PERSPECTIVE

Gluten Reduction Strategies for Wheat and Barley

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Celiac disease is a T-cell mediated autoimmune disorder triggered by ingestion of cereal gluten found in wheat (gliadins and glutenins), barley (hordeins), and rye (secalins). Clinical symptoms of celiac disease are diverse and include flatulence, bloating, fatigue, indigestion, diarrhea, abdominal distension/pain, weight loss, low bone mineral density, anemia, irritability, anxiety, depression, and neurological disorders (23). Celiac disease affects approximately 70 million people or approximately 1% of the global population (15), and an additional 5–10% of the population is affected by non-celiac gluten sensitivity (2,22). Although the roles of gluten and the epitopes that trigger celiac disease are becoming well understood (34,37), the cause of non-celiac gluten sensitivity is not yet understood, with some studies suggesting a role for gluten (5,12), while others point to fermentable short-chain carbohydrates (6,38) or α-amylase/trypsin inhibitors (25).

For both conditions strict avoidance of gluten-containing grains is the recommended treatment. In addition to these two conditions there is a growing number of consumers who believe gluten-free diets are healthier for them, despite the fact there is no apparent clinical reason to avoid gluten. Greater awareness of celiac disease and non-celiac gluten sensitivity has led to rapid growth in the gluten-free market segment. In 2013 the global gluten-free market was estimated at approximately US$3.8 billion and anticipated to grow to US$6.9 billion by 2019 (28).

This rapid growth in the market has led to a greater number and more diverse range of offerings entering the market, which gives consumers who avoid gluten greater choice. These foods are still expensive, however, and may be nutritionally inferior to gluten-containing products. A recent study in the United Kingdom compared the cost and nutritional value of 679 gluten-free products and 1,045 comparable regular (gluten-containing) products (16). On average the price premium for the gluten-free foods was 159%, and more of the gluten-free foods were classified as containing medium or high levels of salt, sugar, fat, and saturated fat compared with the regular foods. Additionally the gluten-free items were more likely to be lower in fiber and protein than the regular foods.

These differences in the nutritional properties of gluten-free foods, as well as anecdotal reports of the poor taste and texture of gluten-free foods, have led a number of research groups to investigate ways in which gluten could be reduced or removed from wheat and barley to provide new higher fiber and better tasting options for those who must or who choose to avoid gluten in their diet.

Wheat

In wheat the majority of the known epitopes that are immunogenic for those with celiac disease are present in the gliadins (34,37), and thus, much of the research effort has been focused on strategies to identify or develop lines with lower gliadin contents. Initial studies focused on identification of lines with lower gliadin content. In early studies lines that lacked the locus on chromosome 6A that encoded the α-gliadins were identified (26); however, adverse reactions, similar to those expected upon ingesting gluten, were noted in celiac patients fed bread made from this wheat (8,9). This suggests that loss of this locus alone is insufficient to make wheat suitable for people with celiac disease.

Lines that lacked the locus that encodes α-gliadins on chromosome 6D were shown to have a significantly reduced number of T-cell stimulatory epitopes present. However, it was demonstrated that the reduction in these epitopes resulted in a loss of dough functionality (41). Based on this result it was hypothesized that durum (tetraploid) wheat might contain lower levels of celiac disease-active epitopes. Screening of tetraploid varieties with antibodies to two α-gliadin epitopes, Glia-α9 and Glia-α20, identified three durum varieties with significantly reduced levels of both epitopes (39). The same research group also compared the relative abundance of these two epitopes in modern cultivars and landraces. The modern cultivars tended to have higher levels of these epitopes than did the landraces, with a trend for the Glia-α9 epitope to be present in higher levels in modern cultivars (40).

In addition to the classical approach to identifying and then breeding for lines with reduced celiac disease immunogenic epitope contents, other research groups have focused on the use of gene technology to reduce the gliadin content in wheat. Suppression of α-gliadins by more than 60%, using RNA interference (RNAi), was compensated for by an increase in other gliadin components, low molecular weight glutenin subunits (LMW-GS), albumins, and globulins; the net result was a reduction in total gluten content of approximately 10%. When compared with the controls there was little difference in dough extensibility or resistance, but a slight decrease in loaf volume was observed (3,4).

