Published in final edited form as:
J Mol Recognit. 2010 ; 23(3): 271–282. doi:10.1002/jmr.967.

Binding site on human immunoglobulin G for the affinity ligand HWRGWV

Haiou Yang[a], **Patrick V. Gurgel**[a], **D. Keith Williams Jr**[b], **Benjamin G. Bobay**[c,d], **John Cavanagh**[c], **David C. Muddiman**[b], and **Ruben G. Carbonell**[a,*]

[a]Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC 27695, USA

[b]W.M. Keck FT-ICR Mass Spectrometry Laboratory, Department of Chemistry, North Carolina State University, Raleigh, NC 27695, USA

[c]Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, NC 27695, USA

[d]North Carolina Research Campus, Kannapolis, Kannapolis, NC 28081, USA

Abstract

Affinity ligand HWRGWV has demonstrated the ability to isolate human immunoglobulin G (hIgG) from mammalian cell culture media. The ligand specifically binds hIgG through its Fc portion. This work shows that deglycosylation of hIgG has no influence on its binding to the HWRGWV ligand and the ligand does not compete with Protein A or Protein G in binding hIgG. It is suggested by the mass spectrometry (MS) data and docking simulation that HWRGWV binds to the pFc portion of hIgG and interacts with the amino acids in the loop Ser383–Asn389 (SNGQPEN) located in the C_H3 domain. Subsequent modeling has suggested a possible three-dimensional minimized solution structure for the interaction of hIgG and the HWRGWV ligand. The results support the fact that a peptide as small as a hexamer can have specific interactions with large proteins such as hIgG.

Keywords

hexamer ligand; immunoglobulin G; glycosylation; mass spectrometry; computational docking; binding site

INTRODUCTION

Human immunoglobulin G (hIgG, IgG) is a glycoprotein with variable (V) and constant (C) domains (Figure 1). The hinge region of the IgG molecule is prone to hydrolyzation with enzymes, forming different fragments such as Fab, Fc, pFc, among others (Figure 1). Two consensus binding sites exist on the Fc portion of IgG, the hinge proximal region of the C_H2 domain for FcγRs binding and the junction of the C_H2 and C_H3 domains where Protein A, Protein G, neonatal Fc receptor (FcRn), and some rheumatoid factors are retained (Nezlin and Ghetie, 2004). The degree of antibody glycosylation influences the binding at the hinge proximal site but has little influence on interactions at the inter-C_H2-C_H3 binding site (Sarmay *et al*., 1992; Jefferis *et al*., 1995; Radaev and Sun, 2002). However, the existence of

*Correspondence to: R. G. Carbonell, Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC 27695, USA. ruben@ncsu.edu.

both C_H2 and C_H3 domains is necessary for the binding at the inter-C_H2-C_H3 binding site (Nardella *et al*., 1985). The binding of the receptor at the hinge proximal site is usually monovalent due to the structural change of Fc upon binding. However, bivalency is allowed for bindings at the junction of the C_H2 and C_H3 domains (Jefferis *et al*., 1998). For both consensus binding sites, most of the amino acids in contact with the ligand molecules are located in loops of Fc domains. The flexibility of the loop structures is considered an important factor for the consensus binding sites to interact with a variety of different molecules (DeLano *et al*., 2000; Sundberg and Mariuzza, 2000).

Small peptides designed for drug applications or for the capture and purification of IgG have been found to have the ability to mimic the binding specificity of large proteins. The cyclic Fc-III peptide (DCAWHLGELVWCT) (DeLano *et al*., 2000), identified by screening a phage display library for binding to Fc, was found to compete effectively with Protein A. Crystallography studies showed that the peptide targeted the C_H2-C_H3 interface of Fc. The cyclic tripeptide $(CFHH)_2KG$, selected against mouse IgG and named FcRM, resembled the loop structure of Fc-RIII in binding the lower hinge region (Pro230–Ser239) of the Fc fragment as determined by nuclear magnetic resonance (NMR) (Verdoliva *et al*., 2005). Linear peptides with sequences taken from the loop residues of the Fc receptor for bovine IgG2 were tested and modified, with the minimum sequence Phe82–Val85 (FIGV) finally identified for its ability to inhibit the binding of the receptor to bovine IgG2 (Zhang *et al*., 2006). A synthesized peptide whose sequence represented the segment Thr256–Pro 271 in the C_H2 domain of human IgG1 was able to compete with hIgG in binding to the FcγRIIa receptor (Uray *et al*., 2004) and displayed some of the functions associated with whole hIgG molecules (Cendron *et al*., 2008). Although the exact binding sites on the proteins for some of these peptides were not identified the concept that a small peptide can play the same role as large proteins in specific protein-protein interactions is clear.

Hexamer ligand HWRGWV has demonstrated the ability to isolate hIgG from complex protein mixtures (Yang *et al*., 2008). It was found that the ligand specifically selected the Fc fragment over Fab and $F(ab')_2$ of hIgG (Yang *et al*., 2005), presenting another example of a small peptide having specific interactions similar to those of a large protein with IgG. This paper describes efforts to determine the possible interaction site of the Fc-binding peptide HWRGWV on hIgG through the use of SDS–PAGE, isothermal adsorption, column chromatography, mass spectrometry (MS), and molecular modeling. The understanding of interactions between the small peptide and IgG might shed a light on protein-ligand interactions or potential antibody functionality.

METHODS

Materials

Peptide resin HWRGWV at a ligand density of 0.08 meq/g was synthesized directly on Toyopearl AF-Amino-650 M (average particle size 65 μm) (Tosoh Bioscience, Inc., Montgomeryville, PA, USA) using fluorenylmethyloxycarbonyl (Fmoc) chemistry by Peptides International (Louisville, KY, USA). Soluble Protein A from *Staphylococcus aureus* and recombinant Protein G were purchased from Rockland (Gilbertsville, PA, USA) and GE Healthcare (Piscataway, NJ, USA), respectively. ECL Plus Western blotting detection reagents were also from GE Healthcare. Endoglycosidase peptide:N-glycosidase F (PNGase F) was obtained from New England Biolabs, Inc. (Ipswich, MA, USA). Human IgG, pepsin, endoproteinase Lys, endoproteinase Glu, and all chemicals unless otherwise mentioned were purchased from Sigma (St. Louis, MO, USA). Fc fragment of hIgG was obtained from Calbiochem (San Diego, CA, USA). NuPAGE gels, buffers, reducing agent, molecular weight markers, polyvinylidene difluoride (PVDF) membrane, staining kits, and WesternBreeze Chromogenic Western blot immunodetection kit were all from Invitrogen

(Carlsbad, CA, USA). Biotinylated *Galanthus nivalis* lectin (GNL) and peroxidase-labeled streptavidin were from Vector Laboratories (Burlin-game, CA, USA). Kodak Biomax MR autoradiography film was purchased from Fisher (Atlanta, GA, USA). MicroCon YM-3 filter (regenerated cellulose, 3000 MWCO) and Durapore 0.22 μm filter were purchased from Millipore (Billerica, MA, USA). Micro-BCA assay kit was from Pierce (Rockford, IL, USA). A Protein Pak 300 SW (7.5 × 300 mm) column and a Waters 626 LC system including a UV detector were used for the chromatography separations (Waters, Milford, MA, USA). An MGW Lauda RM6 circulating bath from Brinkmann (Westbury, NY, USA) was employed for temperature control. Empty PEEK-lined Omega columns with a volume of 0.1 ml were from Upchurch (Oak Harbor, WA, USA). An Alltech Adsorbosphere UHS C18 column (150 × 4.6 mm, 5 μm particle size, Alltech, Nicholasville, KY, USA) was used for reverse-phase chromatography (RP-HPLC). Instruments and accessories for mass spectrometric analysis are as follows: mass spectra were collected using a hybrid Linear Ion Trap Fourier Transform Ion Cyclotron Resonance (LTQ-FT-ICR) (Thermo Finnigan, San Jose, CA, USA); Nano-flow reverse phase chromatography was performed using a 75 μm i.d. PicoFrit capillary column (New Objective, Woburn, MA, USA), with a 5 μm C18 silica stationary phase (Agilent, Palo Alto, CA); PAL Autosampler (LEAP Technologies, Carrboro, NC, USA), custom built C18 OPTI-PAK trap cartridge (Optimize Technologies, Oregon City, OR, USA), 10 port switching valve (VICI, Houston, TX), and Chorus 220 nano-flow pump (CS Analytics, Zwingen, Switzerland) were used for online nanoLC-MS. HPLC-grade acetonitrile (ACN) used in MS analysis was purchased from Burdick and Jackson (Muskegon, MI, USA) while ACN for any other experiments was from Sigma. A Savant SpeedVac concentrator was provided by Thermo Fisher Scientific (Waltham, MA, USA).

Deglycosylated hIgG binding to HWRGWV

Human IgG was deglycosylated by incubating with 7500 units of PNGase F at a protein to enzyme ratio of 6 μg to 75 U in a total of 600 μl volume adjusted with phosphate buffered-saline (PBS, 10 mM phosphate buffer, 2.0 mM KCl and 138 mM NaCl, pH 7.4) for 8 h at 37°C, with the completeness of the reaction being checked by lectin blot. A parallel control experiment was carried out at the same conditions without PNGase F. The hIgG-PNGase F mixture was separated on a Protein Pak 300 SW size exclusion column (SEC) running by PBS at 0.5 ml/min. The collected deglycosylated hIgG from SEC was either directly used for isothermal adsorption measurements or concentrated down to 100 μl to be loaded to the HWRGWV column.

