DEVELOPMENT OF HYPOALLERGENIC FOOD PRODUCT AND IN-HOUSE ELISA TO DIFFERENTIATE DISTINCT SEVERITY OF COW'S MILK ALLERGY AS BISCOTTI, BAKED, FERMENTED AND PASTEURIZED MILK REACTIVE AND TOLERANT ONES

by

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Koc University

Graduate School of Health Sciences

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ABSTRACT

Development of Hypoallergenic Food Product and in-House ELISA to Differentiate Distinct Severity of Cow's Milk Allergy as Biscotti, Baked, Fermented and Pasteurized Milk Reactive and Tolerant Ones

Duygu Yazici

Doctor of Philosophy in Cellular and Molecular Medicine, September 14, 2021

Avoidance of culprit allergen is still the mainstay of treatment in food allergy. However, the difficulties such as adherence to elimination diet or accidental allergen exposure have increased the research in food allergy treatment in recent years. Oral immunotherapy (OIT) is a treatment model of food allergy, and it is defined as the administration of the allergen in slowly increasing doses at certain time intervals until the daily-consumed dose is reached. Although immunotherapy with baked milk products seems safe, anaphylaxis can occur in some patients. Therefore, developing a hypoallergenic product that has less allergenicity than baked milk would open a new horizon in the desensitization treatment of cow's milk allergy. Here, we mainly aimed to produce a more hypoallergenic product than conventional cake, develop indirect in-house ELISA to check the tolerance status with milk, cheese, and yogurt, to check IgE reactivity of patients' sera via western blotting, indirect in-house ELISA and basophil activation test (BAT).

First, a hypoallergenic product candidate was developed by baking the cake twice, which was called "biscotti". The proteins were isolated from the new hypoallergenic product (biscotti), conventional baked milk (cake) and pasteurized milk and the total protein concentration was determined by Bradford assay. The protein content for each product was studied by SDS-PAGE and proteomics. Serum samples were collected from patients with milk allergy who applied to the Pediatric Allergy Division in Koc University School of Medicine. Milk specific IgE (sIgE) binding assays were performed by Western blotting, indirect in-house ELISA by using patients' sera and BAT by using patients' whole blood.

In the band intensity analysis, the casein band intensity was observed lower in the hypoallergenic product candidate than in the conventional cake ($p=0.014$). Proteomics analysis showed that the intensities of each casein fraction and β-lactoglobulin were decreased as the cooking time increased in all milk products (biscotti 1h, biscotti 2h and biscotti 3h, conventional cake). The low binding capacity of milk sIgE to the newly developed hypoallergenic product compared to the conventional cake in the sera of

children with milk allergy was shown first by western blotting (p=0.0012) and then by indirect in-house ELISA (p=0.0001). Biscotti supernatant was used as a stimulating allergen in BAT and the results indicated that it could be used for further experiment before oral food challenge test.

In this study, the low allergenicity of the newly developed hypoallergenic product "biscotti" has been demonstrated by *in vitro* experiments. Our hypoallergenic product could be a safe and useful treatment option in patients with severe milk allergy who are reactive to baked milk. We aim to confirm the low allergenicity of this product by in vivo experiments (oral food challenge tests) and show the effects in tolerance induction in the further steps.

OZETCE

Hipoalerjenik süt ürünü ve inek sütü alerjisi şiddetini reaktif ve toleranslı bireylerde ayırt etmek için biskoti, pişmiş, fermente ve pastörize süt kullanarak dolaylı in-house ELISA geliştirilmesi

Duygu Yazıcı

Hücresel ve Moleküler Tıp Doktora 14 Eylül 2021

Besin alerjilerinde tetikleyici ajanlardan kaçınmak hala tedavinin temel dayanağıdır. Ancak eliminasyon diyetine uyum veya kazara alerjen maruziyeti gibi zorluklar, son yıllarda besin alerjisi tedavisinde araştırmaları artırmıştır. Oral immünoterapi (OIT), alerjenin günlük tüketilen doza ulaşılıncaya kadar belirli zaman aralıklarında artan dozlarda uygulanması olarak tanımlanır. Fırınlanmış süt ürünleri ile immünoterapi güvenli görünse de bazı hastalarda anafilaksi oluşabilir. Bu nedenle, fırınlanmış süt ürünü ‘’kek’’ten daha az alerjeniteye sahip hipoalerjenik bir ürün geliştirmek, inek sütü alerjisinin duyarsızlaştırma tedavisinde yeni bir ufuk açabilir. Bu çalışmamızda esas olarak geleneksel kekten daha hipoalerjenik bir ürün yapmak ve çeşitli süt ürünlerinekarşı tolerans durumunu belirlemek için dolaylı in-house ELISA testi geliştirmeyi hedefledik., Western blot, dolaylı in-house ELISA ve bazofil aktivasyon testi (BAT) ile süt alerjili hastalarda farklı şiddetlerde süt alerjisini IgE reaktivitesi ile karşılaştırabilmek amaçlandı. İlk olarak kek iki kez pişirilerek “biskoti” adı verilen hipoalerjenik bir ürün adayı geliştirildi. Proteinler, aday ürün (biskoti) kek ve pastörize sütten izole edildi ve toplam protein konsantrasyonu Bradford tahlili ile belirlendi. Tüm ürünler için farklı süt protein içerikleri SDS-PAGE ve proteomiks ile gösterildi. Koç Üniversitesi Hastanesi Çocuk Alerji Bölümüne başvuran süt alerjisi olan hastalardan serum örnekleri alındı. Süt spesifik IgE (sIgE) bağlanma deneyleri Western blot, dolaylı in-house ELISA ile hasta serumları kullanılarak ve BAT hastaların tam kanları kullanılarak yapıldı.

Farklı süt proteinleri bant yoğunluğu analizinde, hipoalerjenik ürün adayında kazein bant yoğunluğunun keke göre daha düşük olduğu gözlendi ($p=0.014$). Proteomiks analizler, tüm süt ürünleri için (biskoti 1 saat, biskoti 2 saat ve biskoti 3 saat, geleneksel kek) pişirme süresi artıkça tüm kazein fraksiyonlarının ve β-laktoglobulinin yoğunluğunun azaldığını ortaya koydu. Normal keke kıyasla yeni geliştirilen hipoalerjenik ürüne (biskoti) karşı süt alerjisi olan çocukların serumlarındaki süt sIgE'lerinin düşük bağlanma kapasitesi, önce western blotlama ($p=0.0012$) ve ardından dolaylı in-house ELISA ($p=0.0001$) ile gösterildi. Süt, peynir ve yoğurt ile yapılan dolaylı in-house ELISA

deneylerinde, toleranslı hastaların süt, peynir ve yoğurt için OD değerlerinin reaktif olanlara göre düşük olduğu bulundu. Son olarak, BAT için biskoti üst sıvısı uyarıcı alerjen olarak kullanıldı ve sonuçlar bu testin besin yükleme testi testi öncesi ileri deneylerde kullanılabileceğini gösterdi.

Bu çalışmada yeni geliştirilen hipoalerjenik ürün "biskoti"nin düşük alerjenitesi *in vitro* deneylerle kanıtlanmıştır. Hipoalerjenik ürünümüz, şiddetli süt alerjisi olan ve pişmiş süte tepki gösteren hastalarda güvenli ve faydalı bir tedavi seçeneği olabilir. Bu ürünün düşük alerjenitesini *in vivo* deneylerle (besin yükleme testleri) doğrulamayı ve sonraki adımlarda tolerans indüksiyonundaki etkilerini göstermeyi amaçlıyoruz.

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ABBREVIATIONS

APS	Ammonium persulfate
BAT	Basophil activation test
BSA	Bovine serum albumin
BM	Breast milk
CM	Cow’s milk
CMPA	Cow's milk protein allergy
CBB	Coomassie Brilliant Blue G-250
CD	Cluster of differentiation
CRD	Component Resolved Diagnosis
DBPCFC	Double-blind placebo-controlled food challenge
DTT	Dithiothreitol
ECL	Enhanced chemiluminescent
ELISA	Enzyme-Linked ImmunoSorbent Assay
FA	Food allergy
FcεRI	Fragment crystallizable epsilon receptor 1
FDR	False discovery rate
FITC	Fluorescein isothiocyanate
fMLP	N-formylmethionine leucyl-phenylalanine
FPE	Food protein induced enteropathy
FPIAP	Food protein induced allergic proctocolitis
FPIES	Food protein induced enterocolitis
FSC-A	Forward scatter area
FSC-H	Forward scatter height
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
HSA	Human serum albumin

IL	Interleukin
ILC	Innate lymphoid cells
KU-FA	Koç University - Food Allergy
LC-qTOF-MS	Liquid chromatography–hybrid quadrupole time-of-flight mass spectrometry
µg	Microgram
ml	Milliliter
NHANES	National Health and Nutrition Examination Survey
OD	Optical density
OFC	Oral food challenge
PBS	Phosphate buffered saline
PE	Phycoerythrin
PI	Protease inhibitor
PVDF	Polyvinylidene fluoride
RT	Room temperature
SBPCOFC	Single-blind placebo-controlled oral food challenge
SDS-PAGE	Sodium Dodecyl Sulphate - Polyacrylamide Gel
sIgE	Specific Immunoglobulin E
SPT	Skin prick test
SSC	Side scatter
TEMED	Tetramethylethylenediamine
TGS	Tris-Glycine-EDTA
Th	T helper
TMB	Tetramethylbenzidine peroxidase
TNF	Tumor necrosis factor
Treg cells	T regulator cells
TSLP	Thymic stromal lymphopoietin

Chapter 1

INTRODUCTION

1.1. Food Allergy

Food allergy (FA) is a major public health issue and a leading cause of morbidity. A food reaction is any aberrant response that occurs after consuming food. Adverse food reactions divided into two categories: immune-mediated and non-immune-mediated. While food intolerance refers to the category of non-immune mediated reactions, FA requires an immune-mediated reaction to be diagnosed. (Figure 1.1).

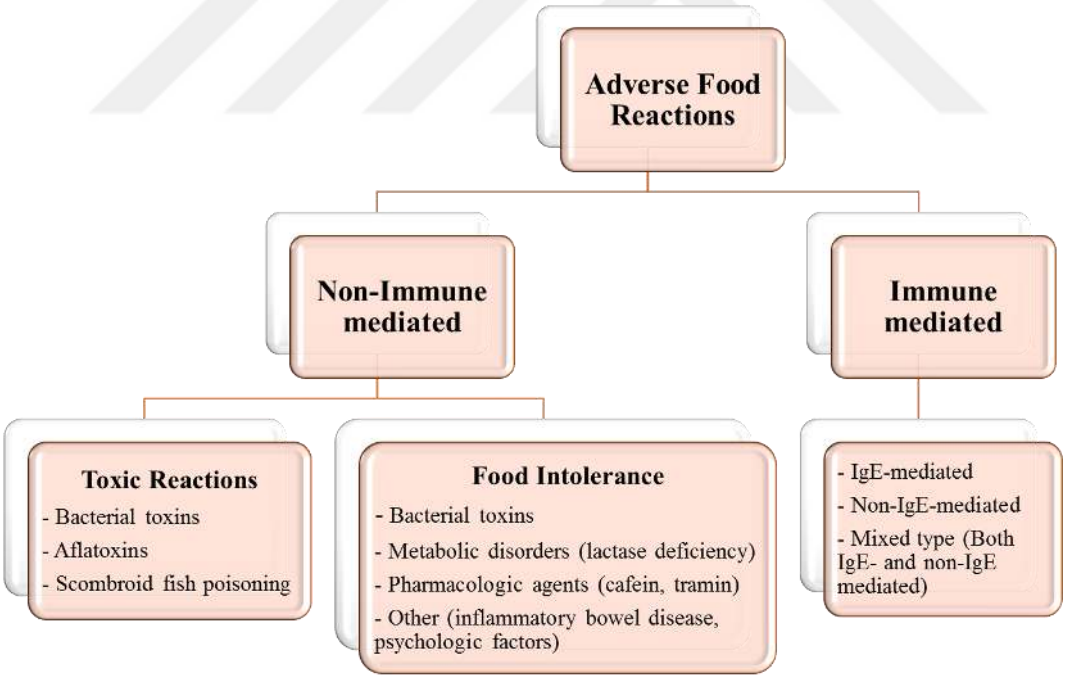

Figure 1.1 Adverse food reactions (Altintas et al., 2017)

The diagnosis of FA has a negative impact on patients' and their families' quality of life, as well as a major financial burden. FA is thought to impact 2% to 10% of the population, according to previous reports (Chafen et al., 2010). FA was detected at a

frequency of 9.7% in adolescents and 6.5% in children between 2007 and 2010, as per data from the National Health and Nutrition Examination Survey (NHANES) (McGowan & Keet, 2013). Cross-sectional studies have reported that the prevalence of FA and anaphylaxis has increased in several countries over the last 10 years (Flom & Sicherer, 2019). Despite the lack of a 10-year interval study in our country, paediatricians agree that the number of patients with FA has increased in the last decade. Egg, milk, hazelnut, peanut, walnut, and wheat allergies are the most common food allergies among children with FA who apply to paediatric allergy clinic in Turkey (Yavuz et al., 2011).

Cow's milk protein allergy (CMPA) is a term used to describe immune system reactions to the proteins found in cow's milk (Johansson et al., 2004; Untersmayr & Jensen-Jarolim, 2006). CMPA is one of the most common food allergies in children constituting 40% of the FAs followed in paediatric allergy clinics. It is estimated that 3.8% of children under five years of age have cow's milk allergy (Flom & Sicherer, 2019). The severity of the reaction and the degree of sensitivity depend on the amount of milk taken, the way it is prepared and milk protein specific IgE levels of the patient. Cow's milk ranks third in food-induced anaphylaxis (10-19%), and in fatal or near-fatal food-induced anaphylaxis (8-15%) (Colver, Nevantaus, Macdougall, & Cant, 2005; Järvinen, Sicherer, Sampson, & Nowak-Wegrzyn, 2008; Pumphrey & Gowland, 2007).

Cow's milk contains approximately 30-35 g/L of cow's milk protein, and the portion that causes allergic reactions is the protein content of the milk (El-Agamy, 2020). Cow's milk is divided into two parts by the effect of chymosin or acidification of milk at pH=4.6: Lactoserum 20% [whey proteins: globular proteins (β-lactoglobulin, α-lactalbumin, immunoglobulins, bovine serum albumin, lactoferrin)] and coagulum 80% (casein). Major cow's milk allergens are casein, β-lactoglobulin and α-lactalbumin (El-Agamy, 2020).

1.2. The Pathogenesis of the Cow's Milk Protein Allergy

Cow's milk protein allergen is first exposed to epithelial cells in the intestine, which release cytokines called "alarmin" such as Interleukin (IL)-25, IL-33, and Thymic stromal lymphopoietin (TSLP) (Berin & Sampson, 2013; Ramsey & Berin, 2021). Overproduction of these cytokines induces $CD103^+$ dendritic cells in the intestinal mucosa to move to lymph nodes, where they induce $CD4^+$ T cells, resulting in the initiation of the immune response. Studies showed that intestinal epithelial cells of mice with food allergies secrete more IL-25 than healthy ones (Schulz et al., 2009). Excessive secretion of IL-25 inhibits T regulator (Treg) cells, which controls immune responses, and causes the onset of Th-2 (T helper) response-dominated allergic inflammation (C. A. Akdis & Akdis, 2009). In recent years, innate lymphoid cells type 2 (ILC2) has been shown to play a role in allergic inflammation. IL-33, IL-25 and TSLP released from skin and gastrointestinal epithelial cells lead to the production of Th2 cytokines (IL-4, IL-5, IL-9 and IL-13) from ILC2 and basophils (Ramsey & Berin, 2021). It has been shown that ILC2s play a role in the pathogenesis of food allergy through the production of Th2 cytokines in the mouse model (Burton et al., 2018) (Figure 1.2).

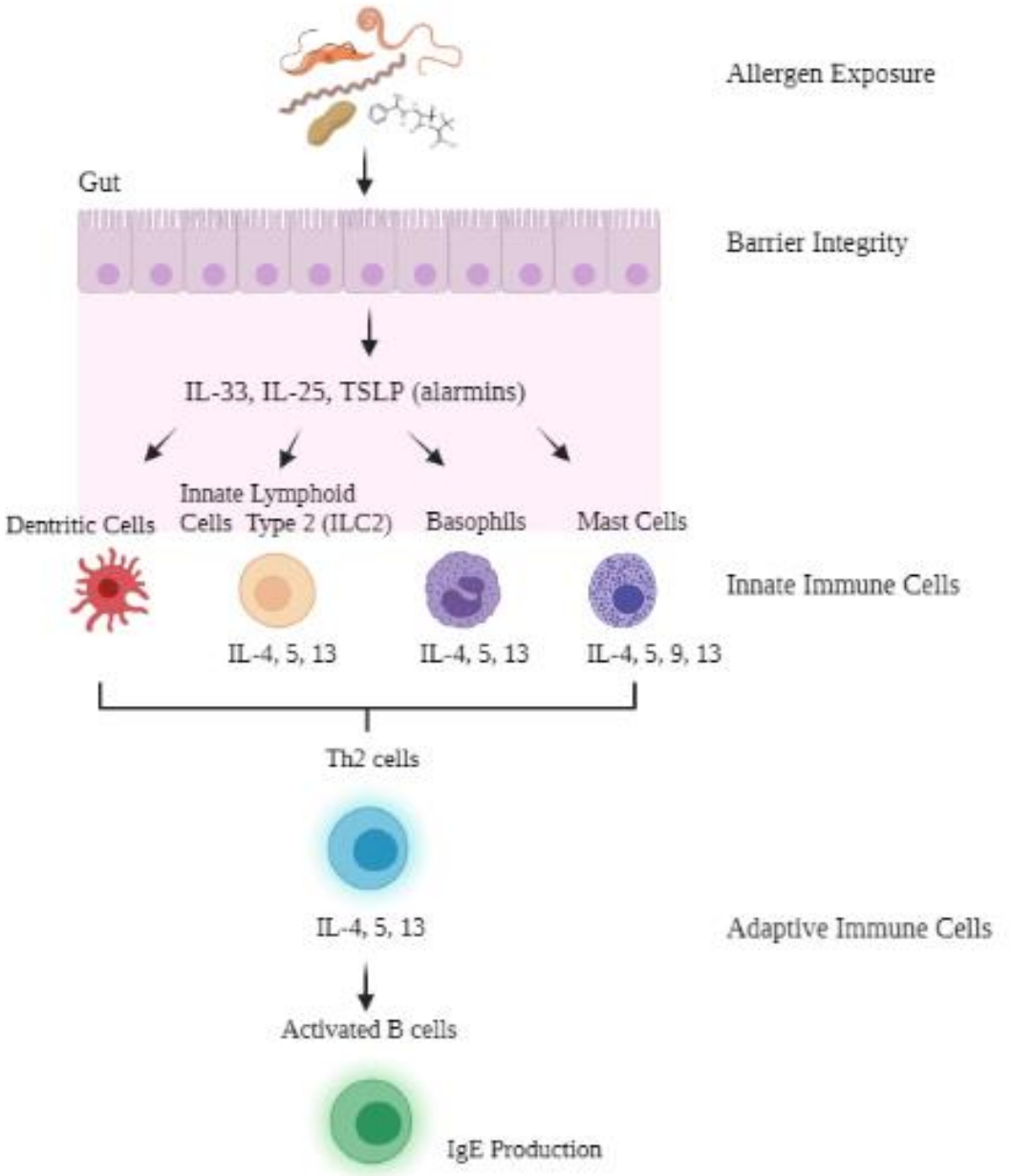

Figure 1.2 The pathogenesis of food allergy

The immune system response in CMPA was classified in three sub-groups: IgE-mediated, non-IgE-mediated, or a combination of both (mixed). The IgE-mediated immune response develops when the production of specific (s) IgE against the allergen occurs. Within the first two hours, the immune system reacts to cow's milk allergen, and the reactions that take place here are produced by an inappropriate type 2 response to the allergen (Komata, Söderström, Borres, Tachimoto, & Ebisawa, 2007; Sampson, 2001). Th2 cells that produce type 2 response secrete large amounts of IL-4, IL-13 and IL-5 cytokines and activate B cells in the germinal center. The immune system enables B cells

to produce sIgE by recognizing specific regions called epitopes in milk proteins (αS1-, αS2-, β-, and κ-caseins, α-lactalbumin and β-lactoglobulin), and B cells can produce sIgE by 2 different mechanisms. One of them is the conversion of the IgM molecule to IgG or IgA molecules as an intermediate step and then the production of IgE indirectly by isotype switching, and the other is the production of IgE directly from the IgM. Indirectly produced IgE molecule has higher affinity than directly produced IgE molecule. However, only the high-affinity IgE molecule causes the onset of allergic reactions (Xiong, Dolpady, Wabl, Curotto de Lafaille, & Lafaille, 2012). The high-affinity IgE molecule can bind to the surfaces of mast cells in the tissue or to the surfaces of basophils in the blood. Mast cells have receptors (FcεRI) on their surface to which IgE can bind (Reber, Hernandez, & Galli, 2017). When individuals with CMPA consume milk, the immune system produces IgE in response to the protein and secretes it into the blood. IgE, which is secreted into the blood binds to the receptors on the surfaces of mast cells and initiates the allergic reaction (Figure 1.3). Likewise, basophils, which are less than 1% of the total blood cells, also have IgE receptors on their surface and cause an allergic reaction as similar with the response of mast cells. Later, the activated mast cells and basophils secrete various molecules such as histamine, tryptase, leukotrienes and cytokines including Tumor necrosis factor (TNF)-α, and create a systemic reaction that affects many organs at the same time, which is called as anaphylaxis (Gilfillan & Tkaczyk, 2006).

