 mM NaCl.

Agyare et al. (2010) reported that Tgase application caused an increase in denaturation temperature of β-lactoglobulin without any major structural changes and altered the pH-stability profile of whey proteins with minimum solubility in the pH range 4.0-4.5. The hydrophilic-hydrophobic balance of the protein surface decreased because of elimination of the positive charge on lysyl residues, and precipitation occured at pH 4.0-4.5. (Agyare et al., 2010). Hunt and Dalgleish (1994) reported a decrease in emulsion stability at pH 4.0 due to changes in the particle size distribution of the WPI emulsions. In the present study, the decrase was especially observed at pH 4.5 which is close to pI values of β-lactoglobulin and α-lactalbumin.

The long-term stability of the CMP emulsions was determined by measuring the creaming index after 24 hours. As seen in Figure 3.6; there was no difference in the creaming index of the samples at pH 6.5. At pH 3.5 and 4.5, CMP without enzyme

had higher creaming index than the other samples. Surprisingly, heat treated sample without enzyme had lower creaming index value than unheated CMP sample. This might be related to denaturation and limited aggregation of some whey proteins in CMP isolate. Heat treatment applied after the enzyme treatment was effective in reducing the creaming index. Dickinson and Hong (1994) reported that heating β-lactoglobulin to 70°C enhances adsorbed layer surface viscoelasticity and emulsion stability improves due to decrease in coalescence rate of β-lactoglobulin-stabilized emulsions (Das & Kinsella, 1990).

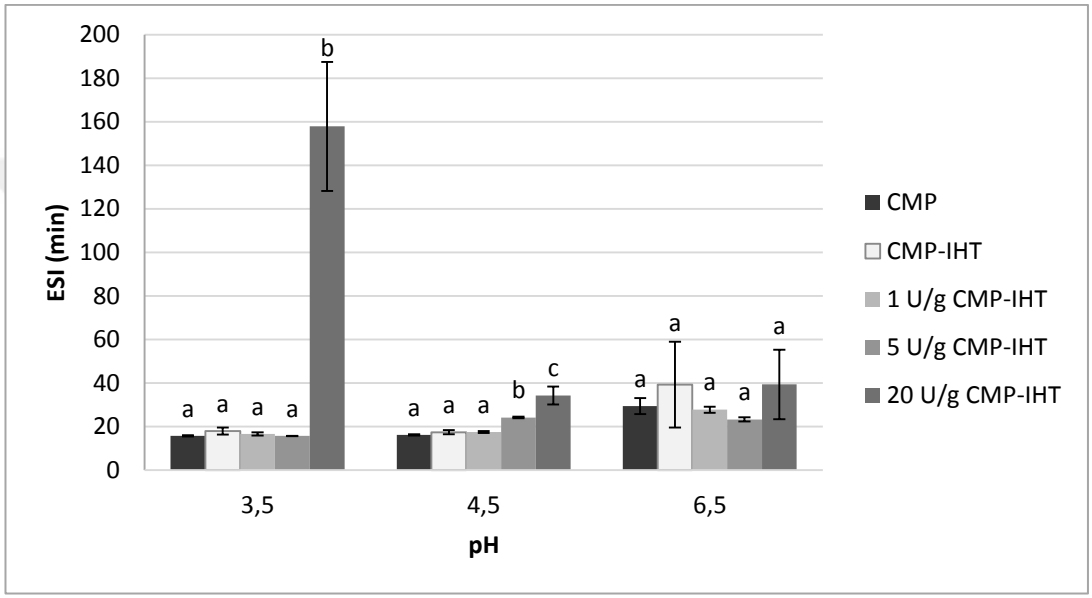


Figure 3. 5: Effect of Tgase enzyme application on ESI values of CMP. All emulsions were prepared with 0.5% (w/v) CMP concentration, 0.25 oil fraction and 10 mM NaCl.

The emulsion capacity denotes the maximum amount of oil that is emulsified under specified conditions by a standard amount of protein (Pearce & Kinsella, 1978). It is defined as the amount of oil that can be emulsified by a standard amount of protein under specific conditions (mL oil/g protein). Application of Tgase to CMP at activity of 1 U/g CMP increased the emulsion capacity (Figure 3.7). However, the application at higher activity of enzyme did not affect the emulsion capacity positively. In addition, it was observed that the enzyme inactivation heat treatment for 80^0C/2 min applied to CMP reduced the emulsion capacity. This result might be related to denaturation of some whey proteins present in the CMP isolate and reduction in their emulsification capacity (Demetriades & McClements, 1998).