A volume of 100 μl PNGase F digestion solution or purified deglycosylated hIgG solution at a concentration of 1 mg/ml was loaded and eluted at the same conditions determined previously for the peptide HWRGWV column (Yang *et al*., 2005). Briefly, samples were loaded at a flow rate of 50 μl/min for 5 min, followed by flushing with another 3 ml PBS; the column was then washed in succession with 4 ml each of PBS + 0.5 M NaCl, pH 4 PB (phosphate buffer), and 2% acetic acid (AcOH) at a flow rate of 0.2 ml/min. The bound and unbound fractions were collected and analyzed by lectin blot analysis.

SDS–PAGE and lectin blot

Samples of PNGase F digestion and fractions from the HWRGWV column were separated on 4–12% Tris-Bis SDS–PAGE gels running in morpholinepropanesulfonic acid (MOPS) running buffer under reducing conditions. Pre-stained Seeblue plus2 protein marker was included to indicate the molecular weight of the proteins. Invitrolon PVDF membranes were employed to blot proteins separated by electrophoresis at a voltage of 100 V for 80 min. The blocking and washing solutions for the lectin blot detection were WesternBreeze blocking solution and WesternBreeze washing solution + 0.01% SDS, respectively. All the steps

executed later were at room temperature unless otherwise mentioned. Protein blotted membranes were blocked in 10 ml blocking solution for 1 h and then incubated with 10 ml of 2 μg/ml biotinylated GNL diluted in blocking solution overnight at 4°C. After washing in washing solution three times for 10 min each, membranes were incubated with 0.05 μg/ml peroxidase-labeled streptavidin for 1 h. After the same washing step, membranes were developed using ECL plus detection solution and exposed to a Kodak Biomax MR autoradiography film.

Isothermal adsorption measurement

Isotherms of deglycosylated and normal hIgG adsorption to HWRGWV resins were carried out in centrifugal filters (0.5 ml) with 0.22 μm Durapore membranes in duplicate. Four hundred microliters of protein solutions with concentrations ranging from 0.010 to 10 mg/ml in PBS were prepared by serial dilution. The most diluted protein solution was first added to the adsorption vessel containing 10 mg (in dry weight) resin washed and equilibrated in PBS after being pre-swelled in 20% methanol. The protein solution was incubated with resins in an orbital shaker for 2 h. After spin removal of the solution, the solution with the second to the lowest concentration (400 μl) was added and incubated with resins in the same way as mentioned. The procedure continued with all protein solutions from low to high IgG concentration. The unbound hIgG concentration in each supernatant was determined using a Micro-BCA assay. The accumulated amount of bound hIgG was calculated by mass balance. The data were fit to a Langmuir isotherm model

$$q=\frac{q_m C}{K_d+C} \quad (1)$$

where q, C, K_d, and q_m are the concentration of the bound protein (mg-protein/g-resin), the concentration of the free protein (mg-protein/ml-solution), the dissociation constant (mg/ml), and the maximum capacity (mg protein/g resin), respectively.

Ligand binding competition

Two approaches were employed to study the competition between Protein A or Protein G and HWRGWV in binding hIgG or its fragments. One method was to inject the mixture of Protein A and hIgG or its fragments in PBS to the HWRGWV column under the chromatographic conditions described above. The molar ratio of Protein A to IgG or its fragments was 2:1. The other approach was to load pure hIgG at a concentration of 1 mg/ml in PBS + 0.5 M NaCl to a HWRGWV column and then to perform a stepwise elution of the bound antibody with Protein A reconstituted in PBS + 0.5 M NaCl at 1, 2, 4, and 6 times the molar amount of injected hIgG. The column was finally cleaned with 2% AcOH. Similar experiments were also conducted by using Protein G at a molar ratio to IgG of 5:1.

Enzymatic fragmentation of hIgG and Fc

For limited proteolysis with pepsin, hIgG lyophilized powder was solubilized in 0.1 M citrate buffer at pH 4 and incubated with pepsin at a protein-to-enzyme ratio of 25:1 (w:w) at 37°C. At set times ranging from 30 min to 22 h, 24 μl samples were removed and added to a mixture of SDS–PAGE sample buffer (15 μl) and reducing agent (3 μl). The samples were immediately boiled for 10 min and then frozen for later use. Another set of 150 μl samples were removed and pepsin was inactivated by adding 220 μl 0.1 M Tris-HCl buffer at pH 8.4 for binding studies on an HWRGWV column. The same procedure was applied to the Fc fragment of hIgG.

For endoproteinase Lys-C and Glu-C cleavage, the Fc fragment was reconstituted in 0.1 M Tris-HCl buffer at pH 8.4 and incubated with the enzymes at 37°C for 24 h. The protein:enzyme ratios were 100:1 (w:w) for Lys-C and 50:1 for Glu-C, respectively.

Enzymatic digests binding to HWRGWV

One hundred microliters of inactivated pepsin digestion solution or the direct digestion solutions of all other enzymes were loaded to a 0.1 ml HWRGWV column at 0.05 ml/min, the flow rate then being increased to 0.1 ml/min after 6 min. The column was washed with loading buffer PBS for 20 min and bound proteins or fragments were eluted with 2% AcOH. Both flow through (FT) and elution peaks were collected and analyzed by SDS–PAGE as to be stated later.

RP-HPLC to separate the enzymatic digests

An Alltech Adsorbosphere UHS C18 column was used to separate the digests and fractions eluted from the HWRGWV column at ambient temperature. The solvents were as follows: solvent A: 0.1% (v:v) trifluoroacetic acid (TFA) in water; solvent B: 0.1% (v:v) TFA in ACN. The column was equilibrated with 100% A. Samples were loaded at 0.1 ml/min, and after 5 min the flow rate was increased to 0.5 ml/min and kept at this level for the rest of the run. The gradient was linearly increased to 27% B over 100 min, then to 100% B over 60 min, and subsequently held at 100% B for 10 min before being changed back to the initial condition. Chromatograms were monitored at 280 and 214 nm, respectively. The manually collected peaks were either directly resolved by SDS–PAGE or dried in a Savant SpeedVac and subsequently used for MS analysis.

To analyze the fragments generated by enzymatic digestions, 12% Tris-Bis SDS–PAGE gels loaded with target samples were run in MES running buffer under reducing or non-reducing conditions. Mark 12 non-staining protein marker was included to indicate the molecular weight of proteins and the gels were visualized by silver-staining. The target samples could be the digestions, the bound and unbound fractions from the HWRGWV column, or collected RP-HPLC peaks.

MS analysis of HWRGWV-bound fragments

All mass spectra were acquired on a hybrid LTQ-FT-ICR mass spectrometer in the positive-ion mode. Automatic gain control (AGC) was set to 1×10^6 and the capillary temperature was set at 250°C. Both direct infusion and nano-flow LC-MS/MS were utilized in this investigation. Reversed phase liquid chromatography was performed at room temperature using a 75 μm i.d. × 100 mm PicoFrit capillary column with a 15 μm emitter tip packed in-house with 5 μm C18 silica stationary phase. The mobile solvents A and B were respectively 2% ACN and 98% ACN in water with 0.2% formic acid as the ion pairing reagent in both. Samples were injected using a PAL Autosampler and over the course of 10 min trapped and washed on a custom built C8 OPTI-PAK trap cartridge with 100% A at 1 μl/min until a 10 port switching valve was triggered to move the sample in line with the gradient. Elution was carried out by a Chorus 220 nano-flow pump at 500 nl/min with the gradient as follows: 2% B for 6 min followed by a linear gradient from 2–55% in 30 min and then 55–59% B in 5 min.

Mass spectrometric data was utilized to determine peptide sequences by matching the known human IgG1 (hIgG1) amino acid sequence from a protein database (PDB: 2IG2) to the mass determined by the instrument. Expasy.org (http://ca.expasy.org/tools/findpept.html) was used to search the sequences that could match the fragments with molecular weight larger than 10 kDa within a mass measurement accuracy of 5 ppm. For smaller species, Bioworks software v2.1 (Beckman Coulter, US) was used for interrogation

of this data and each positive search result was confirmed by manual interpretation of the MS and MS/MS data.

Molecular modeling

The coordinate file for hIgG was obtained from the RCSB Protein Data Bank (PDB, 1FCC) (Sauereriksson *et al*., 1995). The coordinate file for peptide HWRGWV was generated using PYMOL (http://pymol.sourceforge.net). Molecular modeling was accomplished using the program HADDOCK (de Vries *et al*., 2007). Protein residues located in the predicted interaction interface were defined as "active". These assignments were based on MS data generated via LC-MS/MS peptide analysis of enzymatic digestions. All active residues exhibit a relative solvent accessibility >40% as delineated by the program NACCESS (Hubbard and Thornton, 1993). The following residues were defined as active for the hIgG molecule: 384–387, 389,390, 413–416, and 418–422. For each peptide all residues were classified active. The following residues in the protein were defined as being "passive" for the hIgG molecule (residues with solvent accessibility >40% surrounding active residues identified from MS data): 383, 388, 412, 417, and 423. Default HADDOCK parameters were used in the docking procedure. Final structures were grouped using a minimum cluster size of 4 and an RMSD <7.5 Å using the program ProFit (http://www.bioinf.org.uk/software/profit/). The resulting clusters were analyzed and ranked according to their average interaction energies.