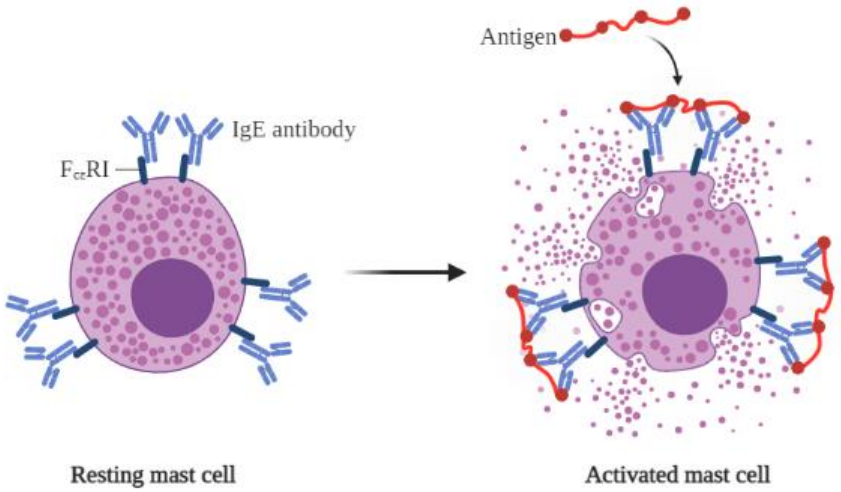

Figure 1. 3 The activation of mast cells

1.3. Cow's Milk Protein Allergens and Allergenic Properties

Cow's milk protein fractions are allergenic and include more than 20 antigens. Caseins and whey proteins are two types of fractions found in cow's milk. Caseins (Bos d 8) are made up of the αs1- (Bos d 9) and αs2- (Bos d 10) types, as well as the β- (Bos d 11), κ- (Bos d 12), and λ - types, and account for 80% of cow milk proteins; α-lactalbumin (Bos d 4), β-lactoglobulin (Bos d 5), bovine serum albumin and small amounts of lactoferrin, transferrin, lipase and esterase are whey proteins (El-Agamy, 2020).

Casein, β-lactoglobulin, and α-lactalbumin are the main allergens in cow's milk. Caseins are contained in milk as micelles, which are presented to the immune system through Peyer's plaques, whereas whey proteins are highly soluble and pass quickly through the intestinal epithelium (Matsuo, Yokooji, & Taogoshi, 2015). Whey proteins are more heat sensitive than caseins, while caseins are more likely than whey proteins to elicit antibody reactions, including IgE (El-Agamy, 2020).

1.4. Effect of Heat, Chemical Reactions and Fermentation on the Allergic Properties of the Proteins

Cooking or flavouring processes (heat, fermentation, and matrix effect) may reduce the allergenicity of the proteins; thus the frequency and the severity of the allergic reactions can be reduced. The recent studies showed that 70-80% of the children with milk allergy are reactive to pasteurized cow's milk but may tolerate conventional baked milk products (matrix with wheat, sugar and baked at 180-200°C for 30 minutes, examples are muffin, cake) (Sackesen et al., 2019).

The effect of heat on allergens is variable and depends on the food. Casein and α-lactalbumin are more heat resistant than whey proteins (β-lactoglobulin, bovine serum albumin). Boiling the milk at 120°C for 15 minutes does not impair the antigenic properties of casein proteins. The casein has linear epitopes and the protein chains that break down into smaller pieces when denatured by heat maintain their epitope property and continue to bind to IgE. However, β-lactoglobulin has conformational epitopes and loses its ability to bind IgE when denatured with heat (Figure 1.4). Thus, exposure to high (200°C) and prolonged (30 minutes) temperatures, such as baking, leads to the destruction of conformational epitopes and deterioration of the three-dimensional structure of

allergens, thereby reducing the allergenicity of the protein (Thomas et al., 2007). High pressure also causes structural changes such as denaturation of whey proteins and allows enzymes to access potential hydrophobic sites. At the same time, aggregates formed by enzymatic degradation of allergenic regions reduce the allergenic potential of milk proteins (Peñas, Préstamo, Luisa Baeza, Martínez-Molero, & Gomez, 2006). Because of the effects that disrupt such antigenic properties, many milk allergic patients can tolerate baked milk products.

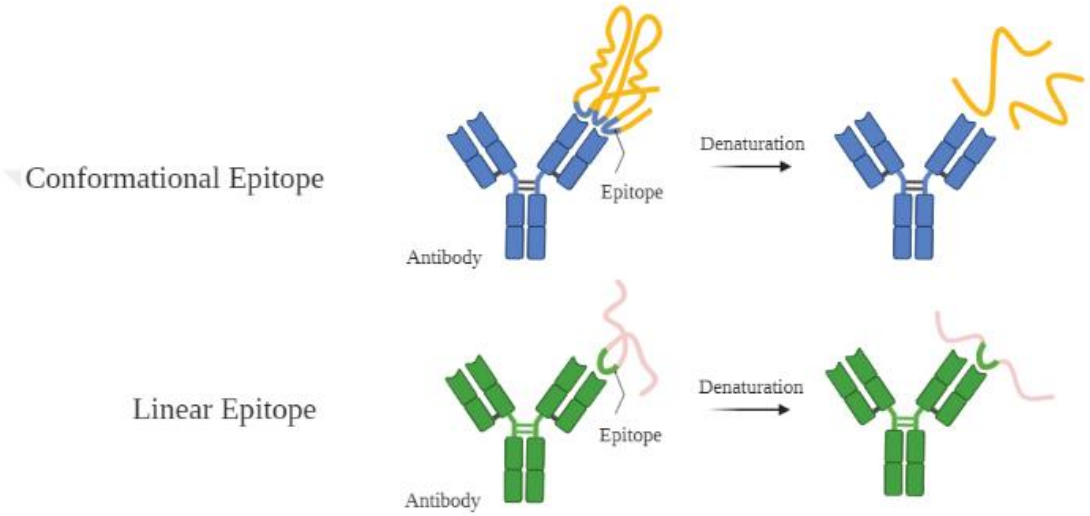

Figure 1.4 The types of epitopes

Another effect that contributes to baked food tolerance is the matrix effect (Nowak-Wegrzyn & Fiocchi, 2009). It is thought that protein, fat and sugar interactions that make up the matrix reduce IgE binding by closing allergen epitopes. Moreover, if proteins are exposed to heat in the presence of sugar, a reaction called “Maillard reaction” or “glycation” occurs, which modifies the structure and the aggregation/allergenic effects of milk proteins in different ways depending on the different combinations of protein, sugar and temperature (Pastorello et al., 2007; Teodorowicz, Van Neerven, & Savelkoul, 2017). By doing so, the Maillard reaction gives brown colour, flavour, and aroma to the nutrition (Figure 1.5). Glycation stands for the breakdown of proteins without enzymes, therefore Maillard reaction also disrupts epitopes by degrading the proteins.

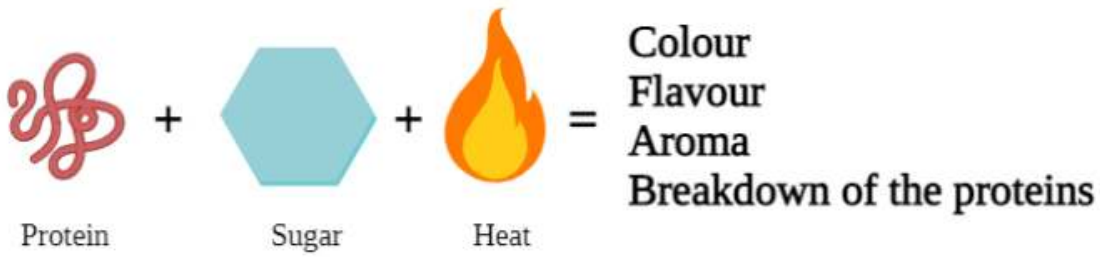

Figure 1.5 The mechanism of the Maillard reaction

Apart from baked dairy products, fermented products such as yogurt and cheese are the products that some patients with milk allergy can tolerate. Studies have shown that during fermentation process certain lactic acid bacteria release proteolytic enzymes, which destroy antigenic epitopes and reduce the allergenic properties of both β-lactoglobulin and casein in milk (El-Ghaish et al., 2011; Pescuma et al., 2011). This could be an explanation for the patients who can consume yoghurt but allergic to milk (Sackesen et al., 2019).

1.5. The Clinical Features of Cow's Milk Protein Allergy

CMPA is one of the most common food allergies in children, and it can be IgE-mediated, non-IgE-mediated, or a combination of the two (mixed). The time interval between ingesting an allergen and experiencing a reaction is important. Depending on the underlying immunological reaction, clinical symptoms vary (IgE-mediated, non-IgE and mixed type).

It's crucial to take a detailed clinical history and determine the symptoms and the underlying immunologic mechanism when making the diagnosis, deciding for the diagnostic tests, and planning the treatment.

1.5.1. IgE-mediated Food Allergy

IgE-mediated reactions occur within a few minutes to 2 hours after allergen exposure and typically occur with every exposure to the allergen. Patients could exposure to cow's milk directly (most commonly) or via other routes such as baked milk, fermented products (yoghurt, cheese), drugs and inhalation.

Cutaneous, respiratory and gastrointestinal systems are the most commonly involved organs, cardiovascular and neurologic systems might also be affected. Among these, the skin is the most affected organ (50-70%). Following that, gastrointestinal system abnormalities (50-60%), respiratory system findings (20-30%), and anaphylaxis (1-4%) are seen (Host & Halken, 2014). The severity of the reaction may vary depending on the degree of sensitivity, the amount of milk consumed, and the form of the food (fresh/fermented/baked). Urticaria, which is often accompanied by angioedema, is the most common skin finding. The most common gastrointestinal symptom is vomiting. While acute respiratory symptoms are mediated by IgE; persistent symptoms are mediated by both IgE and non-IgE mechanisms. Lower respiratory symptoms are uncommon, however they can be associated with deadly outcomes. After consuming cow's milk protein, the most serious IgE-mediated reaction is anaphylaxis, which is a life-threatening systemic allergic reaction.

1.5.2. Non-IgE-mediated Food Allergy

The exact mechanism of non-IgE-mediated food allergy is still unknown. IgE does not contribute to such immune reactions. It can affect the gastrointestinal tract [food protein induced enterocolitis (FPIES), food protein induced allergic proctocolitis (FPIAP), food protein induced enteropathy (FPE)], skin (dermatitis herpetiformis), and respiratory system (Heiner syndrome). Reactions usually develop 2-72 hours after ingestion of the food (Sharma, Bansil, & Uygungil, 2015).

1.5.3. Mixed type Food Allergy

Symptoms of both IgE-mediated and non-IgE-mediated reactions may coexist in mixed-type reactions. Mixed type food allergies include eosinophilic esophagitis, allergic eosinophilic gastroenteritis, and atopic dermatitis.

1.6. The Diagnosis of IgE-mediated Cow’s Milk Protein Allergy

The patient's clinical and nutritional history is taken in a detailed manner as the first step in the diagnosis of CMPA. The symptoms, the order in which they appear, the time interval between drinking cow's milk and occurrence of the symptoms, the number and severity of the previous reactions, the amount that caused the reaction, and the form of

the consumed food should all be queried. The history should be followed by a complete physical examination.

The skin prick test (SPT) and sIgE measurement in blood are two common diagnostic assays. Because they indicate the IgE reaction to food, SPT or serum sIgE tests are usually expected to be positive in IgE-mediated food allergies.

The oral food challenge (OFC) test is the “gold standard” for the diagnosis of food allergies as well as to show if the patient outgrown food allergy. The OFC test, on the other hand, is a time-consuming and expensive approach. Additionally, anaphylaxis may occur during food challenges, necessitating the development of safer and less expensive alternatives to reduce the need of OFC tests.

1.6.1. Skin Prick Test

Skin prick test (SPT) is an easy, low-cost, fast, and reproducible test that shows IgE-mediated sensitization and has been used for many years in the diagnosis of food allergy (Kido et al., 2016; Muraro et al., 2014). Skin prick tests in CMPA are performed by intraepidermal application of allergens into the skin with the prick technique. Oedema of 3 mm or more in size relative to the negative control is considered positive and SPTs can be done at any age.

The sensitivity of SPTs with cow's milk is highly variable (50-100%), with a positive predictive value of less than 50% and a negative predictive value of 95%. In patients who describe a mild reaction with consumption of cow's milk but negative SPTs, the diagnosis should be confirmed by oral food challenge tests (Martorell-Aragonés et al., 2015). In addition to the fact that negative SPT is not sufficient to exclude milk allergy, positive SPT may also show sensitization without any allergic reaction, therefore oral food challenge tests may be required to determine clinical reactivity (especially in patients with a suspicious clinical history).

1.6.2. Serum Specific IgE

Enzyme-Linked ImmunoSorbent Assay (ELISA) is used to measure serum sIgE levels of milk and/or milk protein fractions, which determine the milk and/or milk fraction sensitization (Figure 1.6). A result of sIgE greater than 0.35 kUA/L is considered positive in clinical practice (Martorell-Aragonés et al., 2015). If milk-sIgE is positive, it would be

appropriate to examine other milk protein fractions with prognostic significance. It's suggested that a decrease in casein and β-lactoglobulin sIgE levels throughout follow-up may address tolerance/outgrown.

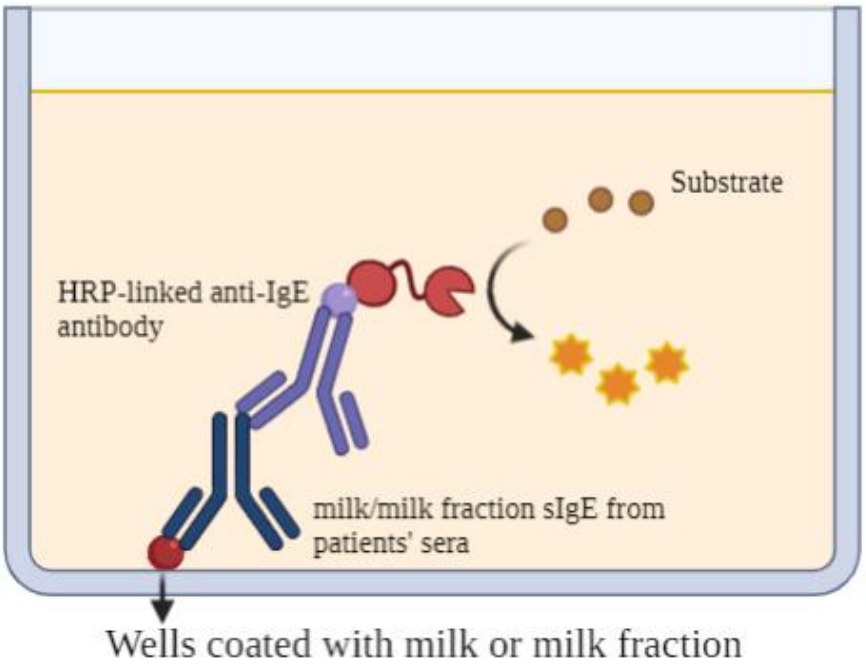

Figure 1.6 The working principle of ELISA that measures the milk specific IgE levels from blood samples of the milk allergic patients

1.6.3. Component Resolved Diagnosis

Component Resolved Diagnosis (CRD) is a method of detecting sIgE antibody binding to individual allergenic molecules (casein, β-lactoglobulin etc) using purified native or recombinant allergens instead of allergen extracts (Calamelli et al., 2019). In the diagnosis of food allergies, CRD plays three crucial functions. First, it can distinguish the true sensitization from the cross-reactive sensitization by identifying the responsible allergen and the probability of developing a reaction related to this allergen. Second, it reduces the need for food challenge testing and the patient anxiety by distinguishing between severe systemic or mild local reactions. Third, it enables the identification of triggering agents for specific immunotherapy (Ansoteguia, Meliolib, & Canonica, 2020).

The stability of the molecule and the patient's clinical history assist the clinician in assessing the risk of systemic or local reactions. Labile allergens are associated with local reactions such as oral symptoms and cooked foods are generally tolerated. In contrast,

stable allergens trigger severe systemic reactions (such as anaphylaxis) in addition to local reactions. Unlike whey proteins, which can be heat-denatured, casein in milk is heat-stable and retains its allergenicity despite high temperatures. High casein and β-lactoglobulin sIgE levels are associated with the frequency of reaction to baked milk and persistent milk allergy. Low levels of casein and β-lactoglobulin sIgE are helpful in demonstrating that baked milk products can be tolerated. In one cohort, the value of casein-sIgE to accurately predict reactivity to baked milk was higher than that of milk-sIgE and β-lactoglobulin-sIgE. In the same cohort, it has been reported that the risk of developing a reaction in the oral food challenge test with baked milk is high if the casein-sIgE is above 20.2 kU/L (Caubet et al., 2013). In addition, cut-off values of 4.94 kU/L and 9.97 kU/L for casein and milk-sIgE, respectively, were shown to have 54% and 60% positive predictive values, respectively, for patients who can tolerate baked milk. While the casein and milk-sIgE values are helpful in identifying the baked milk tolerant group, this needs to be demonstrated by an oral food challenge test in the majority of the patients.

Studies have tried to determine cut-off levels for SPT, sIgE and CRD levels that will predict clinical reactivity in children with food allergies (Buyuktiryaki et al., 2016; Yanagida et al., 2018; Yavuz et al., 2011). Although these values are useful in making the diagnosis of food allergy or in making the decision of OFC in patients with food allergy who have diet elimination, a positive reaction can be seen even at very low values. In addition, the relationship between the levels of these tests and the "severity" of the allergic reaction has not been fully demonstrated. While some patients can consume food with high SPT, sIgE and CRD values, some patients can develop very severe reactions despite very low values (Buyuktiryaki & Santos, 2020). For this reason, research continues to find a biomarker that can be used safely in clinical practice.

1.6.4. Serum Specific IgG4

B cells classically are involved in immune responses as antigen presentation to T cells, cytokine production, differentiation to plasma cells and immunoglobulin production. B cells express four groups of IgGs (IgG1, IgG2, IgG3, and IgG4) and all have different biological functions. IgG4 constitutes 4% of total IgG, but this rate may increase to 75% after long-term exposure to antigens after allergen immunotherapy (M. Akdis & Akdis, 2014; Shamji et al., 2015). The anti-inflammatory properties of specific IgG4 are as follows; (1) inhibiting immune complex formation, (2) low affinity for the activation of

Fcγ receptors, (3) acting as a blocking antibody by competing with IgE in allergen binding, (4) cross-linking of FcεRI and FcγRIIb with IgE and IgG4 results in mast cells and basophils suppression (van de Veen & Akdis, 2016).

