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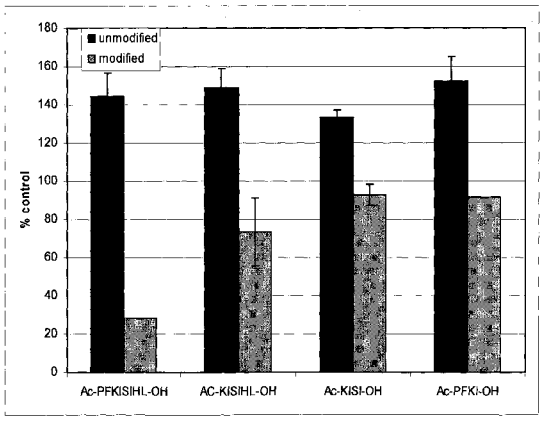
(54) Title: ANTI-INFLAMMATORY PROTEINS AND PEPTIDES AND METHODS OF PREPARATION AND USE THEREOF

FIGURE 11A PFKISIHL (SEQ ID NO: 18)

FIGURE 11B Ac-PFKISIHL-OH (SEQ ID NO: 19)
Ac-KISIHL-OH (SEQ ID NO: 20)
Ac-KISI-OH (SEQ ID NO: 21)
Ac-PFKI-OH (SEQ ID NO: 22)

FIGURE 11C



(57) Abstract: The present disclose relates to anti-inflammatory proteins/peptides, their uses, methods of preparation and methods of their detection. In particular, the invention relates to major royal jelly proteins modified by methyglyoxal and fragments thereof from a Leptospermum derived honey and royal jelly.

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ANTI-INFLAMMATORY PROTEINS AND PEPTIDES AND METHODS OF PREPARATION AND USE THEREOF

RELATED APPLICATION

[0001] This application claims the benefit of New Zealand provisional application NZ 600847 filed 22 June 2012, the entirety of which is incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present disclosure relates to anti-inflammatory proteins and peptides, their uses, and methods of their detection.

BACKGROUND OF THE INVENTION

[0003] Honey has been used for centuries by cultures through the world for its multiple health benefits. Two of the most important health benefits of honey are its anti-bacterial and anti-inflammatory properties. Manuka honey, which is produced by bees that collect nectar from *Leptospermum scoparium*, a plant native to New Zealand and southern Australia, has been identified as being a variety of honey that exhibits particularly effective anti-bacterial and anti-inflammatory properties. Jelly Bush honey, which is produced by bees that collect nectar from *Leptospermum polygalifolium*, a plant native to Australia, has also been identified as being a variety of honey that exhibits particularly effective anti-bacterial and anti-inflammatory properties.

[0004] Recently, it was discovered that the chemical, methylglyoxal (MGO, also called 2-oxopropanal and pyruvaldehyde), is a major component of the anti-bacterial activity of Leptospermum derived honey, such as manuka honey and jelly bush honey. Manuka honey and jelly bush honey samples contain greater concentrations of MGO, and have a higher amount of anti-bacterial activity as compared to honey samples with lower concentrations of MGO. MGO is believed to confer antibacterial properties on honey because MGO is a highly chemically reactive compound, and MGO can readily react with cellular molecules. The chemical reactions between MGO and cellular molecules in bacteria damage bacterial molecules that are important for viability. In this way, MGO functions as an antibacterial agent.

[0005] The presence of high levels of MGO in the honey is a feature that distinguishes manuka honey and jelly bush honey from other varieties of honey. While most varieties of honey exhibit some anti-bacterial activity, the anti-bacterial activity in most varieties of honey is primarily a result of the presence of hydrogen peroxide in the honey. *Leptospermum* derived honey, in contrast, exhibits anti-bacterial activity primarily because of the presence of MGO in the honey.

[0006] In 2004, Kohno et. al. examined the anti-inflammatory effects or actions of royal jelly at a cytokine level. The results suggest that royal jelly has anti-inflammatory actions brought about by an inhibition of pro-inflammatory cytokine production, such as TNF-α, IL-6 and IL-1, by activated macrophages. The study further suggests that the active fractions or components from the royal jelly extracts are sized between 5 kDa and 30 kDa. Thus, most honeys may have a weak anti-inflammatory effect due to royal jelly proteins that occur in the honey.

[0007] While multiple mechanisms of action of the anti-bacterial activity of manuka honey have been elucidated, the mechanisms whereby manuka honey functions as an anti-inflammatory agent have remained unknown. There is a need to develop anti-inflammatory agents based on honey, as many anti-inflammatory agents currently available have major drawbacks to their use. For example, COX-2 inhibitors, a form of non-steroidal anti-inflammatory drug (NSAID), may increase the risk of heart attack and stroke in patients, and aspirin may increase the risk of gastrointestinal bleeding. Additionally, corticosteroids are reported to inhibit the growth of epithelial cells and NSAIDs are reported as being cytotoxic so both of these classes of anti-inflammatory agents are unsuitable for use in wound care. Anti-inflammatory agents derived from honey may have fewer toxic side effects in one or more areas than drugs currently available, and may also offer different possible uses than anti-inflammatory drugs currently available.

[0008] Described in the co-pending application PCT/NZ/2011/000271 is a modified apalbumin of approximately 55 – 75 kDa from manuka honey that results from the high levels of methylglyoxal found in manuka honey. The inventors have identified that the modified apalbumin or major royal jelly protein has significantly greater anti-inflammatory properties than an unmodified apalbumin or major royal jelly protein.

SUMMARY OF THE INVENTION

[0009] Described herein are apalbumins, also known as major royal jelly proteins, and functional fragments thereof, which have at least one lysine or arginine amino acid chemically modified by methylglyoxal (MGO), and which exhibit enhanced anti-inflammatory effects.

[0010] In a first aspect the present invention provides a method of reducing inflammation in a cellular tissue, comprising the step of contacting the cellular tissue with a functional fragment of a major royal jelly protein (MRJP); wherein the functional fragment has been isolated, enriched, synthesized, or recombinantly produced; wherein the functional fragment comprises 2 to 20 amino acids of the last 20 amino acids at the C-terminus of the protein; and wherein a lysine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO). In one embodiment the major royal jelly protein (MRJP) is selected from the group consisting of MRJP1 (SEQ ID NO: 1), MRJP2 (SEQ ID NO: 2), MRJP3 (SEQ ID NO: 3), MRJP4 (SEQ ID NO: 4), MRJP5 (SEQ ID NO: 5), MRJP6 (SEQ ID NO: 6), MRJP7 (SEQ ID NO: 7), MRJP8 (SEQ ID NO: 8), and MRJP9 (SEQ ID NO: 9).

[0011] In another aspect the present invention provides a method of reducing inflammation in a cellular tissue, comprising the step of contacting the cellular tissue with a functional fragment of a major royal jelly protein (MRJP); wherein the functional fragment has been isolated, enriched, synthesized, or recombinantly produced; wherein the functional fragment comprises an amino acid sequence selected from the group consisting of: LVK (SEQ ID NO: 84), LIR (SEQ ID NO: 86), FDR (SEQ ID NO: 127), HNIR (SEQ ID NO: 128), FTK (SEQ ID NO: 130), and QNGNK (SEQ ID NO: 137); and wherein a lysine or arginine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO). In one embodiment the inflammation is associated with one or more of the group consisting

of: an inflammatory disorder, a cardiovascular disorder, a neurological disorder, a pulmonary disorder, a proliferative disorder, an infectious disease or associated syndrome, an allergic, immunological or autoimmune disorder, and inflammation associated with a wound.

[0012] In a further aspect there is provided a method of inhibiting Cathepsin B activity in a cellular tissue, comprising the step of contacting the cellular tissue with a functional fragment of a major royal jelly protein (MRJP); wherein the functional fragment has been isolated, enriched, synthesized, or recombinantly produced; wherein the functional fragment comprises an amino acid sequence selected from the group consisting of: LVK (SEQ ID NO: 84), LIR (SEQ ID NO: 86), FDR (SEQ ID NO: 127), HNIR (SEQ ID NO: 128), FTK (SEQ ID NO: 130), and QNGNK (SEQ ID NO: 137); and wherein a lysine or arginine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO).

[0013] In a further aspect, there is provided an isolated functional fragment of a major royal jelly protein (MRJP), wherein the functional fragment comprises an amino acid sequence selected from the group consisting of:

i. KISIHL (SEQ ID NO: 10);
ii. KNNNQNDN (SEQ ID NO: 11);
iii. KLH (SEQ ID NO: 12);
iv. KSNNRHNNND (SEQ ID NO: 13);
v. KHNN (SEQ ID NO: 14);
vi. KNQAHLD (SEQ ID NO: 15);
vii. KNTRCISP (SEQ ID NO: 16);
viii. KTNFFSIFL (SEQ ID NO: 17); and

wherein a lysine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO).

[0014] In another aspect the present invention provides an isolated functional fragment of a major royal jelly protein (MRJP); wherein the functional fragment comprises an amino acid sequence selected from the group consisting of: LVK (SEQ ID NO: 84), LIR (SEQ ID NO: 86), FDR (SEQ ID NO: 127), HNIR (SEQ ID NO: 128), FTK (SEQ ID NO: 130), and QNGNK (SEQ ID NO: 137); and wherein a lysine or arginine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO).

[0015] In another aspect there is provided a method of producing an anti-inflammatory molecule that is an apalbumin protein or functional fragment thereof by modifying royal jelly, the method including the step of reacting royal jelly with at least 0.1% MGO at between 18 and 37 degrees Celsius.

[0016] In another aspect there is provided a method of enriching the anti-inflammatory molecules in a *Leptospermum* genus derived MGO containing honey comprising the step of adding Major Royal Jelly Protein to the honey.

[0017] In a further aspect there is provided a method of identifying (i) the anti-inflammatory capacity or (ii) MGO-modified major royal jelly protein concentration of a sample of honey, comprising the step of: assaying the Cathepsin B inhibition levels of the honey sample.

[0018] In another aspect there is provided a method of inhibiting Cathepsin B activity in a cellular tissue, comprising the step of contacting the cellular tissue with a functional fragment of a major royal jelly protein (MRJP); wherein the functional

fragment has been isolated, enriched, synthesized, or recombinantly produced; wherein the functional fragment comprises 2 to 20 amino acids of the last 20 amino acids at the C-terminus of the protein; and wherein a lysine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO). In one embodiment the major royal jelly protein (MRJP) is selected from the group consisting of MRJP1 (SEQ ID NO: 1), MRJP2 (SEQ ID NO: 2), MRJP3 (SEQ ID NO: 3), MRJP4 (SEQ ID NO: 4), MRJP5 (SEQ ID NO: 5), MRJP6 (SEQ ID NO: 6), MRJP7 (SEQ ID NO: 7), MRJP8 (SEQ ID NO: 8), and MRJP9 (SEQ ID NO: 9).

[0019] The foregoing brief summary broadly describes the features and technical advantages of certain embodiments of the present invention. Further technical advantages will be described in the detailed description of the invention and Examples that follows. Novel features that are believed to be characteristic of the invention will be better understood from the detailed description of the invention when considered in connection with any accompanying figures and examples. However, the Figures and Examples provided herein are intended to help illustrate the invention or assist with developing an understanding of the invention, and are not intended to limit the invention's scope.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] Figures 1A-1B: SDS PAGE analysis of MRJP. Figure 1A: Bolt® 4-12% Bis-Tris Plus gel. Lane 1: Protein marker (SeeBlue Plus2 Pre-stained Standard. Lane 2: Crude royal jelly is dissolved in double distilled water (10 µl load of approximately 5 mg/mL crude). Lane 3: Crude royal jelly is dissolved in double distilled water (10 µl load of approximately 2 mg/mL crude). Figure 1B: NuPAGE® Novex® 4-12% Bis-Tris gel. Lane 3: Crude royal jelly dissolved in double distilled water (soluble fractions, pH 4). Lane 4: Fraction 2 of 5 mL HiTrap™ de-salt column in 6 M urea containing no PMSF, pH 4, 48 h at room temperature. Lane 5: Fraction 2 of 5 mL HiTrap™ de-salt column in 6 M urea containing 1 mM PMSF, pH 4, 48 h at room temperature. Lane 6: Fraction 2 of 5 mL HiTrap™ de-salt column in 6 M urea containing no PMSF, pH 8, 48 h at room temperature. Lane 7: Fraction 2 of 5 mL HiTrap™ de-salt column in 6 M urea containing 1 mM PMSF, pH 8, 48 h at room

temperature. Lane 8: Fraction 2 of 5 mL HiTrap™ de-salt column in 6 M urea containing no PMSF, pH 8, 168 h at room temperature. Lane 9: Fraction 2 of 5 mL HiTrap™ de-salt column in 6 M urea containing 1 mM PMSF, pH 8, 168 h at room temperature. Lane 10: Protein marker.

[0021] Figure 2: Native PAGE™ Novex® 4-16% Bris-Tri gel of MGO-modified MRJP at pH 4.0 and 7.0, day 1 and day 2. Lane 1: Protein Standard contains protein bands 20-12,000 kDa]. Lane 2: MGO alone in PBS buffer, pH 7.4 after 24 h. Lane 3: MGO alone in 0.2 M sodium acetate buffer, pH 4.0 after 24 h. Lane 4: MRJP in PBS buffer, pH 7.4 after 24 h. Lane 5: MRJP in 0.2 M sodium acetate buffer, pH 4.0 after 24 h. Lane 6: MRJP reacted with MGO in PBS buffer, pH 7.4 after 24 h. Lane 7: MRJP reacted with MGO in 0.2 M sodium acetate buffer, pH 4.0 after 24 h. Lane 8: MGO alone in PBS buffer, pH 7.4 after 48 h. Lane 9: MGO alone in 0.2 M sodium acetate buffer, pH 4.0 after 48 h. Lane 10: MRJP in PBS buffer, pH 7.4 after 48 h. Lane 11: MRJP in 0.2 M sodium acetate buffer, pH 4.0 after 48 h. Lane 12: MRJP reacted with MGO in PBS buffer, pH 7.4 after 48 h. Lane 13: MRJP reacted with MGO in 0.2 M sodium acetate buffer, pH 4.0 after 48 h. Lane 14: BSA control. Lane 15: Protein marker (same as Lane 1).

[0022] Figure 3: Native PAGE™ Novex® 4-16% Bris-Tri gel of MGO-modified MRJP at pH 4.0 and 7.0, day 3 and day 4. Lane 1: Protein Standard contains protein bands 20-12,000 kDa. Lane 2: MGO alone in PBS buffer, pH 7.4 after 72 h. Lane 3: MGO alone in 0.2 M sodium acetate buffer, pH 4.0 after 72 h. Lane 4: MRJP in PBS buffer, pH 7.4 after 72 h. Lane 5: MRJP in 0.2 M sodium acetate buffer, pH 4.0 after 72 h. Lane 6: MRJP reacted with MGO in PBS buffer, pH 7.4 after 72 h. Lane 7: MRJP reacted with MGO in 0.2 M sodium acetate buffer, pH 4.0 after 72 h. Lane 8: MGO alone in PBS buffer, pH 7.4 after 96 h. Lane 9: MGO alone in 0.2 M sodium acetate buffer, pH 4.0 after 96 h. Lane 10: MRJP in PBS buffer, pH 7.4 after 96 h. Lane 11: MRJP in 0.2 M sodium acetate buffer, pH 4.0 after 96 h. Lane 12: MRJP reacted with MGO in PBS buffer, pH 7.4 after 96 h. Lane 13: MRJP reacted with MGO in 0.2 M sodium acetate buffer, pH 4.0 after 96 h. Lane 14: BSA control. Lane 15: Protein marker (same as Lane 1).

[0023] Figure 4: Native PAGE™ Novex® 4-16% Bris-Tri gel of MGO-modified MRJP at pH 4.0 and 7.0, day 5 and day 6. Lane 1: Protein Standard contains

protein bands 20-12,000 kDa. Lane 2: MGO alone in PBS buffer, pH 7.4 after 120 h. Lane 3: MGO alone in 0.2 M sodium acetate buffer, pH 4.0 after 120 h. Lane 4: MRJP in PBS buffer, pH 7.4 after 120 h. Lane 5: MRJP in 0.2 M sodium acetate buffer, pH 4.0 after 120 h. Lane 6: MRJP reacted with MGO in PBS buffer, pH 7.4 after 120 h. Lane 7: MRJP reacted with MGO in 0.2 M sodium acetate buffer, pH 4.0 after 120 h. Lane 8: MGO alone in PBS buffer, pH 7.4 after 144 h. Lane 9: MGO alone in 0.2 M sodium acetate buffer, pH 4.0 after 144 h. Lane 10: MRJP in PBS buffer, pH 7.4 after 144 h. Lane 11: MRJP in 0.2 M sodium acetate buffer, pH 4.0 after 144 h. Lane 12: MRJP reacted with MGO in PBS buffer, pH 7.4 after 144 h. Lane 13: MRJP reacted with MGO in 0.2 M sodium acetate buffer, pH 4.0 after 144 h. Lane 14: BSA control. Lane 15: Manuka honey (crude)

[0024] Figure 5: Native PAGE™ Novex® 4-16% Bris-Tri gel of MGO-modified MRJP at pH 4.0 and 7.0, day 7 and day 9. Lane 1: Protein Standard contains protein bands 20-12,000 kDa. Lane 2: MGO alone in PBS buffer, pH 7.4 after 168 h. Lane 3: MGO alone in 0.2 M sodium acetate buffer, pH 4.0 after 168 h. Lane 4: MRJP in PBS buffer, pH 7.4 after 168 h. Lane 5: MRJP in 0.2 M sodium acetate buffer, pH 4.0 after 168 h. Lane 6: MRJP reacted with MGO in PBS buffer, pH 7.4 after 168 h. Lane 7: MRJP reacted with MGO in 0.2 M sodium acetate buffer, pH 4.0 after 168 h. Lane 8: MGO alone in PBS buffer, pH 7.4 after 216 h. Lane 9: MGO alone in 0.2 M sodium acetate buffer, pH 4.0 after 216 h. Lane 10: MRJP in PBS buffer, pH 7.4 after 216 h. Lane 11: MRJP in 0.2 M sodium acetate buffer, pH 4.0 after 216 h. Lane 12: MRJP reacted with MGO in PBS buffer, pH 7.4 after 216 h. Lane 13: MRJP reacted with MGO in 0.2 M sodium acetate buffer, pH 4.0 after 216 h. Lane 14: No sample loaded here but there was a sample overflow from lane 13. Lane 15: No sample (empty)

[0025] Figure 6A: Inhibition of Cathepsin B with 0.5% MGO at pH 3.8, incubation for 1 day. Figure 6B: Inhibition of Cathepsin B with 0.15% MGO and 0.5% MGO modification of MRJP at 22°C (set temperature) or RT (ambient room temperature).

[0026] Figure 7: Schematic chart of MRJP modification by acetic anhydride (reaction with epsilon amino group), N-ethyl maleimide (NEM) (blocking of cysteine residues) and MGO (reaction with Arg, Lys and Cys residues).

[0027] Figure 8: Cathepsin B inhibition after treatment of MRJPs with NEM, acetic anhydride, and MGO.

[0028] Figure 9: Native PAGE™ Novex® 4-16% Bris-Tri gel of MGO-modified BSA and Cathepsin B hydrolysis of MGO-modified MRJP. Cathepsin B hydrolysis was performed on MGO-modified MRJP prepared at either pH 4.0 or 7.0. Lane 1: Protein standard contains protein bands 20-12,000 kDa. Lane 2: BSA control in PBS, pH 7.4, 37°C, overnight. Lane 3: MGO-modified BSA reaction in PBS pH 7.4, 37°C, overnight. Lane 4: MGO-modified BSA reaction in 0.2 M sodium acetate buffer pH 4.0, 37°C, overnight. Lane 5: MGO-modified BSA reaction in PBS pH 7.4, room temperature, overnight. Lane 6: MGO-modified BSA reaction in 0.2 M sodium acetate buffer pH 4.0, room temperature, overnight. Lane 7: Desalted fraction of MGO-modified MRJP at pH 4/9 days without Cathepsin B digestion. Lane 8: Desalted fraction of MGO-modified MRJP at pH 4/9 days with Cathepsin B digestion. Lane 9: Desalted fraction of MGO-modified MRJP at pH 7.5/9 days without Cathepsin B digestion. Lane 10: Desalted fraction of MGO-modified MRJP at pH 7.5/9 days with Cathepsin B digestion. Lanes 11 to Lane 15: Same as Lane 2 to Lane 6 but desalted before loading into the gel.

[0029] Figure 10: Amino acid sequence of MRJP1, SEQ ID NO: 1.

[0030] Figures 11A-C: Inhibitory activity of peptides derived from the C-terminus of MRJP1. Figure 11A: C-terminal sequence of MRJP1. MGO-modified lysine residue is indicated by underlining. Figure 11B: Synthetic peptides tested for Cathepsin B inhibition. Figure 11C: Cathepsin B inhibition by unmodified and MGO-modified peptides.

[0031] Figures 12A-C: Inhibitory activity of peptides derived from the C-terminus of MRJP2. Figure 12A: C-terminal sequence of MRJP2. MGO-modified lysine residue is indicated by underlining. Figure 12B: Synthetic peptides tested for Cathepsin B inhibition. Figure 12C: Cathepsin B inhibition by unmodified and MGO-modified peptides.

[0032] Figures 13A-C: Inhibitory activity of peptides derived from the C-terminus of MRJP3. Figure 13A: C-terminal sequence of MRJP3. MGO-modified lysine residue is indicated by underlining. Figure 13B: Synthetic peptides tested for

Cathepsin B inhibition. Figure 13C: Cathepsin B inhibition by unmodified and MGO-modified peptides.

[0033] Figures 14A-C: Inhibitory activity of peptides derived from the C-terminus of MRJP4. Figure 14A: C-terminal sequence of MRJP4. MGO-modified lysine residue is indicated by underlining. Figure 14B: Synthetic peptides tested for Cathepsin B inhibition. Figure 14C: Cathepsin B inhibition by unmodified and MGO-modified peptides.

[0034] Figures 15A-C: Inhibitory activity of peptides derived from the C-terminus of MRJP5. Figure 15A: C-terminal sequence of MRJP5. MGO-modified lysine residue is indicated by underlining. Figure 15B: Synthetic peptides tested for Cathepsin B inhibition. Figure 15C: Cathepsin B inhibition by unmodified and MGO-modified peptides.

