The inventors have carefully examined SpeB and IdeS activity on IgG and have made the surprising discovery that SpeB in fact cleaves IgG at a different site in the hinge region than IdeS. Developing this surprising discovery, the inventors realised that tandem cleavage of immunoglobulin with SpeB and IdeS allows the investigation of the hinge region of immunoglobulin molecules in more detail than was previously thought possible. This is important because the hinge region is a key site for post-translational modification and for conjugation of therapeutic agents in antibody-drug conjugates. Accordingly, in one aspect, there is provided:

a method for analysing a sample of ~~immunoglobulin~~ human IgG molecules, comprising contacting the sample with a first polypeptide that cleaves the hinge region of human IgG between positions 238 and 239 according to the Kabat numbering system (positions 225 and 226 according to EU numbering system) and/or~~and~~ a second polypeptide that cleaves the hinge region of human IgG between positions 249 and 250 according to the Kabat numbering system (positions 236 and 237 according to EU numbering system) and analysing the resulting mixture, wherein the first polypeptide and the second polypeptide are cysteine protease enzymes ~~which each cleave a different target site in the hinge region of human IgG~~.

In another aspect, there is provided an isolated peptide consisting of the amino acid sequence CPPCPAPELLG (SEQ ID NO: 3), or a variant of said sequence ~~comprising~~ consisting of one or two conservative modifications, preferably wherein said modifications occur only within positions 2 to 10 of said sequence; optionally wherein said peptide is conjugated to a therapeutic agent.

In yet another aspect, there is provided a kit for use in a method of analysing a sample of immunoglobulin molecules, the kit comprising a first polypeptide and a second polypeptide as defined above.

Brief Description of the Figures

Figures 1A and 1B show results from SDS-PAGE following cleavage of the human monoclonal IgG1 antibody herceptin with IdeS (FabRICATOR) alone, SpeB (Fpn-1) and IdeS together, or SpeB alone.

Figure 2 shows a schematic overview of the different cleavage sites for SpeB (Fpn-1) and IdeS for human monoclonal IgG1 (Herceptin), as determined by mass spectrometry (LC/MS).

Figure 3 shows a schematic overview of cleavage of human IgG by SpeB (Fpn-1) giving rise to Fab and Fc fragments. The novel SpeB cleavage site at the hinge region is also indicated.

Figures 4A and 4B show a RP chromatogram with overlay of the hinge regions of peptides from avastin, herceptin and adcetris antibodies. In Figure 4B, a control synthetic hinge peptide has been added.

CLAIMS

1. A method for analysing a sample of immunoglobulin molecules, comprising contacting the sample with a first polypeptide and a second polypeptide and analysing the resulting mixture, wherein the first polypeptide and the second polypeptide are cysteine protease enzymes which each cleave a different target site in the hinge region of human IgG.

2. A method according to claim 1, wherein the first polypeptide cleaves the hinge region of IgG between positions 238 and 239 according to the Kabat numbering system (positions 225 and 226 according to EU numbering system) and/or the second polypeptide cleaves the hinge region of IgG between positions 249 and 250 according to the Kabat numbering system (positions 236 and 237 according to EU numbering system).

3. A method according to claim 1 or 2, wherein the first polypeptide comprises or consists of the amino acid sequence of SEQ ID NO:1, or a variant or fragment thereof, and the second polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 2, or a variant or fragment thereof.

4. A method according to any one of the preceding claims, wherein a variant of a said sequence is an amino acid sequence having at least 80% identity to said sequence, and a fragment of a said sequence comprises up to 300 contiguous amino acids of said sequence.

5. A method according to any one of the preceding claims, wherein at least one immunoglobulin molecule in said sample is an IgG molecule, preferably a human IgG molecule.

6. A method according to claim 5, wherein at least one said IgG molecule is conjugated to a therapeutic agent, preferably via the thiol group of a cysteine residue of the IgG molecule.

7. A method according to claim 6, wherein said cysteine residue is in the hinge region of the IgG molecule.

8. A method according to claim 6 or 7, wherein the therapeutic agent is a cytotoxin, preferably selected from avristatin, a calicheamicin, CC-1065, doxorubicin, maytonsinoid, methotrexate and a vinca alkaloid.

9. A method according to any one of the preceding claims, which comprises the steps:

(a) contacting said sample with said first polypeptide;
(b) isolating Fc fragments from the resulting mixture;
(c) contacting said isolated Fc fragments with said second polypeptide; and
(d) analysing the resulting mixture.

10. A method according to any one of claims 1 to 8, which comprises the steps:

(a) contacting said sample with said second polypeptide;
(b) isolating Fab fragments from the resulting mixture;
(c) contacting said isolated Fab fragments with said first polypeptide; and
(d) analysing the resulting mixture.

11. A method according to any one of claims 1 to 8, which comprises the steps:

(a) contacting said sample with both said first and said second polypeptide; and
(b) analysing the resulting mixture.

12. A method according to any one of claims 8 to 11, wherein analysing the resulting mixture comprises determining the molecular weight of at least one molecule in the mixture, preferably via the use of high performance liquid chromatography (HPLC) and/or mass spectrometry.