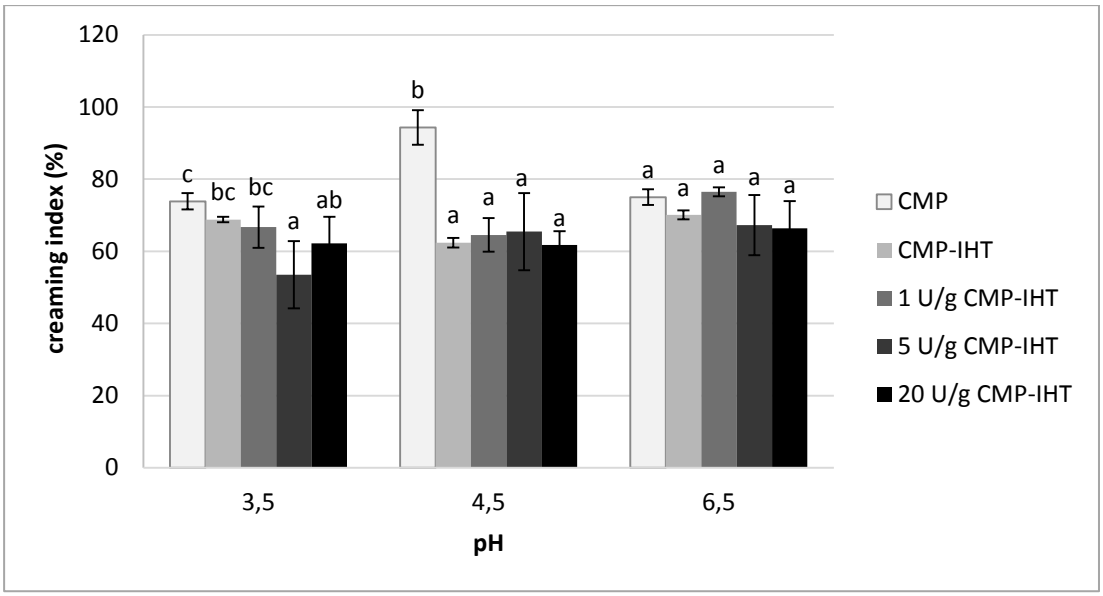


Figure 3. 6: Tgase effect on creaming index after 24h of CMP treated with different enzyme units. All emulsions were prepared with 0.5% (w/v) CMP concentration, 0.25 oil fraction and 10 mM NaCl.