[0035] Figures 16A-B: Additional peptides tested for Cathepsin B inhibition. Figure 16A: Synthetic peptide sequences. Figure 16B: Cathepsin B inhibition by unmodified and MGO-modified peptides.

DETAILED DESCRIPTION OF THE INVENTION

[0036] The following description sets forth numerous exemplary configurations, parameters, and the like. It should be recognized, however, that such description is not intended as a limitation on the scope of the present invention, but is instead provided as a description of exemplary embodiments.

Definitions

[0037] In each instance herein, in descriptions, embodiments, and examples of the present invention, the terms "comprising", "including", etc., are to be read expansively, without limitation. Thus, unless the context clearly requires otherwise, throughout the description and the claims, the words "comprise", "comprising", and the like are to be construed in an inclusive sense as to opposed to an exclusive sense, that is to say in the sense of "including but not limited to".

[0038] "Royal jelly" is a honey bee secretion that is secreted from the glands in the hypopharynx of worker bees. Aside from water, protein is the major component of royal jelly and comprises the Major Royal Jelly proteins.

[0039] An "apalbumin protein" is a glycoprotein found in honey and in royal jelly. The major apalbumin found in honey is Apalbumin 1 (Apa1) also known as Major Royal Jelly Protein 1 (MRJP1) or royalactin. While the specification focuses on the major apalbumins found in honey, it is to be appreciated that the other apalbumins found in honey may also exhibit similar modification potential and similar anti-inflammatory capacity because they are all glycoproteins with a high mannose type of glycosylation as reported in 2000 by Kimura et. al. in Biosci. Biotechnol. Biochem. There are approximately nine major royal jelly proteins and the sequences of major royal jelly proteins 1-9 are shown in the Sequence Listing.

[0040] It should be understood that the terms "MRJP" (e.g., any one of MRJP1-9), "peptide" (e.g., peptide derived from any one of MRJP1-9), and "SEQ ID NO:" (e.g., any one of SEQ ID NO: 1-145, and other such terms, for simplicity, are used to identify the molecules described herein and not to provide their complete characterization. Thus, a protein or peptide may be characterized herein as having a particular amino acid sequence, a particular 2-dimensional representation of the structure, but it is understood that the actual molecule claimed has other features, including 3-dimensional structure, mobility about certain bonds and other properties of the molecule as a whole. It is the molecules themselves and their properties as a whole that are encompassed by this disclosure.

[0041] "Modification" of a primary amino acid sequence is understood to include "deletions" (that is, polypeptides in which one or more amino acid residues are absent), "additions" (that is, a polypeptide which has one or more additional amino acid residues as compared to the specified polypeptide), "substitutions" (that is, a polypeptide which results from the replacement of one or more amino acid residues), and "fragments" (that is, a primary amino acid sequence which is identical to a portion of the primary sequence of the specified polypeptide).

[0042] "Modified apalbumin" is to be understood to include any apalbumin or major royal jelly protein or fragment thereof that has been modified by the chemical reaction of methylglyoxal on the amino acids or the chemical reaction of methylglyoxal on the side chains of the amino group that make up the protein. Methylglyoxal modifications occur at free amino groups of lysine, arginine and/or cysteine amino acid moieties within the apalbumin, including the terminal amino acid, and such MGO

modifications may occur on approximately 1-40 sites within the protein. For example, modified apalbumin1 means Apa1 modified at one or more sites on its amino acid sequences to provide a MGO-modified Apa1.

[0043] As described herein, MRJP "fragments" (i.e., fragments derived from one or more MRJP) will be taken to include peptides obtained from any source, e.g., isolated naturally occurring peptides, recombinant peptides, and synthetic peptides. These include peptides having the naturally occurring sequences as well as modified peptide sequences. Of particular interest are functional fragments of MRJPs, i.e., fragments that retain one or more of the activities of the starting protein, or analogues thereof. Such activities are described in detail herein. Thus, it will be understood that a "fragment" is not limited to a peptide obtained directly from a polypeptide, for example, by digestion of the original polypeptide by a peptidase.

[0044] As used herein, the term "analogue" of a protein or peptide means a protein or peptide that includes a modification as described herein, or a peptide or fragment thereof that includes one or more non-amino acid substituents replacing amino acids, while the analogue still provides the necessary activity and respective stability of the peptide or peptide fragment. The analogues of the invention may include an acetylated N-terminus (Ac) and/or an amidated C-terminus (NH_2), as well as a C-terminal hydroxyl residue (OH). Other analogues are also possible, including those that stabilize the domain necessary for Cathepsin B inhibition. Functional analogue are specifically encompassed by the present invention, i.e., analogues that retain one or more of the activities of the starting sequence. It will be understood that where a peptide analogue is specifically noted (e.g., Ac-Xaa-Xaa-Xaa-OH; 5), the amino acid sequence itself is also considered to be disclosed (e.g., Xaa-Xaa-Xaa). Similarly, where an unmodified peptide is specifically noted (e.g., Xaa-Xaa-Xaa), the analogue is also considered to be disclosed (e.g., Ac-Xaa-Xaa-Xaa-OH).

[0045] "C-terminus" or "C-terminal region" is to be understood to be the amino acid region that is proximate the end of an amino acid chain carrying the free alpha carboxyl group of the last amino acid.

[0046] "Lysine modified by MGO" is to be understood as a lysine amino acid covalently bound to MGO. "Arginine modified by MGO" is understood as an arginine amino acid covalently bound to MGO.

[0047] Amino acid "sequence similarity" or "sequence identity" refers to the amino acid to amino acid comparison of two or more polypeptides at the appropriate place, where amino acids are identical or possess similar chemical and/or physical properties such as charge or hydrophobicity. Sequence similarity and identity are typically determined by sequence alignments at the regions of highest homology. Sequence alignment algorithms are well known and widely used in the art. Based on the sequence comparison, a "percent identity" can be determined between the compared polypeptide sequences.

[0048] Tissue that is "inflamed" is defined as tissue in which an immune response has occurred in response to injury or infection in the tissue, and in which the tissue has one or more symptoms of pain, swelling, heat, sensitivity, and redness.

[0049] As used herein, "anti-inflammatory capacity" is defined as the capacity to clinically reduce inflammation or the symptoms of inflammation in cellular tissue. Anti-inflammatory capacity may be determined using the phagocytosis inhibition assay (PIA) described in PCT/NZ PCT/NZ/2011/000271 or the DCFDA assay also described in PCT/NZ/2011/000271 or the anti-inflammatory capacity may also be determined by the inhibition of Cathepsin B as described in detail below.

Brief Description of the sequences

[0050] SEQ ID NO: 1 - amino acid sequence of Apa1 (also known as Major Royal Jelly Protein 1) obtained from http://www.uniprot.org/uniprot/O18330. See Figure 10.

[0051] SEQ ID NO: 2 - amino acid sequence of Major Royal Jelly Protein 2 obtained from http:// http://www.uniprot.org/uniprot/O77061 is shown in the Sequence Listing.

[0052] SEQ ID NO: 3 - amino acid sequence of Major Royal Jelly Protein 3 obtained from http://www.uniprot.org/uniprot/Q17060-1 is shown in the Sequence Listing.

[0053] SEQ ID NO: 4 - amino acid sequence of Major Royal Jelly Protein 4 obtained from http://www.uniprot.org/uniprot/Q17060-1 is shown in the Sequence Listing.

[0054] SEQ ID NO: 5 - amino acid sequence of Major Royal Jelly Protein 5 obtained from http://www.uniprot.org/uniprot/097432 is shown in the Sequence Listing.

[0055] SEQ ID NO: 6 - amino acid sequence of Major Royal Jelly Protein 6 obtained from http://www.uniprot.org/uniprot/ Q6W3E3 is shown in the Sequence Listing.

[0056] SEQ ID NO: 7 - amino acid sequence of Major Royal Jelly Protein 7 obtained from http://www.uniprot.org/uniprot/ Q6IMJ9 is shown in the Sequence Listing.

[0057] SEQ ID NO: 8 - amino acid sequence of Major Royal Jelly Protein 8 obtained from http://www.uniprot.org/uniprot/ Q6TGR0 is shown in the Sequence Listing.

[0058] SEQ ID NO: 9 - amino acid sequence of Major Royal Jelly Protein 9 obtained from http://www.uniprot.org/uniprot/Q4ZJX1 is shown in the Sequence Listing.

[0059] Exemplary fragments of MRJPs include a fragment of SEQ ID NO: 1 that comprises -Lys-Ile-Ser-Ile-His-Leu **(SEQ ID NO: 10)** or an analogue thereof; a fragment of SEQ ID NO: 2 that comprises -Lys-Asn-Asn-Asn-Gln-Asn-Asp-Asn **(SEQ ID NO: 11)** or an analogue thereof; a fragment of SEQ ID NO: 3 that includes -Lys-Leu-His **(SEQ ID NO: 12)** or an analogue thereof; a fragment of SEQ ID NO: 4: that includes -Lys-Ser-Asn-Asn-Arg-His-Asn-Asn-Asn-Asp **(SEQ ID NO: 13)** or an analogue thereof; a fragment of SEQ ID NO: 5 that includes -Lys-His-Asn-Asn **(SEQ ID NO: 14)** or an analogue thereof; a fragment of SEQ ID NO: 6 that includes -Lys-Asn-Gln-Ala-His-Leu-Asp- **(SEQ ID NO: 15)** or an analogue thereof; a fragment of SEQ ID NO: 8 that includes -Lys-Asn-Thr-Arg-Cys-Ile-Ser-Pro **(SEQ ID NO: 16)** or an analogue thereof; a fragment of SEQ ID NO: 9 that includes -Lys-Thr-Asn-Phe-Phe-Ser-Ile-Phe-Leu **(SEQ ID NO: 17)** or an analogue thereof. In certain aspects, the lysine residue of these fragments is modified by MGO.

[0060] Other fragments are described in detail herein, including those shown as SEQ ID NO: 18-145. The Sequence Listing and all the sequences included therein are hereby incorporated herein in their entirety.

MGO-modified major royal jelly proteins

[0061] Methylglyoxal or MGO is a highly chemically reactive compound with the formula $C_3H_4O_2$. MGO is formed by multiple metabolic pathways in living organisms. Certain preparations of manuka honey, which are referred to as "active" manuka honey, contain much higher concentrations of MGO than other varieties of honey. Active manuka honey has been determined to contain MGO concentrations up to 1000-fold greater than the MGO concentration in other varieties of honey (E. Mavric et al, 2008).

[0062] MGO can participate in a variety of chemical reactions in living organisms, including the formation process of advanced glycation endproducts (AGEs). MGO can modify proteins by reacting with the free amino groups of the amino acids arginine, lysine, and /or cysteine and the terminal amino group, and thereby can chemically modify proteins that contain arginine and/or lysine.

[0063] MGO-modified major royal jelly proteins (MRJPs) and fragments thereof can be derived by isolation of the molecules from active manuka honey. The modified proteins and fragments can be isolated from honey and/or enriched from honey by biochemical techniques. These techniques include but are not limited to filtration, centrifugation, and chromatography, such as ion-exchange, affinity, hydrophobic interaction, size exclusion, and reverse-phase chromatography. MGO-modified MRJP and fragments can also be purified from various sources or chemically synthesized by addition of MGO to royal jelly. In another approach, the major royal jelly may be added to honey derived from the *Leptospermum* genus, such as manuka honey and jelly bush honey to achieve enrichment of MGO-modified MRJP and fragments in the honey.

[0064] A MGO-modified MRJP or a fragment thereof may also be derived by obtaining a sequence coding for the amino acid sequences of SEQ IDs NO: 1-145, cloning the coding sequence into an appropriate vector, transforming a cell line with the vector, causing the polypeptide or peptide to be expressed, purifying the polypeptide or peptide, mixing the polypeptide or peptide with MGO to allow for chemical reaction between MGO and the polypeptide or peptide, and purifying the MGO-modified polypeptide or peptide.

[0065] Expression systems may contain control sequences, such as promoters, enhancers, and termination controls such as are known in the art for a variety of hosts (See e.g. Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Ed., Cold Spring Harbor Press (1989) which is incorporated herein in its entirety). The expression systems may also contain signal peptide and prep-pro-protein sequences that facilitate expression of the coding sequence and/or folding of the protein.

[0066] Synthetic production or peptides may be carried out using the solid-phase synthetic method described by Goodman M. (ed.), "Synthesis of Peptides and Peptidomimetics" in Methods of organic chemistry (Houben-Weyl) (Workbench Edition, 2004; Georg Thieme Verlag, Stuttgart, New York). This technique is well understood and is a common method for preparation of peptides. Peptides may also be synthesized using standard solution peptide synthesis methodologies, involving either stepwise or block coupling of amino acids or peptide fragments using chemical or enzymatic methods of amide bond formation. These solution synthesis methods are well known in the art. See, e.g. H. D. Jakubke in The Peptides, Analysis, Synthesis, Biology, Academic Press, New York, 1987, p. 103-165; J. D. Glass, ibid., pp. 167-184; and EP 0324659 A2, describing enzymatic peptide synthesis methods. Commercial peptide synthesizers, such as the Applied Biosystems Model 430A, may also be used.

[0067] MGO-modified forms of amino acid variants of MRJPs and their fragments may also exhibit anti-inflammatory capacity. As would be understood by one of ordinary skill in the art, minor modification of the primary amino acid sequence of SEQ ID NO: 1 may result in a polypeptide which has substantially equivalent or enhanced anti-inflammatory activity as compared to SEQ ID NO: 1. A peptide may be also modified to provide substantially equivalent or enhanced anti-inflammatory activity as the original peptide. When modification includes one or more substitutions, preferred substitutions are those that are of a conservative nature, i.e., wherein the residue is replaced by another of the same general type.

[0068] In making modifications to the protein or peptide, the hydropathic index of amino acids may be considered (See, e.g., Kyte. et al., J. Mol. Biol. 157, 105-132 (1982), herein incorporated by reference in its entirety). It is known in the art that certain amino acids may be substituted by other amino acids having a similar

hydropathic index or score and still result in a molecule having similar biological activity.

[0069] In particular aspects, the MGO-modified MRJP variant or fragment variant exhibits at least about 75% sequence identity to the non-variant sequence, preferably at least about 80% identity, more preferably at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to any wild type or reference sequence described herein. It is of particular interest where the MGO-modified MRJP variant or fragment variant exhibits anti-inflammatory capacity substantially comparable to or increased over that of non-variant molecule.

[0070] In certain aspects, the MRJP fragment is derived from the C-terminal sequence of the protein. The fragment may be derived from the last 20 amino acids, last 18 amino acids, last 16 amino acids, last 14 amino acids, last 12 amino acids, last 10 amino acids, last 8 amino acids, last 6 amino acids, last 4 amino acids, or last 2 amino acids of the C-terminal sequence of the protein. The functional fragment may comprise 2 to 20 amino acids, 2 to 18 amino acids, 2 to 16 amino acids, 2 to 14 amino acids, 2 to 12 amino acids, 2 to 10 amino acids, 2 to 8 amino acids, 2 to 6 amino acids, 2 to 4 amino acids, or 2 amino acids of this C-terminal region. Thus, the MRJP fragment may be 20 amino acids, 18 amino acids, 16 amino acids, 14 amino acids, 12 amino acids, 10 amino acids, 8 amino acids, 6 amino acids, 4 amino acids, or 2 amino acids in length. Longer fragments may also be useful. In particular aspects, the MGO-modified lysine is at least three residues from the C-terminus, or no more than six residues from the C-terminus of the protein.

[0071] Of particular interest are functional fragments of the C-terminus of MRJPs as described in detail herein. However, other fragment of MRJPs may also be obtained, for example, fragments corresponding to internal amino acid sequences of the protein. In some aspects, a fragment may include a MRJP-derived amino acid sequence which is fused to (i.e., contiguous with) one or more heterologous amino acid sequences. Thus, an MRJP fragment may be presented as a portion of a major royal jelly protein that is included as part of a larger peptide or polypeptide. This may provide better stability and or expression capabilities, or provide options for cleavage or tagging. In certain aspects, the MRJP fragment may be chemically linked to other molecules, for example, one or more chemical moieties. While MGO-modified

fragments are specifically embraced, other chemical modifications of are also possible. The MRJP fragment may also be linked to a substrate such as beads, catheters, needles, sutures, stents, implantable medical devices, contact lenses, root canal fillers, wound dressings, burn dressings, tissue culture plates, fibers, and paper. The fragment may be prepared as a peptide conjugate in accordance with known methods.

[0072] The formation of MGO-modified MRJP or its fragments in honey can be stimulated by (i) prolonged storage at ambient temperature, or (ii) incubation of honey at elevated temperatures (30-40 Celsius), thereby increasing the anti-inflammatory capacity of a sample of honey. Addition of MGO or an MGO precursor, such as dihydroxyacetone (DHA) to a sample of honey, along with sufficient time and/or heating to convert the MGO precursor to MGO, may also stimulate the formation of MGO-modified MRJP or fragments thereof in that sample of honey, and may also increase the anti-inflammatory capacity of the sample of honey, by the generation of MGO-modified MRJP in the honey sample.

[0073] MRJP1 and MRJP3 with enhanced anti-inflammatory properties can also be formed outside of honey. Completely or partially purified MRJP has been found to be treatable with MGO, in order to yield MGO-modified MRJP. The MGO-modified MRJP1 and MRJP3 exhibits enhanced anti-inflammatory properties when compared to the unmodified MRJP1 and MRJP3.

[0074] MGO-modified MRJP, fragments thereof, and variants thereof may be included in therapeutically-effective amounts in pharmaceutical compositions. It is also to be appreciated that a peptide having MGO-modified lysine and/or arginine may be prepared synthetically for use in the compositions.

[0075] The pharmaceutical compositions of the present disclosure may be specially formulated for administration in solid or liquid form, including those adapted for the following: oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, e.g., those targeted for buccal, sublingual, and systemic absorption, boluses, powders, granules, pastes for application to the tongue; parenteral administration, for example, by subcutaneous, intravitreal, intramuscular, intravenous or epidural injection as, for example, a sterile solution or suspension, or sustained release formulation.

[0076] Also encompassed is topical application, for example, as an eye drop, or as a cream, ointment, or a controlled release patch or spray applied to the skin; as well as administration intravaginally or intrarectally, for example, as a pessary, cream or foam; administration sublingually; administration ocularly; administration transdermally; administration pulmonarily, or nasally.

[0077] When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given per se or as a pharmaceutical composition containing, for example, about 0.1 to 99%, or about 1 to 50%, or about 10 to 40%, or about 10 to 30, or about 10 to 20%, or about 10 to 15% of active ingredient in combination with a pharmaceutically acceptable carrier or excipient.

[0078] Wetting agents, emulsifiers, and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the pharmaceutical compositions described herein. These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents, and dispersing agents.

[0079] Prevention of the action of microorganisms upon the compounds of the present invention may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents that delay absorption such as aluminum monostearate and gelatin.

Methods of use for MGO-modified MRJPs or fragments thereof

[0080] Cysteine proteases such as Cathepsin B are involved in the normal lysosomal degradation and processing of proteins. The increased expression or enhanced activation of cysteine proteases is associated with a number of medical conditions. The MGO-modified MRJPs or fragments, or a MGO-modified MRJP- or fragment-containing composition may thereby be used in the prevention, delay, mitigation, or treatment of the following disorders.

[0081] Included are inflammatory disorders such as rheumatoid arthritis, polyarthritis, and other inflammatory arthritidies, inflammatory bowel disease, and inflammatory bowel syndrome, inflammatory peritonitis, uveitis, sepsis, systemic inflammatory response syndrome, multiple organ failure; cardiovascular disorders such as ischemia reperfusion injury from transplantation and/or vascular surgery, angiogenesis, neovascularization, acute cardiac allograft dysfunction, ischemic cardiac damage, chemotherapy-induced myocardial suppression; musculoskeletal disorders such as osteoarthritis, osteoporosis, muscular dystrophy, myositis; neurological disorders such as multiple sclerosis, stroke, Alzheimer's disease, progressive multifocal leukoencephalopathy (PML), prion-associated disorders, ataxia telangiectasia, central nervous system injury; pulmonary disorders such as asthma, bronchitis, chronic obstructive pulmonary disease (COPD), adult respiratory distress syndrome, Wegeneres granulomatosis, and emphysema.

[0082] Included as well are proliferative disorders, including those involving solid tumors, lymphomas, leukemias and other malignancies, for example, acute and chronic myelogenous leukemia, neuronal cancer, cancer invasion and metastasis, tumor angiogenesis, B and T cell lymphomas, acute and chronic lymphocytic leukemia, resistance to chemotherapy, cancer associated coagulopathies (including deep venous thrombosis, coronary artery disorder, pulmonary embolism, disseminated intravascular coagulation), Hodgkin's disease, carcinomas of the colon, liver, lung, breast, kidney, stomach, pancreas, esophagus, oral pharynx, intestine, thyroid, prostate, bladder, brain; osteosarcoma, chondrosarcoma and liposarcoma; neuroblastoma; melanoma; and carcinomas derived from amnion and/or chorion).

[0083] Additionally included are infectious diseases and associated syndromes such as septic shock (including Gram-negative sepsis), HIV infection and AIDS, genital herpes, zoster, chickenpox, molluscum contagiosum, EBV infections, encephalitis including EBV associated encephalitis, choreoretinitis, cytomegalovirus (CMV) associated choreoretinitis or encephalitis, cytomegalovirus infections in neonates (including related pneumonitis), opportunistic infections in immunocompromised individuals (including AIDS and transplant patients), dysentery, hepatitis C, hepatitis A, keratoconjuctovitis, bronchopneumonia (including pneumonia in immunocompromised individuals), gastroenteritis, malaria, rhinovirus, polio, enterovirus infections, common cold, aseptic meningitis, foot and mouth disease,

Klebsiella pneumoniae infection, *Escherichia coli* or *Staphylococcus epidermidis*, leprosy bacteremia, otitis media, lambliasis, non-atopic sinusitis, and fulininant hepatitis.

