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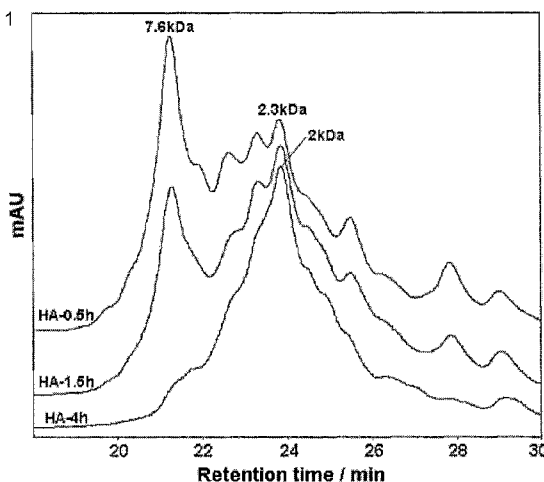
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(54) Title: BARLEY HORDEIN AND GLUTELIN PEPTIDES HAVING ANTIOXIDANT ACTIVITY

FIG. 1



(57) Abstract: The invention is directed to a method of producing a peptide having antioxidant activity from barley hordein by treating barley hordein with alcalase at a sufficient time and temperature suitable for hydrolysis; and recovering the peptide from the hydrolyzed barley hordein. The invention is also directed to a method of producing a peptide having antioxidant activity from barley glutelin by treating barley glutelin with alcalase or flavourzyme at a sufficient time and temperature suitable for hydrolysis; and recovering the peptide from the hydrolyzed barley glutelin.

BARLEY HORDEIN AND GLUTELIN PEPTIDES HAVING ANTIOXIDANT ACTIVITY

Field of the Invention

5 [0001] The present invention relates to peptides derived from barley hordein and glutelin, and having antioxidant activity.

Background of the Invention

10 [0002] At high levels of oxidative stress, the significant imbalance between free radicals and the antioxidant defense system can lead to cell injury (Willcox *et al.*, 2004). Studies correlating diet and some chronic diseases have demonstrated the potential of antioxidant supplementation to support or improve health. Additionally, oxidative reactions in lipid-containing food products generate undesirable flavor and aroma compounds during processing and storage. Free radical chain reactions propagate and engage not only the lipid molecules but also other valuable food
15 components such as proteins and vitamins (Halliwell *et al.*, 2002). Prevention of oxidation can thus improve the nutritional and economical value of sensitive food products.

[0003] Alpha-tocopherol, carotenoids, and polyphenol compounds are examples of natural antioxidants (Liebler *et al.*, 1990; Huang *et al.*, 1997; Urizzi *et al.*, 1999). Similarly, it has been suggested that protein and peptides may be potentially excellent food additive antioxidants which
20 may inhibit oxidation through multiple pathways including scavenging free radicals, chelating prooxidative transition metal ions, reducing hydroperoxides and inactivating reactive oxygen species (Hook *et al.*, 2001). Unique amphipathicity of most antioxidative peptides allows them to prohibit oxidation in both aqueous and lipoid systems, making such peptides applicable to the food industry (Wu *et al.*, 2003). A protein's antioxidative activity is limited by its tertiary
25 structure, since many peptides and amino acids with antioxidative potential are buried within the protein core inaccessible to pro-oxidants.

[0004] Enzymatic hydrolysis is an effective method to release these antioxidative peptides from protein molecules. Antioxidative activity has been identified in several food protein

hydrolysates, including those derived from whey protein (Pena-Ramos *et al.*, 2004), fish protein (Dong *et al.*, 2008), egg yolk (Park *et al.*, 2001), porcine haemoglobin (Chang *et al.*, 2007), zein protein (Kong *et al.*, 2006), chickpea protein (Li *et al.*, 2008) and wheat gluten (Kong *et al.*, 2008). The antioxidant activities of peptides are closely related to their amino acid constituents and their sequences. Several amino acids, such as histidine, tyrosine, methionine, lysine, tryptophan and phenylalanine are generally accepted as antioxidants in spite of their pro-oxidative effects in some cases (Pihlanto, 2006). Peptides with more hydrophobic amino acids have been reported to be related to antioxidative properties (Rajapakse *et al.*, 2005).

[0005] Higher solubility of hydrolysates and increases in amino acid exposure enhance the potential functional and bioactive properties of peptides in food products (Cheng *et al.*, 2010; Elias *et al.* 2006). Antioxidant peptides suppress oxidative damage by quenching the free radical chain reactions, thereby extending the shelf-life of food product. The odorless, colorless nature of peptides enables them to be used as neutral food additives, and their amphipathic nature confers their compatibility with multicomponent or polyphasic food systems. Isolated pure peptides can be incorporated in pharmaceutical and personal care formulations because peptides are considered as safe therapeutic agents (Agyei *et al.*, 2011). Different assays and models have been developed to demonstrate the antioxidant potential of protein hydrolysates (Kong *et al.*, 2006). However, the relationships between antioxidant properties and compositional features of hydrolysates are unclear, and the impacts of peptides conformation on their antioxidant effects are unknown. An important element of nutritional and biofunctional discovery is to gain insight into molecular mechanisms which facilitate the purification process and ultimately improve the design and development of creative health-directed nutraceuticals and functional foods.

[0006] As the fourth most widely cultivated cereal in the world after wheat, rice and corn, barley represents a potential abundant and affordable source of plant proteins. The overall barley grain protein content is 8-13% (w/w) depending on the variety (Wang *et al.*, 2010). Hordein is a barley prolamin and comprises approximately 35-55% of the total barley grain protein, and is the main storage protein for barley. Barley hordeins are divided into four groups based on their electrophoretic mobilities and amino acid compositions: the B (30-50 kDa, sulfur-rich) and C (55-80 kDa, sulfur-poor) hordeins (70-80% and 10-20% of the hordein fraction, respectively)

and the D (80-90 kDa) and A (15 kDa) hordeins (less than 5% of the total hordein fraction). The A hordeins are likely alcohol-soluble albumins or globulins, or breakdown products of larger hordeins rather than true hordeins. C and some B hordeins appear as monomers, while most B and D hordeins are linked by inter-chain disulfide bridges.

5 [0007] Hordein is rich in non-polar and hydrophobic residues (Pro, Leu, Val) and low levels of charged amino acids (Kong *et al.*, 2006). Peptides containing hydrophobic residues have shown strong effects in retarding oxidation processes by scavenging free radicals (Rival *et al.*, 2001; Tang *et al.*, 2008). Enzymatic treatment of hordein with three proteases (alcalase, flavourzyme and pepsin) yielded hydrolysates exhibiting a broad range of molecular structures
10 and antioxidant activities (Bamdad *et al.*, 2011). The alcalase hydrolysates were effective in scavenging lipophilic radicals and entrapping metal ions.

[0008] Glutelin comprises approximately 35-40% of the total barley grain protein, and is one of the main storage proteins of barley. Glutelin is characterized by high proportions of glutamine (glutamic acid, 20.2%), proline (11.2%) and glycine (8.4%) (Lásztity, 1984; Wang *et al.*, 2010),
15 and is enriched in hydrophobic amino acids (around 35%), with the highest levels corresponding to proline, leucine, alanine and valine (Bamdad *et al.*, 2011). Glutelin thus exhibits high surface hydrophobicity which markedly reduces protein solubility, hindering glutelin applications.

[0009] Barley endosperm proteins such as hordein or glutelin are typically regarded as contaminants by the brewing industry and are precipitated out in the spent grains for use as
20 animal feed. Barley proteins exhibit relatively low digestibility, limiting their nutritive value. Development of barley hydrolysates and the subsequent characterization of their functional properties may facilitate the diversified opportunities for barley proteins in food and non-food applications, and identify value-added applications for barley proteins.

[00010] Therefore, there is a need in the art for converting barley byproducts into
25 useful products.

Summary of the Invention

[00011] The present invention relates to peptides derived from barley hordein or glutelin and having antioxidant activity.

[00012] In one aspect, the invention comprises a method of producing a peptide having antioxidant activity from barley hordein or glutelin, comprising the steps of:

- a) hydrolyzing barley hordein or glutelin; and
- b) recovering the peptide from the hydrolyzed barley hordein or glutelin.

5 [00013] In one embodiment, the barley hordein is hydrolyzed with alcalase at a sufficient time and temperature for hydrolysis.

[00014] In one embodiment, the method further comprises the step of fractionating the hydrolyzed barley hordein by ultra-filtration to yield at least a first fraction, a second fraction, a third fraction, and a fourth fraction.

10 [00015] In one embodiment, the first fraction comprises peptides having molecular weights greater than 10 kDa.

[00016] In one embodiment, the second fraction comprises peptides having molecular weights between 5 to 10 kDa.

15 [00017] In one embodiment, the third fraction comprises peptides having molecular weights between 1 to 5 kDa.

[00018] In one embodiment, the fourth fraction comprises peptides having molecular weights less than 1 kDa.

20 [00019] In one embodiment, the method comprises fractionating the second fraction by reversed-phase high performance liquid chromatography to yield at least a fifth fraction comprising at least one antioxidant peptide.

[00020] In one embodiment, the peptide comprises an amino acid sequence selected from QPYPQ; QSYVPVQPQ; QQTPLPQ; QPQPYPQ; TQQPYPQ; SPLQPQ; QQPYPQ; QPVLSQ; QVPQ; LLPQ; or HVLQ.

25 [00021] In one embodiment, the method comprises fractionating the fourth fraction by reversed-phase high performance liquid chromatography to yield at least a sixth fraction comprising at least one antioxidant peptide.

[00022] In one embodiment, the peptide comprises an amino acid sequence selected from KPFPQQPPF; QPPFWQ; SVNPLY; AELIIPQ; or YRIVPL.

[00023] In one embodiment, the barley glutelin is hydrolyzed with alcalase or flavourzyme at a sufficient time and temperature for hydrolysis.

5 [00024] In one embodiment, in step (a), hydrolysis is conducted using alcalase.

[00025] In one embodiment, the method further comprises the step of fractionating the hydrolyzed barley glutelin by ultra-filtration to yield at least a first fraction and a second fraction.

[00026] In one embodiment, the first fraction comprises peptides having molecular weights greater than 10 kDa.

10 [00027] In one embodiment, the second fraction comprises peptides having molecular weights less than 1 kDa.

[00028] In one embodiment, the method comprises fractionating the second fraction by reversed-phase high performance liquid chromatography to yield at least a third fraction comprising at least one antioxidant peptide.

15 [00029] In one embodiment, the peptide comprises an amino acid sequence selected from Gln-Lys-Pro-Phe-Pro-Gln-Gln-Pro-Pro-Phe; Pro-Gln-Ile-Pro-Glu-Gln-Phe; Leu-Arg-Thr-Leu-Pro-Met; or Ser-Val-Asn-Val-Pro-Leu.

[00030] In one embodiment, the duration of hydrolysis is at least between about 0.5 hours to about 4 hours. In one embodiment, the duration of hydrolysis is at least about 1.5 hours. In one
20 embodiment, hydrolysis is conducted at a temperature of about 50° C. In one embodiment, hydrolysis is conducted at a pH in the range of about 7.0 to about 8.0.

[00031] In one embodiment, hydrolysis of hordein is conducted using alcalase at an enzyme/substrate ratio of about 0.24 AU/g protein.

[00032] In one embodiment, hydrolysis of glutelin is conducted using flavourzyme at an
25 enzyme/substrate ratio of about 40 LAPU/g protein.

[00033] In one embodiment, hydrolysis of glutelin is conducted using alcalase at an enzyme/substrate ratio of about 0.12 AU/g protein.

[00034] In another aspect, the invention comprises a barley hordein or glutelin peptide produced by the above method and having antioxidant activity.

[00035] In another aspect, the invention comprises a barley hordein peptide having antioxidant activity and comprising an amino acid sequence selected from QPYPQ; QSYPVQPQ; QQTPLPQ; QPQPYPQ; TQQPYPQ; SPLQPQ; QQPYPQ; QPVLSQ; QVPQ; LLPQ; HVLQ; KPFPQQPPF; QPPFWQ; SVNPLY; AELIIPQ; or YRIVPL.

[00036] In another aspect, the invention comprises a barley hordein peptide having a molecular weight greater than 10 kDa, and exhibiting DPPH scavenging activity and reducing power.

[00037] In another aspect, the invention comprises a barley hordein peptide having a molecular weight less than 1 kDa, and exhibiting ferrous ion chelating activity.

[00038] In another aspect, the invention comprises a barley glutelin peptide having antioxidant activity and comprising an amino acid sequence selected from Gln-Lys-Pro-Phe-Pro-Gln-Gln-Pro-Pro-Phe; Pro-Gln-Ile-Pro-Glu-Gln-Phe; Leu-Arg-Thr-Leu-Pro-Met; or Ser-Val-Asn-Val-Pro-Leu.

[00039] In another aspect, the invention comprises a barley glutelin peptide having a molecular weight greater than 10 kDa, and exhibiting DPPH scavenging activity and reducing power.

[00040] In another aspect, the invention comprises a barley glutelin peptide having a molecular weight less than 1 kDa, and exhibiting ferrous ion chelating activity and hydroxyl scavenging activity.

[00041] In another aspect, the invention comprises a food, cosmetic or pharmaceutical product comprising the above barley hordein or glutelin peptide, or as produced by the above method.

[00042] In yet another aspect, the invention comprises use of a barley hordein or glutelin peptide produced by the above method.

[00043] Additional aspects and advantages of the present invention will be apparent in view of the description, which follows. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description. — — — — —

Brief Description of the Drawings

[00044] The invention will now be described by way of an exemplary embodiment with reference to the accompanying simplified, diagrammatic, not-to-scale drawings:

[00045] Figure 1 shows size exclusion chromatograms of hordein hydrolyzed by alcalase after 0.5, 1.5 and 4 h of hydrolysis.

[00046] Figure 2 shows size exclusion chromatograms of membrane-fractionated hordein hydrolysate (HA-1.5h).

[00047] Figure 3A shows the circular dichroism spectra of peptide fractions obtained from membrane separation of the hordein hydrolysate, HA-1.5h.

[00048] Figure 3B shows the FTIR deconvoluted spectra of peptide fractions obtained from membrane separation of the hordein hydrolysate, HA-1.5h.

[00049] Figure 4 is a graph showing the surface hydrophobicity of hordein hydrolysate (HA-1.5h) and its peptide fractions (bars with different superscript letters differ significantly; $p < 0.05$).

[00050] Figure 5A is a graph showing DPPH radical scavenging activity (EC_{50} values) of the unfractionated and the membrane-fractionated hordein hydrolyzed by alcalase (values with different lowercase letters in the same hydrolysis time are significantly different; $p < 0.05$).

[00051] Figure 5B is a graph showing superoxide radical scavenging activity (percent scavenging at 1.0 mg/ml) of the unfractionated and the membrane-fractionated hordein hydrolyzed by alcalase (values with different lowercase letters in the same hydrolysis time are significantly different; $p < 0.05$).

[00052] Figure 6 is a graph showing ferrous ion chelating activity of the hordein hydrolysates and their fractions obtained after membrane fractionation (EC_{50} values) (values with different lowercase letters in the same hydrolysis time are significantly different; $p < 0.05$).

[00053] Figure 7 is a graph showing reducing power of unfractionated and membrane-fractionated hordein hydrolysates (absorbance at 700 nm at 2 mg/ml). Values with different lowercase letters in the same hydrolysis time differ significantly ($p < 0.05$).

[00054] Figure 8 is a graph showing reversed phase chromatograms of membrane fractions of hordein hydrolyzed by alcalase for 1.5 h.

[00055] Figure 9 is a graph showing the degree of hydrolysis of barley glutelin treated by flavourzyme and alcalase.

[00056] Figures 10A (panels a and b) are SE-HPLC chromatograms of barley glutelin hydrolysates after 0.5 and 4 hours of hydrolysis treated by a) flavourzyme (FH) and alcalase (AH). FH1 and FH2 are flavourzyme hydrolysates after 0.5 and 4 hours of hydrolysis, respectively; AH1 and AH2 are alcalase hydrolysates after 0.5 and 4 hours of hydrolysis, respectively.

[00057] Figures 10B (left and right panels) are graphs showing the relative area (%) of the peptide peaks in barley glutelin hydrolysate fractions prepared with flavourzyme (FH) and alcalase (AH) after 0.5, 2 and 4 hours of hydrolysis.

[00058] Figure 11 is a graph showing the changes in surface hydrophobicity of barley glutelin hydrolysates during hydrolysis.

[00059] Figures 12A-E are graphs showing DPPH radical scavenging activity (1.0 mg/ml) (Figure 12A); superoxide radical ($O_2^{\cdot -}$) scavenging activity (2.0 mg/ml) (Figure 12B); hydroxyl radical ($OH^{\cdot}$) scavenging activity (1.0 mg/ml) (Figure 12C); ferrous ion chelating activity (1.0 mg/ml) (Figure 12D); and reducing power (2.0 mg/ml) of barley glutelin hydrolysates treated by flavourzyme and alcalase (Figure 12E).

[00060] Figure 13 shows size exclusion chromatograms of three fractions (I, II and III) obtained from membrane fractionation of glutelin hydrolysate.

[00061] Figure 14 is a semi-preparative phase reverse chromatogram of Fraction III by membrane fractionation. This fraction was further separated into four fractions based on peptide hydrophobicity as indicated on the graphs.

Detailed Description of Preferred Embodiments

5 [00062] When describing the present invention, all terms not defined herein have their common art-recognized meanings. To the extent that the following description is of a specific embodiment or a particular use of the invention, it is intended to be illustrative only, and not limiting of the claimed invention. The following description is intended to cover all alternatives, modifications and equivalents that are included in the spirit and scope of the invention, as
10 defined in the appended claims.

[00063] The present invention relates to peptides derived from barley hordein or glutelin and having antioxidant activity. As used herein, the term "peptide" means a short polymer of amino acid monomers linked by peptide bonds, typically containing less than fifty monomer units. As used herein, the term "barley hordein" refers to prolamin proteins which may be extracted from
15 barley with ethanol, and includes hordeins A, B, C and D. As used herein, the term "barley glutelin" refers to a storage protein which is found in barley seeds, is soluble in dilute acids or bases, and has high proportions of glutamine, proline, glycine, and hydrophobic amino acids. As used herein, the term "antioxidant activity" means the ability to inhibit oxidation through one or more pathways including, but not limited to, scavenging free radicals, chelating pro-oxidative
20 transition metal ions, reducing hydroperoxides, and inactivating reactive oxygen species.

