separation columns, which utilise the Fc-binding properties of the bacterial proteins A andG.

Many different glycoproteins have utility in therapeutic applications. Methods to analyse the glycosylation of such proteins have utility in the research and development of the proteins as therapeutics. It may also be desirable to provide deglycosylated versions of these proteins.

## Summary ~~of the Invention~~

The inventors have identified a novel endoglycosidase from serotype M49 *Streptococcus pyogenes,* referred to herein as EndoS49. EndoS49 was isolated from strain NZ131, a nephritogenic and highly transformable strain of serotype M49. NZ131 strain is a clinical isolate from a case of acute post-streptococcal glomerulonephritis in New Zealand. At a protein level, EndoS49 has less than 40% identity to EndoS, and is a 90kDa protein, compared to the 108kDa of EndoS. EndoS49 has deglycosylation activity for a broader range of proteins than EndoS.

The enzyme is a 90kDa enzyme, having a family 18 glycoside hydrolase catalytic domain. EndoS49 hydrolyzes glycan on human glycoproteins, and in particular IgG1-4, and alpha-1-microglobulin. EndoS49 can be used in the hydrolysis of glycans on human glycoproteins including IgG and alpha-1-microglobulin. EndoS49 can thus be used in glycoprofiling analysis in which the enzyme is contacted with a glycoprotein, and the products produced are separated for analysis of the glycans and the protein. EndoS49 can also be used to prepare deglycosylated proteins. The enzyme can be modified to reduce or remove endoglycosidase activity.

The modified EndoS49 polypeptide which lacks endoglycosidase activity can be used in methods for isolating glycosylated and/or functionally active IgG. By using such a modified EndoS49 polypeptide in combination with an additional IgG-binding reagent which is capable of binding denatured and/or deglycosylated IgG, the inventors have also identified a method for assessing the glycosylation state or functional quality of an IgG-containing sample.

In accordance with ~~the present invention~~a first aspect, there is thus provided a polypeptide comprising

(a) the amino acid sequence of SEQ ID NO: 1;

(b) a variant thereof having at least 50% identity to the amino acid sequence of SEQ ID NO: 1 and having endogycosidase activity; or

(c) a fragment of either thereof having endoglycosidase activity. ~~The invention also provides~~ In a second aspect, there is provided a polypeptide capable of binding to IgG and which does not have endoglycosidase activity comprising

(a) the amino acid sequence of SEQ ID NO: 2;

(b) a variant thereof having at least 50% identity to the amino acid sequence of SEQ ID NO: 1, in which the amino acid equivalent to glutamic acid at position 186 is substituted; or

(c) a fragment of either thereof.

The ~~invention~~ disclosure also provides polynucleotides, expression vectors and host cells encoding or expressing the polypeptides of the ~~invention~~disclosure. The ~~invention~~ disclosure also relates to the use of the polypeptides of the ~~invention~~ disclosure in a method of determining or analysing the glycosylation state of the protein, and in particular of an antibody, in particular an IgG antibody.

In a third aspect, there is provided a polynucleotide comprising a sequence which encodes a polypeptide as defined herein.

In a fourth aspect, there is provided an expression vector, comprising a polynucleotide as defined herein.

In a fifth aspect, there is provided a method for hydrolyzing glycan of a glycoprotein comprising incubating the glycoprotein with a polypeptide as defined herein.

In a sixth aspect, a method for assessing the glycosylation status of a glycoprotein comprising incubating a glycoprotein with a polypeptide as defined herein, and analysing the products produced.

The ~~invention~~ disclosure also provides a method for isolating IgG from an IgG- containing sample, which method comprises:

(a) contacting said IgG-containing sample with a modified EndoS49 polypeptide which lacks IgG endoglycosidase activity

(b) separating said EndoS49 from the contacted sample;

thereby obtaining isolated IgG.

In seventh aspect, there is provided a method for isolating IgG from an IgG-containing sample, which method comprises

(a) contacting said IgG-containing sample with an EndoS49 polypeptide which lacks IgG endoglycosidase activity, to thereby allow formation of a IgG-EndoS49 polypeptide complex;

(b) separating said EndoS49 from the contacted sample; and thereby obtaining isolated IgG.

In an eighth aspect, there is provided a method for determining the presence or absence of IgG in a sample, which method comprises

(a) contacting said sample with an EndoS49 polypeptide which lacks IgG endoglycosidase activity, to thereby allow formation of a IgG-EndoS49 polypeptide complex;

(b) optionally separating said EndoS49 from the contacted sample; and

(c) determining whether the separated EndoS49 is bound to IgG; and thereby determining the presence or absence of IgG in the sample.

Additionally there is provided method of assessing the glycosylation state or functional quality of an IgG-containing sample, which method comprises taking a first and a second sub-sample of the IgG-containing sample, and wherein steps (a) and (b) according to the method above are applied to the first sub-sample, and wherein steps (a) and (b) as above are applied to the second sub-sample except the EndoS49 polypeptide is substituted with an alternative IgG-binding reagent which is capable of binding denatured and/or deglycosylated IgG, and further comprising:

(c) quantifying the amount of IgG bound to the EndoS49 polypeptide in the first sub-sample, and the amount of IgG bound to the alternative IgG-binding reagent in the second sub-sample; and

(d) comparing both the amounts of bound IgG determined in (c);

and thereby assessing the glycosylation state or functional quality of an IgG containing sample.