[0084] Further included are allergic, immunological, and autoimmune disorders such as house dust mite allergy, transplant rejection, graft verses host disease, Type 1 diabetes mellitus, autoimmune thyroiditis, psoriasis, dermatitis (e.g., contact dermatitis), antibody-mediated autoimmune diseases, lupus erythematosus, Sjogren's syndrome, autoimmune encephalomyelitis; kidney disorders such as polycystic kidney disease, glomerulonephritis; as well as other disorders such as periodontal disease, alcohol hepatitis, prostate hypertrophy, trauma, cutaneous mastocytosis, radiation- and HIV-induced diarrhea, cachexia (including accompanying cancer and malnutrition), and inflammation associated with wounds, e.g., puncture wounds, diabetic ulcers, fungating wounds, cuts, bites, and surgical wounds.

[0085] Indications of note include: rheumatoid arthritis, polyarthritis, Alzheimer's disease, multiple sclerosis, especially relapsing-remitting multiple sclerosis, progressive multifocal leukoencephalopathy (PML), asthma, bronchitis, adult respiratory distress syndrome, Wegeneres granulomatosis, emphysema, and COPD, melanoma, genital herpes, EBV infection, encephalitis, including EBV associated encephalitis, choreoretinitis, including CMV choreoretinitis, bronchopneumonia, gastroenteritis, uveitis, psoriasis, dermatitis, and various wounds.

[0086] In a specific aspect, the MGO-modified MRJPs or fragments thereof may be formulated as a wound dressing. This includes any covering that can be applied to a lesion. This encompasses infected and non-infected abrasions, cuts, bites, bums, wounds, ulcers, abscesses, surgical wounds, fungating tumors, and pressure sores. The lesion is preferably external, for example resulting from damage or injury to the skin.

[0087] The MGO-modified MRJP or fragment, or the MGO-modified MRJP- or fragment-containing composition may be posited on a surface of a substrate, such as a wound dressing substrate. The composition may include water and optionally another solvent. For such compositions, the water and optional solvent may be allowed to evaporate. The MGO-modified MRJP or fragment, or the MGO-modified MRJP- or fragment-containing composition may be incorporated into a natural or synthetic fiber. For example, the polypeptide or peptide may be incorporated on or into fibers used for

cloth, synthetic or paper dressings. Paper may be used as part of a temporary wound dressing.

[0088] The treated substrate may include natural or synthetic materials, and implantable devices. The biocompatibility of the substrate may be evaluated by any suitable methodology known in the art, including one or more viability/cytotoxicity assays known to those of ordinary skill in the art. The treated substrate may be in contact with an aqueous environment, such as water or the inside of a patient. Alternatively, the treated substrate may be contact with air or air and/or air borne bacteria in an external environment or an enclosed bodily organ, such as a lung.

[0089] Thus, inflammation in tissue may be reduced by administering one or more purified MGO-modified MRJPs or fragments, or a MGO-modified MRJP- or fragment-containing composition to inflamed tissue. Inflammation from various injuries, infections, and medical diseases may be treated. Included are acute and chronic inflammatory diseases as noted above.

[0090] Regarding rheumatoid arthritis, it is noted that peptidyl fluoromethyl ketones with the amino acid sequence Phe-Ala held constant but with variable N-terminal groups were effective in inhibiting Cathepsin B activity *in vitro* and also inhibiting the severity of inflammation and the extent of cartilage and bone damage in adjuvant-induced arthritis. See Ahmed et al., 1992. It is considered that the MGO-modified MRJP peptides or fragments or a MGO-modified MRJP-containing composition of the invention can be used in similar treatments for rheumatoid arthritis.

[0091] MGO-modified MRJPs or fragments, or a MGO-modified MRJP- or fragment-containing composition may reduce inflammation in tissue by reducing the rate of phagocytosis by immune cells, and by blocking the mannose receptor or other receptors on immune cells, which trigger phagocytosis. Immune cells include macrophages, monocytes, dendritic cells, and granulocytes.

[0092] Phagocytosis is a cellular response or process of engulfing solid particles and cellular uptake in the immune system. It is a major mechanism used to remove pathogens and cell debris. Bacteria, dead tissue cells, and small mineral particles are all examples of objects that may be phagocytosed or engulfed by a cell. Phagocytosis occurs at the beginning of the inflammatory response of leukocytes to a

trigger of inflammation. Reactive oxygen species and cytokines are produced by cells after phagocytosis to recruit and activate more phagocytes as part of a cascade of cellular events which start with phagocytosis. Thus, inhibition of phagocytosis stops the inflammatory response at the start of the cascade.

[0093] MGO-modified MRJPs or fragments, or an MGO-modified MRJP- or fragment-containing composition may be administered to inflamed tissue in various different forms. The MGO-modified molecule may be purified from other components. For administration, it may be desirable to include one or more other types of compounds such as pharmaceutically acceptable carriers, adjuvants, or other therapeutic molecules. Other anti-inflammatory agents may be used for co-administration with the MGO-modified MRJPs or fragments, or an MGO-modified MRJP- or fragment-containing composition.

[0094] MGO-modified MRJPs or functional fragments thereof may be isolated from active manuka honey, or from manuka honey, or any other type of honey to which MGO or an MGO precursor has been added to modify the apalbumin, or it may be purified from royal jelly or a system in which apalbumin is recombinantly expressed and then treated with MGO.

[0095] MGO-modified MRJPs or fragments may be included in a composition containing one or more other types of compounds. This includes MGO-modified MRJPs or fragments contained in honey or a honey extract that has been enriched for MGO-modified components, and MGO-modified MRJPs or fragments in extracts from the recombinant production of an apalbumin or its peptides, and the chemical modification of the apalbumin or its peptides with MGO.

[0096] MGO-modified MRJPs or fragments or an MGO-modified MRJP- or fragment-containing composition may be administered to inflamed tissue in various different forms, including but not limited to: creams, lotions, liquid solutions, or poultices. MGO-modified MRJPs or fragments may also be administered to inflamed tissue as by inclusion of MGO-modified MRJP peptides or fragments in an edible product. Such products include but are not limited to beverages, candies, syrups, lozenges, pills, and foods.

Methods of detecting MGO-modified MRJPs or MRJP fragments, and characterizing properties of honey

[0097] The anti-inflammatory capacity of a sample of honey may be determined through detection of MGO-modified MRJPs or fragments thereof. The chemical modification of MRJPs by MGO generates molecules that exhibit Cathepsin B inhibition activity. Because MRJPs are present in honey, by measuring the Cathepsin B inhibition activity of a sample of honey, a measurement of the relative concentration of MGO-modified MRJPs or fragments in the honey sample can be obtained. A measurement of the concentration of MGO-modified MRJPs or fragments in a sample of honey directly relates to the anti-inflammatory capacity of the sample of honey.

[0098] Because high MGO concentration is a feature unique to manuka honey among all honey varieties, honey producers may try to simulate active manuka honey by adding MGO to samples of honey that do not naturally contain a desired concentration of MGO. Consumers prefer naturally occurring honeys over treated honeys. Purified, active MGO is readily available from commercial chemical producers (e.g. Sigma-Aldrich, St. Louis, MO, sells a solution of ~ 40% methylglyoxal in water), and honey producers may add MGO to a sample of honey that does not naturally contain a desired concentration of MGO, in order to raise the concentration of MGO in the honey sample to a desired level.

[0099] In particular aspects, the MGO-modified MRJPs or functional fragments thereof are obtained by one or more processes independent from natural honey formation. A process independent from natural honey formation includes any activity not performed by bees, and it therefore includes activities such as addition of purified MGO to a honey sample. A process independent from natural honey formation does not include activities such as bees collecting nectars, pollens, or other plant products that contain high levels of MGO or MGO-precursor molecules.

[00100] The Cathepsin B inhibition activity of honey may also be used to determine an appropriate time to harvest honey from a hive or to store harvested honey in order to obtain honey with desired anti-inflammatory properties. Because the modification of MRJPs by MGO in honey may occur over a period of time, a honey producer may choose to keep honey in the hive until it contains a desired anti-inflammatory capacity and concentration of MGO-modified MRJPs. By measuring the

Cathepsin B inhibition activity of samples of honey from the hive at different time intervals, a honey producer can use the measurement of the Cathepsin B inhibition activity of the honey as a method for determining the optimal time to harvest honey from the hive in order to obtain a honey having desired anti-inflammatory properties in the honey.

[00101] Similarly, a honey producer may also measure the Cathepsin B inhibition activity of honey stored outside of the hive, in order to determine if the honey sample has a desired level of anti-inflammatory properties. By measuring the Cathepsin B inhibition activity of honey samples, a honey producer seeking to obtain a honey sample containing a desired anti-inflammatory capacity can store honey until it has developed a desired level of anti-inflammatory capacity by the formation of MGO-modified MRJPs.

EXAMPLES

The examples described herein are provided for the purpose of illustrating specific embodiments of the invention and are not intended to limit the invention in any way. Persons of ordinary skill can utilize the disclosures and teachings herein to produce other embodiments and variations without undue experimentation. All such embodiments and variations are considered to be part of this invention.

Example 1: Preparation and analysis of MGO-modified MRJP proteins 1 and 3

[00102] Royal jelly obtained from Watson & Son/ManukaMed, was dissolved at 50 mg/mL in phosphate buffered saline pH 7.4 or in 100 mM disodium hydrogen phosphate pH 9.4. The pH value of the final solutions was 3.5-4.0 when PBS was used, or pH 7.4 when 100 mM disodium hydrogen phosphate pH 9.4 was used.

[00103] The protein solution was mixed for 10 minutes before centrifugation of the solution at 10,000 rpm in SS34 rotor at 4 °C. The supernatant was passed through a 0.45 micron filter and then further fractionated using ion exchange chromatography and heparin Sepharose™ to purify MRJP1. Alternatively the crude mixture was fractionated using a 5 mL HiTrap™ desalting column (GE), or separated using size exclusion chromatography on a Sephadex™ G75 column (GE).

[00104] The crude mixture was also reacted with MGO at final concentrations of 0.05%, 0.1%, 0.15%, 0.5%, and 1% at pH 7.4 and 3.5-4.0 as well as pH 6.9, and pH

5.0. Reactions were performed at room temperature, 22°C, 37°C, 40°C and 60°C. The reactions with respect of MGO coupling were performed over the following time periods: 1 day, 2 day, 3 day, 4 day, 5 day, 6 days, 7 days, 8 days, 9 days and 14 days.

[00105] Analysis of the MGO-modified proteins was performed using native PAGE analysis, SDS PAGE analysis, absorbance at 280 nm (protein concentration), absorbance at 330 nm (Arg-MGO adduct formation), size exclusion chromatography (SEC) on Sephadex™ G75, HiTrap™ desalting column and reactions with trinitrobenzene sulphonic acid (TNBS) reaction with lysine residues, fluorescence analysis excitation at 330 nm and emission at 410 nm (Arg-MGO adduct formation) due to the formation of argpyrimidine (Kim et al ., 2010).

[00106] SDS PAGE analysis was performed on a Bolt® 4-12% Bis-Tris Plus gel. A 10 well system (BGO4412BOX, Life technologies, NZ) was used in the gel electrophoresis analysis of samples following the manufacturer's protocol. 2-mercaptoethanol (M3148, Sigma Aldrich, NZ) was used as a reducing agent instead of DTT. The protein marker was SeeBlue Plus2 Pre-stained Standard (LC5925, Life Technologies, NZ). Further SDS PAGE analysis was performed using NuPAGE® Novex® 4-12% Bis-Tris gels, 1.0 mm, 10 well (NP0321BOX, Life technologies, NZ). Gel electrophoresis analysis was carried out for samples dissolved in 6M urea followed by manufacturer's protocol. As before, 2-mercaptoethanol (M3148, Sigma Aldrich, NZ) was used as a reducing agent instead of DTT, and the protein marker was SeeBlue Plus2 Pre-stained Standard (LC5925, Life Technologies, NZ). This analysis revealed the presence of two major royal jelly proteins MRJP1 and MRJP3 (Figures 1A and 1B).

[00107] MGO modification reactions were carried out at different pH conditions for a total of 9 days, as detailed above. Samples were collected every 24 hours and analyzed on 4-16% Native PAGE™ Novex® Bis-Tris gels 1.0 mm, 15 well (BN1004BOX, Life Technologies, NZ) following instructions provided in the manual. Typically, 4 μL of NuPAGE™ LDS sample buffer (4X) (Invitrogen, Carlsbad USA Cat. No. NP0007) was mixed with 11 μl of protein sample plus 1 μl of Native PAGE™ 5% G-250 sample additive. Then, 12 μl of this sample mix was loaded into native PAGE gels. Gels were run at 150 V for 120 minutes following manufacturer's protocols. Protein bands were visualized after de-staining for 2-4 hours following

manufacturer's instructions. Gels were scanned using the UVITEC Cambridge, UK Gel-doc system.

[00108] The native PAGE results are presented in Figures 2-5. At pH 4.0 but not at pH 7.0, the formation of MRJP high molecular weight adducts is seen after 24 hours of MGO treatment (Figure 2, Lane 7). At day 9, it is apparent that the majority of the 300 kDa hexamer is the likely source for the higher molecular weight adducts (Figure 5, Lane 13).

[00109] Changes in the lower molecular weight species are less apparent, but it is expected that MGO modification on the royalactin monomeric form of MRJP1 has also occurred. It may not be as readily apparent due to preclusion of higher molecular weight adducts from the gel. Visible precipitate was observed on the bottom of the micro centrifuge tube upon the centrifugation.

Example 2: Incubation of major royal jelly proteins in the presence of MGO and resulting inhibitory activity

[00110] After partial thawing, the MRJP mixture was dissolved in PBS (pH 7.4) at a concentration of 50 mg/ml. This mixture was stirred for 30 to 60 minutes. The resulting cloudy solution was clarified by centrifugation (10,000 rpm for 10 minutes). The protein content was determined as 5.0 ± 0.5 mg/ml. The pH of the solution was 3.8.

[00111] For the modification with MGO, 900 µl protein solution was mixed with 100 µl aqueous MGO solution (5 %) The 5% MGO stock solution was produced by adding 125 µL of 40% MGO to 875 µL of water. To obtain the final MGO concentration of 0.15% reaction, 30 µL of 5% MGO was added to 970 µL of protein solution and incubated at 22°C (set temperature) or allowed to sit out at ambient room temperature (RT) for 1 to 10 days. The set temperature of 22°C was maintained using a water bath.

[00112] After each incubation interval, unreacted MGO was removed on a HiTrap™ desalting column (5 ml, GE healthcare). The protein was eluted with PBS buffer (25 mM, pH 7.4) at a flow rate of 2 ml/min. Eluting compounds were detected by UV absorbance at 214, 280 and 330 nm. Alternatively, the elution was carried out

in sodium acetate buffer (10 mM, pH 4.0). The chromatography was carried out on an Äkta-900™ system (GE-healthcare) under the control of UNICORN software.

[00113] To test for activity, MGO was removed by passing 500 µl of reaction mixture (MRJP + MGO) through a 5 ml HiTrap™ desalting column. 1 ml fractions were collected and tested in the DCFDA oxidation assay (see Carter WO, Narayanan PK, Robinson JP. Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. J Leukoc Biol. 1994 Feb;55(2):253-8) and in the Cathepsin B assay (see below) to determine for the presence of reactive species as well as Cathepsin B inhibitors.

[00114] The desalting fractions 1-3 containing the high molecular weight proteins were also analyzed for the presence of free amines using TNBS and absorbance at 280 and 330 nm determined as well as fluorescence profile. The numbers were normalized to protein concentration and difference between the starting MRJP and MGO-modified MRJP determined to identify amino acids involved in the activity associated with Cathepsin B inhibition.

[00115] Activity is observed after overnight treatment under the following conditions: 0.5% MGO, room temperature incubation at pH 3.8, with 5 mg/ml MRJP (Figure 6A). Such treatment produces the most rapid production of Cathepsin B inhibitory activity. Inhibition of Cathepsin B is also found using modification with 0.15% MGO and 0.5% MGO at room temperature. See Figure 6B. When the lower MGO concentration of 0.15% is used for MRJP modification, activity is only apparent after longer incubation periods . The reaction products were separated using GE Healthcare desalting column and the high MW fractions that were free of MGO were tested for cathepsin B activity. The gel filtration was performed either at pH 7.5 in PBS or at pH 4.5 in 100 mM sodium acetate buffer. Fractionation was performed under conditions that corresponded to the conditions used for MGO modification either at pH 7.5 or at pH 3.5. This is in contrast to the higher MGO concentration. A similar trend was observed for pH, whereby acidic pH produced inhibitory activity at a faster rate by comparison to pH 7.4. See Figure 6B.

Example 3: Assay for Cathepsin B activity

[00116] Cathepsin B was obtained from Sigma (C6286-25UN Cathepsin B from bovine spleen). The enzyme was prepared by adding 1 mL of 100 mM sodium acetate pH 4.5 containing 2 mM EDTA and 2 mM 2-mercaptoethanol to the enzyme vial. This activated the Cathepsin B. For assays, 50 µl of activated Cathepsin B was diluted with 5 mL of the assay buffer (100 mM sodium acetate pH 4.5 containing 2 mM EDTA and 2 mM 2-mercaptoethanol). 50 µl was used per well in a 96 well plate.

[00117] Assays were performed in duplicate and repeated in replicates of four for active material. To each well of a 96 well plate, 100 µl of assay buffer was added, followed by 50 µl of sample and 50 µl of Cathepsin B (stock 5.25 mg/mL having 12.5 U/mg) diluted 1:100 to 52.5 µg/mL for use in the assay. Total amount of cathepsin B in the assay was 2.625 µg. The plate was allowed to incubate at 37 °C for up to 10 minutes. However, routine analysis was performed with 2-5 minutes of incubation.

[00118] The addition of the substrate was used to initiate the reaction, Na-CBZ-L-lysine p-nitrophenyl ester (CLN) (Sigma C 3637) (Z-Lys-pNP). The protocol was performed similar to that described by O'Neil et al., 1996. 27 mg of substrate was dissolved in 1 mL of DMSO and then 50 µl of the stock substrate solution was mixed with 5 mL of assay buffer (100 mM sodium acetate pH 4.5 containing 2 mM EDTA and 2 mM 2-mercaptoethanol). 50 µl of the substrate was added to each well of a 96 well plate. A row of eight wells of the 96 well plate was assayed during each run.

[00119] The plate was read in a SpectraMax® M4 plate reader incubated at 37 °C. The plate was agitated for 10 seconds and for 3 seconds between each read. The Vmax rate change in absorbance at 330 nm was read over 2 minutes with 10 s interval between reads. A delay of 30 seconds was used for the analysis. The linear portion of the curve was used to calculate the Vmax (milli units/min). The average of duplicate readings was performed. Active fractions were tested again to confirm activity.

Example 4: Identification of amino acid residues involved in Cathepsin B inhibition

[00120] The following methods were employed to demonstrate which amino acids residues were important for Cathepsin B inhibition. The MRJP were either treated with MGO (product 77), acetic anhydride (blocking lysine residues) (product 83) or N-ethylmaleimide (NEM) (product 81). These last two samples were then further

treated with MGO (yielding products 89 and 82, respectively). A sample of the acetylated MRJP (product 83) was taken and further reacted with NEM (yielding product 84). This sample was then further treated with MGO (yielding product 85). See reaction schematic in Figure 7.

Modification of MRJP mixture with MGO to give product 77

[00121] After partially thawing, the MRJP mixture was dissolved in PBS (pH 7.4) at a concentration of 50 mg/ml by stirring for 30 to 60 minutes. The resulting cloudy solution was clarified by centrifugation (10,000 rpm for 10 minutes). The protein content was determined as 5.0 ± 0.5 mg/ml. The pH of the solution was 3.8.

[00122] For the modification with MGO, 900 µl protein solution was mixed with 100 µl aqueous MGO solution (5 %) and incubated at 22°C for 1 to 10 days. The temperature was maintained by incubation in a water bath.

[00123] After each incubation interval, unreacted MGO was removed on a HiTrap™ desalting column (5 ml, GE healthcare). The protein was eluted with PBS buffer (25 mM, pH 7.4) at a flow rate of 2 ml/min. Eluting compounds were detected by UV absorbance at 214, 280 and 330 nm. Alternatively, the elution was carried out in sodium acetate buffer (10 mM, pH 4.0). The chromatography was carried out on an Äkta-900™ system (GE-healthcare) under the control of the UNICORN software.

Modification of MRJP mixture with NEM to give product 81

[00124] The MRJP mixture was dissolved in sodium phosphate buffer (pH 9.6) at a concentration of 50 mg/ml by stirring for 30 to 60 minutes. The resulting slightly cloudy solution was clarified by centrifugation (10,000 rpm for 10 minutes). The protein content was determined as 5.0 ± 0.5 mg/ml. The pH of the solution was 7.5.

[00125] To 1 ml of the protein solution 53 µl of NEM solution (200 mM in PBS) were added and the mixture was stirred for 1 hour at room temperature. Unreacted NEM was removed on a HiTrap™ desalting column (5 ml, GE healthcare). The protein was eluted with sodium acetate buffer (10 mM, pH 4.0) at a flow rate of 2 ml/min. Eluting compounds were detected by UV absorbance at 214, 280 and 330 nm.

Modification of NEM-treated MRJP with MGO to give product 82

[00126] Protein containing fraction from the desalting column (fraction 2) was modified with MGO as described above. Briefly, 900 µl of fraction 2 were reacted with 100 µl MGO (5 %) at 22°C overnight. Unreacted MGO was removed on a HiTrap™ desalting column (5 ml, GE healthcare). The protein was eluted with PBS buffer (25 mM, pH 7.4) at a flow rate of 2 ml/min. Eluting compounds were detected by UV absorbance at 214, 280 and 330 nm. Alternatively, the elution was carried out in sodium acetate buffer (10 mM, pH 4.0).

Acetylation of MRJP mixture to give product 83

[00127] 1.2814 g of defrosted RJP mixture was dissolved in 50 mL of Tris/HCl (0.1 M, pH 8.5) containing 6 M urea. The solution was cooled down on an ice water bath. Acetic anhydride was added every 20 minutes in aliquots of 0.5 ml over a period of 2 hours. The pH of the solution was determined before every addition and adjusted to a pH above 7.5 using Tris/HCl buffer (1 M, pH 8.5).

[00128] The sample was transferred to a dialysis tubing (molecular weight cut off 10 kDa) and dialysed over night against 1.6 litres of water with three changes of dialysate. The content of the dialysis tubing was frozen and freeze dried. 239.9 mg of a white slightly sticky material was recovered.

