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(54) **RECOMBINANT DEAMIDATED GLIADIN ANTIGEN**

REKOMBINANTES DESAMIDIERTES GLIADIN-ANTIGEN

ANTIGÈNE DE GLIADINE RECOMBINANTE DÉAMIDÉE

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**Description**

## BACKGROUND OF THE INVENTION

5 **[0001]** Celiac disease (CD) is a severe gastrointestinal disease that has a strong genetic component. CD is characterized by a permanent intolerance of proteins from wheat, barley, rye, and oats. Although the physiopathology of CD is not completely understood it is clear that the presence of the toxic proteins in the patient's diet causes a total or partial damage of intestinal mucosa (Brandtzaeg, P. 1997. Mechanisms of gastrointestinal reactions to food. Environmental Toxicology and Pharmacology 4;9-24) leading to severe malabsorption syndromes and causing diarrhea, vomiting, abdominal pain, anorexia, retarded growth, malnutrition and anemia. CD has been associated with a higher risk for intestinal cancer in non-diagnosed and untreated patients (Holmes GKT, 1989. Malignancy in coeliac disease-effect of a gluten-free diet, Gut 30;333-338). CD affects mainly children under three years old, but it is also common in adults, and sometimes is clinically atypical or asymptomatic (Ferguson A, et al. 1992. Definitions and diagnostic criteria of latent and potential coeliac disease. Ed by Auricchio S, Visakorpi J K, in Epidemiology of CD. Dyn Nutr Res, Basel, Karger 15 2;119-127). CD is more frequent in patients with other genetic or autoimmune disease, such as insulin dependent diabetes mellitus, Down syndrome, selective IgA deficiency, and dermatitis herpetiformis (Sirgus N et al. 1993. Prevalence of coeliac disease in diabetic children and adolescents in Sweden. Acta Paediatr 66;491-494; Zubillaga P et al. 1993. Down syndrome and coeliac disease. J Paediatr Gastroenterol Nutr 16:168-171; Boyce N 1997).

20 **[0002]** The clinical symptoms of CD could be confused with those produced by other gastrointestinal diseases. In these cases CD is misdiagnosed and patients do not receive the specific treatment, that is, a complete elimination of gluten in their diet. On the other hand, if a non-celiac patient is wrongly diagnosed as celiac, he would undergo an unnecessary gluten free diet for his whole life. Accordingly, a precise diagnosis of CD is essential. Currently the standard for CD diagnosis is intestinal biopsy, repeated three times: at the onset of the clinical symptoms, after several months on a gluten free diet, and after a challenge with gluten.

25 **[0003]** Because intestinal biopsy is an invasive method and precise serological tests have been developed, the above criteria have been revised (Walker-Smith et al. 1990. Revised criteria for diagnosis of coeliac disease. Report of Working group of European Society of Pediatric Gastroenterology and Nutrition. Arch Dis Child 65:909-911). Currently, serological tests can be done at the onset of clinical symptoms and when they are positive, a confirmatory intestinal biopsy will be indicated. The response to the treatment with a gluten-free diet can also be followed by serological tests. If discrepancies occur between the clinical response to the treatment and the result of serological tests a second intestinal biopsy would be indicated. Several serological tests have been developed for celiac disease diagnosis, such as the detection of antibodies to cellular antigens, or antibodies to food antigens, like gliadins. There are diagnostic kits for the detection of anti-endomysial antibodies, anti-reticulin antibodies, anti-gliadin antibodies, and anti-tissue transglutaminase antibodies.

30 **[0004]** Anti-gliadin antibodies (AGA) have been extensively used for serological diagnosis of CD (Stern M et al. 1996. Validation and standardization of serological screening tests for coeliac disease in 1996. 3rd EMRC/ESPGAN Workshop, Dec 5-8, 1996, Molsheim, France, pp:9-24; Catassi C et al. 1999. Quantitative antigliadin antibody measurement in clinical practice: an Italian multicenter study. Ital J Gastroenterol Hepatol 31; 366-370). AGA are mainly detected by ELISA (Enzyme-Linked Immunosorbent Assay), a simpler, more objective method than IFA (indirect immunofluorescent antibody analysis), and can be used for the analysis of a large number of samples. Nevertheless AGA are less specific for CD than endomysal antibodies (EMA) and the detection of antibodies to either IgA or IgG isotypes requires two independent assays. Recently a visual immunoassay for the detection of AGA, which solves some of these problems, has been reported (Garrote J A, Sorell L, Alfonso P et al 1999. A simple visual immunoassay for the screening of coeliac disease. Eur. J Clin Invest 29; 697-699; Spanish Office for Patents and Marks No. 9801067).

35 **[0005]** In 1997, Dietrich *et al.* identified tissue transglutaminase (tTG), an 85 kDa protein, as the major auto antigen detected by anti-endomysial antibodies (Dietrich W et al. 1997. Identification of tissue transglutaminase as the auto antigen of celiac disease. Nat Med. 3:797-801). Detection of anti-tTG antibodies had been reported lately in ELISA or radio-ligand (RLA) formats based on tTG from guinea pig liver extracts or recombinant human tTG cloned from different tissues (Sulkanen S et al. 1998. Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. Gastroenterology 115:1322-1328; Siessler J et al. 1999. Antibodies to human recombinant tissue transglutaminase measured by radioligand assay: Evidence for high diagnostic sensitivity for celiac disease. Horm Metab Res 31; 375-379).

40 **[0006]** Prior art methods for detection of celiac disease use specific gliadin epitopes or pieces of the gliadin protein in an assay, that lead to both false-negatives and false-positives. What is needed is an assay that provides new antigens containing a more inclusive set of epitopes that provides a more accurate assay for celiac disease. Surprisingly, the present invention meets this and other needs.

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## SUMMARY OF THE INVENTION

5 [0007] The present invention provides an antigen for detecting celiac disease. The antigen includes a recombinant or synthetic deamidated gliadin having a hexamer of peptides each having SEQ ID NO:1. In one embodiment the recombinant or synthetic deamidated gliadin is covalently linked to a tag to form a gliadin fusion protein. The gliadin fusion protein may be immobilized on a solid support, and the recombinant or synthetic deamidated gliadin is capable of binding to anti-deamidated gliadin antibodies.

[0008] In other embodiments, the present invention provides use of the antigen of the invention for determining whether a subject has celiac disease

10 [0009] In some other embodiments, the present invention provides a method for diagnosing celiac disease in a subject. The method includes contacting a sample of bodily fluid from the subject with an antigen of the present invention. The method also includes detecting any antibody that has become specifically bound to the antigen, thus indicating the presence of celiac disease in the subject.

15 [0010] In another embodiment, the present invention provides a kit including an antigen of the present invention, a detection reagent, and optionally at least one of buffers, salts, stabilizers and instructions. The present invention further provides an isolated nucleic acid encoding the antigen of the invention, as well as an expression vector comprising such isolated nucleic acid and a host cell comprising such expression vector.

## BRIEF DESCRIPTION OF THE DRAWINGS

20 [0011]

**Figure 1** shows purification of D2-hexamer.

**Figure 2** shows coating titration of a DGP hexamer: Calibrator Cutoff signal relative fluorescence intensity (RFI).

25 **Figure 3** shows coating titration of a DGP hexamer with a lysine substituted for the glutamic acid residue at position 14: Calibrator Cutoff signal RFI.

**Figure 4** shows the rDGP hexamer has improved sensitivity as compared to the rDGP trimer.

## DETAILED DESCRIPTION OF THE INVENTION

## I. Definitions

30 [0012] As used herein, the term "contacting" refers to the process of bringing into contact at least two distinct species such that they can react. The resulting reaction product is either produced directly from a reaction between the added reagents or from an intermediate from one or more of the added reagents which can be produced in the reaction mixture.

35 [0013] As used herein, the term "bodily fluid" refers to fluids of a mammal including aqueous humour, bile, blood and blood plasma, breast milk, interstitial fluid, lymph, mucus, pleural fluid, pus, saliva, serum, sweat, tears, urine, cerebrospinal fluid, synovial fluid or intracellular fluid. One of skill in the art will appreciate that other bodily fluids are useful in the present invention.

40 [0014] As used herein, the term "cross-linker" refers to a bifunctional or multi-functional chemical or biological moiety that is capable of linking two separate moieties together. Examples of cross-linkers useful in the present invention are described below.

[0015] As used herein, "antibody" includes reference to an immunoglobulin molecule immunologically reactive with a particular antigen, and includes both polyclonal and monoclonal antibodies.

45 [0016] As used herein, the term "subject" refers to animals such as mammals, including primates (e.g., humans), cows, sheep, goats, horses, dogs, cats, rabbits, rats and mice.

[0017] As used herein, the term "immobilized" refers to the association of the tTG, the gliadin fusion protein or the tTG-gliadin fusion protein complex with a solid support material through covalent bond formation, ionic bond formation, hydrogen-bonding, dipole-dipole interaction or via Van der Waals interactions. The immobilization can be temporary or permanent.

50 [0018] As used herein, the term "antigen" refers to a molecule that is capable of stimulating an immune response such as by production of antibodies. Antigens of the present invention include solid support immobilized gliadin fusion protein and solid support immobilized tTG-gliadin fusion protein complex. The gliadin fusion protein of the present invention can include both a recombinant deamidated gliadin and a tag, such as Glutathione S-transferase (GST) protein.

55 [0019] As used herein, the term "buffers" refers to any inorganic or organic acid or base that resists changes in pH and maintains the pH around a desired point. Buffering agents useful in the present invention include sodium hydroxide, dibasic sodium phosphate anhydrous, and mixtures thereof. One of skill in the art will appreciate that other buffering agents are useful in the present invention.

[0020] As used herein, the term "tissue Transglutaminase (tTG)" refers to an enzyme of the transglutaminase family that crosslinks proteins between an amino group of a lysine residue and a carboxamide group of a glutamine residue. This creates an intermolecular or intramolecular bond. tTG can be used to detect celiac disease.

5 [0021] As used herein, the term "gliadin fusion protein" refers to a gliadin protein linked to a tag, such as Glutathione S-transferase (GST) or a His tag. The gliadin protein includes a recombinant gliadin protein or a synthetic gliadin protein. The gliadin protein is deamidated. Tags are typically other proteins or compounds that can be used as affinity tags for purification, for solubilization, chromatography, as epitope tags and fluorescence tags. Tags useful in the present invention include BCCP, c-myc-tag, Calmodulin-tag, FLAG-tag, HA-tag, His-tag, Maltose binding protein-tag, Nus-tag, Glutathione-S-transferase (GST) tag, Green fluorescent protein-tag, Thioredoxin-tag, S-tag, Streptag II, Softag 1, Softag 3, T7-tag, 10 Elastin-like peptides, Chitin-binding domain, and Xylanase 10A. One of skill in the art will appreciate that other proteins are useful in fusion proteins of the present invention.

