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Measuring Gluten in the Food Chain

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Abstract

Gluten is a protein that is primarily found in grains such as wheat, rye, barley, and malt. Measuring the gluten content in food items and beverages is vital to those who have Celiac Disease or have gluten sensitivities in order to stop the reaction that occurs when gluten is consumed. There are various methods used in order to detect the gluten protein, as well as the different wheat strains in a given sample. To measure the total amount of gluten protein in breadcrumbs, multiple analyses used in food industry and clinical research are used, including the Bradford assay, the Lateral Flow assay, the R5 BioPharm enzyme-linked immunosorbent assay (ELISA), the Kjeldahl method, and the Dumas method. The Kjeldahl and Dumas methods were performed in an outside laboratory, while the three other techniques were performed in house. The protein content recorded from each method was then compared to one another and used to estimate the amount of gluten in the breadcrumb samples. By conducting this research, the results from the five different techniques allowed for a better understanding of the correlation between the specificity of the methods and which is method is best suited for testing certain food items.

Review of Literature

Celiac Disease is an autoimmune disorder that occurs in genetically predisposed individuals where the consumption of gluten leads to the damage and deterioration of the small intestine. This disease affects about one percent of the population and can express itself throughout anyone's life [15]. Symptoms that can be experienced include extreme headaches, bloating, constipation, anxiety, nausea, and weight loss when gluten, a protein found in wheat, rye, barley, or malt, is ingested [16]. These symptoms result from the intestine attacking the gluten, a foreign antigen in the Celiac patient's body. In order to avoid discomfort and permanent damage to the villi, the patient must adhere to a gluten free diet, excluding gluten-containing food such as pastas, cakes, and breads.

Gluten is a "rubbery mass" that is left when the wheat dough is washed and purified [12]. The gluten protein determines the baking quality of wheat due to its influence on capacity, cohesivity, viscosity, and elasticity on dough. The protein can be divided into two main parts: the soluble gliadins and insoluble glutenins. The structure of the gluten protein is important because knowing the chemical makeup will allow for an easier breakdown of the molecule. Gliadins can be categorized into four primary groups that include the α-, β-, Y-, and ω- gliadins. These groups of gliadins can then be separated into more than hundred smaller fractions through two-dimensional electrophoresis. When the analysis of amino acids and molecular weights is complete, the gliadins are further grouped into ω5-, ω1,2-, α, β, and Y– gliadin groups. These groups differ slightly in structural differences with the addition, elimination, or substitution of certain amino acids. ω –gliadins have high contents of glutamine, proline, and phenylalnine and lack cysteine, so they are unable to have disulphide crosslinks.

In contrast to the ω-gliadins, α / β and Ɣ– gliadins have lower contents of glutamine and proline, which allow them to have similar molecular weights around 28,000-35,000. However, α / β and Ɣ– gliadins differ in their N- and C- terminal domains. The N-terminal domain typically includes glutamine, proline, phenlalanine, and tyrosine, with α / β- gliadins having sequences such as QPQPFPQQPYP and Ɣ– gliadins having a sequence of QPQQPFP. The C- terminal domains of the these two groups are similar but have less glutamine and proline then the N-terminal domain.

Glutenins are proteins that are linked through interchain disulphide bonds [12]. Because glutenin is one of the largest proteins in nature, with its molecular weight ranging from 500,000 to more than 10 million, the addition of this protein in the gluten protein allows for it to have a great effect on dough properties. The N- terminal domain contains glutamine and proline units such as QQQPPFS, where as the C- terminal domain is similar to α / β and Ɣ– gliadins. High molecular weight glutenin subunits are contained within each wheat variety that can be grouped in either the x- or the y- type with molecular weights 83,000-88,000 and 67,000-74,000. Each domain of high molecular weight glutenin subunit is made of a non-repetitive N-terminal domain, a repetitive central domain, and a C- terminal domain, with the first and third domains characterized by the occurrence of charged residues and presence of cysteines. The x- and y- type glutenins differ in cysteine content within the first and second domains.