A similar approach was used to suppress expression of γ-gliadins (24). Suppression of γ-gliadins by approximately 65% was accompanied by a small increase in α-gliadins, a 20–25% increase in LMW-GS, and a 40–50% increase in high molecular weight glutenin subunits (HMW-GS). Small-scale rheological analysis revealed that these changes had no impact on water absorption, and the dough produced was very strong with high stability but was not nearly as extensible as the control lines.

In a series of more comprehensive studies γ-gliadins were suppressed by up to 95% (19,30), which resulted in increased dough strength and enhanced stability. The same research group also developed lines in which total gliadin content was suppressed by up to 90% (20,21). Dough made from these lines was weaker, but more stable, than dough made from the controls, and loaf volume decreased by 20–30% (17,18).

More recently, gene editing has been used to modify the α-gliadins in wheat, with 35 of 45 different α-gliadin genes in one wheat line modified, resulting in a reduction in α-gliadin con-

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tent of up to 82%. In some lines γ- and ω-gliadin expression was also reduced. Analysis of the total gluten content using the R5 and G12 antibodies revealed a three- to fourfold reduction in total gluten content in these lines (31).

Rather than targeting suppression or modification of gliadins and glutenins directly, an alternative strategy is to target genes that control expression of glutenins and gliadins. Use of RNAi to suppress the gene encoding Demeter, a demethylase enzyme involved in the regulation of expression of gliadins and glutenins, resulted in a reduction of these proteins of up to 75% (42). The impact of these changes on functionality was not reported. More recently TILLING (targeting induced local lesions in genomes), a reverse genetics technique, has been used to identify mutations in all three homeoforms of the DNA-binding with one zinc finger (DOF) domain transcription factor that is involved in expression of gliadins and LMW-GS. When mutations in all three homeoforms of the DOF domain were combined in a single wheat line, the gliadin and LMW-GS contents were reduced by 50–60%. The impact on functionality was not determined (29).

Although all of these studies identified strategies to reduce the gluten content of wheat, production of a celiac-safe wheat line remains a challenge that is unlikely to be overcome in the near future. To be safe for consumption by all people with celiac disease, all epitopes that elicit a response need to be removed, and it has been shown that all classes of glutenins and gliadins contain these epitopes (34,37). Bread wheat is hexaploid and contains three related genomes that contain 20–30 glutenin genes and on the order of 100 gliadin genes across 9 loci, which together make up the gluten component of the grain (32). Thus, it is highly improbable that a gluten-free or celiac disease epitope-free wheat can be produced.

Barley

Given the complexity of the gluten-reduction problem in wheat, our research group has focused its efforts on the genetically simpler diploid species barley. The aim is to develop a novel gluten-free cereal that has a higher fiber content than currently available gluten-free cereals and that can be used to produce whole grain foods and malted beverages. In barley there are only four hordein (gluten) protein families: B-, C-, D-, and γ-hordeins. The dominant hordeins comprise two multigene families consisting of at least 13 B-hordein genes (27) and 20–30 C-hordein genes (33). Lines that do not accumulate B-hordeins, Risø 56 (27); C-hordeins, Risø 1508 (13,14); and D-hordeins, Ethiopian-derived landrace R118 (7), were identified. Using a conventional breeding strategy the low-hordein trait from each of these lines was combined into a single line, ULG 3.0 (35), which is now known as Kebari®.

In the Kebari line the gluten content has been reduced to approximately 5 ppm, which is below the 20 ppm level recommended for classification as gluten-free by the Codex Alimentarius Commission (10). Compositional analysis of Kebari flour showed there was little or no change in starch or monosaccharide content, while β-glucan content was reduced approximately threefold. Surprisingly, the protein content remained unchanged despite the loss of the hordeins that normally comprise approximately 50% of the grain protein content. This was partially due to a 10- to 15-fold increase in the level of free amino acids in the grain (35), and subsequent work has shown that the levels of some globulins increased (M. L. Colgrave, *unpublished data*). Levels of α-amylase in the grain were similar or slightly higher than in commercial controls, whereas β-amylase levels were reduced approximately 50-fold.