[0022] As used herein, the term "tTG-gliadin fusion protein complex" refers to a complex formed when the tTG and the gliadin fusion protein become linked together. The tTG and the gliadin fusion protein can be linked in a variety of ways, under a variety of reactions. The tTG can be linked to either or both of the tag and the recombinant deamidated 15 gliadin of the gliadin fusion protein.

[0023] As used herein, the term "recombinant deamidated gliadin" refers to a deamidated gliadin protein prepared via genetic engineering. Deamidated proteins are those that have had some or all of the free amide functional groups hydrolyzed to carboxylic acids, such as conversion of glutamines to glutamic acid. Recombinant deamidated gliadins useful in the present invention comprise peptides having SEQ ID NO:1 or comprise a hexamer having at least 95% 20 sequence identity to SEQ ID NO:3.

[0024] As used herein, the term "crosslinked" refers to the formation of more than one bond between two different chemical moieties. In the present invention, the chemical moieties can be biological species such as proteins, enzymes and antibodies, or solid support materials. The chemical functionality that links the individual chemical moieties that are crosslinked, is termed a "crosslinker". A crosslinker is typically a bifunctional compound that reacts with one reactive 25 functional group on one chemical moiety and one reactive functional group on another chemical moiety, thereby linking the two chemical moieties to each other. The crosslinkers can be homobifunctional crosslinkers or heterobifunctional crosslinkers. Homobifunctional crosslinkers are those where the functional groups of the homobifunctional crosslinker that react with each chemical moiety are the same. Heterobifunctional crosslinkers are those where the functional groups of the heterobifunctional crosslinker that react with each chemical moiety are different. Preferred homobifunctional and 30 heterobifunctional crosslinkers of the present invention are described in greater detail below.

[0025] As used herein, the terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (*i.e.*, 60% identity, preferably 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity over a specified region), when 35 compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition also refers to the complement of a test sequence.

[0026] The phrase "substantially identical," in the context of two nucleic acids or polypeptides, refers to a sequence or subsequence that has at least 40% sequence identity with a reference sequence. Alternatively, percent identity can be any integer from 40% to 100%. Preferred is an identity of at least: 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% compared to a reference 40 sequence using the programs described herein; preferably BLAST using standard parameters, as described below.

[0027] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, 45 subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters. For sequence comparison of nucleic acids and proteins, the BLAST and BLAST 2.0 algorithms and the default parameters discussed below are used.

50 [0028] Preferred examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., Nuc. Acids Res. 25:3389-3402 (1977) and Altschul et al., J. Mol. Biol. 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information 55 (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer

HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word length of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

**[0029]** The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, Proc. Nat'l. Acad. Sci. USA 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

**[0030]** An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions. Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequence.

**[0031]** As used herein, the terms "nucleic acid" and "polynucleotide" are used synonymously and refer to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, nucleic acid analogs may be used that may have alternate backbones, comprising, e.g., phosphoramidate, phosphorothioate, phosphorodithioate, or O-methylphosphoroamidite linkages (see Eckstein, *Oligonucleotides and Analogues: A Practical Approach*, Oxford University Press); and peptide nucleic acid backbones and linkages. Other analog nucleic acids include those with positive backbones; non-ionic backbones, and non-ribose backbones. Thus, nucleic acids or polynucleotides may also include modified nucleotides, that permit correct read through by a polymerase. "Polynucleotide sequence" or "nucleic acid sequence" includes both the sense and antisense strands of a nucleic acid as either individual single strands or in a duplex. As will be appreciated by those in the art, the depiction of a single strand also defines the sequence of the complementary strand; thus the sequences described herein also provide the complement of the sequence. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid may contain combinations of deoxyribo- and ribo-nucleotides, and combinations of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine and isoguanine.

**[0032]** As used herein, the phrase "a nucleic acid sequence encoding" refers to a nucleic acid which contains sequence information for a structural RNA such as rRNA, a tRNA, or the primary amino acid sequence of a specific protein or peptide, or a binding site for a trans-acting regulatory agent. This phrase specifically encompasses degenerate codons (*i.e.*, different codons which encode a single amino acid) of the native sequence or sequences that may be introduced to conform with codon preference in a specific host cell.

**[0033]** As used herein, the term "specifically bound" refers to the capturing or entrapment of the antigen of the present invention by an antibody that is indicative of the presence of celiac disease. Thus, under designated immunoassay conditions, an antibody (e.g., an anti-deamidated gliadin antibody) binds an antigen of the present invention at at least two times over background level and more typically at at least 5, 10, 20, 30, 40, or 50 times over background level. A variety of immunoassay formats may be used to determine whether an antibody specifically binds an antigen of the present invention. For example, solid-phase ELISA immunoassays are routinely used to determine whether an antibody is specifically immunoreactive with a protein (see, e.g., Harlow & Lane, *Using Antibodies, A Laboratory Manual* (1998) for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity).

## II. Antigen

**[0034]** The present invention provides an antigen and method for detection of celiac disease. The antigen may include

a gliadin fusion protein immobilized on a solid support material. The gliadin fusion protein includes both a recombinant deamidated gliadin and a tag. The antigen can optionally include tissue Transglutaminase (tTG). When present, the gliadin fusion protein and tTG can be covalently linked prior to immobilization on the solid support, such as via transamidation, to form a tTG-gliadin fusion protein complex. Following immobilization of the tTG-gliadin fusion protein complex on the solid support, the gliadin fusion protein and the tTG can be cross-linked using suitable cross-linkers.

**[0035]** In some embodiments, the present invention provides an antigen for detecting celiac disease. The antigen may include the solid support bound gliadin fusion protein described below.

**[0036]** In other embodiments, the present invention provides an antigen for detecting celiac disease. The antigen includes a recombinant deamidated gliadin having a hexamer of peptides each having the sequence of SEQ ID NO:1, wherein the recombinant deamidated gliadin is covalently linked to a tag to form a gliadin fusion protein. The gliadin fusion protein may be immobilized on a solid support, and the recombinant deamidated gliadin is capable of binding to anti-deamidated gliadin antibodies.

#### A. Gliadin Fusion Protein

**[0037]** The gliadin fusion protein useful in the present invention includes a recombinant deamidated gliadin that is expressed as a tagged protein. One of skill in the art will recognize that many recombinant gliadin proteins are useful in the method of the present invention. The recombinant gliadin protein of the present invention is a hexamer of D2 (Aleanzi et al, Clin Chem 2001, 47 (11), 2023), peptide sequence: QPEQPQSFPEQERPF (SEQ ID NO:1). In some embodiments, the recombinant gliadin protein is a hexamer of D2, separated by any suitable spacer, such as GGGGS (SEQ ID NO:2). One of skill in the art will appreciate that other spacers are useful in the present invention.

**[0038]** Typical peptide spacer sequences contain Gly, Ser, Ala and Thr residues. Useful spacer include glycine-serine polymers including, for example, (GGGGS)<sub>n</sub> (SEQ ID NO:13), (GS)<sub>n</sub>, (GSGGS)<sub>n</sub> (SEQ ID NO:14), and (GGGS)<sub>n</sub> (SEQ ID NO:15), where n is an integer of at least one; glycine-alanine polymers; alanine-serine polymers; and other flexible linkers.

**[0039]** In some embodiments, the hexamer includes a spacer separating each peptide having the sequence of SEQ ID NO:1. In other embodiments, each spacer can have the sequence of SEQ ID NO:2.

**[0040]** In some embodiments, the recombinant deamidated gliadin is a D2 hexamer (SEQ ID NO:3). In some other embodiments, the present invention provides any nucleotide sequence that encodes the polypeptide in the sequence of SEQ ID NO:1 or SEQ ID NO:3. The recombinant deamidated gliadin proteins of the present invention bind to anti-deamidated gliadin antibodies, and are thus able to diagnose subjects having gluten related disorders such as celiac disease. One of skill in the art will appreciate that other recombinant deamidated gliadin proteins are useful in the present invention.

**[0041]** The gliadin fusion protein also includes a tag. Any tag known in the art is useful in the gliadin fusion proteins of the present invention. Tags suitable in the antigen of the present invention include a Glutathione S-transferase (GST), His-tag, FLAG, Streptag II, HA-tag, Softag 1, Softag 3, c-myc, T7-tag, S-tag, Elastin-like peptides, Chitin-binding domain, thioredoxin, Xylanase 10A, Maltose binding protein and NusA. In some embodiments, the tag is a Glutathione S-transferase (GST) or a His-tag. One of skill in the art will appreciate that other tags are useful in the present invention. The tag is typically attached to the recombinant gliadin protein via covalent linkage.

**[0042]** The His-tag useful in the present invention can be any suitable His-tag. His-tags suitable in the present invention include the sequence of SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:6. In some embodiments, the His-tag can be the sequence of SEQ ID NO:5 or SEQ ID NO:6. In other embodiments, the recombinant deamidated gliadin can be the sequence of SEQ ID NO:7 or SEQ ID NO:8.

**[0043]** In another embodiment, the tag is a Glutathione S-transferase (GST) protein. The GST protein (SEQ ID NO:10) serves many functions, including enabling the purification of the recombinant gliadin protein and the presentation of epitopes represented in the recombinant gliadin protein.

**[0044]** When the gliadin fusion protein includes GST and the recombinant deamidated gliadin is the D2 hexamer, the gliadin fusion protein is represented by the sequence of SEQ ID NO:11. The gliadin fusion protein of the present invention can be prepared by a variety of methods, including via recombinant methods such as those described.

**[0045]** Immobilization of the gliadin fusion protein on the solid support can be achieved by any method known in the art. The immobilization of the gliadin fusion protein to the solid support can be via covalent or ionic bond formation, hydrogen bonding, Van der Waals forces, as well as via antibody-antigen interactions. One of skill in the art will appreciate that other immobilization methods are useful in the present invention.

**[0046]** In some embodiments, the antigen also includes tissue Transglutaminase (tTG). When tTG is present, the tTG and gliadin fusion protein form a tTG-gliadin fusion protein complex. The tTG and the gliadin fusion protein can be linked in a variety of ways, such as by the formation of covalent bonds, ionic bonds, hydrogen bonding, or by Van der Waals interactions. When the tTG and the gliadin fusion protein are linked covalently, the covalent bonds can be formed by a variety of reactions, such as transamidation. The transamidation can occur under a variety of conditions, such as in the

presence of Ca<sup>2+</sup>. The tTG can be linked to either or both of the tag and the recombinant deamidated gliadin of the gliadin fusion protein. The tTG is immobilized to the solid support under the same conditions, and at the same time as immobilization of the gliadin fusion protein. Tissue transglutaminase is known to one of skill in the art and has been described previously, see NCBI RefSeq NP\_004604 and NP\_945189 (April 13, 2008).

