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Effect of proteolysis during Cheddar cheese aging on the detection of milk protein residues by enzyme-linked immunosorbent assay

Katherine O. Ivens,* Joseph L. Baumert,* Robert L. Hutkins,† and Steve L. Taylor*[1]
*Food Allergy Research and Resource Program, Department of Food Science and Technology, University of Nebraska-Lincoln, PO Box 886207, Lincoln 68588
†Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln 68588

ABSTRACT

Cow milk is a common allergenic food, and cow milk-derived cheese retains an appreciable level of allergenicity. The specific and sensitive detection of milk protein residues in foods is needed to protect milk-allergic consumers from exposure to undeclared milk protein residues contained in foods made with milk or milk-derived ingredients or made on shared equipment or in shared facilities with milk or milk-derived ingredients. However, during cheese ripening, milk proteins are degraded by chymosin and milk-derived and bacterial proteases. Commercial allergen-detection methods are not validated for the detection of residues in fermented or hydrolyzed products. The objective of this research was to evaluate commercially available milk ELISA kits for their capability to detect milk protein residues in aged Cheddar cheese. Cheddar cheese was manufactured at a local dairy plant and was aged at 5°C for 24 mo, with samples removed at various time points throughout aging. Milk protein residues and protein profiles were measured using 4 commercial milk ELISA kits and sodium dodecyl sulfate-PAGE. The ELISA data revealed a 90% loss of milk protein residue signal between the youngest and oldest Cheddar cheese samples (0.5 and 24 mo, respectively). Sodium dodecyl sulfate-PAGE analysis showed protein degradation throughout aging, with the highest level of proteolysis observed at 24 mo. Results suggest that current commercial milk ELISA methods can detect milk protein residues in young Cheddar cheese, but the detection signal dramatically decreases during aging. The 4 evaluated ELISA kits were not capable of detecting trace levels of milk protein residues in aged cheese. Reliable detection of allergen residues in fermented food products is critical for upholding allergen-control programs, maintaining product safety, and protecting allergic consumers. Furthermore, this research suggests a novel use of ELISA kits to monitor protein degradation as an indication of cheese ripening.
Key words: ELISA, milk, cheese, proteolysis

INTRODUCTION

Cow milk represents one of the most common allergenic foods around the world (Sampson, 2004). Cow milk contains several major allergenic proteins, including casein, β-LG, and α-LA (Wal et al., 2001). Milk-allergic individuals are advised to avoid all milk-derived food products and ingredients to mitigate the risk of accidental consumption (Sicherer and Sampson, 2010). The level of risk of an allergic reaction is related to the dose of exposure to milk proteins. However, milk-allergic individuals vary widely with respect to their threshold doses for milk proteins (Skripak et al., 2008). Fermented milk products, including cheeses, are assumed to retain their allergenicity, and empirical evidence indicates that milk-allergic individuals can react to ingestion of cheese (Alessandri et al., 2012). The proteolysis that occurs during cheese aging is likely to affect the level and nature of milk allergens especially in aged cheeses. However, only one clinical study has addressed the allergenicity of cheeses, as compared with milk. In that study, approximately 58% of milk-allergic patients tolerated fully matured Parmigiano-Reggiano cheese (Alessandri et al., 2012). Thus, despite the advice milk-allergic individuals are given to avoid cheeses, the degree of tolerance to various cheeses among milk-allergic individuals has not been thoroughly examined.

Proteolysis plays a fundamental role in cheesemaking. Initially, chymosin (or other coagulants) hydrolyzes κ-CN into *para*-κ-CN and glycomacropeptide, destabilizing the milk micelle, thereby causing formation of the cheese matrix and giving cheese its gel-like structure (Fox, 1989). Proteases from the coagulant, starter culture, and other sources also play a significant role in developing characteristic flavors and texture during ripening. Depending on the length and extent of cheese

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[1]Corresponding author: staylor2@unl.edu

ripening, substantial proteolysis can occur. Collectively, proteolytic activity is attributed to chymosin (or rennet), indigenous milk proteases, and the proteases of starter and nonstarter microorganisms.

Proteolysis in cheese is divided into 3 major events: coagulation of curd, primary proteolysis, and secondary proteolysis. Coagulation of the curd as a result of destabilization of the milk micelle and formation of the cheese matrix is initiated by the hydrolytic action of chymosin on κ-CN. Primary proteolysis is the action of proteolytic enzymes to degrade native caseins into peptide fragments (Grappin et al., 1985). Secondary proteolysis is the continued degradation of peptide fragments into smaller casein-derived peptides and free AA (Rank et al., 1985). Primary proteolysis is largely dominated by the activities of chymosin and the indigenous milk protease plasmin, and to a somewhat lesser extent by lactic acid bacteria. Secondary proteolysis, however, is characterized by the diverse specificities of the peptidases of starter and nonstarter microorganisms.