Specific IgG4 measurement can be made with the same technique (ELISA) as the specific IgE measurement. It is thought that IgG4 production in patients with milk allergy is a normal physiological response after milk consumption (Homburger et al., 1986). Its role in diagnosing cow's milk allergy is controversial. Studies have shown that milk-sIgG4 antibodies are at a higher level in patients who tolerate cow's milk (Ruiter et al., 2007; Savilahti, Saarinen, & Savilahti, 2010), therefore it has been stated that the use of IgE and IgG4 antibodies together can be used to predict the development of tolerance (Savilahti et al., 2010). In addition, it has been reported that milk-sIgG4 antibodies may have a role in preventing the development of milk allergy, possibly by blocking antigen presentation to allergen-specific T cells (Ito et al., 2012; van Neerven, Knol, Ejrnaes, & Würtzen, 2006).

1.6.5. Oral Food Challenge Tests

The gold standard for diagnosing IgE-mediated and non-IgE-mediated food allergies is the oral food challenge (OFC) tests. It is frequently used to make a diagnosis, monitor the progress of food allergy if the patient outgrown food allergy, or determine the threshold dose (Calvani, Bianchi, Reginelli, Peresso, & Testa, 2019). While a single-blind placebo-controlled food challenge (SBPCFC) test (the doctor knows the content of the given food, the patient does not know) and the open challenge test (both the patient and the doctor know the content of the given food) are usually sufficient for diagnosis. When atypical and subjective findings were developed a double-blind placebo-controlled food challenge (DBPCFC) test (both the patient and the doctor do not know the content of the given food) should be performed. The DBPCFC test minimizes false positive results and is the gold standard in diagnosis (Bird et al., 2020). However, it is time-consuming, labor-intensive and expensive.. A plenty of variables affect the decision of OFC tests. These include the form of the food used, the amount of dose, the number of doses, the time between doses, age of the patient, the initial reaction, the type and masking of the placebo. The form of milk can be fresh, heated, boiled, baked with different content as flour, sugar, oil etc. Since there are differences in terms of allergenicity, the form of food used in the test is important. For example, some children who cannot tolerate pasteurized milk can

consume baked milk products without any allergic reaction (Koletzko et al., 2012). Before the food challenge tests are performed, the type of food allergy and the severity of the previous reaction should be reviewed, and the tests should be planned accordingly. In IgE-mediated food allergies, open or single/double-blind placebo-controlled OFC tests are usually administered in 6-9 steps, gradually increasing the dose as two or three times the previous dose, up to the daily amount that the child should consume according to his age (Figure 1.7). It is started from 0.1% to 1% of the total food and the time between doses is on average 15-30 minutes (the shortest can be 10; the longest can be 60 minutes). If objective symptoms occur during challenge test, the test should be terminated. In the presence of subjective symptoms, the patient should be carefully monitored. Results should be well documented. In terms of allergic reactions, the patient should be monitored for at least 2 hours after the test, and at least 4 more hours if a reaction is observed, preferably until all signs of the reaction are completely resolved. Those with moderate to severe reactions should be hospitalized and monitored overnight.

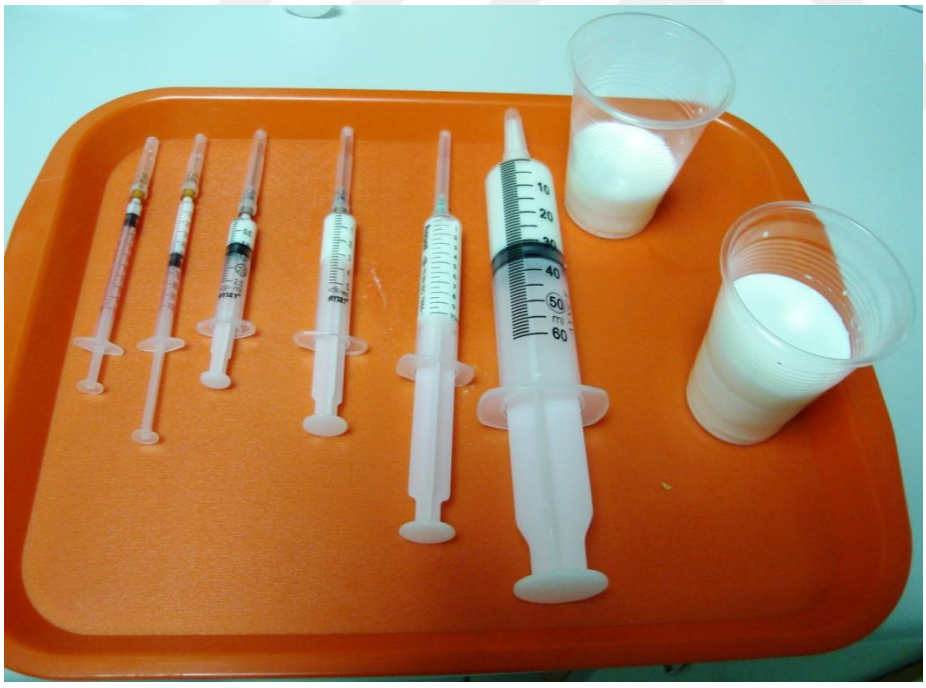

Figure 1.7 The example of 8 doses of the pasteurized milk that was used for open OFC test

1.6.6. Basophil Activation Test

Basophils have markers such as CCR3, CD45 and HLA-DR on their surface. Basophils, activated by the presence of allergen, begin to express activation markers (CD63, CD203c) on their cell surface (Figure 1.8). By determining these markers, activated basophils can be counted with the help of flow cytometry. This method is called basophil activation test (BAT). When basophil activation test is performed from whole blood, test should be performed within a maximum of 4 hours after blood collection in order to maximize the viability of basophils and preserve their functionality. Since the response of everyone's basophils to the allergen is different, dose response study is important to find the correct dose in the basophil activation test. Dose response study provides information about the reactivity and sensitivity of basophils (Hemmings, Kwok, McKendry, & Santos, 2018; McGowan & Saini, 2013).

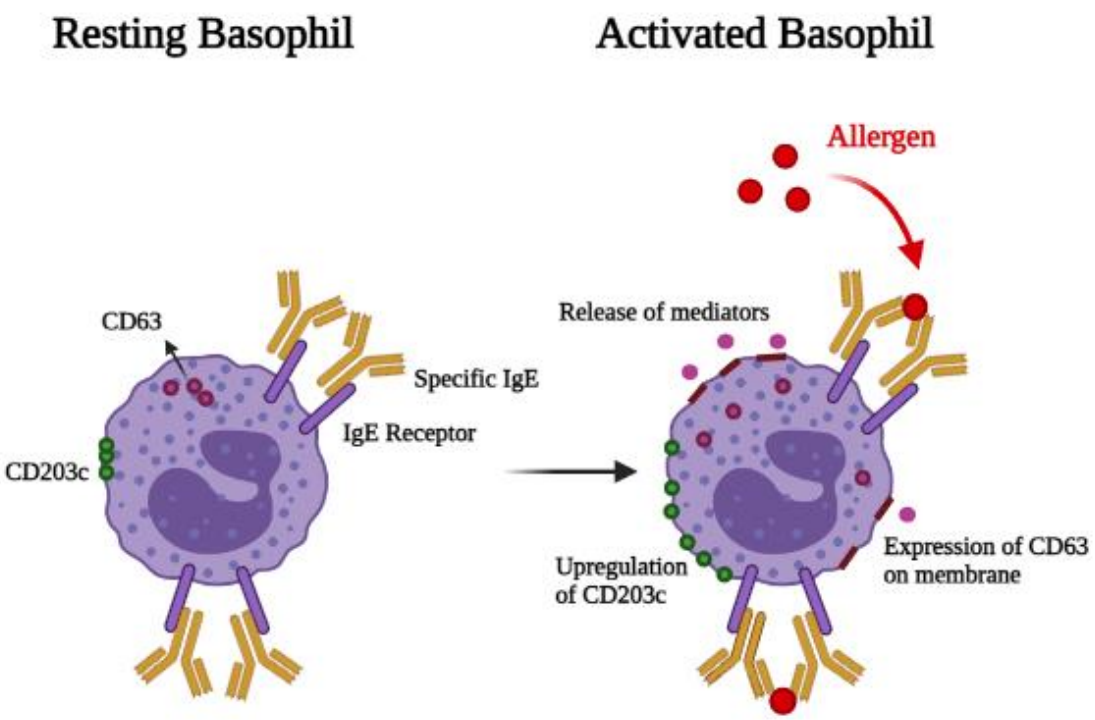

Figure 1.8 The mechanism of basophil activation test

Skin prick test and sIgE measurement used in the diagnosis of food allergy are diagnostic methods with high sensitivity but low specificity. Studies have shown that BAT is more accurate than sIgE measurement in the diagnosis of food allergy (Santos et al., 2014). In

the treatment of CMPA, the patient may develop a tolerance to cow's milk proteins over time. Therefore, the OFC test is used to determine whether the one is tolerant or still reactive to this protein. However, this method may cause the individual to have anaphylaxis, and it is also an expensive and time-consuming method. In diagnostic studies performed with BAT, it was stated that tolerance and reactive phenotypes could be easily distinguished from each other with a specificity of 75-100% and sensitivity of 77-98% (Santos et al., 2014). In a large population study with peanut allergic individuals, it was shown to have 100% specificity in the diagnosis of peanut allergy (Santos et al., 2014). However, BAT has not yet been routinely used in the diagnosis of CMPA, and more research is needed.

A recently published review mentioned that when using BAT as part of a standard diagnostic work-up, there are some practical concerns to consider (Figure 1.9) (Santos et al., 2021). Although BAT to peanut had the highest overall diagnosis accuracy of all current tests (Santos et al., 2014), SPTs or sIgE are faster and more cost-effective, and hence these tests can be employed as a first line. Before recommending patients for OFC, BAT has been advocated as a second-line test in patients with equivocal results after clinical history and sIgE sensitization tests (Santos & Shreffler, 2017). In a prior study of peanut allergy, this proposed strategy reduced the amount of OFC by 67%.

Figure 1.9 Suggested sequence of tests to support the diagnosis of food allergy, before referring patients for an oral food challenge (Santos, Alpan, & Hoffmann, 2021)

As a conclusion, the BAT can be seen as an *in vitro* surrogate for acute allergic reactions and assisting in the diagnosis of allergic illnesses.

1.7. Natural Course of the Cow's Milk Protein Allergy

The course of CMPA may change over time. Generally, CMPA starts in the first two years of life and decreases in the following periods. Studies have shown that 80% of patients develop tolerance to CMPA at the age of 3-4 years (Fiocchi et al., 2010). In addition, it was observed that milk allergy persisted in 21% of children with a severe phenotype when they reached the age of 16 years (Skripak, Matsui, Mudd, & Wood, 2007).

Patients with CMPA are followed in terms of tolerance development or newly formed food allergies. For follow-up, the history of reactions to foods is taken from the patients intermittently or the SPTs and sIgE test are performed. However, these tests are insufficient to distinguish reactive and tolerant phenotypes from each other. This situation requires the elimination of cow's milk in the diets of children with CMPA, and long-term elimination causes a decrease in the growth of children with CMPA.

1.8. Cow's Milk Protein Allergy Treatment

Cow's milk, eggs and wheat are the main nutrients in the daily diet of many cultures. The strict avoidance and preparation to promptly treat allergic reactions are the current standard care for food allergy. However, strict elimination diets and the fear of potentially severe reactions upon allergen exposure can cause significant anxiety among the parents. For this reason, there is a need for safe treatment methods in children with cow's milk allergy, and research on this issue continues (Muraro et al., 2014). Moreover, avoidance of cow's milk products to prevent the development of allergic reactions may cause nutritional deficiencies in children and may have a negative psychosocial effect and lead to a decrease in the quality of life (Leonard & Nowak-Węgrzyn, 2016).

Oral immunotherapy (OIT) is one of the most promising strategies, which employs the introduction of gradually increasing doses of specific food allergens into the diet (Hussey Freeland, Fan-Minogue, Spergel, Chatila, & Nadeau, 2016). The aim of this approach was to produce a state of desensitization during the treatment period and establish a persistent immunological tolerance in the long term. Interestingly, a proportion of children with CMPA can tolerate extensively heated forms of milk. Furthermore, continued ingestion of baked-milk products in these children has been shown to

accelerate the outgrowing of milk allergy and induce milk-specific IgG4 production which suppress the IgE-mediated allergic inflammation (J. S. Kim et al., 2011).

1.9. The Use of Baked Milk Products in Clinical Practice and Treatment

Baked milk products are less allergenic because of the heat and matrix affects as mentioned earlier. There are studies showed that tolerance to baked-milk products in children with IgE-mediated CMPA is a good predictor of unheated-milk tolerance development (J. S. Kim et al., 2011; Leonard & Nowak-Węgrzyn, 2016; Sackesen et al., 2019; Vilar et al., 2021). About 75% of children with milk allergies can tolerate high heat-treated baked goods. Patients with CMPA reactive to baked milk products constitute the severe phenotype group. More crucially, compared to a rigid avoidance diet, which is now the "gold standard of care," adding baked-milk products to the diet appears to significantly accelerate the development of tolerance to unheated-milk.

Furthermore, introducing dietary baked milk into the diet is both safe and convenient. Patients and their parents are eager and enthusiastic about this approach. Introduction of baked-milk products into the diet in milk-allergic children mark a significant shift in the treatment paradigm for milk allergies. Due to the risk of anaphylaxis in children who are reactive to baked-milk products, such interventions should only be added under the guidance of a food allergy specialist (Vilar et al., 2021).

Beforementioned clinical observations gave raise a sequence called "milk ladder" for the reintroduction of milk and dairy products into the diets of infants and children with mild to moderate CMPA. It is a plan to re-introduce milk products gradually and in stages, starting with foods less allergenic and proceeding with more allergenic, respectively (Figure 1.10). Today there is not an option as a less hypoallergenic product than conventional baked milk product (cake, muffin) to induce desensitization by oral route for the baked milk reactive ones.

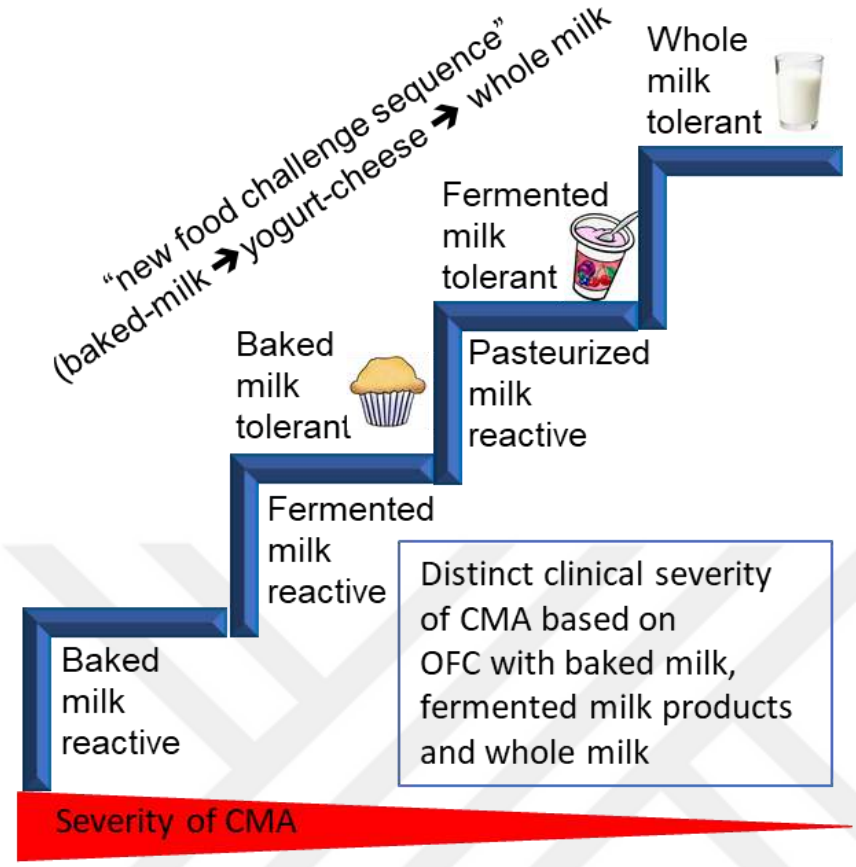

Figure 1.10 The sequence of the Milk Ladder (Sackesen et al., 2019)

1.10. The Aim of the Study

Previous studies indicate that consumption of baked milk products facilitate the tolerance development. Our hypothesis was that "if the milk-allergic patient can consume a hypoallergenic product, the development of the tolerance formation would be accelerated". Our aims within the scope of this hypothesis were as follows:

1- to develop a new hypoallergenic food product that contains hypoallergenic milk proteins with low reaction risk
2- to determine the total protein concentration and milk protein content of diary milk products
3- to evaluate reliable indirect in-house ELISA for the detection of sIgE antibodies against less hypoallergenic baked milk product

4- to check the IgE reactivity with western blotting and newly evaluated indirect in-house ELISA and compare its binding capacity of new hypoallergenic product with conventional cake, fermented milk products and pasteurized milk.
5- to assess the BAT with milk proteins and the proteins of the new developed hypoallergenic food product

For this purposes, 45 milk allergic patients, who were reactive or tolerant to milk, were included into the study.

1- Development of a new hypoallergenic product

To develop a less hypoallergenic milk product than conventional baked milk product with the effect of high and longer duration of temperature and different mixtures of matrix were tested.

2- Determining the total protein concentration and milk protein content

The total protein concentrations were evaluated with Bradford assay. Then the total proteins were run in SDS-PAGE and casein band intensities were compared among all milk products (milk, baked cake, and hypoallergenic product candidate). Moreover, the milk protein content [casein (α-S1 casein, α-S2 casein, β casein and κ casein) and β-lactoglobulin] was checked by proteomics analysis.

3- Evaluation of indirect in-house ELISA

Indirect in-house ELISA was conducted to detect milk protein sIgE. For each product (milk, cheese, yogurt, baked milk, hypoallergenic product candidate), the checkerboard titration was performed to decide the optimum dilutions.

4- Evaluation of IgE reactivity

The IgE reactivity of hypoallergenic product was checked by western blotting, indirect in-house ELISA and BAT by using patients' sera/whole blood who are allergic to milk. As previous studies suggested BAT as a diagnostic tool before oral food challenge test, we also aimed to check the usefulness of BAT by using hypoallergenic product candidate.

Chapter 2

MATERIALS AND METHODS

2.1. Study Population

Forty-five patients aged between 6 months and 15 years with IgE-mediated cow's milk allergy who applied to the Division of Paediatric Allergy, School of Medicine Koç University were included from May 16, 2017 to November 30, 2020. Children were diagnosed as having IgE-mediated milk allergy according to the following criteria:

1. A consistent and clear-cut history of milk-related symptoms that developed within minutes to hours following the ingestion of milk,

2. Positive serum levels of sIgE (sIgE >0.35 kUA/L), or

3. Positive skin prick test (SPT) for milk (a wheal diameter of 3 mm or greater than the negative control)

Blood samples were taken from milk allergic patients who agreed to participate in the study. Serum samples were stored at -80°C until it was studied. Total serum IgE and sIgE levels (both milk and casein) were measured with the ImmunoCAP system (Phadia, Sweden). Children who could consume milk products were called tolerant, those who could not consume milk products at all were called reactive.

Approval was obtained from the Koç University Ethics Committee for this study (Ethics committee number: 2017. 090.IRB1.010). The study was conducted according to the tenets of the Declaration of Helsinki. Prior to the initiation of the study, an informed consent was obtained from subjects/parents.

