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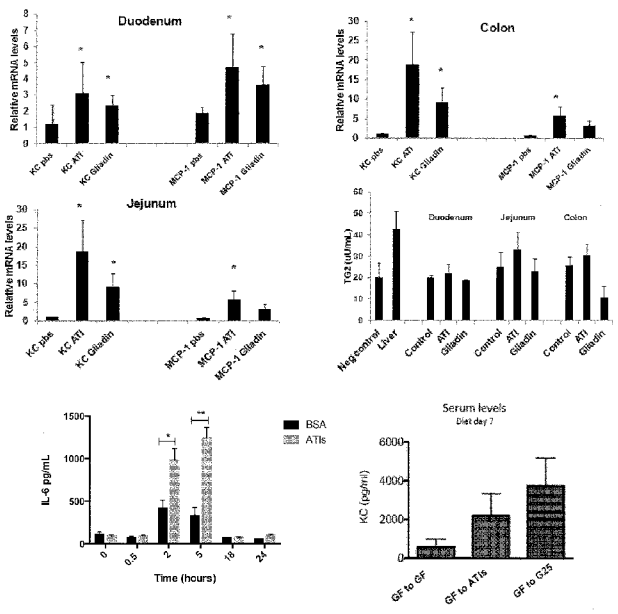


FIG. 1A

(57) Abstract: The present invention features methods of extracting amylase trypsin inhibitors (ATIs) from processed and unprocessed foodstuff, determining bioactivity of ATIs, quantifying the amount of ATIs in a foodstuff, and reducing the content of ATIs in a foodstuff.

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METHODS FOR DETERMINATION OF BIOACTIVITY, QUANTITY, REMOVAL, OR INACTIVATION OF CEREAL AMYLASE TRYPSIN INHIBITORS IN CEREALS, FLOURS, PLANTS, AND COMPLEX FOODS

BACKGROUND

Ingestion of wheat, barley, or rye triggers small intestinal inflammation in patients with celiac disease. Specifically, the storage proteins of these cereals (gluten) elicit an adaptive Th1-mediated immune response in individuals carrying HLA-DQ2 or HLA-DQ8 as a major genetic predisposition. This well-defined role of adaptive immunity contrasts with an ill defined component of innate immunity in celiac disease. We have identified the alpha-amylase/trypsin inhibitors (ATIs) CM3 and 0.19, pest resistance molecules in wheat and related cereals, as strong activators of innate immune responses in monocytes, macrophages, and dendritic cells. ATIs engage the TLR4–MD2–CD14 complex and lead to up-regulation of maturation markers. ATIs then elicit release of proinflammatory cytokines from cells inceliac and non-celiac patients. Mice deficient in TLR4 or TLR4 signaling are protected from intestinal and systemic immune responses upon oral challenge with ATIs. These findings define cereal ATIs as contributors to celiac disease. Moreover, and importantly, ATIs are strong nutritional candidates that exacerbate and thus may fuel inflammation and adverse immune reactions in other intestinal and non-intestinal immune disorders. In this vein, ATIs are the most likely triggers of non-celiac-non-allergy wheat sensitivity, currently a diagnosis of exclusion in the hands of experts, in which patients suffer from intestinal and/or intestinal diseases in tight association with the consumption of gluten containing foods. This condition may affect up to about 10% of most wheat consuming populations worldwide. Therefore, there exists a great need for novel technologies and methods that are able to remove ATI from foods and/or permit the production of foods that maintain the desired properties of gluten containing foods but are low in ATI-content and bioactivity. The development of such techniques is tightly linked to analytical methods to quantify ATI content and bioactivity in foods before and after processing for consumption.

SUMMARY OF THE INVENTION

The present invention features methods of extracting ATIs from processed and unprocessed foodstuff, determining bioactivity of ATIs, quantifying the amount of ATIs in a foodstuff, reducing the content of ATIs in a foodstuff, and supporting the production of a foodstuff with reduced ATI content.

In a first aspect, the invention features a method of reducing the ATI content of a foodstuff, wherein the method includes incubating the foodstuff in an extraction buffer for a duration sufficient to reduce the ATI content of the foodstuff by at least 50% followed by removing the extraction buffer from the foodstuff.

In some embodiments, the extraction buffer is a neutral, near neutral, or alkaline solvent or buffer. In some embodiments, the extract buffer is selected from the group consisting of PBS, Tris-buffer, water, and ammonium or sodium bicarbonate. In some embodiments, the extraction buffer contains salt (e.g., 0.1-1 M NaCl).

In some embodiments, the extract buffer further includes a detergent or detergent mixture. In some embodiments, the detergent or detergent mixture is selected from the group consisting of sodiumdodecyl sulfate, Triton X-100, Tween-20, and sodium deoxycholate.

In some embodiments, the extraction buffer further includes a reducing agent. In some embodiments, the reducing agent is selected from the group consisting of mercapto-ethanol, dithioerythritol (DTT), cysteine, glutathione (GSH) balanced with oxidized glutathione (GSSG), sulfite, and thioredoxin.

In some embodiments, the extraction buffer further includes at least one protease and/or a synthetic enzyme engineered to reduce and/or degrade ATIs. Examples of synthetic enzyme engineered to reduce and/or degrade ATIs are described in detail further herein. In some embodiments, the protease is pepsin, trypsin, chymotrypsin, elastase, thermolysin, bromelain, papain, subtilisin, an alkaline protease, a neutral protease, an acidic protease, a fungal protease, a yeast protease, or a bacterial protease. In some embodiments, the alkaline protease is a serine protease.

In some embodiments, the extraction buffer further includes an alkylating agent. In some embodiments, the alkylating agent is iodoacetamide and the alkylating agent is further quenched by the addition of excess reducing agent.

For food technology, such as bulk preparation of ATI-deenriched flours, etc., the extracted foodstuff (e.g., dough) can be used directly after separation of supernatant, which contains the extracted ATIs. For example, wet doughs are often frozen and shipped to bakeries directly.

In some embodiments, the foodstuff is separated mechanically prior to incubation. In some embodiments, the mechanically separated foodstuff is dried prior to incubation.

In some embodiments, the drying is air-drying or freeze drying. In some embodiments, prior to the drying, the mechanically separated foodstuff is contacted with an aqueous solvent or an organic solvent having a lower boiling point than water. In some embodiments, the organic solvent is selected from the group consisting of acetone and ethanol.

In a second aspect, the invention features a method of extracting amylase trypsin inhibitors (ATIs) from a foodstuff, wherein the method includes A) incubating the foodstuff with an extraction buffer in a mixture; B) centrifuging the mixture to form a pellet and supernatant; C) drying the supernatant; and D) reconstituting the dried supernatant in a reconstitution buffer, wherein extracted ATIs are present in the reconstitution buffer.

In some embodiments, the foodstuff is unprocessed prior to extraction. In some embodiments, the foodstuff is a processed foodstuff. In some embodiments, the food stuff is gluten.

In some embodiments, prior to step A, the foodstuff is ground. In some embodiments, prior to step A, the foodstuff is defatted. In some embodiments, the defatting is performed by the incubation of the foodstuff with methanol/diethylether. In some embodiments, the methanol/diethyl ether is present at a 1:1 ratio. In some embodiments, the extraction buffer is ammonium or sodium bicarbonate, chloroform/methanol, or an acid. In some embodiments, the acid is acetic acid.

In some embodiments, the extraction buffer is at a concentration between 5 mM to 100 mM.

In some embodiments, the drying is freeze-drying or drying under a stream of gas, e.g., air.

In some embodiments, steps A-C are repeated prior to step D. In some embodiments, the extraction buffer in steps A-C is first chloroform/methanol and the extraction buffer is ammonium or

sodium bicarbonate when steps A-C are repeated or wherein the extraction buffer is ammonium or sodium bicarbonate in both extractions.

In some embodiments, prior to step A, the foodstuff is digested with at least one protease and/or a synthetic enzyme engineered to reduce and/or degrade ATIs. In some embodiments, the protease is pepsin, trypsin, chymotrypsin, elastase, thermolysin, bromelain, papain, subtilisin, an alkaline protease, a neutral protease, an acidic protease, a fungal protease, a yeast protease, or a bacterial protease. In some embodiments, the alkaline protease is a serine protease.

In some embodiments, the foodstuff is first digested with pepsin followed by digestion with trypsin and/or chymotrypsin.

In some embodiments, after the foodstuff is digested with a protease, the foodstuff is centrifuged to form a supernatant and the supernatant is incubated with the extraction buffer of step A. In some embodiments, the incubation is performed as a dialysis incubation, step B is omitted, and the supernatant, after dialysis is dried in step C.

In a third aspect, the invention features a method of determining the bioactivity of ATIs in a sample, wherein the method includes incubating ATI-reactive cells with an extract suspected of including ATIs and determining an ATI-response in the ATI-reactive cells, thereby determining the bioactivity of the ATIs in a sample.

In some embodiments, the extract suspected of including ATIs is prepared by the method described in the first and second aspects of the invention.

In some embodiments, the ATI-reactive cells express TLR4, TLR4-MD2, or TLR4-MD2-CD14 signaling complex. In some embodiments, the ATI-reactive cells are selected from the group consisting of monocytes/macrophages, dendritic cells, and cells transfected with a TLR4, TLR4-MD2, or TLR4-MD2-CD14 oligomeric signaling complex.

In some embodiments, the cells are stable cell lines, e.g., THP-1 and U937 cells. In some embodiments, the cells are cells (e.g., HeLa or HEK293 cells) transfected with TLR4-signaling complex.

In some embodiments, the determination of ATI-response includes quantification of one or more cytokines released by the cells. In some embodiments, the one or more cytokines are selected from the group consisting of, e.g., IL-8, IL-15, TNFα, CCL2 (MCP-1), and CCL5 (RANTES).

In some embodiments, the quantification of released chemokines and/or cytokines or related factors is performed by ELISA.

In some embodiments, the cell includes a reporter gene under the control of a TLR4 (ATI)-responsive promoter and the determination of an ATI-response includes detection of expression of the reporter gene. In some embodiments, the ATI-responsive promoter is the IL-8 promoter. In some embodiments, the reporter gene encodes a luminescent or fluorescent protein.

In some embodiments, the determination of ATI-response includes measuring a level of one or more RNAs in the cells associated with an ATI-response. In some embodiments, the one or more RNAs corresponds to those of chemokines, cytokines, Hsp27, Hsp70, and/or Hsp90.

In some embodiments, the determination of ATI-response includes detecting one or more activation markers on or in the cells. In some embodiments, the one or more activation markers is

selected from the group consisting of CD80, CD86, MHC class II, IL-12, IL-23, and interferon gamma (release or intracellular stain).

In some embodiments, the detecting includes flow-cytometry.

In some embodiments, the determination of ATI-response includes detecting an alteration is a signaling pathway associated with an ATI response. In some embodiments, the alteration in a signaling pathway is detected based on the detection of IRF-3, NFkB (p65), or NFkB nuclear translocation.

In some embodiments, the determination of ATI-response includes contacting ovalbumin or gluten specific T cells in the presence of the sample suspected of containing ATIs and measuring the proliferation of the cells in the presence of ATI-reactive antigen presenting cells.

In a fouth aspect, the invention features a method of determining the bioactivity of ATIs in a sample, wherein the method includes A) incubating an immobilized TLR4, TLR4-MD2, or TLR4-MD2-CD14 with an extract suspected of including ATIs, wherein the immobilized TLR4, TLR4-MD2, or TLR4-MD2-CD14 is immobilized to a solid phase and bound to a labeled ATI, B) removing the extract, and C) determining the amount of labeled ATI in the extract or bound to said immobilized TLR4, TLR4-MD2, or TLR4-MD2-CD14 that is immobilized to the solid phase.

In some embodiments, the labeled ATI is labeled with a fluorophore. In some embodiments, the labeled ATI is labeled with an enzyme (e.g., horseradish peroxidase or alkaline phosphatase).

In a fifth aspect, the invention features a method of determining the bioactivity of ATIs in a sample, the method including A) incubating an immobilized TLR4, TLR4-MD2, or TLR4-MD2-CD14 with an extract suspected of including ATIs, B) removing the extract suspected of including ATIs, and C) determining the amount of ATIs that are bound to the immobilized TLR4 using an antibody in an ELISA.

In some embodiments, the antibody recognizes and binds to at least one ATI selected from the group consisting of CM3, CM2, 0.19, 0.28, and 0.53.

In a sixth aspect, the invention features a method of quantifying the amount of ATIs in a foodstuff, wherein the method includes A) incubating the foodstuff in an extraction buffer thereby forming an extract; B) optionally incubating the extract with an alkylating agent; C) optionally separating the ATIs from the extract; and D) quantifying the ATIs.

In some embodiments, the extraction buffer is a neutral, near neutral, or an alkaline solvent or buffer. In some embodiments, the extract buffer is selected from the group consisting of PBS, Tris-buffer, and ammonium or sodium bicarbonate.

In some embodiments, the extract buffer further includes a detergent or detergent mixture. In some embodiments, the detergent or detergent mixture is selected from the group consisting of sodium dodecyl sulfate, Triton X-100, Tween-20, and sodium deoxycholate.

In some embodiments, the extraction buffer further includes a reducing agent. In some embodiments, the reducing agent is selected from the group consisting of mercapto-ethanol, dithioerythritol (DTT), cysteine, glutathione (GSH) balanced with oxidized glutathione (GSSG), sulfite, and thioredoxin.

In some embodiments, the extraction buffer further includes an alkylating agent. In some embodiments, the alkylating agent is iodoacetamide and the alkylating agent is further quenched by the addition of excess reducing agent.

In some embodiments, the method further includes adding at least one protease and/or a synthetic enzyme engineered to reduce and/or degrade ATIs to the extract. Examples of synthetic enzyme engineered to reduce and/or degrade ATIs are described in detail further herein. In some embodiments, the protease is pepsin, trypsin, chymotrypsin, elastase, thermolysin, bromelain, papain, subtilisin, an alkaline protease, a neutral protease, an acidic protease, a fungal protease, a yeast protease, or a bacterial protease. In some embodiments, the protease is pepsin.

In some embodiments, the ATIs are quantified by mass spectrometry, quantitative western blotting, or ELISA.

In some embodiments, an assay involving immobilized TLR4 (e.g., TLR4-MD2, TLR4-CD14, or TLR4-MD2-CD14) and labeled ATI may be used in methods of quantifying the amount of ATIs in a foodstuff and/or determining the bioactivity of ATIs in a foodstuff. In this assay, TLR4 is immobilized on a solid support (i.e., wells of a 96-well plate, or beads). Labeled ATIs (i.e., fluorophore labeled ATIs) are then added to the immobilized TLR4 (e.g., TLR4-MD2, TLR4-CD14, or TLR4-MD2-CD14) to form a complex of TLR4 (e.g., TLR4-MD2, TLR4-CD14, or TLR4-MD2-CD14)/labeled ATI in the wells of the 96-well plate. Further, a sample containing extracted ATIs from a foodstuff is added before, concomitantly, or afterwards to the wells of the 96-well plate containing the TLR4 (e.g., TLR4-MD2, TLR4-CD14, or TLR4-MD2-CD14)/labeled ATI complex. The extracted ATIs in the sample compete with the labeled ATIs in binding to the immobilized TLR4 (e.g., TLR4-MD2, TLR4-CD14, or TLR4-MD2-CD14). After incubating the sample containing extracted ATI with the TLR4 (e.g., TLR4-MD2, TLR4-CD14, or TLR4-MD2-CD14)/labeled ATI complex, the plate is washed to remove unbound labeled ATIs. The more extracted ATIs are in the sample, the more labeled ATIs are competed off the TLR4 (e.g., TLR4-MD2, TLR4-CD14, or TLR4-MD2-CD14)/labeled ATI complex and remain unbound. If the labeled ATIs are attached to specific fluorophores, the amount of fluorescent (luminescent) signal that remains bound to the solid support, or the amount of fluorescent signal (luminescent) in the wash may serve as an indication of the amount of extracted ATIs in the sample. In other embodiments, the labeled ATIs may be detected by a secondary agent (i.e., an antibody) that is coupled to an enzyme, i.e., horseradish peroxidase or alkaline phosphatase, or a fluorescent or luminescent protein, which produces a spectrometric signal upon reaction with its specific substrate. In some embodiments, the antibody recognizes and binds to at least one ATI selected from the group consisting of e.g. CM3, CM2, 0.19, 0.28, and 0.53.

In some embodiments, the measurements of ATI bioactivity can be replaced by the determination of the quantities of extracted ATIs by conventional ELISAs, such as sandwich ELISAs, or related assays. While this is state of the art technology, we demonstrated that once a selection of key or dominant native ATIs or certain ATI fragments are determined, their overall quantity correlates well with the measured bioactivity. In some embodiments, such ELISAs or related techniques recognize and bind to at least one ATI selected from the group consisting of, e.g., CM3, CM2, 0.19, 0.28, and 0.53.

The analytical methods to measure ATI-bioactivity/quantity are centrally linked to the selection and preparation of ATI-containing or ATI-de-enriched foodstuff. These analytic assays used to measure ATI-bioactivity/quantity help to produce foodstuffs in the needed quantities. An estimate is that the disease promoting effect of ATIs is abolished by reducing the average ATI consumption in wheat (e.g., barley, rye) based diets of 0.5-1.0 gram by approximately 90% (see, e.g., Catassi et al., *Nutrients* 7:4966-4977, 2015; Fasano et al., *Gastroenterology* 148:1195-1204, 2015; Schuppan et al., *Best Practice & Research: Clinical Gastroenterology* 29:469-76, 2015).

In a seventh aspect, the invention features a method of producing a foodstuff having a reduced ATI content or is free of ATIs, wherein the method includes A) determining the bioactivity of ATIs in the foodstuff, and/or B) quantifying the amount of ATIs in the foodstuff, and C) reducing the amount of ATIs in the foodstuff by extracting and degrading ATIs using one or more reducing agents, proteases, and/or synthetic enzymes engineered to reduce and/or degrade ATIs in the food stuff.

In a seventh aspect, the invention features a method of reducing the reactivity of a foodstuff, wherein the method includes cultivating a cereal with oxidized glutathione (GSSG) and/or protein bound, oxidized glutathione (PSSG). In some embodiment, the cereal is also cultivated with a balanced amount of reduced glutathione (GSH).

In an eighth aspect, the invention features a method of reducing the ATI/TLR4-reactivity of a foodstuff, wherein the method includes cultivating a cereal with sulfur rich or sulfur depleted fertilizers.

In a ninth aspect, the invention features a method of reducing the ATI content of a foodstuff, wherein the method includes fermenting the foodstuff with a bacteria with high disulfide reducing and/or proteolytic ability. In some embodiments, the bacteria is selected from the group consisting of *Lactobacillus sanfranciscensis* and *L. reuteri LTH2584*.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows the induction of low grade innate inflammation in the intestine of healthy C57BL6 mice at 12 hours after oral gavage of 200 μg of purified wheat ATIs in 200 μl PBS compared to PBS alone or 1 mg of crude Sigma gliadin (containing about 15% wt/wt of ATIs) in 200 μl PBS. Determination of inflammatory genes from the harvested intestine was performed using quantitataive RT-PCR normalized to GAPDH mRNA as housekeeping gene. ATIs caused also a mild upregulation of transglutaminase 2 (TG2) mRNA which is central to celiac disease pathogenesis. **(A):** Transcript levels of inflammatory markers (MCP-1=CCL2 and KC=IL-8) and of TG2 in the intestine were up-regulated after feeding a single dose of ATIs or ATI-containing gliadin (commercial product from Sigma, with about 15% wt/wt ATIs) to C57BL/6 mice kept on a gluten-free diet. n=5 per group; *$p<0.05$. Kinetics of serum IL-6 after one oral ATI vs BSA challenge, and serum IL-8 levels in mice changed from a gluten free diet composed of 20% casein as protein source with defined carbohydrates and fats to either a diet in which 1% wt/wt of the casein was replaced by ATIs (GF to ATI) or a diet in which 25% of the protein was replaced by gluten containing about 5% w/w of ATIs (GF to G25), vs mice continued on the GF diet (GF to GF) for 7 days. **(B):** F4-80 positive cells (macrophages, x200) were higher in the group challenged with ATIs or ATI-containing gliadin. Macrophages show a dark colour (peroxidase-staining). Quantitative assessment counting positive cells in 4 high power fields per sample. n=5 per group.

Figure 2 shows that an ATI containing diet exacerbates inflammatory bowel disease. Dextran sodium sulfate (DSS) colitis (Lauroui et al., *PLoS One* 7:e32084, 2012; Wirtz et al. *Nat. Protoc.* 2:541-546, 2007) was induced by adding a low dose of 1.5% DSS (w/v) in the drinking water of female C57BL/6 mice on a gluten-free diet (GFD, purely casein-based protein source) or "normal" chow containing 25% of gluten (with about 15% w/w as ATIs)/75% casein as protein source. Mouse body weight changes that are strong indicators of the severity of colitis were recorded every two days (data by Dr. G. Pickert in the Ps group). Changes were significant from day 12 on ($p<0.05$ ATI-free vs. ATI-containing chow).