[00064] The peptides of the present invention are produced from barley using the methods described herein. In one embodiment, the method generally involves at least the steps of treating barley hordein with alcalase at a sufficient time and temperature for hydrolysis; and recovering the peptide from the hydrolyzed barley hordein. In one embodiment, the method generally
25 involves at least the steps of treating barley glutelin with alcalase or flavourzyme at a sufficient time and temperature for hydrolysis; and recovering the peptide from the hydrolyzed barley glutelin. The physicochemical properties of the resultant hordein or glutelin peptides may have functional properties and suitability for particular applications such as, for example, as antioxidants in food, cosmetic and pharmaceutical products.

[00065] The detailed steps of the process are as follows. Barley is used as the starting material. As used herein, the term "barley" means a grass in the genus *Hordeum*. Barley proteins may be extracted using the method described by Wang *et al.* (2010). Briefly, barley is pearled and milled. Pearling is used to remove the grain's outer layers (mainly bran and germ) so that the barley cytoplasmic proteins (albumin and globulin) are enriched in the pearling flour, while endosperm proteins (hordein and glutelin) are enriched in the pearled grain flour. The pearled grain flour is treated with an alcohol. The alcohol may be ethanol, methanol or propane, and is preferably a 50% - 70% ethanol. In one embodiment, the ratio of alcohol to flour is 6:1 (v/w). In one embodiment, the solution is stirred for about two hours at about 60 °C. After extraction, the insoluble solids are separated by centrifugation. The alcoholic supernatant is collected, and hordein is isolated by cold precipitation and may be freeze-dried for storage and future use.

[00066] The barley endosperm flour residue which is insoluble in alcohol is mixed with an alkaline solution to extract glutelin. In one embodiment, the alkaline solution is a solution of NaOH and has a pH of about 10 or higher. The ratio of the alkaline solution to flour may be 10:1 (v/w). The mixture is stirred for about half an hour at about room temperature. After extraction, the insoluble solids are separated by centrifugation. The supernatants collected from the alkaline extracts are adjusted to an acidic pH to precipitate the proteins. Glutelin protein isolates are then obtained by centrifugation.

[00067] The hordein or glutelin protein isolates are hydrolyzed using proteases at a particular enzyme/substrate ratio, pH and temperature to yield peptides having antioxidant activity. In one embodiment, the protease comprises alcalase. As used herein, the term "alcalase" refers to an endo-protease which cleaves peptide bonds at the interior of the polypeptides chain to produce small (M_w lower than 1 kDa) and medium (M_w between 1-10 kDa) sized peptides. In one embodiment, alcalase hydrolysis of hordein is conducted at an enzyme/substrate ratio of about 0.24 AU/g protein. In one embodiment, alcalase hydrolysis of glutelin is conducted at an enzyme/substrate ratio of about 0.12 AU/g protein.

[00068] In one embodiment, the protease comprises flavourzyme. As used herein, the term "flavourzyme" refers to an endo- and exopeptidase enzyme mixture having broad specificity to

produce small (M_w lower than 1 kDa) sized peptides and free amino acids. In one embodiment, flavourzyme hydrolysis of glutelin is conducted at an enzyme/substrate ratio of about 40 LAPU/g protein.

[00069] In one embodiment, hydrolysis is conducted at a pH in the range of about 7.0 to about 8.0, and at a temperature of about 50° C. In one embodiment, hydrolysis is conducted between about 0.5 hours to about 4 hours. In one embodiment, hydrolysis is conducted for about 1.5 hours. After adjusting the pH to 7.0, the hydrolysate solutions are heated to inactivate the proteases. In one embodiment, the hydrolysate solutions are heated at 95° C for about five minutes. The hydrolysate solutions are then centrifuged to separate the soluble hydrolysates from the non-soluble substances. The soluble hydrolysates may be freeze-dried for storage and future use.

[00070] Hydrolysates exhibiting the highest antioxidant activities may be further fractionated. In one embodiment, the hydrolysates are fractionated based on molecular weight using membrane fractionation. In one embodiment, the hydrolysates are further fractionated based on surface hydrophobicity using reversed phase-high performance liquid chromatography.

[00071] In one embodiment, the soluble hordein hydrolysates are further fractionated based on molecular weight using membrane fractionation to yield at least a first fraction, a second fraction, a third fraction, and a fourth fraction.

[00072] In one embodiment, the first fraction comprises peptides having molecular weights greater than 10 kDa. The first fraction comprises predominantly hydrophobic amino acid residues, and exhibits greater 1,1-diphenyl-2-picryl hydrazyl (DPPH) scavenging activity and reducing power compared to the second, third, and fourth fractions. Without being bound by any theory, peptides having molecular weights greater than 10 kDa have poly-L-proline helix, β -turn and β -sheet as the dominant structural elements which confer high surface hydrophobicity. The exposed hydrophobic amino acids play an important role in DPPH scavenging activity and reducing power.

[00073] In one embodiment, the second fraction comprises peptides having molecular weights between 5 to 10 kDa.

[00074] In one embodiment, the third fraction comprises peptides having molecular weights between 1 to 5 kDa.

[00075] In one embodiment, the fourth fraction comprises peptides having molecular weights less than 1 kDa, and exhibiting ferrous ion chelating activity. Without being bound by any theory, peptides having molecular weights less than 1 kDa assume unordered random coil conformation accompanied by a large amount of ionized carboxyl groups produced as a result of partial deamidation of glutamine residues to glutamic acid during hydrolysis. Metal binding via such exposed charged groups together with Lys and Met may be the main mechanism responsible for the ferrous ion chelating activity.

[00076] In one embodiment, the second fraction is further fractionated by reversed-phase high performance liquid chromatography to yield at least a fifth fraction comprising at least one peptide. The fifth fraction comprises predominantly hydrophobic amino acid residues, and exhibits greater DPPH and superoxide radical scavenging activities compared to the other fractions. In one embodiment, the fifth fraction comprises a peptide comprising an amino acid sequence selected from QPYPQ; QSYVQPPQ; QQTPLPQ; QPQPYPQ; TQQPYPQ; SPLQPQ; QQPYPQ; QPVLSQ; QVPQ; LLPQ; or HVLQ. The QPYPQ (Gln-Pro -Tyr-Pro-Gln) sequence was identified as the most repeated sequence in potent fractions. Without being bound by any theory, the intermittent Pro and Gln residues and the QPYPQ sequence may be particular structural motifs which play key roles in radical scavenging activity.

[00077] In one embodiment, the fourth fraction is further fractionated by reversed-phase high performance liquid chromatography to yield at least a sixth fraction comprising at least one peptide. The sixth fraction comprises predominantly hydrophobic amino acid residues, and exhibits greater ferrous ion chelating activity compared to the other fractions. In one embodiment, the sixth fraction comprises a peptide comprising an amino acid sequence selected from KPFPQPPF; QPPFWQ; SVNPLY; AELIIPQ; or YRIVPL. Without being bound by any theory, the presence of diverse amino acids (for example, Ser, Ala, Glu, Val, and Pro) and the exposed charged groups may enhance ferrous ion chelating activity.

[00078] In one embodiment, the soluble glutelin hydrolysates are further fractionated using ultra-filtration to yield at least a first fraction and a second fraction. In one embodiment, the first

fraction comprises peptides having molecular weights greater than 10 kDa, and exhibiting 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity and reducing power. In one embodiment, the second fraction comprises peptides having molecular weights less than 1 kDa, and exhibiting ferrous ion chelating activity and hydroxyl scavenging activity.

- 5 [00079] In one embodiment, the second fraction is further fractionated by reversed-phase high performance liquid chromatography to yield a third fraction comprising at least one peptide. The third fraction comprises predominantly hydrophobic amino acid residues, and exhibits greater ferrous-chelating and hydroxyl radical scavenging activities compared to the first and second fractions. In one embodiment, the third fraction comprises a peptide having an amino
10 acid sequence selected from Gln-Lys-Pro-Phe-Pro-Gln-Gln-Pro-Pro-Phe; Pro-Gln-Ile-Pro-Glu-Gln-Phe; Leu-Arg-Thr-Leu-Pro-Met; or Ser-Val-Asn-Val-Pro-Leu.

- [00080] The physicochemical properties of the resultant barley hordein or glutelin hydrolysates and fractions thereof may be evaluated to assess their suitability for particular applications. Such properties may include, but are not limited to, degree of hydrolysis, structural
15 and conformational studies, surface hydrophobicity, molecular weight, amino acid composition, peptide sequence, and antioxidant properties including DPPH, superoxide radical, and hydroxyl radical scavenging activities, ferrous-chelating activity, and reducing power.

- [00081] Accordingly, the barley hordein or glutelin peptides produced by the methods described herein may be used for example, as antioxidants in food, cosmetic and pharmaceutical
20 products. In one embodiment, the invention comprises use of barley hordein or glutelin peptides in a food, beverage, cosmetic or pharmaceutical product. In one embodiment, the invention comprises a food, beverage, cosmetic or pharmaceutical product comprising the barley hordein or glutelin peptides.

- [00082] In other embodiments, the invention may comprise products such as nutraceuticals,
25 agricultural, personal care, coatings, or other compositions, which comprises barley hordein or glutelin peptides.

[00083] Exemplary embodiments of the present invention are described in the following Examples, which are set forth to aid in the understanding of the invention, and should not be

construed to limit in any way the scope of the invention as defined in the claims which follow thereafter.

[00084] **Example 1 – Materials**

[00085] Barley grains (Falcon variety, with 13.2% protein w/w) were provided by James
5 Helm (Alberta Agricultural and Rural Development, Lacombe, Alberta, Canada). Barley hordein and glutelin were extracted according to Wang *et al.* (2010). After pearling and milling, the pearled grain flour was treated with ethanol solution (60%, v/v) to isolate the hordein fraction. The protein content of isolated hordein was 91.6%.

[00086] The residue was then treated with alkaline solution (pH 11.5) to extract glutelin. The
10 protein content of isolated glutelin was determined by combustion with a nitrogen analyzer (Leco Corporation, St. Joseph, MI, USA) calibrated with analytical reagent grade EDTA. A factor of 5.83 was used to convert the nitrogen to protein.

[00087] Alcalase 3.0T (3 AU/g solid) was obtained from Novo Nordisk, Bagsvaerd, Denmark and Novozymes China Inc. Flavourzyme (from *Aspergillus oryzae*, 500L), 2-deoxy-D-
15 ribose (DR), ethylenediaminetetraacetic acid (EDTA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), glutathione (GSH), 2,4,6 trinitrobenzene sulfonic acid (TNBS), butylated hydroxytoluene (BHT), 1-anilino-8-naphthalene-sulfonate (ANS), hydrogen peroxide (30%) and the standard molecular markers for HPLC analysis (thyroglobulin, 670 kDa; ferritin, 440 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; cytochrome C, 13.6 kDa and aprotinin, 6.5 kDa) were purchased from
20 Sigma-Aldrich (St. Louis, MO, USA). Potassium ferricyanide, 3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonic acid)-1,2,4-triazine (ferrozine), trichloroacetic acid (TCA), 2-Thiobarbituric acid (TBA), pyrocatechol violet, pyrogallol, and L-ascorbic acid were obtained from Fischer Scientific (Edmonton, AB, Canada). All other chemicals were of analytical grade.

[00088] **Example 2 – Enzymatic hydrolysis of barley hordein and glutelin**

[00089] Hordein was hydrolyzed according to Bamdad *et al.* (2011). Hordein dispersion
25 (2%, w/v) was hydrolyzed by alcalase (0.24 AU/g protein) at pH 8.0 and 50° C for 0.5 to 4 h at a moderate rate, which resulted in a degree of hydrolysis of 5.93% after 4 h of incubation. At the

end of the hydrolysis period, the hydrolysate solution was heated to inactivate the enzyme, adjusted to pH 7.0, and centrifuged to recover the soluble hydrolysate.

[00090] Glutelin was hydrolyzed by alcalase and flavourzyme at an optimized enzyme/substrate ratio according to preliminary experiments for each enzyme. Glutelin (2.0 g) was first dispersed in 100 ml deionized water using a homogenizer (PowerGen-1000, Fisher Scientific, Fairlawn, NJ, USA). After pH and temperature adjustments, proteases were added to the protein suspensions to initiate hydrolysis. Alcalase hydrolysis was conducted at the enzyme/substrate ratio of 0.12 AU/g protein, at pH 8.0 and 50° C. Flavourzyme hydrolysis was performed at the enzyme/substrate ratio of 40 LAPU/g protein, at pH 7.0 and 50° C. The pH value of the hydrolysis mixture was readjusted and optimized every 10 min during hydrolysis with 0.5M NaOH and 0.5M HCl. Hydrolysis was continued for 4 h and the hydrolysate samples were taken out at different time intervals (0.5, 1, 1.5, 2, 3 and 4 hours). At the end of the hydrolysis period, the pH was brought to 7.0. All the hydrolysate solutions were then heated at 95° C for 5 minutes to inactivate the enzyme and centrifuged at $5,000 \times g$ at 23° C for 10 min (Beckman Coulter Avanti J-E Centrifuge System, CA, USA) to separate the soluble hydrolysates from the non-soluble substances. The soluble hydrolysates were lyophilized and stored at 4° C until analyzed. Protein content of the hydrolysates was determined by the nitrogen analyzer.

[00091] The degree of hydrolysis (DH) was determined during the proteolytic reaction by measuring free primary amines and using L-leucine as the standard according to the TNBS method (Adler-Nissen, 1979) with modifications. Hydrolysate samples (0.25 ml, 0.8 g/l) were pipetted into test tubes containing 2.0 ml of sodium phosphate buffer (0.2 M, pH 8.2), then 2.0 ml of TNBS reagent (0.01%) was added, followed by mixing and incubation at 50° C for 60 min in a covered water bath (to avoid exposure to light). At the end of incubation, the reaction was terminated by addition of 4.0 ml HCl (0.1 M) to each tube. The solutions were cooled to 23° C for 30 minutes, and the absorbance was measured at 340 nm with a UV-visible spectrophotometer (model V-530, Jasco, CA, USA). The total number of amino groups was determined in a sample completely hydrolyzed with 6N HCl at 110° C for 24 h. L-Leucine (0.25×10^{-3} M) was used to construct a standard curve. The free amino content in hydrolysate

samples were expressed as Leu amino acid equivalents, based on the equation of the Leu standard curve generated. The DH values were calculated using the following formula:

$$\text{DH (\%)} = (h/h_{\text{tot}}) \times 100 \quad (1)$$

where h is the number of peptide bonds broken during hydrolysis expressed as mmol/g of protein and h_{tot} is the total amount of peptide bonds in the protein substrate determined from the amino acid composition. For barley glutelin, h_{tot} was 7.93 mmol/g of protein.

[00092] **Example 3 - Fractionation of hydrolysates**

[00093] The hordein hydrolysates were separated through a series of ultra-filtration membranes with molecular weight (M_w) cutoffs of 10, 5, and 1 kDa using a membrane-based tangential flow filtration unit (Centramate™ PE, Pall Life Sciences, Mississauga, ON, Canada). The M_w distribution of the most effective sample and its membrane fractions was evaluated using size exclusion chromatography (Agilent 1100 series HPLC system equipped with a Biosuite™ 125Å/5µm HR-SEC column, 7.8 × 300 mm, Waters Corp., Mass., USA). A calibration curve was obtained from the log M_w of the standard molecular markers and their respective elution times ($R^2 = 0.99$).

[00094] For glutelin, protein samples hydrolyzed for 2 hours with alcalase demonstrated the highest activities and were selected for further analysis. The lyophilized barley glutelin hydrolysate was dissolved in deionized water and passed through an Ultra/Diafiltration system equipped with Centramate Cassettes filtration system (T-series Omega, PALL Life Science, Ann Arbor, MI, USA) using membranes with molecular weight cutoff values of 10 and 1 kDa. The fractions with M_w distribution of > 10 kDa, 1-10 kDa and <1 kDa were collected, lyophilized and stored at 4° C. The antioxidant properties of the AH fractions were evaluated. The concentration of the peptides used was 1.0 g/l for all tests.

[00095] **Example 4 – Characterization**

[00096] **i) Circular Dichromism (CD) Spectra**

[00097] CD spectra of samples at 0.25 mg/mL were recorded on a Jasco™ J-810 spectropolarimeter. For far-UV measurement (180-260 nm), a 0.1 cm quartz cell was used in a nitrogen atmosphere. Ellipticity was recorded at scan speed of 50 nm/min, 0.5 nm resolution, 1.0 nm bandwidth and 10 accumulations. Solvent (water) background was subtracted from the spectra.

[00098] **ii) Fourier Transform Infrared Spectroscopy (FTIR)**

[00099] Samples (5%, w/v) were dissolved in deuterium oxide (D₂O) and placed between two CaF₂ windows separated by a 25 µm polyethylene terephthalate film spacer. Infra-red spectra were recorded at 23° C using a Nicolet™ 6700 spectrometer (Thermo Scientific, Madison, WI, USA) at a resolution of 4 cm⁻¹ and a total of 128 scans for each sample. The spectrometer was continuously supplied with nitrogen. To study the amide I region (1600-1700 cm⁻¹) of the protein, Fourier self-deconvolutions were performed using the Omnic™ 8.1.210 software. Band narrowing was achieved with a full width at half maximum of 20-25 cm⁻¹ and with a resolution enhancement factor of 2.0-2.5 cm⁻¹.

[000100] **iii) Surface hydrophobicity**

[000101] Surface hydrophobicity (H_0) was determined using the apolar fluorescent dye ANS (Kato & Nakai, 1980; Tang *et al.*, 2008). ANS solution (20 µL, 8.0 mM in phosphate buffer) was added to serial dilutions of samples containing 0.0025 to 0.0375% (w/v) protein. Twenty µL ANS solution (8.0×10^{-3} M in 0.1 M phosphate buffer, pH 7.4) was added to 4 mL sample. For hordein samples, fluorescence intensity (FI) was determined using a fluorescence spectrophotometer (Shimadzu RF-5301 spectrofluorometer, Tokyo, Japan) with 390 nm and 470 nm set as the respective excitation and emission wavelengths, with a constant excitation and emission slit of 5 nm. For glutelin samples, FI was measured within 5-15 min after mixing using the Jasco™ FP-6300 spectrofluorometer (Tokyo, Japan) with excitation and emission wavelengths set at 390 nm and 510 nm, respectively. The initial slope of the fluorescence

intensity versus protein concentration plot was calculated by linear regression analysis and used as an index of the protein surface hydrophobicity (H_o).

[000102] **iv) Molecular weight**

[000103] The average molecular weight (M_w) of the glutelin hydrolysates was
5 determined by high-performance size exclusion chromatography (SE-HPLC) (Agilent series 1100, California, US) equipped with a Biosuite™ 125/5 μ m HR-SEC column (7.8 $\times$ 300 mm; Waters Corporation, Massachusetts, USA) at 25 $\pm$ 0.5° C. The elution buffer contained 0.05M Na₂HPO₄/NaH₂PO₄ and 0.1M NaCl and its flow rate was 0.5 ml/min. Sample solution (20 μ l) was injected into the HPLC system and the protein elution was monitored at the UV wavelength
10 of 280 nm. Standard molecular markers were used to calculate M_w of the hydrolysates. A calibration curve was made from the log M_w of the markers and their respective elution times (R^2 = 0.99).