Modification of acetylated MRJP mixture with NEM to give product 84

[00129] The concentrated protein solution (500 µl) was diluted with 500 µl PBS (25 mM, pH 7.4), 53 µl of NEM solution (200 mM in PBS) were added and the mixture was stirred for 1 hour at room temperature. Unreacted NEM was removed on a HiTrap™ desalting column (5 ml, GE healthcare). The protein was eluted with sodium acetate buffer (10 mM, pH 4.0) at a flow rate of 2 ml/min. Eluting compounds were detected by UV absorbance at 214, 280 and 330 nm.

Modification of NEM-treated acetylated MRJP with MGO to give product 85

[00130] Protein containing fraction from the desalting column (fraction 2) was modified with MGO as described above. Briefly, 900 µl of fraction 2 were reacted with 100 µl MGO (5 %) at 22 °C overnight. Unreacted MGO was removed on a HiTrap™ desalting column (5 ml, GE healthcare). The protein was eluted with PBS buffer (25

mM, pH 7.4) at a flow rate of 2 ml/min. Eluting compounds were detected by UV absorbance at 214, 280 and 330 nm. Alternatively, the elution was carried out in sodium acetate buffer (10 mM, pH 4.0).

Results from NEM, acetylation, and MGO modifications

[00131] The samples after treatment with MGO were desalted with a 5 mL HiTrap™ desalting column and fraction 2 containing the high molecular weight proteins were analysed for their ability to inhibit Cathepsin B. The assay for Cathepsin B activity is detailed further above.

[00132] MRJP treated with MGO produced inhibitory activity (Figure 8). Inhibitory activity was also observed for the NEM plus MGO treated MRJP sample. However, upon acetylating MRJP and treating with MGO (product 89; see schematic in Figure 7), all inhibitory activity was lost, providing evidence that MGO modification of lysine residues is important for Cathepsin B inhibitory activity (Figure 8). After acetylation of the lysine residues of MRJP, the proteins appeared to stimulate rather than inhibit Cathepsin B activity (Figure 8).

[00133] It is noted that NEM is also a cysteine protease inhibitor. Our results showed that NEM modification followed by MGO modification produced greater inhibition of Cathepsin B. This was attributed to residual NEM remaining in the samples of MGO-modified protein, allowing the NEM to act in combination with the MGO-modified protein and to further inhibit Cathepsin B activity.

[00134] Thus, in summary, MGO reaction with MRJP produces a Cathepsin B inhibitor. Blocking thiol groups with NEM followed by MGO treatment also produces an active inhibitor of Cathepsin B. Acetylation of MRJP followed by reaction with MGO prevented the production of the Cathepsin B inhibitory activity. Acetylation followed by NEM treatment to block both lysine and cysteine residues on MRJPs followed by MGO treatment also failed to produce an active Cathepsin B inhibitor.

[00135] It has been demonstrated in PCT/NZ/2011/000271 that acetylation selectively blocks lysine residues. As noted above, the acetylated MRJP sample was found to lack activity after addition of MGO. This indicated that the MGO modification of lysine residues is important for the functional activity seen by modified

MRJP in relation to the formation of a reactive form of MGO on the surface of the protein.

[00136] To confirm these results, another sample prepared in an identical fashion was tested for its ability to inhibit Cathepsin B. The acetylated proteins from royal jelly were unable to inhibit Cathepsin B, even after optimal MGO treatment, i.e., 0.5% MGO at pH 3.5, 22 °C overnight. The speed by which this could be done making it preferred over the other methods outlined in Figure 6B which took from 3-7 days to generate similar levels of cathepsin B inhibitory activity. It was concluded that MGO modification of lysine is a key factor in Cathepsin B inhibitory activity.

Example 5: Cathepsin B digestion of MGO-modified MRJP

[00137] Cathepsin B working reagent solution was prepared (1:50) from Cathepsin B stock solution in the buffer system containing 100 mM sodium acetate, 2 mM EDTA and 2.5 mM 2-mercaptoethanol, pH 5.0.

[00138] Cathepsin digestion was performed on selected samples of SEC fractions. Typically, 10 µl of Cathepsin B working solution was mixed with 20 µl of protein sample and digested at 37°C for overnight. After hydrolysis, the digests were prepared for native PAGE analysis. The gel was stained with Coomassie® G250 as shown in Figure 9, see Lanes 7-10.

[00139] Samples used in the Cathepsin B digestion were: 1) Crude MRJP protein; 2) Desalted fraction (first big peak) of MGO-modified MRJP at pH 4.0 for 9 days sample; 3) Desalted fraction (first big peak) of MGO-modified MRJP in PBS (pH 7.5) for 9 days sample; 4) SEC fractions of pH 5.0 fractions; 5) SEC fractions of reaction 221.

[00140] Results are depicted in Figure 9. Lane 1 shows native PAGE molecular weight markers, Lane 7 shows the undigested MGO-modified MRJP at pH 4.0, Lane 8 shows the Cathepsin B digested MGO-modified MRJP at pH 4.0, Lane 9 shows undigested MGO-modified MRJP at pH 7.0, and Lane 10 shows Cathepsin B digested MGO-modified MRJP at pH 7.0.

[00141] At pH 4.0, which is the anticipated pH of honey, high molecular weight adducts (>480 kDa) of MRJPs are formed (see Figure 9, Lane 7 and Figure 2, Lane 5).

These bands are not evident when the MGO modification is performed at pH 7.0 (see Figure 9, Lanes 9-10). Thus, Cathepsin B is unable to hydrolyze the protein at pH 7.0 but was able to hydrolyze the lower molecular weight species produced at pH 4.0. The higher molecular weight species at pH 4.0 appeared to be more resistant to proteolysis than the lower molecular weight species. However, the species produced by MGO-modification of the MRJP at pH 7.0 appeared also to be resistant to hydrolysis.

[00142] Without wishing to be limited by theory, the inability of Cathepsin B to hydrolyze MRJP is proposed to be due to inhibition of this enzyme. We refer to Figure 9. In contrast, the native hexamer form of MRJP1 and the monomeric form of royalactin were readily degraded by this enzyme (data not shown).

[00143] Notably, the level of protein loaded in the gel varied where 25% less protein was loaded for the hydrolyzed samples. For hydrolysis, 10 μL of a 1 mg/mL solution (10 μg) of Cathepsin B was used. Thus, a very high level of enzyme activity was employed for these experiments. Moreover, the reaction was performed over two days at 37°C. Under these conditions, we expected to see complete or near complete hydrolysis of the MRJP proteins.

[00144] As Cathepsin B has an exopeptidase activity at pH 4.0, it was expected that dipeptides would be generated from the hydrolysis that cannot be seen by native PAGE analysis. The incomplete hydrolysis we observed suggests that some Cathepsin B inhibition occurred, and/or the length of time and concentration of Cathepsin B was higher than the inhibitory activity of the MGO-modified MRJP proteins. This may have allowed partial hydrolysis of the 58 kDa protein (MRJP1) and the band present around 242 kDa.

[00145] By comparison, the larger cross-linked complexes did not appear to be susceptible to Cathepsin B cleavage at pH 4.0. Cross-linking of proteins with MGO occurs between lysine residues or lysine and Arg residues. At pH 7.0, the hydrolysis of the oligomeric complex of MRJP proteins was not hydrolyzed by Cathepsin B. This was attributed to the substrate specificity of Cathepsin B at this pH being limited to cleavage at Arg-Arg | Xaa sequences within the MRJP proteins, i.e., endopeptidase activity.

[00146] Yet, this site within MRJPs does not appear to be available for cleavage. The protein level observed on the gel after substantial hydrolysis with Cathepsin B is essentially the same as that observed without the addition of Cathepsin B. It is possible that Cathepsin B was completely inhibited under these conditions. MGO modification of Arg residues is also likely. This would prevent recognition of the MRJP cleavage site for Cathepsin B at pH 7.0. It is also possible that the native gel electrophoresis failed to separate the protein fragments sufficiently.

Example 6: Proposed mechanism of Cathepsin B inhibition

[00147] Cathepsin B is a thiol protease that participates in intracellular degradation and turnover of proteins. It is an important enzyme in the phagocytosis process and it has also been implicated in tumor invasion and metastasis.

[00148] The primary specificity of Cathepsin B is for cleavage sites Arg-Arg-|-Xaa (where | indicates the point of cleavage). The MRJPs would be predicted to be suitable substrates for this enzyme due to the presence of an Arg-Arg peptide sequence in MRJP1-5. However, the significance of this cleavage site has been questioned by our results. In particular, MGO-modified bovine serine albumin that has the same Arg-Arg peptide sequence shows no anti-inflammatory activity, in contrast to the MGO-modified MRJP1 and MRJP3 proteins (results not shown). The results indicate that Arg-Arg cleavage may only be relevant for plasma membrane bound Cathepsin B at optimal conditions of pH 7 (data not shown).

[00149] Cathepsin B also has some C-terminal dipeptidase activity (Brömme et al., 1987 and Chapman et al., 1994). Without wishing to be bound by theory, it is proposed that the C-terminal peptide regions of MGO-modified MRJP1 and MGO-modified MRJP3 are inhibiting the activity of Cathepsin B. It is proposed that the MGO-modified lysine, which can be involved in thiol lysine cross-links, is in close proximity to the thiol active site of Cathepsin B. In this way, the MGO on the lysine is reacting with the thiol active site of Cathepsin B, and inhibiting its action.

[00150] Notably, MRJP1 has a lysine (see SEQ ID NO: 1 at 427) that is 3 cleavage sites from the C-terminus end. MRJP3 has a lysine residue at the first cleavage site at 542 (see SEQ ID NO: 3). Similarly, MRJP2 has a lysine residue at the fourth cleavage site at 445 (see SEQ ID NO: 2) and MRJP 4 has a lysine residue at the

fifth cleavage site at 455 (see SEQ ID NO: 4). MRJP 5 has a lysine residue at the second cleavage site at 595 (SEQ ID NO: 5). MRJP6 has a lysine residue at the 3rd cleavage site at 431 (see SEQ ID NO: 6). MRJP8 has a lysine at the 4th cleavage site at 409 (see SEQ ID NO: 8) and MRJP 9 has a lysine at the fourth cleavage site at 415 (see SEQ ID NO: 9).

[00151] Bovine serum albumin does not have a lysine at the C-terminus until the fifth cleavage point of Cathepsin B. In this way, the lysine residue in MGO-modified BSA may be too far away to inhibit Cathepsin B. Moreover, the lysine residue at 573 in BSA is involved in an alpha helical structure and not readily available for cleavage by Cathepsin B. It is therefore postulated that the inhibitory activity of MGO-modified MRJP1 and MRJP3 is seen because of the relatively close proximity of the lysine to the C-terminus end of the peptide. It is anticipated also that MGO-modified MRJP2, MRJP4, MRJP5, MRJP6, MRJP8, and MRJP9 will also show Cathepsin B inhibitory activity.

[00152] The presence of a reactive MGO species on the surface of the protein was also detected using DCFDA oxidation to fluorescein (data not shown). The reaction of hydrogen peroxide with MGO led to a reaction in the level of DCFDA oxidized and therefore leads to a reduction in the level of fluorescence detected (data not shown). Isolation of high molecular weight proteins using gel filtration or HiTrap™ desalting led to the identification of a highly reactive species on the surface of the protein. This species as seen by DCFDA analysis correlates to the Cathepsin B inhibitory activity (data not shown).

[00153] It is known that the Arg-MGO adduct formation is favored under lower MGO concentrations and this leads to an irreversible adduct that can be detected with absorbance at 330 nm or fluorescence Ex330nm and Em410nm. The reaction with Cys residues forms a hemiacetyl that is reversible and unstable leading to release of MGO off the surface of the protein. The reaction with lysine can form both reversible and irreversible reactions with MGO.

[00154] The initial adduct formed is slowly rearranged to form a stabilized covalently linked MGO attached to the epsilon amino group of lysine. Further rearrangements can occur, leading to the generation of various chemical functionality including N(ε)-(carboxyethyl)lysine (CEL) acid group, hydroxyl group, aldehyde or

ketone functionality as well as the irreversible reactions with other protein to form cross-links.

[00155] MGO modification can lead to reactive species such as aldehydes bound to the protein, and aldehydes can react with the thiol of the Cathepsin B active site. From this, it is possible that inhibition of Cathepsin B may occur between MGO-modified lysine residues with an aldehyde functionality. Lysines are also of interest in Cathepsin B inhibition, as bound MGO can undergo a cannizzaro rearrangement forming CEL and a hydroxyl group, which has carboxylic acid functionality. Two aldehydes react, where one aldehyde is reduced to a corresponding alcohol, while the second aldehyde is oxidized to carboxylic acid.

[00156] At pH 4.0, Cathepsin B has a C-terminal exodipeptidase activity. It is hypothesized that the acid functionality present in the CEL, carboxylic acid moiety may be recognized by Cathepsin B as a C-terminus. This would place the peptide in an orientation that prevents cleavage. Both structures are potentially formed by MGO upon reaction with lysine. Various reaction pathways are likely to be favored under various conditions.

[00157] The chemical reactivity of MGO under acidic conditions, as present in Manuka honey, appears to enable formation of high molecular weight cross-links. This would appear to favor aldehyde formation, which, in turn, continues to react to form protein cross-links. The cannizzaro rearrangement is favored under basic conditions, which is more likely to occur when MRJP proteins are reacted at neutral pH 7.0. The cannizzaro rearrangement prevents further cross-linking between proteins. The aldehyde functional group that is involved in cross-linking is replaced with an acid group and a hydroxyl group. The hydroxyl provides further diversification of the chemistry on the surface of lysine residues after MGO modification and this can provide changes in inhibitory selectivity and affinity.

[00158] As noted above, we observed that blocking lysine residues with acetic anhydride followed by MGO-modification blocks Cathepsin B inhibition. This highlights the importance of lysines in producing inhibitory activity. The reaction of lysine epsilon amino groups with acetic anhydride produces an amide bond. This removes the features required for recognition by the C-terminal exopeptidase activity of Cathepsin B. This, in turn, abolishes enzyme interaction and inhibition.

Example 7: MGO modification of synthetic peptides and their inhibitory activity

[00159] Thirty-seven synthetic peptides were supplied by Mimotopes Pty Ltd (Melbourne, Australia). The structures were based on peptide sequences in MRJP 1-5. Particular focus was placed on the C-terminus of these proteins.

[00160] MGO modification was performed as follows. The incubation mixture included 180 µl peptide solution (5 mM) and 20 µl MGO solution (5%). Incubation was carried out overnight at room temperature. MGO was removed using C18 SPE cartridges. Eluted peptides were concentrated and solutions made up to original concentration.

[00161] The test for inhibition of Cathepsin B activity was carried out with solutions containing 2 mM of unmodified or modified peptide according to the previously described procedure. The synthetic peptides and results are shown in Figures 11A-C, 12A-C, 13A-C, 14A-C, and 15A-C, and Tables 1-5, below.

Table 1: Synthetic peptides derived from the C-terminus of MRJP1

peptide	SEQ ID NO:	unmodified	MGO-modified
Ac-PFKI-OH	22	152.05 ± 13.20	91.45 ± 0.11
Ac-KISI-OH	21	133.11 ± 4.29	92.82 ± 5.44
Ac-KISIHL-OH	20	149.04 ± 9.65	73.23 ± 17.90
Ac-PFKISIHL-OH	19	144.22 ± 12.20	28.11 ± 0.06

Table 2: Synthetic peptides derived from the C-terminus of MRJP2

peptide	SEQ ID NO:	unmodified	MGO-modified
Ac-KN-OH	28	149.55 ± 4.42	111.84 ± 6.21
Ac-NQKN-OH	27	282.39 ± 19.66	102.36 ± 6.36
Ac-NQKNNN-OH	26	146.89 ± 14.87	87.48 ± 4.31
Ac-NQKNNNQN-OH	25	290.25 ± 12.34	171.38 ± 9.10
Ac-NQKNNNQNDN-OH	24	254.04 ± 22.31	123.18 ± 12.85

Table 3: Synthetic peptides derived from the C-terminus of MRJP3

peptide	SEQ ID NO:	unmodified	MGO-modified

Ac-HSSKLH-OH	30	121.50 ± 2.05	102.36 ± 6.36
Ac-SKLH-OH	31	146.35 ± 2.91	67.57 ± 1.73
Ac-SK-OH	32	137.62 ± 9.65	115.94 ± 1.24
Ac-HSSK-OH	33	164.77 ± 3.87	134.68 ± 1.13
Ac-SSKS-OH	34	118.71 ± 9.09	107.49 ± 4.29

Table 4: Synthetic peptides derived from the C-terminus of MRJP4

peptide	SEQ ID NO:	unmodified	MGO-modified
Ac-KS-OH	41	135.26 ± 7.09	110.69 ± 0.89
Ac-SSKSNNRHNNND-OH	36	164.01 ± 8.57	109.55 ± 3.11
Ac-SSKSNN-OH	39	129.06 ± 6.31	107.043 ± 8.64
Ac-SSKSNNRH-OH	38	125.43 ± 9.56	101.92 ± 6.31
Ac-SSKSNNRHNN-OH	37	131.11 ± 6.491	104.11 ± 7.46

Table 5: Synthetic peptides derived from the C-terminus of MRJP5

peptide	SEQ ID NO:	unmodified	MGO-modified
Ac-KH-OH	45	230.49 ± 16.21	115.95 ± 5.94
Ac-QNKHNN-OH	43	167.90 ± 10.09	51.30 ± 5.31
Ac-KHNN-OH	44	129.26 ± 7.90	111.90 ± 2.07
Ac-QNKH-OH	46	137.67 ± 17.04	97.77 ± 3.70

[00162] From this analysis, the synthetic peptides of interest include: MRJP1: Ac-PFKISIHL-OH (SEQ ID NO: 19); MRJP2: Ac-NQKNNNQNDN-OH (SEQ ID NO: 24); Ac-NQKN-OH (SEQ ID NO: 27); MRJP3: Ac-SKLH-OH (SEQ ID NO: 31); and MRJP5: Ac-QNKHNN-OH (SEQ ID NO: 43); Ac-KH-OH (SEQ ID NO: 45).

[00163] Additional synthetic peptides were prepared based on the amino acid sequences of MRJP1, MRJP3, and MRJP5, in accordance with the procedure noted above.

[00164] Table 6: MRJP1 derived synthetic peptides

sample	SEQ ID NO:	unmodified	MGO-modified
H-FDR-OH	50	174.56 ± 11.96	71.18 ± 2.22

Ac-FDR-OH	53	85.57 ± 3.51	44.78 ± 1.54
H-HNIR-OH	54	151.51± 0.62	103.38 ± 3.65
Ac-HNIR-OH	59	171.58 ± 11.29	106.82 ± 3.02
H-YINR-OH	85	142.33 ± 3.64	79.21 ± 2.12
Ac-YINR-OH	87	93.14 ± 2.42	99.05 ± 7.78
H-FTK-OH	88	147.63 ± 0.70	76.59 ± 2.24
Ac-FTK-OH	91	148.84 ± 16.13	105.35 ± 5.73
H-IFVTMLR-OH	92	80.48 ± 4.25	78.70 ± 3.67
Ac-IFVTMLR-OH	93	88.31 ± 12.62	92.63 ± 2.00
H-MQK-OH	94	127.00 ± 0.93	99.48 ± 2.95
Ac-MQK-OH	95	121.24 ± 3.57	69.15 ± 4.87
H-CDR-OH	96	165.10 ± 0.38	96.01 ± 6.45
Ac-CDR-OH	97	120.52 ± 8.17	78.51 ± 2.51
H-MTR-OH	98	143.48 ± 2.33	104.39 ± 2.68
Ac-MTR-OH	99	126.30 ± 8.12	118.90 ± 5.62
TNBS-FAK-pNA	100	99.27 ± 3.8	122.48 ± 7.65

TNBS = N-terminus blocked with trinitrobenzene sulphonic acid. pNA = paranitroanalide.

Table 7: MRJP3 derived synthetic peptides

sample	SEQ ID NO:	unmodified	MGO-modified
H-QNDNK-OH	101	104.37 ± 4.99	68.42 ± 0.22
Ac-QNDNK-OH	102	167.26 ± 7.64	122.35 ± 3.05
H-QNDNR-OH	103	162.38 ± 16.36	116.16 ± 3.66
Ac-QNDNR-OH	104	161.75 ± 9.25	132.44 ± 4.77
H-QNGNK-OH	105	170.73 ± 5.14	118.80 ± 11.41
Ac-QNGNK-OH	106	187.34 ± 9.14	89.21 ± 10.96
H-QNGNR-OH	107	74.41 ± 4.98	84.99 ± 4.08
Ac-QNGNR-OH	108	101.30 ± 6.00	88.67 ± 4.38

Table 8: MRJP5 derived synthetic peptides

sample	SEQ ID NO:	unmodified	MGO-modified
H-TNR-OH	109	102.61 ± 5.34	91.86 ± 3.50
Ac-TNR-OH	110	96.48 ± 2.03	91.72 ± 0.84

H-MDK-OH	111	104.25 ± 2.73	70.62 ± 12.34
Ac-MDK-OH	112	109.67 ± 3.13	99.38 ± 1.63
H-MDR-OH	113	101.73 ± 2.37	74.46 ± 0.97
Ac-MDR-OH	114	107.34 ± 1.46	88.56 ± 2.14
H-TDK-OH	115	103.18 ± 4.50	87.53 ± 3.26
Ac-TDK-OH	116	100.51 ± 10.69	92.75 ± 2.41
H-IDR-OH	117	98.02 ± 2.54	85.60 ± 5.32
Ac-IDR-OH	118	93.46 ± 12.24	81.75 ± 3.26
H-VNR-OH	119	97.75 ± 10.62	82.63 ± 7.88
Ac-VNR-OH	120	99.83 ± 3.40	68.49 ± 8.73
H-MHR-OH	121	96.77 ± 9.26	80.66 ± 6.19
Ac-MHR-OH	122	101.26 ± 4.59	90.13 ± 17.28
H-MNR-OH	123	112.08 ± 6.03	78.56 ± 5.37
Ac-MNR-OH	124	100.83 ± 2.26	86.94 ± 3.01
H-LQK-OH	125	102.47 ± 4.27	95.26 ± 5.54
Ac-LQK-OH	126	102.05 ± 10.32	90.15 ± 1.52

[00165] Further synthetic peptides derived from MRJP1-5 were tested as described herein. The synthetic peptides and results are shown in Figures 16A and 16B, and Table 9, below.