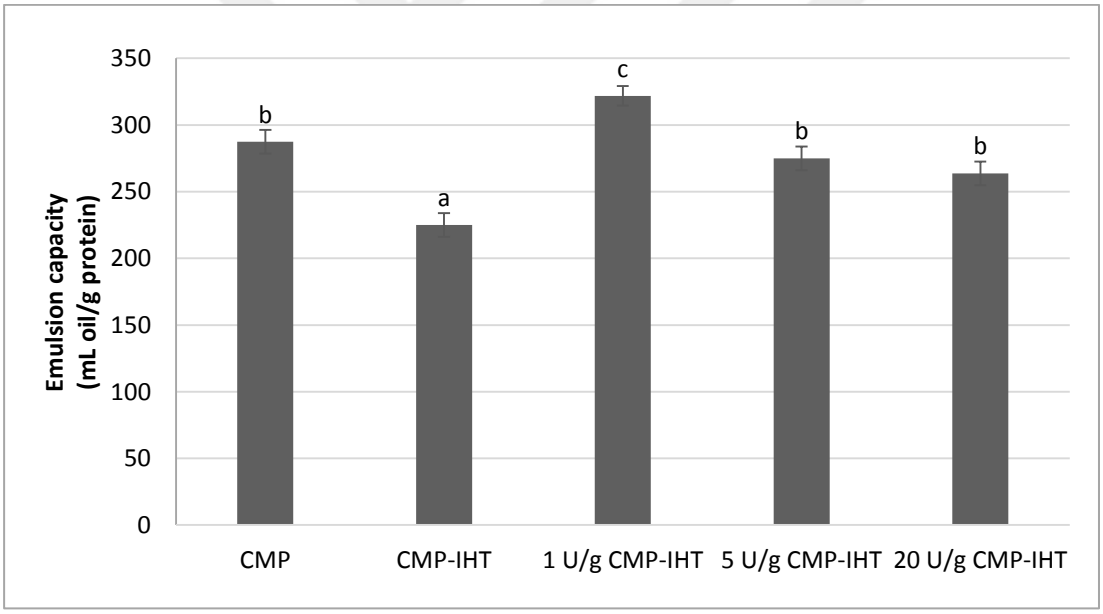


Figure 3. 7: Tgase effect on emulsion capacity of CMP treated with different enzyme units. Each emulsions were prepared with 2% (w/v) peptide concentration (pH 6.7).

3.3.2 Effect of inactivation heat treatment on emulsifying properties of CMP

For determining the inactivation heat treatment effect on emulsifying properties, emulsion samples were prepared from enzyme-treated CMP with or without application of inactivation heat treatment.

As seen Figure 3.8, it was determined that only heat treatment had no significant effect on EAI values. At pH 3.5, the enzyme was found to enhance emulsion activity independently of heat treatment. Martinez et al. (2010) reported that CMP could

form a gel at pH 3.5 and there were interactions between CMP and β-lactoglobulin at this pH because of the difference in their electrical charges. Interactions between CMP molecules and between CMP and β-lactoglobulin could have caused the increase in EAI. CMP has net negative charge at acidic pH (Martinez et al., 2009) and β-lactoglobulin has positive charge. Enhanced steric stabilization or stronger cohesive interaction may be the cause of the enhanced emulsion stability (Martinez et al., 2009).

At pH 6.5, the enzyme-treated sample that the inactivation heat treatment applied had higher EAI than those of the other samples. This showed that application of both enzyme and inactivation heat treatment improved emulsification by CMP.

Native α-lactalbumin can be cross-linked by Tgase directly and although β-lactoglobulin is not an active substrate of Tgase, it can be active after heat treatment because of denaturation (Rodriguez-Nogales, 2006). Prabakaran and Damodaran (1997) reported that when thermal denaturation and aggregation of β-lactoglobulin reaches a critical level, then polymerization reaction was started by Tgase. Færgemand et al. (1998) reported that limited cross-linking of milk proteins reduced the droplet size of oil droplets of emulsion compared to that obtained by excessive cross-linked and native proteins.

Truong et al. (2004) reported that extensive intra- and inter-chain bonds resulted from Tgase crosslinking caused formation of large molecules which are stable against heat treatment and it caused an increase in denaturation temperature of WPI. Wang et al. (2013) also reported that Tgase treatment caused increase zeta potential and surface hydrophobicity of WPI. These changes reduced protein aggregation resulted from increasing denaturation temperature. As a result, decreased aggregation of cross-linked denatured whey proteins may improve emulsion properties at pH 6.5. CMP cannot form gel at pH 6.5 which might reduce its contribution to emulsion (Martinez et al., 2010).

The use of 20 U Tgase/g CMP increased the emulsion stability at pH 3.5 in the heat-treated sample (Figure 3.9). Although there was an increase in ESI values with enzyme and heat treatment, this increase was not found statistically significant at pH 6.5. F**e**rgemand et al. (1998) and Hinz et al. (2007) reported that limited crosslinking with Tgase of milk proteins improved their emulsion stability against coalescence and flocculation.

