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Abstract

Background: Accurate quantitation of gluten in fermented-hydrolyzed foods is challenging due to the lack of appropriate calibrants and variable proteolysis. A multiplex-competitive ELISA was recently developed that recognized the protein/peptide profile differences among the different types of fermented foods such as wheat beers, barley beers, sourdough breads or soy-based sauces.

Purpose: Using gluten-incurred yogurt as a model fermented food, this study evaluated the similarities/differences in protein/peptide profiles arising due to differences in fermentation conditions as recognized by the multiplex-competitive ELISA. This is essential for the selection of appropriate calibration standards for accurate quantitation of gluten in fermented-hydrolyzed foods.

Methodology: Yogurts prepared by incurring 0, 20, 100, and 500 µg/g gluten and by varying certain fermentation conditions (fermentation time, incurred gluten concentrations and starter culture type and concentrations) were analyzed by the multiplex-competitive ELISA. Western blot was performed in order to evaluate similarities/differences in gluten protein-antibody binding patterns. Cluster analysis of the apparent gluten concentration values was performed by hierarchical clustering in order to evaluate similarities/differences in protein/peptide profiles due to differences in the fermentation conditions.

Results: Analysis indicated epitope specific responses with glutenin epitopes being less susceptible to longer fermentation time and higher starter culture concentration compared to gliadins. Incomplete proteolysis was observed after 24 hours of fermentation but became more efficient as fermentation time was increased. Western blot confirmed the results of the ELISA. Cluster analysis indicated a similar gluten protein/peptide profile among yogurts prepared by changing the incurred gluten concentrations, starter culture type and concentrations; however, differences in protein/peptide profiles due to changes in fermentation time were observed.

Conclusion: For yogurt or products with similar fermentation chemistry, fermentation time could make a difference in protein/peptide profiles, especially if the initial proteolysis is incomplete. It will likely require appropriate calibrants with similar protein/peptide profile as both intermediate and complete proteolyzed products for accurate quantitation of gluten.

Introduction

Celiac disease (CD) has a global prevalence rate of 1.4% and affects approximately 1 in 141 in the United States. CD is an immune-mediated disorder triggered by the interaction of gluten with the intestinal mucosa of sensitive individuals. Wheat and related gluten-containing grains are commonly used in fermented and hydrolyzed foods such as beers, soy sauces, vinegars and sourdough breads. Enzymatic action during the fermentation process results in hydrolysis of gluten into peptides, some of which may retain immunopathogenic activity. Currently there are no commercially available methods that can accurately quantitate hydrolyzed gluten derived from 20 ppm (µg/mL) intact gluten in foods subjected to fermentation. Further complicating the quantitative analysis of hydrolyzed gluten is the lack of appropriate standards/calibrants that reflect the protein / peptide profiles characteristic of the various forms of fermentation. To aid in characterizing gluten in different fermented-hydrolyzed foods, a multiplex-competitive ELISA was recently developed utilizing multiple antibodies from established gluten ELISA kits that recognize different gluten epitopes (gliadin, deamidated gliadin, and glutenin) (Panda et al., 2017). The multiplex-competitive ELISA successfully recognized peptide profile differences in the fermented-hydrolyzed foods and was capable of classifying foods based on the types of fermentation. In this study, gluten-incurred yogurt was used as a model food to further evaluate the performance of the multiplex-competitive ELISA. By varying certain fermentation conditions, this research examined additional criteria necessary for selection of appropriate calibrants for the quantitation of gluten in fermented-hydrolyzed foods using the multiplex-competitive ELISA. The differences/similarities in protein/peptide profiles arising due to differences in incurred-gluten concentrations, fermentation time, starter culture types, and concentrations were evaluated.