Table 9: Additional synthetic peptides tested for inhibitory activity

peptide	derived from MRJP	SEQ ID NO:	unmodified	MGO-modified
Ac-LVK-OH	MRJP3 (see Leu170)	48	143.92 ± 3.15	80.84 ± 3.07
Ac-LVK-NH_2	MRJP3 (see Leu170)	49	92.71 ± 7.78	80.03 ± 0.10
Ac-LIR-OH	MRJP2, 4 (see Leu408)	51	128.28 ± 2.50	0.07 ± 3.96
Ac-LIR-NH_2	MRJP2, 4 (see Leu408)	52	65.39 ± 2.75	72.56 ± 9.33
Ac-LLK-OH	MRJP1, 2, 4 (see Leu146; Leu104; Leu169)	56	141.56 ± 13.90	147.60 ± 5.98
Ac-LLK-NH_2	MRJP1, 2, 4 (see Leu146; Leu104; Leu169)	57	159.34 ± 2.34	136.90 ± 14.42

Ac-KI-OH	MRJP1-5^{+}	58	108.98 ± 9.03	92.08 ± 5.79

+ = MRJP1: Lys75, Lys358, Lys427; MRJP2: Lys121, Lys127, Lys356, Lys385; MRJP3: Lys132, Lys361, Lys390; MRJP4: Lys131, Lys356; MRJP5: Lys100, Lys130, Lys310, Lys518.

[00166] Another synthetic peptide is FAK (Phe-Ala-Lys; e.g., Ac-FAK-OH; SEQ ID NO: 60) which includes a sequence found in MRJP1, MRJP3, and MRJP4.

[00167] A table to summarize the amino acid sequences from this Example is provided as follows.

Table 10: Summary of amino acid sequences assessed for inhibitory activity

sequence	SEQ ID NO:	sequence	SEQ ID NO:	sequence	SEQ ID NO:
PFKI	61	HSSKLH	70	KH	80
KISI	62	SKLH	71	QNKHNN	81
KISIHL	63	SK	72	KHNN	82
PFKISIHL	64	HSSK	73	QNKH	83
KN	65	SSKS	74	LVK	84
NQKN	66	KS	75	LIR	86
NQKNNN	67	SSKSNNRHNNND	76	LLK	89
NQKNNNQN	68	SSKSNN	77	KI	90
NQKNNNQNDN	69	SSKSNNRH	78		
		SSKSNNRHNN	79		

sequence	SEQ ID NO:	sequence	SEQ ID NO:	sequence	SEQ ID NO:
FDR	127	MTR	134	TNR	139
HNIR	128	FAK	135	MDK	140
YINR	129	QNDNR	136	MDR	142
FTK	130	QNGNK	137	TDK	143
IFVTMLR	131	QNGNR	138	IDR	144
MQK	132			VNR	145
CDR	133			MHR	47
				MNR	141
				LQK	55

[00168] The results show that both C-terminal and internal peptides can provide inhibition of Cathepsin B after modification of lysine with MGO. The peptide analogue LIR (SEQ ID NO: 51) has particularly potent activity.

[00169] The presence of endogenous protease activity in honey has been determined previously ((Larocca et al., 2012)), and we demonstrate that this protease leads to degradation of MRJP proteins in the presence of urea. The release of peptides from MRJP proteins is therefore expected during prolonged incubation of Manuka honey leading to release of MGO-modified peptides that have enhanced Cathepsin B inhibition.

[00170] Certain peptides show stimulatory activity in the absence of MGO treatment. This activation is in most cases lost after treatment with MGO. In some cases, longer peptides are more active. For specific peptides, the lysine is positioned at least 3 amino acid away from the C-terminal amino acid, and no more than 6 amino acids away from the C-terminal amino acid. For inhibition, a free C-terminus is typically present. This allows a favorable interaction with Cathepsin B and potentially increased inhibition.

[00171] For the assayed peptides, an acid functional end is more likely to produce increased activity as compared to an amidated end. This is consistent with internal protease digestion having the specificity similar to that of pancreatic trypsin, i.e., cleavage C-terminal to arginine and lysine residues, as long as the following amino acid is not proline. The preference for a C-terminal carboxylate group is understandable in context of the structure of Cathepsin B and its exopeptidase activity that acts on a C-terminal end.

[00172] Notably, the occlusive loop of Cathepsin B contains two histidine residues that are believed to coordinate to the C-terminus of the peptide. See, e.g., Musil, D., Zucic, D., Turk, D., Engh, R.A., Mayr, I., Huber, R., Popovic, T., Turk, V., Towatari, T., Katunuma, N., Bode, W. (1991) The refined 2.15 A X-ray crystal structure of human liver cathepsin B: the structural basis for its specificity. EMBO J. 10: 2321-2330. This positions the peptide bond between amino acid 2 and 3 up from the C-terminus of the peptide. In addition, the orientation of peptides with respect to

Cathepsin B may be inverted 180° if the primary amine or epsilon amino group of lysine has reacted with MGO. As noted above, this can undergo a cannizzaro rearrangement to produce CEL. This may also occur on the side chain epsilon amino group of lysine.

[00173] Regarding the FAK peptide analogue (SEQ ID NO: 100), this showed limited inhibitory activity with the C-terminus containing paranitroanilide (pNA). It was postulated that this could block recognition of the peptide by Cathepsin B. To test this, the pNA group was removed under basic conditions in the presence of NaOH. The peptide was reassessed for activity before and after MGO-modification (data not shown). Inhibitory activity was still not observed, suggesting that the position of lysine within the peptide sequence can affect activity. From this, it is concluded that it may be beneficial to position the lysine such that it sits closer to the active site of Cathepsin B.

[00174] The present invention and its embodiments have been described in detail. However, the scope of the present invention is not intended to be limited to the particular embodiments of any process, manufacture, composition of matter, compounds, means, methods, and/or steps described in the specification. Various modifications, substitutions, and variations can be made to the disclosed material without departing from the scope and/or essential characteristics of the present invention.

[00175] Accordingly, one of ordinary skill in the art will readily appreciate from the disclosure that later modifications, substitutions, and/or variations performing substantially the same function or achieving substantially the same result as embodiments described herein may be utilized according to such related embodiments of the present invention. Thus, the invention is intended to encompass, within its scope, the modifications, substitutions, and variations to processes, manufactures, compositions of matter, compounds, means, methods, and/or steps disclosed herein.

References

[00176] K. Kohno, I. Okamoto, O. Sano, N. Arai, K. Iwaki, M. Ikeda, and M. Kurimoto (2004) Royal jelly inhibits the production of proinflammatory cytokines by activated macrophages, Biosci. Biotechnol. Biochem., 68(1):138-145.

[00177] Kimura Y, Miyagi C, Kimura M, Nitoda T, Kawai N, Sugimoto H. (2000Structural features of N-glycans linked to royal jelly glycoproteins: structures of high-mannose type, hybrid type, and biantennary type glycans. Biosci. Biotechnol. Biochem. 64(10):2109-20.

[00178] J. Simuth, K. Bilikova, E. Kovacova, Z. Kuzmova, and W. Schroder (2004) Immunochemical approach to detection of adulteration in honey: physiologically active Royal Jelly protein stimulating TNF-alpha release is a regular component of honey, Journal of Agriculture and Food Chemistry. 52(8):2154-8.

[00179] B. Lerrer, K. Zinger-Yosovich, B. Avrahami, and N. Gilboa-Garber (2007) Honey and royal jelly, like human milk, abrogate lectin-dependent infection-preceding *Pseudomonas aeruginosa* adhesion. ISME Journal. 1:149-155.

[00180] E. Mavric, S. Wittmann, G. Barth, and T. Henle (2008) Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (*Leptospermum scoparium*) honeys from New Zealand. Mol. Nutr. Food Res. 52.

[00181] Auwerx J. (1991). The human leukemia cell line, THP-1: a multifaceted model for the study of monocyte-macrophage differentiation. Experientia. 47:22-31.

[00182] Wan, C. P., C. S. Park, et al. (1993). A rapid and simple microfluorometric phagocytosis assay. Journal of Immunological Methods 162(1):1-7.

[00183] Allen, K. L., P. C. Molan, et al. (1991). A survey of the antibacterial activity of some New Zealand honeys. Journal of Pharmacy and Pharmacology. 43(12):817-22.

[00184] White, J. (1975). Honey: a comprehensive survey. London, Heinemann.

[00185] Schmitt, A., J. Schmitt, et al. (2005). Characterization of advanced glycation end products for biochemical studies: side chain modifications and fluorescence characteristics. Analytical Biochemistry. 338:201-215.

[00186] Jonathan W. C. Brock, William E. Cotham, Suzanne R. Thorpe, John W Baynes1 and Jennifer M. Ames. (2007). Detection and identification of arginine

modifications on methylglyoxal-modified ribonuclease by mass spectrometric analysis. J. Mass Spectrom. 42:89-100.

[00187] Kim J, Kim NH, Sohn E, Kim CS, Kim JS. (2010). Methylglyoxal induces cellular damage by increasing argpyrimidine accumulation and oxidative DNA damage in human lens epithelial cells. Biochem Biophys Res Commun. 391(1):346-51. Epub 2009 Nov 12.

[00188] Brömme D, Steinert A, Fittkau S, Kirschke H. (1987). Action of rat liver cathepsin B on bradykinin and on the oxidized insulin A-chain. FEBS Lett. Jul 27;219(2):441-4.

[00189] Chapman HA Jr, Munger JS, Shi GP. (1994). The role of thiol proteases in tissue injury and remodeling. Am J Respir Crit Care Med. 1994 Dec;150(6 Pt 2):S155-9.

[00190] June O'Neil, George Hoppe, Lawrence M. Sayre, and Henry F. Hoff (1997). Inactivation of cathepsin B by oxidised LDL involves complex formation induced by binding of putative reactive sites exposed at low pH to thiols on the enzyme. Free Radical Biology & Medicine. 23(2):215–225.

[00191] Ahmed NK, Martin LA, Watts LM, Palmer J, Thornburg L, Prior J, Esser RE. (1992) Peptidyl fluoromethyl ketones as inhibitors of cathepsin B. Implication for treatment of rheumatoid arthritis. Biochem Pharmacol. Sep 25;44(6):1201-7.

[00192] Rossano R, Larocca M, Polito T, Perna AM, Padula MC, et al. (2012) What Are the Proteolytic Enzymes of Honey and What They Do Tell Us? A Fingerprint Analysis by 2-D Zymography of Unifloral Honeys. PLoS ONE 7(11): e49164. doi:10.1371/journal.pone1149164

WHAT WE CLAIM IS:

1. A method of reducing inflammation in a cellular tissue, comprising the step of contacting the cellular tissue with a functional fragment of a major royal jelly protein (MRJP); wherein the functional fragment has been isolated, enriched, synthesized, or recombinantly produced; wherein the functional fragment comprises 2 to 20 amino acids of the last 20 amino acids at the C-terminus of the protein; and wherein a lysine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO).

2. The method of claim 1, wherein the functional fragment comprises 2 to 10 amino acids of the last 10 amino acids at the C-terminus of the protein.

3. The method of claim 1 or claim 2, wherein the major royal jelly protein (MRJP) is selected from the group consisting of MRJP1 (SEQ ID NO: 1), MRJP2 (SEQ ID NO: 2), MRJP3 (SEQ ID NO: 3), MRJP4 (SEQ ID NO: 4), MRJP5 (SEQ ID NO: 5), MRJP6 (SEQ ID NO: 6), MRJP7 (SEQ ID NO: 7), MRJP8 (SEQ ID NO: 8), and MRJP9 (SEQ ID NO: 9).

4. The method of any one of claims 1 to 3, wherein the functional fragment comprises an amino acid sequence selected from the group consisting of:

i. KISIHL (SEQ ID NO: 10);

ii. KNNNQNDN (SEQ ID NO: 11);

iii. KLH (SEQ ID NO: 12);

iv. KSNNRHNNND (SEQ ID NO: 13);

v. KHNN (SEQ ID NO: 14);

vi. KNQAHLD (SEQ ID NO: 15);

vii. KNTRCISP (SEQ ID NO: 16); and

viii. KTNFFSIFL (SEQ ID NO: 17).

5. The method of any one of claims 1 to 3, wherein the functional fragment comprises an amino acid sequence selected from the group consisting of:

i. PFKISIHL (SEQ ID NO: 64);

ii. NQKNNNQNDN (SEQ ID NO: 69); NQKN (SEQ ID NO: 66);

iii. SKLH (SEQ ID NO: 71); and

iv. QNKHNN (SEQ ID NO: 81); KH (SEQ ID NO: 80).

6. The method of any one of claims 1 to 3, or 5, wherein the functional fragment is a peptide analogue selected from the group consisting of:

i. Ac-PFKISIHL-OH (SEQ ID NO: 19);

ii. Ac-NQKNNNQNDN-OH (SEQ ID NO: 24); Ac-NQKN-OH (SEQ ID NO: 27);

iii. Ac-SKLH-OH (SEQ ID NO: 31);

iv. Ac-QNKHNN-OH (SEQ ID NO: 43); and Ac-KH-OH (SEQ ID NO: 45).

7. A method of reducing inflammation in a cellular tissue, comprising the step of contacting the cellular tissue with a functional fragment of a major royal jelly protein (MRJP); wherein the functional fragment has been isolated, enriched, synthesized, or recombinantly produced; wherein the functional fragment comprises an amino acid sequence selected from the group consisting of: LVK (SEQ ID NO: 84), LIR (SEQ ID NO: 86), FDR (SEQ ID NO: 127), HNIR (SEQ ID NO: 128), FTK (SEQ ID NO: 130), and QNGNK (SEQ ID NO: 137); and wherein a lysine or arginine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO).

8. The method of claim 7, wherein the functional fragment is a peptide analogue selected from the group consisting of: Ac-LVK-OH (SEQ ID NO: 48), Ac-LIR-OH (SEQ ID NO: 51), Ac-FDR-OH (SEQ ID NO: 53), Ac-HNIR-OH (SEQ ID NO: 59), H-FTK-OH (SEQ ID NO: 88), and Ac-QNGNK-OH (SEQ ID NO: 106).

9. The method of any one of claims 1 to 8, wherein the method reduces the rate of phagocytosis by immune system cells.

10. The method of any one of claims 1 to 8, wherein the method inhibits receptors for phagocytosis on immune system cells.

11. The method of any one of claims 1 to 8, wherein the method reduces respiratory burst and release of reactive oxygen species from inflammatory cells.

12. The method of any one of claims 1 to 11, wherein the inflammation is associated with one or more of the group consisting of: an inflammatory disorder, a cardiovascular disorder, a neurological disorder, a pulmonary disorder, a proliferative disorder, an infectious disease or associated syndrome, an allergic, immunological or autoimmune disorder, and inflammation associated with a wound.

13. The method of claim 12, wherein the inflammation is associated with one or more of the group consisting of: rheumatoid arthritis, polyarthritis, Alzheimer's disease, progressive multifocal leukoencephalopathy (PML), multiple sclerosis, asthma, bronchitis, adult respiratory distress syndrome, Wegeneres granulomatosis, emphysema, chronic obstructive pulmonary disease (COPD), melanoma, genital herpes, Epstein–Barr virus (EBV) infection, encephalitis, EBV associated encephalitis, choreoretinitis, cytomegalovirus (CMV) associated choreoretinitis, bronchopneumonia, gastroenteritis, uveitis, psoriasis, and dermatitis.

14. The method of claim 12, wherein the wound is one or more of the group consisting of: a diabetic ulcer, fungating wound, puncture wound, cut, bite, and surgical wound.

15. A method of treating rheumatoid arthritis in a patient, comprising the step of administering to the patient a composition comprising a functional fragment of a major royal jelly protein (MRJP); wherein the functional fragment has been isolated, enriched, synthesized, or recombinantly produced; wherein the functional fragment comprises 2 to 20 amino acids of the last 20 amino acids at the C-terminus of the protein; and wherein a lysine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO).

16. The method of claim 15, wherein the functional fragment comprises 2 to 10 amino acids of the last 10 amino acids at the C-terminus of the protein.

17. The method of claim 15 or claim 16, wherein the major royal jelly protein (MRJP) is selected from the group consisting of MRJP1 (SEQ ID NO: 1), MRJP2 (SEQ ID NO: 2), MRJP3 (SEQ ID NO: 3), MRJP4 (SEQ ID NO: 4), MRJP5 (SEQ ID NO: 5), MRJP6 (SEQ ID NO: 6), MRJP7 (SEQ ID NO: 7), MRJP8 (SEQ ID NO: 8), and MRJP9 (SEQ ID NO: 9).

18. The method of any one of claims 15 to 17, wherein the functional fragment comprises an amino acid sequence selected from the group consisting of:

i. KISIHL (SEQ ID NO: 10);

ii. KNNNQNDN (SEQ ID NO: 11);

iii. KLH (SEQ ID NO: 12);

iv. KSNNRHNNND (SEQ ID NO: 13);

v. KHNN (SEQ ID NO: 14);

vi. KNQAHLD (SEQ ID NO: 15);

vii. KNTRCISP (SEQ ID NO: 16); and

viii. KTNFFSIFL (SEQ ID NO: 17).

19. The method of any one of claims 15 to 17, wherein the functional fragment comprises an amino acid sequence selected from the group consisting of:

i. PFKISIHL (SEQ ID NO: 64);

ii. NQKNNNQNDN (SEQ ID NO: 69); NQKN (SEQ ID NO: 66);

iii. SKLH (SEQ ID NO: 71);

iv. QNKHNN (SEQ ID NO: 81); and KH (SEQ ID NO: 80).

20. The method of any one of claims 15 to 17, or 19, wherein the functional fragment is a peptide analogue selected from the group consisting of:

i. Ac-PFKISIHL-OH (SEQ ID NO: 19);

ii. Ac- NQKNNNQNDN-OH (SEQ ID NO: 24); Ac-NQKN-OH (SEQ ID NO: 27);

iii. Ac-SKLH-OH (SEQ ID NO: 31);

iv. Ac-QNKHNN-OH (SEQ ID NO: 43); and Ac-KH-OH (SEQ ID NO: 45).

21. A method of treating rheumatoid arthritis in a patient, comprising the step of administering to the patient a composition comprising a functional fragment of a major royal jelly protein (MRJP); wherein the functional fragment has been isolated, enriched, synthesized, or recombinantly produced; wherein the functional fragment comprises an amino acid sequence selected from the group consisting of: LVK (SEQ ID NO: 84),

LIR (SEQ ID NO: 86), FDR (SEQ ID NO: 127), HNIR (SEQ ID NO: 128), FTK (SEQ ID NO: 130), and QNGNK (SEQ ID NO: 137); and wherein a lysine or arginine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO).

22. The method of claim 21, wherein the functional fragment is a peptide analogue selected from the group consisting of: Ac-LVK-OH (SEQ ID NO: 48), Ac-LIR-OH (SEQ ID NO: 51), Ac-FDR-OH (SEQ ID NO: 53), Ac-HNIR-OH (SEQ ID NO: 59), H-FTK-OH (SEQ ID NO: 88), and Ac-QNGNK-OH (SEQ ID NO: 106).

23. The method of any one of claims 15 to 22, wherein administration is one or more of oral, topical, and parenteral administration.

24. The method of any one of claims 15 to 23, wherein administration is intravenous, intravitreal, or intramuscular administration.

25. An isolated functional fragment of a major royal jelly protein (MRJP), wherein the functional fragment comprises an amino acid sequence selected from the group consisting of:

i. KISIHL (SEQ ID NO: 10);

ii. KNNNQNDN (SEQ ID NO: 11);

iii. KLH (SEQ ID NO: 12);

iv. KSNNRHNNND (SEQ ID NO: 13);

v. KHNN (SEQ ID NO: 14);

vi. KNQAHLD (SEQ ID NO: 15);

vii. KNTRCISP (SEQ ID NO: 16);

viii. KTNFFSIFL (SEQ ID NO: 17); and

wherein a lysine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO).

26. An isolated functional fragment of a major royal jelly protein (MRJP), wherein the functional fragment comprises an amino acid sequence selected from the group consisting of:

i. PFKISIHL (SEQ ID NO: 64);

ii. NQKNNNQNDN (SEQ ID NO: 69); NQKN (SEQ ID NO: 66);

iii. SKLH (SEQ ID NO: 71);

iv. QNKHNN (SEQ ID NO: 81); KH (SEQ ID NO: 80); and

wherein a lysine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO).

27. The isolated functional fragment of claim 26, wherein the functional fragment is a peptide analogue selected from the group consisting of:

i. Ac-PFKISIHL-OH (SEQ ID NO: 19);

ii. Ac-NQKNNNQNDN-OH (SEQ ID NO: 24); Ac-NQKN-OH (SEQ ID NO: 27);

iii. Ac-SKLH-OH (SEQ ID NO: 31);

iv. Ac-QNKHNN-OH (SEQ ID NO: 43); and Ac-KH-OH (SEQ ID NO: 45).

28. An isolated functional fragment of a major royal jelly protein (MRJP); wherein the functional fragment comprises an amino acid sequence selected from the group consisting of: LVK (SEQ ID NO: 84), LIR (SEQ ID NO: 86), FDR (SEQ ID NO: 127), HNIR (SEQ ID NO: 128), FTK (SEQ ID NO: 130), and QNGNK (SEQ ID NO:

137); and wherein a lysine or arginine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO).

29. The isolated functional fragment of claim 28, wherein the functional fragment is a peptide analogue selected from the group consisting of: Ac-LVK-OH (SEQ ID NO: 48), Ac-LIR-OH (SEQ ID NO: 51), Ac-FDR-OH (SEQ ID NO: 53), Ac-HNIR-OH (SEQ ID NO: 59), H-FTK-OH (SEQ ID NO: 88), and Ac-QNGNK-OH (SEQ ID NO: 106).

30. The isolated functional fragment of any one of claims 25 to 29, which is isolated from *Leptospermum scoparium* derived manuka honey.