Analyzing gluten samples can be difficult because the gluten protein consists of many different parts. When analyzing food samples for a specific gluten protein, there are various methods that can be used based on the protein or amino acid of interest. This will be done whenever there is a food or beverage item that is trying to be certified as gluten

free by the FDA. The methods include the total protein methods such as the Dumas method and the Kjeldahl method, the Bradford protein assay, ELISAs, and the Lateral Flow assay. The Dumas and Kjeldahl methods are most similar in that the goal for both is to determine the quantity of nitrogen in specific chemical substances. However, the Dumas method is now the standard and preferred method when compared to the Kjeldahl method in determining protein concentration because it is much faster, easier to use, and does not require conversion factors. The Dumas method combusts a known sample of mass in a high temperature (800-900 °C) with oxygen in order to release carbon dioxide, water, and nitrogen [3]. This allows for columns to absorb the excess carbon dioxide and water, such that the thermal conductivity detector is able to separate the nitrogen. The signal received from the detector is able to be directly converted into an exact nitrogen content.

The Kjeldahl method is able to determine the quantity of nitrogen in organic substances, including the inorganic substances ammonia and ammonium. In order to initiate the process of quantification, a given sample is heated between 360-410°C with concentrated sulfuric acid [2]. This acid starts the decomposition of the original sample through oxidation, changing the reduced nitrogen to ammonium sulfate. A condenser is dipped into a weak acid, such as boric acid, after the substance is clarified with the liberation of fumes. The solution is then distilled with sodium hydroxide in order react with ammonium to cause the sample to change to ammonia. The ammonium ion concentration is measured through titration and is converted from the total Kjeldhal nitrogren to protein concentration. This determination is based upon the amount of nitrogenous amino acids in the sample.

Next, the Bradford protein assay is able to measure the concentration of a protein in a given solution based upon the amino acid composition of the sample and the absorbance shift of the dye Coomassie Brilliant Blue G-250 from 465 nm to 595nm when protein binding occurs [4]. Coomassie Brilliant Blue G-250 has three forms influenced by acidity: anionic (blue), neutral (green), and cationic (red) [9][14]. When the protein binds, the dye changes from red to blue during acidic conditions. However, if there is no protein to bind, the solution stays brown [5]. In order to complete this process, Coomassie Brilliant Blue G-250 is dissolved in ethanol. Phosphoric acid is added, the solution is diluted, and samples with five to one hundred micrograms of either albumin or gamma globulin are prepared. The dye reagent is added and then the assay is incubated for five minutes, with the absorbance being measured at 595nm. When the dye is added, the red form of the Coomassie dye donates its electron to the protein, which causes hydrophobic pockets that bind to the nonpolar region of dye. The binding of the protein allows for the stabilization of the dye, thus, allowing the measurement of the amount of dye still present.

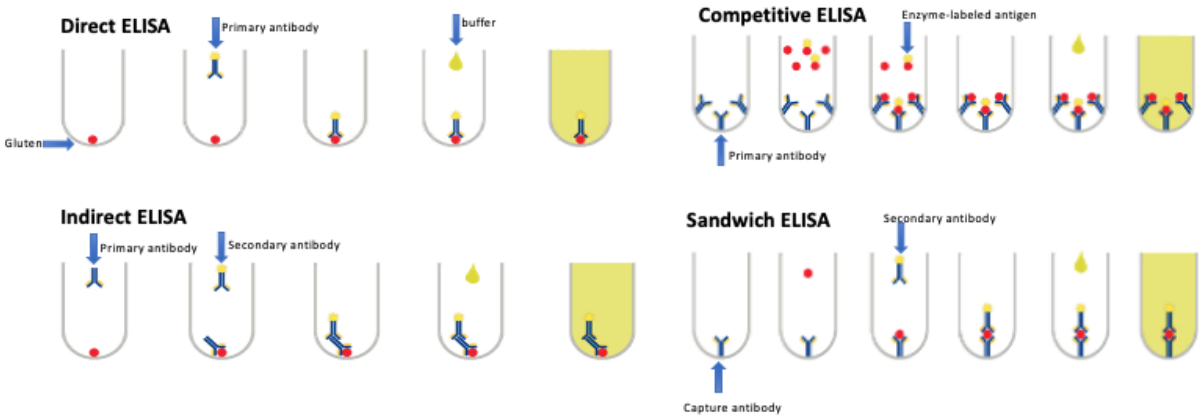
A standard calibration curve of absorbance at 595 nanometers versus the concentration in nanograms per milliliter is created which allows for the determination of the amount of protein within the sample. A known concentration sample of bovine serum albumin (BSA) is diluted to specific measurements such as having just a concentration of Coomassie Brilliant Blue and then having dilated concentrations of 2ng/ml, 4ng/ml, 6ng/ml, 8ng/ml. Bovine serum albumin is used as the standard a part of the standard absorbance curve because it does not affect the quality, quantity, or function of the proteins in the solution. The absorbance and transmittance for every sample is measured