[000104] **v) Amino acid analysis**

[000105] For amino acid analysis, the extracted barley glutelin and the prepared
15 peptides were hydrolyzed under vacuum in 4 M methanesulfonic acid with 0.2% (w/v) tryptamine according to the method of Simpson, Neuberger and Liu (1976) with slight modifications. Glass sample tubes (6 $\times$ 50 mm) were used in the reaction vial assembly, which were then placed in the Work Station™ (Waters, Milford, MA, USA). After treating as suggested in the Work Station manual, the contents were hydrolyzed at 115° C for 24 h, and the
20 pH was adjusted to neutral with 3.5 M NaOH. Amino acid analysis was performed using the Waters ACCQ-Tag™ method. The high-performance liquid chromatography (HPLC) system (Agilent series 1100, Palo Alto, CA, USA) consisted of an autosampler and a binary pump, a control system with a column heater maintained at 37° C, and a UV detector set at a wavelength of 254 nm. A reversed-phase AccQ.Tag 150 $\times$ 3.9 mm C18 column with a three-eluent gradient
25 solvent system (AccQ.Tag eluent, acetonitrile, and water) at a flow rate of 1.5 ml/min was used. Data acquisition was controlled by ChemStation™ software.

[000106] **Example 5 – Antioxidant Properties**

[000107] **i) Determination of median effective concentration (EC₅₀ value) in antioxidant activity assays**

[000108] The EC₅₀ value is defined as the concentration of hydrolysate (mg/mL) required to achieve 50% decrease in initial radical concentration. It has the advantage of displaying the antioxidant activity and its correlation with peptide concentration. For the following antioxidant activity assays, the EC₅₀ value was calculated from linear regression analysis of antioxidant activities at different peptide concentrations, if applicable. The lower EC₅₀ values indicate stronger scavenging ability in an antioxidant sample.

10 [000109] **ii) DPPH scavenging activity**

[000110] DPPH free radical scavenging activity of hordein or glutelin hydrolysates was assessed according to the method of Tang *et al.* (2010) with slight modifications (Bamdad *et al.*, 2011). Aliquots of samples were mixed 1:1 (v/v) with 0.1 mM DPPH in anhydrous ethanol. After incubation at 25° C in the dark for 30 min, the reduction of DPPH free radicals was determined by measuring the absorbance at 517 nm with a UV-visible spectrophotometer (Model V-530, 15 Jasco, CA, USA). Butylated hydroxyl toluene and tocopherol were used as positive controls. Radical scavenging activity of the hydrolysates was calculated as follows:

$$\% \text{DPPH free radical scavenging} = 1 - (A_s / A_c) \times 100 \quad (2)$$

where A_s and A_c represent the absorbances of the sample and the control, respectively. The control contained everything except the hydrolysates.

[000111] **iii) Superoxide radical (O₂^{•-}) scavenging activity**

[000112] The superoxide radical scavenging activity was estimated at 25° C by spectrophotometrically monitoring the inhibition of pyrogallol autoxidation (Li *et al.*, 2008). This assay is dependent on the reducing activity of the test compound by an O₂^{•-}-dependent reaction, which releases chromophoric products. Eighty µL of hordein or glutelin hydrolysates (1.0 mg/mL) was mixed with 80 µL of 50 mM Tris-HCl buffer (pH 8.3) in a 96-well microplate followed by the addition of 40 µL of 1.5 mM pyrogallol in 10 mM HCl. The rate of O₂^{•-}-induced

polymerisation of pyrogallol ($\Delta A_s/\text{min}$) was measured as increase in absorbance at 320 nm for 5 min at 23° C (Marklund *et al.*, 1974). BHT was applied as a positive control and Tris-HCl buffer was used instead of hydrolysates in blank experiments ($\Delta A_c/\text{min}$). Blank contained all the solutions except the hydrolysate. The $\text{O}_2^{\bullet-}$ scavenging activity of hydrolysates was calculated as follows:

$$\text{O}_2^{\bullet-} \text{ scavenging activity} = [(\Delta A_c/\text{min} - \Delta A_s/\text{min})] / (\Delta A_c/\text{min}) \times 100 \quad (3)$$

[000113] **iv) Hydroxyl radical (OH $\cdot$) scavenging activity**

[000114] The hydroxyl radical scavenging assay was carried out using the method described by de Avelar *et al.* (2004) after minor modifications. Barley glutelin hydrolysate (1.0 g/l, 250 μl), EDTA (2.4×10^{-3} M, 42 μl) and FeCl_3 (0.5×10^{-4} M, 400 μl) were dissolved in phosphate buffer (pH 7.4) and mixed thoroughly. Ascorbic acid (0.1 M, 1 μl), DR (0.2 M, 14 μl) and H_2O_2 (0.01%, 142 μl) were added in and the mixture was incubated at 37° C for 60 min. Then the mixture was boiled for 15 min with TBA (10.0 g/l, 1ml) and TCA (28.0 g/l, 1ml). Afterwards, the mixture absorbance was measured at 532 nm (A_{s2}) with the UV-visible spectrophotometer. Phosphate buffer (250 μl) was used as blank control. Hydroxyl radical scavenging ability was evaluated as the inhibition rate of 2-deoxy-D-ribose oxidation by hydroxyl radicals. BHT (0.01 and 0.1 g/l) was used as the positive control. Results were determined using the following equation:

$$\text{Hydroxyl (OH)} \text{ Scavenging Activity (\%)} = (A_{s2} - A_{c2}) / A_{c2} \times 100\% \quad (4)$$

where A_{s2} and A_{c2} represent the absorbance of the sample and the control, respectively. Control contained everything except the hydrolysates (phosphate buffer was used instead of hydrolysates).

[000115] **v) Ferrous ion chelating activity**

[000116] The ferrous (Fe^{2+}) chelating activity of barley protein hydrolysates was measured as described by Kong *et al.* (1990). One mL of 20 mM FeCl_2 was added to 0.5 mL of hordein or glutelin hydrolysates and the reaction was initiated by the addition of 1 mL ferrozine

(0.5 mM). The mixture was vortexed and kept at 23° C for 10 min prior to measuring the absorbance at 562 nm. Ferrozine-Fe²⁺ is a pink chromophore that absorbs strongly at 562 nm. EDTA, a strong metal chelator, was used as a positive control. The chelating ability was calculated as follows:

$$5 \quad \% \text{ Ferrous ion chelating ability} = 1 - (B_s - B_c) \times 100 \quad (5)$$

where B_s and B_c represent the absorbances of the sample and the control, respectively. The control contained everything except the hydrolysates.

[000117] **vi) Reducing power**

[000118] The reducing power of hordein or glutelin hydrolysates was measured
 10 according to the method of Zhang *et al.* (2009). One mL of hordein or glutelin hydrolysates (2 mg/mL) was added to a solution containing 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50° C for 20 min, followed by addition of 2.5 mL of 10% trichloroacetic acid to stop the reaction. After centrifugation at 5000 × g for 10 min, 2.5 mL of the upper layer was diluted with 2.5 mL deionised water and 0.5 mL of
 15 0.1% FeCl₃. After a 10 min reaction, the absorbance was recorded at 700 nm. The blank experiment contained everything except the sample. An increased absorbance of the reaction mixture indicated the increased reducing power. L-ascorbic acid (0.01 and 0.1 g/l) was used as a positive control.

[000119] **Example 6 – Fractionation of peptides from hordein and glutelin**
 20 **hydrolysates by reversed phase - high performance liquid chromatography**

[000120] Since hordein has a high content of hydrophobic residues (33.12%), hydrophobicity may play a key role in peptide bioactivity. The most potent antioxidant peptides from the hydrolysate after 1.5 h of digestion were thus fractionated based on their hydrophobicity. Fractionation was conducted using the Agilent 1100 series HPLC system on an
 25 analytical scale reversed phase column (Eclipse XBD-C18 column, 4.6 × 150 mm, 5µm) with a linear gradient mixture composed of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). The gradient elution conditions were: 5% solvent B for 5 min, 5-40% B over 30 min, 40-90% B over 10 min and then 5 min at 90% B. Based on their hydrophobicity, the most

potent membrane fractions were further purified using the same HPLC system on a Zorbax™ 300SB-C8 semi-preparative column (4.6 × 250 mm) with a similar elution profile at a flow rate of 1.5 mL/min. Several runs were performed to collect adequate volumes of fractions which were then freeze-dried prior to further analysis.

- 5 [000121] The AH fraction with $M_w < 1$ kDa was selected for further fractionation by reversed-phase high performance liquid chromatography (RP-HPLC). The lyophilized sample (100 g/l) was reconstituted in 0.1% trifluoroacetic acid (TFA) solution and injected into a HPLC system equipped with a Zorbax™ SB-C18 column (5 μm, 4.6 × 150 mm; Agilent, CO, USA). The gradient elution was performed at a flow rate of 1.0 ml/min with eluent A as 0.1% TFA in
10 distilled water and eluent B as 0.1 % TFA in acetonitrile (ACN). Separation was performed using a linear gradient elution of 5 to 40% B in 30 min and the peptide peaks were monitored at UV wavelengths of 280 nm. The peaks corresponding to peptides were collected as four fractions and freeze-dried. The antioxidant properties of these fractions were evaluated. The concentration of the peptides used was 1.0 g/l for all the tests.

15 [000122] **Example 7 – Identification of peptides by mass spectroscopy (LC-MS/MS)**

- [000123] The most potent antioxidant fractions were analyzed by LC-MS/MS to identify the peptide sequence. The peptides were subject to LC-MS/MS analysis on a UPLC (Waters, Milford, MA) coupled with q-ToF premier mass spectrometer (Waters, Milford, MA). 5
20 μL of sample was loaded onto a nanoAcquity™ UPLC system with peptide trap (180 μm × 20 mm, Symmetry® C18 nanoAcquity™ column, Waters, Milford, MA) and an analytical column (75 μm × 150 mm, Atlantis™ dC18 nanoAcquity™ column, Waters, Milford, MA). The column was connected to a Q-ToF premier (Waters Corporation) for ESI-MS/MS analysis. Peptides were separated with a gradient of 1-65% solvent A (acetonitrile, 0.1% formic acid) over 35 min at a
25 flow rate of 300 nL/min. The flow entered directly into the mass spectrometer via a nanoLockspray™ ionization source in a positive ion mode (capillary voltage of 3.80 kV and source temperature of 100° C). Spectra were recorded over the mass/charge (m/z) ranges of 100-1000 in MS mode and 50-1500 in MS/MS mode. The signal threshold to perform auto-MS/MS in the data-dependent acquisition was 20 counts/s in total ion current, and the precursor ions

were isolated within a range of m/z 3.0. Instrumental control and data analysis were performed using MassLynx™ software (Micromass U.K. Ltd., Manchester, U.K.). Peaks Viewer™ 4.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) was used in combination with manual de novo sequencing to process the MS/MS data and to perform peptide sequencing. Confidence of positive protein identification was judged by high protein and peptide scores in the search results. Manual inspection of the original MS/MS spectra was often performed to ensure major peaks in the MS/MS spectra were matched and explained.

[000124] **Example 8 - Statistical analysis**

[000125] For the hordein studies, all experiments were performed at least in three independent trials and the results were reported as mean $\pm$ standard deviation. Results were subjected to the analysis of variance using the SAS (SAS Institute, Inc., Cary, NC) and statistical significance of differences ($p < 0.05$) was evaluated by the least significant difference procedure.

[000126] For the glutelin studies, each type of hydrolysate was prepared in two independent batches. The hydrolysate structure characterization and antioxidant property measurements were conducted in triplicate for each batch. Data are represented as the mean of two batches $\pm$ SD. For hydrolysate fractionation with ultra-filtration and reverse phase HPLC, one batch of the sample was randomly selected and the antioxidant data are the mean of three independent determinations $\pm$ SD. Statistical significance of the differences was determined by Student's t-test ($p < 0.05$).

[000127] **Example 9 - Antioxidant peptides derived from barley hordein**

[000128] Hordein was hydrolyzed as described in Example 2. The peptide size decreased as the incubation time increased (FIG. 1). Hydrolysate after 0.5 h treatment was characterized by one major peak (7.6 kDa) and a group of smaller peaks ranging from 6.7 to 2.3 kDa. Further hydrolysis for 1.5 h resulted in evident elevation of 6.7 to 2.3 kDa peaks, while the larger peptides of 7.6 kDa still remained. After 4 h, the major peak shifted to M_w of approximately 2 kDa, with a concomitant decrease in all large peptide peaks.

[000129] FIG. 2 shows the M_w distribution of each fraction. Since hordein hydrolysate after 1.5 h digestion (denoted as "HA-1.5h") exhibited a wide-ranging distribution of peptides in

terms of M_w from 7.6 to lower than 2 kDa, it was selected for further characterization to assess its potential antioxidant activity.

[000130] The CD spectra are shown in FIG. 3A. All the samples showed a broad negative peak at 200-203 nm, albeit with different intensities. These spectra have been assigned to repeated β -turn and poly-L-proline structures (Tatham *et al.*, 1995; Shewry *et al.*, 1995). B-hordeins, the major component of barley hordein, have N-terminal domains composed of repeated short sequences of proline and glutamine which favor the β -reverse turns and poly-L-proline II helices as the secondary structure (Shewry *et al.*, 1995). The >10 kDa fraction showed a similar spectrum as previously reported for cereal prolamins (Tatham *et al.*, 1995; Shewry *et al.*, 1995). This spectrum implies that this fraction contains large peptides which retain much of the native hordein conformation. Further hydrolysis of these large peptides resulted in medium-sized peptides with more unordered and flexible conformations (Mills *et al.*, 2003). This explains the significantly reduced signal intensity ($p < 0.05$) for medium-sized fractions (5-10 and 1-5 kDa). The significant loss of ellipticity can also be explained by its peptide length dependence (Petrella *et al.*, 1996). The small-sized peptides (<1 kDa) exhibited low ellipticity due to the effect of terminal residues, both electrostatically and conformationally, showing a typical spectrum of irregular structure (weak negative signal at 195 nm) (Petrella *et al.*, 1996; Ranjbar *et al.*, 2009).

[000131] The FTIR spectra are shown in FIG. 3B. In the highest M_w fraction (>10 kDa) the band at 1638 (β -sheet) was accompanied by two shoulders at 1620 (poly-L-proline) and 1663 cm^{-1} (β -turn structure) (Lefevre *et al.*, 1999), suggesting the existence of β -sheets as another major secondary structure. Medium-sized peptides (5-10 and 1-5 kDa) showed notably reduced poly-L-proline helices (1620 cm^{-1}) and β -sheet structures (1638 cm^{-1}), while the unordered coils at 1649 cm^{-1} became a distinct peak. This result confirms the formation of peptides with more unordered and flexible conformations. As the smaller-sized peptides (1-5 and <1 kDa) were concentrated in fractions, the band at 1592 cm^{-1} enhanced. Ionized carboxyl groups in D_2O have strong absorption bands at around 1590 cm^{-1} (Venyaminov *et al.*, 1990). Similar to other prolamins storage proteins, hordein is enriched with glutamine. Without being bound by any

theory, glutamine residues may have been exposed as a result of peptide bond cleavage and then converted to glutamic acid (deamidation) during hydrolysis.

[000132] FIG. 4 shows the surface hydrophobicity of hordein membrane fractions compared to the unfractionated sample (HA-1.5h). The highest hydrophobicity was observed in the largest peptide fraction (>10 kDa). Without being bound by any theory, the tertiary structure of hordein resulted in a high molecular surface hydrophobicity with hydrophilic core surrounded by hydrophobic segments. Large peptides probably maintain the major structures in original hordein, thus exhibited rather high surface hydrophobicity. With decreasing of the M_w , peptide surface hydrophobicity decreased progressively due to exposure of the polar residues. Even the medium-sized peptide fraction of 5-10 kDa had higher surface hydrophobicity than the unfractionated hydrolysate. Without being bound by any theory, this may suggest that most of the peptides with high surface hydrophobicity have been concentrated in large M_w fractions. Surface hydrophobicity of peptides strongly depends upon the conformation as well as the hydrophobic contribution of amino acids in the peptide (Mahn *et al.*, 2009).

[000133] As demonstrated in FIG. 5A, the alcalase hydrolysates (HA) and the membrane fractions exhibited very strong DPPH radical scavenging activity, with EC_{50} values ranging from 0.5 to 3.8 mg/mL. Zein hydrolysates exhibited lower than 50% DPPH scavenging activity at 10 mg/mL (Tang *et al.*, 2010) and porcine plasma hydrolysates showed DPPH scavenging effect of 19-76% at 40 mg/mL (Liu *et al.*, 2009). BHT and tocopherol have much stronger DPPH scavenging effects (EC_{50} values of 5 and 8 μ g/mL, respectively) than protein hydrolysates. However, peptides can be used in both hydrophilic and hydrophobic systems at higher concentrations. The lowest EC_{50} value was observed for the largest peptides (>10 kDa) and with M_w decreasing, the EC_{50} values increased significantly ($p < 0.05$). This result emphasizes the importance of peptide size in contributing to DPPH radical scavenging effect. High DPPH scavenging capacity of peptides relies on a proper balance between high levels of hydrophobicity and good diffusivity in the reaction medium (Bamdad *et al.*, 2011). Partial unfolding of hordein during hydrolysis exposed the inside hydrophilic core, leading to a dramatic improvement in protein solubility, and at the same time, a significant decrease in surface hydrophobicity (Bamdad *et al.*, 2011). The largest soluble peptides thus exhibited the highest

DPPH scavenging capacity. Peptides with high surface hydrophobicity have been concentrated in large M_w fractions and the exposed hydrophobic residues may have a close contact with DPPH radicals to exert strong scavenging effect.

[000134] In the superoxide radical scavenging assay, no clear correlation was observed
5 between peptide concentration and scavenging activity. The percent scavenging activity at hydrolysate concentration of 1 mg/mL was thus reported in FIG. 5B. Significant increments were observed upon fractionation of hydrolysates, particularly after 1.5 to 4 h enzymatic treatment. The fractionated samples exhibited higher superoxide scavenging activity compared to many other protein hydrolysates, such as rice endosperm (46.4% at 6 mg/mL) and zein protein (11.5%
10 at 10 mg/mL) (Tang *et al.*, 2010; Zhang *et al.*, 2009). His, Pro, Tyr, and Trp are the most important residues in radical scavenging activity of antioxidant peptides (Saito *et al.*, 2003). These amino acids comprise more than 26% of the total residues in hordein. However, the correlation of M_w and superoxide scavenging activity was not evident. Without being bound by any theory, most peptide fractions (regardless of their molecular sizes) are able to inactivate the
15 superoxide anion radicals.