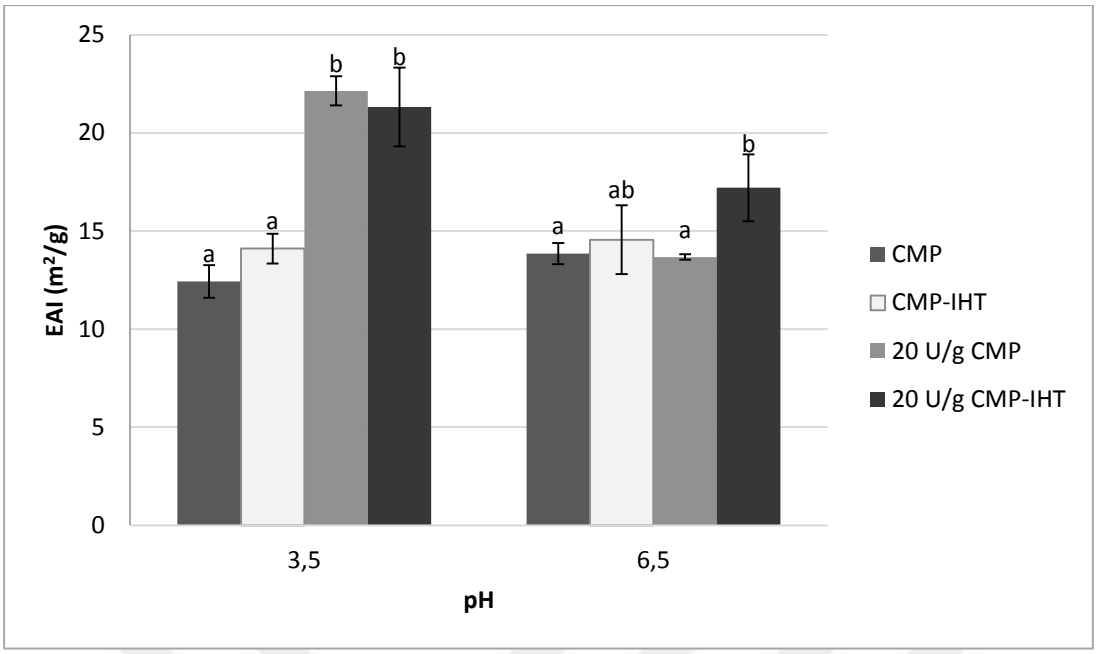


Figure 3. 8: Identifying heat treatment on EAI values of CMP. All emulsions were prepared with 0.5% (w/v) CMP concentration, 0.25 oil fraction and 10 mM NaCl.

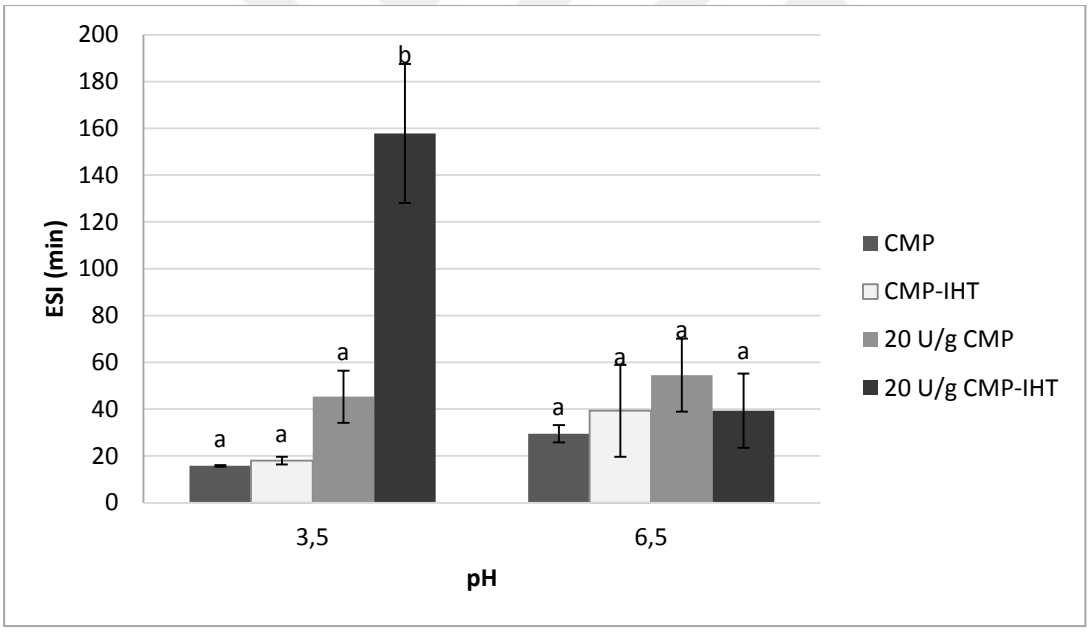


Figure 3. 9: Identifying heat treatment on ESI values of CMP. All emulsions were prepared with 0.5% (w/v) CMP concentration, 0.25 oil fraction and 10 mM NaCl.

Both heat-treated and enzyme-treated samples showed less creaming than the enzyme-free sample (Figure 3.10). At pH 6.5, there was no effect of enzyme and heat treatment.