RESULTS

Deglycosylated hIgG binding to HWRGWV

To examine the effect of glycosylation on hIgG binding to the HWRGWV peptide resin, hIgG was treated with endoglycosidase peptide:N-glycosidase F (PNGase F) and the deglycosylated hIgG was then applied to HWRGWV resins for binding assessment. A lectin blot was employed for glycosylated hIgG detection, where the glycosylated hIgG binds the biotinylated GNL, which can be visualized by chemiluminescence detection after labeling with peroxidase-conjugated streptavidin. In contrast, the deglycosylated IgG cannot be detected due to the lack of the carbohydrates that interact with the GNL.

The completion of the deglycosylation reaction after 8 h of incubation in PNGase F was checked by SDS–PAGE (Figure 2A) and lectin blot (Figure 2B) which were also employed for HWRGWV-bound and -unbound fraction analyses (lanes 6–8). Complete removal of glycans on the γ-chain by PNGase F led to a size shift of about 4 kDa in the IgG heavy chain (Figure 2A, lane 3), compared to the molecular weight of the heavy chain before the reaction (Figure 2A, lane 2). It also can be seen that the size of the heavy chain was not reduced for normal hIgG incubated at the same conditions without PNGase F (Figure 2A, lanes 4 and 5), indicating that the decrease of the heavy chain molecular weight in lane 3 was due to the action of PNGase F. Lectin blots (Figure 2B) verified the success of the deglycosylation where the heavy chain band in lane 3 disappeared due to the lack of glycans, while the heavy chains in all other lanes (2, 4, and 5) were still visible. Therefore, the deglycosylation reaction of hIgG was completed within 8 h of incubation with PNGase F.

Binding of deglycosylated hIgG to the HWRGWV column at a peptide density of 0.08 meq/g was investigated with either pure hIgG purified by SEC or the protein mixture with PNGase F. The chromatograms are shown in Figure 3 and the corresponding SDS–PAGE and lectin blot analyses are shown in Figure 2 (lanes 6–8). As shown in Figure 3, HWRGWV showed no difference in retention and release between the normal and deglycosylated hIgG, with the majority of IgG being eluted by pH 4 PB in a sharp peak (P2) and small amounts remaining in the FT and 0.5 M NaCl wash peaks. These are the same

chromatographic patterns observed for normal hIgG and hIgG incubated in PBS without PNGase F for 8 h at 37°C. The presence of the eluted deglycosylated hIgG with pH 4 PB was confirmed by the lectin blot analysis (Figure 2B, lane 6). It is also evident that PNGase F was not retained by the ligand column since it was present only in the FT (Figure 2A, lane 7) but not in the peak P2 (lane 8) when the IgG-PNGase F mixture was loaded directly.

The isotherm of deglycosylated hIgG adsorption on HWRGWV was found to overlap with that of normal hIgG (Figure 4). Deglycosylated hIgG was prepared by digestion of hIgG with PNGase F followed with SEC purification. To avoid excessive protein sample preparation, deglycosylated hIgG was only examined at the low concentration range. The similarity of the isotherms between deglycosylated and normal hIgG suggested the same dissociation constant for both IgG forms on HWRGWV, assuming the same binding capacity. These results were also confirmed by the chromatographic elution experiments, where it was impossible to separate the two forms of hIgG on HWRGWV using either a salt gradient or pH gradient (data not shown).

Competition of the peptide with Protein A or Protein G for binding hIgG

The possibility that HWRGWV binds to the consensus inter-C_H2-C_H3 interaction site on Fc was explored through competitive binding experiments with Protein A and Protein G. Competition between Protein A and the hexamer ligand HWRGWV for IgG binding was examined with two different approaches in a column format, where Protein A was in solution while the HWRGWV ligand was immobilized on Toyopearl AF-Amino 650 M. The first approach was aimed to investigate the ability of HWRGWV to retain a Protein A-hIgG complex. To do this, pure Protein A, as well as its mixtures with hIgG or hIgG fragments in PBS, were injected into the HWRGWV column (Figure 5). The ratio of Protein A to antibodies in the mixtures was 2:1. At this ratio Protein A is in great excess since the Protein A-hIgG binding stoichiometry in solution is approximately 1:3.3 as reported by Jungbauer and Hahn (2004). The stoichiometry of Protein A binding to Fab is unknown, although Protein A–Fab interactions have been confirmed in several different studies (Inganas *et al*., 1980; Sasso *et al*., 1991; Ljungberg *et al*., 1993; Potter *et al*., 1996). It is shown in Figure 5 that Protein A, when injected alone into the column, was non-specifically retained by the ligand column and could be washed off with 0.5 M NaCl in PBS. The salt wash peak was significantly smaller when Protein A was applied to the HWRGWV column in mixtures with antibodies, suggesting the association of Protein A with hIgG and its fragments prevents the Protein A from binding to the column. When the mixtures were injected, the majority of Fc and hIgG molecules were retained and eluted in pH 4 phosphate buffer together with Protein A; this is the same behavior as that of pure IgG on the HWRGWV column. The SDS–PAGE gel analysis confirmed that HWRGWV bound antibody-Protein A complexes were not eluted until using pH 4 PB (data not shown). Fab fragments in the Protein A mixture remained unbound (Figure 5), the same as when only the pure fragment was loaded to the column (Yang *et al*., 2005).

The second approach was to employ Protein A in PBS with 0.5 M NaCl as an elution agent for hIgG bound on the HWRGWV column (Figure 6). The solvent PBS + 0.5 M NaCl was used to prevent Protein A from binding to the HWRGWV resin. It was shown in the chromatogram (Figure 6) that the major amount of hIgG was eluted by the 2% AcOH elution step but not by Protein A which was applied in a large molar excess (6 times the amount of hIgG). When Protein A was applied in a stepwise fashion (data not shown), most of the IgG was also stripped only with the 2% AcOH elution.

Similar studies of both approaches were also performed using recombinant Protein G (data not shown). Similar to Protein A, Protein G had no influence on the retention patterns of hIgG and its fragments.

Pepsin digestion of hIgG

Pepsin was selected as a fragmentation enzyme to generate pFc fragments for HWRGWV binding study. Pepsin at pH 4 is known (Nezlin, 1998) to hydrolyze hIgG at the lower hinge region into a $F(ab')_2$ fragment and a Fc′ fragment which is further digested into a pFc fragment (C_H3 domain, 12 kDa) and numerous small pieces from the C_H2 domain. The optimal peptic incubation time was selected as 2 h since at this time point pepsin produced most fragments of about 12 kDa that can bind to the HWRGWV column. Human IgG was incubated with pepsin for 2 h at pH 4 before the digestion was neutralized and loaded to the HWRGWV column. Bound peptide fragments were eluted by 2% AcOH and analyzed by SDS–PAGE (Table 1). The HWRGWV-bound fraction was separated by RP-HPLC before MS analysis.

The RP-HPLC separation was completed on an Alltech Adsorbosphere UHS C18 column with a gradient of ACN. A distinct peak appeared at 116 min, which mainly contains fragments of 12 kDa (Table 1).

MS analysis found two species in the RP-HPLC peak of 116 min with molecular weights of 11.698 and 12.698 kDa, respectively corresponding to the sequences Leu79–Leu194 and Glu333–His435 of hIgG. The first species matches part of the Fab portion, and the second matches the pFc moiety of hIgG (Figure 7). The existence of the Glu333–His435 fragment suggests that HWRGWV may bind Fc through interactions with the residues within the pFc region. The detection of Leu79–Leu194 fragments was probably due to the ability of the HWRGWV ligand to bind a small amount of $F(ab')_2$ fragment (Yang *et al*., 2005).

Pepsin digestion of Fc

To eliminate the influence of Fab, Fc was investigated the same way as for hIgG and the results are listed in Table 1. Pepsin cleaves Fc into 2 major bands at 25 and 12 kDa, believed to be Fc′ and pFc fragments. The digest was injected into a HWRGWV column and the bound fraction was collected and then separated by RP-HPLC. A dominant peak at 120 min was identified on RP-HPLC and this peak was further analyzed by MS (Table 1). Two overlapping fragments were resolved, one being Lys334–Gly446 and the other Lys334–His435 with molecular weights of 12.730 and 11.732, respectively (Figure 7). The first fragment is the whole pFc fragment. The second one belongs to the first one and is only one amino acid different from the peptide detected in the peptic digest of hIgG. The finding of these two fragments confirmed the binding of HWRGWV to the pFc fragment of hIgG. The mass spectrometer did not detect the larger fragment Fc′, possibly due to the inherent biases in electrospray ionization toward smaller analytes if the Fc′ and other smaller detected peptides co-eluted. This problem was also encountered, to a different extent, when analyzing all other samples, where smaller fragments were detected but not the larger ones that were visible in SDS–PAGE gels.