[000135] Peptide fractions obtained from membrane separation showed a significant ($p < 0.05$) enhancement of chelating activity as supported by a dramatic decrease of the EC_{50} values (FIG. 6). For most of the membrane fractionated samples, the EC_{50} values increased initially and then decreased during longer hydrolysis times. At the early hydrolysis stage, poly proline-like
20 structure of the larger peptides confers a spiral superstructure shape (Shewry *et al.*, 1995) which, in combination with some metal chelating residues such as Thr, Glu and Asp, might probably entrap the transition metals to hinder sterically their interaction with lipid hydroperoxides. At later stages, chemical chelating of the ferrous ion was probably achieved by exposed charged groups in small peptides when abundant glutamine residues were exposed and converted to
25 glutamic acid (deamidation). Without being bound by any theory, the much higher chelating capacity observed for <1 kDa peptides than for >10 kDa peptides suggests that the ion chelating via exposed charged groups on hordein molecular chains due to extensive hydrolysis may be the main mechanism for their high metal ion chelating effect. Low M_w fractions (1-5 kDa and < 1

kDa) displayed comparable and even lower EC₅₀ values than EDTA (0.45 mg/mL), a finding which has not been reported for other protein hydrolysates.

[000136] In the reducing power assay, the ability of peptides in electron donation changes the redox status of iron (Fe^{3+} converts to Fe^{2+}). Since the percent activity is not reported in this assay, the EC₅₀ value could not be defined. Separating the large-sized peptides (>10 kDa) enhanced the reducing power to 2.5, 3 and 3.9 fold of the non-fractionated sample treated by 1, 2 and 3-h hydrolysis, respectively (FIG. 7). The reducing power of the large peptides increased significantly with prolongation of the hydrolysis time until 3 h and then decreased, suggesting that the peptides with relatively smaller M_w among the large-sized fraction were the most effective peptides in reducing power. Cys/Met as sulfur containing residues, and Glu by donating the acidic hydrogen atom contribute positively to the reducing power of the peptide (Udenigwe *et al.*, 2011). Trp and Tyr have a high density of electrons on their side chains. Without being bound by any theory, it is possible that the electron-dense side chain groups such as Tyr, Cys, Met and Glu were concentrated in certain peptides in large-sized fractions. Further hydrolysis resulted in spreading of the effective residues all over the smaller peptides. Tuna protein hydrolysates also exhibited the same trend. The largest M_w fraction (>10 kDa) had a significantly higher reducing power compared to the smaller M_w fractions due to high contents of Tyr, Trp, Met, Lys, Cys and His (Ahn *et al.*, 2010).

[000137] As shown in FIG. 8, the fraction with M_w >10 kDa is characterized by a group of peaks (retention times of 19-22 min) at the high hydrophobicity part of the chromatogram. The intensity of these peaks declined gradually in 5-10 kDa and 1-5 kDa fractions and almost disappeared in <1 kDa fraction. Peptides with M_w of 5-10 kDa showed a more balanced distribution of sub-fractions with different hydrophobicity through the elution time course compared to other fractions. This fraction (5-10 kDa, denoted as "HA-1.5h-5-10kDa") was selected for further purification using semi-preparative RP-HPLC. The fraction having the smallest peptides was also selected (<1 kDa, denoted as "HA-1.5h-<1kDa") for exhibiting very strong ferrous ion chelating capacity. Several runs with high separation reproducibility (in terms of retention times, resolutions and peak areas) were performed and the eluents were collected in four fractions (0-6, 6.1-13, 13.1-19 and 19.1-29 min retention times; denoted as "F1 to F4").

[000138] The antioxidant activities of the hordein hydrolysates and fractions were determined. The amino acid compositions were determined using Waters AccQ-Tag™ precolumn method. Dried samples were hydrolyzed under vacuum and after derivatization were loaded on reversed phase column. The AccQ™ Reagent, 6-aminoquinolyl-N-

- 5 hydrozysuccinimidyl carbamate, is an N-hydrozysuccinimide-activated heterocyclic carbamate which converts both primary and secondary amino acids to stable fluorescent derivatives. Tables 1 and 2 summarize the antioxidant activities and amino acid compositions of the hydrolysates (HA-1.5h-5-10kDa; HA-1.5h-<1kDa) and the fractions (F1-F4).

[000139] **Table 1. Antioxidant activities of barley hordein hydrolysates and separated fractions**

Fractions	DPPH radical scavenging activity (EC ₅₀ , mg/ml)	Superoxide radical scavenging activity		Ferrous ion chelating activity (EC ₅₀ , mg/ml)
		EC ₅₀ (mg/ml)	% scavenging (1 mg/ml)	
HA-1.5h-5-10kDa	1.6 ^c	n.d.	32.6 ^c	1.4 ^b
F1	3.54 ^b	1.6 ^b	37 ^a	2.3 ^a
F2	1.78 ^c	1.7 ^b	35.7 ^b	2.1 ^a
F3	1.94 ^c	2.4 ^a	26 ^d	1.6 ^b
F4	4.95 ^a	3.3 ^a	18 ^c	1.2 ^b
HA-1.5h-<1kDa	3.7 ^a	n.d.	31.8 ^a	0.54 ^a
F1	4.1 ^b	4.4 ^b	21.2 ^c	0.024 ^c
F2	3.9 ^b	3.2 ^c	24.8 ^b	0.080 ^b
F3	n.d.	6.1 ^a	19 ^d	0.019 ^c
F4	n.d.	4.8 ^b	14.1 ^c	0.014 ^d

*Values with different lowercase letters in the same test for each fraction set are significantly different ($p < 0.05$)

[000140] **Table 2. Amino acid compositions (mole %) of barley hordein hydrolysates and separated fractions**

Residue	HA-1.5h-5-10kDa	F1	F2	F3	F4	Residue	HA-1.5h-<1kDa	F1	F2	F3	F4
Ala	1.8	4.5	4	1.2	0.48	Ala	4.9	6.3	5.0	2.5	0.68
Arg	1.6	4.1	3	0.89	0.66	Arg	4.7	5.8	4.9	2.7	3.5
Asx	1.9	2.7	3.8	1.6	n.d.	Asx	2.1	2.4	2.4	1.9	0.57
Cys	2.2	0.82	4.7	2.4	0.68	Cys	0.64	0.70	0.96	0.57	n.d.
Glx	33.6	46.5	28.8	32.9	35.6	Glx	28.2	39.1	20.0	18.3	18.5

Gly	1.9	4.9	4.2	1.2	n.d.	Gly	4.1	5.2	5.4	1.9	1.5
His	0.95	2.6	1.4	0.72	n.d.	His	2.2	3.3	2.0	0.93	n.d.
Ile	3.4	n.d.	3.4	3.7	3.5	Ile	5.5	2.8	5.5	9.7	11.9
Leu	5.4	1.1	8.8	5.3	5.1	Leu	10.1	5.9	13.4	14.0	10.8
Lys	0.52	n.d.	0.55	0.47	0.33	Lys	1.2	1.5	0.96	1.5	1.2
Met	1	1.2	2.1	1	0.28	Met	1.5	1.2	1.4	1.4	8.8
Phe	4.8	n.d.	1.6	5.3	8.6	Phe	3.9	n.d.	4.2	8.3	13.0
Pro	22.4	4.4	11.5	26.1	30.2	Pro	10.0	5.1	9.7	17.0	21.7
Ser	10.8	18.4	10.9	9.4	10.2	Ser	6.2	8.3	5.5	3.9	1.5
Thr	1.6	3.1	3.3	1.3	0.42	Thr	3.1	3.1	4.0	1.6	0.70
Tyr	2.3	n.d.	1.5	2.9	2.2	Tyr	2.2	1.1	3.3	3.6	3.0
Val	3.7	5.5	6.3	3.5	1.6	Val	9.4	7.7	11.0	9.9	2.3

^a Asx includes aspartate + asparagine and Glx includes glutamine + glutamate.

[000141] In DPPH radical scavenging analysis, the EC₅₀ value of HA-1.5h-5-10kDa (1.6 mg/ml) was significantly lower than that of HA-1.5h-<1kDa (3.7 mg/ml), although they possess similar contents of hydrophobic residues including Ala, Pro, Val, Leu, Ile, Met and Phe (43 and 45%, respectively) (Tables 1 and 2). Considering that large-sized peptide fractions (>10 kDa) exhibited high surface hydrophobicity, it is possible that surface hydrophobicity of the peptides rather than total hydrophobicity plays an important role in DPPH scavenging.

Fractionation of peptides based on their hydrophobicity yielded noticeable changes in DPPH scavenging activities. For fractionated HA-1.5h-5-10kDa samples, significantly lower EC₅₀ values were obtained for fractions 2 and 3 (1.78 and 1.94 mg/ml, respectively).

[000142] Amino acid composition analysis of the fractions from HA-1.5h-5-10kDa indicates more hydrophobic residues in F2 (37%) and F3 (48%) compared to F1 (15%) which may exist at the peptide surface to perform scavenging effects (Table 2). F4 had the highest hydrophobic amino acid content (52%); however, it was less effective in terms of DPPH radical scavenging activity. Without being bound by any theory, this may be attributed to the lower content of Cys and Met, the well-known oxidant scavenger residues (Levine *et al.*, 1996), compared to F2 and F3. Among the fractions from HA-1.5h-<1kDa, F1 and F2 had high content (52 and 30%, respectively) of charged amino acids (Arg, His, Asx and Glx), whereas the hydrophobic residues (Ile, Leu, Phe, Pro, Val) were all concentrated in F3 and F4 (Table 2). F3 and F4 may possess better DPPH scavenging effects; however, such high hydrophobicity (65 and

63%, respectively) caused turbidity in DPPH radical scavenging reactions, making it impossible to measure the scavenging ability.

[000143] RP-HPLC fractions exhibited a clear correlation between concentration and superoxide radical scavenging, which allowed calculation of the EC₅₀ values (Table 1). This may be explained by the hydrophilic-hydrophobic partitioning of residues in each fraction (Mendis *et al.*, 2005). Hydrophilic fractions (F1 and F2) from both HA-1.5h-5-10kDa and HA-1.5h-<1kDa exhibited significantly lower EC₅₀ values and higher scavenging effect at 1 mg/ml (Table 1). Although the contribution of individual residues in superoxide radical scavenging is unclear, several studies support the major role of His, Pro, Ala, Lys and Leu in scavenging free radicals (Mendis *et al.*, 2005). The significant difference ($p < 0.05$) between RP-HPLC fractions in terms of superoxide radical scavenging ability could be closely related to their amino acid composition (Table 2). F1 and F2 contained significantly higher amounts of Arg, His, Met, Ser, Ala, Val, Asx and Gly. High proportions of polar, charged and small-side chain residues in combination with some portions of bulky residues such as Phe, Pro and Tyr suggest the importance of charged, small and eventually redox-active residues in scavenging superoxide radicals.

[000144] In the ferrous ion chelating assay, short peptides (<1 kDa) showed stronger chelating capacity than medium-sized peptides (5-10 kDa). The difference was even more dramatic after fractionation based on hydrophobicity. Only the most hydrophobic fraction (F4) from HA-1.5h-5-10kDa exhibited a slight decrease in EC₅₀ value. All the fractions of HA-1.5h-<1kDa possessed significant enhancement of chelation capacity (6 to 38-fold decrease in EC₅₀ values). The lowest EC₅₀ value (0.014 mg/ml) was observed for F4 from HA-1.5h-<1 kDa, which is much lower than that of the EDTA (0.45 mg/mL), demonstrating extremely strong ferrous ion chelating capacity. The short peptides possess a greater negative charge density (charge-to-mass ratio) compared to large peptides, which provide more binding sites for metal ions. Amino acid analysis indicates that the smaller-sized peptides (<1 kDa) have more positively charged residues (Arg, His and Lys) in addition to higher content of Met and Thr, which may also contribute to ferrous ion chelating (Cheng *et al.*, 2010; Pacheco *et al.*, 2009). In both medium- and small-sized peptide fractions (5-10 and <1 kDa fractions), the more

hydrophobic HPLC fractions (F3 and F4) exhibited stronger capacity to chelate ferrous ions. The amino acid composition of those fractions shows that in addition to hydrophobic residues, the abundance of Glx and Ser in F3 and F4 of HA-1.5-5-10kDa and higher amount of Lys and Met in F3 and F4 of HA-1.5-<1kDa may explain the higher tendency toward metal chelation.

- 5 Low M_w peptides derived from hordein possess very strong ferrous ion chelating capacity probably due to their unique structure of high charge density and more exposed hydrophobic residues.

[000145] Due to the limited amount of eluted fractions obtained from RP-HPLC, the antioxidant assays were restricted to free radical scavenging and metal ion chelating assays,

- 10 whereas larger amounts of samples are required for reducing power. Moreover, conformational studies of the membrane fractions displayed the unordered random coil as the main component. The RP-HPLC fractions were thus excluded from conformational studies.

[000146] Considering that the F2 fraction of HA-1.5h-5-10kDa exhibited the highest DPPH and superoxide radical scavenging results and the F4 fraction of HA-1.5h-<1kDa

- 15 exhibited the highest metal chelation, they were selected as the most potent antioxidant fractions for peptide sequencing (Table 3).

[000147] **Table 3. Amino acid sequences of potent peptides identified by LC-MS/MS analysis and their matched protein fragments**

Peptide fraction	Sequence	SEQ ID NO.	Suggested fragment	Mass (experimental)	Mass (Calculated)	Average Local Confidence (%)
HA-1.5h-5-10kDa-F2	QSYVPQPQ	1	f 4-11 C-Hordein	946.5	945.5	61.2
	QQTPLPQ	2	f111-117 B1-Hordein	810.417	810.423	52.2
	QPQPYPQ	3	f32-38 B1-Hordein f39-45 B1-Hordein f62-68 B1-Hordein f38-44 B3-Hordein f16-22 C-Hordein f35-41 C-Hordein	856.42	856.40	80.6
	TQQPYPQ	4	f116-122 Gamma Hordein-3	860.41	860.40	74.5
	SPLQPQ	5	f29-34 C-Hordein	669.3	668.3	79.3
	QQPYPQ	6	f117-122 Gamma Hordein-3 f89-94 Gamma Gliadin	759.36	759.35	76.2
	QPVLSQ	7	f70-75 B3-Hordein	671.4	670.4	69.8
	QVPQ	8	f244-247 B1-Hordein	471.3	470.2	77.8

	LLPQ	9	f108-111 Gamma Hordein-1	470.3	469.3	72.8
	HVLQ	10	f172-175 B1-Hordein f143-146 B3-Hordein	496.3	495.3	65.3
HA-1.5h- <1kDa-F4	KPFPQQPPF	11	f87-95 B1-Hordein	1084.565	1084.570	54
	QPPFWQ	12	f80-85 B1-Hordein f63-68 B3-Hordein	801.384	801.381	53
	SVNVPLY	13	F275-281 B1-Hordein F45-51 B3-Hordein	790.423	790.422	61
	AELIIPQ	14	F43-49 C-Hordein	782.456	782.453	43
	YRIVPL	15	F251-256 B3-Hordein	759.45	759.46	

[000148] The evident high frequency of hydrophobic residues (Pro, Leu, Val) intensifies their influence on prohibiting oxidation reactions. Most of the potent peptides have alternate Gln and Pro residues derived from the N-terminal repetitive domain of barley hordein (Shewry *et al.*, 1995). In addition to the chemical structure of the side chain groups, the vicinal residues are important to the chemical reactivity of amino acids (Levine *et al.*, 1996). By radical transfer reactions, the oxidized residues can exchange the radical damage to the vicinal residues and the resulting peptide radical would be more stable (Elias *et al.*, 2006). Pro is a well-known antioxidant residue found in potent peptides derived from different proteins (Sabeena Farvin *et al.*, 2010). Gln may regulate the intracellular oxidative balance, thus alleviating oxidative damage (Marques *et al.*, 2011). Leu and Val in hordein peptides provide high hydrophobicity for the peptides which is important in performing antioxidant mechanisms. Tyr, present in four peptides in close proximity to Pro and Gln, is able to receive the radical exchange and provide stability for the attacked peptide by virtue of its aromatic side chain. The pentapeptide sequence of QPYPQ found in several potent peptides might be considered as the particular structural motif that plays a key role in scavenging free radicals. The sequence PYPQ was also found in potent antioxidant peptides from yogurt (Sabeena Farvin *et al.*, 2010) and casein hydrolysate (Rival *et al.*, 2001). The intermittent Pro and Gln residues were also observed in three out of four antioxidant peptides originating from caseins (Rival *et al.*, 2001; Fornaroli *et al.*, 2001). The tetrapeptide LLPQ shows the importance of the presence of Leu at the N-terminal which was also noted by Ma *et al.* (2010) for buckwheat protein hydrolysate.

[000149] Sequence analysis of the peptides in the F4 fraction of HA-1.5h-<1kDa revealed some peptides originating from the C-terminal region of B1, B3 and C-hordein. The

repeated sequences of Pro and Gln were also present. Hydrophobic amino acids (such as Leu, Ile, Phe, Pro and Val) were identified, which could probably enhance the metal chelation capacity. Despite the amino acid compositional analysis of the short peptides which showed a relatively high content of Glx, Arg, Met and His, the LC-MS/MS results showed only few numbers of Glu, Arg and Lys in peptide sequences. This may be attributed to the predominance of hydrophobic peptides and partial deamidation of Gln residues which resulted in multiple charged ions and complicated the MS spectra (Ji *et al.*, 2005).

[000150] **Example 10 - Antioxidant peptides derived from barley glutelin**

[000151] The protein content of the isolated barley glutelin and the hydrolysates was 86% and 80-84% (w/w) on a dry basis, respectively. After 1h hydrolysis, more than 40% (w/w) hydrolysates were soluble at 2.0 g/l. Two hours of hydrolysis led to hydrolysates completely soluble in water at the same concentration. The antioxidant activity of the protein hydrolysates depends on the protein substrate, the specificity of the enzyme, the conditions used during proteolysis and the degree of hydrolysis. Barley glutelin hydrolysis was thus performed using two selected proteases, alcalase and flavourzyme, at different hydrolysis times. As shown in Figure 9, the degree of hydrolysis (DH) increased rapidly in the first 0.5 h, followed by a slower rate of increase up to 4 h, with the DH value ranging from 12.7 to 22.8%. The DH of flavourzyme hydrolysates (FHs) was higher ($P < 0.05$) than that of alcalase hydrolysates (AHs) at comparable hydrolysis times. Flavourzyme is an endo- and exopeptidase enzyme mixture, which has broad specificity to produce small-size peptides and free amino acids (Ven *et al.*, 2002). As an endo-protease, alcalase cleaves peptide bonds at the interior of the polypeptides chain to produce small- and medium- sized peptides (Klompong *et al.*, 2008). Consequently, flavourzyme treatment results in hydrolysates with higher DH.