Enzyme-treated CMP had higher emulsion activity and stability compared to CMP at pH 3.5. In addition to enzyme, inactivation heat treatment also improved emulsifying properties of CMP. On the other hand, at pH 6.5, while the effects of enzyme and

heat treatment were not significant on emulsion stability, they improved the emulsion activity of CMP.

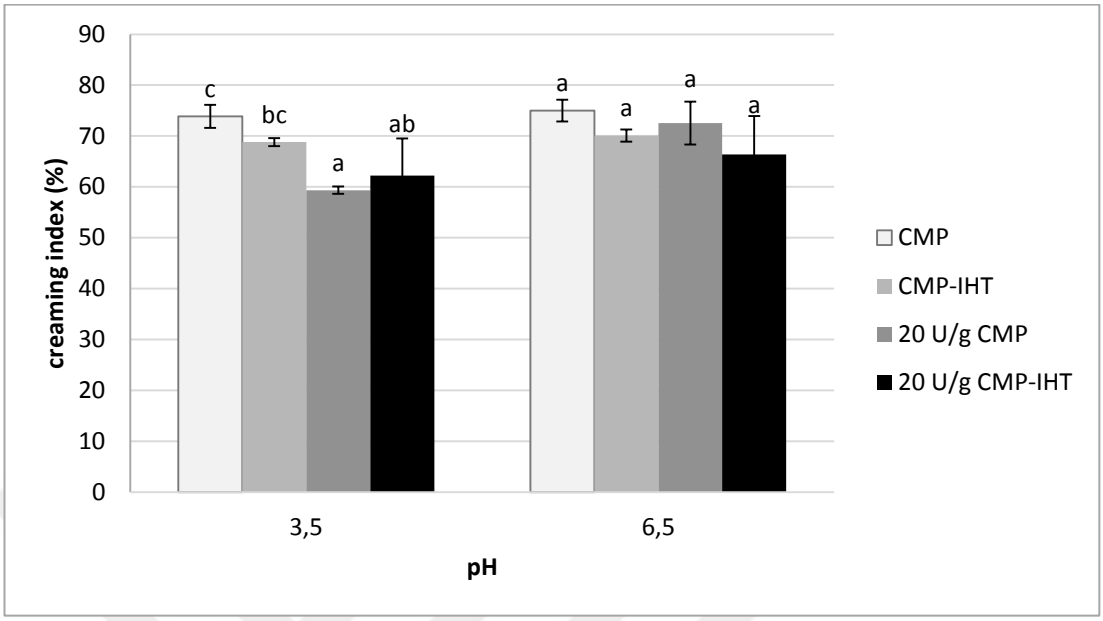


Figure 3. 10: Identifying heat treatment on creaming index values of CMP. All emulsions were prepared with 0.5% (w/v) CMP concentration, 0.25 oil fraction and 10 mM NaCl.

3.3.3 Comparison of emulsion properties of treated-CMP with other milk proteins

Protein solubility is an important prerequisite fo film formation because of criticality of rapid migration and adsorption at the interface (Chobert et al., 1989). Proteins with smaller mass are more effective in producing small droplets and so peptides are expected to be more effective in producing small droplets and better emulsions than proteins (Williams et al., 2002). NaCS and whey protein have higher emulsifying ability than that of aggregated milk protein products (Singh & Ye, 2008; Ye et al., 2000).

Hydrolysis of protein can be improved or worsen emulsion properties depending on degree of hydrolysis. Euston et al. (2001) and Singh & Dalgleish (1998) found that emulsifying properties decrease greatly if the proportion of small peptides increares. In general terms, limited hydrolysis improves emulsifiying properties because of formation of more flexible amphiphilic peptides but emulsifying activity and stability diminish because of extensive hydrolysis (Flanagan & Fitzgerald, 2003). Luo et al. (2014) reported that hydrolysed NaCS showed improved emulsification properties. Hu et al.(2010) reported that while Tgase crosslinking and proteolysis could improve

emulsifying properties of peanut protein, proteolysis followed by Tgase crosslinking showed more improvement. Flanagan and FitzGerald (2003) reported that crosslinking with Tgase of NaCS after hydrolysis resulted in significant improvements in EAI at low pH. On the other hand, extensive crosslinking with Tgase lead to polymerization and it is important to control the polymerization extent for modifying the properties of proteins with Tgase application. Liu and Damodaran (1998) reported that increasing degree of polymerization of β-casein caused improvement in the storage stability of emulsions, whereas decrease in EAI values.