The summary of the study design is given in Figure 2.1

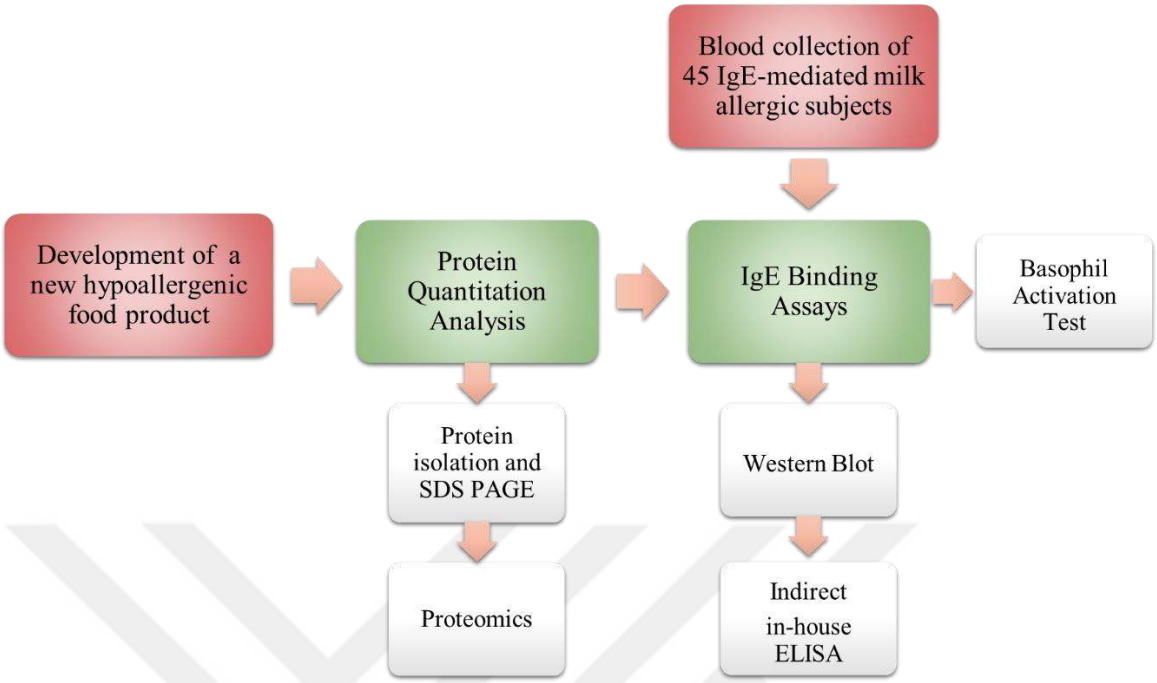

Figure 2.1 Study design

2.2. Development of Hypoallergenic Milk Product

The main aim of this thesis was the development of the hypoallergenic milk product that eventually will contribute to the tolerance induction of the milk allergic subjects. For this purpose, the allergenicity of the developed product was first evaluated with the protein quantitation analysis like SDS-PAGE band analysis and proteomics. IgE binding assays were also performed in order to check the reactivity of the milk allergic patients' sera with the newly developed hypoallergenic product (Figure 2.1).

In all experiments unless it is specified, fresh cow's milk was untreated and used as a natural control allergen. Baked milk was baked at 180°C for 30 minutes in a water bath in an oven.

2.2.1. Matrix Effect

In order to test the effect of matrix; sugar, baking soda, oil, and flour was prepared with different recipes. All ingredients [sugar, baking soda, milk, vegetable oil], except flour were mixed well in a bowl and the mixture was equally divided into four small bowls. Flour was added to the cake base mixtures according to flour/sugar ratio of 4/1, 2/1 (conventional one), 1/1 and 0.5/1, respectively (Figure 2.2). Then each of the cake mixtures were poured into approximately 8 cm diameter cake tins and baked for 30 minutes in 180°C oven. In addition to these different mixtures, other cake mixtures with and without baking soda, oil and sugar were prepared and tested for caseins bands. The flour/sugar ratio of 2/1 was chosen for the following experiments because of the flavour problem with the other ratios.

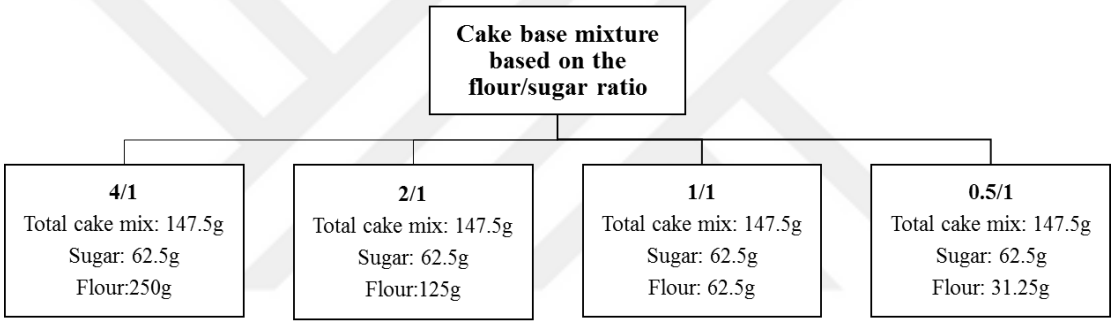

Figure 2.2 Essays of different baked cake recipes

2.2.2. The effect of the temperature and duration in oven

Conventional baked milk cake contains 2/1 of flour/sugar ratio the raw mixture of conventional baked milk cake was prepared and incubated overnight at -20°C. The following day, it was baked for 30 minutes at 180°C, left to cool down and when it was cooled properly it was sliced and baked again at a low temperature (90°C) for 1, 2 and 3 hours (Figure 2.3). Samples were taken hourly to determine if the duration of the exposure had affected the protein bands in the conventional cake, 1h cake, 2h cake and 3h cake. Cakes that were baked once or twice were compared according to their casein contents and IgE binding. Cakes which were baked twice were called “biscotti”.

Figure 2.3 Two steps of baking: First step is the classical/conventional baking of the overnight frozen cake at 180°C, 30 minutes. Second step is the additional baking of the cake slices at 90°C for 3 hours to have biscotti product

2.3. Protein Quantitation Analysis

2.3.1. Protein isolation

Five grams of the conventional cake, 1h, 2h and 3h biscotti samples were crumbled and suspended in 10 ml 1X Phosphate buffered saline (PBS) with protease inhibitor (PI) cocktail (Sigma Aldrich, Missouri, United States) and incubated on an orbital shaker at 4°C overnight. After the centrifugation of all samples at 3750 rpm for 1 hour at 4°C, the supernatants were collected, aliquoted and stored at -20°C until studied (Figure 2.4). Both the cow's milk and baked milk were diluted 1:20 with the same PBS+PI cocktail. The relative protein concentrations of all mentioned above products were measured with Bradford assay according to manufacturer instructions (Bio-Rad Laboratories, California, United States).

2.3.2. Sodium Dodecyl Sulphate- Polyacrylamide Gel (SDS-PAGE) and Band Intensity Analysis

After the protein concentrations were determined, 15 micrograms (µg) of each sample were calculated and mixed with 1:1 ratio 2X Laeemli buffer (Bio-Rad Laboratories, California, United States). A reducing agent like beta-mercaptoethanol (Bio-Rad Laboratories, California, United States) was not added to the Laeemli buffer and additional heating step was not used for the destruction of the proteins. 1X Tris-Glycine-EDTA (TGS) buffer was used as running buffer (Bio-Rad Laboratories, California, United States). 15 µg of each protein were loaded into the lanes of Any-kD Mini-PROTEAN®TGX Stain-Free ™ Protein gel (Bio-Rad Laboratories, California, United

States). As protein ladder, Precision Plus Protein™ Dual Color Standards (Bio-Rad Laboratories, California, United States) was used. Then the gel was run for 60 minutes at 100 mV. After the run was finished, the image of the gel was taken with Bio-Rad Gel Doc XR. Image Lab (Bio-Rad Laboratories, California, United States) program was used to perform the band intensity analysis.

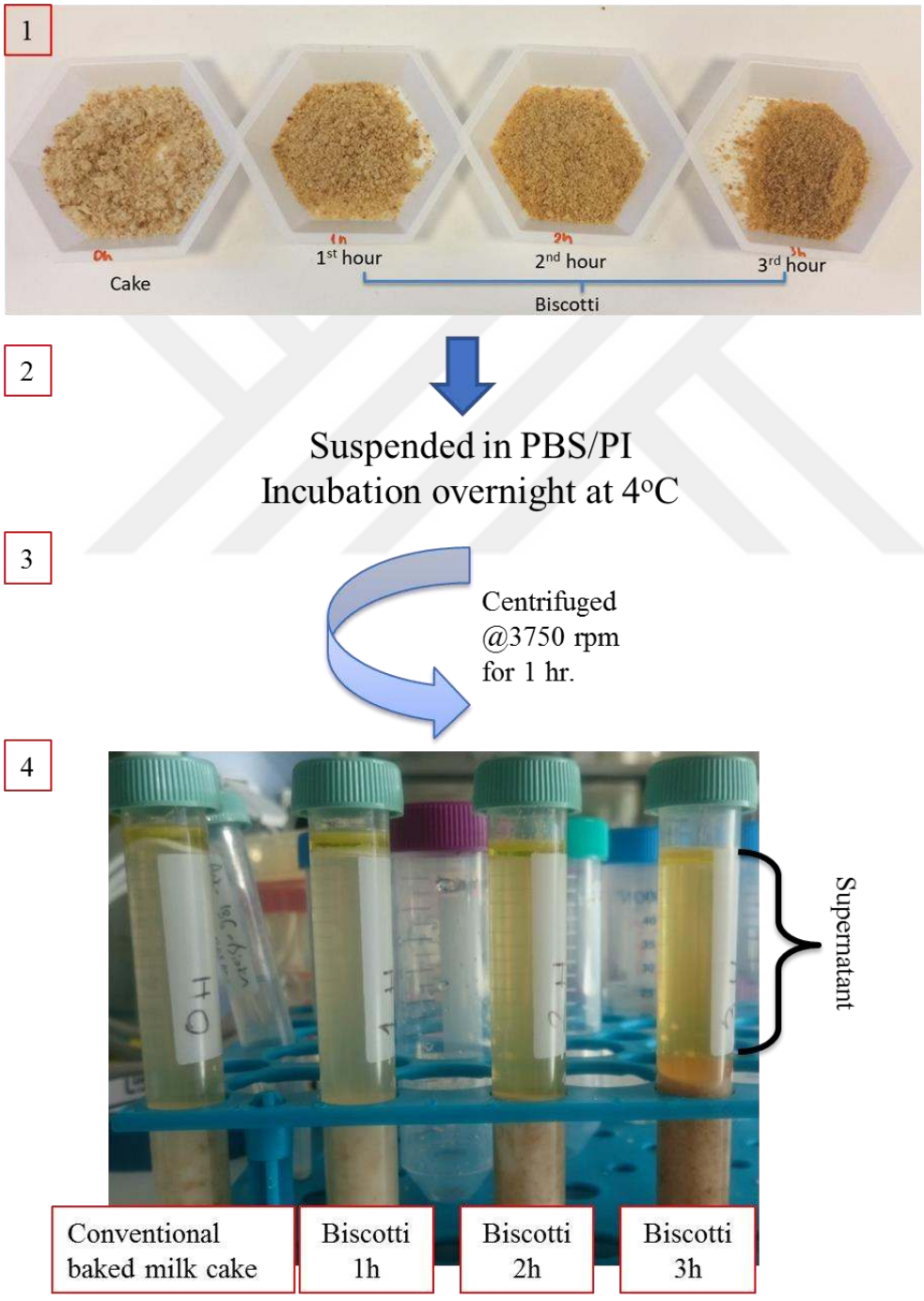

Figure 2.4 Steps of the protein isolation from cake and biscotti samples

2.3.3. Proteomics

Protein samples, which were treated with Dithiothreitol (DTT) and iodoacetamide for alkylation and reduction, were digested with trypsin (1:50 w/w) at 37°C for 16 hours. 30 mg of the proteins was injected for proteomics analysis. These analyses were performed using liquid chromatography–hybrid quadrupole time-of-flight mass spectrometry (LC-qTOF-MS) with a C18 column (150×1 mm, particle size 5 µm, pore size 300 Å, Phenomenex Jupiter, USA). Agilent MassHunter Workstation Software Q-TOF B.08.00 was used to run the Agilent 1260 Infinity series LC and 6530 accurate-mass LC–qTOF-MS instrument (Agilent Technologies, California, United States) in auto MS/MS positive ionization mode. The MS/MS scan data was collected in the m/z range of 100–1700. Reference mass correction was achieved using tuning mix (Agilent 85000 ESI-TOF Tuning Mix, Agilent Technologies, California, United States USA) as in untargeted lipidomic analysis. Maxquant proteomics platform (Maxplanck Institute, Germany, https://www.maxquant.org) was used for protein identification and semi-quantification. Bos Taurus database was downloaded from UniprotKB. Recorded experimental MS/MS data was matched with in silico MS/MS data for peptide and protein identification. In matching process, 20 ppm mass tolerance was used for first and main search. False discovery rate (FDR) was adjusted as 0.01 for reliable identification. Label free quantification algorithm was performed to calculate protein intensity. The proteomics analysis was performed at Hacettepe University, Department of Analytical Chemistry.

2.4. IgE Binding Assays

2.4.1. Western Blotting

2.4.1.1. SDS-PAGE Preparation

Any-kD Mini-PROTEAN®TGX Stain-Free ™ Protein gel was not applicable for western blotting experiments. Handmade gel was prepared as follows. The vertical electrophoresis set was used (Bio-Rad Laboratories, California, United States). Glass parts, separators and comb were cleaned with 70% ethanol and dried. The separating gel and stacking gel were prepared according to recipe that was shown in Table 2.1. For both preparation, ammonium persulfate (APS, Sigma Aldrich, Missouri, United States) and tetramethylethylenediamine (TEMED, Sigma Aldrich, Missouri, United States) were added lastly to prevent early solidification of the gels. The separating gel was poured in between two glasses of vertical electrophoresis set up. One millilitre (ml) of isopropanol was poured on top of separating gel in order to make the top of the gel a straight line and the gel was let to solidify for 30 minutes at room temperature (RT). The isopropanol was removed, and the gel was washed with distilled water 4-5 times and the water was drained completely. The stacking gel was prepared in a 15 ml centrifuge tube. The stacking gel was poured on top of separating gel, the comb was placed, and the gel was left to solidify for 30 minutes in RT.

Table 2.1 The recipes of both handmade separating and stacking gels

	12.5% Separating gel (Total= 10 ml)	***5% Stacking gel (Total= 5 ml)***
1.5 M Tris-HCl, pH 8.8	2.5 ml	-
0.5 M Tris-HCl, pH 6.8	-	1.25 ml
40% Acrylamide *(Bio-Rad Laboratories, California, United States)*	3.04 ml	480 µl
2% bis-Acrylamide *(Bio-Rad Laboratories, California, United States)*	1.68ml	260 µl
10% SDS (w/v)	100 µl	50 µl
ddH$_2$O	2.63 ml	2.92 ml
10% APS (w/v)	50 µl	25 µl
TEMED *(Sigma Aldrich, United States)*	5 µl	5 µl

Cow's milk, conventional cake and 3h biscotti samples were used for western blotting experiments. 3 µg of samples were calculated for each sample and loaded to the gel lanes with Precision Plus Protein™ Dual Color Standards (Bio-Rad Laboratories, California, United States). 1X TGS (Bio-Rad Laboratories, California, United States) was used as running buffer as mentioned above. Then the gel was run for 60 minutes at 100 mV. For each experiment one set of extra gel was conducted as loading control. After running, loading control gel was stained with Coomassie Brilliant Blue G-250 (CBB, PanReac AppliChem, Illinois, United States) for 20 minutes (Table 2.2). The gel was destained for 2 hours and the bands were visualized with Gel-Doc XR (Bio-Rad Laboratories, California, United States). Every 30 minutes the destaining solution was changed to clarify the gel completely (Table 2.2).

Table 2.2 The recipes of Staining and Destaining solutions

	Staining Solution	***Destaining Solution***
0.1% CBB	0.5 g	-
2% Acetic acid	10 ml	10 ml
40% Methanol	200 ml	200 ml
dH_2O	290 ml	290 ml

Figure 2.5 showed the representative image of cow's milk proteins that was run with handmade SDS-PAGE.

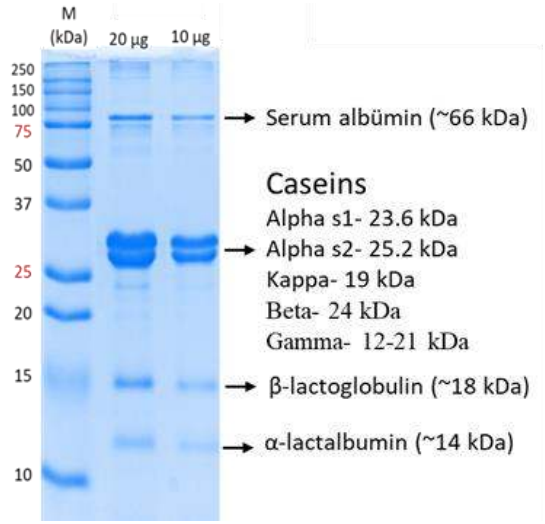

Figure 2.5 Commassie staining of SDS-PAGE for different amounts of the cow's milk protein. M: marker

2.4.1.2. Blotting

After proteins were run in SDS-PAGE, they were transferred to the membrane as follows. PVDF membrane (Bio-Rad Laboratories, California, United States) and filter paper (Bio-Rad Laboratories, California, United States) was cut to the dimensions of the gel. Polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, California, United States) was firstly soaked in the methanol solution (Sigma Aldrich, Missouri, United States) for 5 minutes for activation. Then PVDF membrane, the gel, filter paper and fiber pads were equilibrated in transfer buffer about 15 minutes. The gel sandwich was prepared according to the manufacturer's instructions (Bio-Rad Criterion Blotting System, Bio-Rad Laboratories, California, United States) and it was placed in the module. The ice cooling unit which was stored at -20°C was added, the tank was filled with transfer buffer which was prepared by adding 20% methanol to the 1X TGS. Blotting was run for 70 minutes at 300 mA. After blotting, the gel was stained with staining solution to indicate that the proteins were blotted.

2.4.1.3. Blocking and Antibody Treatment

For washing the PVDF membrane, PBS-T solution was prepared with adding 0.05% Tween-20 to 1X PBS. After blotting, the PVDF membrane was washed once with PBS-T and blocked with 3% Bovine Serum Albumin (BSA, Sigma Aldrich, Missouri, United States) for 1 hour at RT. After blocking, membrane was washed with PBS-T three times for 5 minutes. In order to ensure the places of casein bands, commercially available 1:2500 diluted in PBS-T with 1% BSA polyclonal rabbit anti-casein antibody (Cat no: ab166596, Abcam, Cambridge, United Kingdom) was used. Milk allergic patient sera was used as a dilution of 1:10 in PBS-T with 1% BSA. Membrane was incubated either anti-casein or patient's sera overnight at 4°C. After incubation, membrane was washed with PBS-T three times for 5 minutes. Then, as secondary antibody, 1:2500 diluted horseradish peroxidase (HRP)-linked anti-rabbit antibody (Cat no: 7074, Cell Signaling Technology, Massachusetts, United States) for rabbit anti-casein, and 1:5000 diluted HRP-linked goat anti-human IgE antibody (Cat no: A9667, Sigma Aldrich, Missouri, United States) for patients' sera were used. Secondary antibody incubation was done at RT for 1 hour. After washing membranes 5 times for 5 minutes, Pierce enhanced chemiluminescent (ECL) Western Blotting Substrate (Thermo Fisher Scientific, Massachusetts, United States) was added into the membrane and incubated for 2 minutes. Lastly, the bands were visualized with Gel-Doc XR (Bio-Rad Laboratories, California, United States).