31. The isolated functional fragment of any one of claims 25 to 29, which is a synthetic or recombinant molecule.

32. A composition comprising the isolated functional fragment of any one of claims 25 to 31, or an analogue thereof.

33. A wound dressing comprising a functional fragment of a major royal jelly protein (MRJP); wherein the functional fragment has been isolated, enriched, synthesized, or recombinantly produced; wherein the functional fragment comprises an amino acid sequence selected from the group consisting of:

i. KISIHL (SEQ ID NO: 10);

ii. KNNNQNDN (SEQ ID NO: 11);

iii. KLH (SEQ ID NO: 12);

iv. KSNNRHNNND (SEQ ID NO: 13);

v. KHNN (SEQ ID NO: 14);

vi. KNQAHLD (SEQ ID NO: 15);

vii. KNTRCISP (SEQ ID NO: 16);

viii. KTNFFSIFL (SEQ ID NO: 17); and

wherein a lysine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO).

34. A wound dressing comprising a functional fragment of a major royal jelly protein (MRJP); wherein the functional fragment has been isolated, enriched, synthesized, or recombinantly produced; wherein the functional fragment comprises an amino acid sequence selected from the group consisting of:

i. PFKISIHL (SEQ ID NO: 64);

ii. NQKNNNQNDN (SEQ ID NO: 69); NQKN (SEQ ID NO: 66);

iii. SKLH (SEQ ID NO: 71);

iv. QNKHNN (SEQ ID NO: 81); KH (SEQ ID NO: 80); and

wherein a lysine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO).

35. The wound dressing of claim 30, wherein the functional fragment is a peptide analogue selected from the group consisting of:

i. Ac-PFKISIHL-OH (SEQ ID NO: 19);

ii. Ac-NQKNNNQNDN-OH (SEQ ID NO: 24); Ac-NQKN-OH (SEQ ID NO: 27);

iii. Ac-SKLH-OH (SEQ ID NO: 31);

iv. Ac-QNKHNN-OH (SEQ ID NO: 43); and Ac-KH-OH (SEQ ID NO: 45);

36. A wound dressing comprising a functional fragment of a major royal jelly protein (MRJP); wherein the functional fragment has been isolated, enriched, synthesized, or recombinantly produced; wherein the functional fragment comprises an amino acid sequence selected from the group consisting of: LVK (SEQ ID NO: 84), LIR (SEQ ID NO: 86), FDR (SEQ ID NO: 127), HNIR (SEQ ID NO: 128), FTK (SEQ ID NO: 130), and QNGNK (SEQ ID NO: 137); and wherein a lysine or arginine acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO).

37. The wound dressing of claim 36, wherein the functional fragment is a peptide analogue selected from the group consisting of: Ac-LVK-OH (SEQ ID NO: 48), Ac-LIR-OH (SEQ ID NO: 51), Ac-FDR-OH (SEQ ID NO: 53), Ac-HNIR-OH (SEQ ID NO: 59), H-FTK-OH (SEQ ID NO: 88), and Ac-QNGNK-OH (SEQ ID NO: 106).

38. A method of producing an anti-inflammatory molecule that is an apalbumin protein or functional fragment thereof by modifying royal jelly, the method including the step of reacting royal jelly with at least 0.1% MGO at between 18 and 37 degrees Celsius.

39. The method of claim 38 including the step of reacting the royal jelly with at least 0.5% MGO at between 18 and 37 degrees Celsius.

40. The method of claim 38 or claim 39 including the step of reacting royal jelly with at least 1.0% MGO at between 18 and 37 degrees Celsius.

41. A method of identifying (i) the anti-inflammatory capacity or (ii) MGO-modified major royal jelly protein concentration of a sample of honey, comprising the step of: assaying the Cathepsin B inhibition levels of the honey sample.

42. The method as claimed in claim 41, wherein the honey is derived from *Leptospermum*, including manuka honey or jelly bush honey.

43. The method of claim 41 or claim 42, wherein the method is used to enable a bee keeper to determine the right time to harvest honey from a hive in order to obtain a honey sample containing a desired anti-inflammatory capacity or MGO-modified major royal jelly protein content.

44. The method of claim 41 or claim 42, wherein the method is used to enable a honey producer to determine a desired length of time to store honey, in order to obtain a honey sample with a desired anti-inflammatory capacity and MGO-modified major royal jelly protein content.

45. A method of inhibiting Cathepsin B activity in a cellular tissue, comprising the step of contacting the cellular tissue with a functional fragment of a major royal jelly protein (MRJP); wherein the functional fragment has been isolated, enriched, synthesized, or recombinantly produced; wherein the functional fragment comprises 2 to 20 amino acids of the last 20 amino acids at the C-terminus of the protein; and wherein a lysine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO).

46. The method of claim 45, wherein the functional fragment comprises 2 to 10 amino acids of the last 10 amino acids at the C-terminus of the protein.

47. The method of claim 45 or claim 46, wherein the major royal jelly protein (MRJP) is selected from the group consisting of MRJP1 (SEQ ID NO: 1), MRJP2 (SEQ ID NO: 2), MRJP3 (SEQ ID NO: 3), MRJP4 (SEQ ID NO: 4), MRJP5 (SEQ ID NO:

5), MRJP6 (SEQ ID NO: 6), MRJP7 (SEQ ID NO: 7), MRJP8 (SEQ ID NO: 8), and MRJP9 (SEQ ID NO: 9).

48. The method of any one of claims 45 to 47, wherein the functional fragment comprises an amino acid sequence selected from the group consisting of:

i. KISIHL (SEQ ID NO: 10);

ii. KNNNQNDN (SEQ ID NO: 11);

iii. KLH (SEQ ID NO: 12);

iv. KSNNRHNNND (SEQ ID NO: 13);

v. KHNN (SEQ ID NO: 14);

vi. KNQAHLD (SEQ ID NO: 15);

vii. KNTRCISP (SEQ ID NO: 16); and

viii. KTNFFSIFL (SEQ ID NO: 17).

49. The method of any one of claims 45 to 48, wherein the functional fragment comprises an amino acid sequence selected from the group consisting of:

i. PFKISIHL (SEQ ID NO: 64);

ii. NQKNNNQNDN (SEQ ID NO: 69); NQKN (SEQ ID NO: 66);

iii. SKLH (SEQ ID NO: 71);

iv. QNKHNN (SEQ ID NO: 81); and KH (SEQ ID NO: 80).

50. The method of any one of claims 45 to 47, or 49, wherein the functional fragment is a peptide analogue selected from the group consisting of:

i. Ac-PFKISIHL-OH (SEQ ID NO: 19);

ii. Ac-NQKNNNQNDN-OH (SEQ ID NO: 24); Ac-NQKN-OH (SEQ ID NO: 27);

iii. Ac-SKLH-OH (SEQ ID NO: 31);

iv. Ac-QNKHNN-OH (SEQ ID NO: 43); and Ac-KH-OH (SEQ ID NO: 45).

51. A method of inhibiting Cathepsin B activity in a cellular tissue, comprising the step of contacting the cellular tissue with a functional fragment of a major royal jelly protein (MRJP); wherein the functional fragment has been isolated, enriched, synthesized, or recombinantly produced; wherein the functional fragment comprises an amino acid sequence selected from the group consisting of: LVK (SEQ ID NO: 84), LIR (SEQ ID NO: 86), FDR (SEQ ID NO: 127), HNIR (SEQ ID NO: 128), FTK (SEQ ID NO: 130), and QNGNK (SEQ ID NO: 137); and wherein a lysine or arginine amino acid residue of the functional fragment has been chemically modified by methylglyoxal (MGO).

52. The method of claim 51, wherein the functional fragment is a peptide analogue selected from the group consisting of: Ac-LVK-OH (SEQ ID NO: 48), Ac-LIR-OH (SEQ ID NO: 51), Ac-FDR-OH (SEQ ID NO: 53), Ac-HNIR-OH (SEQ ID NO: 59), H-FTK-OH (SEQ ID NO: 88), and Ac-QNGNK-OH (SEQ ID NO: 106).

53. A method of enriching the anti-inflammatory molecules in a *Leptospermum* genus derived MGO containing honey comprising the step of adding major royal jelly protein to the honey.

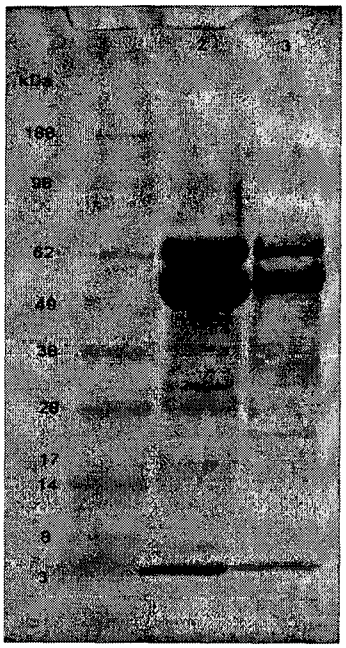

FIGURE 1A

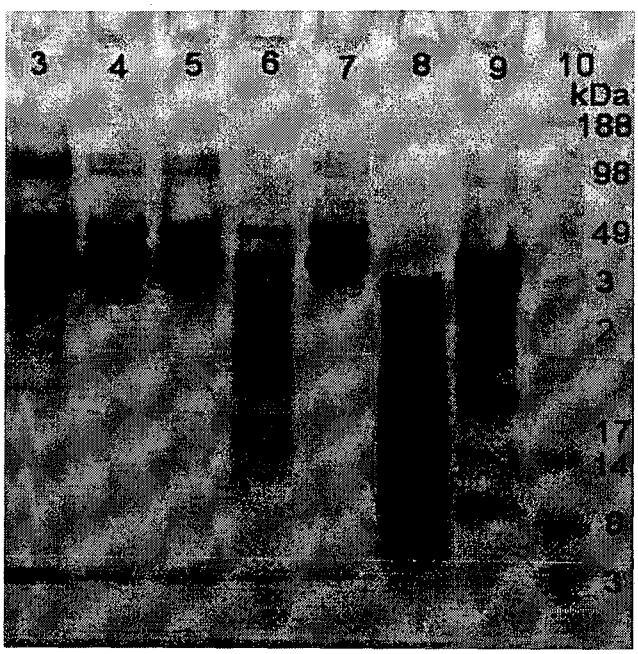

FIGURE 1B

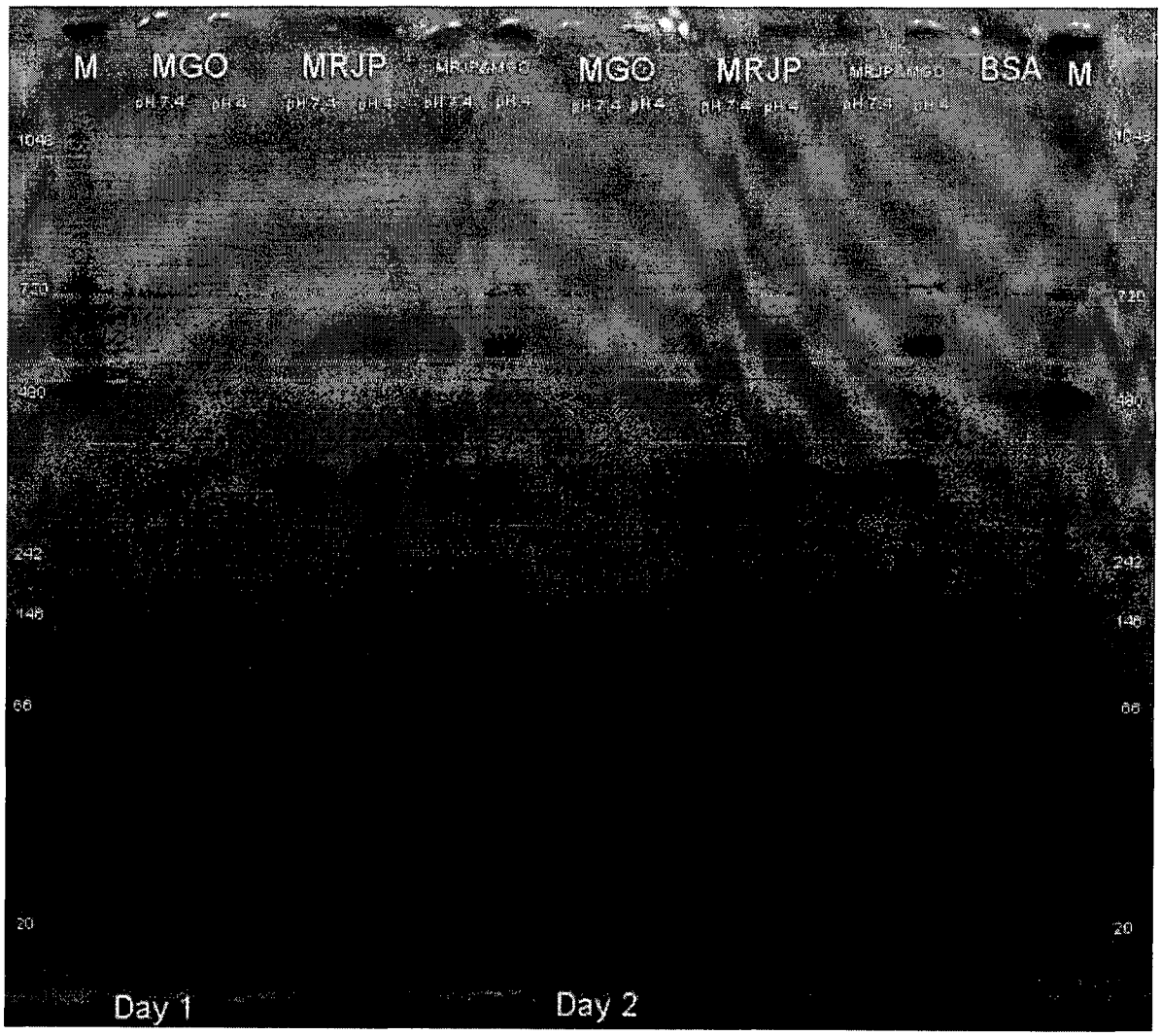

FIGURE 2

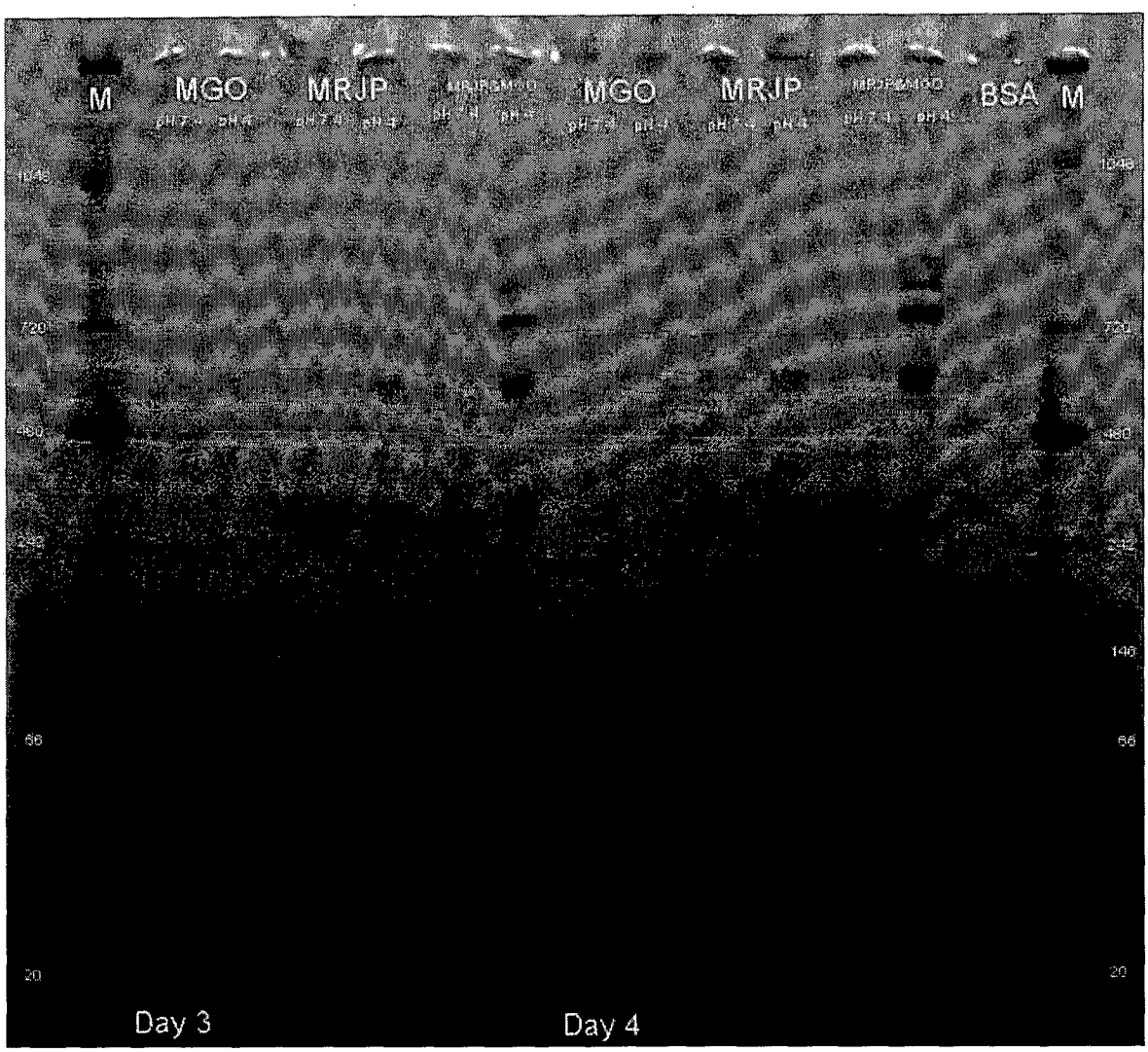

FIGURE 3

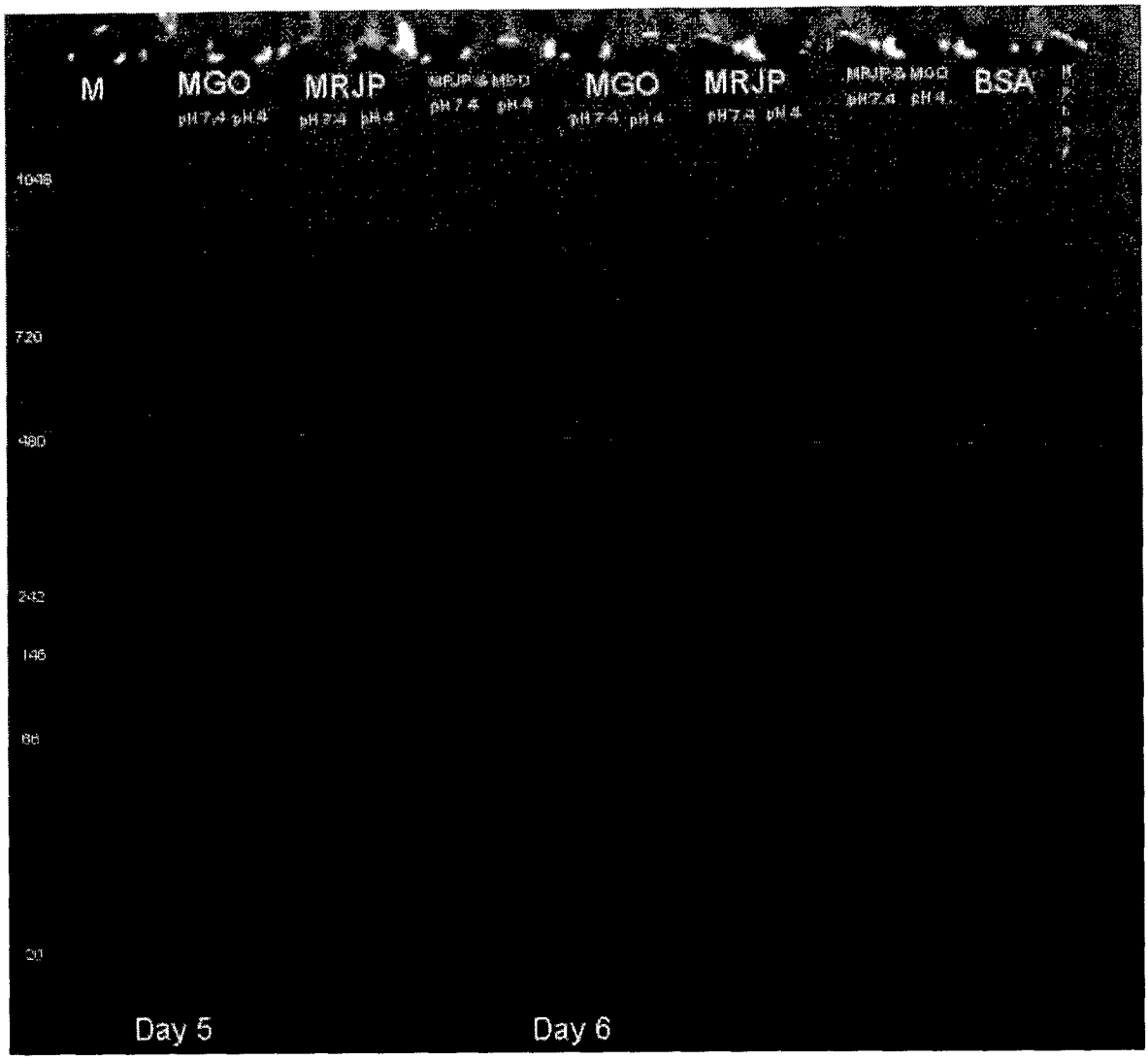
M
MGO
pH7.4 pH4
MRJP
pH7.4 pH4
MRJP & MGO
pH7.4 pH4
MGO
pH7.4 pH4
MRJP
pH7.4 pH4
MRJP & MGO
pH7.4 pH4
BSA
1048
720
480
242
146
66
20
Day 5
Day 6