in the spectrophotometer, an instrument that uses a lamp to provide a light source through a diffraction grating to separate the light into different measurable wavelengths. When the solution is measured, the more it absorbs the light frequency at 595 nanometers, the more of the blue color there is. Therefore, there is more protein in a bluer solution, showing a directly proportional relationship between the color of the solution and the amount of protein in the substance.

The Bradford assay is preferred because it can be done in a single step, allowing the procedure to be easy to follow. In addition, the assay is less expensive than other methods, has a high sensitivity for the dye for a specific protein, and can be done in less than thirty minutes at room temperature conditions. However, this assay has a short-range consisting from 0 ug/mL to 200 ug/ml, such that dilutions, in which errors can occur, are normally necessary. Secondly, certain conditions and detergents tend to interfere with the dye's ability to bind to the protein. If there is not an ideal number of aromatic residues, the dye will not be able to bind correctly.

Another way in which to quantify a protein is through the use of an enzyme-linked immunosorbent assay (ELISA). There are four main types of ELISAs which include the direct, indirect, sandwich, and the competitive ELISA [13]. The direct ELISA is used for proteins of high molecular weights. A sample of an antigen that has been immobilized directly on the well plate is directly conjugated to the horseradish peroxide enzyme, a catalyst enzyme, such that the detection of the desired gluten protein can be recognized. An indirect ELISA consists of a two-step binding process where a primary antibody binds to the target molecule/antigen and then the enzyme labeled antibody binds to the first antibody. The sandwich ELISA uses two specific antibodies to physically

sandwich the added antigen and to capture the antibody on the coated well plate. The resultant is a signal that is proportional to the amount of analyte in the sample. Lastly, the competitive ELISA uses a primary antibody and a secondary antibody that is conjugated with an enzyme specific to the primary antibody that bind to the given antigen on the well plate. A substrate is added to the solution that allows for the determination of the concentration of the given protein, which produces a fluorescent signal that is indirectly related to the of antigen concentration.



Lastly, the Lateral Flow assay is a simple immunoassay diagnostic test used to detect a target protein or analyte [1]. This device typically consists of a control line that confirms whether or not the assay is working correctly, as well as one or more other lines that confirm the detection of the target protein. In order to test for a protein, a running, liquid buffer needs to be placed directly onto the conjugated pad, such that a three-way bond is created between the antibodies and targets to make the lines on the test visible. The Lateral Flow assay is a very popular and commonly used protein detector test used in human health testing because it requires minimal training to use, the results are accurate, and it offers flexibility.

Because there are various methods for analyzation, experimentation must be done in order to determine which method yields the most accurate results. The purpose of the study was to analyze the gluten content within breadcrumbs to evaluate which method would be the most appropriate method for this food item. This study was conducted in order to further the research needed to solidify a universal analyzation technique. The hypothesis was that the R5 ELISA would yield the most accurate results due the fact that it is a sandwich ELISA that requires multiple antibodies, with the null hypothesis being that none of the methods would be successful compared to the others in yielding the most accurate results. In the following section, the methods will explain how the R5 and G12 ELISA's were performed.

Materials and Methods

The Whole Foods 365 Everyday Value Whole Wheat Bread Crumbs were used in this experiment [21]. The whole wheat breadcrumbs are made of whole wheat flour and yeast. One serving is a quarter of a cup with 110 calories.

The methods used in this study were the Biopharm R5 ELISA RIDASCREEN Gliadin sandwich assay, AgraQuant G12 ELISA, Kjeldahl, and Dumas methods. The R5 and G12 ELISA's were performed in-house, while the Kjeldahl, and Dumas methods were performed in an outside laboratory.

The R5 ELISA RIDASCREEN Gliadin sandwich assay manufactured by Biopharm was one of three tests performed according to instructions during this study [17]. The equipment that was used was the microtiter plate spectrophotometer (450 nm), centrifuge, centrifugal vials (e.g. Brand 10742512), shaker, laboratory mincer / grinder, pestle and mortar, ultra-turrax or homogenizer, a water bath (50 °C / 122 °F), graduated pipettes, and variable 20 µl - 200 µl and 200 - 1000 µl micropipettes. The reagents used were the distilled water, cocktail (patented) (R7006 / R7016, 105 ml / 1000ml) and ethanol solution.