Figure 3 shows that an ATI containing diet exacerbates experimental multiple sclerosis. **(A-D):** Experimental autoimmune encephalomyelitis (EAE), a suitable model of multiple sclerosis, was induced by immunization with myelin oligodendrocyte protein (MOG_{35-55} peptide emulsified in complete Freund's adjuvant and pertussis toxin on the first day of immunization and 2 days later) in female C57BL/6 mice on a gluten-free diet (GFD, casein as protein source). Animals were divided in group 1 continuing on the GFD and group 2 changing to a diet containing 25% of gluten (with about 15% w/w as ATIs)/75% casein as protein source). CNS-infiltrating total T cell (A), CD4+ (B) and CD8+ (C) T cell numbers were calculated using FACS-analysis of extracted brain tissue (shown is a representative experiment of three independent experiments with n=4 mice per group). Mice were observed and scored daily for clinical signs of EAE after induction (days post induction, DPI) (D). Data are representative of three independent experiments (n=10 per group, maximal clinical score 3.75). *$p<0.05$ comparing both groups. **(E):** EAE-induction in female C57BL/6 mice on a gluten-free diet (GFD) as in Fig.3A. Animals were divided in four groups: group 1 continued with the GFD (GF to GF), group 2 changed to a diet containing purified ATIs (GF to ATI; casein-based protein source with 1% purified ATIs), group 3 changed to a diet containing 25% gluten (about 15% w/w as ATIs) (GF to G), and group 4 changed to a diet containing 25% gluten with partial de-enrichment ATIs by extraction (reduction of ATI-content by about 70% (GF to G-ATI). Mice were observed and scored daily for clinical signs of EAE after induction (days post induction, DPI) (D). Data represents one independent experiment (n=10 per group, maximal clinical score: 2.5: incipient hindleg paralysis, see below). *$p<0.05$ for the area under curve from baseline to peak of disease for GF/GF vs GF/ATI. Clinical EAE score: 0, no disease; 1, decreased tail tone; 2, abnormal gait (ataxia) and/or impaired righting reflex (hind limb weakness or partial paralysis); 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, hind limb paralysis with partial fore limp paralysis; 6, moribund or dead.

Figure 4 shows that an ATI containing diet exacerbates allergic asthma. **(A-F):** Murine asthma model induced by ovalbumine (OVA) sensitisations and challenge in female C57BL/6 mice on a gluten-free diet (GFD, casein as protein source) or "normal" chow containing 25% of gluten (with about 15% w/w as ATIs)/75% casein as protein source). Serum IgG1 was analysed after the sensitisations **(A).** Animals were sacrificed after three challenges with PBS or OVA. Bronchoalveolar lavage fluid (BALF) was used to prepare cytospins and count cell populations **(B)**. BALF was also used to measure IL-13 and IL-5 secretion **(C and D)**. Histological lung sections were scored after H&E and PAS staining **(E and F)**. **(G-I):** Murine asthma model induced by OVA sensitisations and challenge in female C57BL/6 mice on a gluten-free diet (GFD), as detailed in Fg.4A. Animals were divided in 5 groups: group 1 continued with the GFD and was sensitised with PBS, group 2 continued with the GFD and was

sensitised with OVA, group 3 changed to a diet containing 25% gluten (with about 15% w/w as ATIs)/75% casein as protein source), group 4 changed to a diet containing ATIs (casein-based protein source with 1% purified ATIs), and group 5 changed to a diet containing 25% gluten with low levels of ATIs (de-enriched of ATIs by about 70%). All groups were challenged with OVA and then sacrificed. Splenocytes were isolated from each group and stimulated with 3 µg/mL of OVA for proliferation assays (A). Supernatants were analysed for cytokines IL-13 and IL-5 (B and C). **p<0.01, ***p<0.005.

Figure 5 shows simple method to extract ATIs from wheat (rye, barley) flour, their related products, and from other plant flours for determination of bioactivity. Extraction is incomplete using water, but improved and reproducible using buffered solutions containing low-intermediate concentrations of salt (preferably NaCl). Examples are phosphate buffered saline, pH 7.4, or 5-50 mM ammonium bicarbonate (Abic). Other concentrations of these chemicals in the solvents or related or unrelated chemicals with buffering properties can be also used, such as EDTA, EGTA, sodium bicarbonate, sodium carbonate, and various phosphate buffers. Dialysis of the extracts can be done against water, Abic solutions or acetic acid (AA). Compared to AA, Abic dialysis yields a higher ATI bioactivity in the cellular readout system. Bioactivity can be directly determined without defatting after two sequential extraction of, e.g., 1 h (1st and 2nd extractions). Similarly, extractions for preparation of bulk ATI-de-enriched foodstuffs can be done with the latter or similar simplified methodologies.

Figure 6 shows extraction and bioactivity determination of ATIs from wheat flours. **(A and B):** Total protein extracted sequentially from 10g of flour from modern wheat and from more ancient wheat variants (Iran, Kamut) AA: 0.05% acetic acid; Abic, 50 mM ammonium bicarbonate buffer. Bioactivity assay using the human monocyte-macrophage cell line THP-1 and standardized lyophoilized extracts (combined 1st and 2nd extract representing >90% of total ATIs). **(C, D, and E):** Sequential extraction (3 extractions) with ammonium bicarbonate (ABic) of commercial wheat flour and two more ancient hexaploid wheat variants (Iran and Kamut). Sequential extractions using different time points (from 0.5 to 24 h), different concentrations of Abic, and determination of the ATI bioactivity of each extract.

Figure 7 shows extraction and bioactivity determination of ATIs from flours and foods. **(A, B, C, D, and E):** Bioactivity of gluten containing cereals, gluten-free staples/plants, products manufactured with wheat, different wheat cultivars (all modern hexaploid (kind gift of Dr. F. Longin, Univ. of Hohenheim, Germany)), and commercial beers. In these bioassays, 1 µg of IL-8 release equates to ~2 mg of ATIs.

Figure 8 shows (**A**) resistance of purified ATIs from wheat to a 2 h digestion with pepsin, typsin or the sequence of pepsin and trypsin (enzyme:substrate ration 1:100 at 37°C; bioactivity is reduced by only ~30% after these digestions; BSA served as negative control. (**B and C)** extraction and bioactivity determination of ATIs from different commercial glutens, and from gluten containing bread vs flour compared to gluten free bread and cooked pasta (all after pepsin.trypsin digestion followed by two sequential combined ammonium bicarbonate extractions). **(D):** complete ATI inactivation by reduction

and alkylation at elevated temperature; LPS served as positive control in the used THP1 cell bioassays.

Figure 9 shows other high sensitivity bioassays for bioactivity quantification of extracted ATIs. ATI extracts with Abic were lyophilized and standardized as to protein content. Dose dependent stimulatory response in **(A)** murine bone marrow-derived dendritic cells (BM DCs) and **(B)** a TLR4/MD-2/CD14/IL-8 Prom/LUCPoter™ HeLa cell line. Compared with the THP-1 cells used in Fig.6, these readout cells showed higher sensitivity for ATI-bioactivity (ceiling effect at concentrations above ~50 and ~100 µg of ATI per ml).

Figure 10 shows a summary ATI bioactivity determinations from flours and baked foods. ATI content is usually low in most gluten free foods. Classification of grains and food products according to the amount of bioactive ATI (innate stimulating activity as determined by one of the bioassays). **high:** typical gluten containing cereals and products**, medium:** between about 5-20%, and **low**: below 5% of typical gluten containing cereals or foods.

Figure 11 shows ATIs in extracts from wheat flour on SDS PAGE gels (4-20% gradient). **(A):** Spring wheat extracted with acetic acid (AA) and subject to salting out using different concentrations of sodium chloride (NaCl). ATIs represent most of the band running in the 10-15kD range. Supernatant (s) and pellet (p) were treated with a without a reducing agent (dithiothreitol, dtt). Notably, in the acidic range ATIs are prominently precipitating between at 0.2 M NaCl, while at 0.5M NaCl gluten will precipitate (bands above the 25kD range). **(B):** Three different wheat samples extracted with ammonium bicarbonate (Abic) with or without reducing agent. Abic mostly extracts ATIs (prominent band(s) with molecular weight around 15 kDa) with only minor amounts of gluten and other proteins.

Figure 12 shows **(A):** determination of total ATI-quantity and ATI-species in different wheat extracts using mass spectrometry and **(B):** relative amount of main ATIs in different extracts as well as other ATIs that include chymotrypsin inhibitor WCI, endogenous alpha-amylase/subtilisin inhibitor and CMX1/CMX3.

Figure 13 shows relative quantity of ATI-species in different wheat extracts using mass spectrometry. **(A):** Abic extract, **(B):** chloroform/methanol (CM) extract, **(C):** commercial extract (1520), and **(D):** commercial extract(3535). Lyophilized ATI-extracts (Abic and chloroform-methanol) were reduced/alkylated, digested with trypsin, and subjected to mass spectrometry. "Others" include chymotrypsin inhibitor WCI, endogenous alpha-amylase/subtilisin inhibitor, and the rare ATIs CMX1/CMX3).

Figure 14 shows relative quantity of the major ATI-species CM3 and 0.19 in different wheat extracts using mass spectrometry and Western blotting. **(A and B):** Determination of the percentage of CM3 and 0.19 relative to total protein in commercial extract 1520 and 3535 using mass spectrometry. **(C):** Reducing SDS PAGE gel (4-20% gradient) and Western-blotting using rabbit peptide antibodies specific for CM3 and 0.19, respectively.

Figure 15 shows ATI bioactivity of fermented wheat dough. **(A):** ATI bioactivity in 90% wheat dough fermented with 10% rye malt. Samples were extracted with Abic, lyophilized and reconstituted. Standardized extracts were cultured with the human monocytic cell line THP-1 and cytokines secreted (IL-8, MCP-1 and TNF-α) measured by ELISA. Bars in represent samples fermented with different strains (Lactobacillus sanfranciscensis (mutant), L. Sakei, L. Reuteri and L. Sanfranciscensis). Other bars represent a control sample of unfermented rye malt. Results are from 3-5 samples, and analysed for sugnificance using one way ANOVA and compared against the unfermented sample (control) using Dunnett's test. Level of significance ($p<0.05$) was represented by asterisks. *$p<0.05$, **$p<0.01$, ***$p<0.005$. **(B):** ATI bioactivity of fermented dough prepared with 100% whole wheat. Samples were extracted with Abic and analyzed as described above.

Figure 16 shows inhibition of ATI-bioactivity using the natural TLR4-antagonist *LPS-RS*. TLR4-inhibitory lipopolysaccharide from *Rhodobacter sphaeroides (LPS-RS,* 1.5 μg/mL*)* was pre-incubated with THP-1 cells for 30 min, then ATIs enriched in CM3 and 0.19 (37.5μg/mL) were added and cultured overnight. E.coli LPS (12.5ng/mL) was also cultured overnight with THP-1 cells pre incubated (30 min) with LPS-RS (250ng/mL). E.coli LPS (12.5ng/mL), LPS-RS (1.5μg/mL) or buffer alone served as controls.

DETAILED DESCRIPTION OF THE INVENTION

Amylase trypsin inhibitors (ATIs)

Amylase trypsin inhibitors (ATIs) (as a term for all structurally and especially functionally related molecules) are molecules present in plants that act as triggers of innate immune activation. ATIs affect not only celiac disease activity but other inflammatory conditions. As used herein, ATIs refer to molecules having TLR4-stimulating activities in foodstuffs. In some embodiments, ATIs stimulate responsive cells by mimicking the MD2 protein component of the tetrameric TLR4-signaling complex, which is composed of two molecules of TLR4 and two molecules of MD2.

The newly discovered activity of ATIs as nutritional activators of TLR4 is different from that of gluten in celiac disease and of wheat (rye, barley) proteins in allergy. ATIs can also act as inhalative allergens (Schuppan et al, *Gastroenterology* 2009; Batais et al, *Eur Ann Allergy Clinic* 2008; Catassi et al, *Nutrients* 2013; Uvackova et al, *J Proteome Res* 2013). Notably, the disease causing potency of ATIs ingested with wheat, barley, or rye (gluten-containing cereals), as well as with some non-gluten containing staples, is not limited to the gastrointestinal tract, but is also likely implicated to affect other extraintestinal diseases (Catassi et al., *Nutrients* 7:4966-4977, 2015; Fasano et al., *Gastroenterology* 148:1195-1204, 2015; Schuppan et al., *Best Practice & Research: Clinical Gastroenterology* 29:469-76, 2015). We have accumulated broad evidence that nutritional ATIs induce low grade but significant inflammation in the small intestine, colon, and. the surrounding mesenteric lymph nodes. Notably, nutritional ATIs exacerbate inflammatory diseases in general, as illustrated in mouse models of inflammatory bowel disease, of multiple sclerosis, of systemic lupus erythematosus, of non-alcoholic steatohepatitis, and of allergic asthma. Figs. 1-4 demonstrate this "adjuvant effect" in the mentioned *in vivo* situations and diseases.

In addition to gluten-containing foodstuffs, such as wheat, barley, and rye, we also detected significant TLR4-stimulating activities caused by ATIs in some gluten-free foodstuffs, such as millet, soy, and quinoa. We also observed that foodstuffs cultivated at low altitude and/or with extensive fertilization tend to contain high levels of ATIs, while foodstuffs cultivated at high altitude and/or without fertilization often contain lower levels of ATIs or are even ATI-free. This can be explained by structural similarities of ATIs in wheat, rye, and barley (gluten-containing grains), e.g., the predominant CM3, CM2, 0.19, 0.28, and 0.53 variants in wheat and their homologues in barley and rye. Some of these ATIs are found in gluten-free staples. The methods of the invention can be performed in a variety of foodstuffs suspected of containing ATIs, including both gluten-containing foodstuffs and gluten-free foodstuffs, as well as foodstuffs that have been processed, e.g., by cooking or baking, which only slightly reduces ATI content and bioactivity. The methods of the invention allows the selection of wheat variants that express low levels of ATIs by measuring ATI content and/or bioactivity. Identification of wheat variants that express low levels of ATIs by measuring ATI content and/or bioactivity allows subsequent ATI de-enrichment of these wheat variants using the ATI-reducing/extracting methods described herein.

Some examples of foodstuffs that require ATI removal prior to consumption in order to produce less proinflammatory foodstuffs are shown in Fig. 10.

ATI reducing agents and degrading enzymes

ATIs are resistant to degradation by many known proteases, including digestive enzymes of the human gastrointestinal tract, such as pepsin, trypsin, and chymotrypsin. Much of this resistance is due to the presence of several (i.e., usually five) intramolecular disulfide bonds in ATIs that generate a compact secondary structure. Once the disulfide bonds in ATIs are reduced by reducing agents and ATIs are exposed to modestly elevated temperature, e.g., 60 °C, depending on the buffer system, ATI activity is lost. However, ATIs can refold and regain bioactivity at lower temperatures. Therefore, one strategy to eliminate ATI bioactivity in foodstuffs is to further degrade ATIs using enzymes, thus preventing ATI refolding.

Reducing agents that can be used to reduce the disulfide bonds in ATIs include, but are not limited to, mercapto-ethanol, dithioerythritol (DTT), cysteine, glutathione (GSH), oxidized glutathione (GSSG), protein bound glutathione (PSSG), sulfites, and thioredoxin. In some embodiments, an reducing agent may be balanced by, e.g., reduced glutathione (GSH). In some embodiments, bacteria with high disulfide reducing ability (e.g., *Lactobacillus sanfranciscensis* and *L. reuteri LTH2584*) may be used as a reducing agent. In some embodiments, a foodstuff may be cultivated and/or fermented with bacteria with high disulfide reducing ability (e.g., *Lactobacillus sanfranciscensis* and *L. reuteri LTH2584*). In some embodiments, one or more reducing agents may be added to the extraction buffer used to incubate the foodstuff. In some embodiments, one or more reducing agents may be used to reduce the disulfide bonds in ATIs prior to enzyme degradation. In some embodiments, one or more reducing agents may be used in combination (i.e., at the same time) with enzymes to reduce and degrade ATIs.

Enzymes, such as proteases, may be used to degrade ATIs. Proteases that may be used to degrade ATIs include, but are not limited to, pepsin, trypsin, chymotrypsin, elastase, thermolysin,

bromelain, papain, subtilisin, an alkaline protease, a neutral protease, an acidic protease, a fungal protease, a yeast protease, and a bacterial protease. Alkaline, neutral, and acidic proteases are proteases that are the most active and work most optimally at basic (i.e., at a pH from about 8 to about 14), neutral (i.e., at a pH of about 7), and acidic pH (i.e., at a pH from about 1 to about 6) conditions, respectively. Examples of alkaline proteases that may be used to degrade ATIs include, but are not limited to, serine proteases, endopeptidases, Protease K, Alcalase® (Novozymes), Savinase® (Novozymes), Maxacal® (Genencor), Blap® (Henkel), KAP® (Kao Corporation), and other alkaline proteases known in the art, e.g., the alkaline proteases described in Anwar et al., *Biores. Tech.* 64:175-183, 1998, which is incorporated herein by reference in its entirety. In some embodiments, depending on the pH of the extraction buffer, one or more appropriate proteases (i.e., alkaline, neutral, and/or acidic proteases) may be selected to be added to the extraction buffer to degrade ATIs. For example, an alkaline protease (e.g., a serine protease) may be added to a basic extraction buffer.

In some embodiments, an enzyme may be specifically engineered or evolved to degrade ATIs (i.e., a synthethic enzyme). Techniques that may be used to engineer or evolve an enzyme to have novel capabilities are known in the art, i.e., random mutagenesis, protease library screens, mutant screens, gene recombination, gene site saturation mutagenesis (GSSM) as described in U.S. Patent Nos. 6,171,820 and 6,579,258, and tunable gene assembly (TGR) as described in U.S. Patent No. 6,537,776, each of which is incorporated herein by reference in its entirety. In some embodiments, an enzyme used to degrade ATIs may be engineered to have both functions of a disulfide bond reducing agent and a protease. In some embodiments, the enzyme specifically engineered or evolved to degrade ATIs may be used in combination with one or more proteases, such as pepsin, trypsin, chymotrypsin, elastase, thermolysin, bromelain, papain, subtilisin, an alkaline protease, a neutral protease, an acidic protease, a fungal protease, a yeast protease, and a bacterial protease.

EXAMPLES

Example 1 - Methods to determine the (proinflammatory) bioactivity due to ATIs in unprocessed and processed foods

1.1.Quantitative extraction of ATIs and proteolytic digestion of ATIs

Quantitative ATI extraction from different cereals, grains, and foods for determination of bioactivity

Buffer/solvent extractive method (applicable to most unprocessed foods, see sketch in **Fig. 5**):

1. Dried material is ground and sieved
2. 20 g are defatted with 200 ml methanol/diethylether 1:1 for 2 h at 4-8 °C (stirring)
3. Centrifugation at 5.000 - 10.000 rpm for 10-30 min

Steps 2.-3 can also be omitted, without significant loss of bioactivity

4. Pellet is extracted with 200 ml of 50 mM ammonium bicarbonate (Abic) overnight at 4°C (stirring)
5. Centrifugation at 5.000 - 10.000 rpm for 10-30 min
6. Supernatant is freeze dried
7. Second (third) extraction as above

8. Combined supernatant is freeze-dried

9. Freeze dried material is reconstituted in e.g. 2 ml of PBS or cell culture compatible buffer or media

Other solvents such as chloroform/methanol (CM) can also be used, but they predominantly extract the more hydrophobic CM-class of ATIs, leaving other ATI species largely not extracted. Sequential extraction using CM and the above solvents/buffers or vice versa is possible.

Buffers may vary in composition. Thus ammonium bicarbonate can also may be used in lower (e.g., 5 mM) or higher (e.g., 100 mM) concentrations, other neutral or slightly alkaline buffers can be used. Even acidic extraction is possible, e.g., by using 50 mM acetic acid. However acidic buffers co-extract significant amounts of other proteins such as gluten (further described in detail below).

Notably, ATI bioactivity and ATI quantity can be determined from two exhaustive extracts with the above buffers, e.g., alkaline buffers containing salt (e.g., NaCl up to 1.0 M) usually extract 80-90% of total ATIs (**Fig. 6C-6E**). Here, both extracts can be combined to be used directly or in a dilution for bioassay or ELISA.

In other cases, e.g., in liquid foods, such as beer, ATI bioactivity and ATI quantity can be directly determined from these liquids, without extraction (see **Fig. 7E** for beers).

Quantitative ATI extraction from further processed, e.g. cooked or baked foods such as bread, cookies, pasta or pizza

1. Dried material is ground and sieved
2. 20 g are defatted with 200 ml methanol/diethylether 1:1 for 2h at 4-8 °C (stirring)
3. Centrifugation at 5.000 - 10.000 rpm for 10-30 min

Steps 2.-3 can also be omitted, without significant loss of bioactivity

4. Pellet is digested with (gastrointestinal) proteases that do not significantly degrade the highly disulfide linked ATIs, such as pepsin followed by trypsin to better extract ATIs from the food matrix.

Proteolytic digestion of ATIs

Example of proteolytic digestion (pepsin-trypsin digestion protocol) can be found in Frazer et al, Lancet 1959. A brief example is described below.

1. 1 g of suspended sample is digested with pepsin (10 mg in 0.5 mL of 0.1 M HCL, pH 1.8) at 37ºC for 4 h.
2. Sample is centrifuged at 4,500 rpm for 10 min and the pellet is discarded.
3. Supernatant's pH is adjusted from 1.8 to 7.8 with NaOH and then trypsin (Sigma T9201, 10 mg in 0.5 mL neutral buffer, such as PBS) is added and incubated at 37ºC for 4 h.
4. pH is adjusted to 4.5 with HCl and N-tosyl-chloromethyl-ketone (TLCK, 10 mg in 0.5 mL DMSO) is added to block the trypsin.
5. Precipitate is removed by centrifugation at 4,500 rpm for 10 min.
6. Pellet is discarded and supernatant is dialysed (molecular weight exclusion e.g. 1000 D) in 10 mM ammonium bicarbonate, Ph 7.8 (4L, 2 changes) at 4 ºC.
7. Samples are sterile filtrated and freeze-dried.