2.4.2. ELISA

2.4.2.1. Indirect in-house ELISA for cake and 3h biscotti

The indirect in-house ELISA was assessed for the detection of cake and 3h biscotti sIgE antibodies in the patients' sera (Figure 2.6). A checkerboard titration was conducted to determine the optimum concentrations of the coated antigens and patients' sera. 100 µl of the conventional cake and 3h biscotti proteins were used in duplicate as a coating protein with a concentration of 10 µg/mL in PBS in 96-well flat bottom high binding ELISA plate (Greiner Bio-One, Frickenhausen, Germany) and incubated for overnight at 4°C. Then, the plate was washed with 350 µl PBS-T three times with g ELx50 Auto Strip Washer (BioTek Instruments, Vermont, United States) and blocked with 2% human serum albumin (HSA, Vitrolife, V.Frölunda, Sweden) in PBS-T for 1.5 hours. Subsequently, the plate was washed again three times and 1:20 diluted milk allergic patients' sera were added. The plate was incubated for overnight at 4°C. After washing three times, 1:1000 diluted HRP-conjugated goat-anti-human IgE (Cat no: A9667, Sigma Aldrich, Missouri, United States) was added. The plate was incubated for 1.5 hours at 37°C and washed five times. Then the plate was developed using a tetramethylbenzidine peroxidase (TMB) substrate (Sigma Aldrich, Missouri, United States) which was incubated for 30 minutes at RT. Then the reaction was stopped with 1M hydrochloric acid (HCl) and optical density (OD) values were measured at 450 nm with Synergy-H1 microplate reader (BioTek Instruments, Vermont, United States).

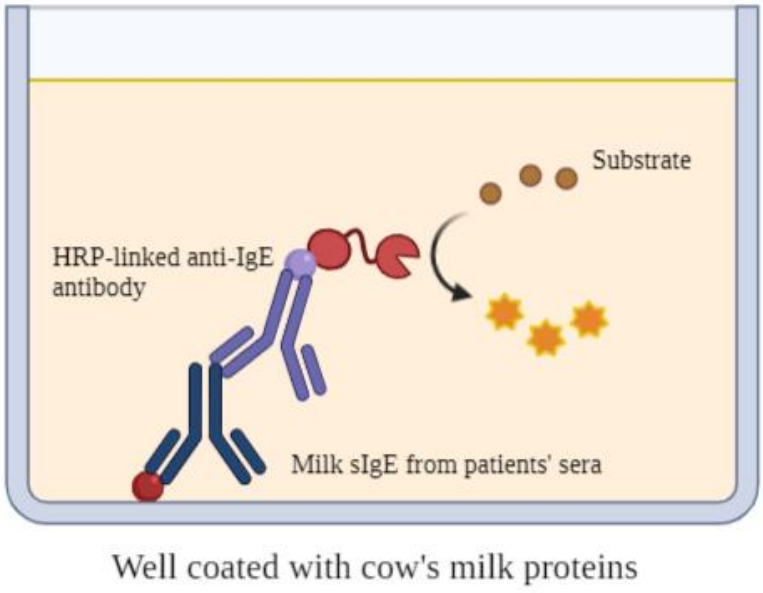

Figure 2.6 Principle of indirect in-house ELISA

2.4.2.2. Indirect in-house ELISA for milk, cheese and yogurt

The indirect in-house ELISA was evaluated for the detection of milk, cheese and yogurt sIgE antibodies in the patients' sera. After determining the concentrations via checkerboard titration, the procedure was done as follows. 100 µl of proteins were used in duplicate as coating protein with a concentration of 10 µg/mL in PBS in 96-well plate and incubated for overnight at 4°C. Then, the plate was washed with 350 µl PBS-T three times and blocked with 2% HSA in PBS-T for 1.5 hours. Subsequently, the plate was washed again three times and diluted milk allergic patients' sera were added. The plates were incubated for overnight at 4°C. After washing three times, 1:1000 diluted HRP-conjugated goat-anti-human IgE (Cat no: A9667, Sigma Aldrich, Missouri, United States) was added. The plates were incubated for 1.5 hours at 37°C and washed five times. Then the plates were developed using TMB substrate which was incubated for 30 minutes at RT. Then the reaction was stopped with 1M HCl and OD values were measured at 450 nm with Synergy-H1 microplate reader.

2.4.3. Basophil Activation Test

This test was performed using fresh whole blood of the subjects and milk allergens with the Flow CAST® kit (BUHLMANN Laboratories AG, Schönenbuch, Switzerland) according to the manufacturer's instructions. Different allergens and doses were tried beforehand to find the appropriate dose and allergen. Optimization doses were as follows; for the casein mixture; 10, 100, 200, 400, 800 ng/ml and for the conventional biscotti protein; 10, 100, 1000, 10000, 100000 ng/ml, respectively. Briefly, 50 µL of homogenized EDTA whole blood was incubated in a water bath at 37°C for 15 minutes with 20 µL of staining reagent [anti-CD63-fluorescein isothiocyanate (FITC) and anti-CCR3-phycoerythrin (PE) mAbs) with added milk allergens. After adding 2 mL of 1X lysis solution, the mixture was incubated for 10 minutes at RT and centrifuged at 500 g for 5 minutes. Monoclonal mouse anti-human high-affinity IgE receptor antibody (Anti-FcεRI mAb) and N-formylmethionine leucyl-phenylalanine (fMLP) were used as positive controls. A buffer containing calcium, heparin and IL-3 was used as a negative control. The positive control and allergen-induced CD63 and CCR3 expressions were evaluated using flow cytometry (Attune NxT Flow Cytometer, Thermo Fisher, MA USA) and the cells were analyzed with FlowJo Software version 10.8 (BD Biosciences, USA). Allergen

stimulation results was evaluated if both positive controls exhibited activation of >10% of basophils and the negative control exhibited activation of <5% of basophils and the patient was classified as responder. The patients who have <10% for CCR3+ and CD63+ of the cells after the stimulation with positive controls (anti-FcεR1 antibody and fMLP) are called as non-responder (Y. H. Kim et al., 2020).

2.5. Statistical Analysis

The sample size calculation was done by using MedCalc Statistical Software version 12.7.7 (Medcalc Software, Ostend, Belgium; http://www.medcalc.org; 2013). Based on the data taken from n=3 results, and an 80% power (1-β) with the type I error (α=0.05), the sample size for both the protein concentration and band intensity analysis was calculated as n=5, for western blotting it was n=4 and for proteomics it was n=3. Statistical analysis of the data was done using SPSS v26.0 (Armonk, NY: IBM Corp), and the graphs were drawn with the GraphPad Prism version 5 (GraphPad Software, San Diego, United States). A Shapiro-wilk test was used to check whether the variables were normally distributed or not. The non-parametric tests (Mann-Whitney U for 2 sample comparison, Kruskal Wallis for more than 2 sample comparison, Wilcoxon rank sum test for paired comparisons) were used due to the data was not normally distributed. Using Spearman/Pearson correlation analysis, the linear relationship between the in-house ELISA results and the level of milk sIgE and total IgE of the patients were determined. Those with a p value less than 0.05 was considered statistically significant.

Chapter 3

RESULTS

3.1. Demographic results of the study participants

Forty-five patients [42.2% female, 3.54 (1.80-5.59) years] were included in this study. Twenty-three patients (51.1%) were allergic to several foods like egg, lentil, nuts, and etc. Thirty of them (66.7%) had a history of anaphylaxis when exposed to milk products. The characteristic features of the patients were presented in Table 3.1.

3.2. Production processes of a hypoallergenic food product

3.2.1. Total milk protein visualization with SDS-PAGE

At the very beginning of the current thesis, the human breast milk (BM) and the cow's milk (CM) were run in SDS-PAGE and stained with Coomassie Brilliant Blue to visualize the proteins that milk contains (Figure 3.1). The protein bands of serum albumin, caseins, β-lactoglobulin and α-lactalbumin in two different samples of BM and CM samples were shown in Figure 3.1 (Bloom et al., 2014). It was detected that CM samples contained α-S2 casein while BM did not. Moreover, we did not observe any β-lactoglobulin bands in BM samples.

Table 3.1 Demographic characteristics of the participants

Characteristic	n= 45
Female/Male, n/n (%)	26/19 (57.8/42.2)
Age, years*	3.54 (1.80-5.59)
Anaphylaxis, n (%)	30 (66.7%)
Total IgE (IU/L)*	281.7 (113.2-1100)
Milk sIgE (kU/L)*	26.1 (3.98-88.33)
Casein sIgE (kU/L)*	18.35 (1.27-82.83)
Eosinophil count, (mm^3)*	335 (200-700)
Eosinophil (%)*	3.95 (2.48-7.28)
Basophil count, (mm^3)*	65 (0-100)
Basophil (%)*	0.5 (0.3-0.7)
Multiple food allergy, n (%)	23 (51.1)
Asthma, n (%)	21 (46.7)
Allergic rhinitis, n (%)	17 (34.8)
Allergic conjunctivitis, n (%)	2 (4.4)
Atopic dermatitis, n (%)	24 (53.3)
Inhalant allergen sensitization, n (%)	12 (26.7)
Family history of allergic disease, n (%)	
Mother	23 (51.1)
Father	15 (33.3)
Sibling	6 (13.3)

*Median (interquartile range), sIgE: specific immunoglobulin E

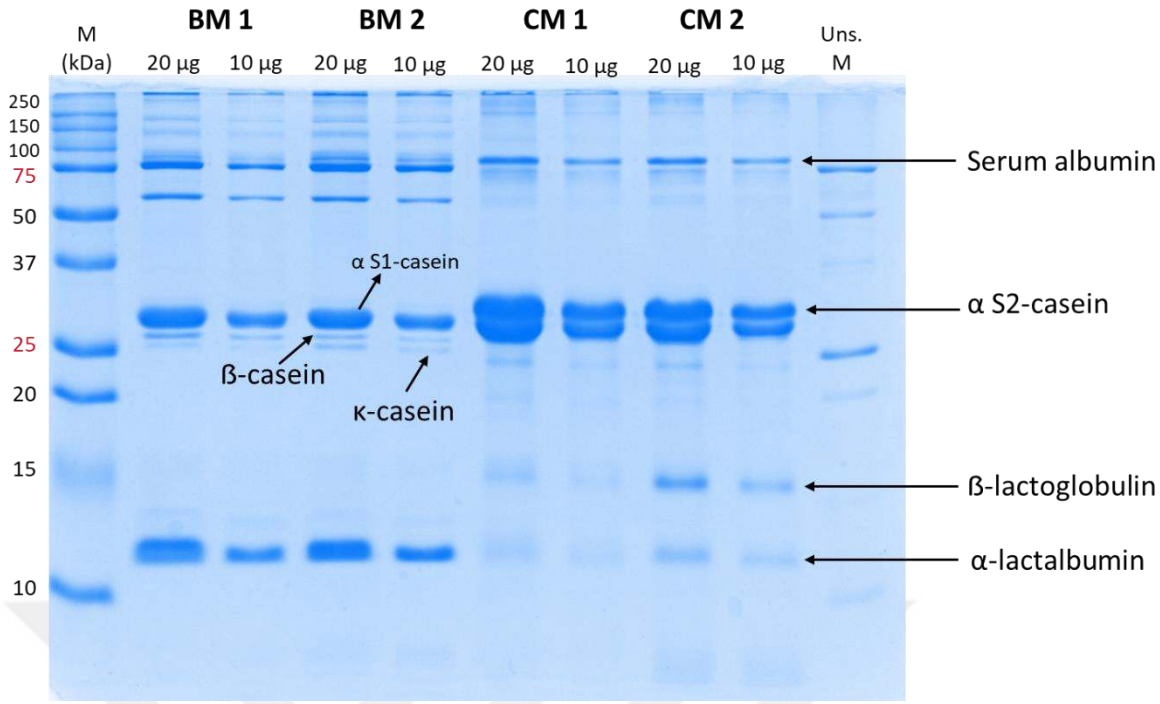

Figure 3.1 Coomassie Brilliant Blue staining of the SDS-PAGE for human breast milk (BM) and cow's milk (CM). M: marker, Uns. M: unstained marker

It is well known that milk allergic infants does not develop allergic reactions with breast milk and additionally, the baked milk product which is also called as the conventional cake is less allergic than pasteurized milk so in order to develop less allergenic milk protein containing product, the multiple combinations of the cow's milk, flour, vegetable oil, baking soda and sugar were assessed to see the matrix effect and to optimize the cake recipe (Table 3.2). The SDS-PAGE result showed that the presence of flour and sugar decreased the intensities of the casein bands as this is expected due to the matrix effect. Then we decided to use the one which contained Cow's milk + Flour +Oil + Baking soda+ Sugar (CM-F-O-BS-S) as basic recipe since this one showed the weakest intensity of the casein bands (Figure 3.2).

Table 3.2 Abbreviations that were used for the optimization of the new hypoallergenic product

Combinations	Abbreviations
Baked Cow's milk	B-CM
Cow's milk + Sugar	CM-S
Cow's milk + Baking soda	CM-BS
Cow's milk + Flour	CM-F
Cow's milk + Flour + Baking soda	CM-F-BS
Cow's milk + Sugar + Baking soda	CM-S-BS
Cow's milk + Flour + Sugar	CM-F-S
Cow's milk + Flour + Baking soda + Sugar	CM-F-BS-S
Cow's milk + Flour +Oil + Baking soda+ Sugar	CM-F-O-BS-S

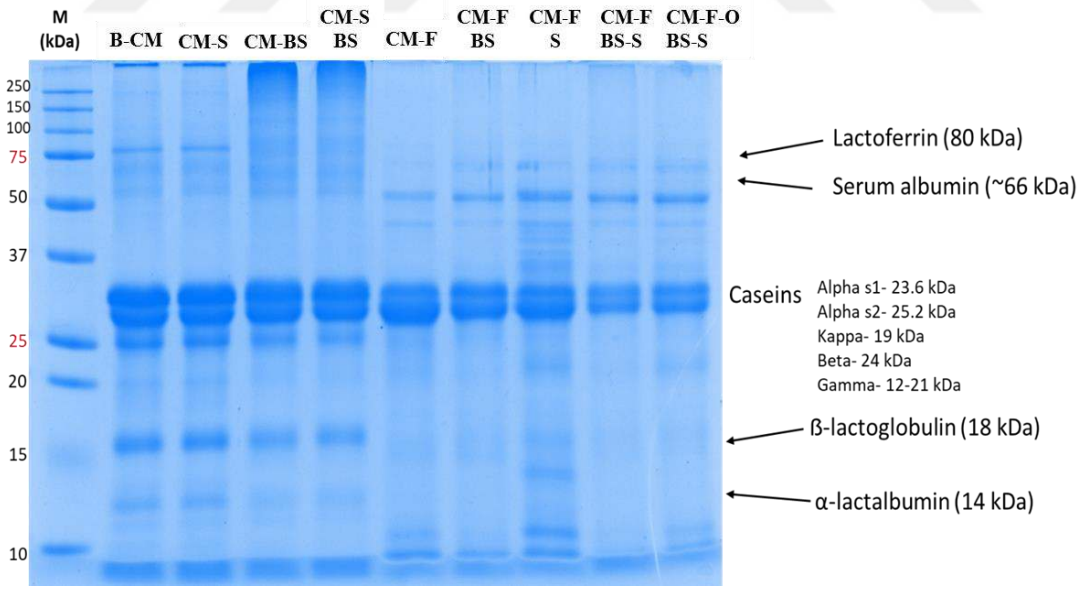

Figure 3.2- Coomassie staining of the SDS-PAGE for cake combinations. M: marker

3.2.2. The effect of vegetable oil and margarine on the casein bands

To see the effect of the flour/sugar ratio and the type of oil (vegetative oil or margarine) on the intensities of the casein bands, the following gel was run (Figure 3.3). The lowest casein bands were observed in the flour/sugar ratio of 0.5/1 cake samples for both vegetable oil and margarine. Despite the fact that the casein band intensity of 0.5/1 ratio cake made with margarine was low, the cake was not edible. Therefore, vegetable oil was chosen for the following experiments.

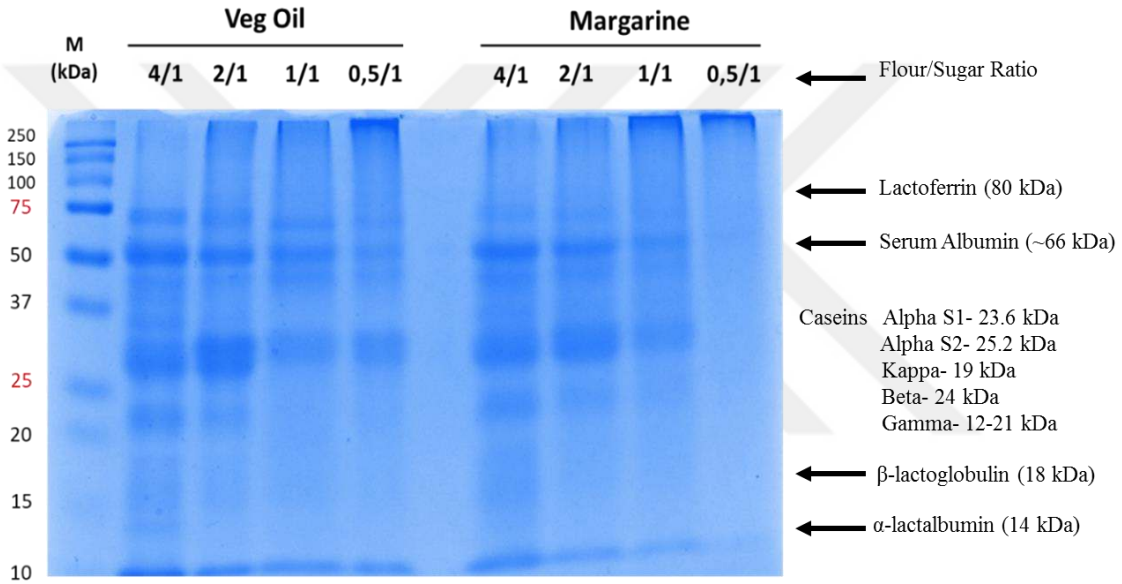

Figure 3.3 Coomassie staining of SDS-PAGE for vegetable oil/margarine optimization. M: marker

3.2.3. The effect of different sugars: glucose and sucrose on the casein bands

To evaluate the effect of the sugar type, glucose and sucrose were used. Glucose is a sugar that acts as the reducing agent and can donate electrons to some other molecule by oxidizing it, while sucrose does not have any reducing capacity. In our daily life, sucrose is commonly used. Reducing sugars can react with other parts of the food, like amino acids, to change the colour or taste of the food as well as may affect the casein band intensity. Figure 3.4 shows that there was no difference between the cakes made with sucrose and glucose in terms of the casein band intensity.

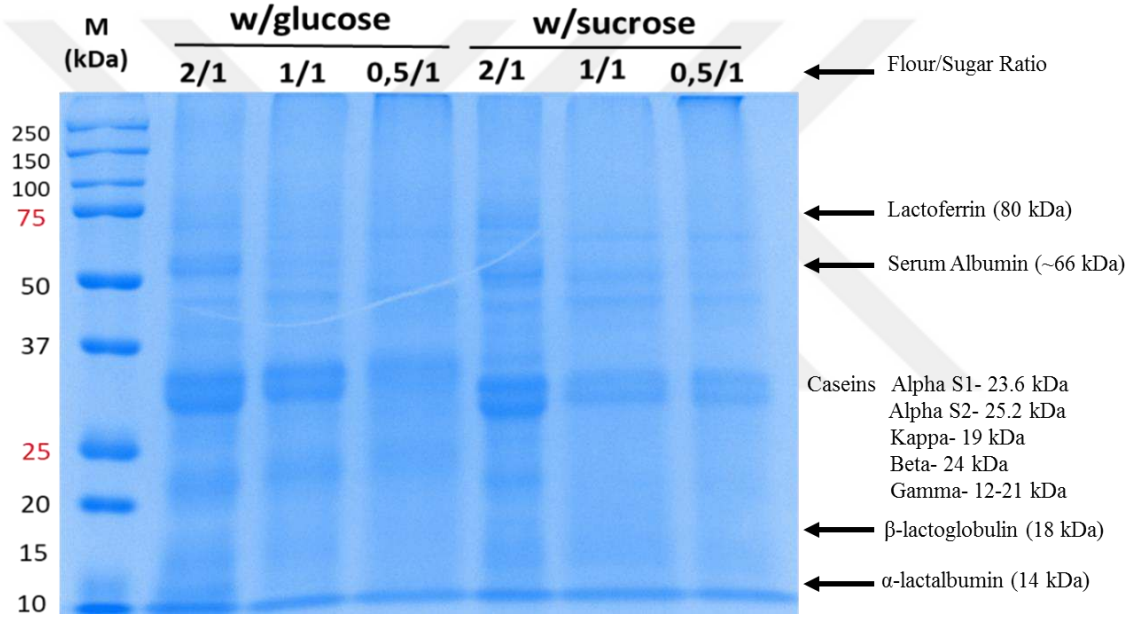

Figure 3.4 Coomassie staining of SDS-PAGE for glucose/sucrose optimization. M: marker

3.2.4. The comparison of oven and microwave on the casein bands

Afterwards, the microwave and the oven were used for baking and the lowest casein band intensities were observed in oven (Figure 3.5). The cake with the flour/sugar ratio of 0.5/1 baked with oven had the lowest intensity of the casein bands.