CMP (enzyme-free), 20 U/g enzyme-treated CMP samples and other emulsifiers (NaCS and WPI) had similar EAI values at their own native pH (pH 7.1 for NaCS, pH 6.4 for WPI and pH 6.7 for CMP) (Figure 3.11). On the other hand, 20 U/g enzyme-treated and inactivation heat treatment applied CMP samples had higher EAI values.

In previous studies, NaCS was found to have higher EAI and ESI values than CMP and WPI, respectively (Kinsella et al., 1978; Kreuβ et al., 2009). On the other hand, Martin-Diana et al. (2005) reported higher EAI and ESI values for WPC than CMP.

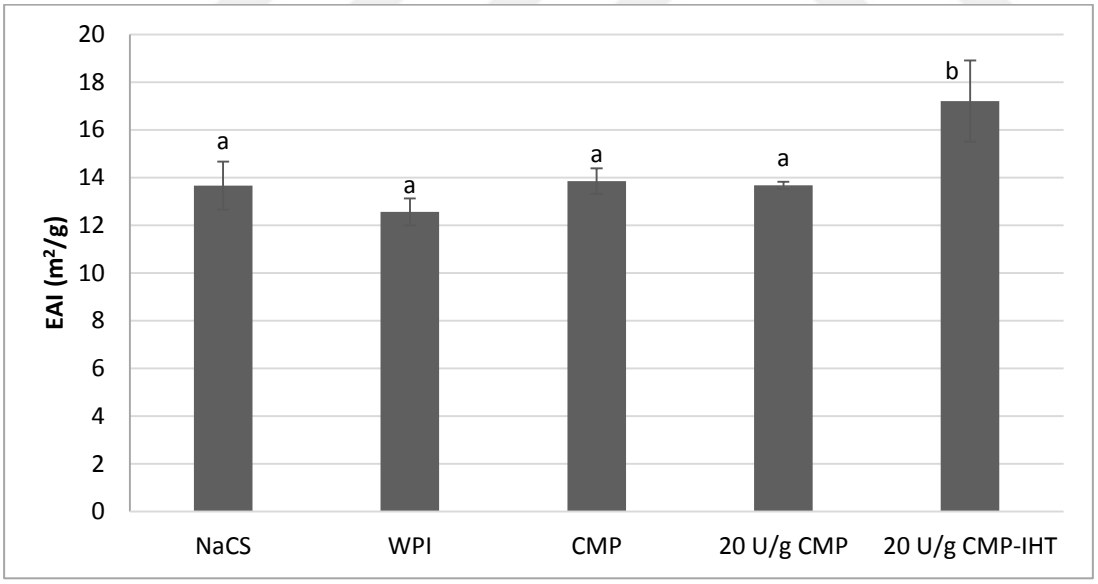


Figure 3. 11: The comprasion of enzyme-treated CMP EAI with other emulsifiers. All emulsions were prepared with 0.5% (w/v) CMP concentration, 0.25 oil fraction and 10 mM NaCl at their own native pH.

As shown in Figure 3.12, the ESI of the enzyme-treated CMP was found to be higher than those of the enzyme-free CMP and other emulsifiers. It means that enzyme application improves the emulsion stability.

