White Paper

The use and limitations of the Competitive ELISA to detect proteins used in protease enzyme fermentation applications

John Deaton, Gina Labellarte, David Allen, Eli Franklin, John Goodwin

DEERLAND Enzymes

ABSTRACT

ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. In an ELISA, an antigen must be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme. Detection of the substance is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product. There are many different types of ELISAs, including the sandwich method and competitive method.

The sandwich ELISA method is commonly used for the detection of proteins such as gluten and milk. This test method requires two epitope binding domains (of 15-20 amino acids in length), which means large fragments of these proteins can be present and not be detected. For fermentations that produce small peptides which can still illicit immune responses, it is necessary for the ELISA to detect smaller fragments. The competitive ELISA method requires only one epitope, allowing it to measure smaller peptides. Because of the increased sensitivity of this method, it is being considered as the preferred method for protein detection in fermentations such as beer, probiotics and enzymes. This paper will focus on the use of the competitive ELISA method to detect proteins (gluten and beta lactoglobulins) used in protease enzyme fermentations.

INTRODUCTION

Enzymes for use in dietary supplements is a growing industry. Supplemental enzymes assist in the digestion of dietary sugars, fats and proteins. The enzymes are typically produced from either fermented bacteria or mold, and plant extraction. Fermentation requires the organism to grow using protein sources such as milk, soy and wheat as “food”. Bacteria and mold utilize the proteins in their environment by secreting enzymes to break down this food so that they can grow[1]. Enzymes are specific to the substances they break down. For instance, the most potent enzymes that are manufactured to break down gluten usually contain wheat in the fermentation media. The majority of these proteins are usually consumed by the organism by the end of the fermentation, and much of the remainder is removed during final processing of the enzymes (i.e. centrifugation/filtration). Testing for these proteins in the final enzyme product has been difficult and no clear validated method has been established.

Both the Food and Drug Administration (FDA) and the Alcohol and Tobacco Tax and Trade Bureau (TTB) appear to be on the same page when it comes to assessing the gluten content of fermented or

hydrolyzed foods. The FDA writes in its August 3, 2011 Federal Register Notice regarding gluten-free labeling that, "FDA recognizes that for some food matrices (e.g., fermented or hydrolyzed foods), there are not currently available validated methods that can be used to accurately determine if these foods contain < 20 ppm gluten." The TTB writes in their interim policy on gluten content statements that they agree "with FDA that there are no scientifically valid methods for accurately measuring the gluten content of fermented products..."[2].

In 2013 the FDA defined the term ''gluten-free'' and identified a gap in the analytical methodology for detection and quantification of gluten in foods subjected to fermentation and hydrolysis. A "gluten-free" claim will be permitted on fermented and hydrolyzed foods or foods containing fermented or hydrolyzed ingredients that meet all of the requirements for bearing a "gluten-free" claim even though the gluten content of the food cannot be reliably measured pursuant to § 101.91(c). Until provisions are established specifically for these foods, through further rulemaking, as is true for all food manufacturers who wish to use "gluten-free" labeling on their food, manufacturers of fermented or hydrolyzed foods or foods that use fermented or hydrolyzed ingredients are responsible for ensuring that the food bearing a "gluten-free" claim is not misbranded for failure to meet all of the requirements of the final rule. Manufacturers can implement measures that are necessary to prevent the introduction of gluten into the food during the manufacturing process to ensure that the finished product will comply with the provisions in § 101.91.

As a common practice, testing laboratories have been using the RIDASCREEN® Gliadin Competitive ELISA (Art. No. R7021), for the analysis of fermented and hydrolyzed foods which are declared as "gluten-free" (e.g. beer, starch syrup, starch, malt extract, sourdough, soy sauce). The competitive ELISA quantitates peptide fragments of prolamins from wheat (gliadins), rye (secalin) and barley (hordein). The R5 monoclonal antibody recognizes (among others) the potentially toxic peptide sequence QQPFP, which occurs repeatedly in the prolamin molecules[3]. The R5 gluten competitive ELISA has been validated for the detection of gluten after beer fermentation[4,5]; however, there is no validation for these competitive ELISA kits on protease containing fermentations.

Another common protein used as ingredient in the food industry for its functional properties is milk protein, which can cause severe reactions for individuals allergic to dairy. For this reason, an ELISA test to detect bovine beta-lactoglobulin is included in this work in addition to the test for gluten.