8. Protein concentration may be determined by using a bicinchoninic acid protein assay (Pierce).

9. Freeze dried material is reconstituted in e.g. 2 ml of PBS or cell culture compatible buffer or media.

Other buffers and buffer and enzyme concentrations can be used, as long as enzyme activity is maintained. In some, e.g., hardly baked, foods, more harsh conditions may be applicable. Also other enzymes such as chymotrypsin or thermolysin can be used exclusively (as neutral protease) or in combination with trypsin, since these can leave ATIs largely intact, while degrading other, major (cereal) proteins such as gluten (Camus and Laporte, Reprod Nutr Dev 1980; Gessendorfer et al, Anal Bioanal Chem 2009; Jos et al, Scand J Gastroenterol 1975; Rombouts et al, Sci Rep 2013).

Due to their resistance to gastrointestinal proteases, ATIs remain largely intact after these proteolytic extraction procedures and maintain bioactivity. Our *in vitro* studies show that even with purified ATIs, the above-described digestion procedure reduces bioactivity by maximally 40%. Moreover, extracted bioactivity of 1 g of wheat flour before baking (10-20 min at 150-180ºC) is reduced by maximally 50% post baking (see examples in **Figs. 7C, 8A, and 8D**).

1.2 Bioactivity assays

Examples of bioactivity determinations are shown in **Figs. 6-10**.

Cell culture

ATI extracts (freeze dried or centrifuged/concentrated) as described in Example 1 and reconstituted in cell-culture compatible buffers, are added to cultures of ATI-reactive (TLR4-bearing) cells, preferably monocytes/macrophages, dendritic cells, or cells transfected with the TLR4-MD2-CD14 oligomeric signaling complex. It is also preferable to use stable cell lines such as human THP-1, U937, or TLR4-MD2-CD14 transfected HEK-293 cells (obtainable e.g. from American Type Culture Collection). Apart from human cells, cells of other species such as mouse or rat can be used. Culture is preferably in complete RPMI or DMEM supplemented with 100 IU/ml penicillin/100 µg/ml streptomycin and 10% fetal calf serum at 37°C in a 5% CO2 atmosphere. Cells should be mycoplasma-free for reproducible results. Usually 1-100 µL of the reconstituted extracts described in Example 1 are added to the cultures of the indicator cells (preferably seeded at densities of 25,000-200,000 cells in 0.2-1 mL medium in 24 well culture plates). Culture supernatants and/or cells are preferably harvested at 12-24 h post addition of the extracts to be tested.

Cytokine/chemokine assays.

The release of several chemokines and/or cytokines into the cell culture media due to addition of ATIs/ATI extracts to the above cells can be quantified to serve as a quantitative measure of stimulatory ATI activity. Examples are IL-8, IL-15, TNFa, CCL2 (MCP-1), CCL5 (RANTES). This can be done using commercial validated ELISAs (e.g. IL-8 and TNFa [BD]; MCP-1, IL-15, RANTES [R&D Systems]) according to the manufacturers' protocols. Apart from TLR4-dependent cytokines and chemokines, other secreted or cell extractable proteins/peptides downstream of TLR4 activation can be measured, including heat shock proteins (such as Hsp27, Hsp70, Hsp90) activation or known from the literature.

Use of cells transfected with TLR4-MD2-CD14 and an indicator gene

The lipopolysaccharide (LPS) receptor complex of TLR4, MD-2 and CD14 has been identified ATI-sensing receptor complex (Junker et al, J Exp Med 2012). Since IL-8 is a major cytokine induced by TLR4, a reporter cell line which stably expresses TLR4, MD-2 and CD14 proteins and the Renilla luciferase reporter gene under the transcriptional control of the IL-8 promoter was developed and is commercially available (R&D Systems, San Diego, CA; Lee et al 2013). We used this cell line to test ATI activity and compared values to those obtained with IL-8, MCP-1 and TNF-α release from ATI-stimulated THP-1 cells (**Fig. 9**). Other indicator systems that, e.g., generate fluorescent products or release soluble products that can be quantified in the cellular supernatant as described in Example 1, or harbor other promoters that are driven by the activation of the TLR4-MD2-CD14 can also be used. These systems are particularly well suited for high throughput or high quantitative measurements of TLR4-complex stimulating ATI-activity.

Other assays for determination of ATI bioactivity

RNA isolation and quantitative RT-PCR

Total RNA is isolated from cells using e.g. TRIzol (Invitrogen). Quantitative RT-PCR is preferably performed using exon–exon boundary-spanning primer sequences and e.g., the SYBR green or Taqman methodology as described for a set of cytokines by Junker et al (2012). Apart from TLR4-dependent cytokines and chemokines, other transcripts downstream of TLR4 activation can be measured, including heat shock proteins (such as Hsp27, Hsp70, Hsp90) but also other transcriptionally activated genes known from the literature.

Flow cytometry

After stimulation with extracted ATIs the above cells can also analyzed using flow cytometry that quantifies activation markers. Activation markers are e.g. CD80, CD86, MHC class II, IL-12, IL-23, interferon gamma (intracellular stain) and many others known from the common literature. Cells are preincubated with FcR blocking reagent (Miltenyi Biotec) for 15 min at 4°C before staining preferably with fluorescent-labeled monoclonal antibodies to these activation markers at predetermined concentrations for e.g. 30 min at 4°C. Cells are then washed with staining buffer (e.g. PBS and 1% BSA), cell viability is assessed by DAPI exclusion (0.1 µg/ml; Roche), and only viable cells are analyzed by flow cytometry using e.g. a four-laser LSRII and FlowJo software (Tree Star) as described (Junker et al, J Exp Med 2012).

Altered signal transduction

Other quantitative readouts that can be used to measure ATI activity in samples are based on activation (or inhibition) of signaling pathways in the responsive cells. Examples are the phosphorylation of certain kinase substrates, such as IRF-3 or NFkB (p65), or NFkB nuclear translocation.

Lymphocyte proliferation assay

This method exploits the ability of TLR4-complex stimulated antigen presenting cells (such as monocytes, macrophages, dendritic or B cells) to permit proliferation or activation of antigen specific T cells in the presence of antigen. An example is the incubation of ovalbumin or gluten specific T cell

lines or T cell clones, and HLA-compatible antigen presenting cells or cell lines loaded with ovalbumin (ova peptide) or gluten (gluten peptide), respectively. An (unknown amount of) ATI added to these cultures will dose-dependently stimulate T cell proliferation which can be measured in standard T cell proliferation assays, using e.g. 3H-thymidine or BrdU incorporation. A simplified version of such assay is the addition of ATI-containing solutions to a classical mixed lymphocyter reaction with white blood cells of peripheral human or non-human, e.g. rodent, blood, or e.g., rodent splenocytes.

Example 2 - Methods to determine the chemical quantity of ATIs in unprocessed and processed foods.

2.2 Quantitative extraction of ATIs from food

These methods permit another, even more thorough extraction of the highly proteolytically resistant ATIs (this resistance is mainly due to the presence of usually 5 intramolecular disulfide bridges), especially from processed, e.g., baked foods. Chemical quantification after reductive extraction is quantitative, but leads to loss of bioactivity due to unfolding of the proteins. However, this does not affect protein quantification. Extraction preferably uses reductive methods with or without detergents, and with or without additional proteolytic digestion.

These methods permit quantification of all ATI-species in a given cereal or staple variant. Thus whole wheat genome sequencing revealed that modern hexaploid wheat contains up to 16 different ATI-species, and about half of these have been characterized as proteins. Moreover, ATIs represent between 2% and 4% of total wheat protein (with 80%-90% being gluten proteins). Importantly, ancient and older (diploid, tetraploid and early hexaploid) wheats often contain fewer ATI genes and proteins (Altenbach et al, BMC Res Notes 2011; Dupont et al, Proteome Sci 2011) (**Figs. 7A-7D and 10**). Finally, a second fertilization cycle (post anthesis fertilization) increases total protein content (mainly gluten), but decreases ATI content (Altenbach et al., Proteome Sci 2011). This is reflected by our bioactivity determinations shown in **Fig. 7D,** which demonstrates dependence of ATI content (bioactivity) per gram of flour on the genetic background of the cereal, on the growing location, and on the extent of fertilization. We also show that the various ATI-variants, such as the predominant CM3 and 0.19 species in wheat, display similar TLR4-stimulating bioactivity on a molar basis (see below).

Importantly, the other major gluten containing cereals (barley and rye) express various amylase/trypsin/chymotrypsin/subtilisin inhibitors with high sequence homology to the wheat ATIs (Pekkarinen et al, J Agricult Food Chem 2003; Junker et al, J Exp Med 2012) and with similar overall TLR4-stimulating bioactivity (**Figs. 7A and 10**).

Importantly, some gluten-free grains and staples contain relevant ATI bioactivity, which can be explained by ATI-species with structures resembling the wheat ATIs. In some embodiments, the ATI bioactivity in some gluten-free grains reaches up to about 20% the bioactivity in modern hexaploid wheat (**Fig. 7B**),

Example: Reductive extraction of ATIs.

One gram of the (dried) food to be analyzed is extracted in neutral, near neutral or alkaline solvents/buffers, such as PBS, Tris-buffer, ammonium bicarbonate, preferably containing a detergent or detergent mixture (e.g., sodiumdodecyl sulfate, Triton X-100, Tween-20, sodium deoxycholate), and

a reducing agent, such as 2-mercapto-ethanol, dithioerythritol (DTT), e.g. at 5-200 mM and at 37°C for 30 min to 4 hrs. For stabilization of free thiol groups, samples should be alkylated, e.g., with iodoacetamide at a final concentration of 20-500 mM for 10-60 min in the dark. Iodoacetamide will be quenched by excess reducing agents, e.g., 200 mM DTT. The cleared supernatant can be separated from small molecular weight contaminants e.g. by dialysis or ultrafiltration. For mass spectrometry the suspension (cleared supernatant) can be further digested, e.g., with trypsin.

For further analysis samples can be freeze-dried, concentrated in solution, e.g., by ultracentrifugation, or protein can be adsorbed to a solid support. For mass spectrometry, peptides are preferably adsorbed to a solid support.

2.2 Quantification of extracted ATIs

Examples of chemical quantification of ATIs in flours are shown in **Figs. 11-14.**

Mass spectrometry

This is preferentially performed in samples enriched in ATIs (quantitative extraction) and performed after proteolytic digest, using standard state of the art quantitatative MS techniques (Prandi B et al, Food Chem 2013). Liquid chromatography-mass spectrometry quantification of 4 different ATI isolates is shown in **Figs. 12-14**.

Quantitative Western blotting

This is performed after reconstitution in appropriate buffers and by using poly- or monoclonal antibodies directed either to total ATIs or to all or major ATI species. This works best without proteolytic digest.

High titer antibodies have been produced against a mixture of ATIs extracted from hexaploid wheat and against several antigenic epitopes of major ATIs such as CM3, CM16, 0.19 or 0.54. Examples of Western blot characterization are shown in **Fig. 14C**.

Up to 16 ATI species have been described in what based on genetic analysis. However, a subset of ATIs usually represent >90% of all ATIs. These are noncovalently linked tetrameric CM2, CM3, CM16, dimeric 0.19, 0.54, and monomers of e.g. 0.19 and 0.54 (Altenbach et al, BMC Res Notes 2011; Dupont et al, Proteome Sci 2011). Our data indicate that TLR4-stimulatory ability as highest for di- and tetrameric ATIs and lowest for monomeric ATIs. Quantification of the major 3-8 ATI species in a given wheat, rye, or barley variant will approximate total ATI quantification. If desired, also the minor species can be quantified.

ELISA or similar quantitative assays

These work best both without and with (limited) proteolytic digest. They are based on monoclonal or polyclonal antibodies produced against antigenic (linear as well as structural epitopes of the major ATIs). In a classical ELISA variant, one of the antibodies is bound to a solid phase, the other is linked to a detection system, such as biotin, alkaline phosphatase, peroxidase, fluorescent, or luminescent indicators, and state-of the art detection systems. This also includes assays based on inhibition which require, e.g., a coated antibody and a labeled ATI standard to compete with ATIs in the unknown sample. Other state of the art assays such as radioimmunoassays (e.g., equilibrium or inhibition format) or bead assays can also be used, including quantification by multiplexed assays.

Notably, we observed that ELISA quantification of 2-3 of the major ATI species (CM3, CM2, 0.19, 0.28, and 0.53/0.54) correlates well with total ATI bioactivity in extracts of wheat and other bioactive ATI containing cereals and foods (data not shown).

Example 3 - Methods to prevent correct intramolecular disulfide formation in ATIs during grain growth or filling

The equilibrium between oxidized glutathione (GSSG) and protein bound glutathione (PSSG) shifts towards PSSG during the grain filling phase, e.g., the accumulation of storage proteins, mainly gluten (Bykova et al., Phytochem 2011; Ferreira et al, Plant Physiol Biochem 2012), prevents premature intra- and intermoleular disulfide bonding. Fixing the growing wheat plant in this redox state, characterized by PSSG will also prevent disulfide bonding within ATI molecules, and thus make the ATIs highly susceptible to denaturation and proteolytic degradation, either before or after further processing of the wheat flour. Similarly, excessive and continuous induction of the plant's early developmental thioredoxin enzymatic system, also in conjunction with enhancement of PSSG complex formation, will keep nascent ATI molecules in the non-disulfide linked state, making them susceptible to proteolytic digestion (Wong et al, Phytochem 2004; Wong et al, Plant Cell Physiol 2004). These measures will also make wheat (rye, baley) flour more susceptible to proteolytic digestion in the human body.

Methods to promote the formation of PSSG bonds are cultivation with excess GSH or GSSG, the cultivation with or induction of enzymes that decrease the plant's redox potential such as superoxide dismutase or catalase activities. An alternative strategy is modulation of sulphur supply to the growing wheat (rye, barley), e.g., via use of sulphur rich vs. sulphur depleted fertilizers, also in sequence. Thus under sulphur depletion, resultant flour proteins including ATIs are more fixed in their disulfide structures and less susceptible to proteolytic degradation by external proteases or proteases if the gastrointestinal tract (Reinbold et al, J Agricult Food Chem 2008), although ATI content is (slightly) decreased.

Example 4 - Methods to remove ATIs or abolish ATI bioactivity from complex foods by thorough extraction from, e.g., wheat (rye, barley, or by fermentation and their ancestral grains) prior to introduction into the food chain

ATIs are quite resistant to degradation by many known proteases, including digestive enzymes of the human gastrointestinal tract such as pepsin, trypsin and chymotrypsin. Much of this resistance is due to the presence of several, usually five, intramolecular disulfide bonds that generate a compact secondary structure which is usually composed of four major alpha helices which are arranged up-and-down, securing a compact structure and hydrophobic interaction surfaces for di- and tetramerization (Oda et al, Biochemistry 1997). Useful methods to significantly decrease (>70%, preferably 80% and more) bioactive ATIs in e.g., flours are therefore based on 1) exhaustive extraction using preferably water based buffers, and 2) disulfide reduction which facilitates unfolding, eliminate bioactivity (i.e., activation of the TLR4-complex) and permit proteolytic digestion either before or during ingestion of the thus prepared foods. Proteolytic digestion of the reduced and unfolded ATIs can be achieved easily with common proteases used in the food industry, including e.g., pepsin at acidic pH,

or e.g. trypsin, chymotrypsin or subtilisin at near neutral or alkaline pH. Enzymes that degrade unfolded or denatured ATIs are also produced during certain fermentation processes, as described below, including sourdough fermentation or reductive fermentation. This also includes fermentations that produce specific enzyme activities to cleave (unfolded, denatured) ATIs, including bacteria that have been engineered to produce such activity for the food industry.

4.1 Chemical Methods (See also Figs..5, 6, and 11)

Solvent extraction of ATIs

ATIs can be extracted from flours and even processed foods using the methods described above (1.1.1 and 1.1.2). Extraction with water-based buffers is preferable due to low costs and lack of potential toxicity of residual solvent. The extracted flour (food) can be separated mechanically, e.g., by sedimentation or centrifugation, and then freeze-dried or (preferably) dried in air or other (inert) gases that permit drying of bulk quantities. Before drying the pellet, other steps, such as washes with water and/or with (organic) solvents that have a lower boiling point than water but bind residual water, such as acetone, ethanol, or similar solvents used in the food industry can be used for removal of traces of salt or acid (from the extraction buffer), and residual water. Residual water can also be retained when the extracted flour is frozen for storage and transport, or directly used for further food processing, such as fermentation or dough preparation.

Removal of 80-90% of bioactive ATIs can be reached with 1-2 extractions, as detailed in Example 1. This reduction would highly significantly reduce the risk of ATI-induced inflammation in the intestine and outside of the intestine in predisposed individuals, since nutritional ATI effects are dose dependent.

Chemical reduction of disulfides in ATIs.

These methods are similar to those detailed for analytical (quantitative) extraction of ATIs (described above). However, here the major aim is reductive inactivation of biologically active ATIs, in order to eliminate their TLR4 stimulatory activity, without toxicity to the consumer.

Example: Reductive inactivation of ATIs.

100 g of the (dried) flour or food is incubated in neutral, near neutral, or alkaline solvents/buffers, such as water or saline with pH adjustment, or with PBS and a reducing agent, such as cysteine, or glutathione (GSH) balanced with oxidized glutathione (GSSG), at temperatures that can range between 4°C and 100°C, for times that can range between one second and 24 hrs. Other usually mildly reducing agents that are known in the food industry can also be used. Reduced ATIs are preferably denatured at temperatures above 50°C and can be locked in this denatured state by disulfide linkage with, e.g., cysteine or GSH, or via mis-folded, newly generated, intra- and intermolecular disulfide bonds. Other free SH-group blocking agents used in the food industry can also be employed.

Fixation of partly reduced and thus severed intramolecular disulfides in ATIs can also be brought about during dough mixing by the addition of vitamin C or other pro-oxidative systems that generate, e.g., activated oxygen. This has been demonstrated for disulfides in glutenins (Koehler P et al, J Agricult Food Chem 2003a and b), but is also effective in ATIs.

4.2 Fermentation strategies to decrease bioactive ATI in foods such as flours

There are several fermentation methods that reduce ATI bioactivity, largely being based on non-reductive or reductive fermentation and/or proteolytic digestion of (disulfide reduced) ATIs.

Fermentation of wheat (rye, barley) dough with lactobacilli is used to enhance the degradation of gluten proteins under acidic conditions. Select lactobacilli that have an active thiol (disulfide reducing) metabolism are particularly suited to reduce and subsequently degrade ATIs proteolytically. An example are Lactobacillus reuteri and L. sanfranciscensis which efficiently deactivate proteins such as the highly disulfide linked ovotransferrin of wheat as well as ATIs. Proteolytic activity is mainly brought about by wheat aspartic acid proteases (Loponen et al, J Agricult Food Chem 2007; Loponen et al, J Agricult Food Chem 2008), but other proteolytic enzymes used in the food industry can be added. Fermentation of wheat flour and other ATI-containing flours can be done e.g. with varying proportions of rye malt in the dough, such as 5-20%. Here, apart from adding beneficial bacteria that are used in the food industry to prepare e.g. sourdough (Lactobacillus species), rye malt serves as source of proteolytic enzymes that were shown to degrade immunogenic gluten proteins (Loponen et al, J Agricult Food Chem 2007). This method can be combined with any of the other ATI-reducing methods described herein.

Reduction of ATI content by non-reductive and reductive fermentation of ATI-containing flours and doughs

The doughs can be prepared with whole wheat flour and without additives. Optimization is possible using refined instead of crude flours, or addition of common agents that are used in the food industry to improve fermentation or dough quality. An example is the addition of reducing agents, such as cysteine or glutathione, either before, during or after fermentation. Another alternative is the use of fermenting bacteria with high disulfide reducing ability. Such strains have been described and comprise, e.g., Lactobacillus sanfranciscensis and L. reuteri LTH2584. **Fig. 15** shows the results of fermentation experiments (24 hrs) performed. ATI activity was determined in two sequential combined ammonium bicarbonate extractions from the fermented and then frozen doughs, using the methodology described above.

The following fermentation protocols were applied for 24 hrs at 37°C:

i) chemically acidified control (low SH (sulfhydryl)-levels in dough, no reduction of disulfide bonds in proteins);

ii) Lactobacillus sakei (low SH);

iii) Lactobacillus sanfranciscensis (high SH-levels in dough and reduction of disulfide bonds in proteins owing to glutahione reductase activity);

iv) Lactobacillus sanfranciscensis delta gsrH (low SH-levels in dough as gene coding for glutathione reductase gene was disrupted);

v) Lactobacillus reuteri LTH2584 (high SH-levels owing to cystathionine gamma lyase);

vi) a second chemically acidified control to match the growth temperature of L. reuteri.

Controls for appropriate fermentation: Doughs were analysed with respect to pH, organic acid levels and SH-groups, as were the numbers of fermenting bacteria. The data demonstrate an up to

~50-60% reduction in ATI-bioactivity in the sourdoughs that were fermented with Lactobacilli in general, either with or without rye malt addition. This indicates significant proteolytic digestion and thus inactivation of ATIs by fermentation in general, without higher degradation activity by disulfide reducing strains.

Notably, an advantage of the thiol-reducing bacteria (L. sanfranciscensis and L. reuteri) is likely. In a preliminary experiment, post fermentation heating of the dough to elevated temperatures that do not yet inactivate the rye (barley) malt or Lactobacillus-derived proteolytic enzymes (time up to 2 hrs) could further reduce ATI bioactivity down to 20% in the doughs subject to thiol reducing Lactobacilli vs. 30% in the non-thiol-reducing bacteria, indicating an advantage of the former strains. This was accompanied by a comparable reduction of major ATI-bands CM3 and 0.19 in a Western blot using our polyclonal antibodies specific for these protein. Overall, reductive fermentation more than conventional fermentation leads to disulfide reduction of ATIs which then become susceptible to proteolytic degradation by enzymes used in the food industry, such as those produced by Lactobacillae or that are present in malt.