The initial version of Kebari was in a hulled background, which was suitable for malting and brewing, but Kebari seeds are smaller and thinner than current commercial cultivars, which creates some processing challenges. We have implemented a breeding program to improve seed size and have also developed a hull-less version that can be used as whole grain and flour in gluten-free products, for which barley has an advantage over grains like rice and corn, because it has a much higher fiber content. Small-scale processing trials have shown that Kebari can be rolled, flaked, and extruded (C. A. Howitt, *unpublished data*).

Gluten-free and Reduced-Gluten Beers

The recent rapid growth in the demand for gluten-free products has also resulted in rapid development of the gluten-free beer market. The global gluten-free beer market in 2017 was valued at US$268 million and is anticipated to grow to US$600 million by 2022 (36). There are three strategies for producing gluten-free and reduced-gluten beers. The first strategy is to use inherently gluten-free cereals (e.g., corn, rice, sorghum, millet) or pseudocereals (e.g., buckwheat). However, these products often lack the distinctive flavor and aroma imparted by malted barley. The second strategy is to remove the gluten during the brewing process, either through filtration or enzymatic digestion during fermentation of the beer. In enzymatic digestion, which is more commonly used, enzymes called prolyl endopeptidases (or PEPs) are used to cut proteins at the amino acid proline, which is present at high levels in hordeins, glutenins, and gliadins. The third strategy is to use gluten-free barley. In 2016 German brewer Radeberger launched Pionier, the first gluten-free barley-based beer brewed using Kebari.

Beers brewed using the latter two strategies are tested using a competitive enzyme-linked immunosorbent assay (ELISA) to determine that the gluten content is below the 20 ppm threshold limit recommended by the World Health Organisation (WHO). However, some questions remain as to the accuracy of these readings. In fact, the U.S. Food and Drug Administration (FDA) has recognized that current analytical methods, including competitive ELISA, are not able to accurately quantify gluten in hydrolyzed or fermented products such as beer. They ruled that beer cannot be labeled "gluten-free" unless it is made from materials, like rice, that are inherently gluten-free. As a result of this ruling, brewers instead label their beers as "gluten-reduced" or "crafted to remove gluten."

A recent study showed that some of these gluten-reduced beers triggered an immune reaction when tested against blood samples from some patients with celiac disease (1), suggesting that gluten-reduced beers may not be suitable for all people with celiac disease. To investigate this further we have used liquid chromatography–mass spectrometry to examine the gluten content in 12 gluten-reduced beers and 4 traditional beers (11). Gluten was detected in all 12 gluten-reduced beers, and in some of these beers, levels were similar to those in the traditional beers; however, in the majority of gluten-reduced beers the level of gluten was reduced (Fig. 1). The lowest level of gluten was observed in the beer brewed from Kebari.

Conclusions

The rapid growth of the gluten-free market over the last decade has led to increased efforts by both research groups and

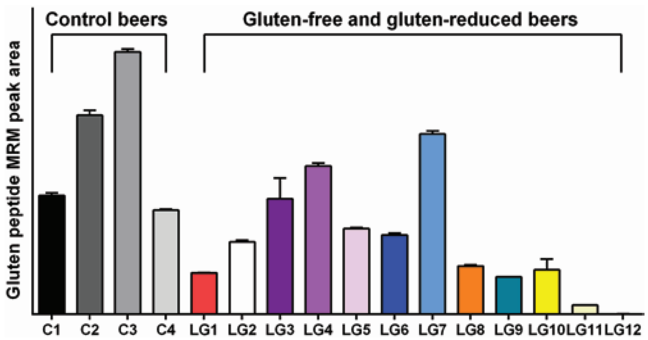

Fig. 1. Liquid chromatography–mass spectrometry analysis of gluten in four commercial gluten-containing beers (C1–C4) and a range of gluten-reduced beers (LG1–LG12). LG12 was brewed from Kebari® barley. The results indicate gluten reduction is variable, and several gluten-reduced beers yielded gluten levels similar to those of traditional barley malt-based beers. MRM = multiple reaction monitoring.