FIGURE 4

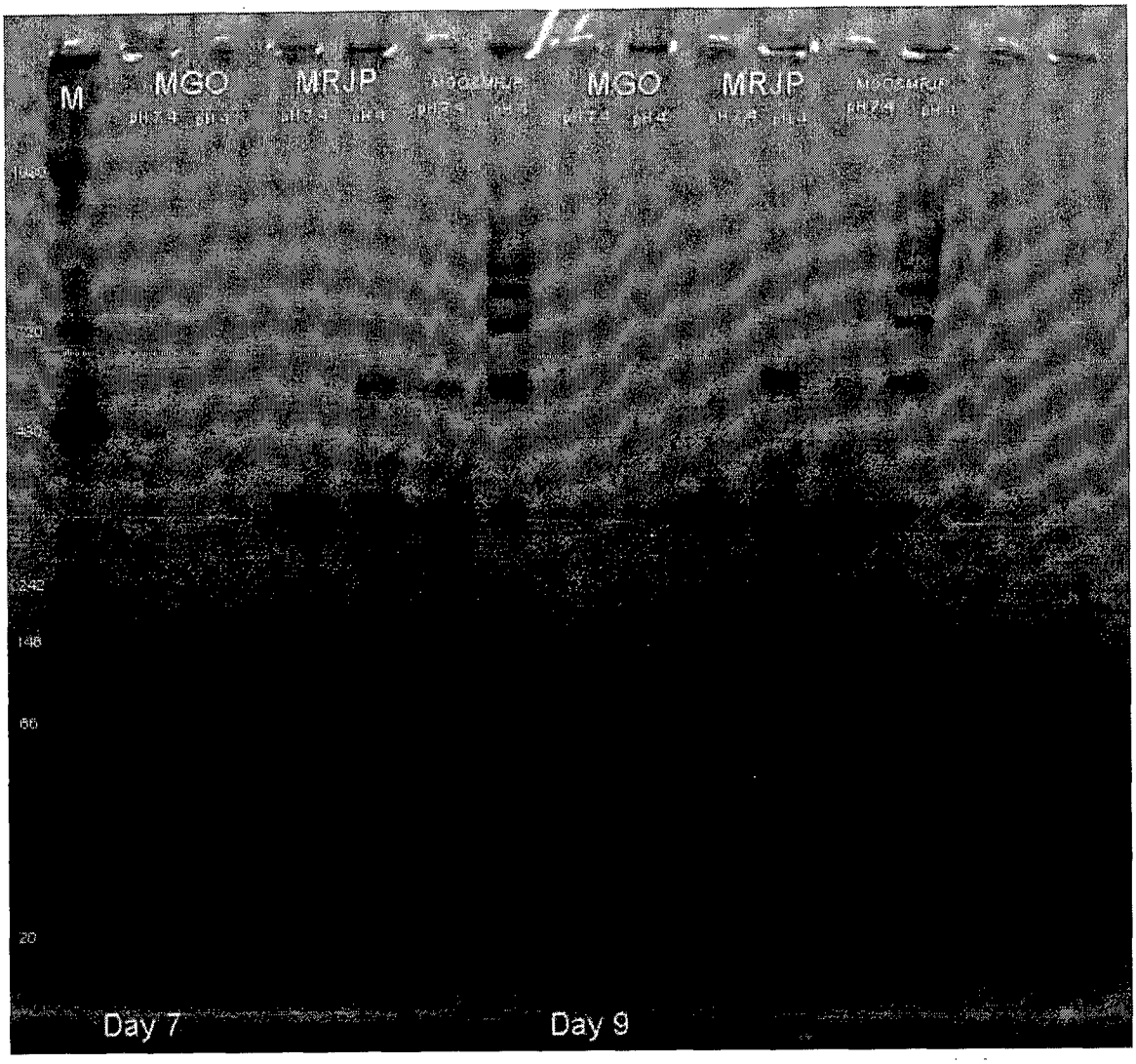

FIGURE 5

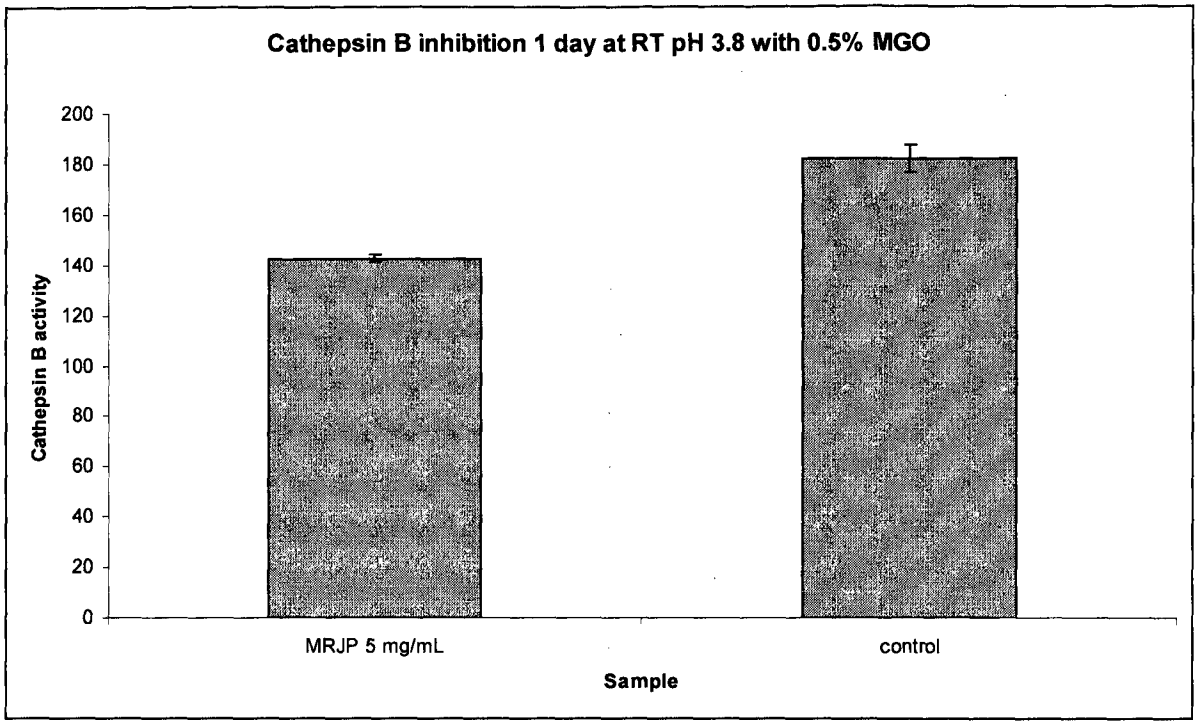

FIGURE 6A

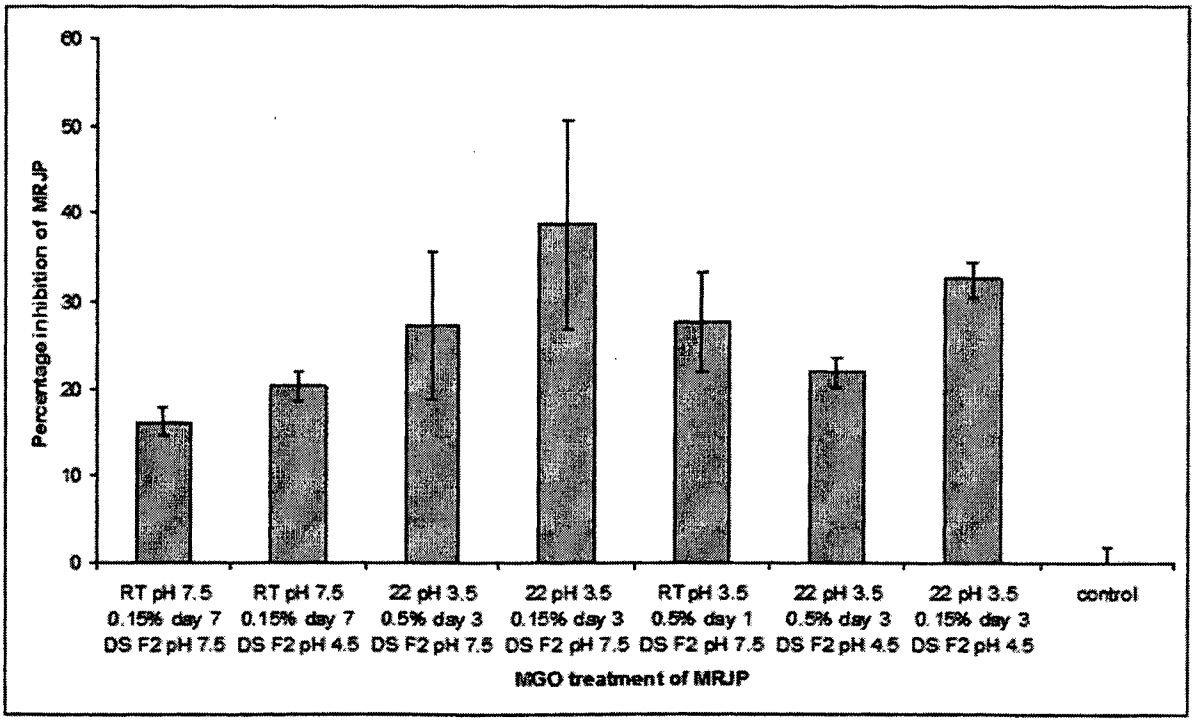

FIGURE 6B

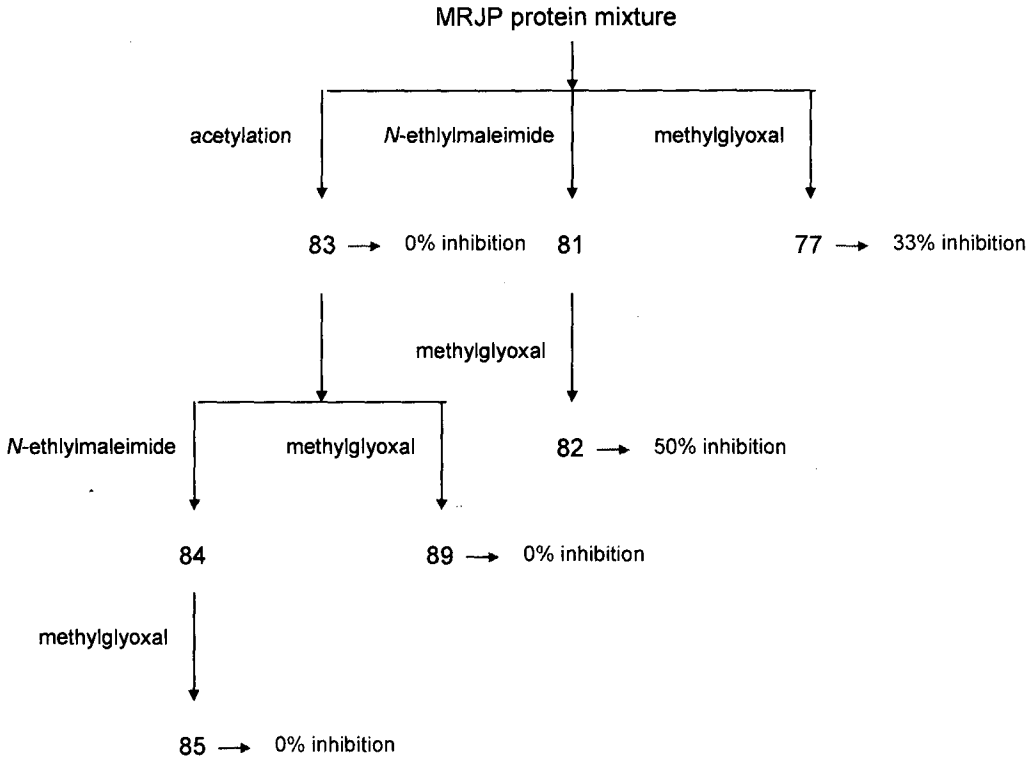
MRJP protein mixture
acetylation
N-ethlylmaleimide
methylglyoxal
83 → 0% inhibition
81
77 → 33% inhibition
methylglyoxal
N-ethlylmaleimide
methylglyoxal
82 → 50% inhibition
84
89 → 0% inhibition
methylglyoxal
85 → 0% inhibition

FIGURE 7

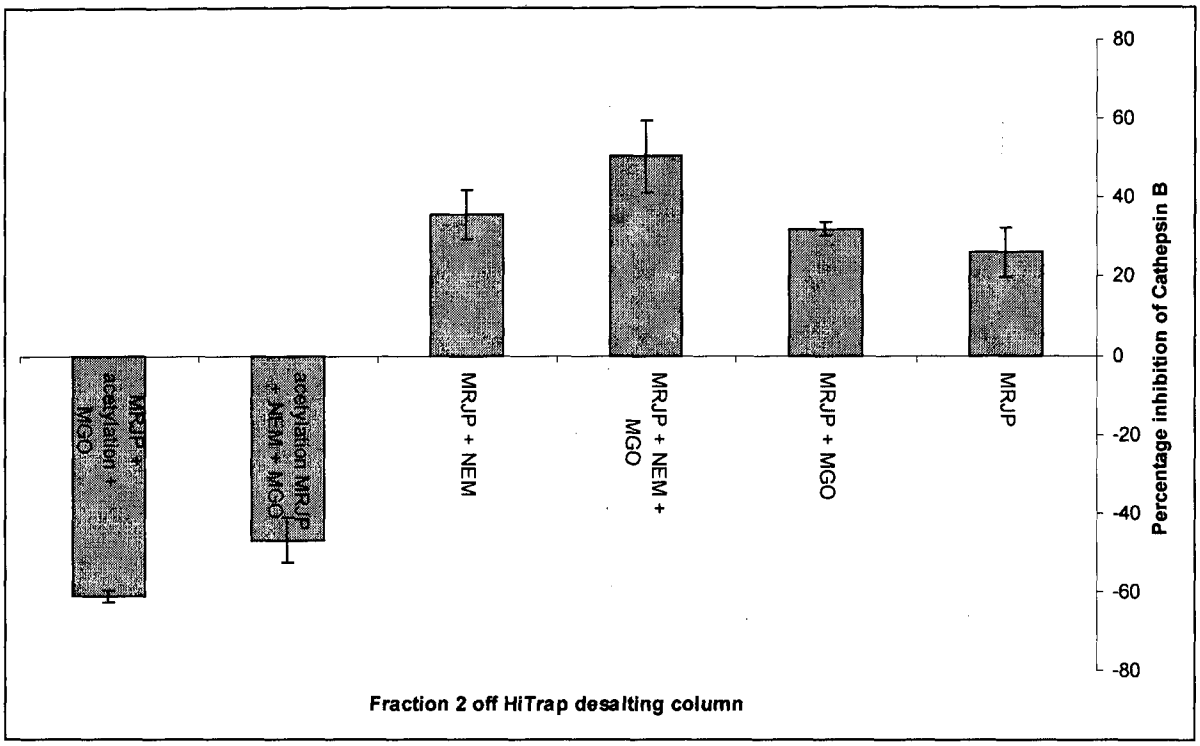
80
60
40
20
0
-20
-40
-60
-80
Percentage inhibition of Cathepsin B
MRJP + acetylation + MGO
acetylation MRJP + NEM + MGO
MRJP + NEM
MRJP + NEM + MGO
MRJP + MGO
MRJP
Fraction 2 off HiTrap desalting column

FIGURE 8

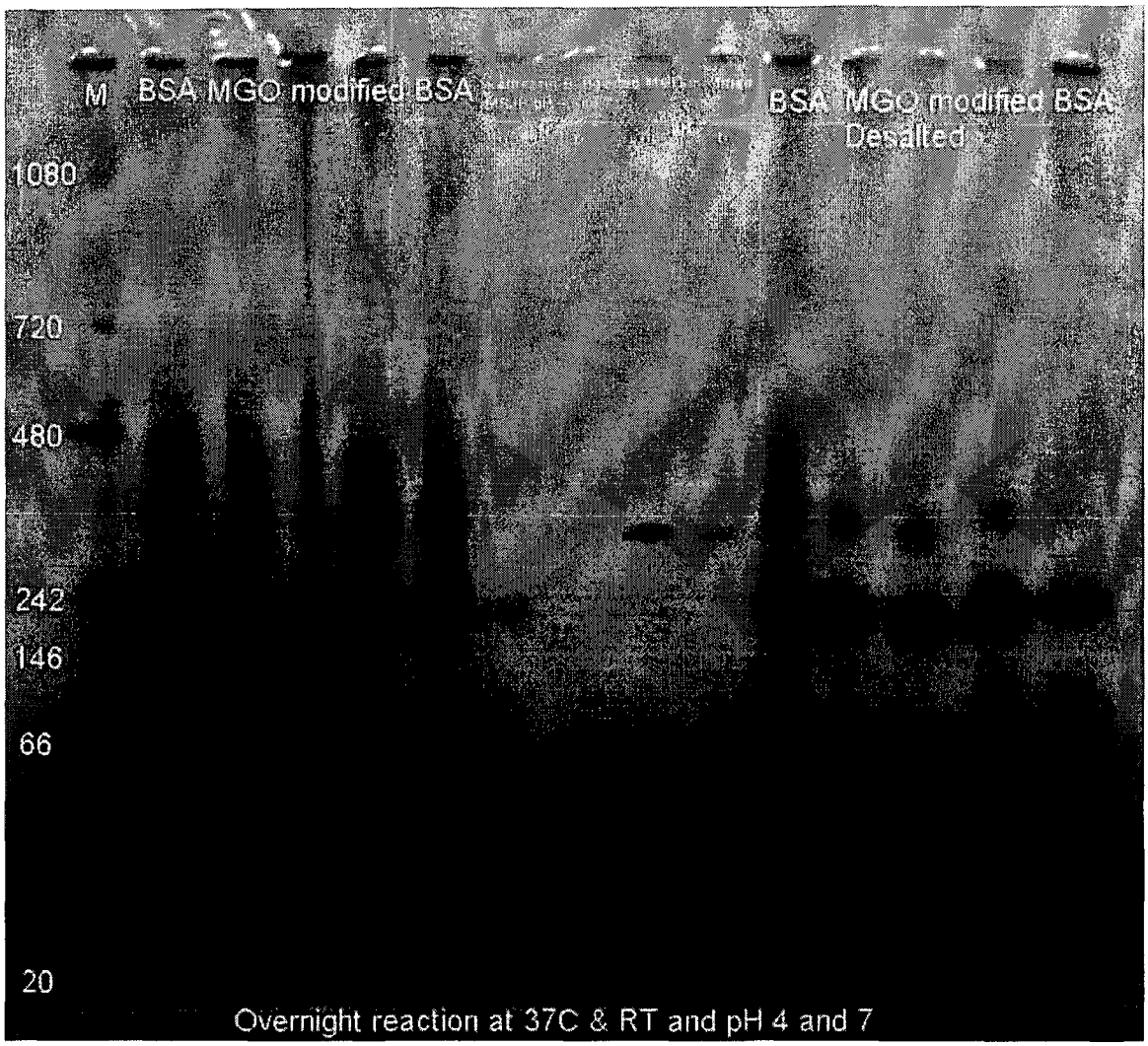
M
BSA MGO modified BSA
BSA MGO modified BSA
Desalted
1080
720
480
242
146
66
20
Overnight reaction at 37C & RT and pH 4 and 7

FIGURE 9

```
        10         20         30         40         50         60
MTRLFMLVCL GIVCQGTTGN ILRGESLNKS LPILHEWKFF DYDFGSDERR QDAILSGEYD

        70         80         90        100        110        120
YKNNYPSDID QWHDKIFVTM LRYNGVPSSL NVISKKVGDG GPLLQPYPDW SFAKYDDCSG

       130        140        150        160        170        180
IVSASKLAID KCDRLWVLDS GLVNNTQPMC SPKLLTFDLT TSQLLKQVEI PHDVAVNATT

       190        200        210        220        230        240
GKGRLSSLAV QSLDCNTNSD TMVYIADEKG EGLIVYHNSD DSFHRLTSNT FDYDPKFTKM

       250        260        270        280        290        300
TIDGESYTAQ DGISGMALSP MTNNLYYSPV ASTSLYYVNT EQFRTSDYQQ NDIHYEGVQN

       310        320        330        340        350        360
ILDTQSSAKV VSKSGVLFFG LVGDSALGCW NEHRTLERHN IRTVAQSDET LQMIASMKIK

       370        380        390        400        410        420
EALPHVPIFD RYINREYILV LSNKMQKMVN NDFNFDDVNF RIMNANVNEL ILNTRCENPD

       430
NDRTPFKISI HL
```

SEQ ID NO: 1

FIGURE 10

PFKISIHL (SEQ ID NO: 18)

FIGURE 11A

Ac-PFKISIHL-OH (SEQ ID NO: 19)
Ac-KISIHL-OH (SEQ ID NO: 20)
Ac-KISI-OH (SEQ ID NO: 21)
Ac-PFKI-OH (SEQ ID NO: 22)

FIGURE 11B

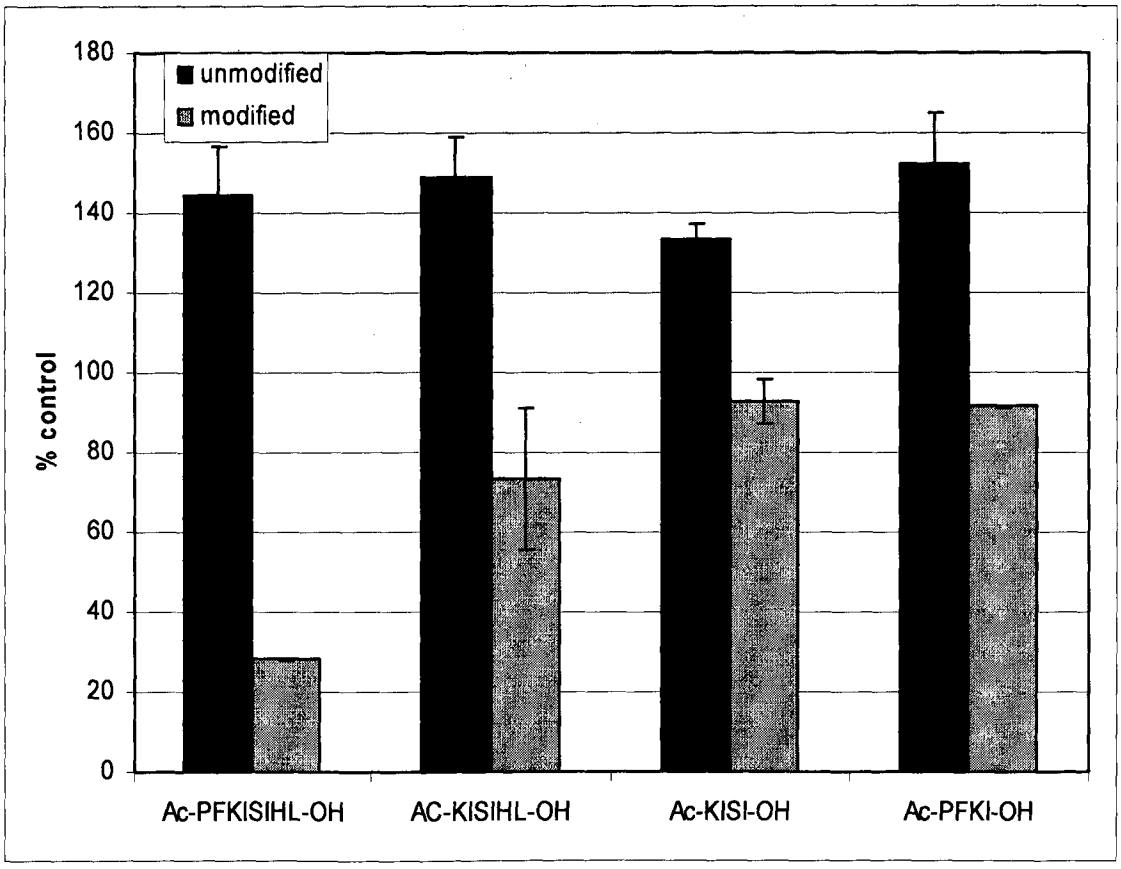

FIGURE 11C

NQKNNNQNDN (SEQ ID NO: 23)