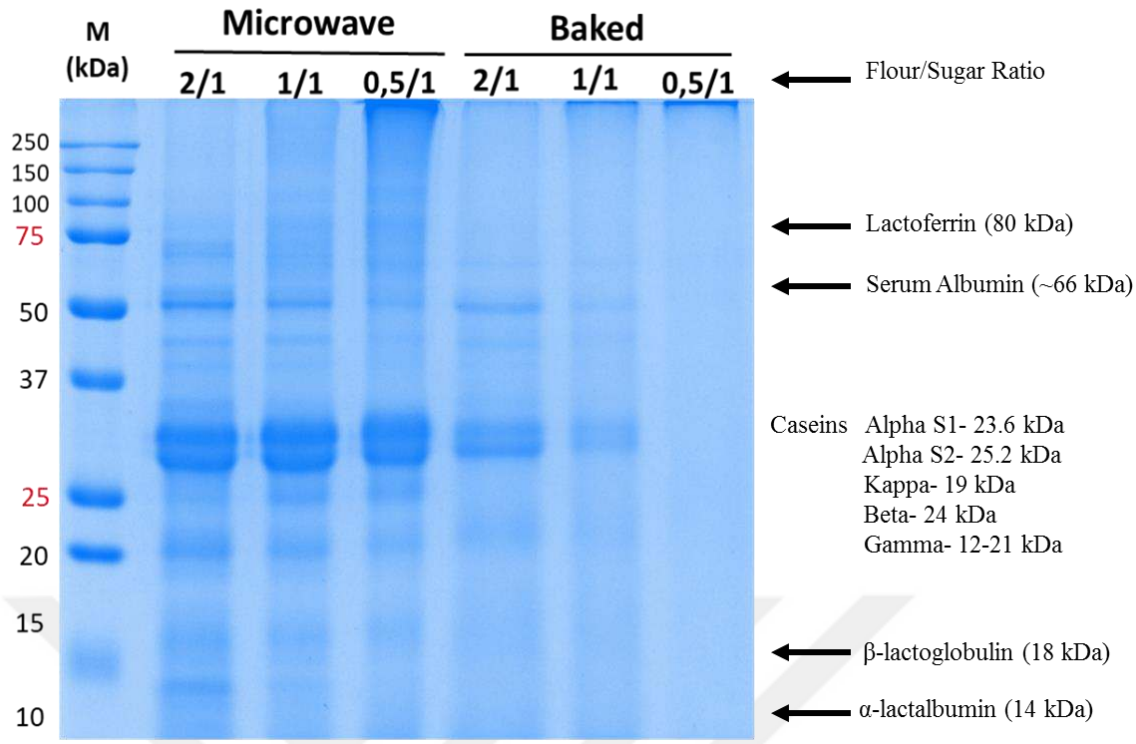

Figure 3.5 Coomassie staining of SDS-PAGE for microwave/oven optimization. M: marker

3.2.5. The effect of the Maillard reaction on the casein bands

Later, we observed that once the colour of the cake becomes browner, then the intensities of the casein bands were decreased more. Brown colour is the marker of the Maillard reaction. If we see browner colour, the more Maillard reaction is occurred and more proteins are nonenzymatically degraded. For that reason, cakes were crumbed and crust parts with the highest Maillard reaction were run separately. We observed that the cake with the flour/sugar ratio of 0.5/1 had the lowest intensity of casein bands (Figure 3.6). Cooking time was also checked with the current experiment (Figure 3.6). As the cooking time increased at 200°C, the casein band density decreased. However, the taste of the cakes was not good.

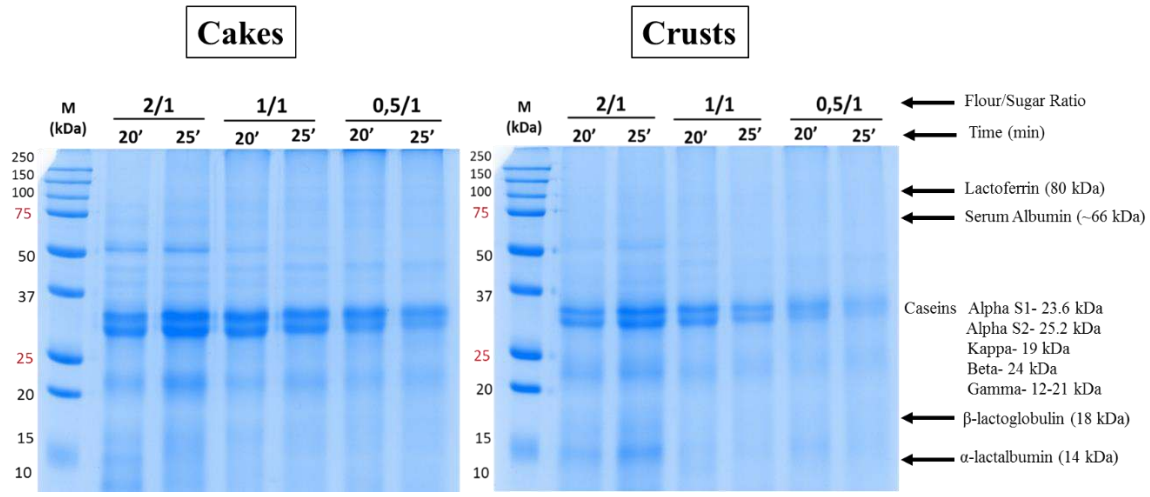

Figure 3.6 Coomassie staining of SDS-PAGE for cake and crust samples. M: marker

3.2.6. The idea of twice-baked cake: Biscotti

Afterward, we thought that the inside of the cake would be brown as like the outside of the cake for the complete Maillard reaction in whole cake. Therefore, we consulted the pastry chefs at the Divan Patisserie. They recommended us that if low temperature with long exposure was used, like biscotti, we could achieve the same inside/outside brown colour. With the conventional cake recipe (2/1 ratio), biscotti was done as you can see in Figure 3.7. You can also see the powder of biscotti samples. When the time passes, the colour of powder becomes browner.

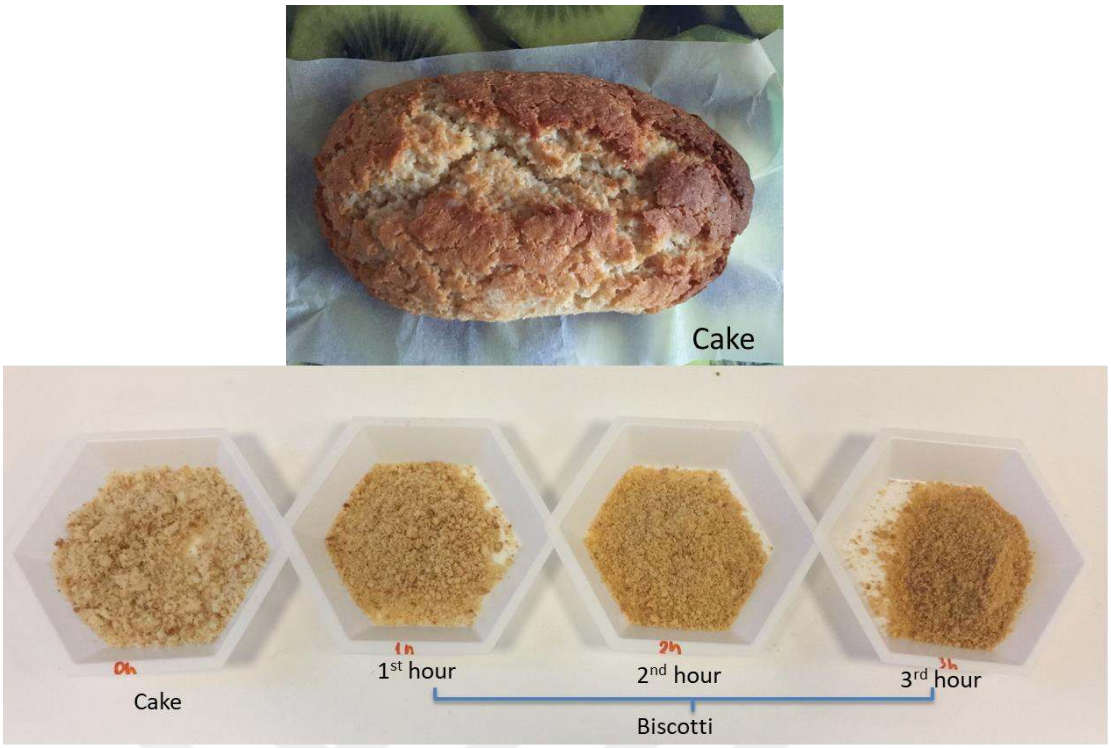

Figure 3.7 Conventional cake and Biscotti after powdered

3.3. Protein quantitation analysis

3.3.1. Total protein concentration in the biscotti and the conventional cake samples

Milk proteins were isolated from five different milk products. The protein concentrations of all products studied in quintuplicate as shown in Table 3.3. When the concentrations were compared, it was seen that concentration of the biscotti-3h was significantly lower than the cake (p=0.002, Figure 3.8).

Table 3.3 Bradford results of milk products showing total protein amount

	Concentrations (mg/ml)					MEAN
Cow's milk (1:20)	1. 2	1.18	1.35	1.3	1.35	**1.28**
Conventional cake (5 g)	1.55	1.41	1.4	1.5	1.6	**1.49**
Biscotti-1h (5 g)	1.43	1.4	1.4	1.3	1.9	**1.49**
Biscotti-2h (5 g)	1.27	1.12	1.19	0.9	1.36	**1.17**
Biscotti-3h (5 g)	1	0.76	0.88	0.9	1.25	**0.96**

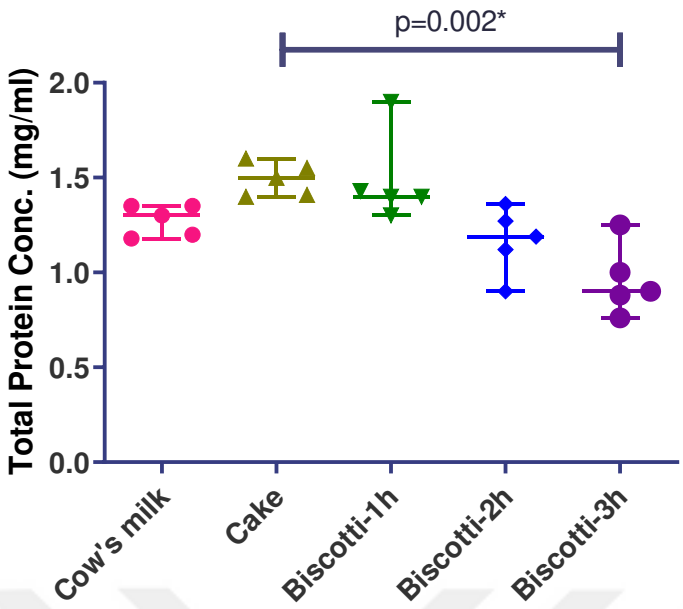

Figure 3.8 Comparison between the protein concentrations of different milk products. (*cake and biscotti-3h- nonparametric post-hoc analysis)

3.3.2. The effect of baking duration on the casein band intensity

SDS-PAGE analysis was performed with the biscotti, which were baked for 1 hour, 2 hours and 3 hours. In Figure 3.9 shows that the casein band intensity was decreasing as the cooking time increasing for biscotti samples.

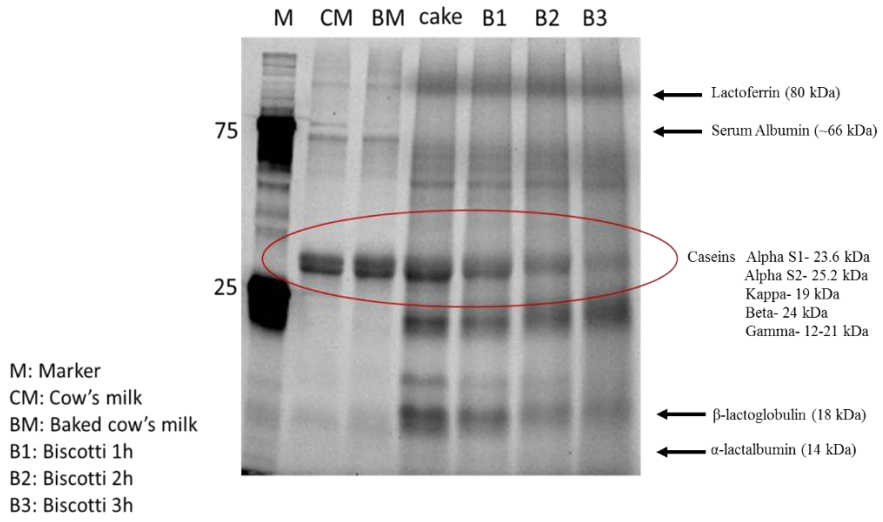

Figure 3.9 SDS PAGE analysis of milk, cake and biscotti samples (M: marker)

Casein band intensities of the milk, cake and biscotti samples were analyzed with Image Lab (Biorad) program in order to get numerical value so that the band intensities can be comparable.

The band intensities of all products were analyzed in quintuplicate. It was found that casein band intensities of the biscotti-3h were significantly lower than the cow's milk (p=0.008, Figure 3.10).

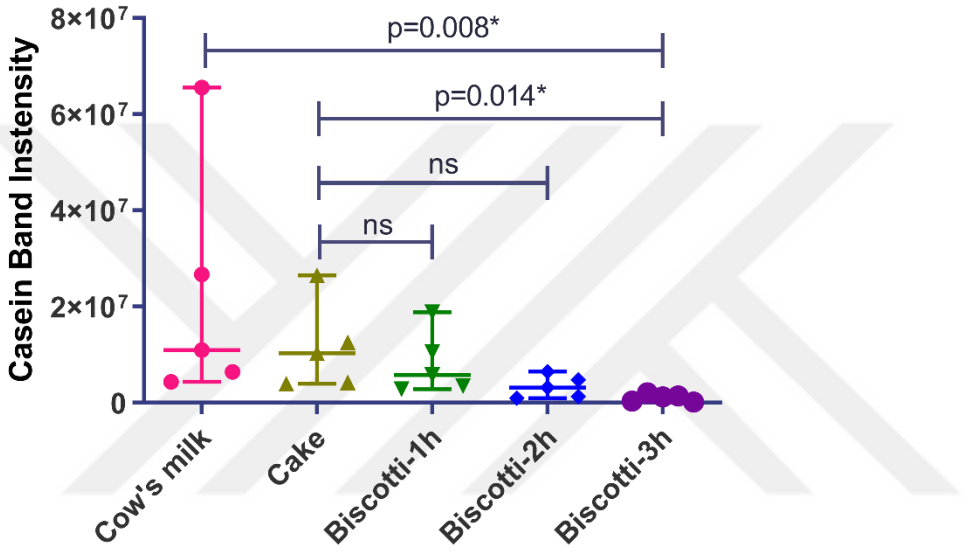

Figure 3.10 Casein band analysis of milk, cake and biscotti samples was performed with Image Lab, Biorad (*cake and biscotti-3h- nonparametric post-hoc analysis) (ns: non-significant)

3.3.3. SDS-PAGE analysis of the fermented milk products: yogurt and cheese

Further, SDS-PAGE analysis was performed for yogurt and cheese. Figure 3.11 shows the SDS-PAGE results of yogurt and cheese samples. Most of the proteins of the milk can also be seen in yogurt and cheese samples.

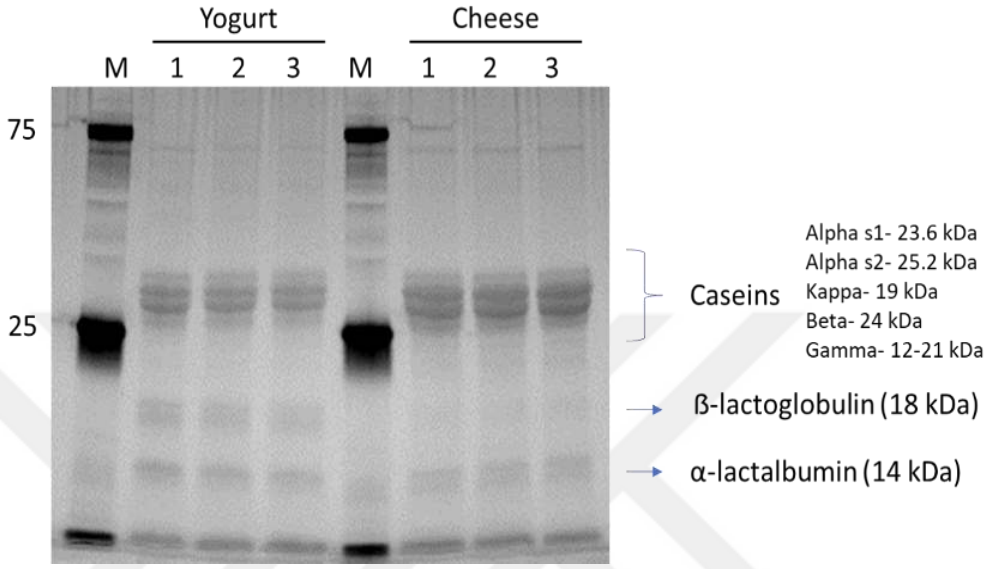

Figure 3.11 SDS PAGE analysis of yogurt and cheese samples (M: marker)

3.3.4. Proteomics analysis of the milk and milk products

To analyse the milk protein contents of the cow's milk and milk products (conventional cake, biscotti 1h, biscotti 2h and biscotti 3h), proteomics analysis was performed using LC-qTOF-MS. The intensities of 4 different subunits of casein (α-S1 casein, α-S2 casein, β casein and κ casein) and β-lactoglobulin were measured for each product. The highest proteins for α-S1 casein, α-S2 casein, β-casein and κ-casein and β-lactoglobulin were obtained in pasteurized milk samples and the lowest protein levels were obtained in 3 hours baked biscotti samples (Figure 3.12).

When the milk protein intensities were compared across all milk products (conventional cake, biscotti 1h, biscotti 2h and biscotti 3h), the intensities of each casein fraction and β-lactoglobulin were decreased as the cooking time increased. The reduction of intensities was found to be statistically significant for each casein fraction and β-lactoglobulin (Figure 3.13).