commercial entities to develop palatable, health-promoting dietary options for those who must or who choose to avoid gluten. This has led to improvements in gluten-free diets; however, these products remain expensive and may have lower fiber contents compared with gluten-containing foods. Much effort has been directed toward understanding the epitopes in gluten that cause celiac disease responses and toward strategies for developing epitope- or gluten-free wheat. In our opinion, development of a wheat line that is safe for all people with celiac disease remains an elusive goal that is unlikely to be realized in the near future. Similar strategies in barley have proven more effective, and a barley variety in which the gluten content has been reduced to approximately 5 ppm has been developed. The first product from this barley, a beer, was launched in 2016, and it is hoped that this will be the first of many new products suitable for people with celiac disease and those who avoid gluten in their diets.

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Conflict of Interest Statement

C. A. Howitt and M. L. Colgrave are coinventors on patents relating to Kebari®.

References

1. Allred, L. K., Lesko, K., McKiernan, D., Kupper, C., and Guandalini, S. The celiac patient antibody response to conventional and gluten-removed beer. J. AOAC Int. 100:485, 2017.
2. Aziz, I., and Hadjivassiliou, M. Coeliac disease: Noncoeliac gluten sensitivity—Food for thought. Nat. Rev. Gastroenterol. Hepatol. 11:398, 2014.
3. Becker, D., Folck, A., Knies, P., Lörz, H., and Weiser, H. Silencing the α-gliadins in hexaploid bread wheat. Page 86 in: *Gluten Proteins*. G. L. Lookhart and P. K. W. Ng, eds. AACC International, St. Paul, MN, 2006.
4. Becker, D., Wieser, H., Koehler, P., Folck, A., Mühling, K. H., and Zörb, C. Protein composition and techno-functional properties of transgenic wheat with reduced α-gliadin content obtained by RNA interference. J. Appl. Bot. Food Qual. 85:23, 2012.
5. Biesiekierski, J. R., Newnham, E. D., Irving, P. M., Barrett, J. S., Haines, M., Doecke, J. D., Shepherd, S. J., Muir, J. G., and Gibson, P. R. Gluten causes gastrointestinal symptoms in subjects without celiac disease: A double-blind randomized placebo-controlled trial. Am. J. Gastroenterol. 106:508, 2011.
6. Biesiekierski, J. R., Peters, S. L., Newnham, E. D., Rosella, O., Muir, J. G., and Gibson, P. R. No effects of gluten in patients with self-reported non-celiac gluten sensitivity after dietary reduction of fermentable, poorly absorbed, short-chain carbohydrates. Gastroenterology 145:320, 2013.
7. Brennan, C. S., Smith, D. B., Harris, N., and Shewry, P. R. The production and characterisation of *Hor* 3 null lines of barley provides new information on the relationship of D hordein to malting performance. J. Cereal Sci. 28:291, 1998.
8. Ciclitira, P. J., Hunter, J. O., and Lennox, E. S. Clinical-testing in celiac patients of bread made from wheats deficient in some α-gliadins. Clin. Sci. 59:25, 1980.