Digestion of Fc with endoproteinases Lys-C and Glu-C

Fc fragments of hIgG were also treated by endoproteinases Lys-C and Glu-C to produce fragments different than those of peptic digests. Lys-C selectively cleaves the peptide bonds at the C-terminal of lysine and Glu-C breaks the bonds at the C-terminal of glutamic acid and aspartic acid residues (Birktoft & Breddam, 1994). After incubation of Fc individually with the endoproteinases, the digests were applied to the HWRGWV column. The HWRGWV-bound and unbound fractions were then separated by RP-HPLC before MS analysis. The RP-HPLC, SDS–PAGE, and MS results are also summarized in Table 1 and Figure 7.

An 11.228 kDa peptide was identified by MS in the HWRGWV-bound Lys-C digestion. The peptide was composed of the amino acid residues Lys334–His435 of hIgG, the same sequence found in the peptic digestion of Fc. This finding further confirmed the interactions between the pFc and the HWRGWV ligand. Some smaller fragments were also found, the sequence Phe275–Lys288 from the C_H2 domain, and sequences Gln362–Lys370 and Gly371–Lys392 located inside the larger fragments Lys334–His435. The finding of these small fragments opens up the possibility of narrowing down the binding site of HWRGWV on pFc.

In the case of the Glu-C digestion of Fc, both the HWRGWV-bound and unbound fractions were analyzed by MS. The unbound fraction was included to identify the fragments that may partially overlap with, and thereof shorten, the bound fragments. The MS was not able to detect any fragments larger than 3 kDa visible in the SDS–PAGE gel. Some small fragments were found: Leu234–Asp249, Leu234–Glu258, Val259–Asp268, and Gly281–Gln294 in P1; Val273–Gln283 and Lys334–Glu357 only in unbound but not bound fraction.

The attempt to utilize MS to reveal the HWRGWV-bound Fc digestion by cyanogen bromide (CNBr) was not successful. MS was also not able to reveal any peptic fragments generated by further digestion of the pFc fragment.

Molecular modeling

Preliminary modeling of the HWRGWV peptide/hIgG (1FCC) complex was accomplished with the program HADDOCK (de Vries *et al*., 2007). The peptide was constructed through PyMol and then energy minimized via GROMACS (Lindahl *et al*., 2001). The energy-minimized configuration of the peptide is found in Figure 8, where it is compared to a non-minimized structure. This energy-minimized configuration was then used to make the initial estimates of the interactions with different parts of the Fc fragment using HADDOCK. The power of the HADDOCK docking program is that the program generates the lowest energy structure(s) via a three-stage process: the first step involves an initial rigid-body docking assignment based on the protein crystal structure and the peptide energy-minimized configuration. This is then followed by a fully flexible assignment (allowing the ligand and target to sample multiple conformations) based on the 20% lowest energy structures from the rigid-body assignment. The final phase of the calculation involves the inclusion of a water refinement protocol (on the top 20% of the fully flexible assignment, again with full flexibility of the ligand and target) to solvate both the target and ligand (general information about the HADDOCK program can be found at: http://www.nmr.chem.uu.nl/haddock/). The binding regions on the Fc fragment were initially selected manually based on the MS data and were then varied randomly among all solvent accessible amino acids in the pFc portion of hIgG, to avoid creating a bias for the resulting structures. It was found that, despite the different binding regions selected, HADDOCK determined the majority of structures were bound to the same pocket of Asn384–Glu388 on Fc (Figure 9A). Furthermore, clustering analysis (based on RMSD calculations between the peptide and binding area) more than 50% of the structures were bound as shown in Figure 9A.

In the predicted binding models, amino acids Gln387–Asn389 are within reasonable interaction distances with the two N-terminal amino acids of the ligand (His1 and Trp2), and this is in line with the finding that the N-terminal amino acids play important role in binding hIgG (Yang *et al*., 2005). A few significant backbone hydrogen bond pairs are predicted by docking among the first four residues of the peptide: Glu388–His1 (amine terminal), Glu388–Trp2 (amide group), Gln386–Arg3 (carbonyl group), and Asn384–Gly4 (carbonyl group). More significantly, potential side chain hydrogen bonds are seen for the first 3 amino acids of the HWRGWV ligand: Asp413–His1 (imidazole ring), Pro387–Trp2 (amino group), and Gln419–Arg3 (δ-guanidino group) (Figure 9B). These amino acids on Fc are not

commonly found in any of the two consensus binding sites of Fc (Nezlin and Ghetie, 2004) and are also different from the amino acids found to interact with an RF which bound human IgG at the C-terminal of Fc (Duquerroy *et al*., 2007).

DISCUSSION

Several approaches were taken in order to determine the binding site of Fc for the HWRGWV ligand. First, hIgG was deglycosylated to see if the binding of IgG to the peptide was affected, where observed influences would suggest that HWRGWV binds near the hinge region or on carbohydrates of hIgG. Second, the binding of hIgG to the peptide column was investigated in the presence of Protein A or Protein G to determine if the peptide competes with these proteins for binding to the IgG molecule. If so, this would indicate that the peptide binds to the Protein A and Protein G binding sites at inter-C_H2-C_H3 domains. Third, enzyme digests of hIgG were adsorbed to HWRGWV columns and the resulting peptides were sequenced by MS to identify the amino acid sequences of HWRGWV-bound fragments. Finally, docking calculations were carried out to confirm the experimental results.

The carbohydrate moiety in the Fc portion had no influence on the interactions between the Fc-binding ligand HWRGWV and hIgG. Similar binding and elution patterns and binding affinity was observed on the HWRGWV ligand for both deglycosylated and normal hIgG. It is evident that the hexamer ligand HWRGWV does not bind to IgG through a lectin-like interaction. It is also possible to conclude that the ligand does not bind to the IgG through the hinge proximal region of the C_H2 domain. Otherwise, deglycosylation would have influenced the interactions between HWRGWV and IgG (Jefferis *et al*., 1998; Sondermann and Oosthuizen, 2002). The ability of the ligand HWRGWV to bind both normal and deglycosylated hIgG is similar to that of Protein A on which normal and carbohydrate-deficient IgG exhibited identical binding abilities (Nose and Wigzell, 1983).

Protein A and Protein G have no effect on hIgG binding to the HWRGWV ligand. Similarity of the chromatography patterns between pure IgG or its fragments and their mixtures with Protein A or G signifies that the adsorption of hIgG and its fragments on HWRGWV column are not influenced by the presence of Protein A or G in the feed streams. Protein A or G was not able to elute hIgG bound on HWRGWV, which indicates that Protein A or G is unable to break the interaction between hIgG and HWRGWV. The observation that Protein A or Protein G exited the peptide column always as the complex with antibodies suggests that HWRGWV does not affect the association between hIgG and Protein A or G. Therefore, a conclusion can be drawn that the interaction sites on hIgG for HWRGWV are different from those for Protein A and Protein G. The similar results obtained with Protein A and Protein G on HWRGWV columns might have been expected since both Protein A and Protein G are known to bind to IgG in a region localized around the junction of the C_H2 and C_H3 domains even though they may interact with different amino acids on Fc at this portion (Eliasson *et al*., 1989; Sauereriksson *et al*., 1995). The phenomenon that existence of antibodies-Protein A or G complexes from HWRGWV columns is understandable because Protein A or Protein G has a higher affinity for all antibody fragments than HWRGWV. For example, the binding affinity of Protein A for hIgG and its Fab fragment are about 10^8 M^{-1} and 10^5–10^6 M^{-1}, respectively (Gouda *et al*., 1998). The affinity of HWRGWV to hIgG is about 10^5 M^{-1} and a much smaller value is expected for the Fab fragment of hIgG (Yang *et al*., 2008).

It is evident that HWRGWV binds to the Fc fragment at a site different from the conventional hinge proximal or the inter-C_H2-C_H3 binding sites. To determine the potential site of hIgG to bind HWRGWV, MS, and MS/MS data were used to sequence the

HWRGWV-bound and unbound fragments of hIgG and Fc generated by enzymatic digestions. Three enzymes (pepsin, Glu-C, and Lys-C) were used for hIgG or Fc fragmentation to produce small fragments consisting of different amino acid residues.

Figure 7 aligns all the segments sequenced by MS that are within the Fc portion of hIgG. It is clearly shown that pFc (Lys334–Gly447) and its sub-fragments have been found in several digestions to be retained by the HWRGWV resins, indicating the interactions between the pFc and the HWRGWV ligand. Inside this portion, fragments Gln362–Lys370 and Gly371–Lys392 were individually found to interact with peptide ligand HWRGWV. As shown in Figure 10, the amino acids in the segment Gln362–Lys370 form a β-strand and all seven residues in the middle of this strand are fully buried inside the Fc 3-D structure. Therefore, the HWRGWV binding site on Fc is unlikely localized in this segment. In contrast, the Gly371–Lys392 portion of Fc contains a loop structure (Ser383–Asn389) that is exposed to the bulk solvent (Figure 10), and hence is able to be in contact with the ligand HWRGWV.