[000152] The SE-HPLC chromatograms of the FH after 0.5h of hydrolysis was characterized by two major peaks (P3 and P4) at M_w of 1.4 kDa and 0.7 kDa, respectively and a broad peak (P1) with rather high M_w (17.2 kDa), and several small peaks (P2) in the M_w range of 5.1-3.2 kDa (Figure 10A (a)). After 4 h hydrolysis, P1 disappeared, whereas the P2 amplitude was dramatically enhanced. For AH chromatogram after 0.5 h of hydrolysis, three major peaks were identified with M_w of 22.5 kDa (P1'), 7.8 kDa (P2') and 1.5 kDa (P3') (Figure 10A (b)).

Meanwhile, several shoulder peaks were found between P2' and P3'. After 4 h hydrolysis, P1' and the shoulder peaks were significantly reduced while P3' was enhanced remarkably.

[000153] The SEC-HPLC profiles were then divided into three fractions based on their apparent M_w . Fraction I corresponds to large-sized peptide fragments ($M_w > 10$ kDa), fraction II to medium-sized peptide fragments ($1 \text{ kDa} < M_w < 10 \text{ kDa}$) and fraction III to small-sized peptide fragments ($M_w < 1 \text{ kDa}$). Figure 10B summarizes the quantitative changes of these three fractions during hydrolysis, represented by the area of each fraction relative to the total area of the SE-HPLC chromatogram. In the case of FHs, with increasing hydrolysis time, fraction II increased significantly ($p < 0.05$) with concurrent reduction of fraction I in the chromatogram, indicating a degradation of large peptides to medium-sized peptides. Hydrolysis with alcalase resulted in the medium-sized peptides as the major fraction which depicts the endoprotease nature of the enzyme.

[000154] Without being bound by any theory, the slight increase in the proportion of fraction I might be due to formation of some aggregates as a result of protein unfolding.

Hydrolysis-induced aggregation of barley glutelin has been observed, in which partial hydrolysis of glutelin during deamidation process induced protein aggregation via intermolecular β -sheet and re-organized β -turn structures (Zhao *et al.*, 2011). Limited denaturation of protein, accompanied by the increment in surface hydrophobicity, promoted the aggregate formation through hydrophobic interactions (Cabra *et al.*, 2007; Zhao *et al.*, 2011). Studies have shown that aggregation of hydrolysate is the consequence of hydrolysis by glutamyl endoprotease enzymes (Creusot *et al.*, 2007). Alcalase performs both subtilisin and glutamyl endoprotease activity, thus can release the peptides with Glu at C-terminal and hydrophobic patches in the sequence, contributing to the high tendency of aggregation for the generated peptides (Spellman *et al.*, 2005). This also explains the gradual decreasing proportion of short peptides ($> 1 \text{ kDa}$) in the glutelin hydrolysate, as they probably co-aggregated with the large peptides. The SE-HPLC data agree with the DH data that barley glutelin was more extensively hydrolyzed by flavourzyme treatment, since both medium (53%) and small-sized (28%) peptides dominated in the FHs after 4 h hydrolysis, whereas medium-size peptides (65%) prevailed in AHs with only 7% small-size peptides observed.

[000155] Due to cleavage of peptide bonds, proteolysis is accompanied by gain or loss in hydrophobicity (Liu *et al.*, 2010). As shown in Figure 11, H_0 of FH decreased sharply when the hydrolysis time was increased to 1.5 h, and then increased gradually in the next 2.5 h. The peptides that were released during the first 1.5 h may have great flexibility to expose more hydrophilic groups outward in the aqueous system (Liu *et al.*, 2010). With a deeper hydrolysis, the released peptides may change their conformations to expose hydrophobic amino acid residues. Enzymatic hydrolysis by alcalase was accompanied by a slight increase of H_0 in the first 1 h, and then it levelled off in the next 3 h. The average surface hydrophobicity of FHs was significantly higher than that of AHs ($p < 0.05$).

10 [000156] The antioxidant properties of the hydrolysates prepared at different incubation times were evaluated based on their radical scavenging capacity (DPPH/ $O_2^{\cdot-}/OH^{\cdot}$), Fe^{2+} -chelating effect and reducing power. Since preliminary data indicated that the antioxidant activity of peptides may be dose-dependent, the peptide concentrations used in each assay were optimized and the lowest effective concentrations (1.0 or 2.0 g/l) were selected. The antioxidant activity of unhydrolyzed glutelin was not tested because it formed a turbid solution due to low solubility.

[000157] DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule; thus, DPPH is often used as a substrate to evaluate the antioxidant activity of an antioxidant. As shown in Figure 12A, FH showed a slight decrease in DPPH scavenging ability during the first 1 h of hydrolysis which thereafter remained almost unchanged. The same trend was also observed for AH, but the decrease was more pronounced during the first 2 h. FHs showed a much higher DPPH radical scavenging activity than AHs, probably due to their significantly higher surface hydrophobicity. The high level of DPPH free radical scavenging activity of protein hydrolysates is associated with a high amount of hydrophobic amino acids or peptide (Rajapakse *et al.*, 2005). FHs exhibited a moderate scavenging capacity against the DPPH radical, reaching a scavenging activity of 56-61% at the concentration of 1.0 g/l, comparable to that of whey, porcine and chickpea protein hydrolysate (Xie *et al.*, 2008). Superoxide anion radical ($O_2^{\cdot-}$) can produce hydrogen peroxide and hydroxyl

radicals through dismutation and other types of reaction. Not only $O_2^{\cdot-}$, but also its derivatives, can cause damage to DNA and cell membranes.

[000158] As shown in Figure 12B, the $O_2^{\cdot-}$ scavenging of AH decreased initially (49 to 44%) in the first 2 h, and then increased slightly afterwards. Flavourzyme hydrolysis was accompanied by a slight increase of $O_2^{\cdot-}$ scavenging capacity. Despite the DPPH radical scavenging activity, in $O_2^{\cdot-}$ scavenging assay AHs exhibited superior activity compared to FHs, and the maximum scavenging value was approximately 50% for AH at the concentration of 2.0 g/l. This value is comparable to and even higher than that of many other protein hydrolysates. Zein and rice endosperm protein hydrolysates exhibited an $O_2^{\cdot-}$ scavenging ability of 11.5% at 10 g/l and < 20% at 0.5 g/l, respectively (Tang *et al.*, 2010; Zhang *et al.*, 2010). Saito *et al.* (2003) indicated that His, Pro, and Tyr are the most important residues in radical scavenging activity of antioxidant peptides. These amino acids comprise more than 16 % of the total residues in barley glutelin (Wang *et al.*, 2010), which may explain the remarkable $O_2^{\cdot-}$ scavenging ability of barley glutelin hydrolysates. Hydroxyl radical can react with biomolecules such as amino acids, proteins, and DNA, as well as trigger lipid peroxidation (Xie *et al.*, 2008); thus, removal of hydroxyl radical is probably one of the most effective defenses of a system against oxidation. In general, the inhibition effect of the AHs upon $OH^{\cdot}$ was significantly greater than that of the FHs and it was even enhanced with prolonged hydrolysis time (47 to 58%) (Figure 12C). The $OH^{\cdot}$ scavenging capacity of FH reached a maximum at 1 h of hydrolysis (58%) at 1.0 g/l, showing potential to protect a food or living system against hydroxyl radical-induced damages.

[000159] Since compounds interfering with the catalytic activity of metal ions could affect the peroxidative process, measuring the chelating ability of the compound is important for evaluating its antioxidant activity (Xie *et al.*, 2008). In Figure 12D, the Fe^{2+} chelating capacity of FHs was low (around 23% to 30%) and not influenced by hydrolysis time. On the other hand, Fe^{2+} chelating capacity of AH increased dramatically (53 to 88%) up to 2 h of hydrolysis, then increased more steadily with prolonged hydrolysis time. The maximum Fe^{2+} -chelating capacity was 90% for AH after 4 h of hydrolysis at 1.0 g/l. This value is much greater than many other protein hydrolysates. Chang *et al.* (2007) reported a chelating ability ranging from 8 to 63% for

hydrolysates derived from porcine hemoglobin at 5.0 g/l assay concentration. Corn zein and chickpea protein hydrolysates showed poor Fe^{2+} -chelating ability, even at 30-40 g/l concentration (Kong *et al.*, 2006; Li *et al.*, 2008). Such a high Fe^{2+} chelating capacity in AH may arise from the exposure of more acidic and basic amino acids by peptide cleavage as the carboxyl and amino groups in their side chains can bind Fe^{2+} (Zhang *et al.*, 2010). In addition, the superior Fe^{2+} chelation ability of AHs may contribute to their high hydroxyl radical scavenging effects due to combined effect of radical scavenging and ion chelation activity.

[000160] Free radicals form stable substances by accepting electrons and therefore the free radical chain reactions are interrupted. The reducing power assay is often used to evaluate the ability of natural antioxidants to donate an electron or hydrogen (Chang *et al.*, 2003). As shown in Figure 12E, AHs possessed significantly higher reducing power than FHs. Reducing power of AHs increased obviously (0.103 to 0.121) with increasing hydrolysis time up to 2 h of incubation, and then leveled off during the next 2.0 h. On the contrary, reducing power of FHs was reduced significantly (0.094 to 0.083) during the first 1.5 h of hydrolysis, and then increased slightly afterwards. The increase or decrease in reducing power for AH and FH may be related to the exposure of electron-dense amino acid side chain groups, such as polar or charged moieties during hydrolysis (Bamdad *et al.*, 2011). On the other hand, the phenolic and indolic groups of tyrosine and tryptophan have been reported to play important roles as hydrogen donors in a redox system (Pihlanto, 2006). At the same concentration (2.0 g/l), hydrolysates from alfalfa leaf protein and chickpea protein showed much greater reducing power with values of 0.69 and 0.2, respectively (Li *et al.*, 2008; Xie *et al.*, 2008).

[000161] The above results demonstrated that the type of enzyme used is a factor in determining the antioxidant activities of barley glutelin hydrolysates. AHs demonstrated significantly higher antioxidant capacity than FHs in most of the selected assays. The DPPH/ $\text{OH}^\cdot$ radical scavenging activity, Fe^{2+} chelating ability and reducing power were related to hydrolysis time, suggesting that peptide size may be related to a certain antioxidant activity. The $\text{O}_2^{\cdot -}$ scavenging ability was less impacted by hydrolysis time.

[000162] Since AHs possessed higher antioxidant activities in most of the assays, they were chosen as sample for analyzing antioxidant activities of the barley glutelin peptides in

relation to their molecular weight. The AH sample was obtained after 2h of hydrolysis and then separated by means of an ultra-filtration into three fractions. Fraction I corresponds to large-sized peptide fragments with M_w exceeding 10 kDa, fraction II to medium-sized peptide fragments with M_w between 1 and 10 kDa, and fraction III to small-sized peptide fragments with M_w lower than 1 kDa. Molecular weight distribution of all fractions was also monitored using size exclusion chromatography (Figure 13). Fraction I contained peptides with M_w ranging from 517 kDa to 650 Da with the major components around 17 kDa. In Fraction II, the main peaks represent the peptides of 3.2 and 1 kDa, while Fraction III represents peptides with M_w smaller than 1 kDa.

[000163] Their antioxidant activities were measured and compared to that of the hydrolysate before fractionation at 1.0 g/l (Table 1A).

Table 1. Antioxidant activities of (A) barley glutelin hydrolysate (2 h of treatment) and the fractions separated by ultra-filtration (Fraction I, II and III) and (B) barley glutelin hydrolysate ($M_w < 1$ kDa) separated by reverse-phase column (Fraction 1, 2, 3 and 4) as well as the positive controls

Antioxidant assay	(A) Fractions separated by ultra-filtration				
	2h-AH	Fraction I (> 10 kDa)	Fraction II (10-1 kDa)	Fraction III <1 kDa	Positive control
DPPH Scavenging %	31.5 ± 0.6	61.9 ± 1.7	40.8 ± 1.0	18.3 ± 0.5	78.9 / 99.0 (BHT)
Reducing Power	0.18 ± 0.004	0.29 ± 0.009	0.065 ± 0.002	0.040 ± 0.004	0.25/0.39 (ascorbate)
O ₂ ⁻ Scavenging %	18.7 ± 0.9	24.4 ± 1.1	16.2 ± 0.9	25.5 ± 0.8	32.0/47.9 (BHT)
Fe ²⁺ Chelating %	86.1 ± 0.8	70.1 ± 1.8	82.5 ± 1.8	85.3 ± 1.3	38.5/98.5 (EDTA)
OH ⁻ Scavenging %	51.1 ± 0.2	60.2 ± 1.0	63.7 ± 0.8	69.1 ± 0.9	42.4/87.2 (BHT)

Antioxidant assay	(B) Fractions separated by reverse-phase column				
	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Positive control
O ₂ ⁻ Scavenging %	18.2 ± 0.9	25.6 ± 1.1	20.4 ± 0.7	21.3 ± 0.9	32.0/47.9 (BHT)
Fe ²⁺ Chelating %	70.1 ± 1.2	82.5 ± 0.8	85.3 ± 1.0	93.0 ± 1.1	38.5/98.5 (EDTA)
OH ⁻ Scavenging %	45.9 ± 0.7	64.1 ± 0.5	65.7 ± 0.3	70.4 ± 0.5	42.4/87.2 (BHT)

*The concentration of barley glutelin hydrolysate fractions was 1.0 mg/ml; the concentration of BHT, ascorbate acid and EDTA was 0.01 and 0.1 mg/ml.

[000164] The large-sized peptides possessed much greater ($p < 0.05$) DPPH scavenging activity and reducing power, whereas small-sized peptides demonstrated significantly greater ($p < 0.05$) OH⁻ scavenging and Fe²⁺ chelating activity. In general, these optimal fractions exhibited

higher antioxidant activities than the hydrolysate before separation. Reducing power was dramatically improved (0.288 at 1.0 g/l) by separating large-sized peptides from barley glutelin hydrolysates, resulting in values comparable to or even greater than those from other proteins (Li *et al.*, 2008; Xie *et al.*, 2008). No relationship between $O_2^{\cdot-}$ scavenging ability and peptide M_w could be found.

[000165] The amino acid composition of these three fractions is set out in Table 2:

Table 2. Amino acid composition (%) of barley glutelin, and their alcalase hydrolysates separated by ultra-filtration (Fraction I, II and III) and reverse-phase column (Fraction 4).

Amino acids	Barley glutelin	Fraction I	Fraction II	Fraction III	Fraction 4
Asx	5.8	6.6	8.6	7.5	5.6
Ser	5.7	8.1	8.2	7.1	5.5
Glx	20.0	22.3	28.5	20.3	18.0
Gly	9.1	12.9	5.8	7.5	5.6
His	2.7	1.2	1.1	2.1	6.6
Arg	4.6	5.0	3.0	5.5	3.0
Thr	4.7	2.2	2.2	3.8	1.8
Ala	6.1	3.5	3.6	6.9	2.5
Pro	10.7	15.5	17.3	7.7	17.6
Cys	0.8	3.6	3.0	2.0	3.2
Tyr	3.5	2.4	2.2	2.6	2.5
Val	6.1	3.4	4.2	6.9	7.1
Met	1.5	n.d.	0.2	0.3	n.d.
Lys	3.7	4.1	2.1	3.7	1.7
Ile	3.3	3.0	3.1	4.1	6.6
Leu	8.0	3.4	4.0	8.2	9.6
Phe	3.8	3.0	3.7	3.5	9.1

*Asx represents Asn and Asp; Glx represents Gln and Glu; "n.d." means not detectable.

*The order of amino acids listed in the table follows the elution order of the amino acids from the reversed-phase HPLC chromatographic column.

*Barley glutelin amino acid composition was analyzed in our previous work (Wang, Tian, Chen, Temelli, Liu & Wang, 2010).

[000166] Fraction I and II had significantly higher Pro residue percentages (15.5 and 17.3%) than that of fraction III (7.7%), confirming that Pro was less prone to cleavage by proteases and peptidases. These fractions possessed a similar amount of hydrophobic amino acid residues (Val, Leu, Ile, Met, Phe, Ala and Pro 31.8-37.6%). No significant change in the proportion of His, Tyr, Mer, Lys, Trp and Phe was observed which are generally accepted as

antioxidant amino acids (Pihlanto, 2006). This confirms that the molecular weight of AH is important to manifest antioxidant activities. The majority of previous antioxidant peptide research has shown that short peptides and amino acids are the most efficient antioxidants because their accessibility to the oxidant/antioxidant test systems is greater than that of large peptides and proteins (Hernandez-Ledesma *et al.*, 2005). It is interesting that the large-sized peptide fraction was more effective in DPPH scavenging activity and reducing power in barley glutelin hydrolysates. Without being bound by any theory, specific hydrophobic clusters of glutelin, with bulky and aromatic side chains, may act as hydrogen donors and as direct radical scavengers (Farvin *et al.*, 2010).

[000167] Peptides of <1 kDa possessed superior $O_2^{\cdot-}$ and $OH^{\cdot}$ scavenging activity and Fe^{2+} chelating capacity. To elucidate further the antioxidant activity of specific peptides in this small-sized peptide fraction, the 1 kDa M_w cutoff ultra-filtration permeate was subjected to further fractionation using a reversed-phase HPLC system. Semi-preparative Chromatography of peptides <1 kDa produced four fractions by elution time (Figure 14). The $O_2^{\cdot-}/OH^{\cdot}$ radical scavenging activity and Fe^{2+} chelating capacity was then evaluated. The results in Table 1B demonstrated that peptides with higher hydrophobicity (fraction 4) exhibited greater $OH^{\cdot}$ radical scavenging and Fe^{2+} chelating capacity, with the maximum being 70.4% and 93.0%, respectively at 1 g/l. Still no relationship between $O_2^{\cdot-}$ scavenging ability and peptide hydrophobicity could be identified. As shown in Table 2, Fraction 4 had a significantly greater percentage of hydrophobic amino acid residues (52.5%) than original barley glutelin and Fraction 3 separated by ultra-filtration (M_w <1 kDa). Peptides with more hydrophobic amino acids play important roles contributing to antioxidation (Rajapakse *et al.*, 2005). In addition, Fraction 4 demonstrated a significantly higher His (6.6%) and Phe (9.1%) content, which have superior proton-donation ability due to their imidazole and benzyl groups, respectively (Rajapakse *et al.*, 2005).