**[0047]** In other embodiments, the tTG and the gliadin fusion protein are covalently linked by a cross-linker. One of skill in the art will appreciate that other methods of cross-linking are available, such as via ionic bonding, hydrogen bonding or via van der Waals forces. One of skill in the art will recognize that any cross-linker is suitable in the instant invention. In some embodiments, the cross-linker is a member selected from a heterobifunctional crosslinker and a homobifunctional crosslinker. In yet other embodiments, the cross-linker is a homobifunctional crosslinker. In still yet other embodiments, the cross-linker is a member selected from bis(sulfosuccinimidyl)suberate (BS3), ethylene glycol bis[succinimidylsuccinate] (EGS), ethylene glycol bis[sulfosuccinimidylsuccinate] (sulfo-EGS), bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone (BSOCOES), dithiobis(succinimidyl)propionate (DSP), 3,3'-dithiobis(sulfosuccinimidyl)propionate (DTSSP), disuccinimidyl suberate (DSS), disuccinimidyl glutarate (DSG), methyl N-succinimidyl adipate (MSA), disuccinimidyl tartarate (DST), 1,5-difluoro-2,4-dinitrobenzene (DFDNB), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC or EDAC), sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), N-hydroxysulfosuccinimide (sulfo-NHS), hydroxylamine and Sulfo-LC-SPDP (N-succinimidyl 3-(2-pyridyldithio)-propionate) and sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate (sulfo-LC-SPDP). In another embodiment, the cross-linker is bis(sulfosuccinimidyl)suberate (BS3).

**[0048]** In a further embodiment, the recombinant deamidated gliadin has 95% identity to SEQ ID NO:3. In some other embodiments, the recombinant deamidated gliadin has the sequence of SEQ ID NO:3.

## B. Solid Support

**[0049]** A solid support material for use in the present invention is characterized by the following properties: (1) insolubility in liquid phases used for screening; (2) capable of mobility in three dimensions independent of all other supports; (3) containing many copies of the gliadin fusion protein or the tTG-gliadin fusion protein complex; (4) compatibility with screening assay conditions; and (5) being inert to the assay conditions. A preferred support also has reactive functional groups, including hydroxyl, carboxyl, amino, thiol, aldehyde, halogen, nitro, cyano, amido, urea, carbonate, carbamate, isocyanate, sulfone, sulfonate, sulfonamide and sulfoxide for attaching the gliadin fusion protein and tTG.

**[0050]** As used herein, solid support material is not limited to a specific type of support. Rather a large number of supports are available and are known to one of ordinary skill in the art. Solid phase supports include silica gels, resins, derivatized plastic films, beads such as glass, plastic, or magnetic beads, cotton, alumina gels and polysaccharides such as Sepharose. Other solid supports can be ELISA microtiter plates. A suitable solid phase support can be selected on the basis of desired end use and suitability for various synthetic protocols. For example, in polyamide synthesis, useful solid phase support can be resins such as polystyrene (e.g., PAM-resin obtained from Bachem Inc., Peninsula Laboratories), POLYHIPE™ resin (obtained from Aminotech, Canada), polyamide resin (obtained from Peninsula Laboratories), polystyrene resin grafted with polyethylene glycol (TentaGel™, Rapp Polymere, Tubingen, Germany), polydimethyl-acrylamide resin (available from Milligen/Bio search, California), or PEGA beads (obtained from Polymer Laboratories). Preferred solid phase synthesis supports for specific syntheses are described below. The solid support may be a bead. One of skill in the art will recognize that many types of solid supports are useful in the present invention.

## C. Process for Preparing Recombinant Deamidated Gliadin Antigen

**[0051]** Enclosed herein is an antigen for detecting celiac disease prepared by the process including contacting a solid support with a gliadin fusion protein, wherein the gliadin fusion protein includes a recombinant deamidated gliadin having a hexamer of peptides each having the sequence of SEQ ID NO:1 and wherein the recombinant deamidated gliadin is covalently linked to a tag, such that the gliadin fusion protein is immobilized on the solid support. Thus, the antigen for detecting celiac disease is prepared.

**[0052]** The process of preparing the recombinant deamidated gliadin antigen can prepare any recombinant deamidated gliadin antigen described above.

**[0053]** The tag is as described above. The tag may be GST or a His-tag. The tag may be GST. In some embodiments, the gliadin fusion protein may be immobilized on the solid support via the tag.

**[0054]** The solid support is as described above. The solid support may be a bead, such as a magnetic bead. The solid support may have a functional reactive group.

**[0055]** When tTG is present, the process can also include forming a covalent bond between the gliadin fusion protein and the tTG prior to the contacting step to form a tTG-gliadin fusion protein complex. The process of forming a covalent bond between the gliadin fusion protein and the tTG can also occur during and/or after the contacting step. The complexing of the gliadin fusion protein and the tTG can occur by any method known in the art. The complexation may occur by

transamidation to form a covalent bond.

**[0056]** The process may further comprise contacting the solid support with a cross-linker to cross-link the gliadin fusion protein and the tTG. The cross-linker may cross-link the GST protein to the tTG. One of skill in the art will appreciate that any cross-linker is useful in this process, such as those described above. The cross-linking can occur via hydrogen-bonding, covalent or ionic bond formation.

## 1. General Recombinant Methods

**[0057]** This invention can employ routine techniques in the field of recombinant genetics for the preparation of recombinant deamidated gliadin polypeptides. Basic texts disclosing the general methods of use in this invention include Sambrook & Russell, *Molecular Cloning, A Laboratory Manual* (3rd Ed, 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel et al., eds., 1994-1999).

**[0058]** A recombinant deamidated gliadin, or a fusion protein, e.g., comprising recombinant deamidated gliadin and a tag such as a GST tag or a His tag, can be expressed using techniques well known in the art. Eukaryotic and prokaryotic host cells may be used such as animal cells, insect cells, bacteria, fungi, and yeasts. Methods for the use of host cells in expressing isolated nucleic acids are well known to those of skill and may be found, for example, in the general reference, *supra*. Accordingly, this invention also provides for host cells and expression vectors comprising the nucleic acid sequences described herein.

**[0059]** Nucleic acids encoding a recombinant deamidated gliadin, or a fusion protein, e.g., comprising recombinant deamidated gliadin and a tag such as a GST tag or a His tag, can be made using standard recombinant or synthetic techniques. Nucleic acids may be RNA, DNA, or hybrids thereof. One of skill can construct a variety of clones containing functionally equivalent nucleic acids, such as nucleic acids that encode the same polypeptide. Cloning methodologies to accomplish these ends, and sequencing methods to verify the sequence of nucleic acids are well known in the art.

**[0060]** The nucleic acids may be synthesized *in vitro*. Deoxynucleotides may be synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage & Caruthers, *Tetrahedron Letts.* 22(20):1859-1862 (1981), using an automated synthesizer, e.g., as described in Needham-VanDevanter, et al., *Nucleic Acids Res.* 12:6159-6168 (1984). The nucleic acids encoding the desired protein may be obtained by an amplification reaction, e.g., PCR.

**[0061]** One of skill will recognize many other ways of generating alterations or variants of a given polypeptide sequence. Most commonly, polypeptide sequences are altered by changing the corresponding nucleic acid sequence and expressing the polypeptide.

**[0062]** One of skill can select a desired nucleic acid or polypeptide based upon the sequences referred to herein and the knowledge readily available in the art regarding recombinant deamidated gliadin structure and function. The physical characteristics and general properties of these proteins are known to skilled practitioners.

**[0063]** To obtain high level expression of a recombinant deamidated gliadin, or a fusion protein comprising recombinant deamidated gliadin and a tag such as a GST tag or a His tag, an expression vector is constructed that includes such elements as a promoter to direct transcription, a transcription/translation terminator and a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, e.g., in the references providing expression cloning methods and protocols cited hereinabove. Bacterial expression systems for expressing ribonuclease are available in, e.g., *E. coli*, *Bacillus sp.*, and *Salmonella* (see, also, Palva, et al., *Gene* 22:229-235 (1983); Mosbach, et al., *Nature* 302:543-545 (1983). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available.

**[0064]** In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for expression of the nucleic acid in host cells. A typical expression cassette thus contains a promoter operably linked to the nucleic acid sequence encoding the recombinant deamidated gliadin or the fusion protein (e.g., a recombinant deamidated gliadin-GST fusion protein), and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. Depending on the expression system, the nucleic acid sequence encoding the recombinant deamidated gliadin or fusion protein (e.g., recombinant deamidated gliadin-GST fusion protein) may be linked to a cleavable signal peptide sequence to promote secretion of the encoded protein by the transformed cell.

**[0065]** As noted above, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

**[0066]** The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET15b, pET23D, pET-22b(+), and fusion expression systems such as GST and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient

methods of isolation, e.g., 6-his. These vectors comprise, in addition to the expression cassette containing the coding sequence, the T7 promoter, transcription initiator and terminator, the pBR322 ori site, a bla coding sequence and a lac1 operator.

5 [0067] The vectors comprising the nucleic acid sequences encoding the RNase molecules or the fusion proteins may be expressed in a variety of host cells, including *E. coli*, other bacterial hosts, yeast, and various higher eukaryotic cells such as the COS, CHO and HeLa cells lines and myeloma cell lines. In addition to cells, vectors may be expressed by transgenic animals, preferably sheep, goats and cattle. Typically, in this expression system, the recombinant protein is expressed in the transgenic animal's milk.

10 [0068] The expression vectors or plasmids of the invention can be transferred into the chosen host cell by well-known methods such as calcium chloride transformation for *E. coli* and calcium phosphate treatment, liposomal fusion or electroporation for mammalian cells. Cells transformed by the plasmids can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as the amp, gpt, neo and hyg genes.

15 [0069] Once expressed, the expressed protein can be purified according to standard procedures of the art, including ammonium sulfate precipitation, column chromatography (including affinity chromatography) and gel electrophoresis (see, generally, R. Scopes, Protein Purification, Springer-Verlag, N.Y. (1982), Deutscher, Methods in Enzymology Vol. 182: Guide to Protein Purification., Academic Press, Inc. N.Y. (1990); Sambrook and Ausubel, both *supra*).

20 [0070] In some embodiments, the present invention provides an isolated nucleic acid including the sequence of SEQ ID NO:9, which encodes a recombinant gliadin protein D2 hexamer sequence. In other embodiments, the isolated nucleic acid is in an expression vector. In some other embodiments, the expression vector is in a host cell.