9. Ciclitira, P. J., Hunter, J. O., and Lennox, E. S. Clinical testing of bread made from nullisomic 6A wheats in celiac patients. Lancet 316:234, 1980.
10. Codex Alimentarius Commission. Standard for foods for special dietary use for persons intolerant to gluten. Standard 118-1979. In: *Codex Alimentarius International Food Standards*. Available online at www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCODEX%2BSTAN%2B118-1979%252FCXS_118e_2015.pdf. FAO/WHO, Rome, 2015.
11. Colgrave, M. L., Byrne, K., and Howitt, C. A. Liquid chromatography-mass spectrometry analysis reveals hydrolyzed gluten in beers crafted to remove gluten. J. Agric. Food Chem. 65:9715, 2017.
12. Di Sabatino, A., Volta, U., Salvatore, C., Biancheri, P., Caio, G., De Giorgio, R., Di Stefano, M., and Corazza, G. R. Small amounts of gluten in subjects with suspected nonceliac gluten sensitivity: A randomized, double-blind, placebo-controlled, cross-over trial. Clin. Gastroenterol. Hepatol. 13:1604, 2015.
13. Doll, H. Inheritance of the high-lysine character of a barley mutant. Hereditas 74:293, 1973.
14. Doll, H. Barley seed proteins and possibilities for their improvement. Page 207 in: *Seed Proteins: Biochemistry, Genetics, and Nutritive Value*. W. Gottschalk and H. P. Mullen, eds. Martinus Nishoff, The Hauge, 1983.
15. Fasano, A., Sapone, A., Zevallos, V., and Schuppan, D. Non-celiac gluten sensitivity. Gastroenterology 148:1195, 2015.
16. Fry, L., Madden, A. M., and Fallaize, R. An investigation into the nutritional composition and cost of gluten-free versus regular food products in the UK. J. Hum. Nut. Diet. 38:108, 2017.
17. Gil-Humanes, J., Piston, F., Altamirano-Fortoul, R., Real, A., Comino, I., Sousa, C., Rosell, C. M., and Barro, F. Reduced-gliadin wheat bread: An alternative to the gluten-free diet for consumers suffering gluten-related pathologies. PLoS One. DOI: https://doi.org/10.1371/journal.pone.0090898. 2014.
18. Gil-Humanes, J., Piston, F., Barro, F., and Rosell, C. M. The shutdown of celiac disease-related gliadin epitopes in bread wheat by RNAi provides flours with increased stability and better tolerance to over-mixing. PLoS One. DOI: https://doi.org/10.1371/journal.pone.0091931. 2014.
19. Gil-Humanes, J., Piston, F., Hernando, A., Alvarez, J. C., Shewry, P. R., and Barro, F. Silencing of γ-gliadins by RNA interference (RNAi) in bread wheat. J. Cereal Sci. 48:565, 2008.
20. Gil-Humanes, J., Piston, F., Shewry, P. R., Tosi, P., and Barro, F. Suppression of gliadins results in altered protein body morphology in wheat. J. Exp. Bot. 62:4203, 2011.
21. Gil-Humanes, J., Pistón, F., Tollefsen, S., Sollid, L. M., and Barro, F. Effective shutdown in the expression of celiac disease-related wheat gliadin T-cell epitopes by RNA interference. Proc. Natl. Acad. Sci. U.S.A. 107:17023, 2010.
22. Golley, S., Corsini, N., Topping, D., Morell, M., and Mohr, P. Motivations for avoiding wheat consumption in Australia: Results from a population survey. Public Health Nutr. 18:490, 2015.
23. Green, P. H. R., Lebwohl, B., and Greywoode, R. Celiac disease. J. Allergy Clin. Immunol. 135:1099, 2015.