FIGURE 12A

Ac-NQKNNNQNDN-OH (SEQ ID NO: 24)
Ac-NQKNNNQN-OH (SEQ ID NO: 25)
Ac-NQKNNN-OH (SEQ ID NO: 26)
Ac-NQKN-OH (SEQ ID NO: 27)
Ac-KN-OH (SEQ ID NO: 28)

FIGURE 12B

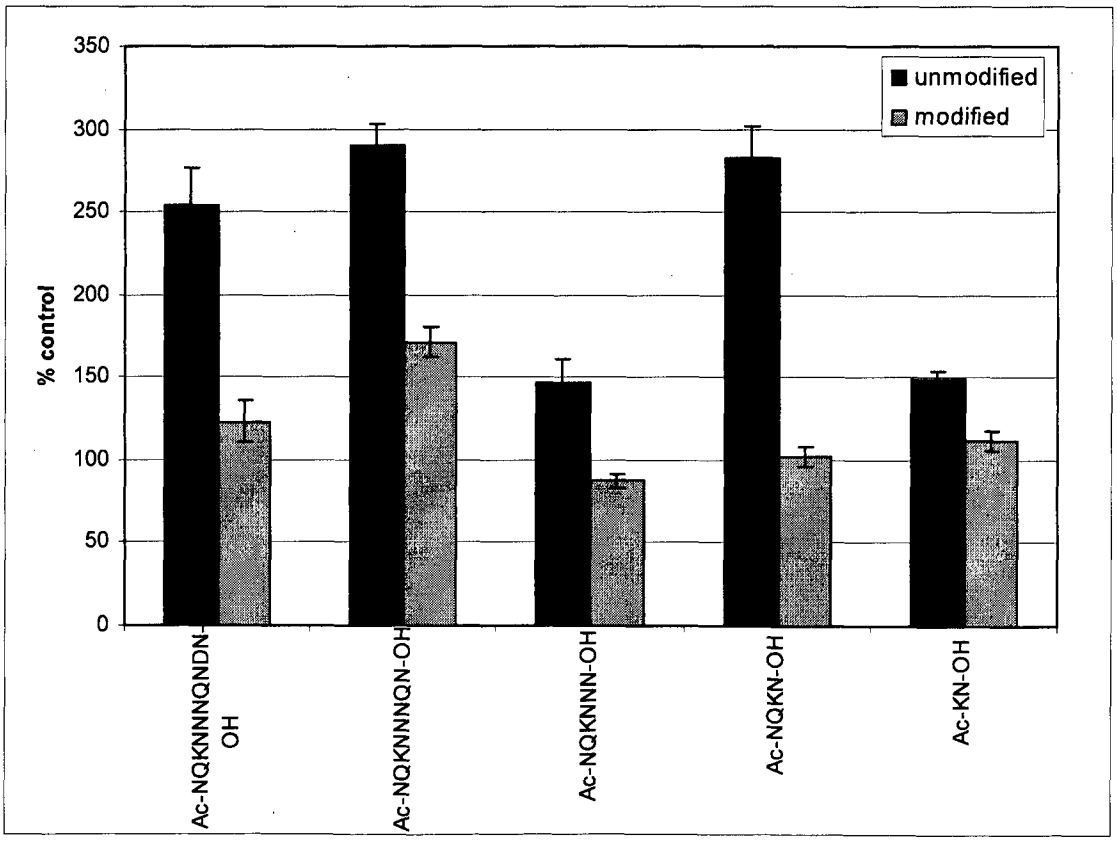

FIGURE 12C

HHSSKLH (SEQ ID NO: 29)

FIGURE 13A

Ac-HSSKLH-OH (SEQ ID NO: 30)
Ac-SKLH-OH (SEQ ID NO: 31)
Ac-SK-OH (SEQ ID NO: 32)
Ac-HSSK-OH (SEQ ID NO: 33)
Ac-SSKS-OH (SEQ ID NO: 34)

FIGURE 13B

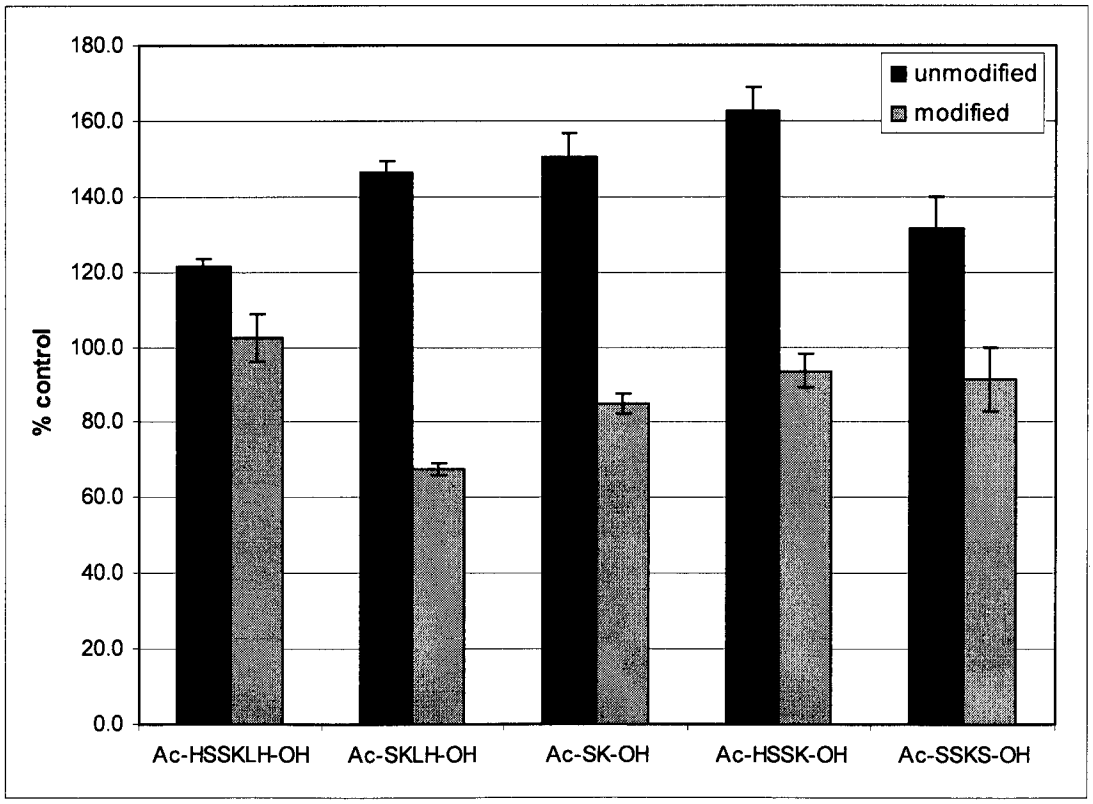

FIGURE 13C

HSSKSNNRHNNND (SEQ ID NO: 35)

FIGURE 14A

Ac-SSKSNNRHNNND-OH (SEQ ID NO: 36)
Ac-SSKSNNRHNN-OH (SEQ ID NO: 37)
Ac-SSKSNNRH-OH (SEQ ID NO: 38)
Ac-SSKSNN-OH (SEQ ID NO: 39)
Ac-SSKS-OH (SEQ ID NO: 40)
Ac-KS-OH (SEQ ID NO: 41)

FIGURE 14B

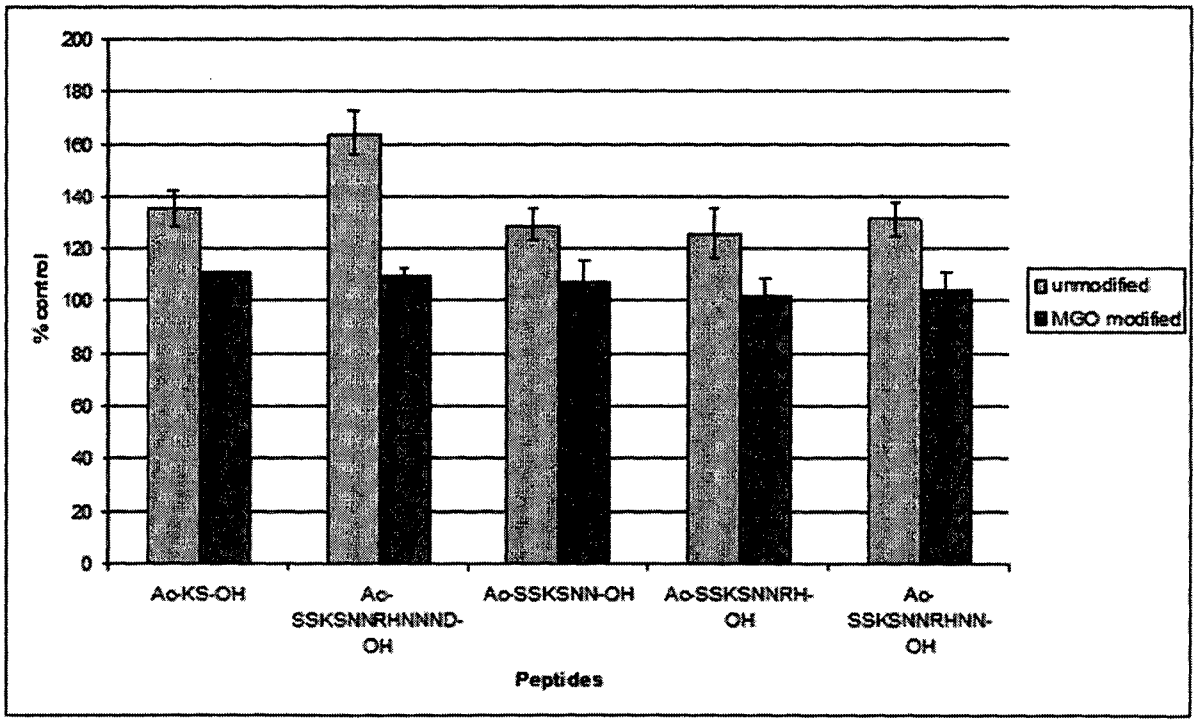

FIGURE 14C

QNKHNN (SEQ ID NO: 42)

FIGURE 15A

Ac-QNKHNN-OH (SEQ ID NO: 43)
Ac-KHNN-OH (SEQ ID NO: 44)
Ac-KH-OH (SEQ ID NO: 45)
Ac-QNKH-OH (SEQ ID NO: 46)

FIGURE 15B

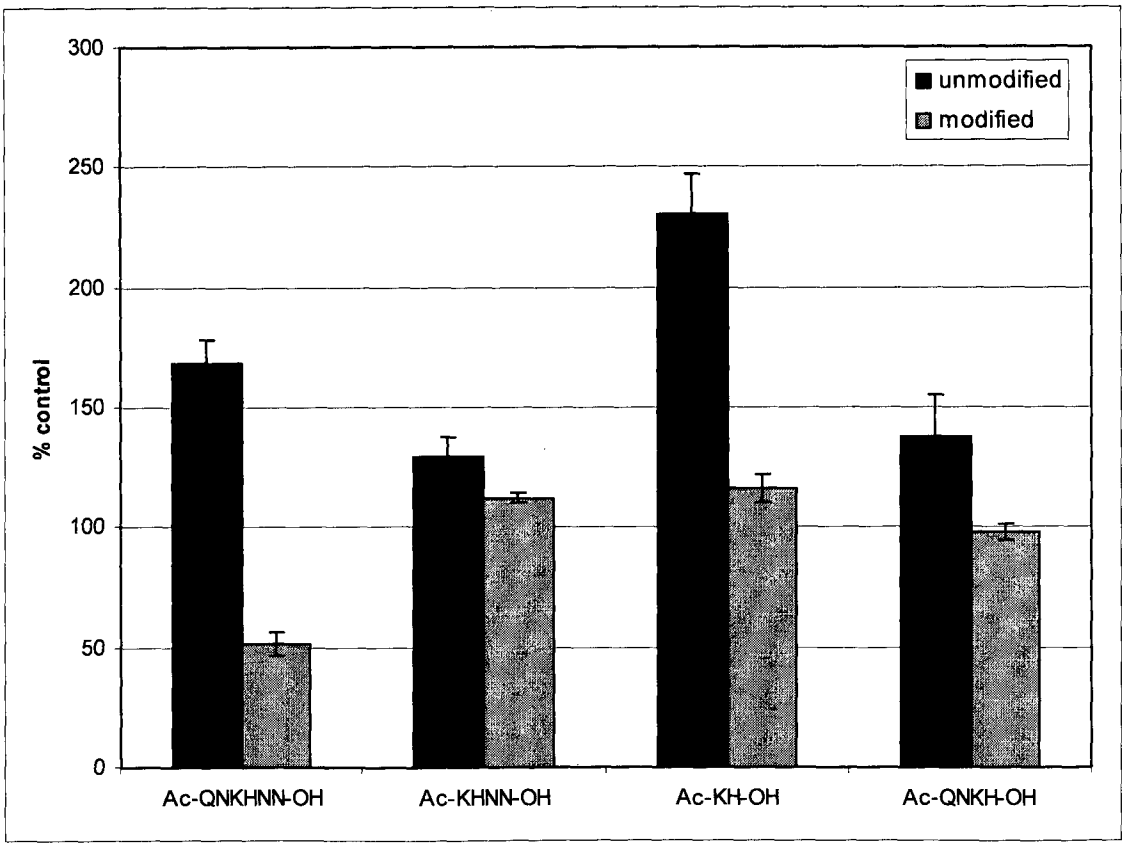

FIGURE 15C

Ac-LVK-OH (SEQ ID NO: 48)
Ac-LVK-NH_2 (SEQ ID NO: 49)
Ac-LIR-OH (SEQ ID NO: 51)
Ac-LIR-NH_2 (SEQ ID NO: 52)
Ac-LLK-OH (SEQ ID NO: 56)
Ac-LLK-NH_2 (SEQ ID NO: 57)
Ac-KI-OH (SEQ ID NO: 58)

FIGURE 16A

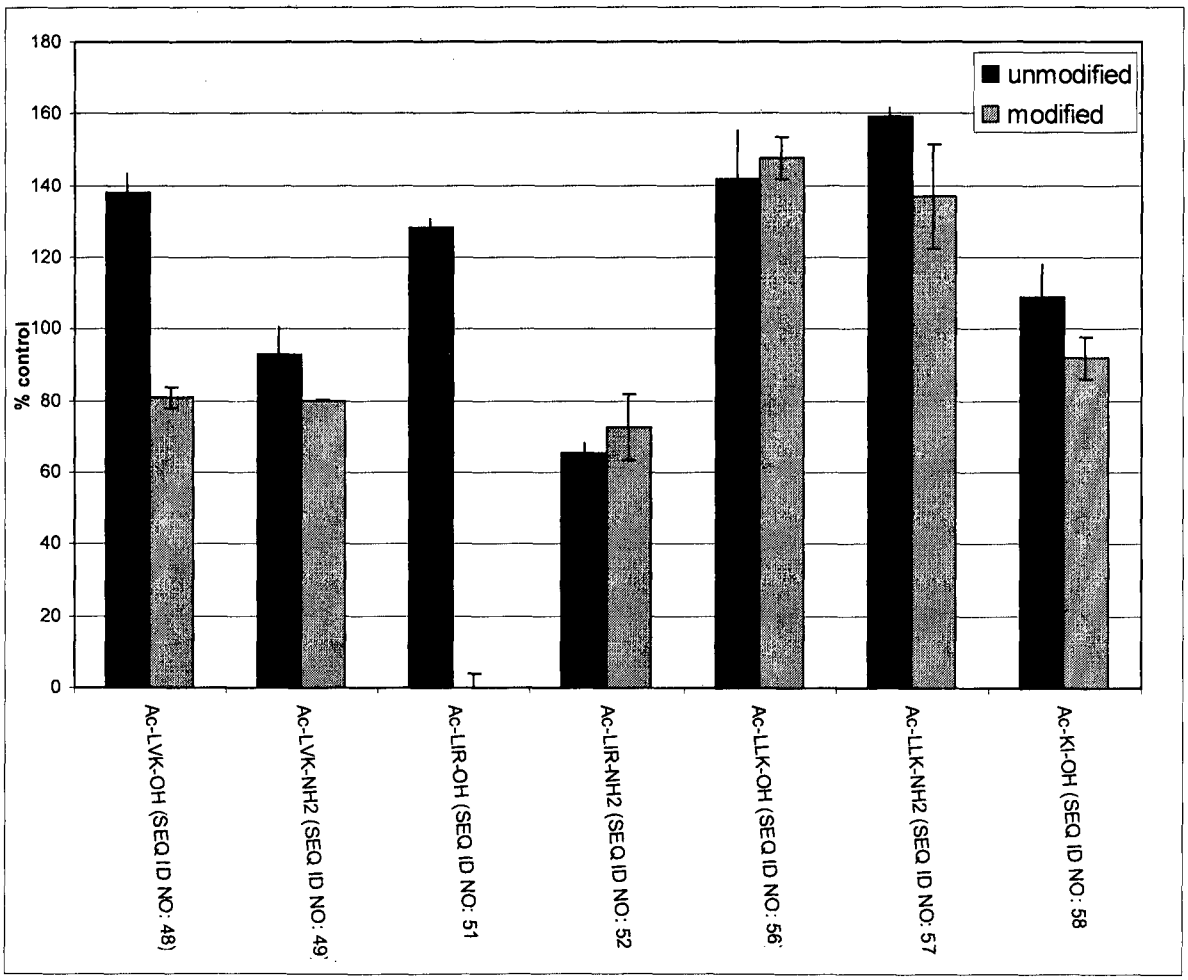

FIGURE 16B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2013/000110

A. CLASSIFICATION OF SUBJECT MATTER

A61K 38/08(2006.01)i, A61K 38/17(2006.01)i, A61K 35/64(2006.01)i, A61P 29/00(2006.01)i

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)
A61K 38/08; A23L 1/08; C07K 14/76; G01N 33/00; A61K 38/38; A23L 1/076; A61K 35/64; A61K 38/17; A61P 29/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: "royal jelly, apalbumin, methylglyoxal, MGO, inflammation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEONG et al., `Indigenous New Zealand honeys exhibit multiple anti-inflammatory activities` Innate Immunity, Vol.18, No.3, pp.459-466 (6 October 2011) See abstract, pages 461, 464, 465 and figure 1.	25-31,33-44,53
A	WO 2010-082846 A1 (COMVITA NEW ZEALAND LIMITED) 22 July 2010 See abstract, claims 1, 10, 28, 34 and page 31.	25-31,33-44,53
A	US 2011-0287059 A1 (STEPHENS et al.) 24 November 2011 See abstract and claim 4.	25-31,33-44,53
A	MAJTAN et al., `The immunostimulatory effect of the recombinant apalbumin 1-major honeybee royal jelly protein-on TNFα release` International Immunopharmacology, Vol.6, pp.269-278 (6 September 2005) See abstract and page 276.	25-31,33-44,53
A	ADAMS et al., `The origin of methylglyoxal in New Zealand manuka (Leptospermum scoparium) honey` Carbohydrate Research, Vol.344, No.8, pp.1050-1053 (26 May 2009) See abstract.	25-31,33-44,53

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
03 September 2013 (03.09.2013)	**04 September 2013 (04.09.2013)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea
Facsimile No. +82-42-472-7140

Authorized officer
KIM Seung Beom
Telephone No. +82-42-481-3371

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2013/000110

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	WO 2012-087160 A2 (MANUKAMED LIMITED) 28 June 2012 See the whole document.	25-31,33-40,53

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2013/000110

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

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

1. ☒ Claims Nos.: 1-24,45-52
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 1-24 and 45-52 pertain to methods for treatment of the human body by therapy and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☒ Claims Nos.: 13,14
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:
Claims 13 and 14 are unclear since they are referring to the multiple dependent claims which do not comply with PCT Rule 6.4(a).

3. ☒ Claims Nos.: 4-6,9-12,18-20,23,24,32,48-50
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required addtional 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 an additional fee, this Authority did not invite payment of any additional fee.

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/NZ2013/000110

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010-082846 A1	22/07/2010	AU 2009-337193 A1	22/07/2010
		CA 2748232 A1	22/07/2010
		EP 2367444 A1	28/09/2011
		EP 2367444 A4	28/11/2012
		US 2012-0021061 A1	26/01/2012
US 2011-0287059 A1	24/11/2011	AU 2009-337192 A1	22/07/2010
		CA 2748230 A1	22/07/2010
		EP 2368111 A1	28/09/2011
		WO 2010-082845 A1	22/07/2010
WO 2012-087160 A2	28/06/2012	AU 2011-345454 A1	09/05/2013
		CA 2822385 A1	28/06/2012
		TW 201245222 A	16/11/2012
		WO 2012-087160 A3	01/11/2012