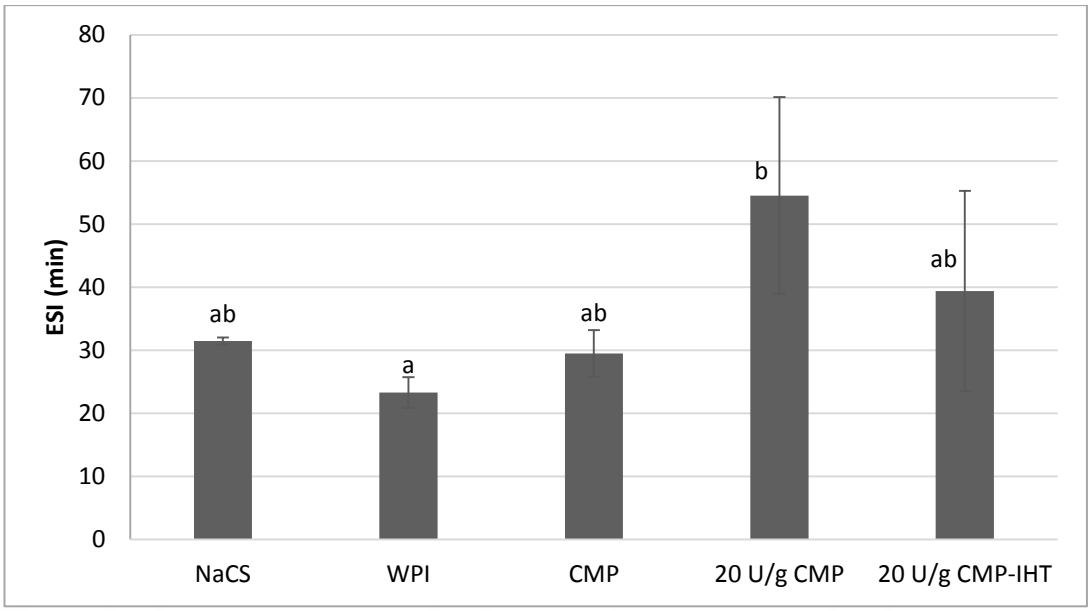


Figure 3. 12: The comprasion of enzyme treated CMP ESI with other emulsifiers. All emulsions were prepared with 0.5% (w/v) CMP concentration, 0.25 oil fraction and 10 mM NaCl at their own native pH.

When comparison was made based on the creaming index values, the creaming index of enzyme-treated CMP sample was lower than those of enzyme-free CMP and other emulsifiers (Figure 3.13).

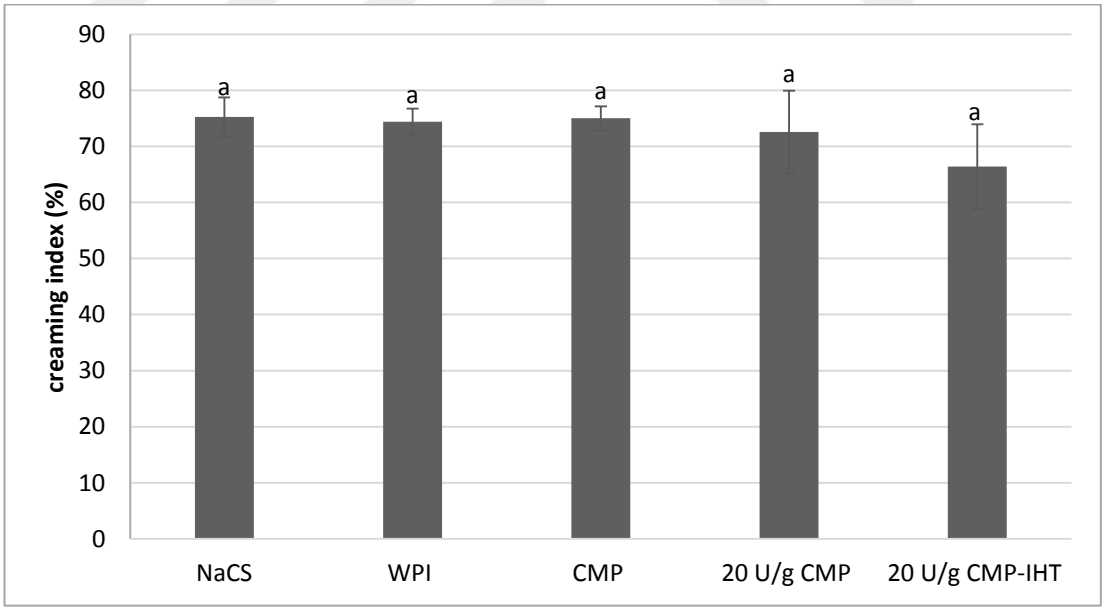


Figure 3. 13: The Comprasion of Enzyme Treated CMP creaming index after 24h values with other emulsifiers. All emulsions were prepared with 0.5% (w/v) CMP concentration, 0.25 oil fraction and 10 mM NaCl at their own native pH.

CMP has higher emulsion capacity value than WPI and NaCS and using enzyme decreased the emulsion capacity (Figure 3.14). Enzyme-treated CMP had higher

emulsion activity and stability. In conclusion, enzyme treatment improved EAI, ESI and creaming index but it was ineffective on emulsion capacity.