Method

Gluten-incurred yogurt preparation: Four different yogurt samples containing 0, 20, 100 and 500 µg/g (ppm) gluten were prepared. Fresh whole milk was purchased from a local grocery store and was spiked with the appropriate amount of gluten. The milk was heated until the temperature reached 185-190 F and then cooled in an ice water bath until the temperature reached 110-115 F. Commercial yogurt starter culture (Culture for Health mild flavor yogurt starter, c) was added at a ratio of 0.05 g/160 mL of milk (A) or 0.2 g/160 mL milk (B) and mixed. Fermentation was carried out at 106 F for up to 72 hours, and samples were collected at 24 hours (S3), 48 hours (S4) and 72 hours (S5). Two different batches of yogurts were prepared on two different days. For comparison among the starter cultures, yogurts were prepared with two additional cultures, Yogourmet original yogurt starter culture (a), Euro cuisine yogurt starter culture (b), by fermenting for 24 hours.

Multiplex-competitive ELISA: Samples were analyzed by the multiplex-competitive ELISA (Panda et al., 2017), which was modified and optimized to include six gluten specific antibodies (Table 1). Two batches of yogurts were analyzed in duplicate and duplicate ELISA plates were analyzed each day.

Western blot analysis: Yogurt samples prepared with starter culture c were analyzed by western blot using a previously described procedure (Panda et al., 2015). The G12 and MioBS antibodies were used at 1:5 dilution, while the R5, Skerritt, Neogen and 2D4 antibodies were used at 1:50 dilution. A Syngene G: Box Chemi XX9 imager (Syngene, Frederick, MD) was used to measure the band chemiluminescence.

Statistical Analysis: The significance of differences between the different yogurt samples was determined by the Student's unpaired t-test using the Prism software. Differences were considered significant if $P < 0.05$. To evaluate the protein/peptide profile differences, Hierarchical Cluster Analysis using Ward's Minimum method (JMP® 12.0.1, SAS Institute, Inc., Cary, NC) was performed on the yogurt samples. The clustering patterns were illustrated using a dendrogram.

Results

Table 1. HRP conjugated detector antibodies from commercial ELISA kits used in the multiplex-competitive ELISA

Antibody (name used)	Type ^a	Target	ELISA kits ^b	Manufacturer	Dilution ^c
G12	MAb	QPQLPY	AgraQuant Gluten G12	Romer Labs	1 to 3
R5	MAB	QQPF, QQQF, LQPF, QLFP	RIDASCREEN Gliadin Competitive	R-BioPharm, AG	1 to 33
Neogen ^d	MAB	Gluten	Veratox for Gliadin (Cat # 8480)	Neogen Corp.	1 to 20
2D4	MAB	Diamidated gliadin	Microbiologique Gluten Sandwich	PI Bioscientific Inc.	1 to 25
MioBS	PAB	Gliadin	Wheat/Gliuten (Gliadin) MioBS	Morinaga Institute of Biological Sciences, Inc.	1 to 2
Skerritt	MAB	HMW ^e glutenin	AllerTek Gluten	ELISA Technologies Inc.	1 to 10

^a Gluten specific monoclonal antibody developed and licensed by the U.S. Department of Agriculture (USDA)
^b MAb- Monoclonal antibody, PAB- Polyclonal antibody
^c HMW-high molecular weight
^d Name of ELISA test kits
^e Dilution was done on a volume/volume basis using PBS