[000168] Fraction 4, obtained by reversed-phase HPLC, which had relatively superior radical scavenging activity, was subsequently subjected to LC-MS/MS for peptide sequence identification. The MassLynx™ software identified four peptides from barley protein: Gln-Lys-Pro-Phe-Pro-Gln-Gln-Pro-Pro-Phe, Pro-Gln-Ile-Pro-Glu-Gln-Phe, Leu-Arg-Thr-Leu-Pro-Met and Ser-Val-Asn-Val-Pro-Leu. The identified peptides exhibited a high content of hydrophobic

amino acid residues such as Pro, Phe, Leu, Ile and Val, which comprise 50-67 % of the total residues. These hydrophobic amino acids present in the sequences of barley glutelin may have significant antioxidant properties. Two peptides contained the aromatic amino acid Phe at the C-terminal end. Phe, by virtue of its aromatic ring, can act as a direct radical scavenger since it donates protons easily to electron deficient radicals and still remain stable via electron resonance over its ring (Rajapakse *et al.*, 2005). In addition, Met was found at the C-terminal end of one peptide. Met is believed to be important in radical scavenging activity since Met is prone to oxidation to its sulfoxide (Hernandez-Ledesma *et al.*, 2005). Metal-chelating amino acid residues such as Met, Glu, Gln, Lys and Arg were detected within the sequences, which have been reported to interact with metal ions through their charged groups and inactivate the prooxidant activity of metal ions (Park *et al.*, 2001; Zhang *et al.*, 2010). This could explain the very strong Fe^{2+} -chelating capacity of barley glutelin peptides which contributed to greater radical scavenging potential. This was not observed in the amino acid sequences despite of its high percentage in the amino acid composition of Fraction 4, and may possibly exist as a free amino acid to manifest antioxidant activities independently.

[000169] Antioxidant activities of 2h alcalase hydrolysate and hydrolysate fractions were then compared to the selected positive controls. As summarized in Table 1, the optimal DPPH scavenging activity (61.9% at 1.0 g/l) was observed for the AH fraction with $M_w > 10$ kDa, comparable to BHT (78.9%) at 0.01 g/l. The $\text{O}_2^{\cdot-}$ -scavenging activities reached a maximum (48.0% at 2.0 g/l) after 0.5 h of hydrolysis by alcalase. This value was similar to BHT (47.9%) at 0.1 g/l. Barley glutelin hydrolysates can thus be considered as effective free radical scavengers. EDTA, a standard metal ion chelator, displayed 98.5% chelating ability at 0.1 g/l. In this test, AHs showed very strong Fe^{2+} -chelating activity with the maximum value at 93.0% at 1.0 g/l, comparable to EDTA at 0.1 g/l. AH fraction with $M_w > 10$ kDa showed good reducing power (0.288 at 1.0 g/l), which is comparable to ascorbic acid (0.245) at 0.01 g/l.

[000170] As will be apparent to those skilled in the art, various modifications, adaptations and variations of the foregoing specific disclosure can be made without departing from the scope of the invention claimed herein.

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[000171] The following references are incorporated herein by reference (where permitted) as if reproduced in their entirety. All references are indicative of the level of skill of those skilled in the art to which this invention pertains.

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WHAT IS CLAIMED IS:

1. A method of producing a peptide having antioxidant activity from barley hordein or glutelin, comprising the steps of:

- 5 a) hydrolyzing barley hordein or glutelin; and
 b) recovering the peptide from the hydrolyzed barley hordein or glutelin.

2. The method of claim 1 wherein the barley hordein is hydrolyzed with alcalase at a sufficient time and temperature for hydrolysis.

10 3. The method of claim 1, further comprising the step of fractionating the hydrolyzed barley hordein by ultra-filtration to yield at least a first fraction, a second fraction, a third fraction, and a fourth fraction.

15 4. The method of claim 3, wherein the first fraction comprises peptides having molecular weights greater than 10 kDa.

5. The method of claim 3, wherein the second fraction comprises peptides having molecular weights between 5 to 10 kDa.

20 6. The method of claim 3, wherein the third fraction comprises peptides having molecular weights between 1 to 5 kDa.

25 7. The method of claim 3, wherein the fourth fraction comprises peptides having molecular weights less than 1 kDa.

8. The method of claim 5, comprising fractionating the second fraction by reversed-phase high performance liquid chromatography to yield at least a fifth fraction comprising at least one antioxidant peptide.

9. The method of claim 8, wherein the peptide comprises an amino acid sequence selected from QPYYPQ; QSYVPVQPQ; QQTPLPQ; QPQPYPQ; TQQPYYPQ; SPLQPQ; QQPYPQ; QPVLSQ; QVPQ; LLPQ; or HVLQ.
- 5 10. The method of claim 7, comprising fractionating the fourth fraction by reversed-phase high performance liquid chromatography to yield at least a sixth fraction comprising at least one antioxidant peptide.
11. The method of claim 10, wherein the peptide comprises an amino acid sequence selected
10 from KPFPPQPPF; QPPFWQ; SVNVPPLY; AELIIPQ; or YRIVPL.
12. The method of claim 1 wherein the barley glutelin is hydrolyzed with alcalase or flavourzyme at a sufficient time and temperature for hydrolysis.
- 15 13. The method of claim 12, wherein in step (a), hydrolysis is conducted using alcalase.
14. The method of claim 1, further comprising the step of fractionating the hydrolyzed barley glutelin by ultra-filtration to yield at least a first fraction and a second fraction.
- 20 15. The method of claim 14, wherein the first fraction comprises peptides having molecular weights greater than 10 kDa.
16. The method of claim 14, wherein the second fraction comprises peptides having molecular weights less than 1 kDa.
- 25 17. The method of claim 16, comprising fractionating the second fraction by reversed-phase high performance liquid chromatography to yield at least a third fraction comprising at least one antioxidant peptide.

18. The method of claim 17, wherein the peptide comprises an amino acid sequence selected from Gln-Lys-Pro-Phe-Pro-Gln-Gln-Pro-Pro-Phe;
Pro-Gln-Ile-Pro-Glu-Gln-Phe;
Leu-Arg-Thr-Leu-Pro-Met; or
5 Ser-Val-Asn-Val-Pro-Leu.

19. The method of claim 1, wherein the duration of hydrolysis is at least between about 0.5 hours to about 4 hours.

10 20. The method of claim 19, wherein the duration of hydrolysis is at least about 1.5 hours.

21. The method of claim 19, wherein hydrolysis is conducted at a temperature of about 50° C.

15 22. The method of claim 21, wherein hydrolysis is conducted at a pH in the range of about 7.0 to about 8.0.

23. The method of claim 22, wherein hydrolysis of hordein is conducted using alcalase at an enzyme/substrate ratio of about 0.24 AU/g protein.

20

24. The method of claim 22, wherein hydrolysis of glutelin is conducted using flavourzyme at an enzyme/substrate ratio of about 40 LAPU/g protein.

25 25. The method of claim 22, wherein hydrolysis of glutelin is conducted using alcalase at an enzyme/substrate ratio of about 0.12 AU/g protein.

26. A barley hordein or glutelin peptide produced by the method of claim 1 and having antioxidant activity.

27. A barley hordein peptide having antioxidant activity and comprising an amino acid sequence selected from QPYYPQ; QSYVPVQPQ; QQTPLPQ; QPQPYPQ; TQQPYYPQ; SPLQPQ; QQPYPQ; QPVLSQ; QVPQ; LLPQ; HVLQ; KPFPQQPPF; QPPFWQ; SVNVPY; AELIIPQ; or YRIVPL.

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28. A barley hordein peptide having a molecular weight greater than 10 kDa, and exhibiting DPPH scavenging activity and reducing power.

29. A barley hordein peptide having a molecular weight less than 1 kDa, and exhibiting
10 ferrous ion chelating activity.

30. A barley glutelin peptide having antioxidant activity and comprising an amino acid sequence selected from Gln-Lys-Pro-Phe-Pro-Gln-Gln-Pro-Pro-Phe; Pro-Gln-Ile-Pro-Glu-Gln-Phe; Leu-Arg-Thr-Leu-Pro-Met; or Ser-Val-Asn-Val-Pro-Leu.

15

31. A barley glutelin peptide having a molecular weight greater than 10 kDa, and exhibiting DPPH scavenging activity and reducing power.

32. A barley glutelin peptide having a molecular weight less than 1 kDa, and exhibiting
20 ferrous ion chelating activity and hydroxyl scavenging activity.

33. A food, cosmetic or pharmaceutical product comprising the barley hordein or glutelin peptide of any one of claims 1-32.

25 34. A food, cosmetic or pharmaceutical product comprising the barley hordein or glutelin peptide as produced by the method of claim 1.

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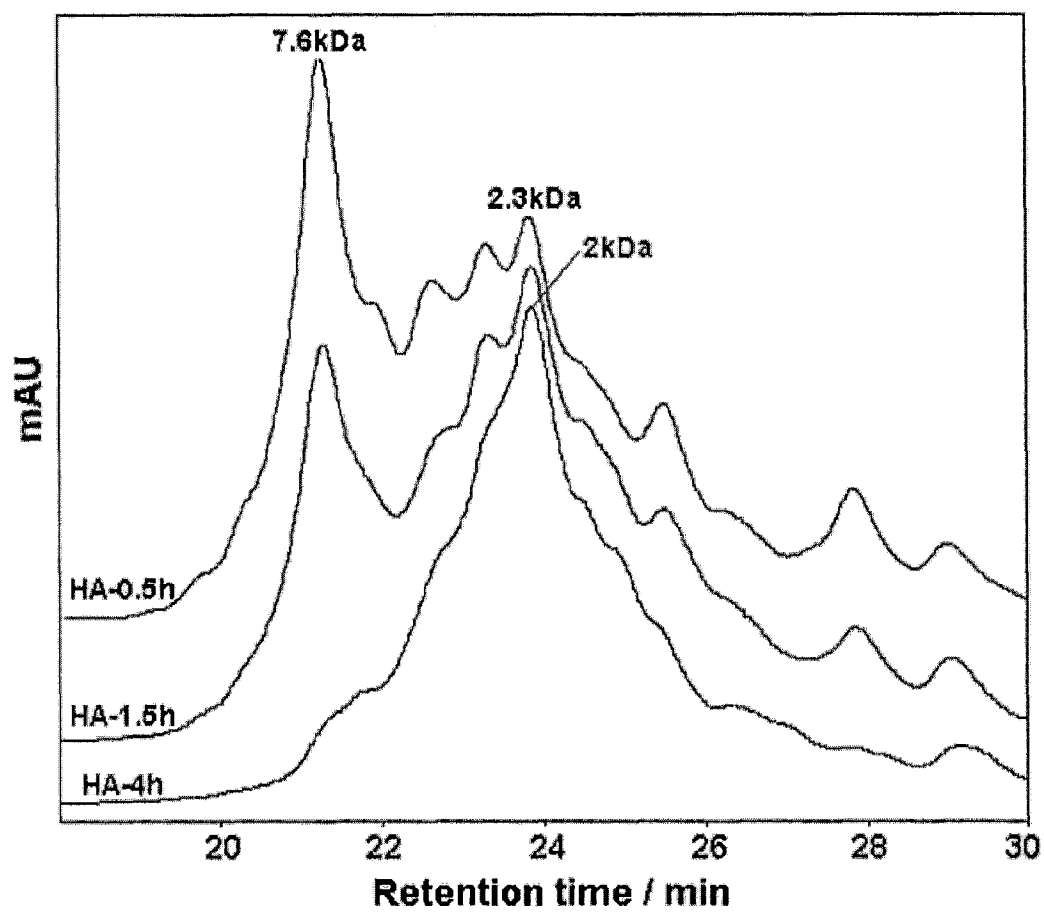


FIG. 1

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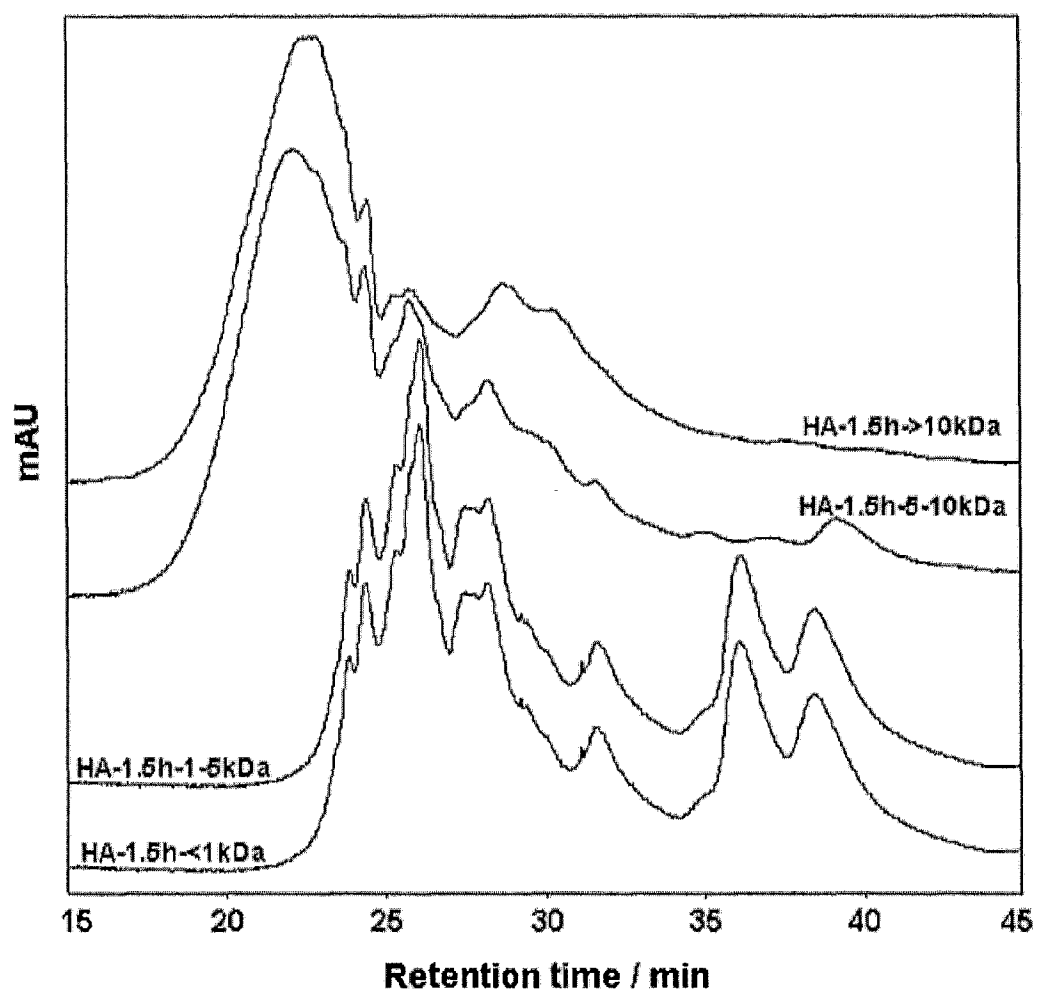


FIG. 2

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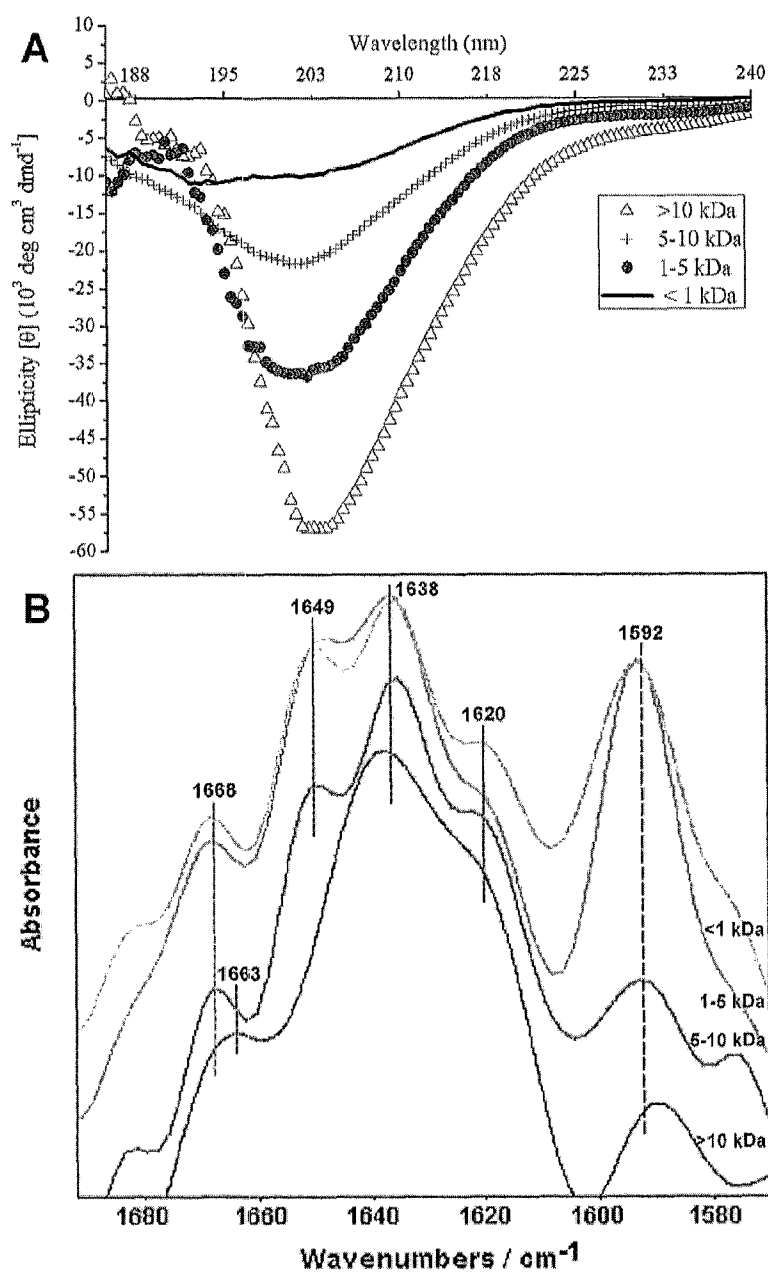