Docking calculation confirmed the binding of this loop (Ser383–Asn389) to the HWRGWV ligand. This binding cleft repeatedly showed the lowest energy of interaction between the ligand and the target (compared to anywhere else on the target) as well as the highest convergence (i.e., number of similar solved structures). However, it has been shown that in solution proteins exist as ensembles and therefore the conformation that is bound is not necessarily the one with the lowest energy. High energy conformers may bind and thereby be stabilized (Ma *et al*., 1999; Tsai *et al*., 1999a, Tsai *et al*., 1999b; Kumar *et al*., 2000; Boehr and Wright, 2008; Lange *et al*., 2008). Nevertheless, this prediction (Figure 9) is consistent with the experimental results that neither hydrophobic nor electrostatic interactions are a dominant force in binding (Yang *et al*., 2008), as well as the MS data provided here in this manuscript. C-terminal amino acids of the ligand in B are pointing outside of the binding pocket on Fc, which would allow for the ligand to be attached to chromatography beads, as was the case in our experimental studies.

Figure 11 displays the sequence alignment results generated using the Nomad multiple sequence alignment tool from Expasy (http://www.expasy.ch/tools/nomad.html). The 31 amino acid segment 362–392 from human IgG1 was compared with other subclasses of human IgG (Figure 11A) and IgGs from rabbit, bovine, goat, and mouse (Figure 11B). Column 2 in Figure 11 indicates the beginning and ending amino acid in all the sequences being compared, and the total number of amino acids in each molecule. The 362–392 fragment of hIgG1 was found by MS to bind HWRGWV, and it embraces the predicted binding loop SNGQPENN (shown in red in Figure 11). All the aligned IgG segments compared in Figure 11 are localized in the C_H3 domain with the start residue being the 86th amino acid from the C-terminal, approximately the same location of the 362–392 segment of hIgG1. The overall alignment scores from Nomad are in the range of 60–83 (the last column in Figure 11). The high degree of sequence homology among the segments suggests that there is a strong structural resemblance among these fragments. The amino acids of the predicted binding loop are in color to help illustrate the similarities between the same regions in comparison to hIgG1. As shown in Figure 11, the amino acids that interact with HWRGWV by HADDOCK simulation (Glu388, Pro387, Gln386, and Asn384) are highly conserved in the IgGs that are being compared. The conserved amino acid residues in the predicted binding loop indicate the similarity of the examined Igs in binding HWRGWV, which agrees very well with the previously published experimental results (Yang *et al*., 2005) showing that HWRGWV binds well to all of these IgG sub-classes and IgG from these other mammals. The sequences of human IgM and IgA were also compared with that of hIgG1 (Figure 11C) where the segments of 31 amino acids beginning with the 107th (for IgM) and 108th (for IgA) amino acids from the C-terminals were aligned with the hIgG1

362–392 segment. The alignment scores were around 62. As can be seen in Figure 11, there are some similarities among the IgG, IgM, and IgA segments, but the resemblance is not nearly as high as with the IgG sub-classes and the IgGs from various animals. In addition to the very large differences in quarternary structure among these molecules, these sequence differences might account for the differences found in the binding of HWRGWV to IgG compared to IgM and IgA (Yang *et al*., 2005). Both IgM and IgA seem to bind more strongly to HWRGWV resin, but it is not clear whether the observed binding is due to non-specific interactions or specific affinity adsorption. In summary, the predicted binding sequence obtained from HADDOCK, the MS experimental results, and the correlation between the sequence homology and experimental binding results with different molecules provides strong additional evidence that HWRGWV binds to the loop SNGQPENN localized in the C_H3 domain of IgG.

Other fragments detected by MS and localized in the hinge region and C_H2 domain are basically belong to two large segments Leu234–Asp265 and Val284–Gln294, considering both the bound and unbound peptide fragments (Figure 7). The former segment is in the hinge proximal portion and the latter localized on the opposite side of the complement C1q binding motif of hIgG (Asp270, Pro329, Pro331, and Lys322) (Idusogie *et al*., 2000). The interactions between HWRGWV and these two segments are less likely since removal of the carbohydrates on hIgG would otherwise influence its binding to HWRGWV. The appearance of the fragments Leu234–Asp265 and Val284–Gln294 in the HWRGWV-bound fraction might be due to non-specific interactions.

The combined data described here indicate the possibility that HWRGWV interacts with the pFc portion of hIgG. It is very likely that the active interaction region is the loop Ser383–Asn389 contained within the segment Gly371–Lys392. Protein A and Protein G make contact with amino acids mainly localized in the two loops formed by Leu251–Ser254 and Met428–Gln438 (Sauereriksson *et al*., 1995). Also, the contacting surface area on IgG for Protein A and Protein G does not cover the loop Ser383–Asn389 which is away from the C_H2-C_H3 interface and more close to the C-termini of the H chains (Figures 9A and 10). This might be the explanation for the non-competition in binding hIgG between HWRGWV and Protein G or Protein A.

CONCLUSIONS

It was found that the deglycosylated hIgG could be retained on the HWRGWV resin as well as the normal hIgG with similar affinities. It is hence concluded that the peptide ligand does not bind hIgG through a lectin-like interaction. It is also unlikely for HWRGWV to interact with hIgG through the sites that are affected by the degree of glycosylation of IgG, for example the hinge proximal region. Meanwhile, there is no competition observed between HWRGWV and Protein Aor Protein G in binding hIgG, indicating that HWRGWV does not bind to hIgG at the inter-C_H2-C_H3 site. HWRGWV has the ability to retain the pFc fragment of hIgG and may interact with the amino acid residues localized in the loop Ser383–Asn389. This loop is away from the C_H2-C_H3 interface and closer to the carboxyl termini of the hIgG heavy chains. The binding of the loop to HWRGWV is specific, involving several forces of hydrogen bonding, hydrophobic, and possibly electrostatic interactions. This finding might suggest a functionality site of antibodies that is different from the two consensus binding sites on Fc.

References

Birktoft, JJ.; Breddam, K. Proteolytic enzymes: serine and cysteine peptidases. In: Barrett, AJ., editor. Methods Enzymol. Vol. 244. Academic Press; San Diego: 1994. p. 114p. 126

Boehr DD, Wright PE. How do proteins interact? Science. 2008; 320:1429–1430.10.1126/science.1158818 [PubMed: 18556537]

Cendron AC, Wines BD, Brownlee RTC, Rarnsland PA, Pietersz GA, Hogarth PM. An Fc gamma RIIa-binding peptide that mimics the interaction between Fc gamma RIIa and IgG. Mol Immunol. 2008; 45:307–319. [PubMed: 17673295]

DeLano WL, Ultsch MH, de Vos AM, Wells JA. Convergent solutions to binding at a protein-protein interface. Science. 2000; 287:1279–1283. [PubMed: 10678837]

de Vries SJ, van Dijk ADJ, Krzeminski M, van Dijk M, Thureau A, Hsu V, Wassenaar T, Bonvin AMJJ. HADDOCK versus HADDOCK: new features and performance of HADDOCK2.0 on the CAPRI targets. Proteins: Struc Funct & Bioinformatic. 2007; 69:726–733.

Duquerroy S, Stura EA, Bressanelli S, Fabiane SM, Vaney MC, Beale D, Hamon M, Casali P, Rey FA, Sutton BJ, Taussig MJ. Crystal structure of a human autoimmune complex between IgM rheumatoid factor RF61 and lgG1 Fc reveals a novel epitope and evidence for affinity maturation. J Mol Biol. 2007; 368:1321–1331.10.1016/j.jmb.2007.02.085 [PubMed: 17395205]

Eliasson M, Andersson R, Olsson A, Wigzell H, Uhlen M. Differential IgG-binding characteristics of staphylococcal protein-A, streptococcal protein-G, and A chimeric protein A/G. J Immunol. 1989; 142:575–581. [PubMed: 2521350]

Gouda H, Shiraishi M, Takahashi H, Kato K, Torigoe H, Arata Y, Shimada I. NMR study of the interaction between the B domain of staphylococcal protein A and the Fc portion of immunoglobulin G. Biochemistry. 1998; 37:129–136. [PubMed: 9425032]

Hubbard, SJ.; Thornton, JM. Department of Biochemistry and Molecular Biology, University College; London: 1993.

Idusogie EE, Presta LG, Gazzano-Santoro H, Totpal K, Wong PY, Ultsch M, Meng YG, Mulkerrin MG. Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc. J Immunol. 2000; 164:4178–4184. [PubMed: 10754313]

Inganas M, Johansson SGO, Bennich HH. Interaction of human polyclonal IgE and IgG from different species with protein-A from Staphylococcus-Aureus—demonstration of protein-A-reactive sites located in the Fab′2 fragment of human-IgG. Scand J Immunol. 1980; 12:23–31. [PubMed: 6158089]

Jefferis R, Lund J, Goodall M. Recognition sites on human-IgG for Fc-gamma receptors—the role of glycosylation. Immunol Lett. 1995; 44:111–117. [PubMed: 7797239]

Jefferis R, Lund J, Pound JD. IgG-Fc-mediated effector functions: molecular definition of interaction sites for effector ligands and the role of glycosylation. Immunol Rev. 1998; 163:59–76. [PubMed: 9700502]

Lange OF, Lakomek NA, Fares C, Schroder GF, Walter KFA, Becker S, Meiler J, Grubmuller H, Griesinger C, de Groot BL. Recognition dynamics up to microseconds revealed from an RDC-derived ubiquitin ensemble in solution. Science. 2008; 320:1471–1475.10.1126/science.1157092 [PubMed: 18556554]

Lindahl E, Hess B, van der Spoel D. GROMACS 3.0: a package for molecular simulation and trajectory analysis. J Mol Model. 2001; 7:306–317.