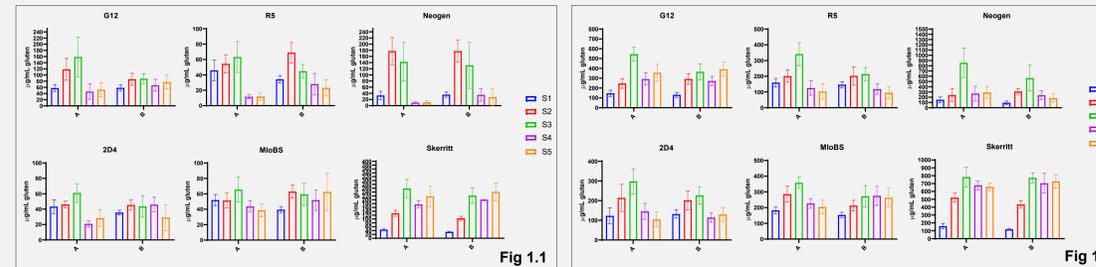


Fig 1.1 Multiplex-competitive ELISA apparent gluten concentration values of the 20 (Fig 1.1), 100 (Fig 1.2) and 500 (Fig 1.3) µg/mL gluten-incurred samples using two different starter culture concentrations, 0.05g (A) and 0.2 gm (B) per 160 mL milk, fermented for 24 (S3), 48 (S4) and 72 (S5) hours. A significant (P<0.05) increase in apparent gluten concentration values was observed in sample S2 (after heat treatment) compared to S1 with all the antibodies and incurred levels with only a few exceptions (R5, 2D4 and MioBS antibodies at 20 µg/mL incurred level, and Neogen antibody at the 100 µg/mL incurred level for yogurts prepared with starter culture concentration A). After fermentation for 24 hours (S3), a significant (P<0.05) increase in gluten concentration was observed with multiple antibodies and incurred levels. The incomplete proteolysis observed at 24 hours became more efficient (the apparent gluten concentration values reduced close to the initial incurred levels) as fermentation was carried out for 48 hours (S4). Increasing the fermentation time to 72 hours (S5) didn't produce a significant reduction in the gluten concentration values compared to sample S4. The effect of increasing the starter culture concentration seemed to enhance proteolysis, especially for the gliadin epitopes. The glutenin epitope, however, remained less susceptible to the effect of increase in the starter culture concentration and longer fermentation time.

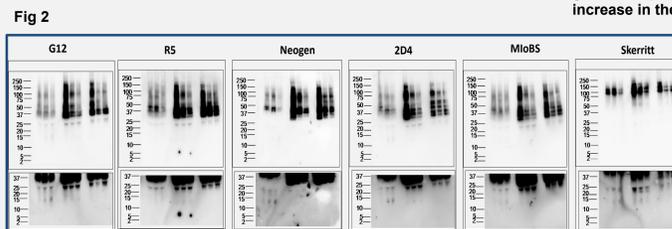


Fig 2. Western blot band patterns of yogurt samples prepared with 0.05 g starter cultures per 160 mL of milk, using the six gluten specific antibodies (G12, R5, Neogen, 2D4, MioBS and Skerritt) used in the multiplex-competitive ELISA. Similar observation was obtained with samples prepared with 0.2 g starter culture per 160 mL milk. Lane information: Lane 1-Molecular weight marker, Lane 2, 3 and 4 - 20 µg/mL gluten incurred yogurt fermented for 24 (S3), 48 (S4) and 72 (S5) hours respectively, Lane 6, 7 and 8 - 100 µg/mL gluten incurred yogurt fermented for 24 (S3), 48 (S4) and 72 (S5) hours respectively, lane 10, 11 and 12- 500 µg/mL gluten incurred yogurt fermented for 24 (S3), 48 (S4) and 72 (S5) hours respectively. The lower panel of each figure represents images obtained at a higher exposure level in order to visualize the low intensity protein/peptide bands. High intensity bindings in the range of 35 to 150 kDa were observed for yogurt samples S3 with all the six gluten-specific antibodies. However, for samples S4 and S5 there was a reduction in the band intensities compared to sample S3 (more evident with the 100 and 500 µg/mL gluten incurred yogurts). No differences in the band distribution pattern were observed among samples S3, S4 and S5. This could be due to the inability of western blot to visualize smaller peptide bands (<12kDa), which are likely to be more abundant in S4 and S5 samples, due to more efficient proteolysis compared to S3. The band distribution pattern of the yogurts containing all the three incurred levels were similar.

Conclusions

- For yogurts, certain fermentation parameters such as incurred gluten concentration, starter culture type, or concentrations may have minimal effect on gluten protein/peptide profiles and, ultimately, on selecting appropriate calibrants.
- Fermentation time is the only parameter that could make a difference in overall gluten protein/peptide profile, especially if the initial proteolysis is incomplete.
- Although this study only investigated yogurt, the same effect may be observed during the fermentation of other fermented products with similar fermentation chemistry.
- For products where fermentation time is relatively short (e.g. yogurt or sourdough), these results suggest it is important to ensure that the calibrant selected for quantitation provides accurate quantitation of gluten in both intermediate and a more efficiently proteolyzed product.