FIG. 3

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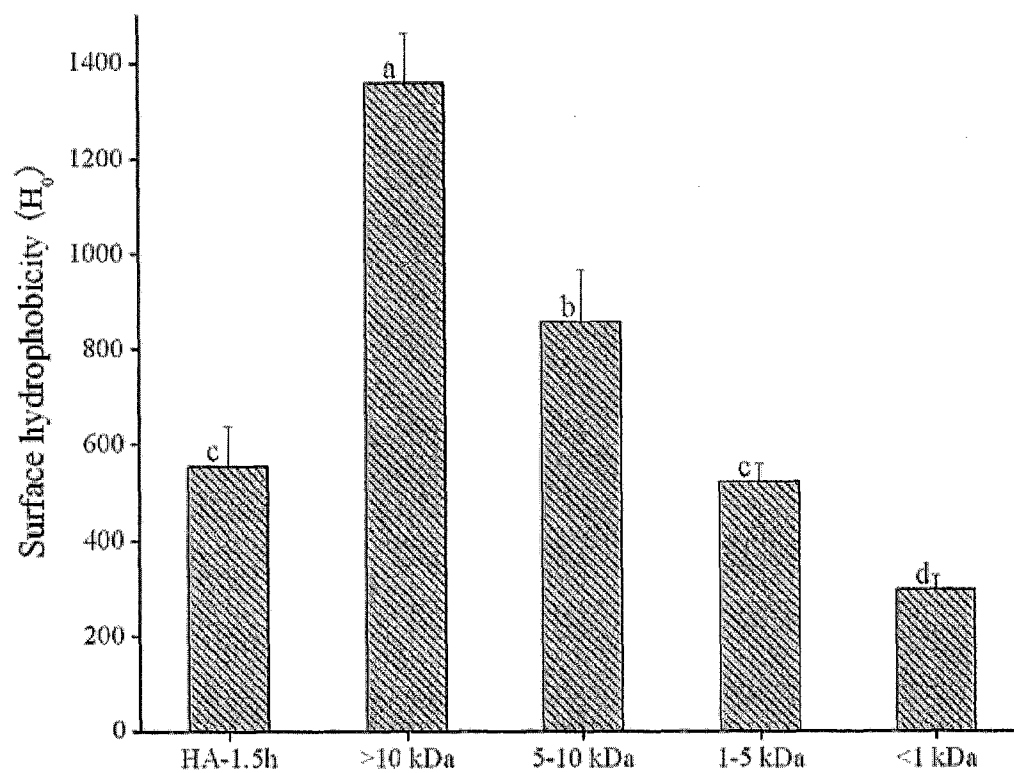


FIG. 4

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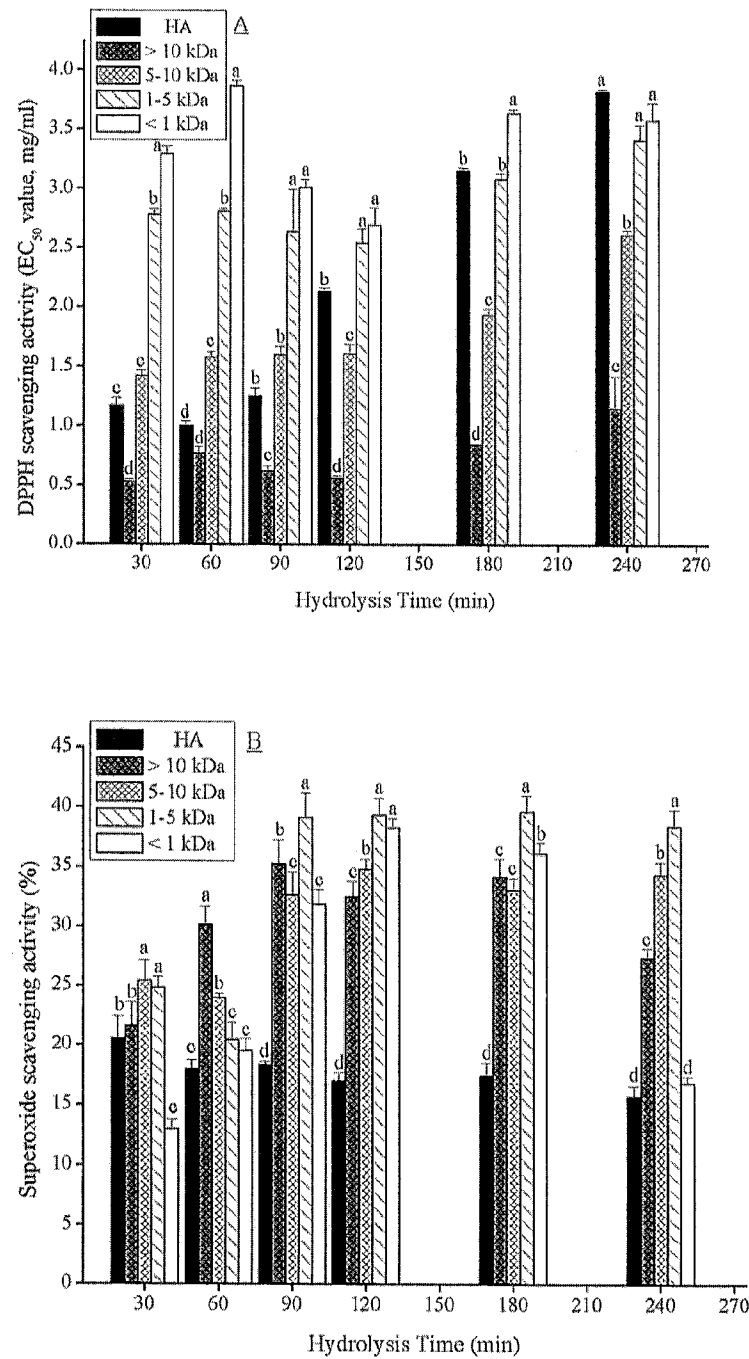


FIG. 5

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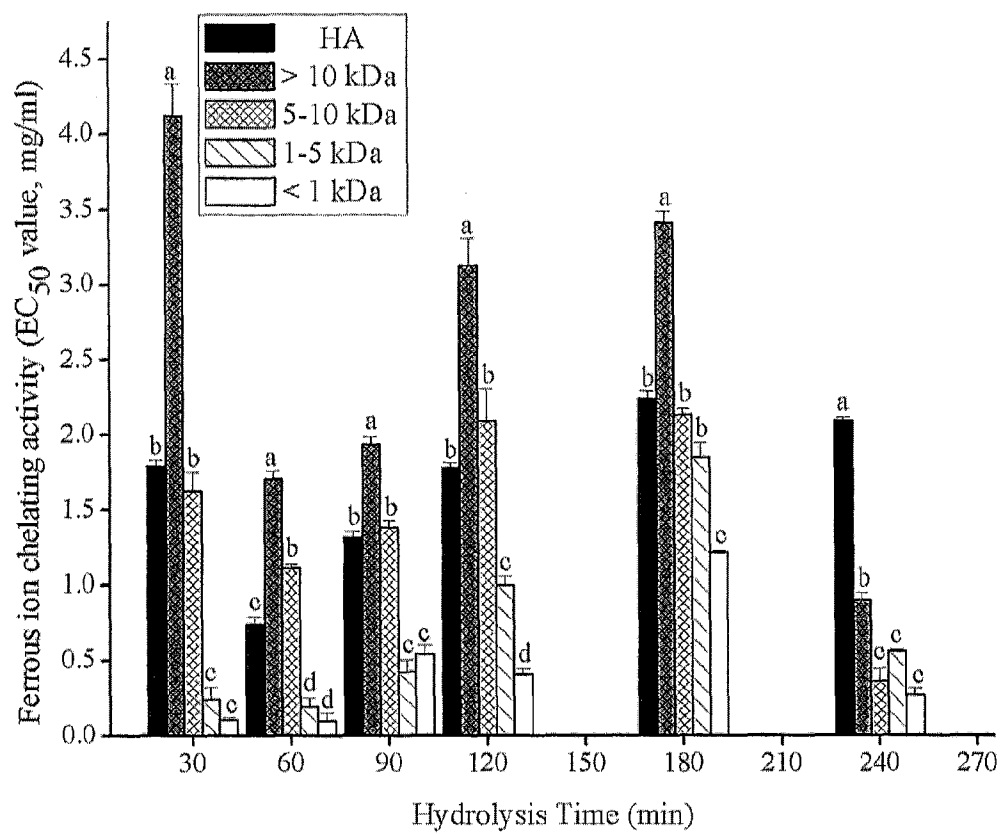


FIG. 6

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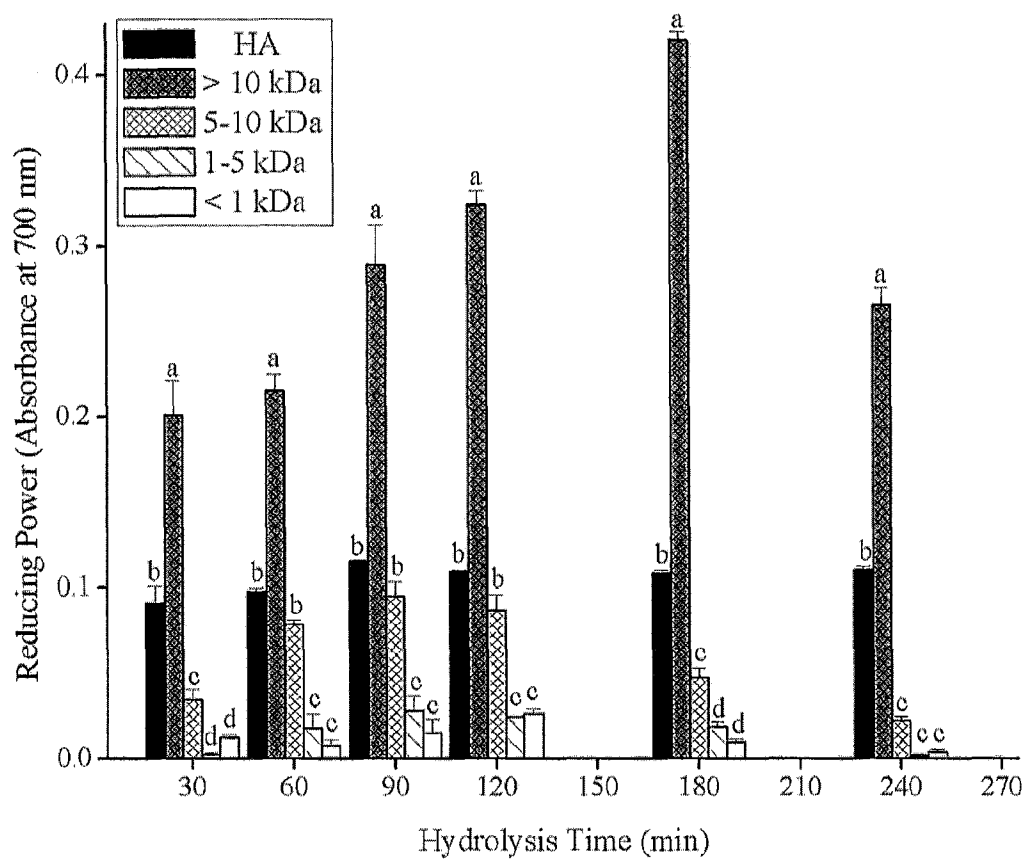


FIG. 7

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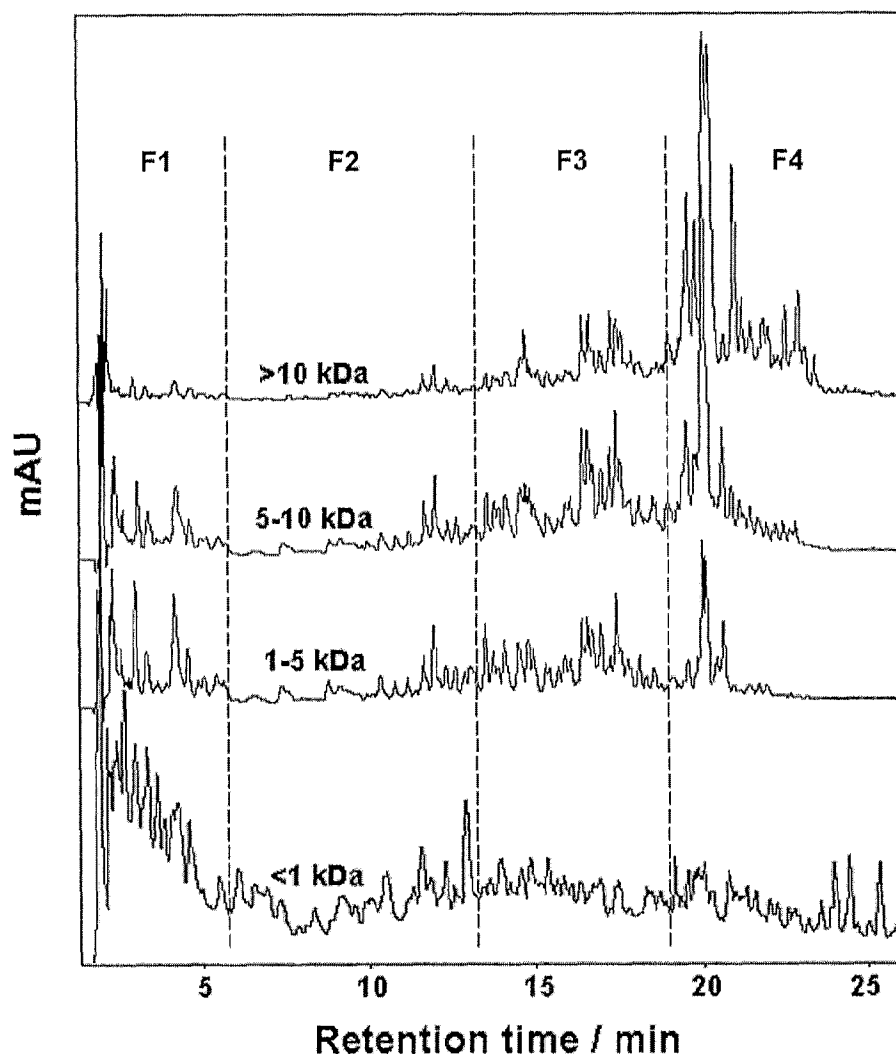


FIG. 8

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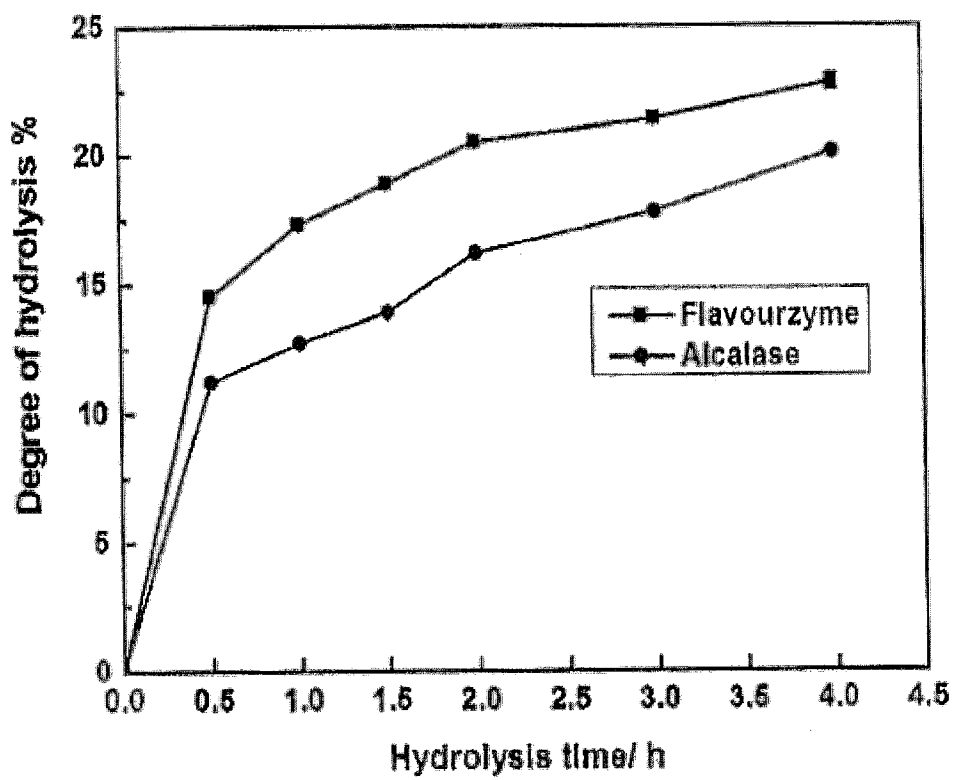


FIG. 9

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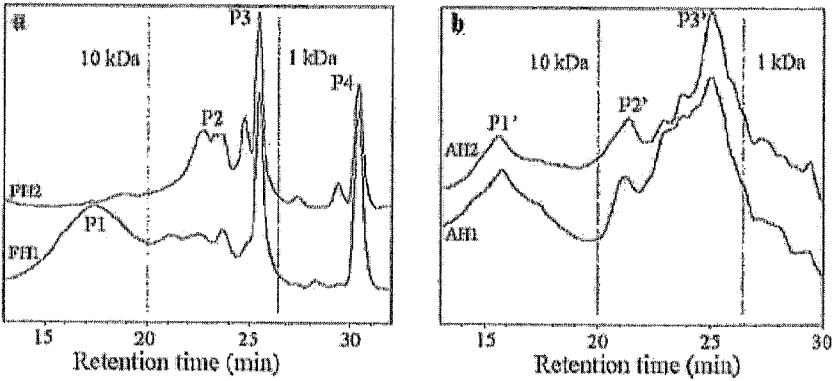


FIG. 10A

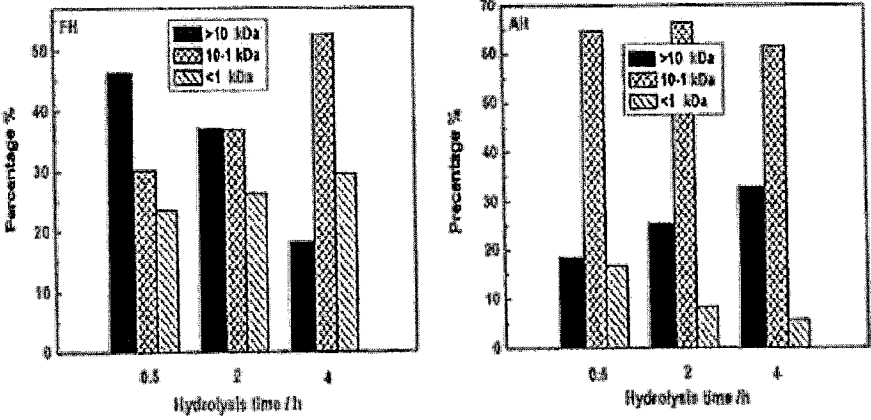


FIG. 10B

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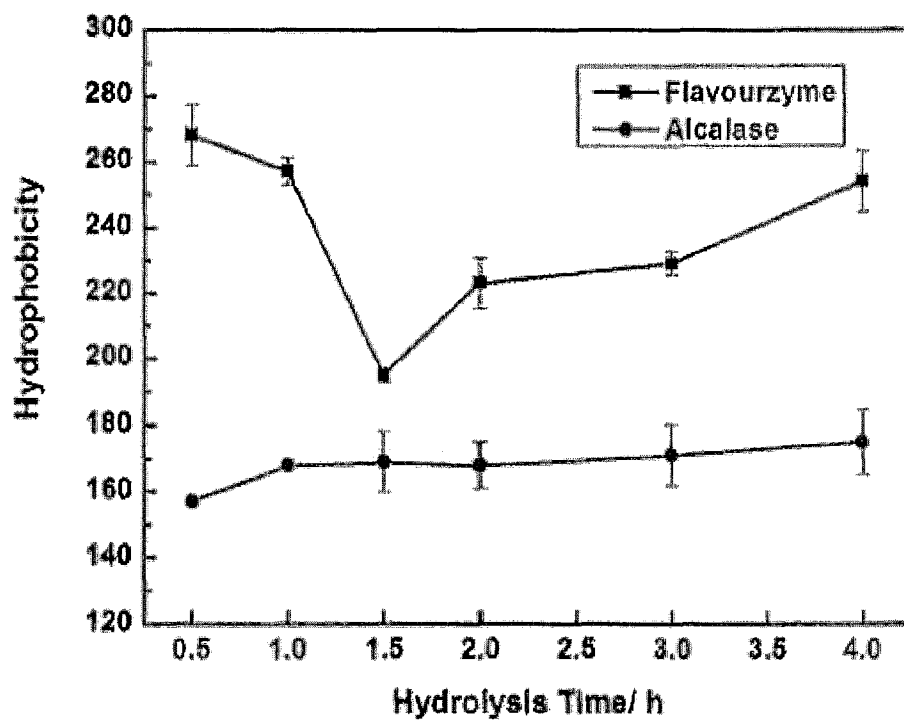