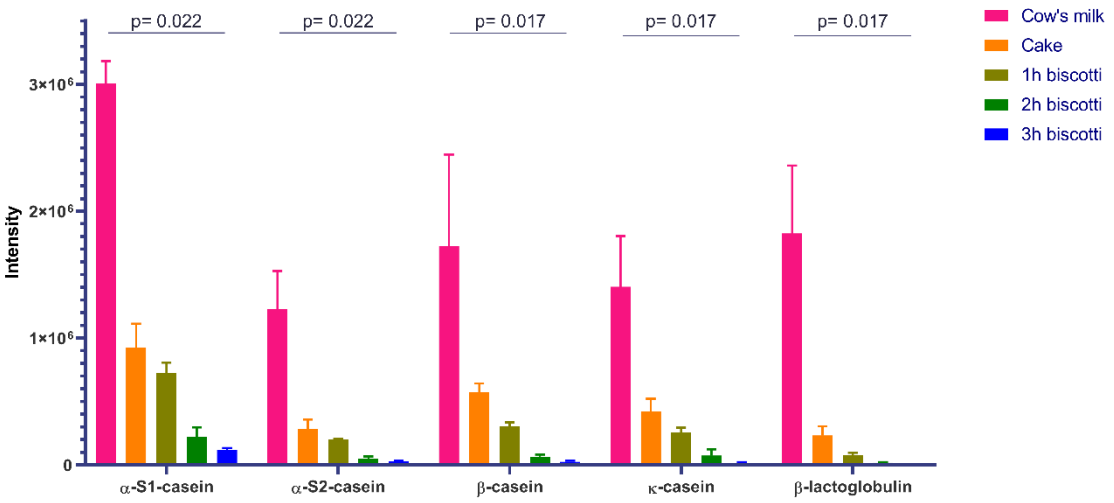

Figure 3.12 LC-qTOF-MS analysis of the milk, conventional cake and biscotti samples (n=3, Analysis was performed with the Related-Samples Friedman's Two-Way Analysis of Variance by Ranks for all groups)

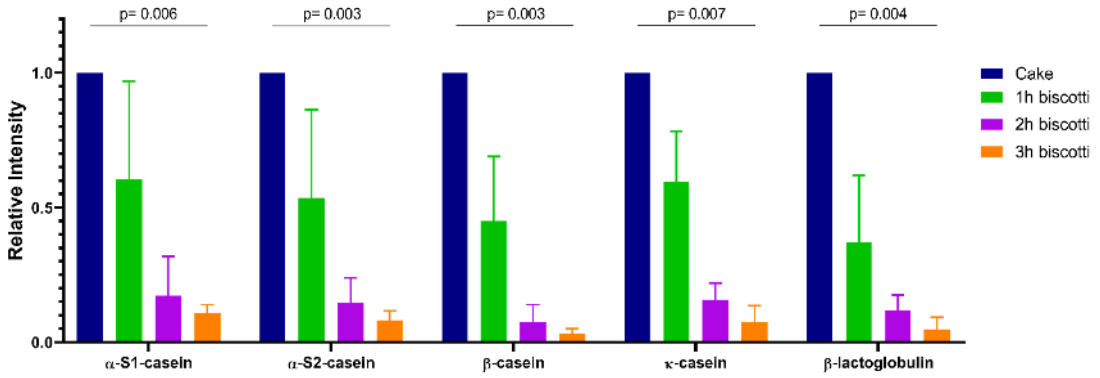

Figure 3.13 LC-qTOF-MS analysis of the cake and biscotti samples (n=5, Biscotti samples were normalized to milk. Analysis was performed with the Related-Samples Friedman's Two-Way Analysis of Variance by Ranks)

3.4. IgE binding assays

3.4.1. Binding of IgE to different milk products by Western Blotting

In this experiment, the serum from the patients who have milk sIgE greater than 100 kU/L were used to analyze the binding capacity of milk sIgE to milk proteins obtained from the pasteurized milk, conventional cake and 3 hours baked biscotti.

It was observed that milk sIgE binding to caseins was significantly lower with biscotti-3 h than conventional cake and pasteurized milk (n=4, p= 0.0012, Figure 3.14).

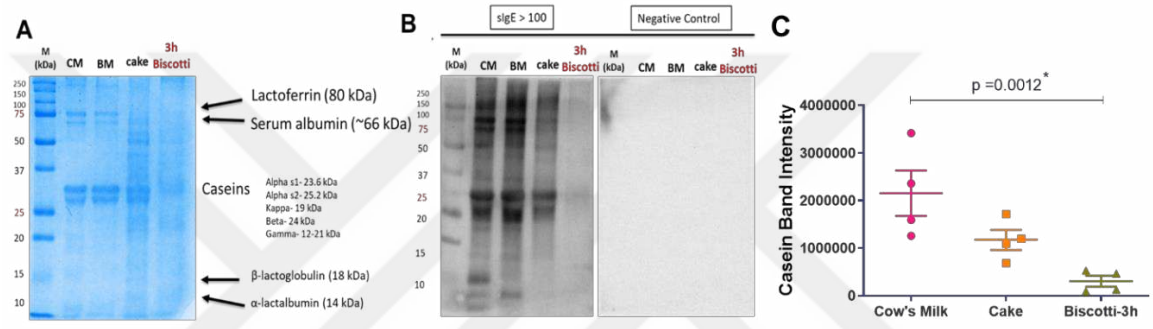

Figure 3.14 Western Blotting Results. A. Loading control, CM: Cow's milk, BM: baked milk. B. PVDF membrane incubated with 3 patients' sera with sIgE higher than 100 kU/L, and negative control. B. Casein band analysis of milk, conventional cake and biscotti-3h with Image Lab (*cake and biscotti-3h- nonparametric post-hoc analysis)

3.4.2. Indirect in-house ELISA results

The reactivity of milk sIgE to the patients' sera was checked with indirect in-house ELISA. Five kinds of ELISA were designed: milk, cheese, yogurt, conventional cake and biscotti-3h were used as coating antigen in order to measure the patients' sIgE levels of the milk, cheese, yogurt, conventional cake and biscotti 3h. For cake and biscotti 3h samples, the checkerboard titration was performed to decide the optimum dilutions of patients' sera and secondary antibody. Table 3.4 shows the results of checkerboard titrations of the conventional cake and biscotti 3h samples done with the patients' serum whose milk sIgE level was >100 kU/L. It was decided that the dilution of 1:20 for patients' sera and 1:1000 dilution of HRP-linked human anti-IgE secondary antibody were going to be used for both the conventional cake and biscotti 3h.

Table 3.4 Checkerboard titrations of the conventional cake and biscotti 3h samples

CAKE							BISCOTTI						
	no sec	1:10.000	1:5000	1:2000	1:1000	1:500		no sec	1:10.000	1:5000	1:2000	1:1000	1:500
no primary	0,048	0,045	0,047	0,046	0,047	0,048	no primary	0,048	0,049	0,045	0,045	0,046	0,049
1:500	0,097	0,104	0,120	0,150	0,228	0,239	1:500	0,091	0,097	0,106	0,132	0,189	0,211
1:250	0,141	0,152	0,179	0,227	0,387	0,405	1:250	0,115	0,138	0,153	0,194	0,308	0,349
1:100	0,225	0,265	0,302	0,427	0,794	0,841	1:100	0,194	0,235	0,271	0,355	0,625	0,721
1:20	0,352	0,392	0,478	0,662	1,257	1,385	1:20	0,298	0,378	0,425	0,582	0,986	1,119
1:10	0,557	0,650	0,742	1,029	1,880	2,126	1:10	0,493	0,605	0,686	0,890	1,495	1,734

The same dilutions were also optimal for milk, cheese and yogurt ELISAs.

3.4.2.1. Development of indirect in-house ELISA to measure sIgE to biscotti and conventional cake

The reactivity of the milk allergic patients' sera was also assessed with indirect in-house ELISA. All patients were coded as KUFA, which represents Koç University Food Allergy. As all serum samples were diluted at the same ratio (1:20), the comparison analysis could be performed. There was statistically significant difference between the OD values of the biscotti-3h and cake ($p<0.0001$, Figure 3.15). Figure 3.15 showed the individual cake and biscotti-3h sIgE ELISA results of each 21 patients.

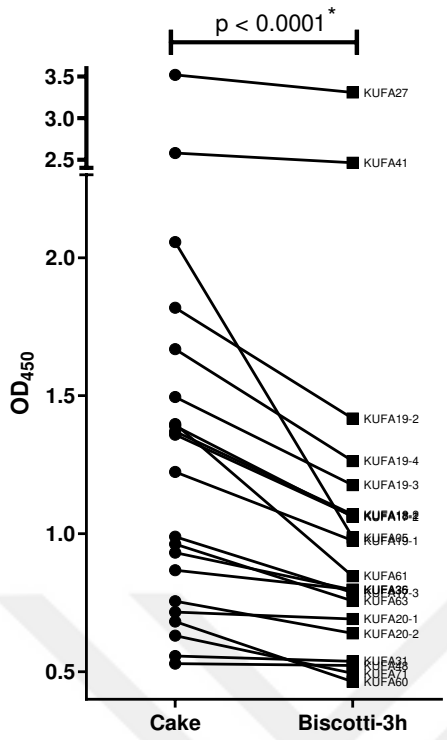

Figure 3.15 The cake/biscotti-3h sIgE ELISA results of 21 patients' sera (*Wilcoxon matched-pairs signed rank test)

3.4.2.2. Development of indirect in-house ELISA to measure sIgE to milk, cheese and yogurt

To investigate the reactivity of the milk allergic patients' sera with milk, cheese and yogurt, indirect in-house ELISA was conducted for each product. The OD values and tolerance status of the milk, cheese and yogurt and the sIgE levels of 35 patients were listed in Table 3.5.

The importance of these experiments was the potential to check the tolerance status of the subjects before OFC test which has a risk of anaphylaxis. After determining the tolerance status of patients (reactive or tolerant), OD values of the reactive and tolerant ones were compared with each other for milk, cheese and yogurt separately (Figure 3.16). The OD values of tolerant patients were low for milk, cheese and yogurt when compared to reactive ones.

Table 3.5 Comparison of OD values and tolerance status of milk allergic subjects (n=3, 1:20 dilution)

	OD Values			Tolerance Status					
	Milk	**Cheese**	**Yogurt**	**Milk**	**Cheese**	**Yogurt**	**milk sIgE (kU/L)**	**Casein sIgE (kU/L)**	**Total IgE (IU/L)**
KUFA-2	0.484	0.379	0.28	T	T	T	0.08	0.17	755.3
KUFA-5	2.53	2.70	1.40	R	R	R	>100	>100	672.2
KUFA-6	0.44	0.371	0.286	T	T	T	11.5	4.51	302
KUFA-8	0.373	0.303	0.19	T	T	T	0.6	-	18.13
KUFA-11	0.327	0.212	0.157	T	T	T	0.27	0.11	14.26
KUFA-12	0.28	0.197	0.139	T	T	T	0.53	0.01	166.3
KUFA-13	0.265	0.203	0.176	T	T	T	-	-	1857
KUFA-17	0.75	0.75	0.66	R	R	R	72.6	92.9	407
KUFA-18	0.78	0.74	0.70	R	R	R	>100	92.1	627
KUFA-19	0.83	0.85	0.73	R	R	R	>100	>100	265
KUFA-21	0.506	0.396	0.242	R	R	R	30.9	22.3	918.7
KUFA-22	0.361	0.265	0.163	T	T	T	6.47	4.58	59.19
KUFA-25	1.56	1.438	0.661	R	R	R	>100	>100	1510
KUFA-27	2.395	2.311	0.611	R	R	R	>100	>100	3549
KUFA-28	0.627	0.421	0.24	R	T	T	26.8	1	84.38
KUFA-29	0.841	0.348	0.352	T	T	T	1.85	0.12	281.7
KUFA-31	0.395	0.346	0.243	T	T	T	24.2	6.39	107.1
KUFA-32	0.758	0.698	0.251	R	R	R	49.2	39.7	149.8
KUFA-34	0.701	0.589	0.236	R	R	R	26	33.6	134
KUFA-35	0.21	0.25	0.18	T	T	T	92.2	77.1	-
KUFA-36	0.931	0.827	0.498	R	R	R	63.5	47.6	144.5
KUFA-38	0.523	0.395	0.172	R	R	R	32.4	20.5	54.61
KUFA-41	1.10	1.10	1.18	R	R	R	>100	>100	548
KUFA-43	0.253	0.213	0.107	R	R	T	0.88	0.1	7.53
KUFA-48	0.377	0.254	0.22	R	T	T	22.6	5.2	3678
KUFA-53	1.698	1.576	0.792	R	R	R	>100	>100	1282
KUFA-60	0.78	0.68	0.29	R	R	R	35.4	25.3	555
KUFA-61	1.61	1.58	0.53	R	R	R	98.5	100	220
KUFA-64	2.425	2.319	0.864	R	R	R	>100	>100	4150
KUFA-65	0.20	0.15	0.12	R	T	T	1.66	0.17	60
KUFA-67	1.644	1.581	0.468	R	R	R	>100	>100	3313
KUFA-68	0.23	0.16	0.12	R	T	T	4.36	1.36	-
KUFA-69	1.37	1.206	0.177	R	R	R	>100	>100	7028
KUFA-70	0.19	0.16	0.11	R	T	T	2.87	1.01	157
KUFA-71	0.60	0.44	0.15	R	T	T	12.3	6.3	517

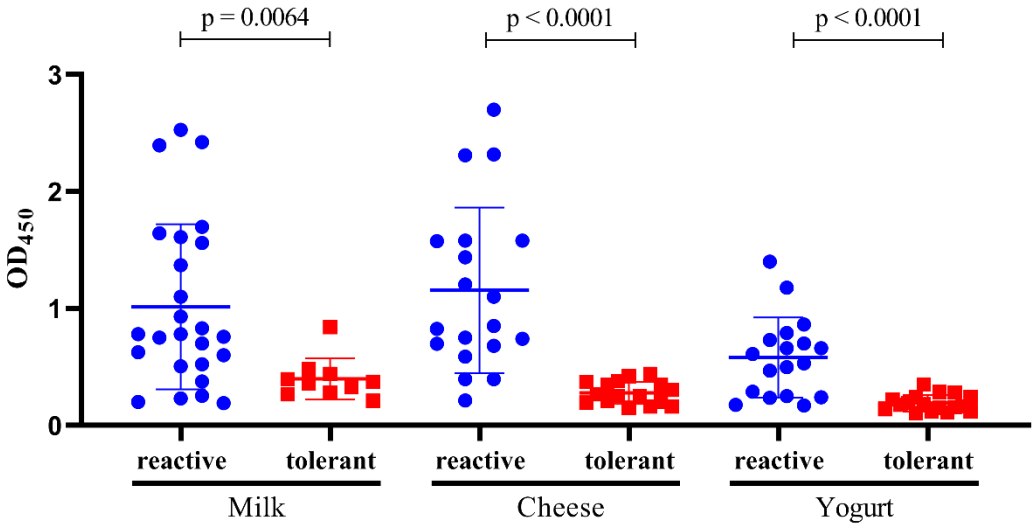

Figure 3.16 Milk, cheese and yogurt sIgE ELISA results (milk reactive n=25, milk tolerant=10, cheese reactive n=19, cheese tolerant=16, yogurt reactive n=18, yogurt tolerant=17, All analysis were performed with nonparametric Mann-Whitney U test)

The milk sIgE levels were also lower in milk, cheese and yogurt than the reactive ones (Figure 3.17). Spearman's rank order correlations were run to examine the relationship between milk sIgE OD values from in-house ELISA, milk sIgE (Uni-CAP, Phadia, Thermo Fisher Scientific, MA, USA), casein sIgE (Uni-CAP, Phadia, Thermo Fisher Scientific, MA, USA), and total IgE levels (Uni-CAP, Phadia, Thermo Fisher Scientific, MA, USA). There were positive and significant correlation between milk sIgE OD values of in-house ELISA and milk sIgE levels (r_s= 0.81, n= 30, p<0.0001), milk sIgE OD values of in-house ELISA and casein sIgE levels (r_s=0.84, n=29, p<0.0001), and milk sIgE OD values of in-house ELISA and total IgE levels (r_s= 0.43, n=30, p=0.0174).

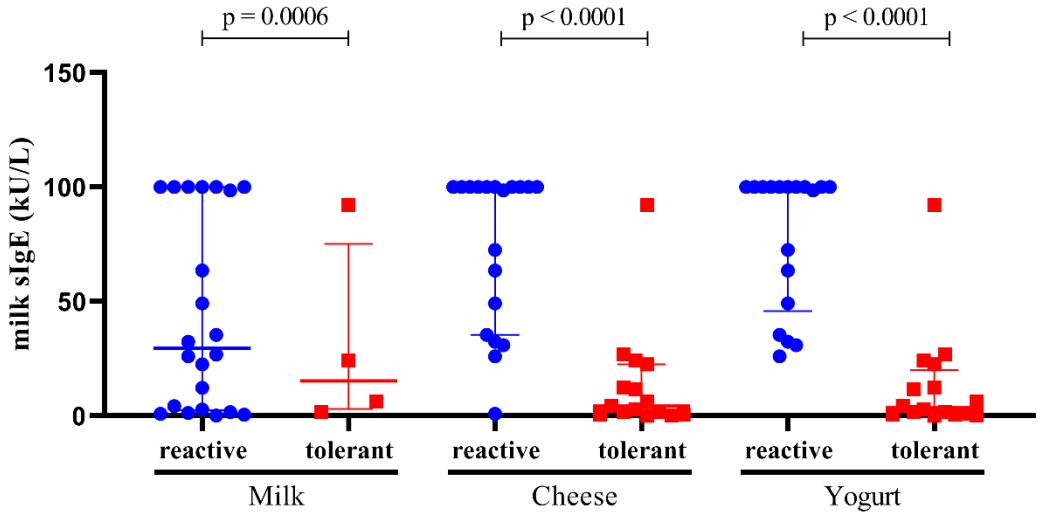

Figure 3.17 Milk sIgE (Uni-CAP, Phadia, Thermo Fisher Scientific, MA, USA) results of milk, cheese and yogurt reactive and tolerant ones (milk reactive n=25, milk tolerant=9, cheese reactive n=19, cheese tolerant=15, yogurt reactive n=18, yogurt tolerant=16, All analysis were performed with nonparametric Mann-Whitney U test)

3.4.3. Basophil Activation Test results done with casein and biscotti

Basophil activation test (BAT) is a functional procedure that measures the degree of degranulation following stimulation with allergen (Y. H. Kim et al., 2020). BAT reduces the need for *in vivo* procedures, such as oral food challenge, which can cause allergic reactions of unpredictable severity. As it closely reflects the patients' phenotype in most cases, it may be used to support the diagnosis and monitoring the natural resolution of food allergies.

Figure 3.18 shows the gating strategy for the detection of $CCR3^+/CD63^+$ basophils. First, total monocytes, lymphocytes and granulocytes were gated. Then single cells were selected with FSC-H and FSC-A graph. Afterwards, $CCR3^+$ and SSC low cells were gated as basophils, and activated basophils were determined as $CCR3^+/CD63^+$ cells by using basophil gate.