Example 5 - TLR4 antagonistic compounds that can be added to ATI containing foods

There are several (weak to moderately active) oral TLR4 antagonists, many of them of herbal origin, with long-term use as spices or food additives. These are e.g. alkaloids, aromatic compounds, polyphenols, peptides or proteins (Rossignal and Lynn 2005; Lucas and Maes, Mol Neurobiol 2013). Examples include, but are not limited to, Bartonella quintana lipopolysaccharide (Popa et al, Infect Immunol 2007); extracts of leaves from Clerodendrum (Kouakou et al, BMC Complement Altern Med 2013); cinnamon extract and its active components (Kanuri et al, J Nutr 2009); eritoran mimicking the MD component of the TLR4-MD2-CD14 complex, formulated to be resistant to gastric or intestinal hydrolysis (Shirey et al, Nature 2013); epigallocatechin-3-gallate from, e.g., green tea (Hong et al, J Immunol 2010; Bao et al, Mol Nutr Food Res 2013); 6-shogaol and 1-dehydro-10-gingerdione (1D10G) from Ginger (Ahn et al, Mol Cless 2009; Park et al, Biochem Biophys Res Communic 2012); Melatonin (Hu et al, Pinela Res 2013); Berberin as TLR4-NFkB pathway inhibitor (Li et al, Acta Pharmacol Sin 2012); Lipopolysaccharide from the photosynthetic bacterium *Rhodobacter sphaeroides* (LPS-RS) (Rallabhandi et al, Coats et al, J Immunol 2005); and Lipopolysaccharide from *Porphyromonas gingivalis* (Coats et al, J Immunol 2005; Curtis et al, Infect Immunol 2011)

Adding these compounds or compound mixtures with proven TLR4 antagonistic activity to ATI-containing foods will antagonize the TLR4-activating activity of ATIs, without need of prior extraction or inactivation. This should be relevant to either completely avoid these prior inactivation or extraction steps, or to "neutralize" residual ATI activity after these steps. We could demonstrate that this neutralization of ATI-induced innate immune activation by using the natural TLR4-antagonist from the photosynthetic bacterium *Rhodobacter sphaeroides* (LPS-RS) (Rallabhandi et al, J Immunol 2005) (**Fig. 16**).

OTHER EMBODIMENTS

All publications, patents, and patent applications mentioned in the above specification are hereby incorporated by reference to the same extent as if each individual publication, patent or patent

application was specifically and individually indicated to be incorporated by reference in its entirety. Various modifications and variations of the described devices and methods of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific embodiments, it will be understood that it is capable of further modifications and that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the art are intended to be within the scope of the invention. This application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known customary practice within the art to which the invention pertains and may be applied to the essential features herein before set forth.

Other embodiments are within the following claims.

CLAIMS

1. A method of reducing the ATI content of a foodstuff, said method comprising incubating said foodstuff in an extraction buffer for a duration sufficient to reduce the ATI content of said foodstuff by at least 50% followed by removing said extraction buffer from said foodstuff.

2. The method of claim 1, wherein said extraction buffer is a neutral, near neutral, or alkaline solvent or buffer.

3. The method of claim 2, wherein said extract buffer is selected from the group consisting of PBS, Tris-buffer, water, and ammonium or sodium bicarbonate.

4. The method of claim 2 or 3, wherein said extract buffer further comprises a detergent or detergent mixture.

5. The method of claim 4, wherein said detergent or detergent mixture is selected from the group consisting of sodiumdodecyl sulfate, Triton X-100, Tween-20, and sodium deoxycholate.

6. The method of any one of claims 1-5, wherein said extraction buffer further comprises a reducing agent.

7. The method of claim 6, wherein said reducing agent is selected from the group consisting of -mercapto-ethanol, dithioerythritol (DTT), cysteine, glutathione (GSH) balanced with oxidized glutathione (GSSG), sulfite, and thioredoxin.

8. The method of any one of claims 1-7, wherein said extraction buffer further comprises at least one protease and/or a synthetic enzyme engineered to reduce and/or degrade ATIs.

9. The method of claim 8, wherein said protease is pepsin, trypsin, chymotrypsin, elastase, thermolysin, bromelain, papain, subtilisin, an alkaline protease, a neutral protease, an acidic protease, a fungal protease, a yeast protease, or a bacterial protease.

10. The method of claim 9, wherein said alkaline protease is a serine protease.

11. The method of any one of claims 1-10, wherein said extraction buffer further comprises an alkylating agent.

12. The method of claim 11, wherein said alkylating agent is iodoacetamide and said alkylating agent is further quenched by the addition of excess reducing agent.

13. The method of any one of claims 1-12, wherein said foodstuff is separated mechanically prior to incubation.

14. The method of claim 13, wherein said mechanically separated foodstuff is dried prior to incubation.

15. The method of claim 14, wherein said drying is air-drying or freeze drying.

16. The method of claim 14 or 15, wherein, prior to said drying, said mechanically separated foodstuff is contacted with an aqueous solvent or an organic solvent having a lower boiling point than water.

17. The method of claim 16, wherein said organic solvent is selected from the group consisting of acetone and ethanol.

18. A method of extracting amylase trypsin inhibitors (ATIs) from a foodstuff, said method comprising:
A) incubating said foodstuff with an extraction buffer in a mixture;
B) centrifuging said mixture to form a pellet and supernatant;
C) drying said supernatant; and
D) reconstituting the dried supernatant in a reconstitution buffer,
wherein extracted ATIs are present in said reconstitution buffer.

19. The method of claim 18, wherein said foodstuff is unprocessed prior to extraction.

20. The method of claim 18, wherein said foodstuff is a processed foodstuff.

21. The method of claim 18, wherein said foodstuff is gluten.

22. The method of any one of claims 18-21, wherein, prior to step A, said foodstuff is ground.

23. The method of any one of claims 18-22, wherein, prior to step A, said foodstuff is defatted.

24. The method of claim 23, wherein said defatting is performed by the incubation of said foodstuff with methanol/diethylether.

25. The method of claim 24, wherein said methanol/diethyl ether is present at a 1:1 ratio.

26. The method of any one of claims 1-25, wherein said extraction buffer is ammonium or sodium bicarbonate, chloroform/methanol, or an acid.

27. The method of claim 26, wherein said acid is acetic acid.

28. The method of claim 26 or 27, wherein said extraction buffer is at a concentration between 5 mM to 100 mM.

29. The method of any one of claims 18-28, wherein said drying is freeze-drying or drying under a stream of gas, e.g., air.

30. The method of any one of claims 18-29, wherein steps A-C are repeated prior to step D.

31. The method of claim 30, wherein the extraction buffer in steps A-C is first chloroform/methanol and the extraction buffer is ammonium or sodium bicarbonate when steps A-C are repeated or wherein the extraction buffer is ammonium or sodium bicarbonate in both extractions.

32. The method of any one of claims 18-31, wherein, prior to step A, the foodstuff is digested with at least one protease and/or a synthetic enzyme engineered to reduce and/or degrade ATIs.

33. The method of claim 32, wherein said protease is pepsin, trypsin, chymotrypsin, elastase, thermolysin, bromelain, papain, subtilisin, an alkaline protease, a neutral protease, an acidic protease, a fungal protease, a yeast protease, or a bacterial protease.

34. The method of claim 33, wherein said alkaline protease is a serine protease.

35. The method of claim 33, wherein said foodstuff is first digested with pepsin followed by digestion with trypsin and/or chymotrypsin.

36. The method of any one of claims 32-35, wherein, after the foodstuff is digested with a protease, the foodstuff is centrifuged to form a supernatant and said supernatant is incubated with the extraction buffer of step A.

37. The method of claim 36, wherein said incubation is performed as a dialysis incubation, step B is omitted, and said supernatant, after dialysis is dried in step C.

38. A method of determining the bioactivity of ATIs in a sample, said method comprising incubating ATI-reactive cells with an extract suspected of comprising ATIs and determining an ATI-response in said ATI-reactive cells, thereby determining the bioactivity of said ATIs in a sample.

39. The method of claim 38, wherein said extract suspected of comprising ATIs is prepared by the method of any one of claims 1-37.

40. The method of claim 38 or 39, wherein said ATI-reactive cells express TLR4, TLR4-MD2, or TLR4-MD2-CD14 signaling complex.

41. The method of any one of claims 38-40, wherein said ATI-reactive cells are selected from the group consisting of monocytes/macrophages, dendritic cells, and cells transfected with a TLR4, TLR4-MD2, or TLR4-MD2-CD14 oligomeric signaling complex.

42. The method of claim 41, wherein said cells are stable cell lines selected from the group consisting of THP-1 and U937 cells.

43. The method of any one of claims 38-42, wherein said determination of ATI-response comprises quantification of one or more cytokines released by said cells.

44. The method of claim 43, wherein said one or more cytokines are selected from the group consisting of IL-8, IL-15, TNFα, CCL2 (MCP-1), and CCL5 (RANTES).

45. The method of claim 43 or 44, wherein said quantification is performed by ELISA.

46. The method of any one of claims 38-42, wherein said cell comprises a reporter gene under the control of a TLR4 (ATI)-responsive promoter and said determination of an ATI-response comprises detection of expression of said reporter gene.

47. The method of claim 46, wherein said ATI-responsive promoter is the IL-8 promoter.

48. The method of claim 46 or 47, wherein said reporter gene encodes a luminescent or fluorescent protein.

49. The method of any one of claims 38-42, wherein said determination of ATI-response comprises measuring a level of one or more RNAs in said cells associated with an ATI-response.

50. The method of claim 49, wherein said one or more RNAs corresponds to Hsp27, Hsp70, and/or Hsp90.

51. The method of any one of claims 38-42, wherein said determination of ATI-response comprises detecting one or more activation markers on or in said cells.

52. The method of claim 51, wherein said one or more activation markers is selected from the group consisting of CD80, CD86, MHC class II, IL-12, IL-23, and interferon gamma (release or intracellular stain).

53. The method of claim 51 or 52, wherein said detecting comprises flow-cytometry.

54. The method of any one of claims 38-42, wherein said determination of ATI-response comprises detecting an alteration is a signaling pathway associated with an ATI response.

55. The method of claim 54, wherein said alteration in a signaling pathway is detected based on the detection of IRF-3, NFkB (p65), or NFkB nuclear translocation.

56. The method of any one of claims 38-42, wherein said determination of ATI-response comprises contacting ovalbumin or gluten specific T cells in the presence of said sample suspected of containing ATIs and measuring the proliferation of said cells in the presence of ATI-reactive antigen presenting cells.

57. A method of determining the bioactivity of ATIs in a sample, said method comprising

A) incubating an immobilized TLR4, TLR4-MD2, or TLR4-MD2-CD14 with an extract suspected of comprising ATIs, wherein said immobilized TLR4, TLR4-MD2, or TLR4-MD2-CD14 is immobilized to a solid phase and bound to a labeled ATI,

B) removing the extract, and

C) determining the amount of labeled ATI in the extract or bound to said immobilized TLR4, TLR4-MD2, or TLR4-MD2-CD14 that is immobilized to the solid phase.

58. The method of claim 57, wherein said labeled ATI is labeled with a fluorophore.

59. The method of claim 57, wherein said labeled ATI is labeled with an enzyme.

60. The method of claim 59, wherein said enzyme is horseradish peroxidase or alkaline phosphatase.

61. A method of determining the bioactivity of ATIs in a sample, said method comprising

A) incubating an immobilized TLR4, TLR4-MD2, or TLR4-MD2-CD14 with an extract suspected of comprising ATIs,

B) removing the extract suspected of comprising ATIs, and

C) determining the amount of ATIs that are bound to said immobilized TLR4 using an antibody in an ELISA.

62. The method of claim 61, wherein said antibody recognizes and binds to at least one ATI selected from the group consisting of CM3, CM2, 0.19, 0.28, and 0.53/0.54.

63. A method of quantifying the amount of ATIs in a foodstuff, said method comprising:

A) incubating said foodstuff in an extraction buffer thereby forming an extract;

B) optionally incubating said extract with an alkylating agent;

C) optionally separating the ATIs from said extract; and

D) quantifying said ATIs.

64. The method of claim 63, wherein said extraction buffer is a neutral, near neutral, or an alkaline solvent or buffer.

65. The method of claim 64, wherein said extract buffer is selected from the group consisting of PBS, Tris-buffer, and ammonium or sodium bicarbonate.

66. The method of claim 64 or 65, wherein said extract buffer further comprises a detergent or detergent mixture.

67. The method of claim 66, wherein said detergent or detergent mixture is selected from the group consisting of sodium dodecyl sulfate, Triton X-100, Tween-20, and sodium deoxycholate.

68. The method of any one of claims 63-67, wherein said extraction buffer further comprises a reducing agent.

69. The method of claim 68, wherein said reducing agent is selected from the group consisting of mercapto-ethanol, dithioerythritol (DTT), cysteine, glutathione (GSH) balanced with oxidized glutathione (GSSG), sulfite, and thioredoxin.

70. The method of any one of claims 63-69, wherein said extraction buffer further comprises an alkylating agent.

71. The method of claim 70, wherein said alkylating agent is iodoacetamide and said alkylating agent is further quenched by the addition of excess reducing agent.

72. The method of any one of claims 63-71, wherein said method further comprises adding at least one protease and/or a synthetic enzyme engineered to reduce and/or degrade ATIs to said extract.

73. The method of claim 72, wherein said protease is pepsin, trypsin, chymotrypsin, elastase, thermolysin, bromelain, papain, subtilisin, an alkaline protease, a neutral protease, an acidic protease, a fungal protease, a yeast protease, or a bacterial protease.

74. The method of claim 73, wherein said protease is pepsin.

75. The method of any one of claims 63-74, wherein said ATIs are quantified by mass spectrometry, quantitative western blotting, or ELISA.

76. A method of producing a foodstuff having a reduced ATI content or is free of ATIs, said method comprising

A) determining the bioactivity of ATIs in the foodstuff, and/or

B) quantifying the amount of ATIs in the foodstuff, and

C) reducing the amount of ATIs in the foodstuff by extracting and degrading ATIs using one or more reducing agents, proteases, and/or synthetic enzymes engineered to reduce and/or degrade ATIs in the food stuff.

77. A method of reducing the reactivity of a foodstuff, said method comprising cultivating a cereal with oxidized glutathione (GSSG) and/or protein bound glutathione (PSSG).

78. The method of claim 77, further comprising cultivating the cereal with a balanced amount of reduced glutathione (GSH).

79. A method of reducing the ATI/TLR4-reactivity of a foodstuff, said method comprising cultivating a cereal with sulfur rich or sulfur depleted fertilizers.

80. A method of reducing the ATI content of a foodstuff, said method comprising fermenting said foodstuff with a bacteria with high disulfide reducing and/or proteolytic ability.

81. The method of claim 80, wherein said bacteria is selected from the group consisting of *Lactobacillus sanfranciscensis* and *L. reuteri LTH2584*.

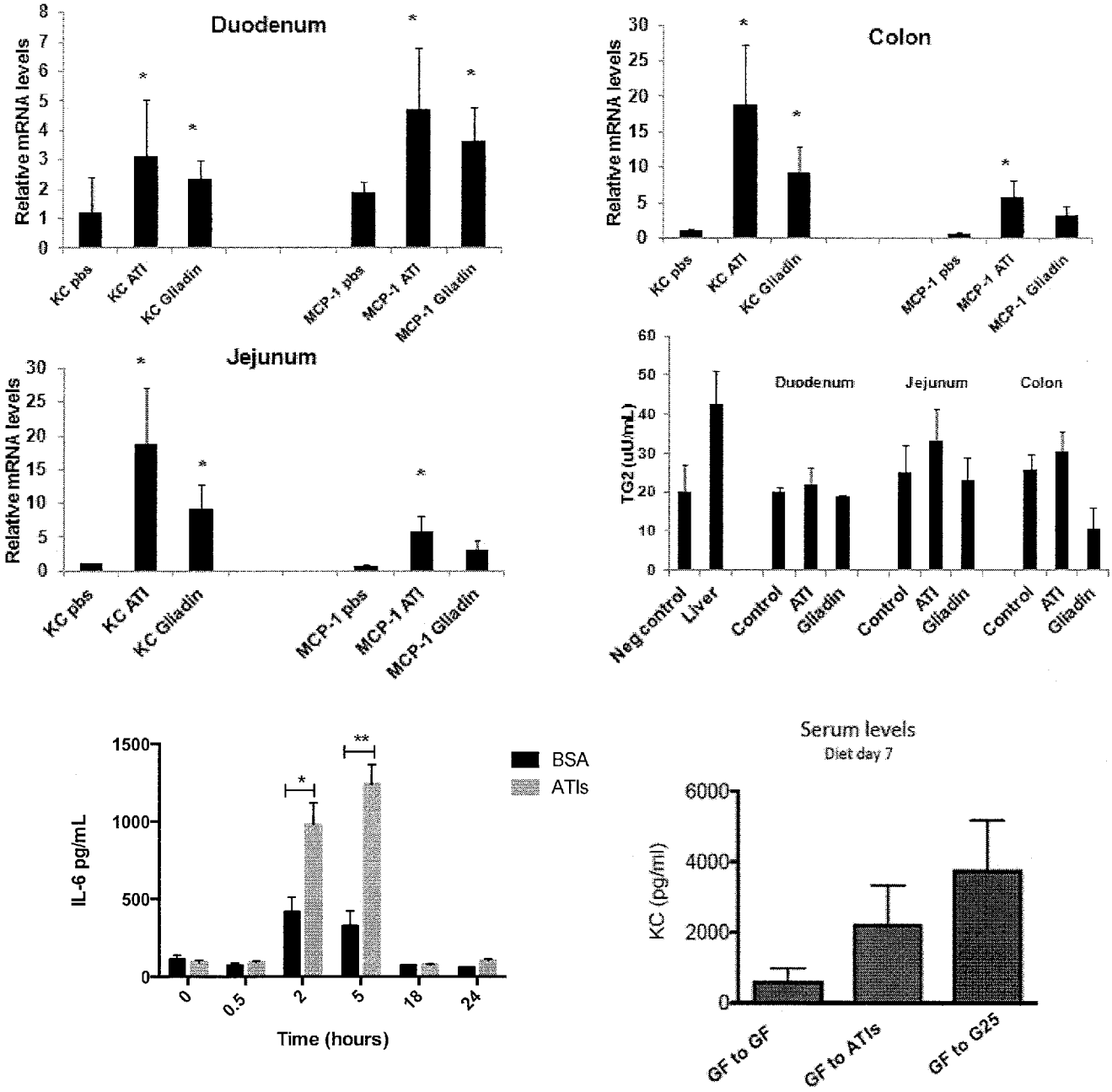

Duodenum
Relative mRNA levels
KC pbs
KC ATI
KC Gliadin
MCP-1 pbs
MCP-1 ATI
MCP-1 Gliadin
Colon
Jejunum
Duodenum
Jejunum
Colon
TG2 (uU/mL)
Neg control
Liver
Control
ATI
Gliadin
IL-6 pg/mL
Time (hours)
BSA
ATIs
Serum levels
Diet day 7
KC (pg/ml)
GF to GF
GF to ATIs
GF to G25

FIG. 1A

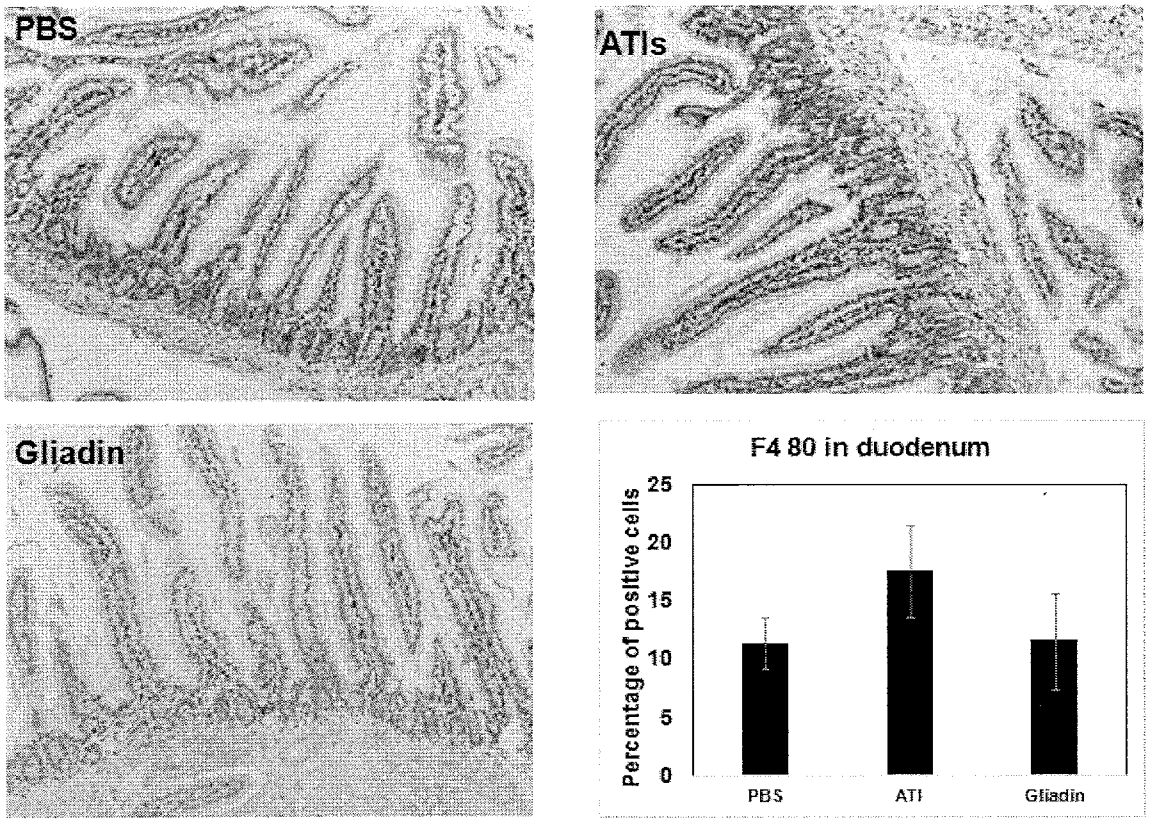

PBS
ATIs
Gliadin
F4 80 in duodenum
Percentage of positive cells
25
20
15
10
5
0
PBS
ATI
Gliadin