Ljungberg UK, Jansson B, Niss U, Nilsson R, Sandberg BEB, Nilsson B. The interaction between different domains of staphylococcal protein-A and human polyclonal IgG, IgA, IgM, and F(ab′)2—separation of affinity from specificity. Mol Immunol. 1993; 30:1279–1285. [PubMed: 8413328]

Jungbauer A, Hahn R. Engineering protein A affinity chromatography. Curr Opin Drug Disc Dev. 2004; 7:248–256.

Kumar S, Ma B, Tsai CJ, Sinha N, Nussinov R. Folding and binding cascades: dynamic landscapes and population shifts. Protein Sci. 2000; 9:10–19. [PubMed: 10739242]

Ma BY, Kumar S, Tsai CJ, Nussinov R. Folding funnels and binding mechanisms. Protein Eng. 1999; 12:713–720. [PubMed: 10506280]

Nardella FA, Teller DC, Barber CV, Mannik M. IgG rheumatoid factors and straphylococcal protein-A bind to a common molecular site on IgG. J Exp Med. 1985; 162:1811–1824. [PubMed: 2415656]

Nezlin, R. Structure and Function. Academic Press; New York: 1998. The Immunoglobulins.

Nezlin R, Ghetie V. Interactions of immunoglobulins outside the antigen-combining site. Adv Immunol. 2004; 82:155–215. [PubMed: 14975257]

Nose M, Wigzell H. Biological significance of carbohydrate chains on monoclonal-antibodies. Proc Natl Acad Sci USA. 1983; 80:6632–6636. [PubMed: 6579549]

Potter KN, Li YC, Capra JD. Staphylococcal protein a simultaneously interacts with framework region 1, complementarity-determining region 2, and framework region 3 on human V(H)3-encoded Igs. J Immunol. 1996; 157:2982–2988. [PubMed: 8816406]

Radaev S, Sun P. Recognition of immunoglobulins by Fc gamma receptors. Mol Immunol. 2002; 38:1073–1083. [PubMed: 11955599]

Sarmay G, Lund J, Rozsnyay Z, Gergely J, Jefferis R. Mapping and comparison of the interaction sites on IgG responsible for triggering antibody dependent cellular cytotoxicity (ADCC) through types of human Fc-gamma receptor. Mol Immunol. 1992; 29:633–639. [PubMed: 1533898]

Sasso EH, Silverman GJ, Mannik M. Human-IgA and IgG F(ab′)2 that bind to staphylococcal protein-A belong to the VHIII-subgroup. J Immunol. 1991; 147:1877–1883. [PubMed: 1909733]

Sauereriksson AE, Kleywegt GJ, Uhl M, Jones TA. Crystal-structure of the C2 fragment of streptococcal protein-G in complex with the Fc domain of human-IgG. Structure. 1995; 3:265–278. [PubMed: 7788293]

Sondermann P, Oosthuizen V. X-ray crystallographic studies of IgG-Fc gamma receptor interactions. Biochem Soc Trans. 2002; 30:481–486. [PubMed: 12196119]

Sundberg EJ, Mariuzza RA. Luxury accommodations: the expanding role of structural plasticity in protein-protein interactions. Structure. 2000; 8:R137–142. [PubMed: 10903952]

Tsai CJ, Ma BY, Nussinov R. Folding and binding cascades: Shifts in energy landscapes. Proc Natl Acad Sci USA. 1999; 96:9970–9972. [PubMed: 10468538]

Tsai CJ, Kumar S, Ma BY, Nussinov R. Folding funnels, binding funnels, and protein function. Protein Sci. 1999; 8:1181–1190. [PubMed: 10386868]

Uray K, Medgyesi D, Hilbert A, Sarmay G, Gergely J, Hudecz F. Synthesis and receptor binding of IgG1 peptides derived from the IgG Fc region. J Mol Recognit. 2004; 17:95–105. [PubMed: 15027030]

Verdoliva A, Marasco D, De Capua A, Saporito A, Bellofiore P, Manfredi V, Fattorusso R, Pedone C, Ruvo M. A new ligand for immunoglobulin G subdomains by screening of a synthetic peptide library. Chembiochem. 2005; 6:1242–1253. [PubMed: 15937987]

Yang H, Gurgel PV, Carbonell RG. Hexamer peptide affinity resins that bind the Fc region of human immunoglobulin G. J Pept Res. 2005; 66:120–137. [PubMed: 16650069]

Yang H, Gurgel PV, Carbonell RG. Purification of human immunoglobulin G via Fc-specific small peptide ligand affinity chromatography. J Chromatogr A. 200810.1016/j.chroma.2008.12.004

Zhang GP, Guo JQ, Zhou JY, Wang XN, Li QM, Yang YY, Shen HG, Zhao D, Zhang H, Xi J, Wang L, Qiao SL, Jin X. Identification of the linear epitope for Fc-binding on the bovine IgG2 Fc receptor (boFc gamma 2R) using synthetic peptides. FEBS Lett. 2006; 580:1383–1390.10.1016/j.febslet.2006.01.060|0014-5793 [PubMed: 16457820]

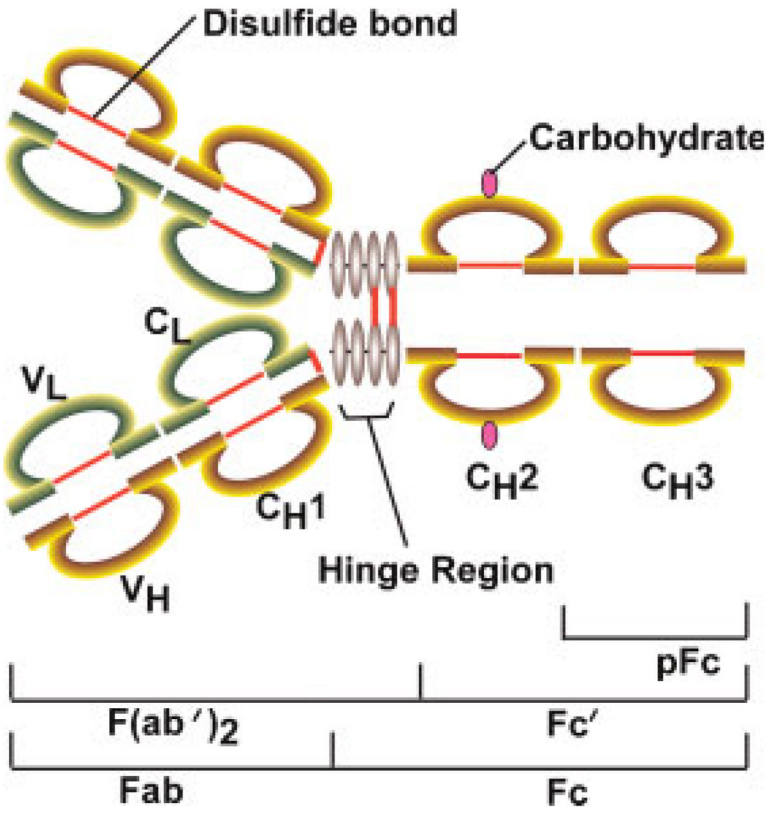

Figure 1.
Structural diagram of human IgG1. Green line: light chain; brown line: heavy chain. Each "C" like structure denotes one of the IgG domains. The enzymatic fragments Fab, Fc, F(ab′)$_2$, Fc′, and pFc are indicated below the diagram.

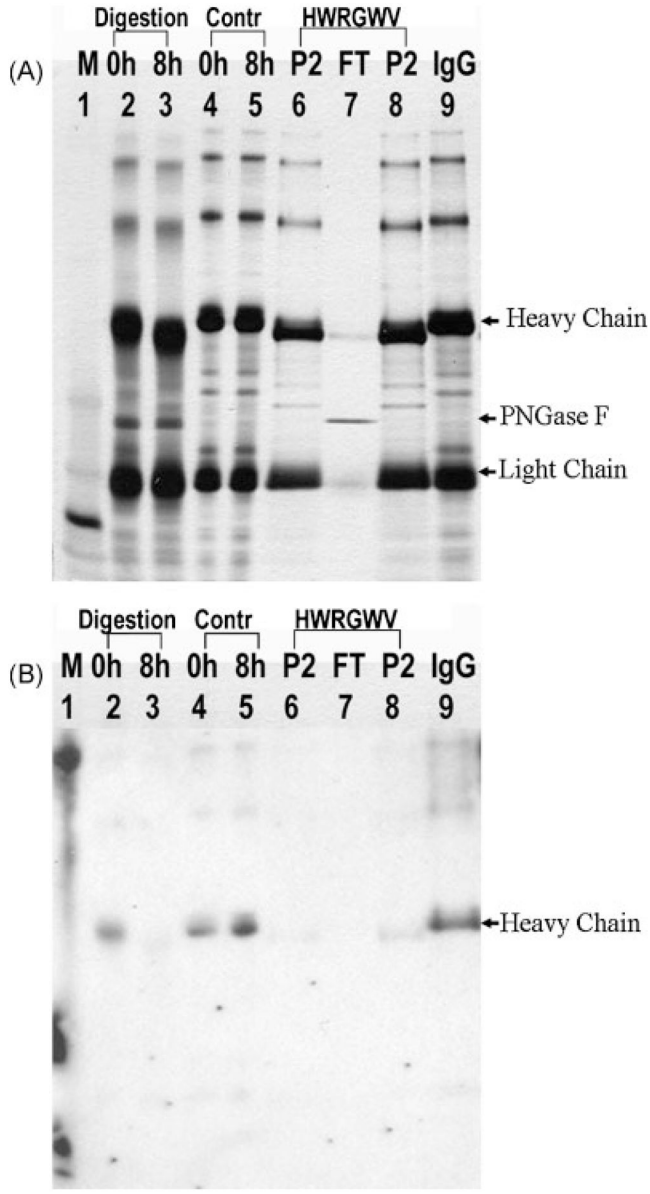

Figure 2.