## 2. Immobilization on the Solid Support

25 [0071] The gliadin fusion protein of the present invention can be immobilized to any useful solid support material by any useful immobilization method known in the art. The immobilization of the gliadin fusion protein to the solid support can be via covalent or ionic bond formation, hydrogen bonding, Van der Waals forces, as well as via antibody-antigen interactions. One of skill in the art will appreciate that other immobilization methods are useful.

[0072] Other compounds have been developed that enable immobilization in a manner similar to antibodies. Certain of these "antibody mimics" use non-immunoglobulin protein scaffolds as alternative protein frameworks for the variable regions of antibodies.

30 [0073] For example, Ladner et al. (U.S. Patent 5,260,203) describe single polypeptide chain binding molecules with binding specificity similar to that of the aggregated, but molecularly separate, light and heavy chain variable region of antibodies. The single-chain binding molecule contains the antigen binding sites of both the heavy and light variable regions of an antibody connected by a peptide linker and will fold into a structure similar to that of the two peptide antibody. The single-chain binding molecule displays several advantages over conventional antibodies, including, smaller size, greater stability and are more easily modified.

35 [0074] Ku et al. (Proc. Natl. Acad. Sci. U.S.A. 92(14):6552-6556 (1995)) discloses an alternative to antibodies based on cytochrome b<sub>562</sub>. Ku *et al.* (1995) generated a library in which two of the loops of cytochrome b<sub>562</sub> were randomized and selected for binding against bovine serum albumin. The individual mutants were found to bind selectively with BSA similarly with anti-BSA antibodies.

40 [0075] Lipovsek et al. (U.S. Patents 6,818,418 and 7,115,396) discloses an antibody mimic featuring a fibronectin or fibronectin-like protein scaffold and at least one variable loop. Known as Adnectins, these fibronectin-based antibody mimics exhibit many of the same characteristics of natural or engineered antibodies, including high affinity and specificity for any targeted ligand. Any technique for evolving new or improved binding proteins may be used with these antibody mimics.

45 [0076] The structure of these fibronectin-based antibody mimics is similar to the structure of the variable region of the IgG heavy chain. Therefore, these mimics display antigen binding properties similar in nature and affinity to those of native antibodies. Further, these fibronectin-based antibody mimics exhibit certain benefits over antibodies and antibody fragments. For example, these antibody mimics do not rely on disulfide bonds for native fold stability, and are, therefore, stable under conditions which would normally break down antibodies. In addition, since the structure of these fibronectin-based antibody mimics is similar to that of the IgG heavy chain, the process for loop randomization and shuffling may be employed *in vitro* that is similar to the process of affinity maturation of antibodies *in vivo*.

50 [0077] Beste et al. (Proc. Natl. Acad. Sci. U.S.A. 96(5):1898-1903 (1999)) discloses an antibody mimic based on a lipocalin scaffold (ANTICALIN®). Lipocalins are composed of a  $\beta$ -barrel with four hypervariable loops at the terminus of the protein. Beste (1999), subjected the loops to random mutagenesis and selected for binding with, for example, fluorescein. Three variants exhibited specific binding with fluorescein, with one variant showing binding similar to that of an anti-fluorescein antibody. Further analysis revealed that all of the randomized positions are variable, indicating that ANTICALIN® would be suitable to be used as an alternative to antibodies.

55 [0078] ANTICALINS® are small, single chain peptides, typically between 160 and 180 residues, which provides several

advantages over antibodies, including decreased cost of production, increased stability in storage and decreased immunological reaction.

**[0079]** Hamilton et al. (U.S. Patent 5,770,380) discloses a synthetic antibody mimic using the rigid, non-peptide organic scaffold of calixarene, attached with multiple variable peptide loops used as binding sites. The peptide loops all project from the same side geometrically from the calixarene, with respect to each other. Because of this geometric confirmation, all of the loops are available for binding, increasing the binding affinity to a ligand. However, in comparison to other antibody mimics, the calixarene-based antibody mimic does not consist exclusively of a peptide, and therefore it is less vulnerable to attack by protease enzymes. Neither does the scaffold consist purely of a peptide, DNA or RNA, meaning this antibody mimic is relatively stable in extreme environmental conditions and has a long life span. Further, since the calixarene-based antibody mimic is relatively small, it is less likely to produce an immunogenic response.

**[0080]** Murali et al. (Cell Mol Biol 49(2):209-216 (2003)) discusses a methodology for reducing antibodies into smaller peptidomimetics, which they term "antibody like binding peptidomimetics" (ABiP) which may also be useful as an alternative to antibodies.

**[0081]** In addition to non-immunoglobulin protein frameworks, antibody properties have also been mimicked in compounds comprising RNA molecules and unnatural oligomers (e.g., protease inhibitors, benzodiazepines, purine derivatives and beta-turn mimics). Alternatively, known binding interactions between, for example, streptavidin and biotin, can be used to bind the gliadin fusion protein to the solid support.

**[0082]** Additional methods for linking the gliadin fusion protein to the solid support include the use of homobifunctional and heterobifunctional linkers. Zero-length cross linking reagents induce the direct conjugation of two ligands without the introduction of any extrinsic material. Agents that catalyze the formation of disulfide bonds belong in this category. Another example is reagents that induce the condensation of carboxy and primary amino groups to form an amide bond, such as carbodiimides, ethylchloroformate, Woodward's reagent K1 and carbonyldiimidazole. Homobifunctional reagents carry two identical functional groups, whereas heterobifunctional reagents contain two dissimilar functional groups. A vast majority of the heterobifunctional cross-linking agents contains a primary amine-reactive group and a thiol-reactive group. A novel heterobifunctional linker for formyl to thiol coupling was disclosed by Heindel, N. D. et al., Bioconjugate Chem. 2, 427-430 (1991). The covalent cross-linking agents may preferably be selected from reagents capable of forming disulfide (-S-S-), glycol (-CH(OH)-CH(OH)-), azo (-N=N-), sulfone (-S(=O)<sub>2</sub>-), or ester (-C(=O)-O-) bridges.

**[0083]** Carboxylic acid groups residing on the surface of paramagnetic latex beads, internally dyed with Luminex dyes, can be converted to N-hydroxysuccinimide esters through the action of N-cyclohexyl-N'-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (CMC) and N-hydroxysuccinimide (NHS). After magnetic separation and washing, a mixture of the gliadin fusion protein and tTG is added in a detergent and buffered saline containing 10mM CaCl<sub>2</sub> at pH 7.4. The suspension is incubated for 1 hour with shaking at room temperature. After washing, the beads are blocked to reduce non-specific binding and then stored in particle diluent.

### III. Method for Diagnosing a Subject with Celiac Disease

**[0084]** In some embodiments, the present invention provides a method for determining whether a subject has celiac disease. The method includes contacting a sample of bodily fluid from the subject with an antigen of the present invention, including a recombinant deamidated gliadin including a hexamer having the sequence of SEQ ID NO:3. The method also includes detecting any antibody that has become specifically bound to the antigen, thus indicating the presence of celiac disease in the subject.

**[0085]** The sample of the present invention can be any bodily fluid. The sample can be aqueous humor, bile, blood and blood plasma, breast milk, interstitial fluid, lymph, mucus, pleural fluid, pus, saliva, serum, sweat, tears, urine, cerebrospinal fluid, synovial fluid or intracellular fluid. In some embodiments, the sample is a blood sample.

**[0086]** The subject of the present invention can be any mammal. The subject can be primates (e.g., humans), cows, sheep, goats, horses, dogs, cats, rabbits, rats and mice. The subject may be a human.

**[0087]** The presence of the antibody bound to the solid support immobilized gliadin fusion protein or tTG-gliadin fusion protein complex can be detected by any means known in the art. In some embodiments, the detecting step can be performed using an assay such as ELISA, a RIA or an immunofluorescence assay. Alternatively, the detecting step can be performed using an enzymatic method. Immunoassays which can be used in the detecting step include, for example, competitive and non-competitive assay systems such as Western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays and protein A immunoassays. See, e.g., Harlow and Lane, Using Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1999).

**[0088]** The antibody specific for the antigen can be any suitable antibody. The antibody can be IgA, IgD, IgE, IgG or IgM. For example, the antibody can be IgG or IgA. One of skill in the art will appreciate that other antibodies are useful in the present invention.

#### IV. Kits

[0089] In some embodiments, the present invention provides a kit including an antigen as described above, wherein the recombinant deamidated gliadin includes a hexamer that is substantially identical to the sequence of SEQ ID NO:3 or having the sequence of SEQ ID NO:3, a detection reagent, and optionally at least one of buffers, salts, stabilizers and instructions.

[0090] Buffers, salts and stabilizers useful in the present invention include those known to one of skill, and can be found in Gennaro, Ed., Remington's Pharmaceutical Sciences, 18th Edition, Mack Publishing Co. (Easton, Pa.) 1990.

#### V. Examples

##### Example 1. Purification of D2-Hexamer with His Tag

[0091] The D2-hexamer with His tag can be purified using either native or denaturing conditions. In this example, the D2-hexamer was purified in the denaturing condition with 8M urea. A "classic" D2 hexamer with His tag was made in which the protein had the sequence of SEQ ID NO:7. Additionally, a "lysine-containing" D2 hexamer with His tag was made in which the protein had the sequence of SEQ ID NO:8.

[0092] **Purification of the classic recombinant hexamer.** The lysed cells from the 6 liter *E. coli* culture over-expressing the hexamer were suspended in the Equilibration Buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 8M Urea, 500 mM NaCl, 10 mM Imidazole, pH 8.0) at about 5 ml/g wet weight. After stirring for 30 min at room temperature, the cellular debris were removed by centrifugation. The about 200 ml supernatant was added to 25 ml of Ni-NTA resin pre-washed with the Equilibration Buffer, and mixed for 60 min at room temperature. The hexamer proteins bound to the resin were separated from the unbound lysate before being washed four times with 100 ml Washing Buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 8M urea, 500 mM NaCl, 20 mM imidazole, 0.5% Triton X100, pH 8.0). After the washing buffer was removed from the resin, the bound hexamer proteins were eluted with four volumes of 20 ml Elution Buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 8M urea, 200 mM NaCl, 250 mM imidazole, pH 7.5). The eluted protein fractions were pooled, concentrated, and dialysed against 10 mM MOPS, 150 mM NaCl (pH7.4). Any precipitations observed were removed by centrifugation at 15k x g. The affinity purified proteins can be further purified with a size-exclusion column with 10 mM MOPS, 150 mM NaCl (pH7.4), monitored with UV at 230 nm. The fractions containing the first main peak were pooled and concentrated.

[0093] The purification method of the lysine-containing D2-hexamer recombinant protein was identical to that of the classic recombinant hexamer.