FIG. 1B

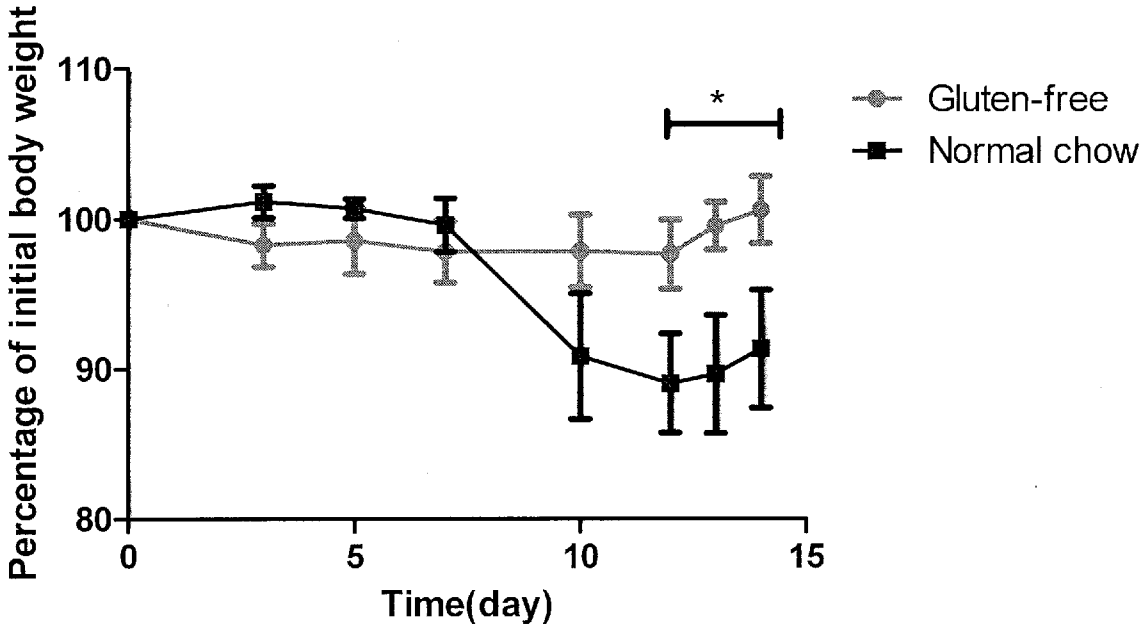

Percentage of initial body weight
110
100
90
80
0
5
10
15
Time(day)
*
Gluten-free
Normal chow

FIG. 2

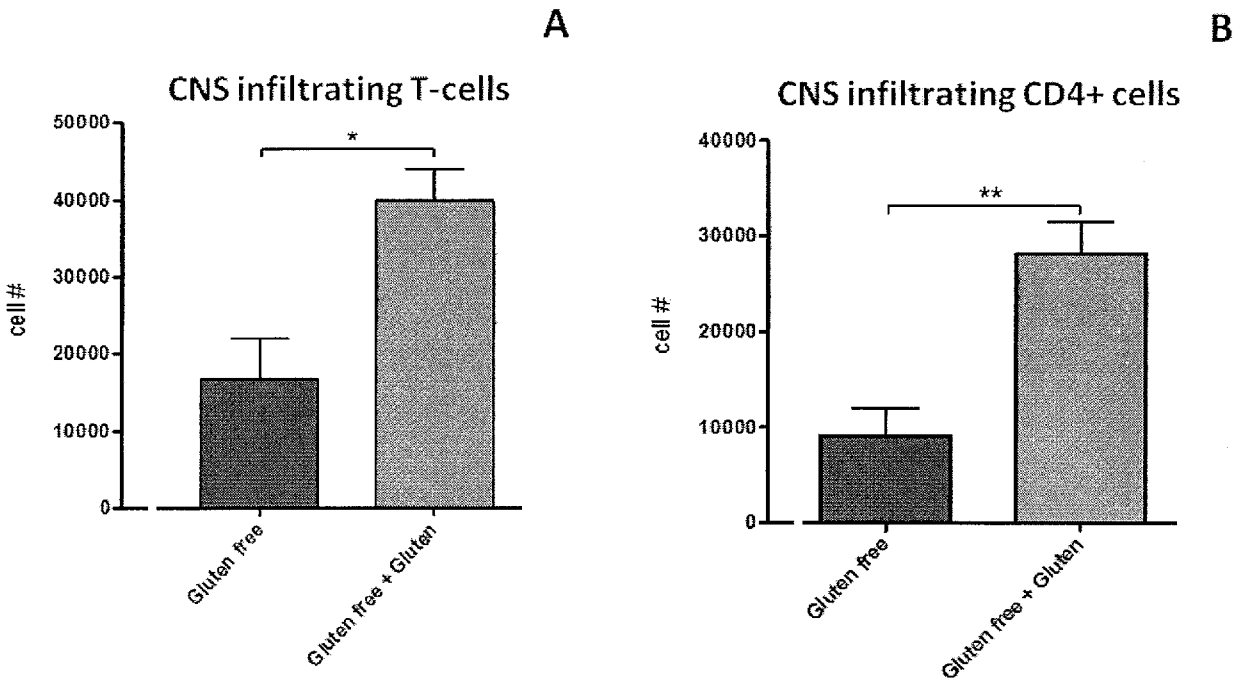

A
CNS infiltrating T-cells
cell #
50000
40000
30000
20000
10000
0
*
Gluten free
Gluten free + Gluten
B
CNS infiltrating CD4+ cells
cell #
40000
30000
20000
10000
0
**
Gluten free
Gluten free + Gluten

FIG. 3A-3B

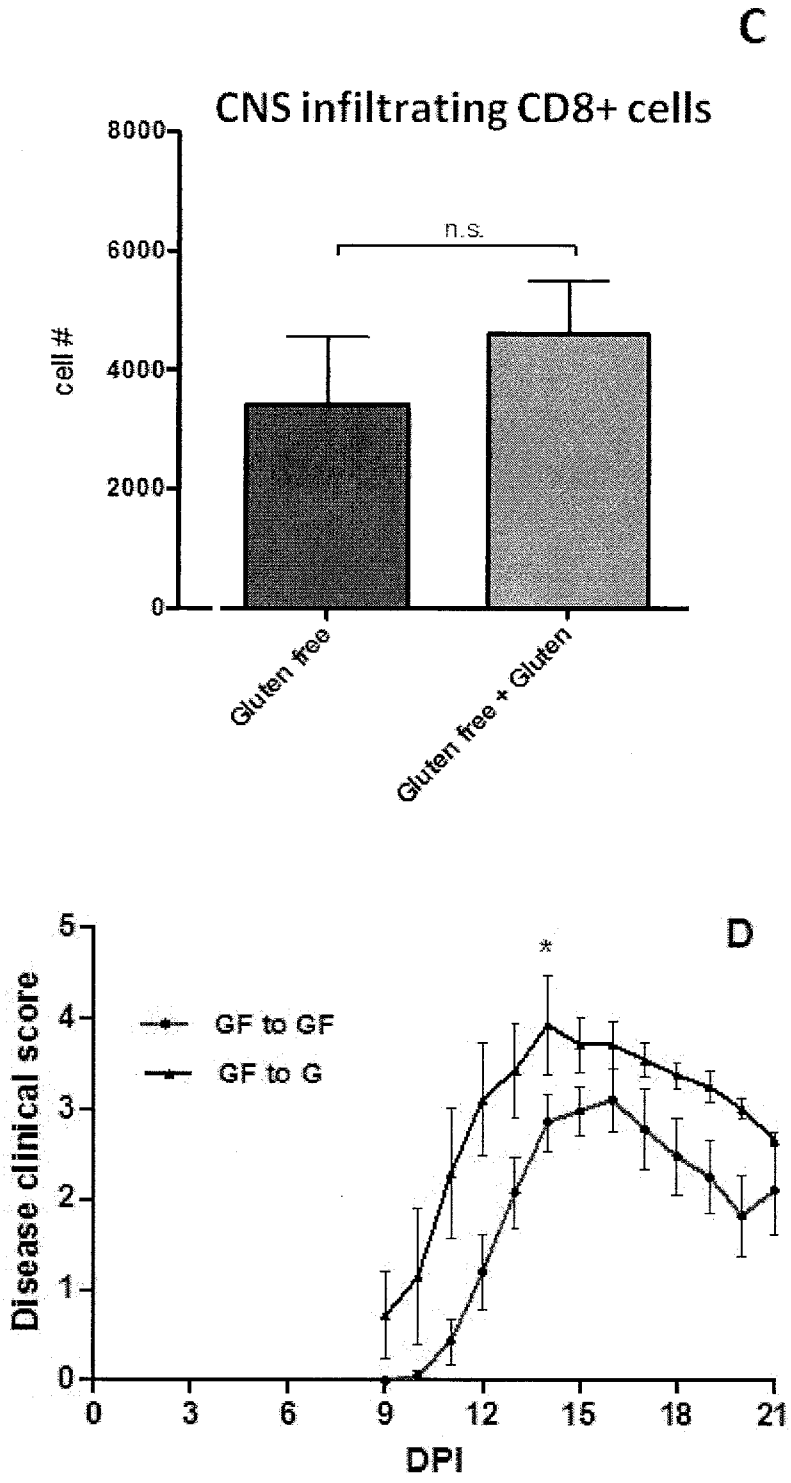

C
CNS infiltrating CD8+ cells
8000
6000
4000
2000
0
cell #
n.s.
Gluten free
Gluten free + Gluten
D
Disease clinical score
5
4
3
2
1
0
GF to GF
GF to G
*
0
3
6
9
12
15
18
21
DPI

FIG. 3C-3D

E

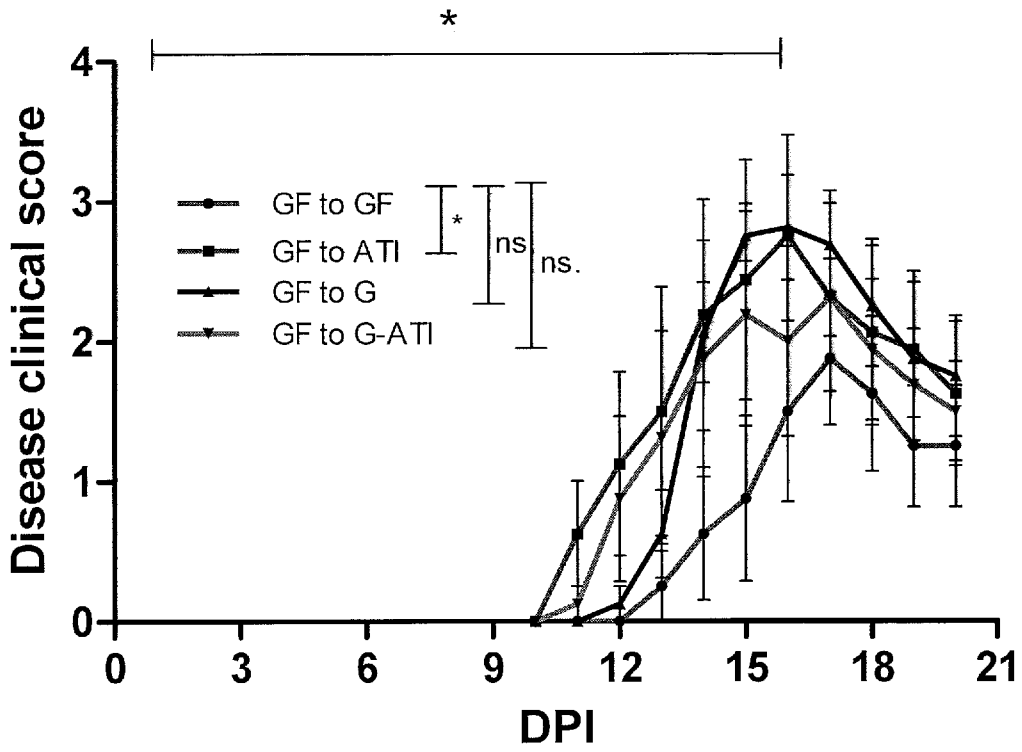

*
Disease clinical score
4
3
2
1
0
GF to GF
GF to ATI
GF to G
GF to G-ATI
*
ns
ns.
0
3
6
9
12
15
18
21
DPI

FIG. 3E

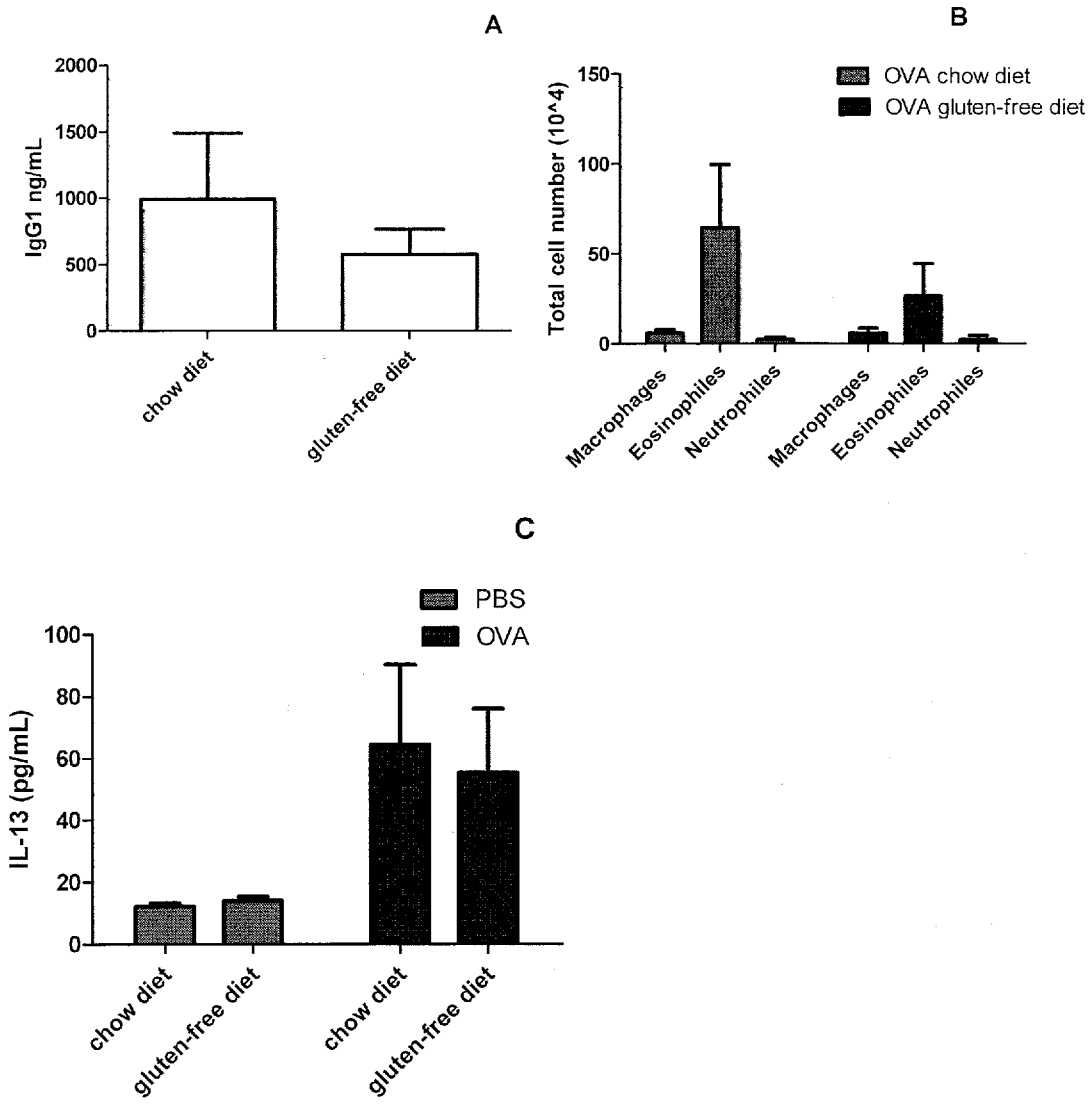

A
IgG1 ng/mL
2000
1500
1000
500
0
chow diet
gluten-free diet
B
Total cell number (10^4)
150
100
50
0
OVA chow diet
OVA gluten-free diet
Macrophages
Eosinophiles
Neutrophiles
Macrophages
Eosinophiles
Neutrophiles
C
IL-13 (pg/mL)
100
80
60
40
20
0
PBS
OVA
chow diet
gluten-free diet
chow diet
gluten-free diet

FIG. 4A-4C

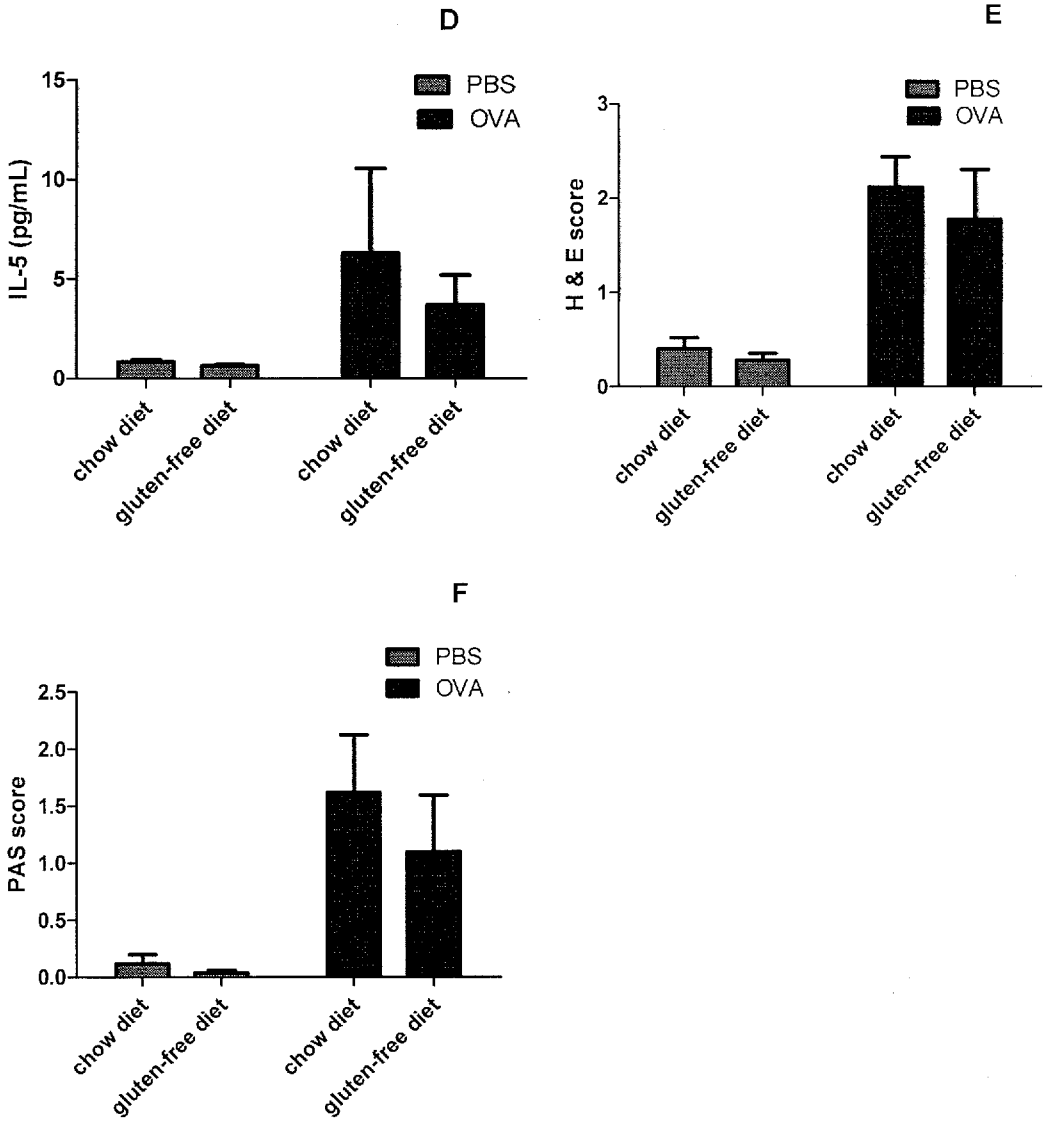

D
PBS
OVA
IL-5 (pg/mL)
15
10
5
0
chow diet
gluten-free diet
chow diet
gluten-free diet
E
PBS
OVA
H & E score
3
2
1
0
chow diet
gluten-free diet
chow diet
gluten-free diet
F
PBS
OVA
PAS score
2.5
2.0
1.5
1.0
0.5
0.0
chow diet
gluten-free diet
chow diet
gluten-free diet