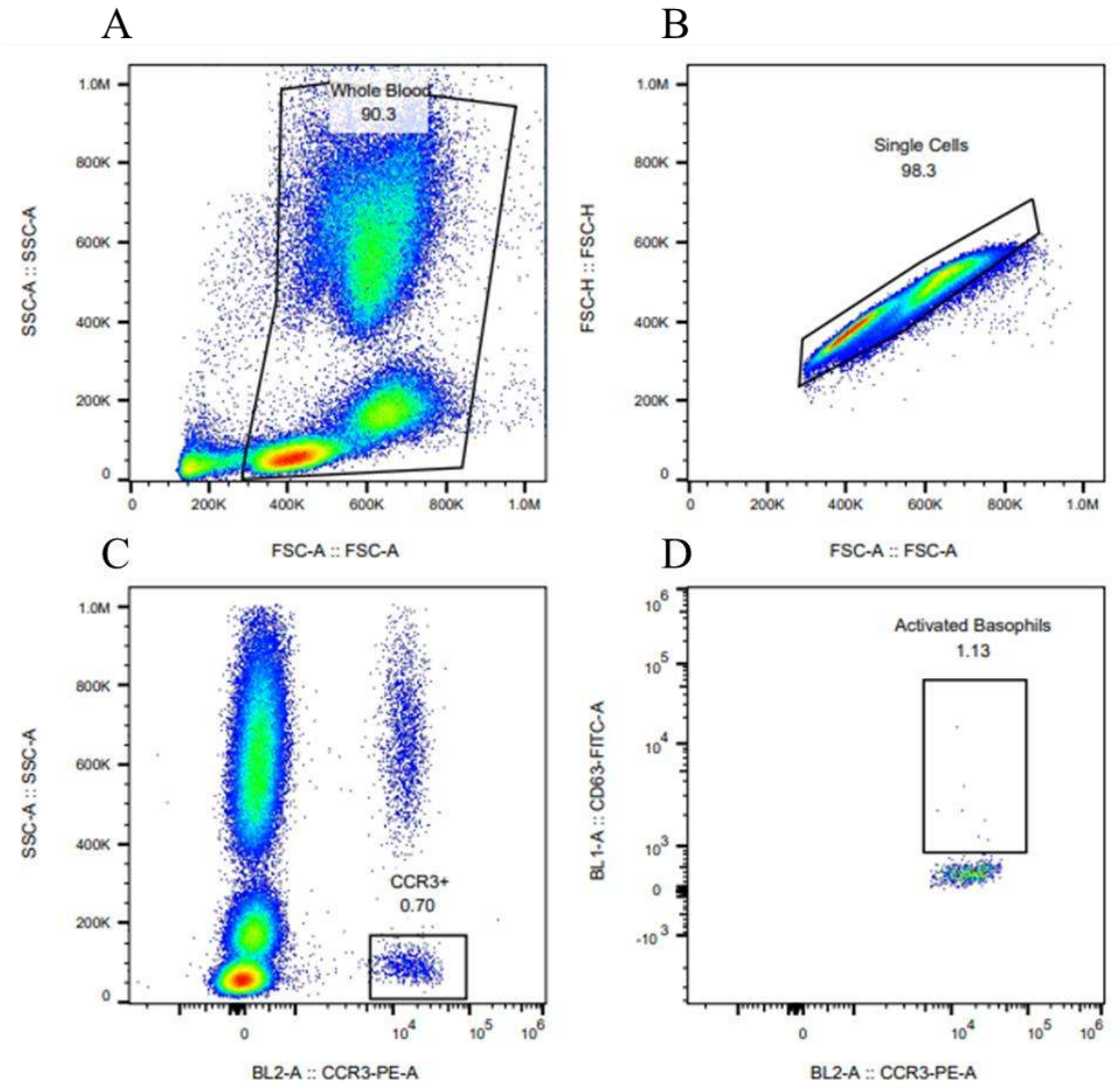

Figure 3.18 Flow Cytometric determination of $CCR3^{+}/CD63^{+}$ Cells. Total monocytes, lymphocytes and granulocytes were gated (A) and single cells were selected (B). $CCR3^{+}$ cells were gated as basophils (C) and from this gate activated basophils were determined as $CCR3^{+}/CD63^{+}$ cells

BAT was performed in ten milk allergic patients and one healthy subject. Figure 3.19 shows the BAT result of patient KUFA-21. Casein mix (10, 100, 200, 400, 800 ng/ml) and biscotti supernatant (10, 100, 1000, 10000, 100000 ng/ml) were used as allergen at different concentrations. The healthy subject was used as a negative control in order to show that biscotti protein do not activate basophils (Figure 3.20)

Figure 3.19 and Figure 3.20 showed the BAT results that negative controls were under 5% and both positive controls were above 10% but caseins/biscotti proteins stimulated the activation of basophils only in the milk allergic patient (Figure 3.19).

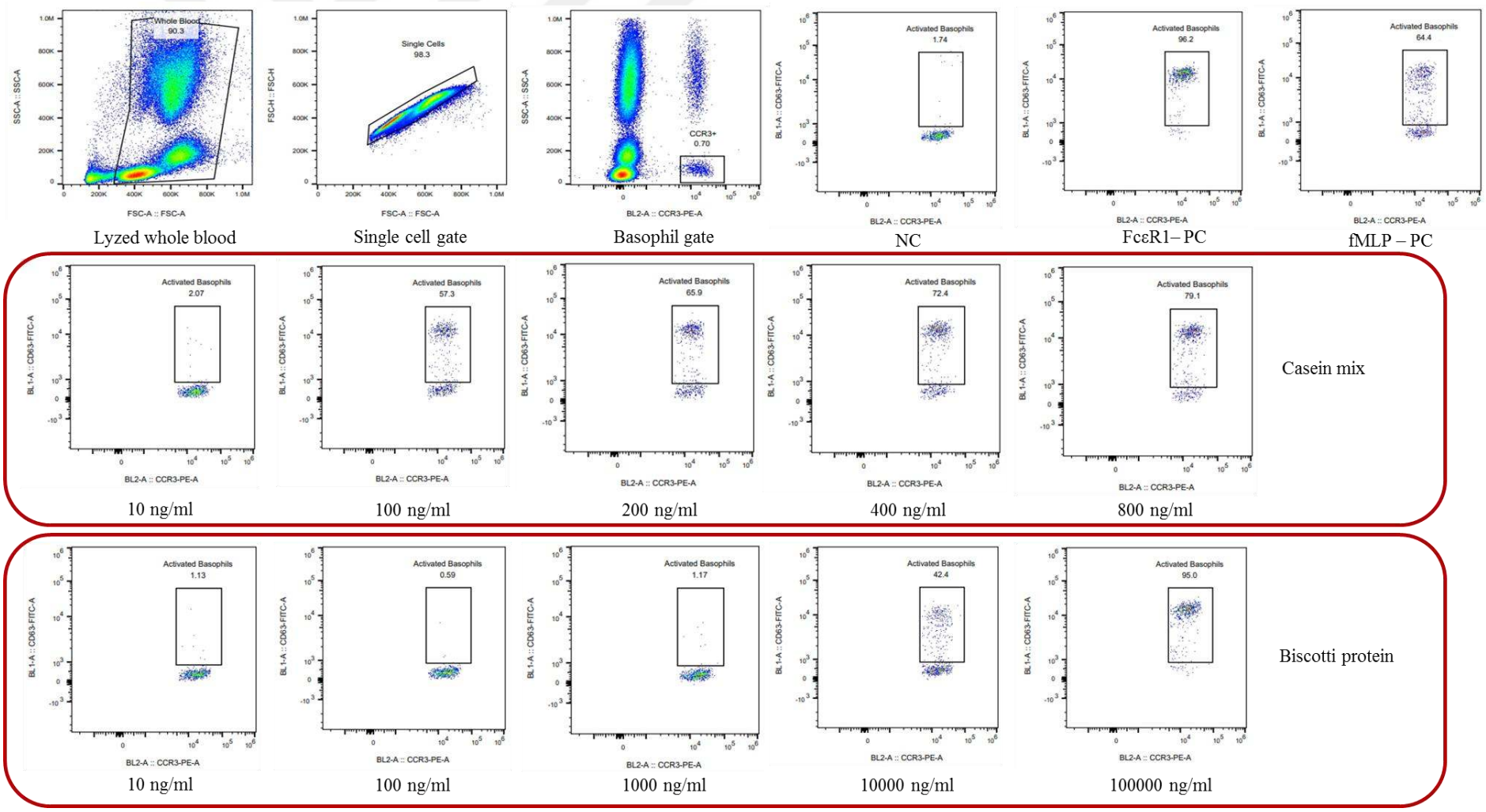

Figure 3.19 BAT result of milk allergic patient KUFA-21 done with casein and biscotti supernatant (NC: negative control, PC: positive control)

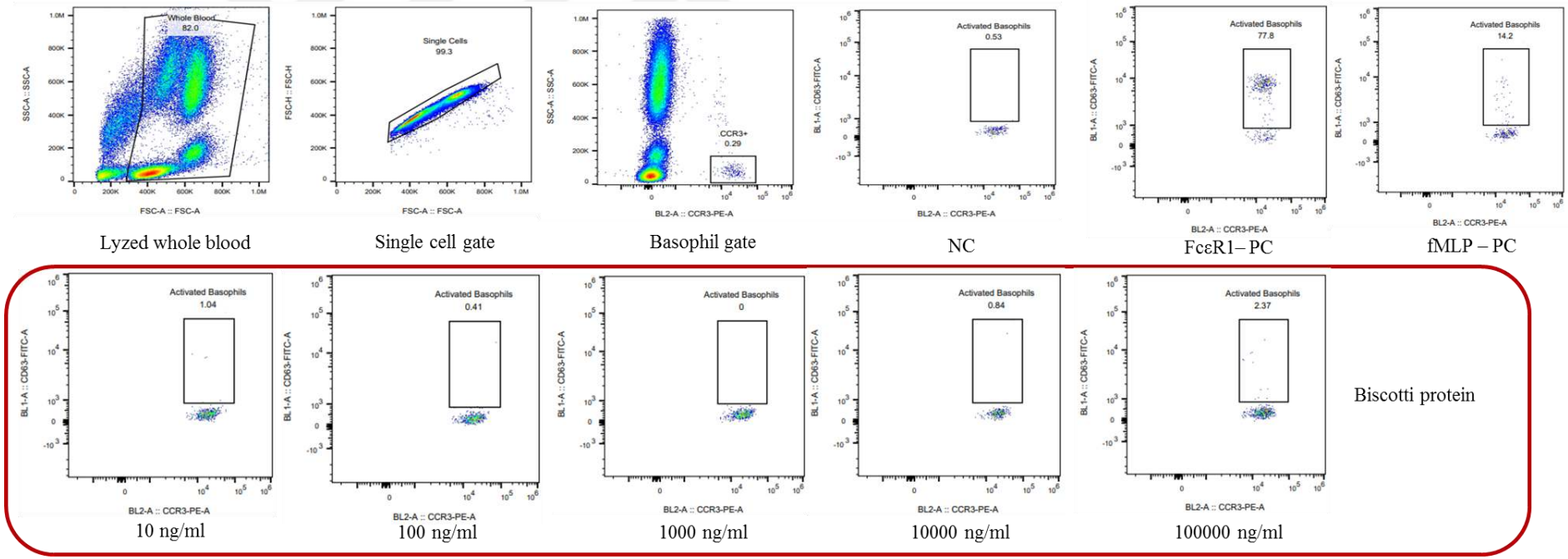

Figure 3.20 BAT result of a non-allergic subject done with biscotti supernatant (NC: negative control, PC: positive control)

Casein mix and biscotti supernatant induced a dose response. Figure 3.21 shows the dose response graphs. As the doses increased, the percentages of activated basophils increased as well.

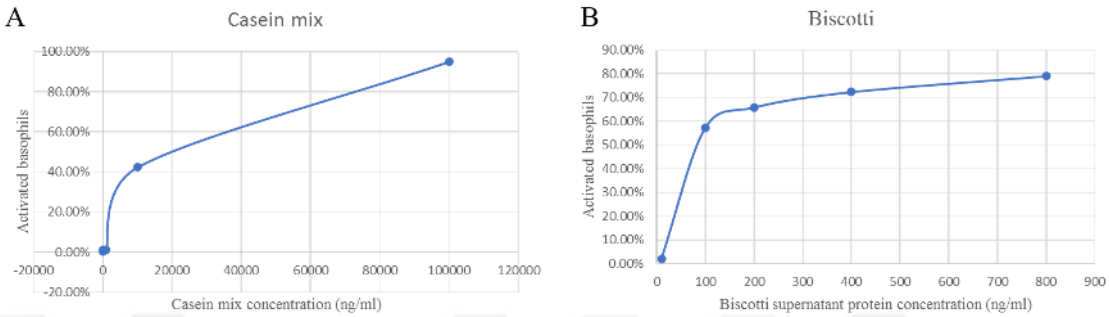

Figure 3.21 Dose response graphs of casein mix (A) and biscotti supernatant (B)

Chapter 4

DISCUSSION

In this study, we aimed i) to develop a new hypoallergenic food product that contains less allergenic milk proteins, ii) to determine the total protein concentration and milk protein content of diary milk products and newly developed hypoallergenic food product, iii) to evaluate reliable indirect in-house ELISA for the detection of sIgE antibodies against milk products, iv) to check the IgE reactivity with the western blotting, newly evaluated indirect in-house ELISA and BAT.

Cow's milk protein allergy is the most common type of food allergy in childhood presenting a variable prevalence worldwide (Flom & Sicherer, 2019). Allergic symptoms occur when immune system reacts with the proteins found in cow's milk (Johansson et al., 2004; Untersmayr & Jensen-Jarolim, 2006). Currently, the traditional therapeutic approach in food allergy is the strict avoidance of the food and preparation to promptly treat allergic reactions, which cause significant anxiety among the parents (Muraro et al., 2014). Therefore, there is an urgent need for safe treatment options for the children with cow's milk allergy.

To develop a new hypoallergenic food product that contains hypoallergenic milk proteins, the optimization experiments were performed with the conventional cake recipe for this project. Type of oil (vegetative or margarine), sugar (sucrose or glucose) and baking tool (oven or microwave) were all checked. The flour/sugar ratio and the matrix affect were also studied. Then, Maillard reaction was observed in the brown crust parts of the cakes and the idea of Biscotti (twice-baked cake) had evolved. “Maillard reaction” or “glycation” occurs if the proteins are exposed to heat in the presence of sugar and water, which modifies the structure and the aggregation/allergenic effects of milk proteins in different ways depending on the different combinations of protein, sugar and temperature. By using the conventional

cake recipe but twice baking procedure, the hypothesis is that inside of the biscotti would be brown like outside meaning there would be Maillard reaction inside of the cake and so the proteins would be disrupted without using any enzyme (Pastorello et al., 2007; Teodorowicz et al., 2017). The proteins of conventional cake and biscotti were isolated and run in SDS-PAGE. It was observed that the casein band intensities were low in biscotti sample even though same amount of the protein was loaded the gels.

In previous studies, Bloom et al. mentioned that the temperature and duration are two important factors that effect on protein allergenicity, along with the presence of wheat. They showed strongly staining casein bands with gel electrophoresis that persisted for up to 60 min of heating while β-lactoglobulin and α-lactalbumin bands became progressively weaker with increasing heating times (Bloom et al., 2014). Sackesen et al. showed that 70-80% of the children with milk allergy are reactive to pasteurized cow's milk but may tolerate conventional baked milk products (matrix with wheat, sugar and baked at 180-200°C for 30 minutes, examples are muffin, cake) (Sackesen et al., 2019). Previous studies indicated that tolerance to baked-milk products in children with IgE-mediated CMPA is a good predictor of the milk tolerance development (J. S. Kim et al., 2011; Leonard & Nowak-Węgrzyn, 2016; Sackesen et al., 2019; Vilar et al., 2021). For the baked milk reactive ones, there is currently no less hypoallergenic choice than a conventional baked milk product (cake, muffin) to promote desensitization via oral route.

To determine the total protein concentration and milk protein content of diary milk products, the Bradford assay and proteomics analysis were performed, respectively. The protein concentration of biscotti sample was lower than the cake. While the same amount of total protein was used for all following experiments, the total protein concentration was low for biscotti sample,. In proteomics analysis, the intensities of 4 different subunits of casein (α-S1 casein, α-S2 casein, β casein and κ casein) and β-lactoglobulin were checked for pasteurized milk, conventional cake, biscotti 1h, biscotti 2h, and biscotti 3h. The pasteurized milk samples showed the highest proteins for α-S1 casein, α-S2 casein, β casein and κ casein and β-lactoglobulin and the lowest protein levels were obtained in 3 hours baked biscotti samples. Due to Maillard reaction, matrix effect and high temperature, the concentrations of total protein found with Bradford assay and the intensities of different protein fractions shown by proteomics were low.

ELISA is performed to detect serum sIgE levels of the total milk and/or milk fractions (casein, β-lactoglobulin and α-lactalbumin) for diagnostic purposes. There is a need for more specific ELISA systems for detecting sIgE reactivities against baked milk products (partially denaturated milk proteins found in conventional cake and biscotti samples) as well as fermented products like cheese and yogurt.

To evaluate reliable indirect in-house ELISA for the detection of sIgE antibodies against milk products (milk, yogurt, cheese, cake and biscotti), the checkerboard titrations were done in current study. The point of doing checkerboard titration is to decide optimum concentration of patients' sera and secondary antibody and to see whether the ELISA system works properly or not. If OD values increase as the concentration increases or the dilution decreases, the appropriate results would be gotten with this system for further experiments. By looking OD values of checkerboard, optimum concentration of patients' sera and secondary antibody were chosen.

IgE reactivity is important for milk allergic subjects. The mechanism that leads IgE-mediated allergy depends on the IgE antibody affinities and the epitopes that antibodies specifically bound. Western blot technique is most frequently used one for checking IgE reactivity. Patients' sera are used and the binding of IgE antibodies that are in the patients' sera to the allergen are visualized. Bloom et al. shows that, the binging of sIgE to the allergens varies based on the temperature, baking duration and the presence of wheat by doing western blotting (Bloom et al., 2014).

Skin prick test is also a diagnostic method along with sIgE measurement, however these tests have high sensitivity with low specificity. Recent studies show that BAT is more accurate than sIgE measurement in the diagnosis of FA (Santos et al., 2014). Milk allergic patients may develop a tolerance to cow's milk proteins over time. Therefore, the OFC test is used to determine whether the one is tolerant or still reactive to the allergen. However, severe anaphylactic reactions may occur during OFC tests. Moreover, OFC test is also an expensive and time-consuming process. Studies indicate that the tolerance and reactive phenotypes can be easily differentiated from each other with a specificity of 75-100% and sensitivity of 77-98% with BAT (Santos et al., 2014). Nevertheless, BAT is not yet routinely used in the diagnosis of CMPA, and more research is needed.

To check the IgE reactivity of milk allergic patients, the western blotting, newly evaluated indirect in-house ELISA and BAT were performed in this study. The western blotting was done with pasteurized milk, cake and biscotti samples. The results show that the milk sIgE bound incompetently to the casein bands of the biscotti compared to the conventional cake and cow's milk. The results are consistent with the literature as the more reactivity is observed with milk and then cake (Bloom et al., 2014) and lastly with biscotti.

Similar results are shown in indirect in-house ELISA (for cake and biscotti) as well. Biscotti indirect ELISA gives lower OD values compared to the cake indirect ELISA. Milk, cheese and yogurt ELISA results demonstrate that tolerant patients have lower OD values than the reactive ones for each three group of patients (milk, cheese and yogurt). It is also found that the milk sIgE OD values of in-house ELISA were positively and significantly correlated with milk sIgE levels, casein sIgE levels, and total IgE levels. There are no such studies in the literature.

A newly published review mentioned that BAT can be used as part of a standard diagnostic work-up, (Figure 1.9) (Santos et al., 2021). Because SPTs or sIgE are easy to perform and more cost-effective; these tests can be employed as a first line prior to BAT (Santos et al., 2014). Before recommending patients for the OFC, BAT has been advocated as a second-line test in patients with equivocal results after clinical history and sIgE sensitization tests (Santos & Shreffler, 2017). In a prior study of peanut allergy, this proposed strategy reduced the amount of OFC by 67%. Our BAT results showed that the casein mix and biscotti protein induce a dose response. Because the biscotti protein supernatant does not react with the individual who has not milk allergy, it is concluded that the biscotti supernatant can be used as an allergen for BAT for further experiments before OFC test with biscotti. Moreover, the dose response obtained in BAT test may drive the clinician to plan the threshold of protein to reach during OFC tests with biscotti 3h. This part is the further aim of our group to study in vivo whether the combination of sIgE binding to biscotti by indirect in house ELISA and BAT test with biscotti proteins may predict the reactivity and tolerance situation of the severe milk allergic patients.

The current study has certain limitations. Firstly, all the IgE reactivity assays (western blotting, indirect in-house ELISA, BAT) was performed using the total protein concentrations. For all milk products, the concentration of caseins should be measured

specifically and then the calculations would be done according to the casein concentration. Further studies are required to perform the casein specific assays. Secondly, it is thought that the Maillard reaction is the reason of the inside and outside brown color of the biscotti. The Maillard reaction can be confirmed by looking the reactions end products via metabolomics (Hrynets, Bhattacherjee, & Betti, 2019). Further experiments should be done to examine the end products of the Maillard reaction. Thirdly, the number of patients should be increased for the BAT experiments.

In conclusion, a new hypoallergenic product called biscotti is developed which will be more hypoallergenic option than the current conventional cake in this current study. The low casein intensity of Biscotti was showed by SDS-PAGE and proteomics analysis. Indirect in-house ELISA is also developed properly and can be used for further experiments as well in order to check the IgE reactivity of patients' sera with milk, cheese, yogurt, cake and biscotti. Biscotti gives low IgE reactivity with patients' sera done with western blotting and ELISA. Lastly, BAT is evaluated with the biscotti protein supernatant and it can be used before OFC tests with biscotti.

For further analysis, we aim to confirm the low allergenicity of the biscotti by *in vivo* experiments (SPT and OFC) and to show the effects in tolerance induction.

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