METHOD

In this technique, antibody is first incubated in solution with a sample containing antigen (protein of interest). The antigen-antibody mixture is then added to an antigen-coated microtiter well. The more antigens present in the sample, the less free antibody will be available to bind to the antigen-coated well. The higher the concentration of antigen in the original sample, the lower the absorbance. In the competitive ELISA, there is an inverse relationship between the intensity of color that results and the amount of test sample antibody bound in the test system. The results are expressed relative to the optical density (OD) of a standard competing antibody.

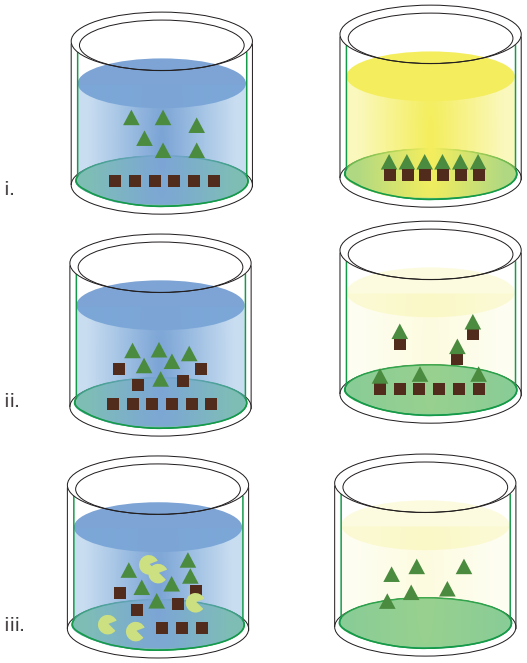

Figure 1.

i. Protein of interest is bound to each well. Additions are made in this order: the sample, antibody, wash, 2-antibody color reagent. Only the antibodies attached to bound protein will result in a yellow color.

ii. Samples that contain the protein of interest will compete for the antibodies, reducing the number of antibodies that are available to bind to the well. This results in a lighter color of yellow. Loss of the yellow color can be quantified and reported as amount of protein present in the sample.

iii. Samples that contain proteases will degrade the protein of interest that's bound in the well. When there's no bound protein, this reduces the number of antibodies that can bind to the well. This yields the same result as having protein present in the sample (lighter color of yellow.) Loss of the yellow color will be quantified and reported incorrectly as the amount of protein in sample.

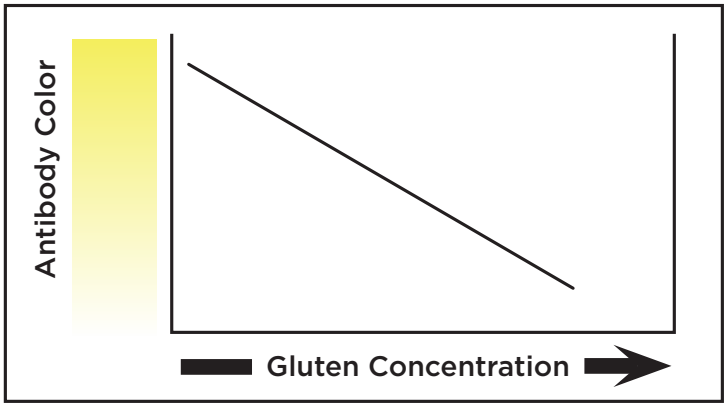

Figure 2.

As the concentration of gluten in the sample increases, the yellow color (absorption nm) in the sample well decreases

Sample Protease A

Protease A was made using wheat in the fermentation media. Protease A* is the same protease, however no wheat was added in the fermentation media. Protease A† is inactivated Protease A. A separate sample of Protease A† was spiked with 20ppm of gluten.

These samples were tested with the RIDASCREEN® Gliadin Competitive ELISA assay (Art. No R7021).

Sample Protease B

Protease B was made using casein in the fermentation media. Protease B* is the same protease, however no milk products were added in the fermentation media. Protease B† is inactivated Protease B. A separate sample of Protease B† was spiked with 90ppm of β-Lactoglobulin.

These samples were tested with the RIDASCREEN® β-Lactoglobulin Competitive ELISA assay (Art. No R4901).

Protease inhibition was done by boiling the sample and/or adding a protease inhibitor cocktail (GE Healthcare Biosciences 80-6501-23, Catalog No. 45001740).