[0094] **Characterization of the purified classic or lysine-containing hexamer proteins.** The affinity purified protein was analyzed by SDS-PAGE gel electrophoresis (Figure 1). The D2-hexamers that were further purified on the size-exclusion column as described above were analyzed by SDS-PAGE (Figure 1). Unexpectedly, both hexamers (classic and the lysine-containing) showed a major band around 45 kd, corresponding to the size of a trimer of hexamer proteins. This aggregation of the hexamers is so strong that it is not dissociated under the denaturing conditions used in SDS-PAGE. Additionally, both hexamer proteins migrated at the position of about 45 kd in a size-exclusion chromatogram. Without being bound to a particular theory, the surprising tendency of the hexamers to aggregate to form a trimer of hexamers may contribute to the improved immunoreactivity of the D2 hexamer.

##### Example 2. Preparation of Recombinant Deamidated Gliadin Antigen

[0095] This example provides a protocol that was used for the preparation of the "classic" His-tagged recombinant deamidated gliadin protein (SEQ ID NO:7).

##### Immobilization of the Recombinant Deamidated Gliadin Peptide (DGP) Antigen on Magnetic Beads

[0096] 10 mg of carboxyl modified magnetic beads are placed in a microfuge tube. 1000  $\mu$ l of 50 mM 2-(N-morpholino) ethanesulfonic acid (MES) pH 6.1 in 70 % ethanol (EtOH) is added to the tube. The tube is vortexed and beads are magnetically separated. The supernatant is pipetted off and discarded. This wash process is repeated one more time.

[0097] 500  $\mu$ l of 120 mM N-hydroxysuccinimide (NHS) in 50 mM MES, pH 6.1 in 70 % EtOH is added into the tube with beads and mixed. 500  $\mu$ l of N-Cyclohexyl-N'-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (CMC) in 50mM MES, pH 6.1 in 70 % EtOH is added into the same tube with beads and mixed. The tube is incubated at room temperature for 30 min while mixing continuously.

[0098] The beads are separated from the supernatant and 1000  $\mu$ l of 5mM MES pH 6.1 in 10 % EtOH is added. The beads are mixed, magnetically separated and the supernatant pipetted off and discarded. This wash process is repeated one more time.

[0099] The washed beads are suspended by adding 250  $\mu$ l of 5 mM MES pH 6.1 and mixed. The recombinant DGP

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antigen (prepared as detailed earlier) is mixed in the bead coupling buffer (buffered saline containing detergents) to obtain a coating concentration of 5 µg/mg that is added to the beads. This mixture is incubated at room temperature for 60 min with continuous mixing.

**[0100]** 1000 µl of post coating wash buffer (buffered saline containing detergents, calcium chloride and preservatives) is added to the tube and mixed. The beads are magnetically separated and the supernatant pipetted off and discarded. This wash process is repeated 3 more times.

### Bead blocking

**[0101]** 1000 µl of blocking buffer (buffered saline containing detergents, calcium chloride, preservatives and blockers) added to the tube. The tube is incubated at 2-8 °C with mixing. The beads are magnetically separated and the supernatant pipetted off and discarded at the end of incubation.

**[0102]** The beads are washed with Particle diluent (buffered saline containing detergents, calcium chloride, preservatives and blockers) by adding 1000 µl of Particle diluent to the tube. The tube is mixed and the beads are magnetically separated and the supernatant pipetted off and discarded. This wash process is repeated 3 more times.

**[0103]** Add 1000 µl of Particle diluent (100 µl/mg particles) into the tube and store at 2-8 °C in this buffer.

### Example 3. Detection of Celiac Disease Using the Recombinant DGP Antigen

**[0104]** This example provides a method that was used for the detection of celiac disease using the classic His-tagged recombinant deamidated gliadin protein (SEQ ID NO:7) as an antigen.

### Summary of the Celiac IgA and IgG immunoassay protocol:

**[0105]** The instrument, BioPlex 2200 (manufactured by Bio-Rad Laboratories) aspirates 5 µl of sample from sample tube and dispenses it into a reaction vessel (RV) chased by 45 µl of Wash buffer (phosphate buffered saline containing detergent and preservatives).

**[0106]** 100 µl of Sample diluent (buffered saline containing detergent, preservatives and blockers) is added to the RV followed by 150 µl of Wash buffer.

**[0107]** The RV is incubated for 130 s at 37 °C.

**[0108]** 100 µl of Particle reagent (a solution of recombinant deamidated gliadin coated beads in particle diluent) is added to the RV. The final sample dilution is 1/80.

**[0109]** The mixture is incubated for 1180 s at 37 °C with intermittent mixing.

**[0110]** The beads are washed 3 times with 600 µl, then 300 µl, then 600 µl of Wash buffer with magnetic separation after each wash.

**[0111]** 50 µl of Conjugate Reagent (a mixture of anti-human IgA/IgG-phycoerythrin in conjugate diluent (buffered saline containing detergent, preservatives and blockers)) is added to the RV.

**[0112]** The mixture is incubated for 600 seconds at 37 °C with intermittent mixing.

**[0113]** The beads are washed 3 times with 600 µl, then 300 µl, then 600 µl of Wash buffer with magnetic separation after each wash.

**[0114]** 50 µl of Wash buffer is added to the RV to re-suspend the beads.

**[0115]** The bead suspension is aspirated into the Luminex Detector module (LDM) and the median fluorescence of particles in each of the specified bead region is measured. Figure 2 shows the coating titration of the DGP classic hexamer.

### Sensitivity in Celiac Testing

**[0116]** Table 1 shows the amount of signal (relative fluorescence intensity, RFI) detected for normal and Celiac positive samples at different concentrations of DGP hexamer.

**Table 1. RFI and Coating Concentration of DGP for "classic" DGP**

Coating Concentration of DGP, µg/mg	RFI of Normal healthy sample		RFI of Celiac positive sample at cutoff	
	IgA	IgG	IgA	IgG
0.0	21	14	20	15
2.0	46	71	750	1152

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(continued)

Coating Concentration of DGP, µg/mg	RFI of Normal healthy sample		RFI of Celiac positive sample at cutoff	
	IgA	IgG	IgA	IgG
5.0	47	76	808	1235
10.0	49	81	831	1262
25.0	51	81	844	1273
40.0	50	80	842	1275

Analysis of Celiac Samples

[0117] The following concordance study was comprised of 62 Celiac samples. Table 2 shows the results of the comparison of DGP hexamer and the predicate method.

**Table 2. Concordance study of 62 Celiac samples using "classic" DGP**

Analyte	Agreement with Predicate Method (INOVA)		
	Positive agreement	Negative agreement	Total agreement
IgA	97 %	91 %	95 %
IgG	100 %	94 %	97 %

[0118] Relative fluorescence intensity (RFI) was measured for normal and Celiac patient samples using a DGP hexamer (Table 3). Patient immunoreactivity was assessed by antibody index (AI) in which positive reactivity is >1.0.

**Table 3. RFI and AI data for Normal and Celiac Patient samples using "classic" DGP**

IgA		
Sample	RFI	AI
2320644	170	0.2
2324881	49	0.0
GA61882J	956.0	2.1
GA61882R	903.0	1.5
GA64089B	809.0	1.6
GA64089G	1111.0	2.3
GA64089O	1753.5	3.6
A-9355	42.0	0.0
15902.0	2030	4.5
16313	14928.0	20.1
13424	5724.0	8.1
13425	5816.0	8.2
IgG		
Sample	RFI	AI
2320644	37.0	0
2324881	75.0	0
GG61791B	1287.0	1.2

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(continued)

<b>IgG</b>		
<b>Sample</b>	<b>RFI</b>	<b>AI</b>
GG61791J	1319.0	1.3
GG66322P	204.0	0.2
GG66322R	3764.0	3.7
GG66322T	4847.0	4.2
A-9355	105.0	0.1
15902	5360.0	4.5
16313	10454.0	8.5
13424	4436.0	3.3
13425	2171.5	1.9

**Example 4. Detection of Celiac disease using the recombinant DGP antigen with an additional lysine**

**[0119]** In this example, the "lysine-containing" D2 hexamer protein (a His-tagged Recombinant Deamidated Gliadin Peptide with a lysine substituted for a glutamic acid residue at position 14 near the N-terminal region) (SEQ ID NO:8) was tested for sensitivity to Celiac disease.

**[0120]** The immobilization of this antigen on magnetic beads was done in the same manner as described in Example 2. Figure 3 shows the coating titration of the lysine-containing DGP hexamer.

**[0121]** The detection of Celiac disease using this antigen by immunoassay was performed in the same manner as described for the "classic" hexamer protein in Example 3.

Sensitivity in Celiac Testing

**[0122]** Table 4 shows the RFI for normal and Celiac positive samples at different concentrations of DGP hexamer.

**Table 4. RFI and Coating Concentration of DGP for "lysine-containing" DGP**

<b>Coating Concentration of DGP, <math>\mu</math>g/mg</b>	<b>RFI of Normal healthy sample</b>		<b>RFI of a Celiac positive sample at cutoff</b>	
	<b>IgA</b>	<b>IgG</b>	<b>IgA</b>	<b>IgG</b>
0.0	21	22	25	27
0.5	45	60	623	1003
2.0	49	61	762	1170
5.0	61	89	814	1279
15.0	65	98	850	1336
45.0	64	95	875	1355

Analysis of Celiac Samples

**[0123]** Table 5 shows the relative fluorescence intensity (RFI) for normal and Celiac patient samples as measured by a DGP hexamer. Patient immunoreactivity was assessed by antibody index (AI) in which positive reactivity is >1.0.

**Table 5. RFI and AI data for Normal and Celiac Patient samples using "lysine-containing" DGP**

IgA		
Sample	RFI	AI
2320644	165	0.2
2324881	55	0
GA61882J	942.0	2.1
GA61882R	814.0	1.5
GA64089B	749.5	1.6
GA64089G	1042.0	2.2
GA64089O	1647.0	3.5
A-9355	45.0	0
15902.0	1920	4.5
16313	15549.0	23
13424	5407.0	8.2
13425	5587.5	8.4
IgG		
Sample	RFI	AI
2320644	47.0	0
2324881	70.0	0
GG61791B	1216.5	1.2
GG61791J	1120.0	1.1
GG66322P	194.0	0.2
GG66322R	3476.0	3.6
GG66322T	4607.0	4.2
A-9355	153.5	0.1
15902	5541.0	4.6
16313	10723	8.7
13424	3993.0	3.2
13425	1990.0	1.8

**Example 5. Comparison Studies of DGP Trimer v. DGP Hexamer**

**[0124]** The following concordance study consisting of 62 Celiac samples compared the predictive value of the DGP hexamer to a previously described gliadin antigen, a D2 trimer recombinant fusion protein (described previously in US 2009/0311727). The DGP hexamer showed improved positive agreement and total agreement versus DGP trimer (Table 6). These results demonstrate the improved sensitivity of the DGP hexamer as compared to the DGP trimer.