Deglycosylated hIgG detection by (A) silver stained SDS–PAGE gel and (B) the corresponding lectin blot. The gel was run under reduced conditions. 0 h (lanes 2 and 4) and 8 h (lanes 3 and 5) indicate the hIgG incubation time in hours with (digestion) and without (contr) PNGase F. Lanes 1 (M) and 9 (IgG) are molecular marker and hIgG standard, respectively. Lanes 6–8 correspond to the peaks indicated in Figure 3. Specifically, lane 6 was the P2 from the HWRGWV column loading of SEC isolated deglycosylated hIgG while lanes 7 and 8 were from the direct loading of PNGase F digestion solution to the column.

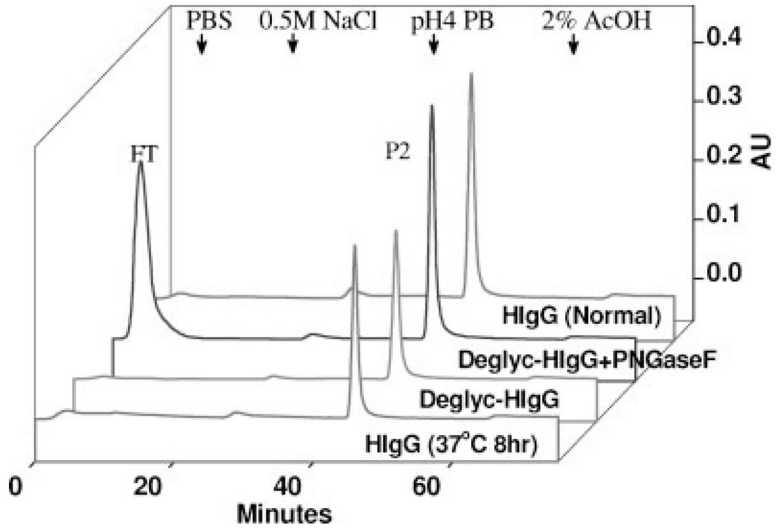

Figure 3.

Deglycosylated hIgG binding on HWRGWV column. Samples were loaded in PBS at pH 7.4, and sequentially washed for 20 min each with 0.5 M NaCl, pH 4 PB, and 2% acetic acid (AcOH). The flow rate was 0.2 ml/min. Both the column volume and the injection volume were 100 μl. The solutions of hIgG (normal), hIgG heated in PBS at 37°C for 8 h (37°C 8 h), the mixture of deglycosylated hIgG with PNGase F (Deglyc-hIgG + PNGaseF), and deglycosylated hIgG purified by SEC (Deglyc-hIgG) were separately loaded to the column. FT and P2 refer to the flow through and hIgG elution peaks from the HWRGWV column.

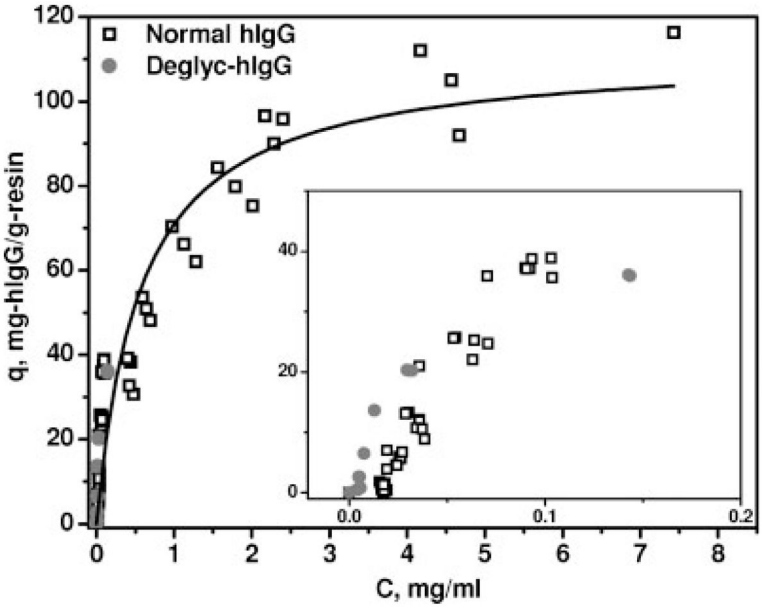

Figure 4.

Isotherms of normal and deglycosylated hIgG (Deglyc-hIgG) binding to HWRGWV resins with a ligand density of 0.08 meq/g. Symbols are the experimental data and the line is from the Langmuir fitting of normal hIgG adsorption data. The data at the origin area are enlarged in the insert.

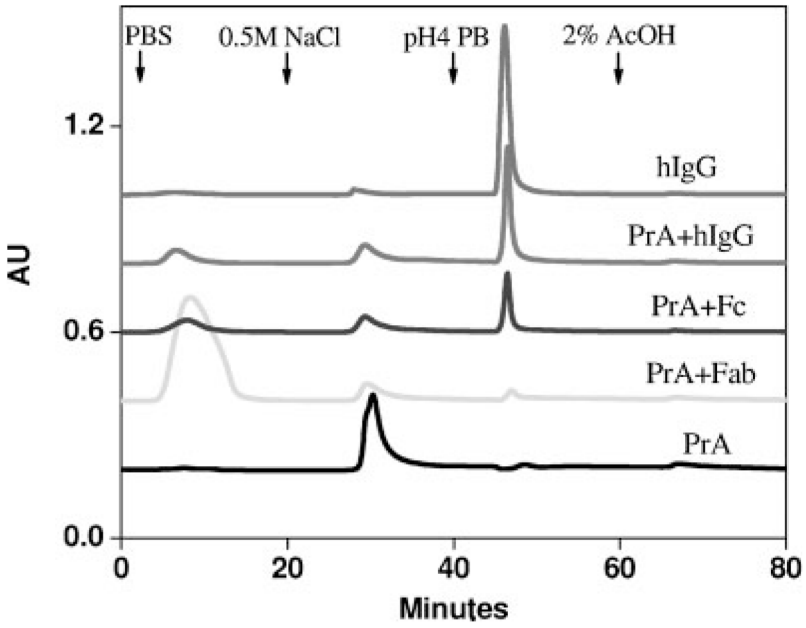

Figure 5.

Chromatograms of Protein A (PrA) mixtures with hIgG and its fragments binding on the HWRGWV column. The loading concentrations of pure Protein A and hIgG were 1 mg/ml. The molar ratio of Protein A to hIgG and its fragments was 2:1 and the concentrations of hIgG, Fc, and Fab in the mixtures were 1, 0.311, and 0.58 mg/ml, respectively.

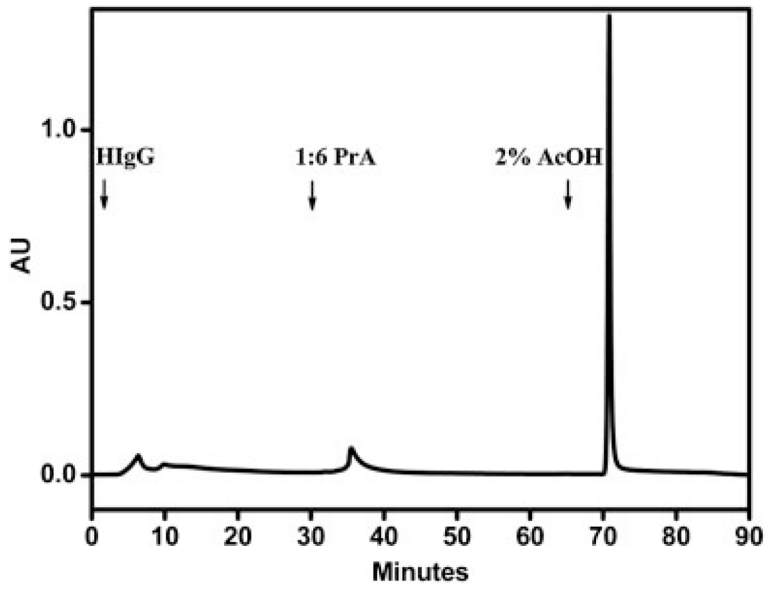

Figure 6.

Chromatogram of HWRGWV-bound hIgG elution by Protein A (PrA) and 2% AcOH. hIgG was injected to the column in PBS + 0.5 M NaCl, washed with PrA in the same buffer, and eluted by 2% AcOH in water. The concentrations of hIgG and Protein A were 1 and 1.68 mg/ml to form a hIgG:PrA molar ratio of 1:6.