FIG. 11

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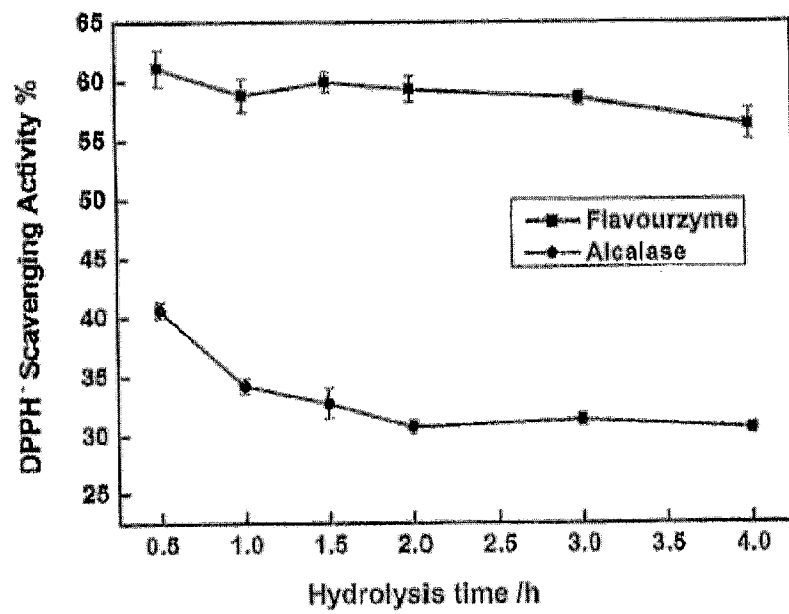


FIG. 12A

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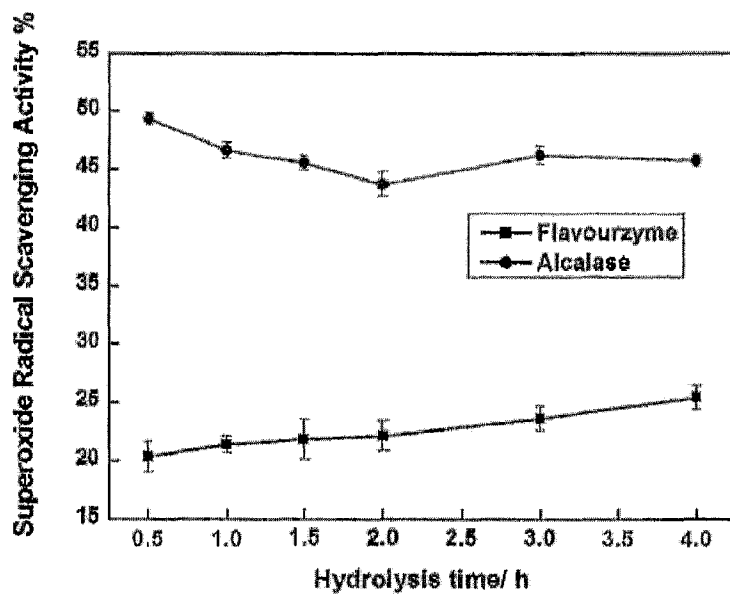


FIG. 12B

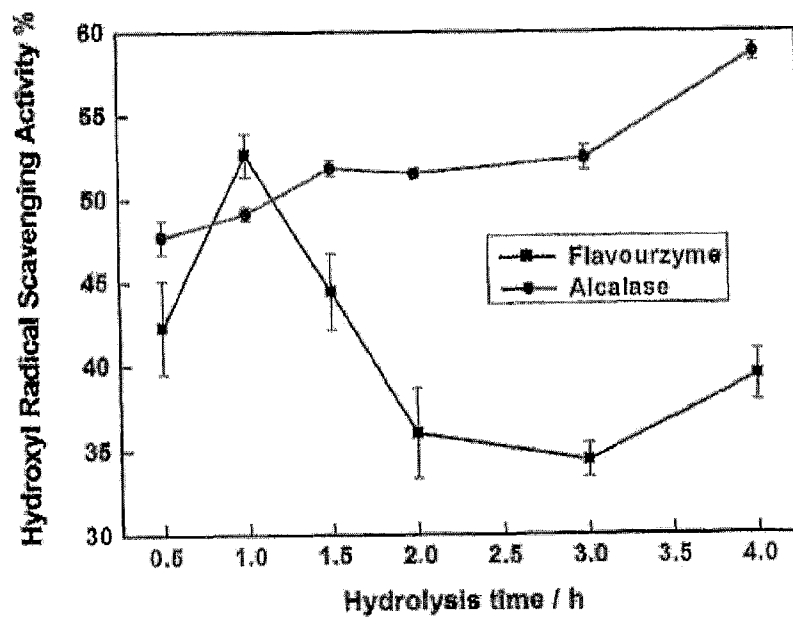


FIG. 12C

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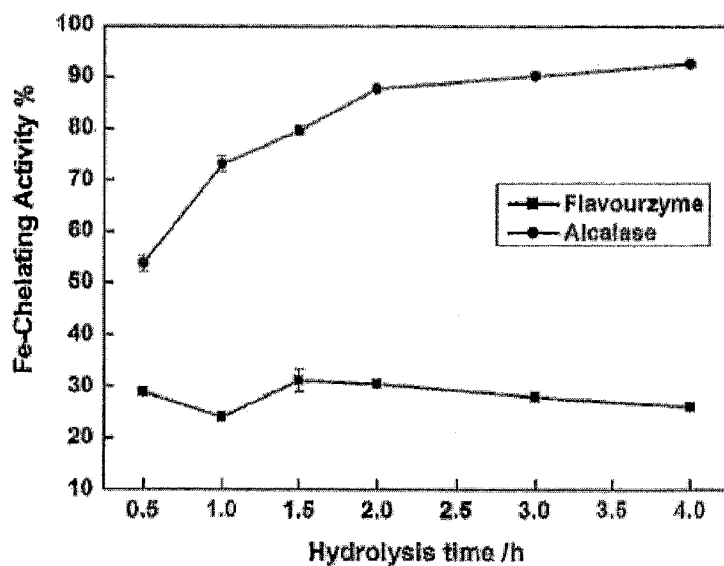


FIG. 12D

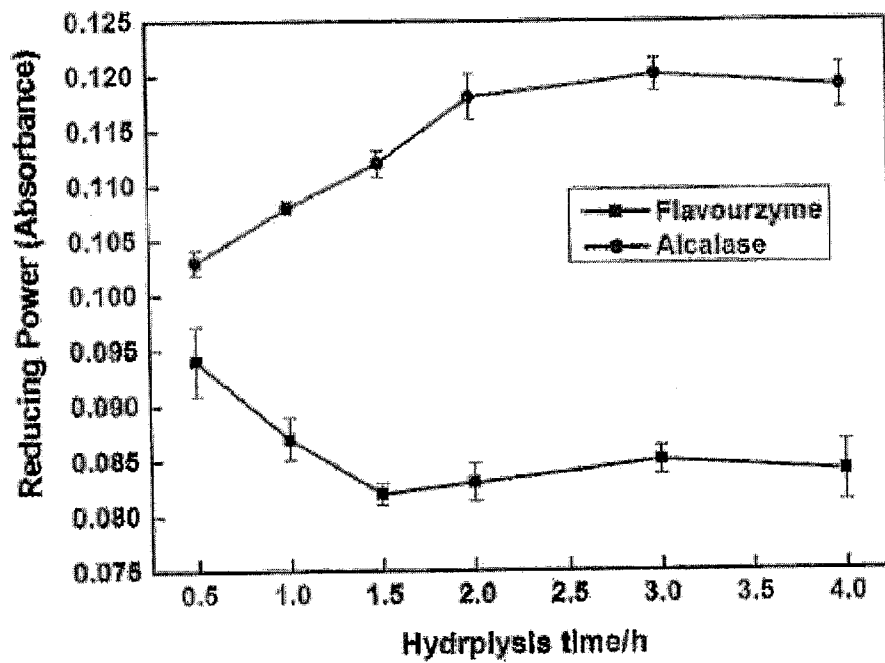


FIG. 12E

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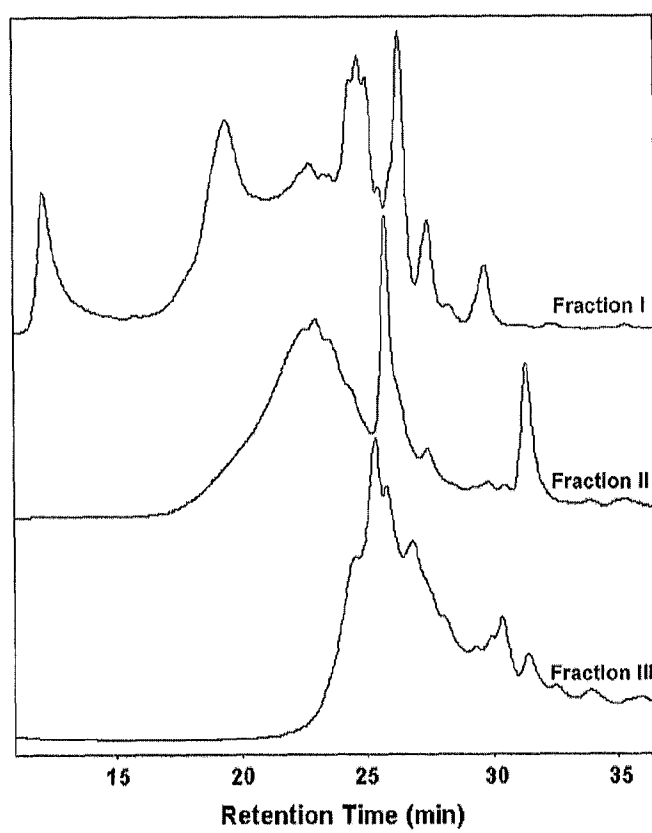


FIG. 13

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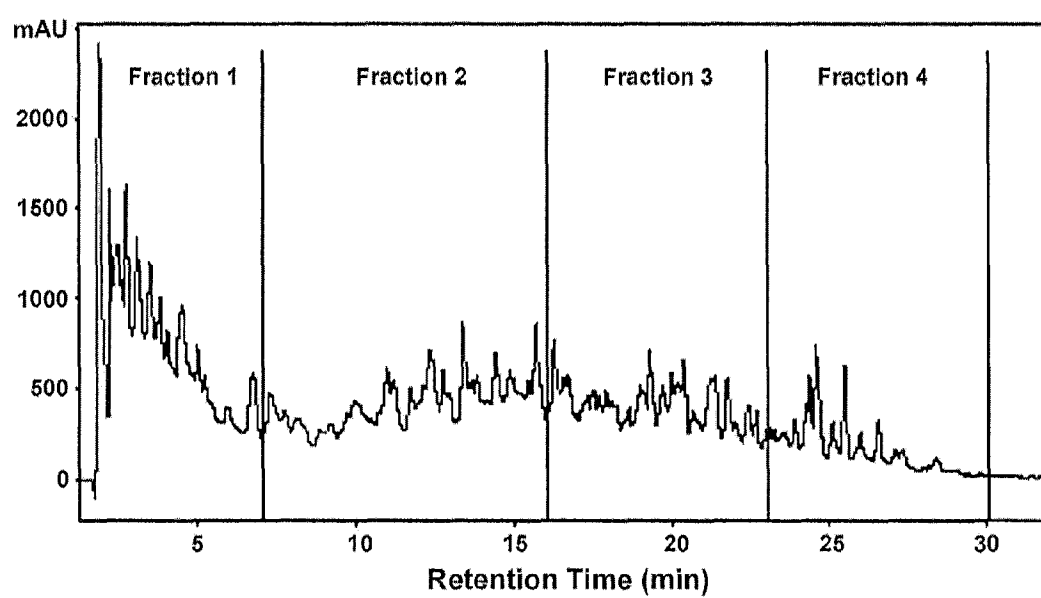


FIG. 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2013/051010

A. CLASSIFICATION OF SUBJECT MATTER

IPC: C07K 14/415 (2006.01), A23L 1/305 (2006.01), A61K 36/8998 (2006.01), A61K 38/17 (2006.01), A61K 8/64 (2006.01), A61K 8/97 (2006.01), A61P 39/06 (2006.01), C07K 1/16 (2006.01), C07K 1/34 (2006.01), C07K 7/06 (2006.01), C12P 21/06 (2006.01). 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)

C07K 14/415 (2006.01), A23L 1/305 (2006.01), A61K 36/8998 (2006.01), A61K 38/17 (2006.01), A61K 8/64 (2006.01), A61K 8/97 (2006.01), A61P 39/06 (2006.01), C07K 1/16 (2006.01), C07K 1/34 (2006.01), C07K 7/06

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
TotalPatent, Pubmed, Google Scholar, Keywords: barley, hordein, glutelin, antioxidant, alcalase, flavourzyme, peptide, hydrolysate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Bamdad, et al., "Effects of enzymatic hydrolysis on molecular structure and antioxidant activity of barley hordein",	1, 2, 19-23, 26, 28, 29, 33 and 34
Y	Journal of Cereal Science, July 2011 Vol. 54, pages 20-28, ISSN: 0733-5210, whole document	3-8 and 10
X	Xia, Y., et al., "Fractionation and characterization of antioxidant peptides derived from barley glutelin by enzymatic hydrolysis",	1, 12-22, 24-26 and 30-34
Y	Food Chemistry, March 2012, Vol. 143, pages 1509-1518, ISSN: 0308-8146, whole document	3-8 and 10

[X] Further documents are listed in the continuation of Box C.

[] See patent family annex.

* Special categories of cited documents :	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier application or patent but published on or after the international filing date	"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
"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)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search

18 February 2014 (18-02-2014)

Date of mailing of the international search report

05 March 2014 (05-03-2014)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 001-819-953-2476

Authorized officer

Yolanda Romsicki (819) 997-1044

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2013/051010

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Kawase, S-I., et al., "Effects of fragmentation and deamidation on the antioxidative activity of C hordein", Journal of Oleo Science, March 2003, Vol. 52, pages 175-184, ISSN: 1345-8957 whole document	1, 19-22, 26-28, 33 and 34
P, X	Bamdad, F., and Chen., L., "Antioxidant capacities of fractionated barley hordein hydrolysates in relation to peptide structures", Molecular Nutrition and Food Research, March 2013, Vol. 57, pages 493-503, ISSN: 1613-4133 whole document	1-11, 19-23, 26-29, 33 and 34
A	Chanput, W., et al., "Antioxidative properties of partially purified barley hordein, rice bran protein fractions and their hydrolysates", Journal of Cereal Science, May 2009, Vol. 49, pages 422-428, ISSN: 0733-5210, whole document	1-34
A	Kasarda, D. D., et al., "Nucleic acid (cDNA) and amino acid sequences of α -type gliadins from wheat (<i>Triticum aestivum</i>)", PNAS USA, August 1984, Vol. 81, pages 4712-4716, ISSN: 0027-8424, whole document	1-34

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CA2013/051010**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

a. (means)

☐ on paper

☒ in electronic form

b. (time)

☐ in the international application as filed

☐ together with the international application in electronic form

☒ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments :

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CA2013/051010**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the 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. ☐ Claim Nos. :
because they relate to subject matter not required to be searched by this Authority, namely :
2. ☐ Claim 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. ☐ Claim Nos. :
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 :

Methods of producing peptides having antioxidant activity from barley hordein or glutelin are known in the art. Therefore, the claims are directed to a plurality of inventive concepts as follows:

Group A - Claims 1-3 (all partly), 4 (entirely), 19-23 (all partly), 26 (partly), 27 (partly), 28 (entirely), 33 (partly), and 34 (partly) are directed to peptides having molecular weights greater than 10 kDa and antioxidant activity from barley hordein, and methods of producing same. (Continued on extra sheet).

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 claim 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 claim 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.

Continuation of Box. No. III

Group B - Claims 1-3 (all partly), 5 (entirely), 8 (entirely), 9 (entirely), 19-23 (all partly), 26 (partly), 27 (partly), 33 (partly) and 34 (partly) are directed to peptides having a molecular weights between 5-10 kDa and antioxidant activity from barley hordein, and methods of producing same.

Group C - Claims 1-3 (all partly), 6 (entirely), 19-23 (all partly), 26 (partly), 27 (partly), 33 (partly) and 34 (partly) are directed to peptides having molecular weights between 1-5 kDa and antioxidant activity from barley hordein, and methods of producing same.

Group D - Claims 1-3 (all partly), 7 (entirely), 10 (entirely), 11 (entirely), 19-23 (all partly), 26 (partly), 27 (partly), 29 (partly), 33 (partly) and 34 (partly) are directed to peptides having molecular weights less than 1 kDa and antioxidant activity from barley hordein, and methods of producing same.

Group E - Claims 1 (partly), 12-14 (all partly), 15 (entirely), 19-22 (all partly), 24-26 (all partly), 30 (partly), 31 (entirely), 33 (partly) and 34 (partly) are directed to peptides having molecular weights greater than 10 kDa and antioxidant activity from barley glutelin, and methods of producing same.

Group F - Claims 1 (partly), 12-14 (all partly), 16-18 (all entirely), 19-22 (all partly), 24-26 (all partly), 30 (partly), 32 (entirely), 33 (partly) and 34 (partly) are directed to peptides having molecular weights less than 1 kDa and antioxidant activity from barley glutelin, and methods of producing same.

Since the claims of Groups A to F are not linked by a single inventive concept, said claims are directed to multiple inventions. It is possible that Groups A-F may be further subdivided based on the peptide sequences indicated in claims 9, 11, 18, 27 and 30.