24. Howitt, C. A., Bowerman, A. F., Newberry, M., Bekes, F., Larroque, O., and Morell, M. K. Suppression of gliadins by RNAi alters grain protein composition and dough functionality. Page 26 in: *Cereals 2008: Proceedings of the 58th Australian Cereal Chemistry Conference*. J. F. Panozzo and C. K. Black, eds. AACC International, DownUnder Section, St. Paul, MN, 2009.
25. Junker, Y., Zeissig, S., Kim, S.-J., Barisani, D., Wieser, H., et al. Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4. J. Exp. Med. 209:2395, 2012.
26. Kasarda, D. D., Bernardin, J. E., and Qualset, C. O. Relationship of gliadin protein components to chromosomes in hexaploid wheats (*Triticum aestivum* L.). Proc. Natl. Acad. Sci. U.S.A. 73:3646, 1976.
27. Kreis, M., Shewry, P. R., Forde, B. G., Rahman, S., and Miflin, B. J. Molecular analysis of a mutation conferring the high-lysine phenotype on the grain of barley (*Hordeum vulgare*). Cell 34:161, 1983.
28. Markets and Markets. Gluten-free products market by type, sales channel, and geography: Global trends and forecasts to 2019. www.marketsandmarkets.com, 2014.
29. Mohes, C. P., Austhill, W. J., Loeffler, D., and Mullenberg, J. Reduced gluten grains and compositions thereof. Patent WO 2016196489 A1, 2018.
30. Pistón, F., Gil-Humanes, J., Rodríguez-Quijano, M., and Barro, F. Down-regulating γ-gliadins in bread wheat leads to non-specific increases in other gluten proteins and has no major effect on dough gluten strength. PLoS One. DOI: 10.1371/journal.pone.0024754. 2011.
31. Sanchez-Leon, S., Gil-Humanes, J., Ozuna, C. V., Gimenez, M. J., Sousa, C., Voytas, D. F., and Barro, F. Low-gluten, non-transgenic wheat engineered with CRISPR/Cas9. Plant Biotechnol. J. 16:902, 2017.
32. Shewry, P. R. Wheat. J. Exp. Bot. 60:1537, 2009.
33. Shewry, P. R., Bunce, N. A., Kreis, M., and Forde, B. G. Polymorphism at the *Hor 1* locus of barley (*Hordeum vulgare* L.). Biochem. Genet. 23:391, 1985.
34. Sollid, L. M., Qiao, S.-W., Anderson, R. P., Gianfrani, C., and Koning, F. Nomenclature and listing of celiac disease relevant gluten T-cell epitopes restricted by HLA-DQ molecules. Immunogenetics 64:455, 2012.
35. Tanner, G. J., Blundell, M. J., Colgrave, M. L., and Howitt, C. A. Creation of the first ultra-low gluten barley (*Hordeum vulgare* L.) for coeliac and gluten-intolerant populations. Plant Biotechnol. J. 14:1139, 2016.
36. Technavio. Global gluten-free beer market 2018–2022. www.technavio.com, 2018.
37. Tye-Din, J. A., Stewart, J. A., Dromey, J. A., Beissbarth, T., van Heel, D. A., et al. Comprehensive, quantitative mapping of T cell epitopes in gluten in celiac disease. Sci. Transl. Med. 2:41, 2010.
38. Uhde, M., Ajamian, M., Caio, G., De Giorgio, R., Indart, A., Green, P. H., Verna, E. C., Volta, U., and Alaedini, A. Intestinal cell damage and systemic immune activation in individuals reporting sensitivity to wheat in the absence of coeliac disease. Gut 65:1930, 2016.
39. van den Broeck, H., Hongbing, C., Lacaze, X., Dusautoir, J. C., Gilissen, L., Smulders, M., and van der Meer, I. In search of tetraploid wheat accessions reduced in celiac disease-related gluten epitopes. Mol. Biosyst. 6:2206, 2010.
40. van den Broeck, H. C., de Jong, H. C., Salentijn, E. M. J., Dekking, L., Bosch, D., Hamer, R. J., Gilissen, L. J. W. J., van der Meer, I. M., and Smulders, M. J. M. Presence of celiac disease epitopes in modern and old hexaploid wheat varieties: Wheat breeding may have contributed to increased prevalence of celiac disease. Theor. Appl. Genet. 121:1527, 2010.
41. van den Broeck, H. C., van Herpen, T., Schuit, C., Salentijn, E. M. J., Dekking, L., Bosch, D., Hamer, R. J., Smulders, M. J. M., Gilissen, L., and van der Meer, I. M. Removing celiac disease-related gluten proteins from bread wheat while retaining technological properties: A study with Chinese spring deletion lines. BMC Plant Biol. 9:41, 2009.
42. Wen, S. S., Wen, N. A., Pang, J. S., Langen, G., Brew-Appiah, R. A. T., et al. Structural genes of wheat and barley 5-methylcytosine DNA glycosylases and their potential applications for human health. Proc. Natl. Acad. Sci. U.S.A. 109:20543, 2012.

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