FIG. 4D-4F

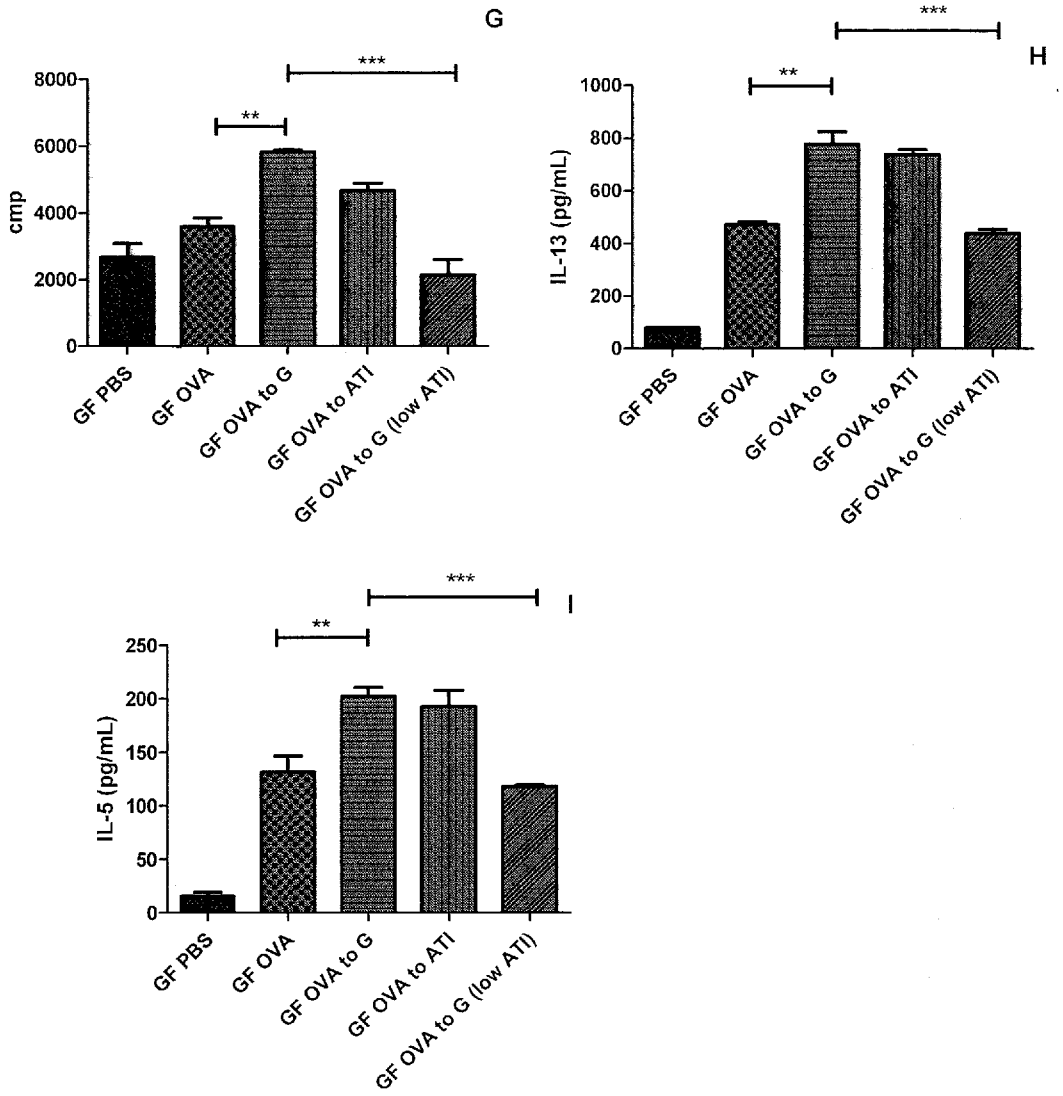

G
cmp
8000
6000
4000
2000
0
**
***
GF PBS
GF OVA
GF OVA to G
GF OVA to ATI
GF OVA to G (low ATI)
H
IL-13 (pg/mL)
1000
800
600
400
200
0
**
***
GF PBS
GF OVA
GF OVA to G
GF OVA to ATI
GF OVA to G (low ATI)
I
IL-5 (pg/mL)
250
200
150
100
50
0
**
***
GF PBS
GF OVA
GF OVA to G
GF OVA to ATI
GF OVA to G (low ATI)

FIG. 4G-4I

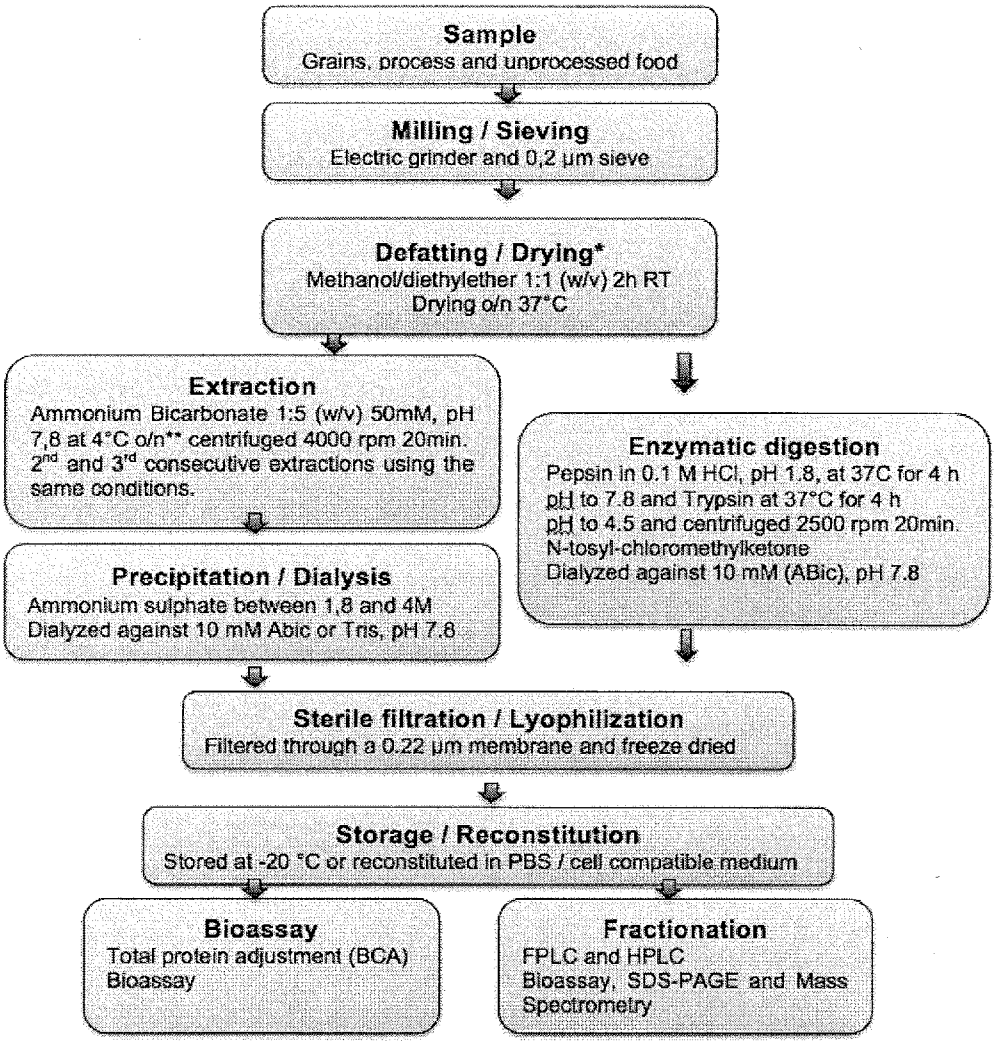
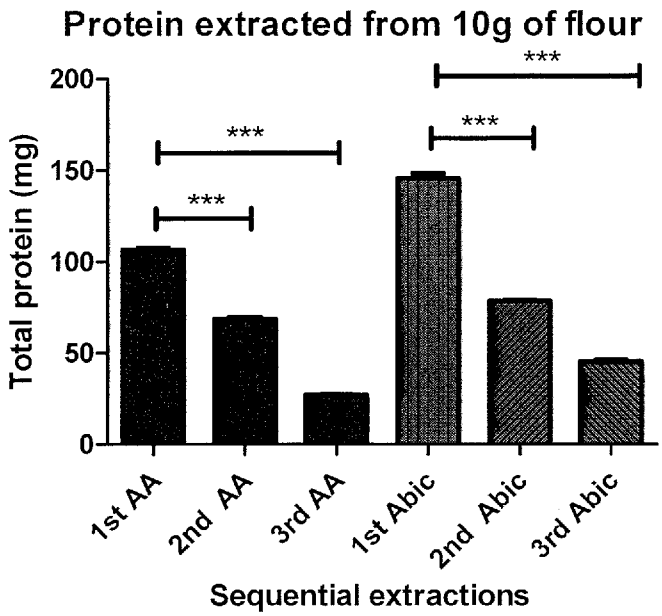

Sample
Grains, process and unprocessed food
Milling / Sieving
Electric grinder and 0,2 µm sieve
Defatting / Drying*
Methanol/diethylether 1:1 (w/v) 2h RT
Drying o/n 37°C
Extraction
Ammonium Bicarbonate 1:5 (w/v) 50mM, pH 7,8 at 4°C o/n** centrifuged 4000 rpm 20min. 2nd and 3rd consecutive extractions using the same conditions.
Enzymatic digestion
Pepsin in 0.1 M HCl, pH 1.8, at 37C for 4 h
pH to 7.8 and Trypsin at 37°C for 4 h
pH to 4.5 and centrifuged 2500 rpm 20min.
N-tosyl-chloromethylketone
Dialyzed against 10 mM (ABic), pH 7.8
Precipitation / Dialysis
Ammonium sulphate between 1,8 and 4M
Dialyzed against 10 mM Abic or Tris, pH 7.8
Sterile filtration / Lyophilization
Filtered through a 0.22 µm membrane and freeze dried
Storage / Reconstitution
Stored at -20 °C or reconstituted in PBS / cell compatible medium
Bioassay
Total protein adjustment (BCA)
Bioassay
Fractionation
FPLC and HPLC
Bioassay, SDS-PAGE and Mass Spectrometry

FIG. 5

A


Protein extracted from 10g of flour
Total protein (mg)
200
150
100
50
0
***
***
***
***
1st AA
2nd AA
3rd AA
1st Abic
2nd Abic
3rd Abic
Sequential extractions

B

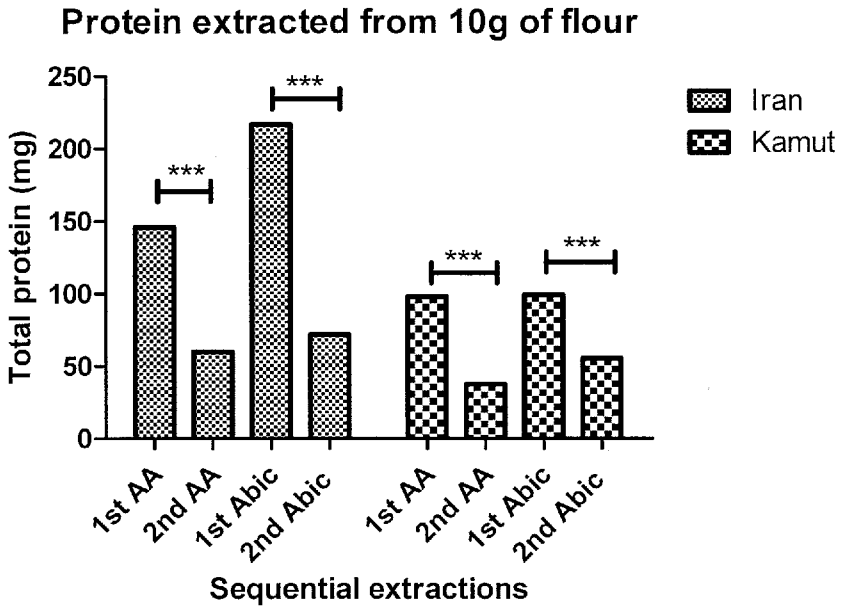

Protein extracted from 10g of flour
Total protein (mg)
250
200
150
100
50
0
***
***
***
***
Iran
Kamut
1st AA
2nd AA
1st Abic
2nd Abic
1st AA
2nd AA
1st Abic
2nd Abic
Sequential extractions

FIG. 6A-6B

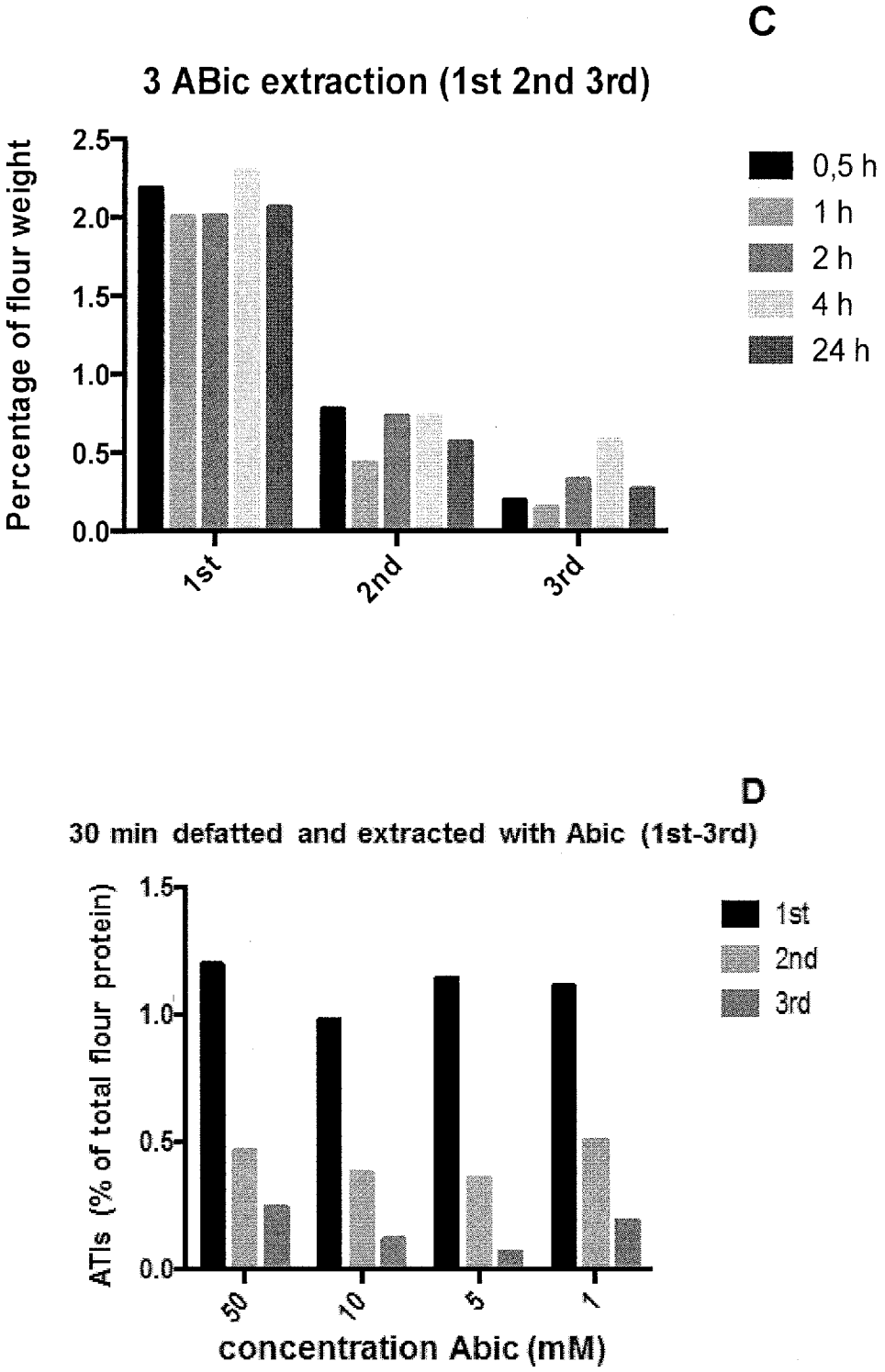

C
3 ABic extraction (1st 2nd 3rd)
Percentage of flour weight
2.5
2.0
1.5
1.0
0.5
0.0
1st
2nd
3rd
0,5 h
1 h
2 h
4 h
24 h
D
30 min defatted and extracted with Abic (1st-3rd)
ATIs (% of total flour protein)
1.5
1.0
0.5
0.0
50
10
5
1
concentration Abic (mM)
1st
2nd
3rd

FIG. 6C-6D

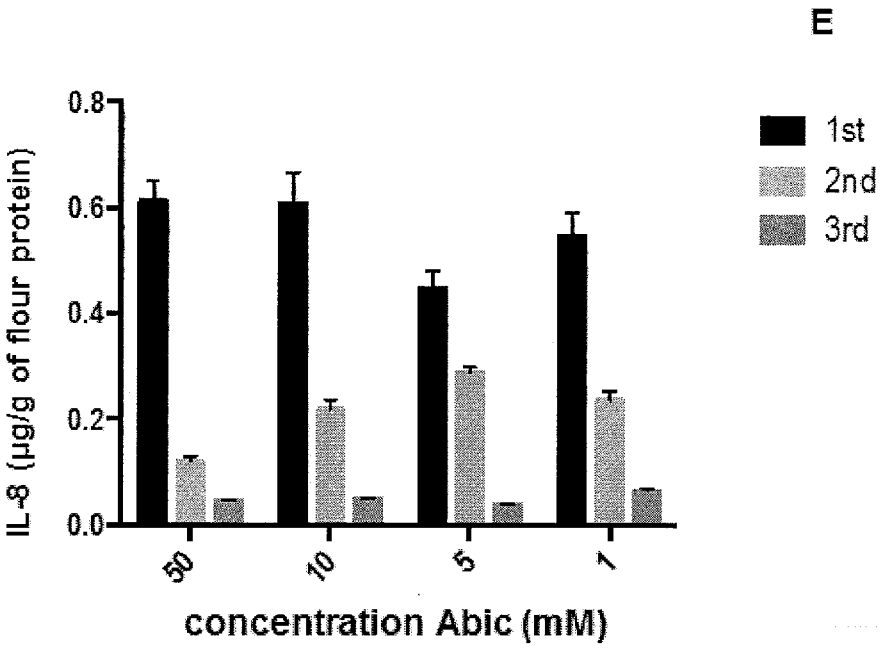

E
1st
2nd
3rd
IL-8 (µg/g of flour protein)
0.8
0.6
0.4
0.2
0.0
50
10
5
1
concentration Abic (mM)

FIG. 6E

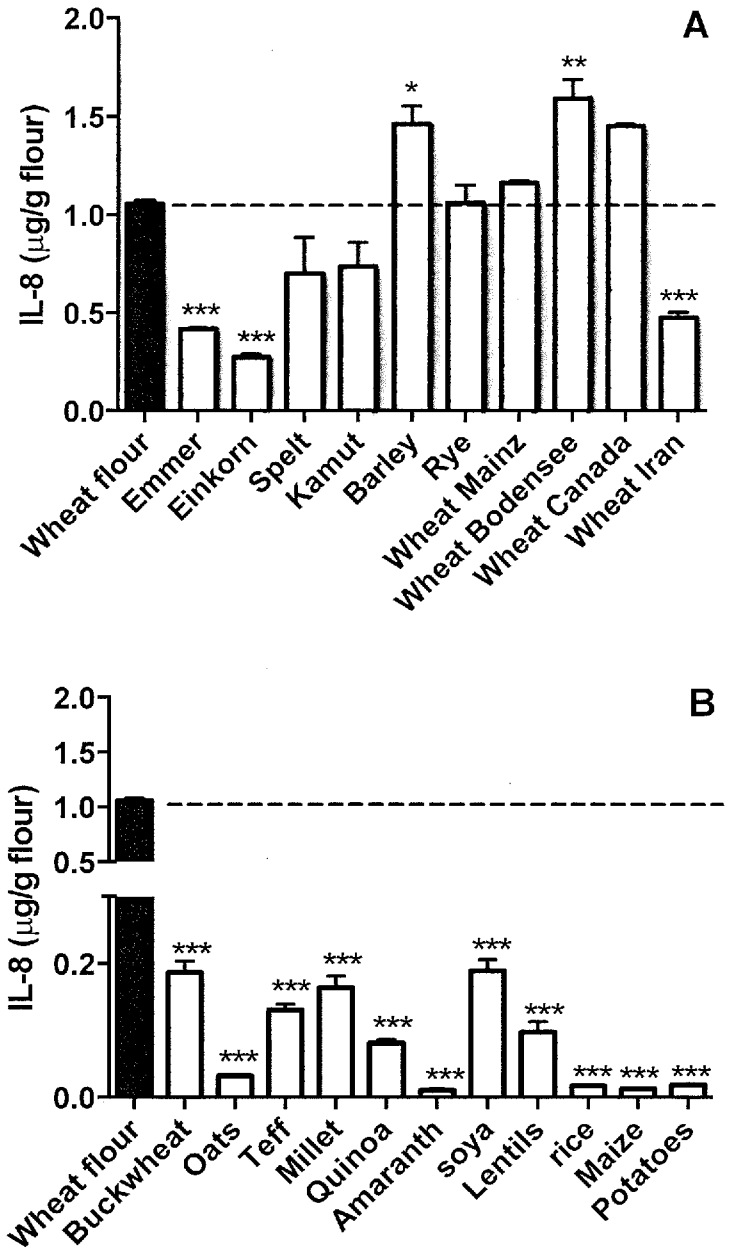
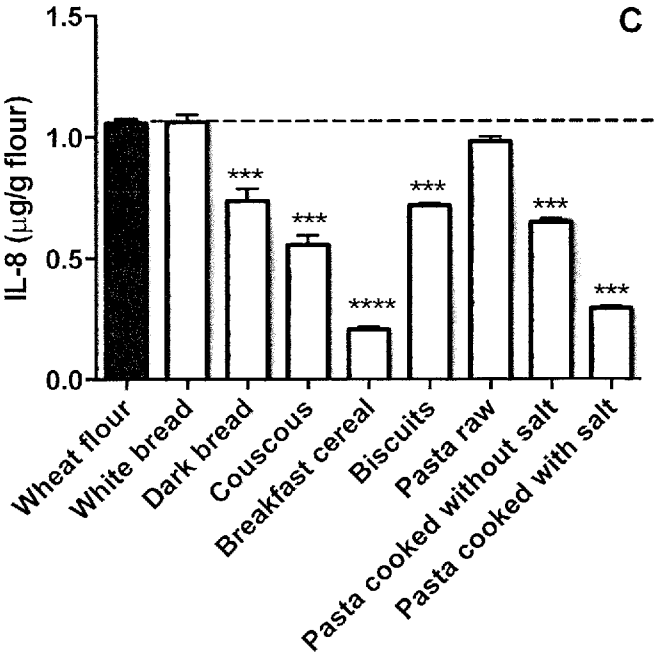