13. A method according to claim 12, wherein said analysis comprises determining the presence, absence, and/or amount of a peptide with a molecular weight of approximately 1096 Da.

14. A method according to claim 13, wherein said peptide consists of the sequence CPPCPAPELLG (SEQ ID NO: 3), or a variant of said sequence comprising one or two conservative modifications, preferably wherein said modifications occur only within positions 2 to 10 of said sequence.

15. A method according to any one of claims 11 to 14, wherein said analysis is carried out to determine:

(a) the proportion of immunoglobulin molecules in the sample to which a therapeutic agent is conjugated and/or unconjugated;

(b) the ratio of therapeutic agent : immunoglobulin molecule; and/or

(c) the presence or absence of post-translational modifications of the amino acid sequence set forth in SEQ ID NO: 3.

16. A peptide consisting of the amino acid sequence CPPCPAPELLG (SEQ ID NO: 3), or a variant of said sequence comprising one or two conservative modifications, preferably wherein said modifications occur only within positions 2 to 10 of said sequence; optionally wherein said peptide is conjugated to a therapeutic agent.

17. The peptide according to claim 16, which is produced by contacting an immunoglobulin containing sample with a first polypeptide and a second polypeptide as defined in claim 1.

18. A kit for use in a method of analysing a sample of immunoglobulin molecules, the kit comprising a first polypeptide and a second polypeptide as defined in claim 1.

CLAIMS

1. A method for analysing a sample of human IgG molecules, comprising contacting the sample with a first polypeptide that cleaves the hinge region of human IgG between positions 238 and 239 according to the Kabat numbering system (positions 225 and 226 according to EU numbering system) and/or a second polypeptide that cleaves the hinge region of human IgG between positions 249 and 250 according to the Kabat numbering system (positions 236 and 237 according to EU numbering system) and analysing the resulting mixture, wherein the first polypeptide and the second polypeptide are cysteine protease enzymes.

2. A method according to claim 1, wherein the first polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 1, or a variant thereof having at least 80% sequence identity to SEQ ID NO: 1 and having cysteine protease activity or fragment thereof comprising up to 300 contiguous amino acids of SEQ ID NO: 1 and having cysteine protease activity, and the second polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 2, or a variant thereof having at least 80% sequence identity of SEQ ID NO: 2 and having cysteine protease activity or fragment thereof comprising up to 300 contiguous amino acids of SEQ ID NO: 2 and having cysteine protease activity.

3. A method according to any one of the preceding claims, wherein at least one said human IgG molecule is conjugated to a therapeutic agent, preferably via the thiol group of a cysteine residue of the human IgG molecule, optionally wherein said cysteine residue is in the hinge region of the human IgG molecule.

4. A method according to claim 3, wherein the therapeutic agent is a cytotoxin, preferably selected from avristatin, a calicheamicin, CC-1065, doxorubicin, maytonsinoid, methotrexate and a vinca alkaloid.

5. A method according to any one of the preceding claims, which comprises the steps:

(a) contacting said sample with said first polypeptide;

(b) isolating Fc fragments from the resulting mixture;

(c) contacting said isolated Fc fragments with said second polypeptide; and

(d) analysing the resulting mixture.

6. A method according to any one of claims 1 to 4, which comprises the steps:

(a) contacting said sample with said second polypeptide;

(b) isolating Fab fragments from the resulting mixture;

(c) contacting said isolated Fab fragments with said first polypeptide; and

(d) analysing the resulting mixture.

7. A method according to any one of claims 1 to 4, which comprises the steps:

(a) contacting said sample with both said first and said second polypeptide; and

(b) analysing the resulting mixture.

8. A method according to any one of claims 4 to 7, wherein analysing the resulting mixture comprises determining the molecular weight of at least one molecule in the mixture, preferably via the use of high performance liquid chromatography (HPLC) and/or mass spectrometry.

9. A method according to claim 8, wherein said analysis comprises determining the presence, absence, and/or amount of a peptide with a molecular weight of approximately 1096 Da, optionally wherein said peptide consists of the sequence CPPCPAPELLG (SEQ ID NO: 3), or a variant of said sequence consisting of one or two conservative modifications, preferably wherein said modifications occur only within positions 2 to 10 of said sequence.

10. A method according to any one of claims 7 to 9, wherein said analysis is carried out to determine:

(a) the proportion of immunoglobulin molecules in the sample to which a therapeutic agent is conjugated and/or unconjugated;

(b) the ratio of therapeutic agent : immunoglobulin molecule; and/or

(c) the presence or absence of post-translational modifications of the amino acid sequence set forth in SEQ ID NO: 3.

11. An isolated peptide consisting of the amino acid sequence CPPCPAPELLG (SEQ ID NO: 3), or a variant of said sequence consisting of one or two conservative modifications, preferably wherein said modifications occur only within positions 2 to 10 of said sequence; optionally wherein said peptide is conjugated to a therapeutic agent.

12. The isolated peptide according to claim 11, which is produced by contacting an immunoglobulin containing sample with a first polypeptide and a second polypeptide as defined in claim 1.

13. A kit for use in a method of analysing a sample of immunoglobulin molecules, the kit comprising a first polypeptide and a second polypeptide as defined in claim 1.