**Table 6. Comparison concordance study**

Analyte	Agreement with Predicate Method (INOVA)		
	Positive agreement	Negative agreement	Total agreement
IgG-DGP Trimer	84 %	94 %	89 %
IgG-DGP Hexamer	100 %	94 %	97 %

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**[0125]** The DGP hexamer also showed surprisingly improved specificity as compared to the DGP trimer, which yielded many false positive results. This problem was eliminated by using the DGP hexamer. Table 7 below shows data from a screen of 407 normal samples. DGP trimer gave 25 % false positives whereas DGP hexamer gave only 0.5 % false positives. The specificity for false positive detection is  $\leq 1$  %.

**Table 7. Screen of normal samples**

	<b>DGP Trimer</b>	<b>DGP Hexamer</b>
Total Samples	407	407
False Positives	102	2

**[0126]** Figures 4a and 4b show the improved performance of the DGP hexamer as compared to the DGP trimer. In these studies, the bead coating concentration of the DGP hexamer was 5 times less than that of the DGP trimer, while the cutoff RFI signal was the same. The recombinant DGP hexamer had improved sensitivity as compared to the recombinant DGP trimer in both IgA (Figure 4a) and IgG (Figure 4b) assays.

SEQUENCE LISTING

**[0127]**

<110> Bio-Rad Laboratories, Inc.

<120> Recombinant Deaminated Gliadin Antigen

<130> 71401-858799

<150> EP 12855209.8

<151> 2012-12-03

<150> US 61/567,060

<151> 2011-12-05

<160> 15

<170> FastSEQ for Windows Version 4.0

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<212> PRT

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<211> 5

<212> PRT

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20 25 30  
Gln Glu Arg Pro Phe Gly Gly Gly Ser Gln Pro Glu Gln Pro Gln  
35 40 45  
Gln Ser Phe Pro Glu Gln Glu Arg Pro Phe Gly Gly Gly Ser Gln  
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25

Pro Glu Gln Pro Gln Gln Ser Phe Pro Glu Gln Glu Arg Pro Phe Gly  
65 70 75 80  
Gly Gly Gly Ser Gln Pro Glu Gln Pro Gln Gln Ser Phe Pro Glu Gln  
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 30 Gly Gly Gly Ser Gln Pro Glu Gln Pro Gln Gln Ser Phe Pro Glu Gln  
 35 40 45  
 Glu Arg Pro Phe Gly Gly Gly Ser Gln Pro Glu Gln Pro Gln Gln  
 50 55 60  
 Ser Phe Pro Glu Gln Glu Arg Pro Phe Gly Gly Gly Ser Gln Pro  
 65 70 75 80  
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 85 90 95  
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 with lysine substituted at position 14  
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	ggctcgggaat	tccagcccga	acaaccgcaa	caatcattcc	ccgagcaaga	aaggccgttc	240
	ggtggcgggtg	gctcgcagcc	cgaacaaccg	caacaatcat	tccccgagca	agaaaggccg	300
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			20					25					30		
Tyr	Glu	Arg	Asp	Glu	Gly	Asp	Lys	Trp	Arg	Asn	Lys	Lys	Phe	Glu	Leu
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Val	Val	Leu	Tyr	Met	Asp	Pro	Met	Cys	Leu	Asp	Ala	Phe	Pro	Lys	Leu
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Val	Cys	Phe	Lys	Lys	Arg	Ile	Glu	Ala	Ile	Pro	Gln	Ile	Asp	Lys	Tyr
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Tyr	Glu	Arg	Asp	Glu	Gly	Asp	Lys	Trp	Arg	Asn	Lys	Lys	Phe	Glu	Leu
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Gly	Ala	Val	Leu	Asp	Ile	Arg	Tyr	Gly	Val	Ser	Arg	Ile	Ala	Tyr	Ser

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 Lys Asp Phe Glu Thr Leu Lys Val Asp Phe Leu Ser Lys Leu Pro Glu  
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 Gly Asp His Val Thr His Pro Asp Phe Met Leu Tyr Asp Ala Leu Asp  
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 Val Val Leu Tyr Met Asp Pro Met Cys Leu Asp Ala Phe Pro Lys Leu  
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### Claims

1. An antigen for detecting celiac disease comprising a recombinant or synthetic deamidated gliadin protein comprising a hexamer of peptides, wherein each peptide has the sequence of SEQ ID NO:1.
2. The antigen of claim 1, wherein the hexamer comprises a spacer separating each peptide, preferably the spacer has the sequence of SEQ ID NO:2.
3. The antigen of claim 1 or claim 2, wherein the recombinant or synthetic deamidated gliadin protein has at least 95% identity to SEQ ID NO:3 or has the sequence of SEQ ID NO:3.
4. The antigen of any one of claims 1 to 3, wherein the recombinant or synthetic deamidated gliadin is covalently linked to a tag to form a gliadin fusion protein.
5. The antigen of claim 4, wherein the tag is selected from a Glutathione S-transferase (GST) and a His-tag, preferably
  - (i) the tag is GST; or
  - (ii) the His-tag has a sequence selected from the group consisting of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6.
6. The antigen of claim 5, wherein the recombinant deamidated gliadin has a sequence selected from the group consisting of SEQ ID NO:7 and SEQ ID NO:8.
7. The antigen of claim 4, wherein the antigen further comprises tissue Transglutaminase (tTG) to form a tTG-gliadin fusion protein complex, preferably the tTG and the gliadin fusion protein are covalently linked by a cross-linker.
8. The antigen of claim 7, wherein the cross-linker is a member selected from a heterobifunctional crosslinker and a homobifunctional crosslinker, preferably the cross-linker is a homobifunctional crosslinker, more preferably the cross-linker is selected from bis(sulfosuccinimidyl)suberate (BS3), ethylene glycol bis[succinimidylsuccinate] (EGS), ethylene glycol bis[sulfosuccinimidylsuccinate] (sulfo-EGS), bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone (BSO-COES), dithiobis(succinimidyl)propionate (DSP), 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP), disuccinimidyl suberate (DSS), disuccinimidyl glutarate (DSG), methyl N-succinimidyl adipate (MSA), disuccinimidyl tartarate

(DST), 1,5-difluoro-2,4-dinitrobenzene (DFDNB), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC or EDAC), sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), N-hydroxysulfosuccinimide (sulfo-NHS), hydroxylamine and Sulfo-LC-SPDP (N-succinimidyl 3-(2-pyridyldithio)-propionate) and sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate (sulfo-LC-SPDP), most preferably the cross-linker is bis(sulfosuccinimidyl)suberate (BS3).

9. An isolated nucleic acid encoding the antigen of any one of claims 1 to 8 or comprising SEQ ID NO:9.

10. An expression vector comprising the isolated nucleic acid of claim 9.

11. A host cell comprising the expression vector of claim 10.

12. A kit comprising:

the antigen of any one of claims 1 to 8;  
a detection reagent; and  
optionally at least one member selected from the group consisting of buffers, salts, stabilizers, and instructions.

13. Use of the antigen of any one of claims 1 to 8 for determining whether a subject has celiac disease.

14. A method for diagnosing celiac disease in a subject the method comprising:

(a) contacting a sample of bodily fluid from the subject with the antigen of any one of claims 1 to 8; and  
(b) detecting any antibody that has become specifically bound to the antigen, as an indication of the presence of celiac disease in the subject.

15. The method of claim 14, wherein

(i) the sample is a blood sample;  
(ii) the detecting step is performed using an assay selected from the group consisting of ELISA, RIA, and an immunofluorescence assay; and/or  
(iii) the antibody specific for the antigen is selected from the group consisting of IgG and IgA.

## Patentansprüche

1. Antigen zum Nachweis von Zöliakie, umfassend ein rekombinantes oder synthetisches deamidiertes Gliadinprotein, das ein Hexamer von Peptiden umfasst, wobei jedes Peptid die Sequenz von SEQ ID Nr. 1 aufweist.

2. Antigen gemäß Anspruch 1, wobei das Hexamer einen Spacer umfasst, der die Peptide jeweils voneinander trennt, wobei der Spacer vorzugsweise die Sequenz von SEQ ID Nr. 2 aufweist.

3. Antigen gemäß Anspruch 1 oder 2, wobei das rekombinante oder synthetische deamidierte Gliadinprotein wenigstens 95% Identität mit SEQ ID Nr. 3 aufweist oder die Sequenz von SEQ ID Nr. 3 aufweist.

4. Antigen gemäß einem der Ansprüche 1 bis 3, wobei das rekombinante oder synthetische deamidierte Gliadinprotein kovalent mit einem Tag verknüpft ist, wobei ein Gliadin-Fusionsprotein entsteht.

5. Antigen gemäß Anspruch 4, wobei das Tag aus einer Glutathion-S-Transferase (GST) und einem His-Tag ausgewählt ist, wobei vorzugsweise

(i) es sich bei dem Tag um GST handelt; oder  
(ii) das His-Tag eine Sequenz aufweist, die aus der Gruppe ausgewählt ist, die aus SEQ ID Nr. 4, SEQ ID Nr. 5 und SEQ ID Nr. 6 besteht.

6. Antigen gemäß Anspruch 5, wobei das rekombinante deamidierte Gliadin eine Sequenz aufweist, die aus der Gruppe ausgewählt ist, die aus SEQ ID Nr. 7 und SEQ ID Nr. 8 besteht.