A
IL-8 (µg/g flour)
2.0
1.5
1.0
0.5
0.0
***
***
*
**
***
Wheat flour
Emmer
Einkorn
Spelt
Kamut
Barley
Rye
Wheat Mainz
Wheat Bodensee
Wheat Canada
Wheat Iran
B
IL-8 (µg/g flour)
2.0
1.5
1.0
0.5
0.2
0.0
***
***
***
***
***
***
***
***
***
***
Wheat flour
Buckwheat
Oats
Teff
Millet
Quinoa
Amaranth
soya
Lentils
rice
Maize
Potatoes

FIG. 7A-7B


C
IL-8 (µg/g flour)
1.5
1.0
0.5
0.0
***
***
****
***
***
***
Wheat flour
White bread
Dark bread
Couscous
Breakfast cereal
Biscuits
Pasta raw
Pasta cooked without salt
Pasta cooked with salt

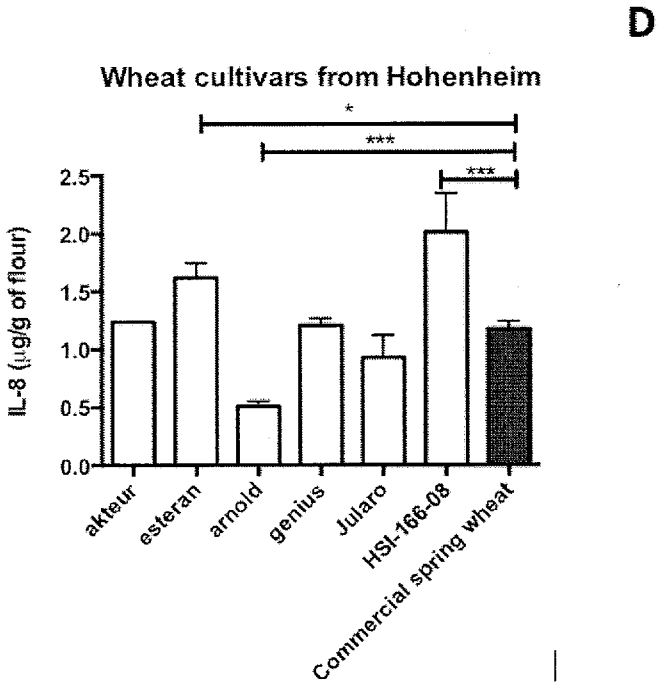

D
Wheat cultivars from Hohenheim
*
***
***
IL-8 (µg/g of flour)
2.5
2.0
1.5
1.0
0.5
0.0
akteur
esteran
arnold
genius
Jularo
HSI-166-08
Commercial spring wheat

FIG. 7C-7D

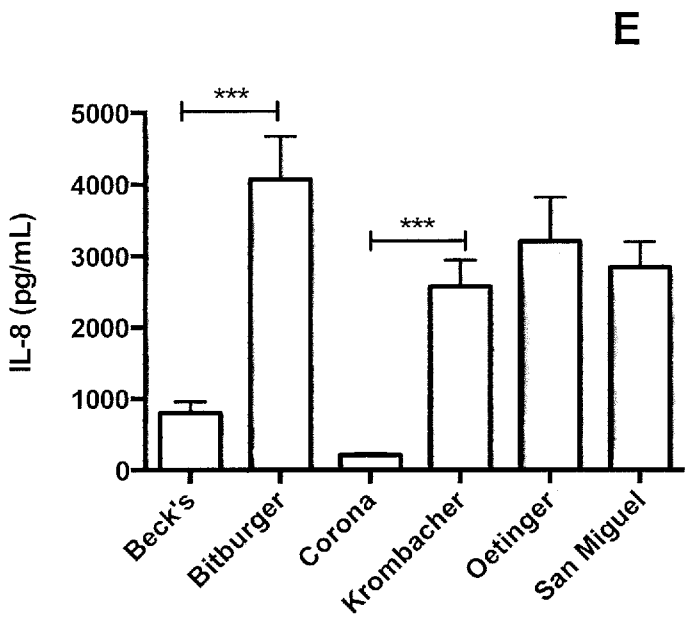

E
***
***
5000
4000
3000
2000
1000
0
IL-8 (pg/mL)
Beck's
Bitburger
Corona
Krombacher
Oetinger
San Miguel

FIG. 7E

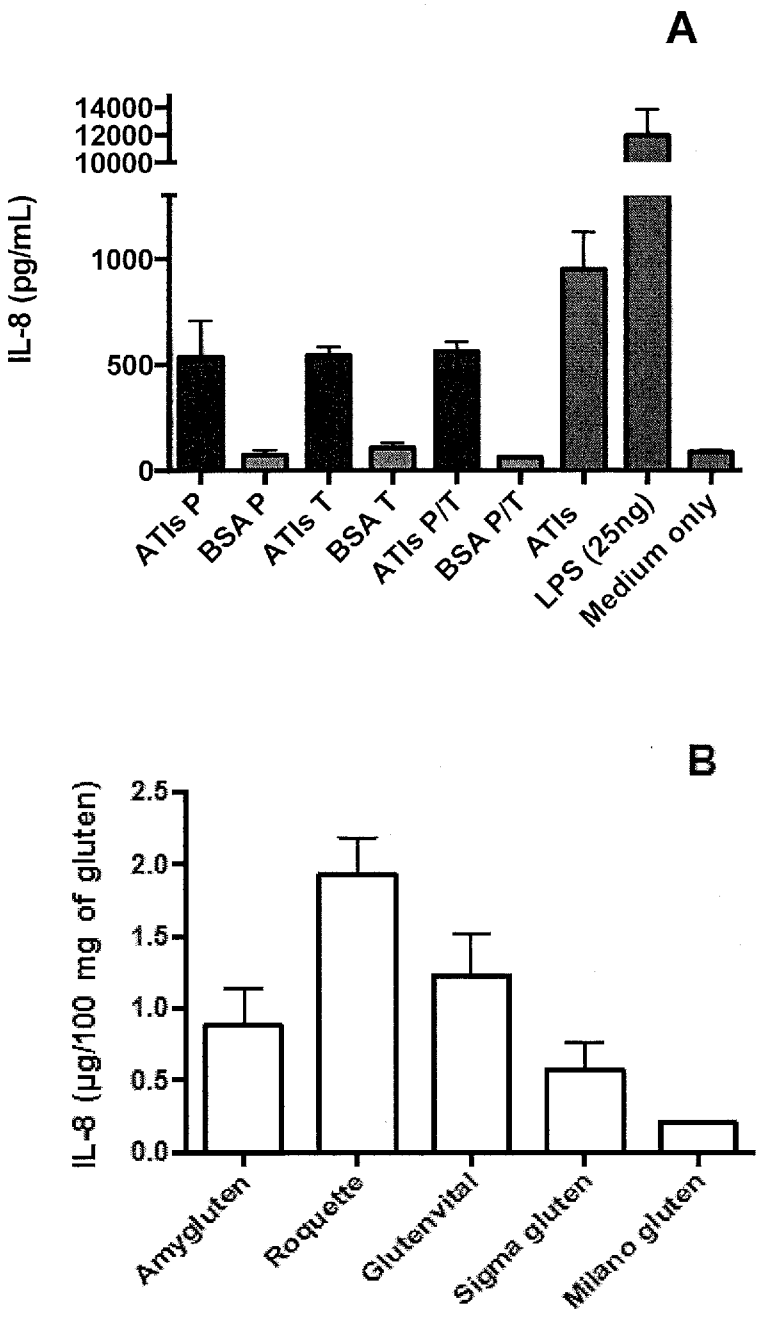

A
IL-8 (pg/mL)
14000
12000
10000
1000
500
0
ATIs P
BSA P
ATIs T
BSA T
ATIs P/T
BSA P/T
ATIs
LPS (25ng)
Medium only
B
IL-8 (µg/100 mg of gluten)
2.5
2.0
1.5
1.0
0.5
0.0
Amygluten
Roquette
Glutenvital
Sigma gluten
Milano gluten

FIG. 8A-8B

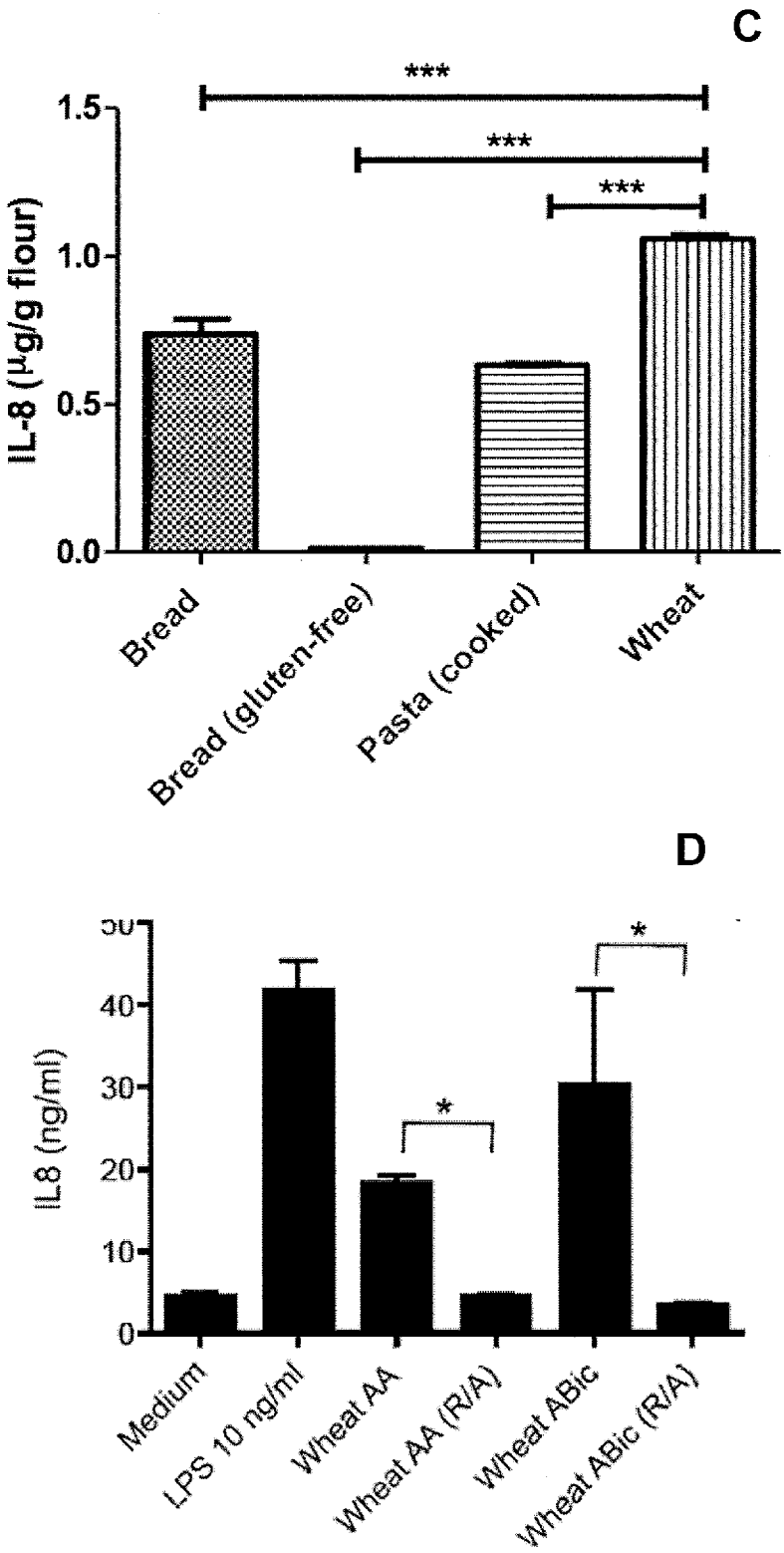
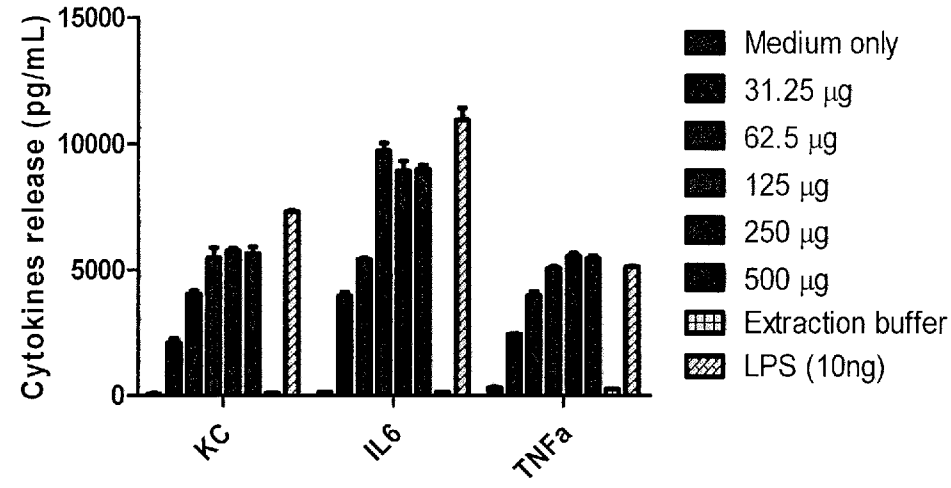

C
***
***
***
1.5
1.0
0.5
0.0
IL-8 (µg/g flour)
Bread
Bread (gluten-free)
Pasta (cooked)
Wheat
D
50
40
30
20
10
0
IL8 (ng/ml)
*
*
Medium
LPS 10 ng/ml
Wheat AA
Wheat AA (R/A)
Wheat ABic
Wheat ABic (R/A)

FIG. 8C-8D

A


Murine Bone Marrow-derived Dendritic Cells
Cytokines release (pg/mL)
15000
10000
5000
0
KC
IL6
TNFa
Medium only
31.25 µg
62.5 µg
125 µg
250 µg
500 µg
Extraction buffer
LPS (10ng)

B

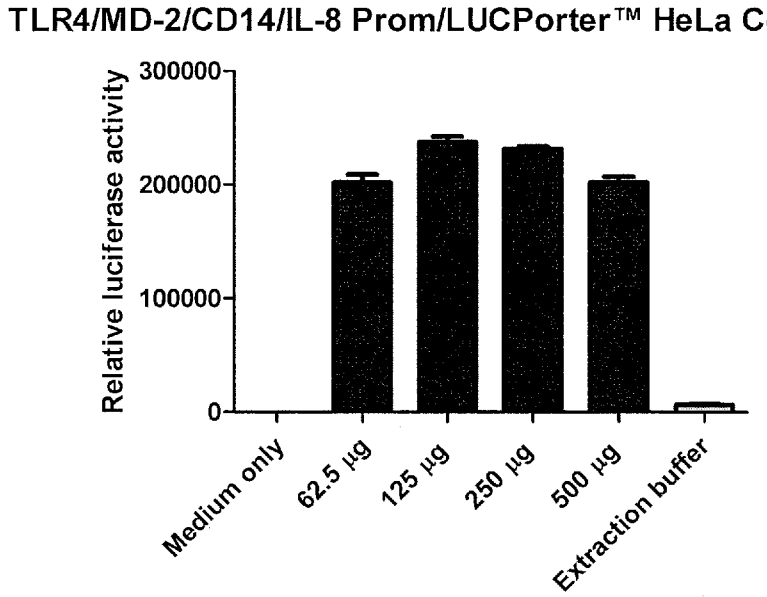

TLR4/MD-2/CD14/IL-8 Prom/LUCPorter™ HeLa Cell Line
Relative luciferase activity
300000
200000
100000
0
Medium only
62.5 µg
125 µg
250 µg
500 µg
Extraction buffer

FIG. 9A-9B

High: gluten containing	
Wheat	*Triticum* aestivum
Barley	*Hordeum vulgare* L.
Rye	*Secale cereale*
Medium: gluten-free (gluten-poor)	
Soya	*Glycine Max*
Quinoa	*Chenopodium* quinoa
Buckwheat	*Fagopyrum esculentum*
Peas	*Pisum sativum*
Early Crops	*Einkorn<Emmer<Spelt*
Low: gluten free	
Rice	*Oryza sativa*
Millet	*Panicum miliaceum*
Oats	*Avena sativa*
Maize	*Zea mays* L.
Amaranth	*Amaranthus caudatus*

High: Gluten containing
Pizza
Pasta
Bread
Low: Gluten-free
Pizza gluten-free
Pasta gluten-free
Bread gluten-free

FIG. 10

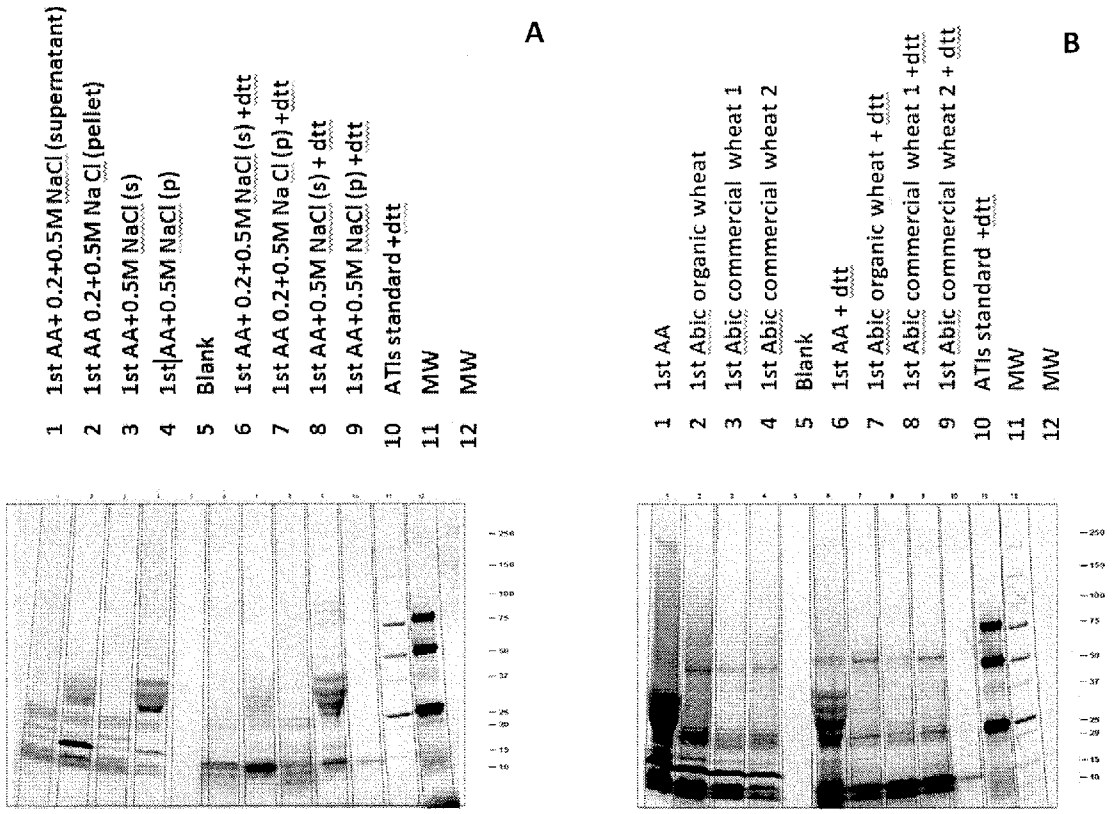
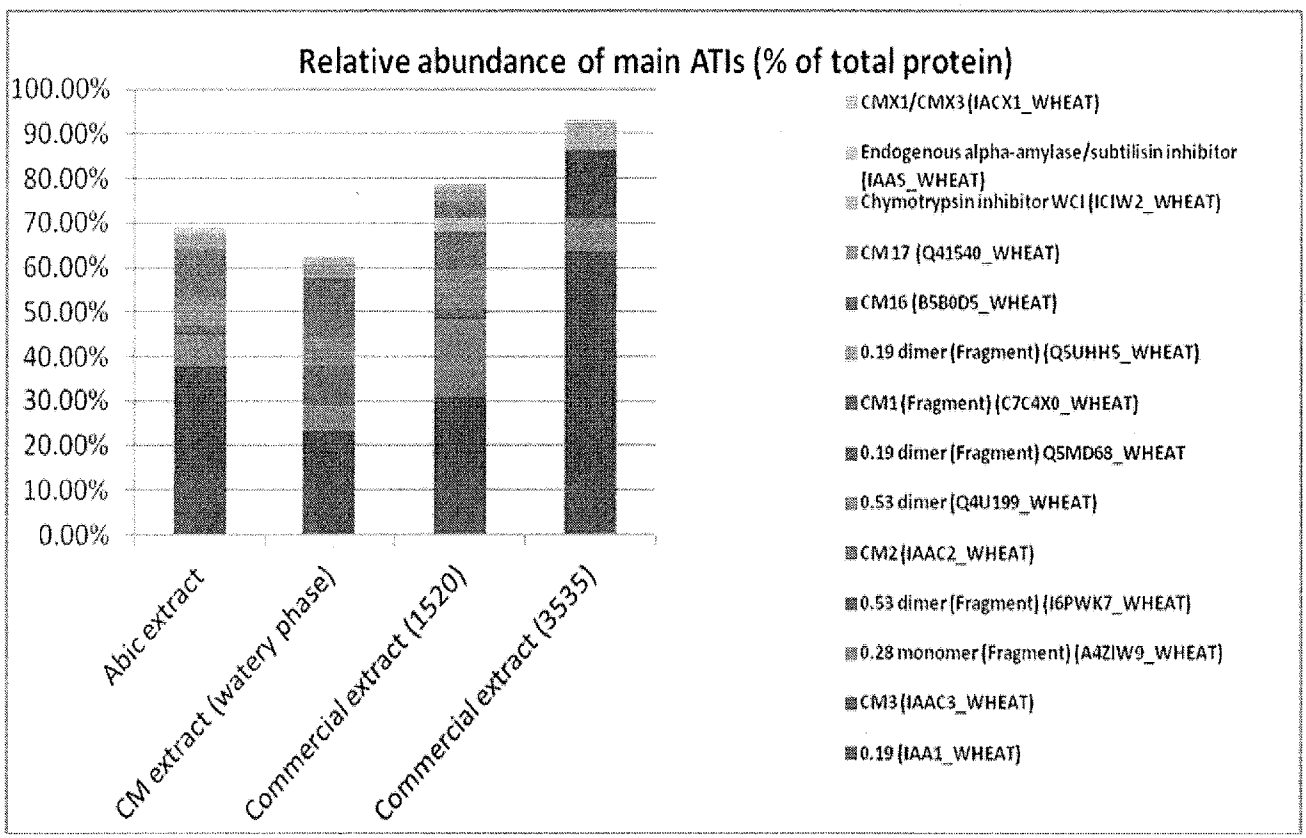