Before the experiment was started, the samples were prepared. Surfaces were cleaned, as well as other equipment with 40 % ethanol. The sample preparation was carried out in a room isolated from the ELISA procedure. The gliadin contamination of reagents and equipment with the test strips RIDA®QUICK Gliadin was checked.

The samples were then extracted in the following steps. They were incubated for 40 min at 50 °C (122 °F), cooled and mixed with 7.5 ml 80 % ethanol, and then shaken for 1 hour upside down at room temperature (20 - 25 °C / 68 - 77 °F). The samples were placed in a centrifuge for 10 min, at room temperature (20 - 25 °C / 68 - 77 °F). The

supernatant was transferred in a screw top vial, and the sample was diluted with the ratio 1:12.5 (1+11.5 / 80 µl + 920 µl) with diluted sample diluent (see 10.1.): the final dilution factor is 500. One hundred microliters was placed in each well in the assay.

The second means of analysis was through the AgraQuant® Gluten G12® ELISA Test Kit. The instructions for the kit were carried as directed in the instructions [18]. A sample of the breadcrumbs was taken and homogenized with of 5 grams in a mortar. 0.25 grams of homogenate was measured of into a test tube and 2.5 mL of extraction buffer (bottle A) was added. The sample was shaken until it was evenly dispersed in the extraction buffer. The extract was incubated in the pre-warmed water bath at 122°F for 40 minutes. The sample extract was taken out of the pre-warmed water bath cooled down (20-25°C/67-77°F). After, 7.5 mL of ethanol 80% was added and then shaken for 60 minutes at room temperature (20-25°C/67-77°F) in a laboratory shaker. After 30 minutes, the sample was checked and placed back onto the shaker for 30 more minutes to complete the rest of the extraction process. The samples were placed into the centrifuge for 10 minutes at 2000 g in order for there to be a clear supernatant. After this process was completed, the supernatant was collected and transferred to a clean vial.
Lastly, the supernatant was diluted with a ratio of 1:10 with the dilution buffer prepared with 100 µL of sample extract with 900 µl dilution buffer.

Standard assay protocol:

Antibody-coated microwells were placed into a microwell strip holder. 100 µL of ready-to-use standards and prepared samples into the appropriate well was added into the wells using a single channel pipette. Every time a new sample was placed into a well a

fresh pipette was used in order to ensure that the samples were uncontaminated. The wells and the samples were incubated at room temperature for 20 minutes. After the 20 minutes passed, the contents of the wells were emptied into a waste container. Each of the wells was then washed with the wash buffer solution, and then the wash buffer solution was discarded. This step was repeated for an additional four times, making a total of five washes with the wash buffer solution. After the fifth wash, layers of paper towels were placed on a flat surface where the microwell were tapped on them to ensure that all the residual buffer was removed – the paper towels were never placed directly into the wells, as this could lead to contamination and could misconstrue the results.

Next, 100 μL of enzyme-conjugate solution was placed into each well with a pipette, and then the well was incubated at room temperature for 20 minutes. The microwell plate was washed five times just as in the above steps. In each of the microwell plates, 100 μL of the substrate solution was placed into each of the wells with a pipette. The wells were incubated at room temperature for 20 minutes in order to allow the reaction to develop. In order to stop the reaction, 100 μL of stop solution was added into each microwell using an 8- channel pipette – the color changed from blue to yellow. Lastly, the absorbance of each well was read at 450 nm with the microwell reader.

Future Research

Because of the various types and sizes of the gluten proteins, more research must be conducted in order to better understand the differences between the different gluten molecules and which methods are best suited for detecting each specific gluten molecule. More knowledge of which method is best suited for detecting gluten will allow for more accurate detection of gluten in food and beverages. This information will allow for the FDA to regulate gluten free foods more precisely, therefore, protecting Celiac Disease patients, as well as those who are suffering from gluten sensitivities. In addition, research needs to be continued in order to determine a universal reference material and analyzation technique for gluten, such that there is a tool agreed upon by the Celiac community, scientific community, and the FDA.

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