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7. Antigen gemäß Anspruch 4, wobei das Antigen weiterhin Gewebe-Transglutaminase (tTG) umfasst, wobei ein tTG-Gliadin-Fusionsprotein-Komplex entsteht, wobei das tTG und das Gliadin-Fusionsprotein vorzugsweise durch einen Vernetzer kovalent miteinander verknüpft sind.
- 5 8. Antigen gemäß Anspruch 7, wobei der Vernetzer ein Vertreter ist, der aus einem heterobifunktionellen Vernetzer und einem homobifunktionellen Vernetzer ausgewählt ist, wobei der Vernetzer vorzugsweise ein homobifunktioneller Vernetzer ist, wobei der Vernetzer besonders bevorzugt aus Bis(sulfosuccinimidyl)suberat (BS3), Ethylenglycol-bis[succinimidylsuccinat] (EGS), Ethylenglycolbis[sulfosuccinimidylsuccinat] (Sulfo-EGS), Bis[2-(succinimidooxy-carbonyloxy)ethyl]sulfon (BSOCOES), Dithiobis(succinimidyl)propionat (DSP), 3,3'-Dithiobis(sulfosuccinimidylpropionat) (DTSSP), Disuccinimidylsuberat (DSS), Disuccinimidylglutarat (DSG), Methyl-N-succinimidyladipat (MSA), Disuccinimidyltartrat (DST), 1,5-Difluor-2,4-dinitrobenzol (DFDNB), 1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimid-Hydrochlorid (EDC oder EDAC), Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexan-1-carboxylat (Sulfo-SMCC), N-Hydroxysulfosuccinimid (Sulfo-NHS), Hydroxylamin und Sulfo-LC-SPDP (N-Succinimidyl-3-(2-pyridyldithio)propionat) und Sulfosuccinimidyl-6-(3'-[2-pyridyldithio]propionamido)hexanoat (Sulfo-LC-SPDP) ausgewählt ist, wobei  
10 es sich bei dem Vernetzer am meisten bevorzugt um Bis(sulfosuccinimidyl)suberat (BS3) handelt.
- 15 9. Isolierte Nucleinsäure, die das Antigen gemäß einem der Ansprüche 1 bis 8 codiert oder SEQ ID Nr. 9 umfasst.
10. Expressionsvektor, der die isolierte Nucleinsäure gemäß Anspruch 9 umfasst.
- 20 11. Wirtszelle, die den Expressionsvektor gemäß Anspruch 10 umfasst.
12. Kit, umfassend:
- 25 das Antigen gemäß einem der Ansprüche 1 bis 8;  
ein Nachweisreagens; und  
gegebenenfalls wenigstens einen Vertreter, der aus der Gruppe ausgewählt ist, die aus Puffern, Salzen, Stabilisatoren und Anweisungen besteht.
- 30 13. Verwendung des Antigens gemäß einem der Ansprüche 1 bis 8 zur Bestimmung, ob ein Proband an Zöliakie leidet.
14. Verfahren zum Diagnostizieren von Zöliakie bei einem Probanden, wobei das Verfahren umfasst:
- 35 (a) das In-Kontakt-Bringen einer Probe einer Körperflüssigkeit von dem Probanden mit dem Antigen gemäß einem der Ansprüche 1 bis 8; und  
(b) Nachweisen von Antikörper, der spezifisch an das Antigen gebunden wurde, was die Anwesenheit von Zöliakie bei dem Probanden anzeigt.
- 40 15. Verfahren gemäß Anspruch 14, wobei
- (i) die Probe eine Blutprobe ist;  
(ii) der Schritt des Nachweisens mit Hilfe eines Assays durchgeführt wird, das aus der Gruppe ausgewählt ist, die aus ELISA, RIA und einem Immunfluoreszenzassay besteht; und/oder  
(iii) der für das Antigen spezifische Antikörper aus der Gruppe ausgewählt ist, die aus IgG und IgA besteht.
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### Revendications

- 50 1. Antigène pour détecter une maladie coeliaque, comprenant une protéine gliadine désamidée recombinante ou synthétique comportant un hexamère de peptides, dans lequel chaque peptide a la séquence de SEQ ID NO:1.
2. Antigène selon la revendication 1, dans lequel l'hexamère comprend un espaceur séparant chaque peptide, de préférence, l'espaceur a la séquence de SEQ ID NO:2.
- 55 3. Antigène selon la revendication 1 ou la revendication 2, dans lequel la protéine gliadine désamidée recombinante ou synthétique a une identité d'au moins 95% avec SEQ ID NO:3 ou a la séquence de SEQ ID NO:3.
4. Antigène selon l'une quelconque des revendications 1 à 3, dans lequel la gliadine désamidée recombinante ou

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synthétique est liée de manière covalente à une étiquette pour former une protéine de fusion de gliadine.

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5. Antigène selon la revendication 4, dans lequel l'étiquette est choisie parmi une Glutathion-S-transférase (GST) et une étiquette His, de préférence

(i) l'étiquette est une GST ; ou  
(ii) l'étiquette His a une séquence choisie dans le groupe constitué par SEQ ID NO:4, SEQ ID NO:5 et SEQ ID NO:6.

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6. Antigène selon la revendication 5, dans lequel la gliadine désamidée recombinante a une séquence choisie dans le groupe constitué par SEQ ID NO:7 et SEQ ID NO:8.

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7. Antigène selon la revendication 4, ledit antigène comprenant en outre une Transglutaminase tissulaire (tTG) pour former un complexe tTG-protéine de fusion de gliadine, de préférence la tTG et la protéine de fusion de gliadine sont liées de manière covalente par un agent réticulant.

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8. Antigène selon la revendication 7, dans lequel l'agent réticulant est un membre choisi parmi un agent réticulant hétérobifonctionnel et un agent réticulant homobifonctionnel, de préférence, l'agent réticulant est un agent réticulant homobifonctionnel, plus préférablement, l'agent réticulant est choisi parmi le subérate de bis(sulfosuccinimidyle) (BS3), le bis[succinimidylsuccinate] d'éthylène glycol (EGS), le bis[sulfosuccinimidylsuccinate] d'éthylène glycol (sulfo-EGS), la bis[2-(succinimidooxycarbonyloxy)éthyl]sulfone (BSOCOES), le dithiobis(succinimidyl)propionate (DSP), le 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP), le subérate de disuccinimidyle (DSS), le glutarate de disuccinimidyle (DSG), l'adipate de méthyl-N-succinimidyle (MSA), le tartrate de disuccinimidyle (DST), le 1,5-difluoro-2,4-dinitrobenzène (DFDNB), le chlorhydrate de 1-éthyl-3-[3-diméthylaminopropyl]carbodiimide (EDC ou EDAC), le 4-(N-maléimidométhyl)cyclohexane-1-carboxylate de sulfosuccinimidyle (sulfo-SMCC), le N-hydroxysulfosuccinimide (sulfo-NHS), l'hydroxylamine et le Sulfo-LC-SPDP (3-(2-pyridyldithio)propionate de N-succinimidyle) et le 6-(3'-[2-pyridyldithio]propionamido)hexanoate de sulfosuccinimidyle (sulfo-LC-SPDP), le plus préférablement, l'agent réticulant est le subérate de bis(sulfosuccinimidyle) (BS3).

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9. Acide nucléique isolé codant pour l'antigène selon l'une quelconque des revendications 1 à 8 ou comprenant SEQ ID NO:9.

10. Vecteur d'expression comprenant l'acide nucléique isolé selon la revendication 9.

- 35  
11. Cellule hôte comprenant le vecteur d'expression selon la revendication 10.

12. Trousse comprenant :

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l'antigène selon l'une quelconque des revendications 1 à 8 ;  
un réactif de détection ; et  
facultativement au moins un membre choisi dans le groupe constitué par les tampons, les sels, les agents stabilisants, et un mode d'emploi.

- 45  
13. Utilisation de l'antigène selon l'une quelconque des revendications 1 à 8 pour déterminer si un sujet est atteint d'une maladie coeliaque.

14. Procédé pour diagnostiquer une maladie coeliaque chez un sujet, ledit procédé comprenant :

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(a) la mise en contact d'un échantillon de fluide corporel prélevé chez le sujet avec l'antigène selon l'une quelconque des revendications 1 à 8 ; et  
(b) la détection de tout anticorps qui s'est spécifiquement lié à l'antigène, comme indication de la présence d'une maladie coeliaque chez le sujet.

- 55  
15. Procédé selon la revendication 14, dans lequel

(i) l'échantillon est un échantillon de sang ;  
(ii) l'étape de détection est réalisée à l'aide d'un test choisi dans le groupe constitué par un test ELISA, un test RIA et un est par immunofluorescence ; et/ou

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(iii) l'anticorps spécifique de l'antigène est choisi dans le groupe constitué par l'IgG et l'IgA.

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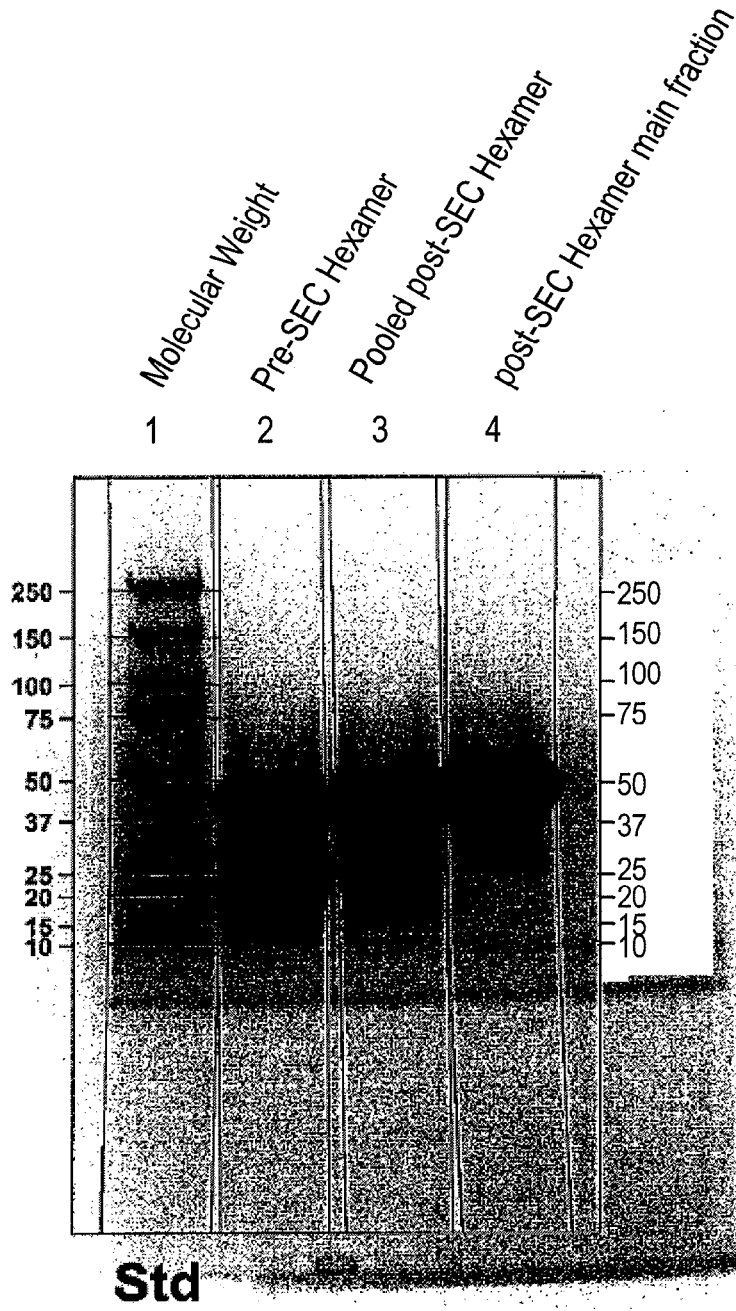
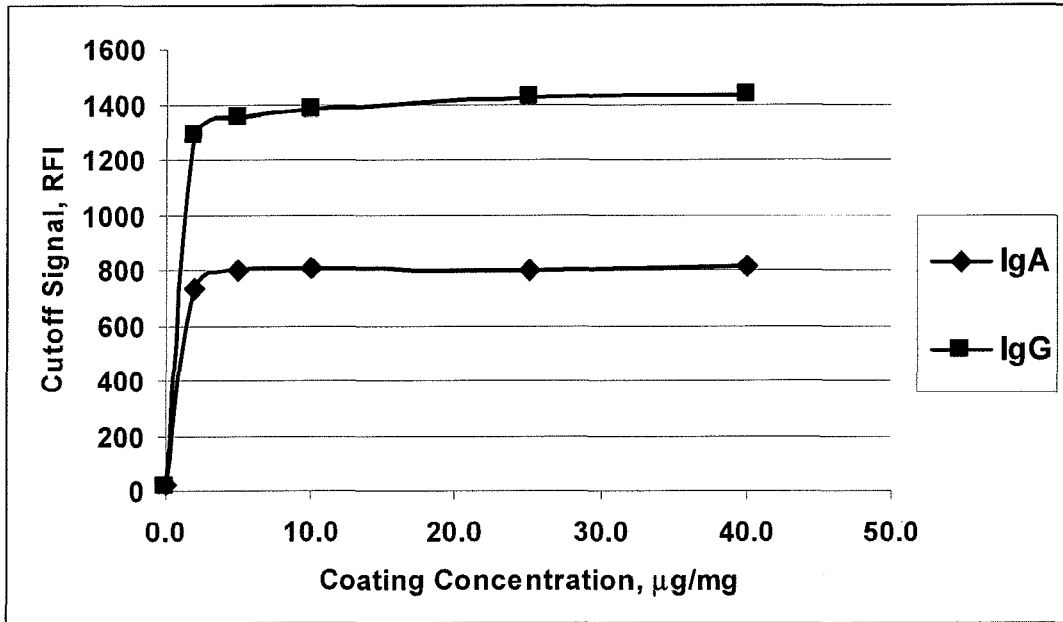