RESULTS

Table 1.

Product	Testing Facility	Test	Results	Limit of Detection
Protease A	Third Party Lab	Gluten Peptide ELISA	>270ppm	10ppm
Protease A	Deerland Enzymes	Gluten Peptide ELISA	>270ppm	10ppm
Protease A*	Third Party Lab	Gluten Peptide ELISA	>270ppm	10ppm
Protease A*	Deerland Enzymes	Gluten Peptide ELISA	>270ppm	10ppm
Protease A†	Third Party Lab	Gluten Peptide ELISA	<10ppm	10ppm
Protease A†	Deerland Enzymes	Gluten Peptide ELISA	<10ppm	10ppm
Protease A† SPIKED	Deerland Enzymes	Gluten Peptide ELISA +20ppm	20ppm	10ppm
Protease B	Third Party Lab	β-Lactoglobulin	>810ppm	5ppm
Protease B	Deerland Enzymes	β-Lactoglobulin	>810ppm	5ppm
Protease B*	Third Party Lab	β-Lactoglobulin	>810ppm	5ppm
Protease B*	Deerland Enzymes	β-Lactoglobulin	>810ppm	5ppm
Protease B†	Third Party Lab	β-Lactoglobulin	<5ppm	5ppm
Protease B†	Deerland Enzymes	β-Lactoglobulin	<5ppm	5ppm
Protease B† SPIKED	Deerland Enzymes	β-Lactoglobulin + 90ppm	90ppm	5ppm

DISCUSSION

The RIDASCREEN competitive ELISA kits for both the gluten peptides and the β-Lactoglobulin indicated very high levels of the respective proteins, as seen in Table 1, denoted by A and B. However, removing the protein of interest from the fermentation media did not change those results, as seen in A* and B*. This suggests that something else is affecting the test besides the protein itself.

After evaluation of the assay, it should be noted that the assay well starts off with bound protein of interest. It is the competitive binding between bound proteins in the well vs. free floating protein in the sample that gives the test its results. Protease enzymes will degrade the bound proteins in the well, removing the competitive part of the

assay and resulting in a false positive (loss of color). This was tested by inhibiting the protease before performing the ELISA. Proteases A† and B† both resulted in a "not detected" result.

To ensure that the process of deactivating the proteases was not altering the sensitivity of the assay, the additional step was taken of testing the A† and B† samples which were spiked with the protein of interest. In these tests, the proteins were indeed detected, as can be seen in Table 1.

In summary, because active proteases degrade the protein of interest in the competitive ELISA, the test is likely to result in a false positive for fermented or hydrolyzed substances. In order to test for gluten or β-Lactoglobulin using the competitive ELISA, the protease must be deactivated for more accurate results.

REFERENCES

1. Secretion, processing and activation of bacterial extracellular proteases. Wandersman, C. Molecular Microbiology, 1989 Dec;3(12): 1825-31
2. Revised Interim Policy on Gluten Content Statements in the Labeling and Advertising of Wine, Distilled Spirits, and Malt Beverages, TTB Ruling, Number 2014-2, February 11, 2014 http://www.ttb.gov/rulings/2014-2.pdf
3. "AACCI approved methods technical committee report: collaborative study on the immunochemical determination of partially hydrolyzed gluten using an R5 competitive ELISA", Koehler P, Schwalb T, Immer U, Lacorn M, Wehlin P, Don C, Cereal Foods World, 2013 May; 58(3):154-158.
4. Competition enzyme-linked immunosorbant assay (ELISA) can be a sensitive method for the specific detection of small quantities of allergen in a complex mixture. Dobrovolskaia E, Gam A, Slater JE (2006) Clin Exp Allergy 36:525–30.
5. Detection and Quantification of Gluten during the Brewing and Fermentation of Beer Using Antibody-Based Technologies. Panda, Rakhi; Zoerb, Hans F.; Cho, Chung Y.; Jackson, Lauren S.; Garber, Eric A.E. Journal of Food Protection®, Number 6, June 2015, pp. 1064-1243, pp. 1167-1177(11)

DEERLAND Enzymes®

3800 Cobb International Boulevard
Kennesaw, GA 30152
770.919.8901
800.697.8179
info@deerlandenzymes.com

DeerlandEnzymes.com