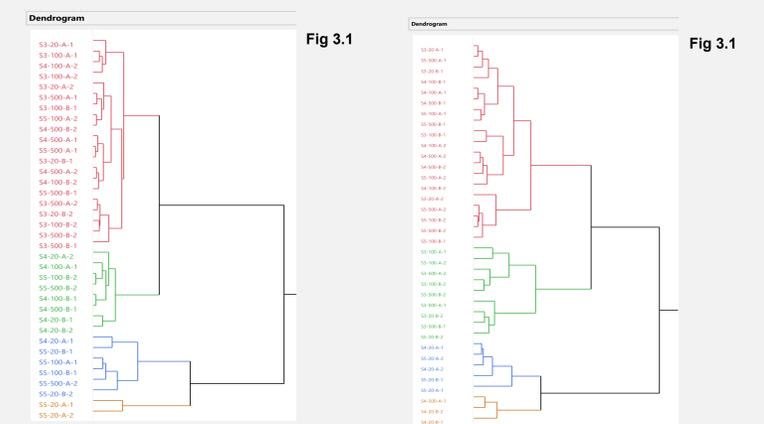


Fig 3: Dendrogram of the hierarchical cluster analysis performed on the apparent gluten concentration values obtained from the analysis of the yogurt samples. Yogurt samples prepared by changing the incurred gluten concentration (20, 100 and 500 µg/mL), fermentation time [24 (S3), 48 (S4) and 72 (S5) hours] and starter culture concentration [0.05 g/160 mL milk (A) and 0.2 g/160 mL milk (B)] were analyzed by the multiplex-competitive ELISA. The average apparent gluten concentration values obtained from the analysis of two different batches of yogurts (1 and 2) were used in cluster analysis. Cluster analysis was performed on the relative response values of the six gluten-specific antibodies to that of the R5 antibody (Fig 3.1) or the G12 antibody (Fig 3.2). The overall protein/peptide profiles among the yogurts prepared by incurring different gluten concentrations and using different starter culture concentrations were similar. However, for yogurt samples collected at different fermentation times (S3, S4 and S5), the majority of the S3 samples clustered separately from S4 and S5 samples (no S3 samples in the green and blue clusters in Fig 3.1, and eight of the S3 samples clustering separately (Green cluster) in Fig 3.2). These results indicate that yogurts fermented for 24 hours have a distinctive protein/peptide profile compared to those fermented for 48 or 72 hours.

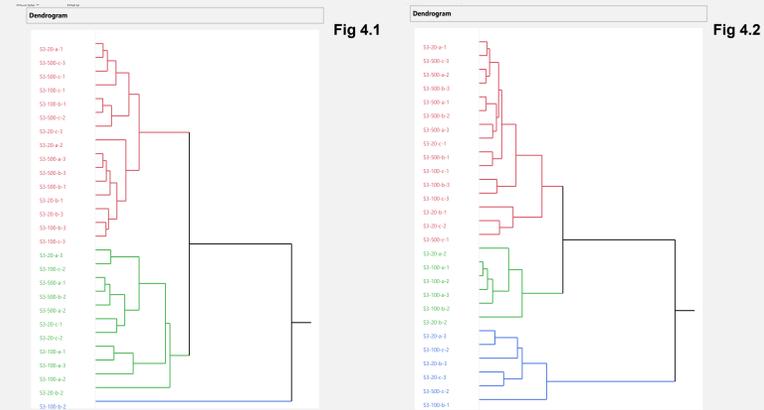


Fig 4. Dendrogram of the hierarchical cluster analysis performed on the apparent gluten concentration values obtained from the analysis of the yogurt samples. Yogurt samples prepared by incurring 20, 100 and 500 µg/mL gluten and using three different types of starter cultures (a, b and c) were fermented for 24 hours (S3) and the samples were analyzed by the multiplex-competitive ELISA. The average apparent gluten concentration values obtained from the analysis of two different batches of yogurts (1, 2) and values obtained after a repeat analysis of the batch 1 yogurt (3) were used in cluster analysis. Cluster analysis was performed on the relative response values of the six gluten-specific antibodies to that of the R5 antibody (Fig 4.1) or the G12 antibody (Fig 4.2). All the major clusters contained yogurts prepared with all the three starter cultures, indicating similar protein/peptide profiles among the yogurts prepared by using different types of starter cultures.

References

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