A
1 1st AA+ 0.2+0.5M NaCl (supernatant)
2 1st AA 0.2+0.5M Na Cl (pellet)
3 1st AA+0.5M NaCl (s)
4 1st AA+0.5M NaCl (p)
5 Blank
6 1st AA+ 0.2+0.5M NaCl (s) +dtt
7 1st AA 0.2+0.5M Na Cl (p) +dtt
8 1st AA+0.5M NaCl (s) + dtt
9 1st AA+0.5M NaCl (p) +dtt
10 ATIs standard +dtt
11 MW
12 MW
B
1 1st AA
2 1st Abic organic wheat
3 1st Abic commercial wheat 1
4 1st Abic commercial wheat 2
5 Blank
6 1st AA + dtt
7 1st Abic organic wheat + dtt
8 1st Abic commercial wheat 1 +dtt
9 1st Abic commercial wheat 2 + dtt
10 ATIs standard +dtt
11 MW
12 MW

FIG. 11A-11B

A



B

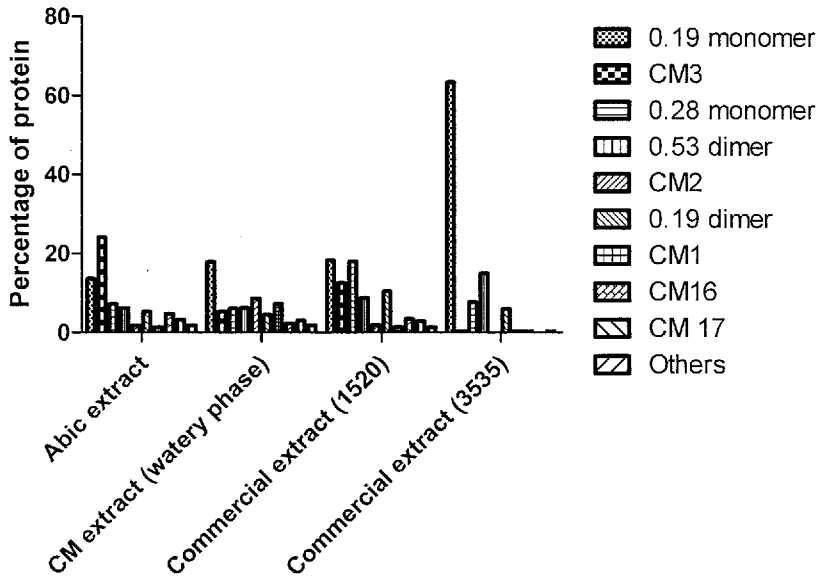
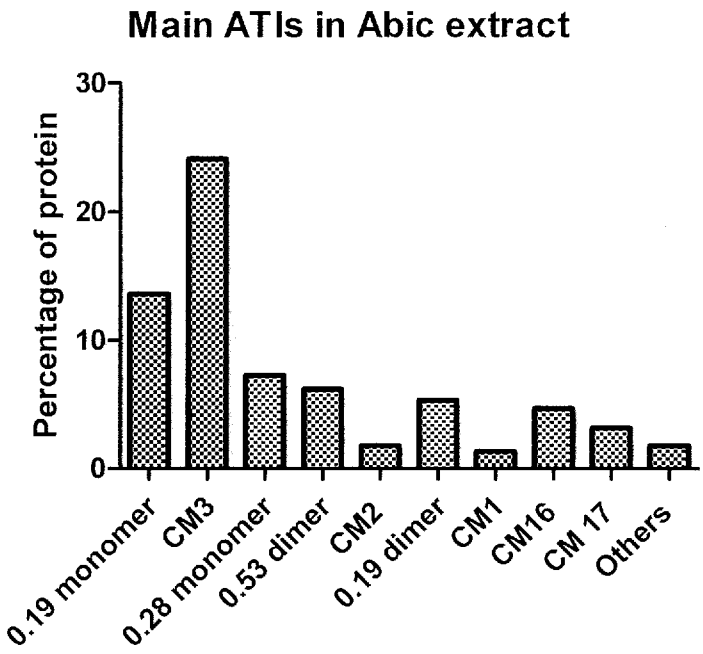


FIG. 12A-12B

A



B

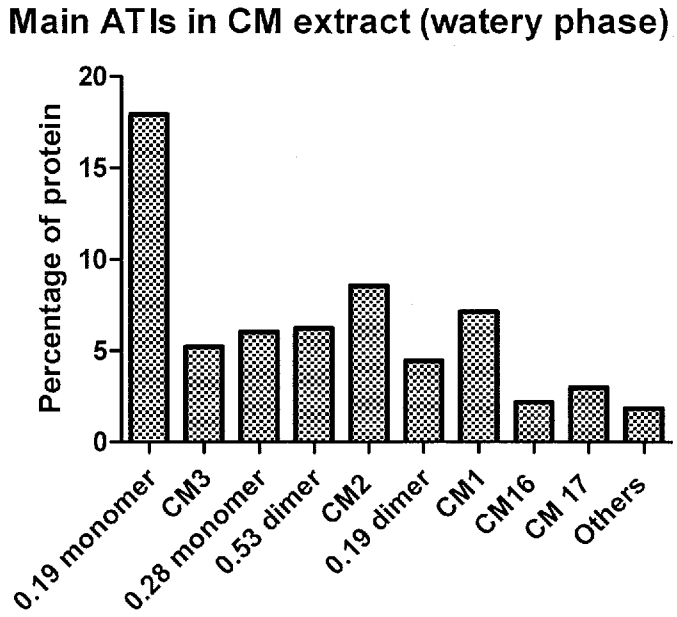
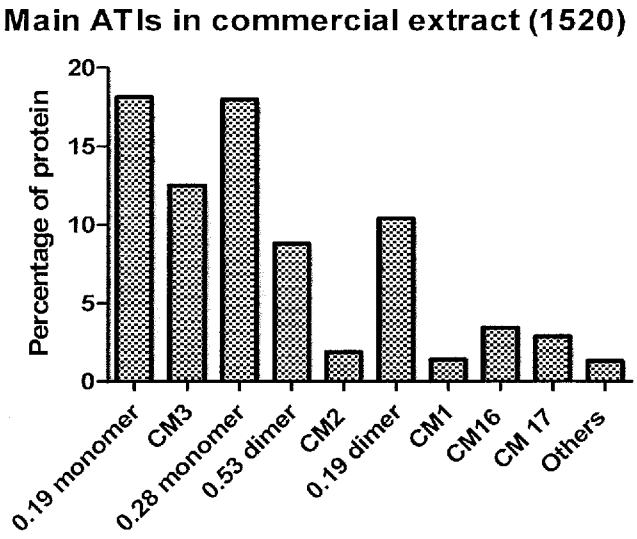


FIG.13A-13B

C



D

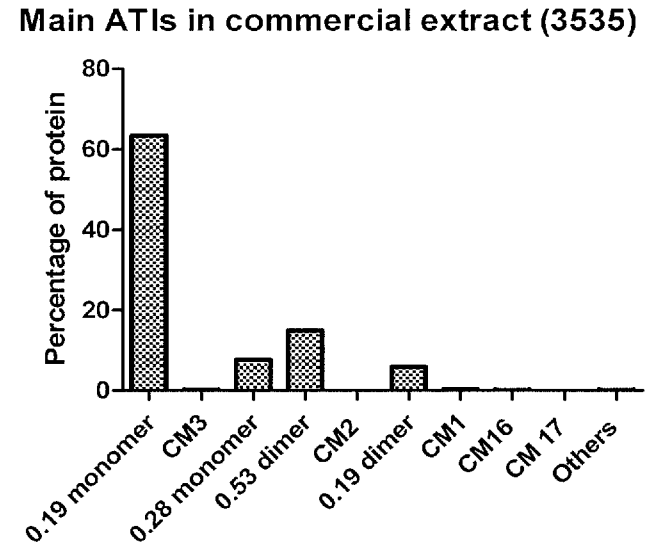


FIG. 13C-13D

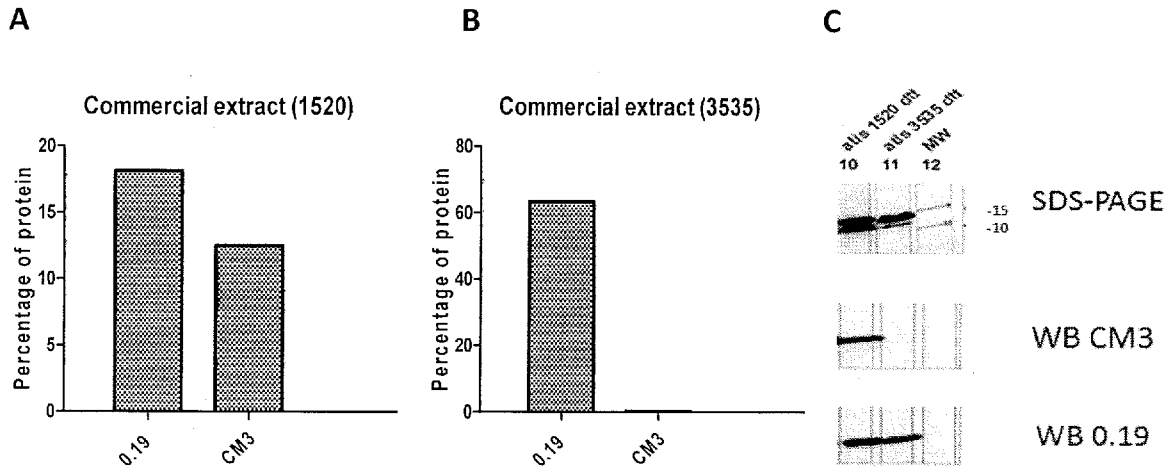

A
Commercial extract (1520)
Percentage of protein
20
15
10
5
0
0.19
CM3
B
Commercial extract (3535)
Percentage of protein
80
60
40
20
0
0.19
CM3
C
atis 1520 dnt
atis 3535 dnt
MW
10
11
12
-15
-10
SDS-PAGE
WB CM3
WB 0.19

FIG. 14

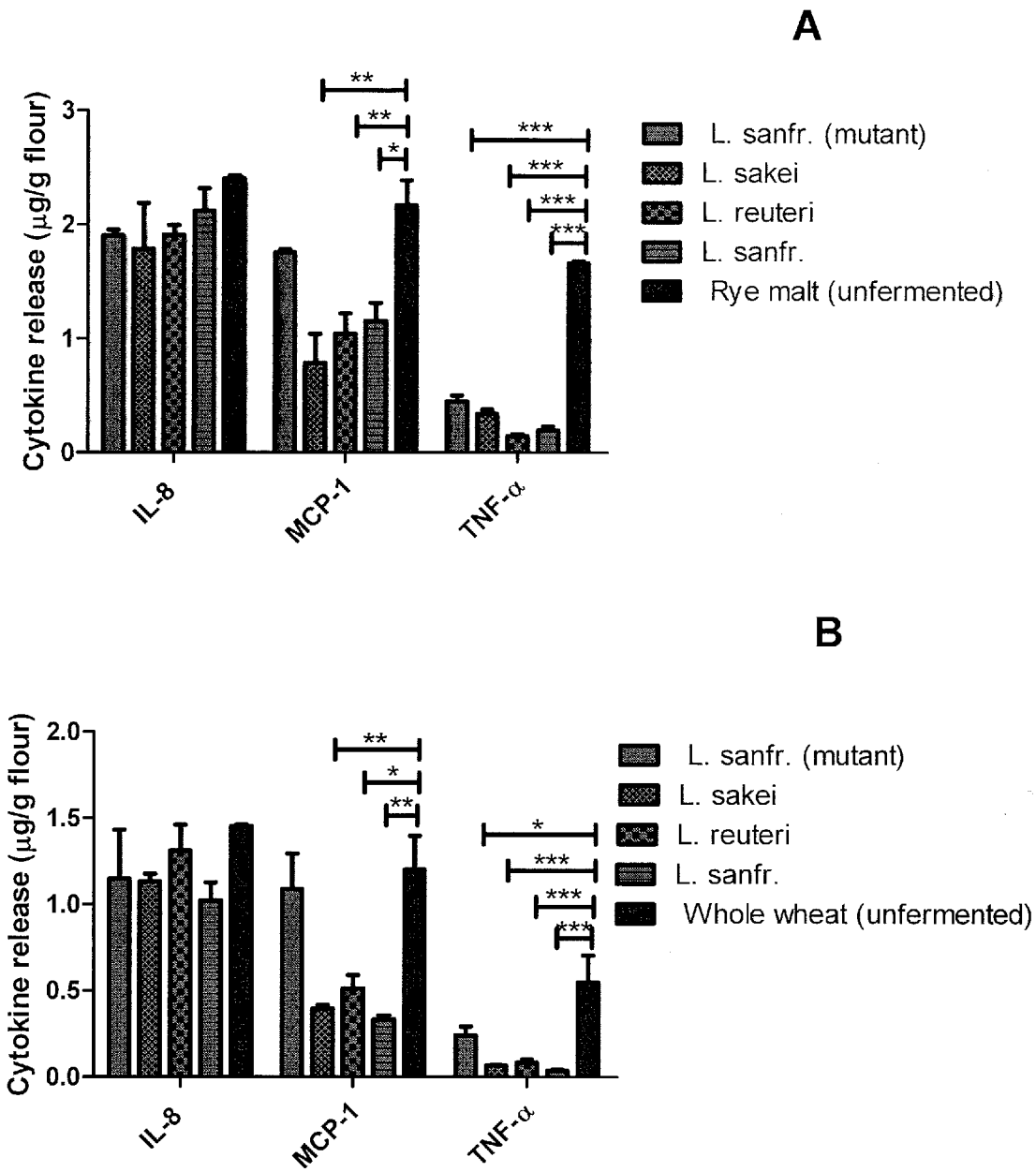

A
Cytokine release (μg/g flour)
3
2
1
0
**
**
*
***
***
***
***
IL-8
MCP-1
TNF-α
L. sanfr. (mutant)
L. sakei
L. reuteri
L. sanfr.
Rye malt (unfermented)
B
Cytokine release (μg/g flour)
2.0
1.5
1.0
0.5
0.0
**
*
**
*
***
***
***
IL-8
MCP-1
TNF-α
L. sanfr. (mutant)
L. sakei
L. reuteri
L. sanfr.
Whole wheat (unfermented)

FIG. 15A-15B

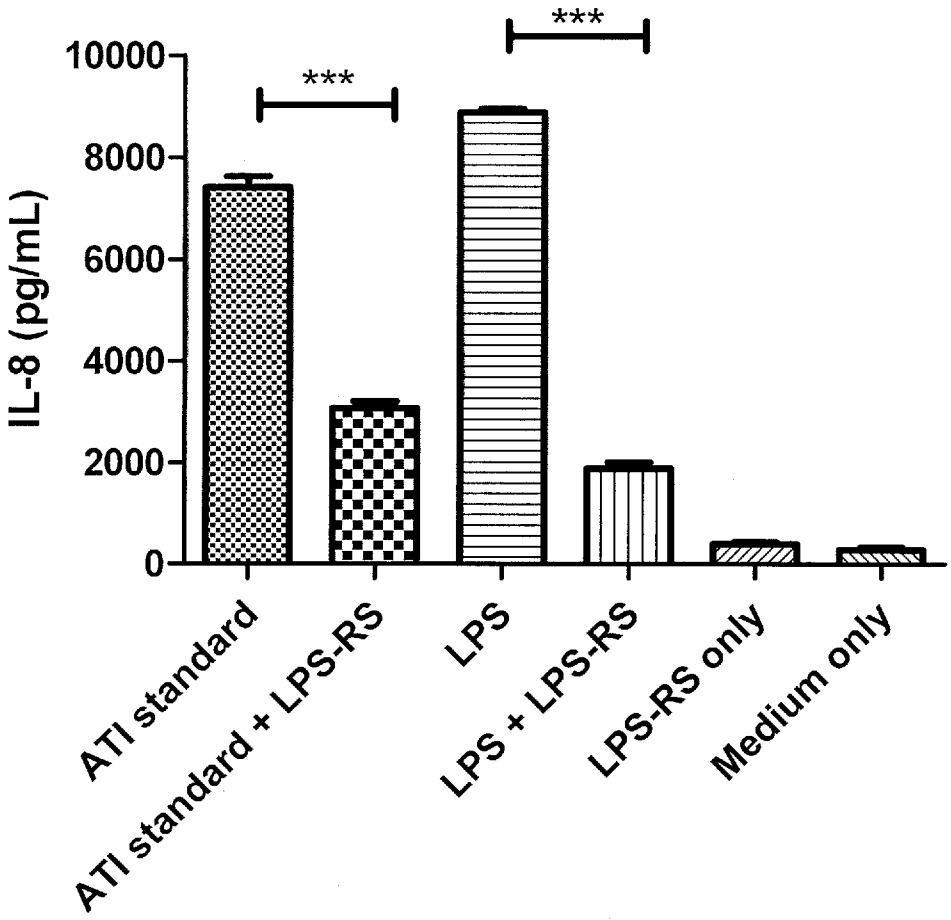

***
***
10000
8000
6000
4000
2000
0
IL-8 (pg/mL)
ATI standard
ATI standard + LPS-RS
LPS
LPS + LPS-RS
LPS-RS only
Medium only

FIG. 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/059473

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A23J 1/12; A23L 7/10; C07K 1/14; C07K 16/18 (2016.01)
CPC - A23J 1/125; A23J 1/12; A23L 7/10; C07K 1/14; C07K 16/18 (2016.11)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A23J 1/12; A23L 7/10; C07K 1/14; C07K 16/18 (2016.01)
CPC - A23J 1/12; A23J 1/125; A23L 7/10; C07K 1/14; C07K 16/18 (2016.11)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/172.1; 530/387.3; 530/388.2; 530/389.8; IPC(8) - A23J 1/12; A23L 7/10; C07K 1/14; C07K 16/18; CPC - A23J 1/12; A23J 1/125; A23L 7/10; C07K 1/14; C07K 16/18 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Orbit, Google Patents, Google Scholar
Search terms used: amylase, trypsin, inhibitor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 2013/0266584 A1 (SCHUPPAN et al) 10 October 2013 (10.10.2013) entire document	1-5, 38, 57-67, 76 18-22
Y	US 2013/0023649 A1 (KELLER et al) 24 January 2013 (24.01.2013) entire document	18-22
A	JUNKER et al., Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4, The Journal of Experimental Medicine, 03 December 2012, Pgs. 2395-2408	1-5, 18-22, 38, 57-67, 76-81
P, X	WO 2015/168416 A1 (BETH ISRAEL DEACONESS MEDICAL CENTER INC) 05 November 2015 (05.11.2015) entire document	1-5, 18-22, 38, 57-67, 76-81

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 December 2016	29 DEC 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/059473

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-17, 23-37, 39-56, 68-75
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.