FIG. 1



**FIG. 2**

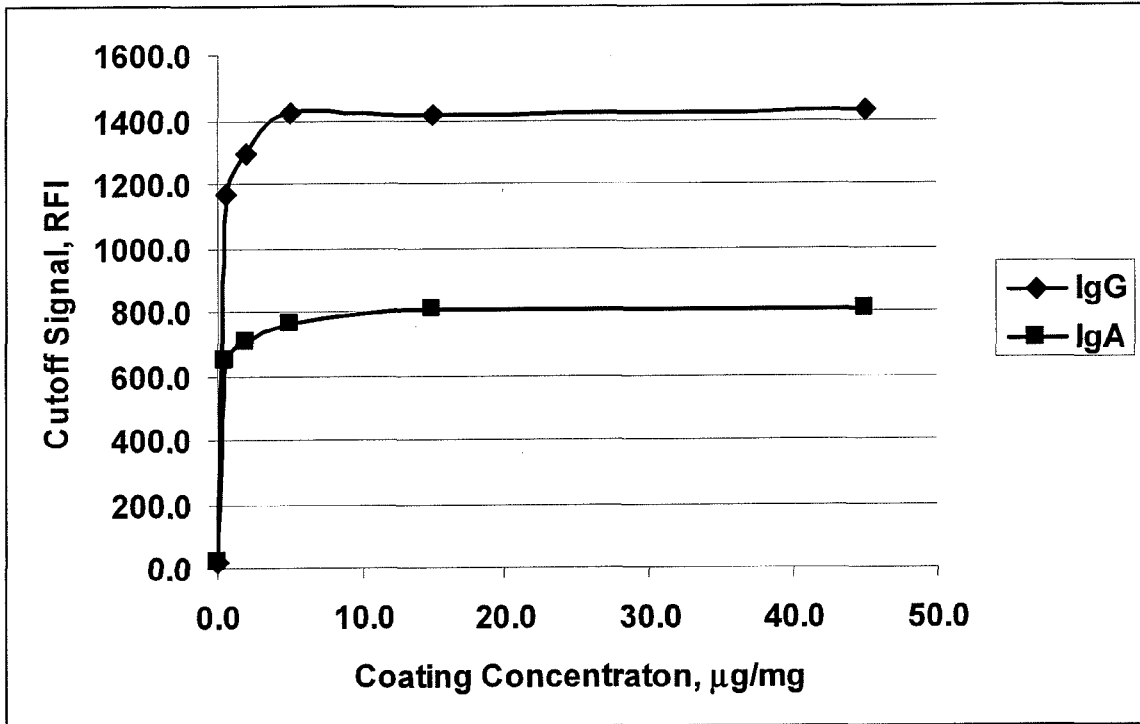
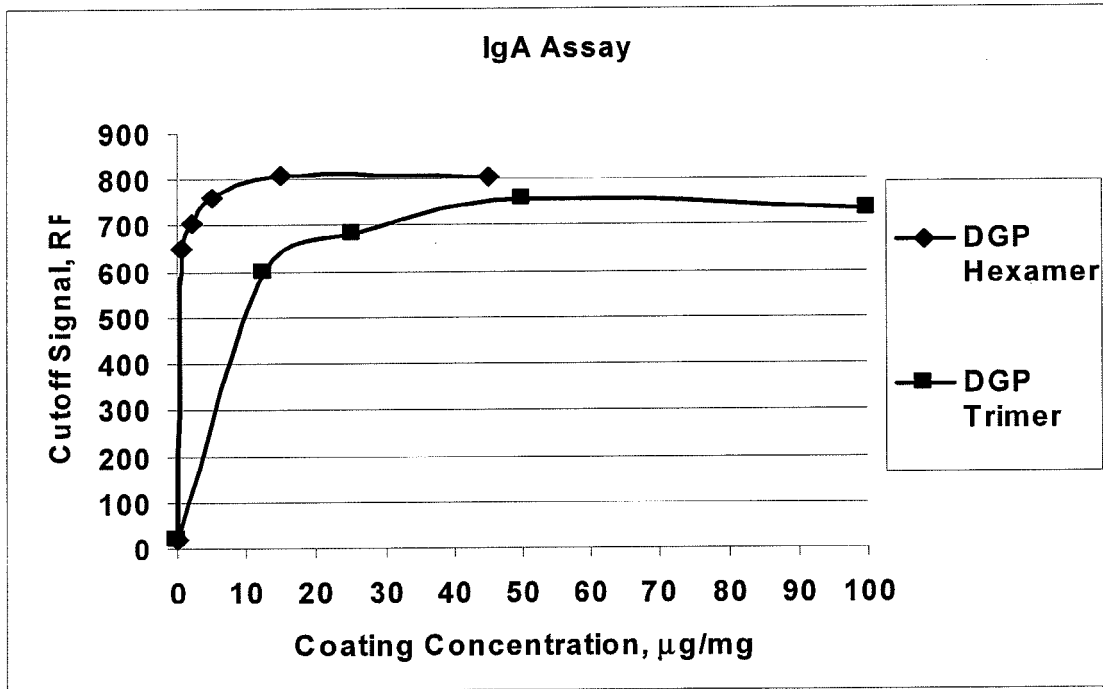
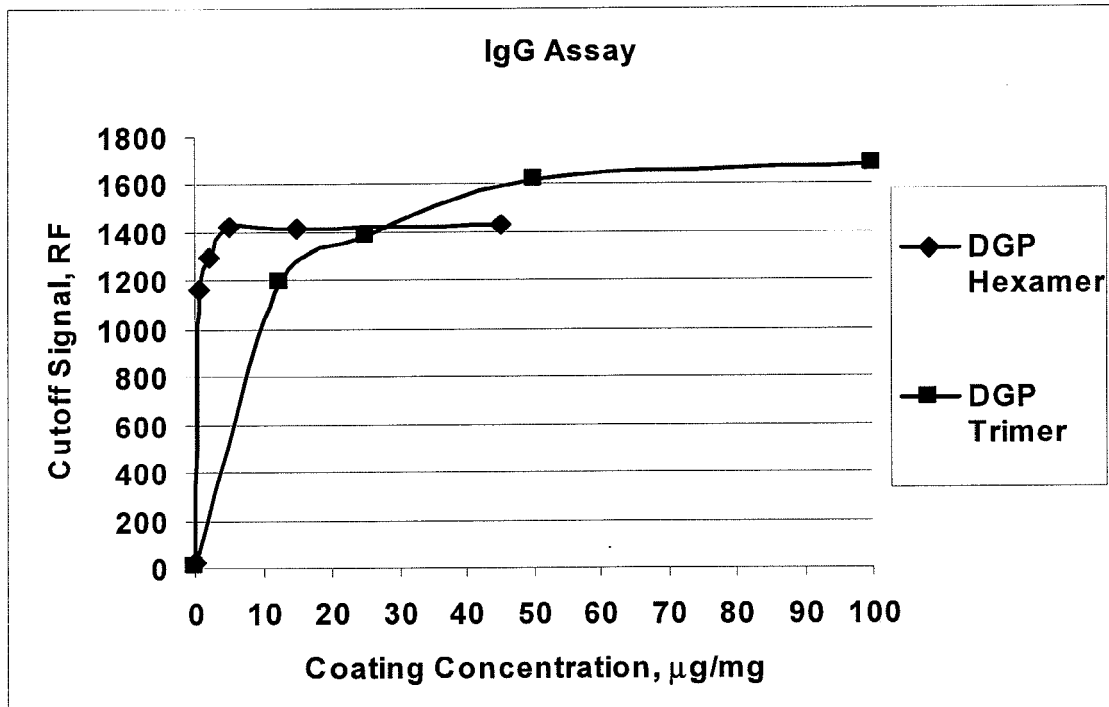


FIG. 3

A



B



**FIG. 4**

## REFERENCES CITED IN THE DESCRIPTION

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专利名称(译)	重组脱酰胺醇麦醇溶蛋白抗原		
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摘要(译)

本发明提供了一种通过使来自受试者的体液样品与固定在固体支持物上的麦醇溶蛋白融合蛋白的六聚体形成的抗原接触来确定受试者是否患有乳糜泻的方法。抗原的麦醇溶蛋白融合蛋白包括与诸如谷胱甘肽-S转移酶 ( GST ) 蛋白的标签连接的重组脱酰胺醇麦醇溶蛋白。通过将麦醇溶蛋白融合蛋白固定在固体支持物上来制备抗原。抗原可以进一步包括与麦醇溶蛋白融合蛋白交联的组织转谷氨酰胺酶 ( tTG )。当存在tTG时，将tTG和重组脱酰胺醇麦醇溶蛋白混合在一起，然后固定化为固相。