In a ninth aspect, there is provided a method of assessing the glycosylation state or functional quality of an IgG containing sample, which method comprises taking a first and a second sub-sample of the IgG-containing sample, and wherein steps (a) and (b) according to the seventh aspect are applied to the first sub-sample, and steps (a) and (b) according to the seventh aspect are applied to the second sub-sample except the EndoS49 polypeptide is substituted with an alternative IgG-binding reagent which is capabale of binding unglycosylated and/or denatured, inactive IgG, and further comprising

(a) quantifying the amount of IgG bound to the EndoS49 polypeptide in the first sub-sample, and the amount of IgG bound to the alternative IgG-binding reagent in the second sub-sample; and

(b) comparing both of the amounts of bound IgG determined in (c); and

thereby assessing the glycosylation state or functional quality of an IgG containing sample.

The modified enzyme of the present ~~invention~~ diclosure may also be used in methods for isolating Fab or Fc fragments of IgG. The methods of the ~~invention~~ disclosure make use of a

highly specific IgG cleaving enzyme from *S. pyogenes,* IdeS (Immunoglobulin G-degrading enzyme of *S. pyogenes),* and an EndoS49 polypeptide.

In an tenth aspect, there is provided a method for isolating Fab fragments from IgG-containing samples, which method comprises (a) contacting said Ig-G containing sample with IdeS, and an EndoS49 polypeptide; (b) separating said IdeS and said EndoS49 polypeptide from the contacted sample; and thereby isolating Fab fragments.

In a eleventh aspect, there is provided a method for isolating Fc fragments from IgG-containing samples, which method comprises (a) contacting said IgG-containing sample with IdeS; (b) separating IdeS from the mixture obtained in step (a), thereby isolating Fab and Fc fragments; (c) contacting said Fab and Fc fragments with an EndoS49 polypeptide to thereby allow formation of a Fc fragment-EndoS49 polypeptide complex; (d) separating the Fc fragment-EndoS49 polypeptide complexes from the mixture obtained in step (c); and (e) isolating Fc fragments from the Fc fragment-EndoS49 polypeptide complexes obtained in step (d).

In one method of the ~~invention~~disclosure, a sample containing IgG is contacted with IdeS and an EndoS49 polypeptide, which is a modified EndoS49 polypeptide which lacks endoglycosidase activity as described above.

In the methods of the ~~invention~~disclosure, typically IdeS cleaves the IgG into Fab and Fc fragments and the EndoS49 polypeptide binds to the Fc fragments. The Fc fragments are then separated from the Fab fragments.

This method is particularly useful for isolating Fab or Fc fragments from samples comprising purified IgG. More specifically, it is useful for isolating Fab or Fc fragments from a sample comprising IgG purified using the modified EndoS49 polypeptide of the disclosure~~invention~~. However, the method can also be adapted for use on samples containing unpurified IgG, such as serum, cell lysate or cell culture medium.

Also provided are kits for carrying out the methods according to the ~~invention~~disclosure.

In a twelfth aspect, there is provided a kit for isolating IgG from an IgG-containing sample, comprising (a) an EndoS49 polypeptide as defined herein; and optionally (b) means for separating said Endo S49 polypeptide from a sample.

In a thirteenth aspect, there is provided a kit for determining the presence or absence of IgG in a sample, comprising (a) an EndoS49 polypeptide as defined herein; and optionally (b) means for separating said Endo S49 polypeptide from a sample.

In a fourteenth aspect, there is provided a kit for assessing the glycosylation

state or functional quality of an IgG containing sample, comprising (a) an EndoS49 polypeptide as defined herein; (b) an alternative IgG-binding reagent which is capable of binding denatured and/or deglycosylated IgG; (c) means for separating said EndoS49 polypeptide and said alternative igG-binding reagent from a sample.

In a fifteenth aspect, there is provided kit for isolating Fab or Fc fragments of IgG comprising (a) IdeS; (b) an EndoS49 polypeptide; and (c) means for separating said IdeS and said EndoS49 polypeptide from a sample.

**Brief Description of the Figures**

**Figure 1.** ClustalW alignment of EndoS49 and EndoS reveals two different proteins. EndoS49 and EndoS was aligned using ClustalW in the software MacVector. GH18 catalytic motif (D**D*D*E) is present at position 179-186 with Glu186 as the catalytic residue.

**Figure 2.** EndoS49 has activity on glycoproteins. A. 1 μg of EndoS49, its catalytic mutant and the truncated versions was incubated with 3 μg human IgG in PBS overnight at 37°C and analyzed on a SDS-PAGE gel and with LCAlectin blot. B. 1 μg EndoS49 and EndoS49(E186L) was incubated with 3 μg of subclasses 1-4 of human IgG and analyzed as above. C. 1 μg EndoS49 and its mutants were incubated with 3 μg Alpha-1-microglobulin and analyzed on a 10 % SDS-PAGE gel.

**Figure 3.** EndoS49 binds to IgG. 4, 2 and 1 μg of EndoS49 and its mutants were immobilized on a PVDF membrane and incubated with human IgG and later with protein-G coupled to HRP.

**Figure 4.** The genomic context of *ndoS49* and *ndoS*. A comparison of the genes surrounding *ndoS49* and *ndo*Sin GAS strains NZ131 (M49) and 5005 (M1) was carried out in MacVector.

**Figure 5.** Phylogenetic analysis of EndoS49 and other bacterial endoglycosidases.