During cheesemaking, the whey protein fraction and its 2 major allergens, α-LA and β-LG, are removed; thus, the main proteolytic substrates are the various fractions of casein. In general, chymosin hydrolyzes α_{S1}-CN at several sites during the early stages of ripening. In studies of Cheddar cheese ripening, less than 15% of the original α_{S1}-CN remained intact after 90 d (Børsting et al., 2012; Møller et al., 2012). Due to its low water activity, β-CN is highly resistant to hydrolysis by chymosin, but about 50% is degraded during Cheddar cheese ripening after 270 d (Børsting et al., 2012; Møller et al., 2012). Plasmin, an indigenous milk protease, preferentially hydrolyzes β-CN during primary proteolysis (Fox et al., 2015). Chymosin has not been documented to degrade para-κ-CN during ripening (Hayaloglu and McSweeney, 2014). The fraction of κ-CN remaining in the cheese after coagulation is largely resistant to proteolysis during the early stages of ripening.

Several extraction and fractionation protocols, based on the liberation of peptide fragments and free AA, have been developed to evaluate the extent of ripening and proteolysis in cheese (Møller et al., 2012; Yu et al., 2012; Karametsi et al., 2014). As aging continues, the number of large peptides isolated from cheese dramatically decreases, and ripening is dominated by the release of small peptides and free AA (Parente et al., 2012; Møller et al., 2013). Larger pH 4.6-soluble peptides are typically derived from the action of chymosin, whereas pH 4.6-insoluble fragments are typically γ-CN derived from the action of plasmin on β-CN (Fox et al., 2015). The γ-CN have been used in several studies as an indicator of ripening, particularly with Parmigiano-Reggiano and Grana Padano cheeses (Cattaneo et al., 2008; Masotti et al., 2010). The small peptides produced during ripening, especially those produced during secondary proteolysis, are particularly difficult to visualize with electrophoretic methods.

Even though these general features characterize cheese proteolysis, dramatic differences occur in proteolysis during the manufacturing of various types of cheese as a result of myriad environmental factors, including pH, water activity, cook temperature, storage conditions, fat content, and homogenization (Larsen et al., 2010; Deegan and McSweeney, 2013; Di Luccia et al., 2013). Microbial proteases vary widely in their specificity for hydrolyzing milk proteins. In a study of Cheddar cheese ripening, it was found that different *Lactococcus* strains had different proteolytic patterns (Gutiérrez-Méndez et al., 2010; Steele et al., 2013; Karametsi et al., 2014). Nonstarter microbial proteases additionally have varying patterns of proteolysis (Steele et al., 2013).

Throughout the aging of Cheddar cheese, several epitopes capable of binding IgE and eliciting an allergic response remain intact on the most abundant caseins, α_{S1}- and β-CN (Hayaloglu and McSweeney, 2014; Karametsi et al., 2014). However, some allergenic epitopes can lose their immunoreactive potential as a result of proteolysis during cheese manufacture and aging. The comparative allergenic potency of various types of cheeses has not been carefully assessed. However, established scientific knowledge indicates that proteolytic enzymes can destroy or reveal allergenic epitopes on proteins (Yao et al., 2014; Verhoeckx et al., 2015).

Methods to detect potentially allergenic milk residues in foods are well developed, although accurate detection by some methods can be affected by forms of proteolysis, including fermentation. The most common method are ELISA, because ELISA specifically detect proteins from the allergenic source and allergens are proteins. Additionally, commercial ELISA are available in formats such as lateral flow devices that are rapid and sufficiently rugged to use within manufacturing facilities. However, the protein targets of the antisera used in ELISA vary even for specific foods, such as milk. For milk, commercial ELISA exist for the detection of total milk, casein, and β-LG (Ivens et al., 2016). Mass spectrometry methods continue to be developed for the detection of food allergen residues. Whereas mass spectrometry methods target the detection of specific proteins or peptides, such proteomics methods are available for milk proteins for only a few food matrices (Weber et al., 2006). Recently developed methods provide improved resolution and allow identification of specific milk proteins and peptides (Møller et al., 2012; Parente et al., 2012; Karametsi et al., 2014). The DNA-based methods to detect allergenic residues in foods