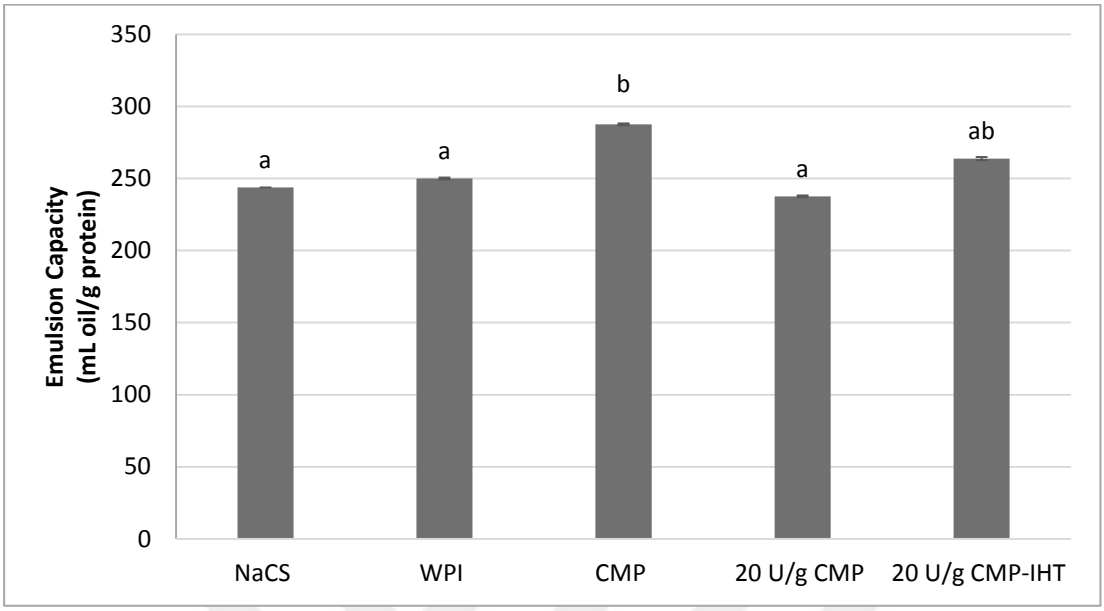


Figure 3. 14: Comprasion emulsion capacity values of CMP and other emulsifiers. Each emulsions were prepared with %2 (w/v) peptide concentration at their own native pH.

4. CONCLUSION

Tgase treatment and pH had significant effects on emulsion properties of CMP. Application of Tgase at a level of 20 U/g CMP was found to improve emulsion activity and stability especially at pH 3.5. In addition to activity of enzyme, heat treatment applied for inactivation of enzyme was also found to improve the emulsification properties of CMP. This effect can be explained by the presence of other whey proteins in the CMP isolate which can be partially denatured and cross-linked by Tgase. Interactions between Tgase cross-linked CMP molecules and partially denatured and Tgase cross-linked whey proteins possibly strengthened emulsions prepared with the CMP isolate.

Emulsifying properties of CMP were found to be similar to those of commonly used milk protein emulsifiers including NaCS and WPI. Application of Tgase at a level of 20 U/g CMP with inactivation heat treatment was found to improve emulsion activity and stability but not emulsion capacity.

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CURRICULUM VITAE

Name Surname **: Gülnaz ARTAN**

Place and Date of Birth **: İstanbul/20.11.1990**

E-Mail **: glnaz.artan@gmail.com**

EDUCATION **:**

- **B.Sc.** **:** 2014, Ankara University, Engineering Faculty, Food Engineering Department

PROFESSIONAL EXPERIENCE AND REWARDS:

- Research & Development Assistant, Pepsico (09.2016-…)

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Kocaman, E., Artan, G., Dastjerd A. K., Akyilmaz, M.** 2016: Influence of pH on Physicochemical Properties of Caseinomacropeptide.-11th International Conference on Protein Stabilisation, June, 2016 İstanbul, Turkey. (Poster Presentation).
- **Kocaman, E., Artan, G., Akyilmaz, M. 2016**: Cross linking of Caseinomacropeptide by Transglutaminase – 11th International Conference on Protein Stabilisation, June, 2016 İstanbul, Turkey. (Poster Presentation).
- **Artan, G., Çınar, A., Eryeşil, C., Candoğan, K.** 2014: Effects Of Addition Guar Gum And Pea Fiber On Rheological Properties Of Salt-Reduced Chicken Meat Protein Gels – 5th Food Engineering Congress - Bolu İzzet Baysal University, April, 2014 Bolu**,** Turkey. (Poster Presentation).