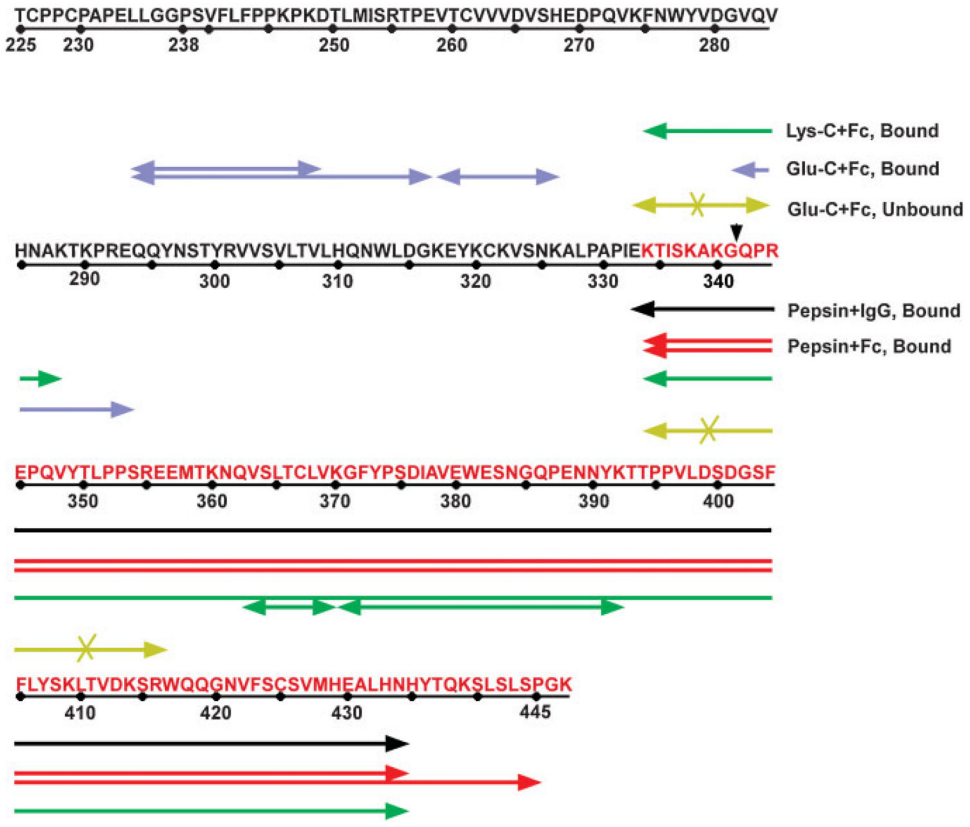

Figure 7.

Alignment of peptide segments obtained from proteinase cleavage of hIgG or its Fc fragment. The Fc sequence and numbering are from PDB 1FCC. HWRGWV- bound and unbound species of pepsin, Lys-C, and Glu-C digestions are indicated with arrowed lines of different colors. The black arrow between 341 and 342 residues indicates the separation point of C_H2 and C_H3 domains.

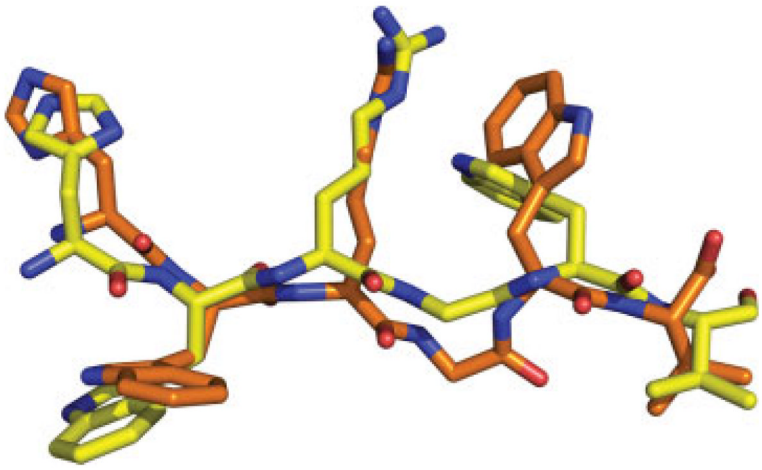

Figure 8.

Multiple conformations of the HWRGWV peptide. The peptide prior to energy minimization through GROMACS is shown as yellow (carbon residues are yellow, nitrogen residues are blue, oxygen residues are red) while the energy minimized structure is shown as orange (carbon residues are orange, nitrogen residues are blue, oxygen residues are red). Hydrogen atoms were removed for clarity, but where included for the docking protocol. The HADDOCK program performs its own energy minimization while allowing for complete flexibility of the peptide throughout the docking protocol, thus sampling all allowed conformations.

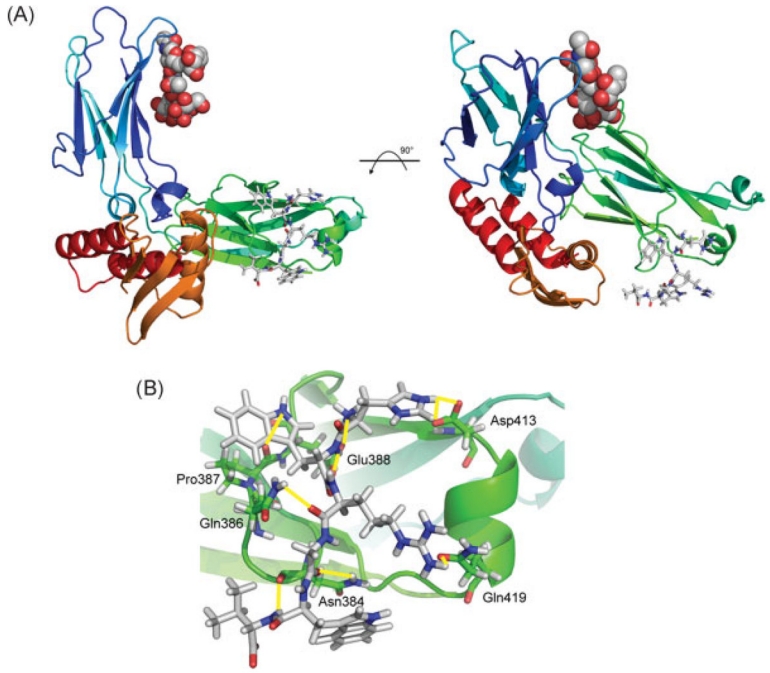

Figure 9.

(A) 3-D view and (B) ligand interaction schematic of HWRGWV binding to Fc of hIgG. In A, the Fc portion of hIgG is show in cartoon form and drawn blue to green. The space filled spheres are the glycosylation of the hIgG. Protein A (red cartoon) and Protein G (orange cartoon) are superimposed on the hIgG structure for clarity as to where the HWRGWV (shown as grey sticks) binding interaction with hIgG takes place. In B, the interaction plot depicts the HWRGWV (shown as grey sticks) binding interaction with the MS identified interaction site of Asn384–Glu388 (shown as green sticks). Possible hydrogen bonds are shown as yellow lines with hIgG residues labeled.

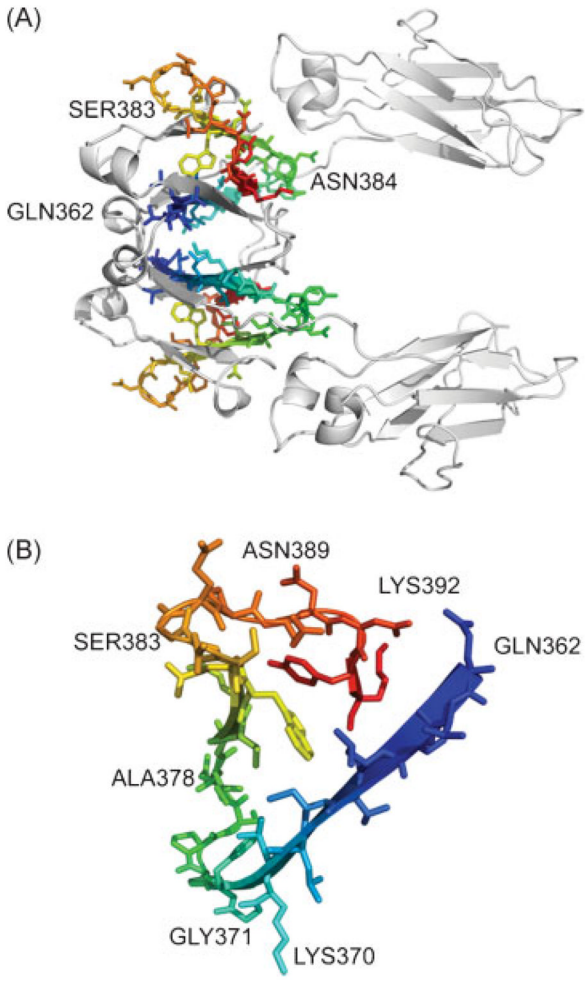

Figure 10.

(A) the position of segments Gln362–Lys370 and Gly371–Lys392 in the Fc portion of hIgG (PDB: 1FCC) and (B) solvent accessibility of the amino acid residues in these two segments. Strands and coils are depicted in blue and red, respectively. The backbones of the polypeptide chains other than in Gln362–Lys392 region are in gray; the amino acid residues in this region are colored according to the solvent accessibility where the color from dark blue to light blue to green attributes to the residues of fully buried to highly exposed.

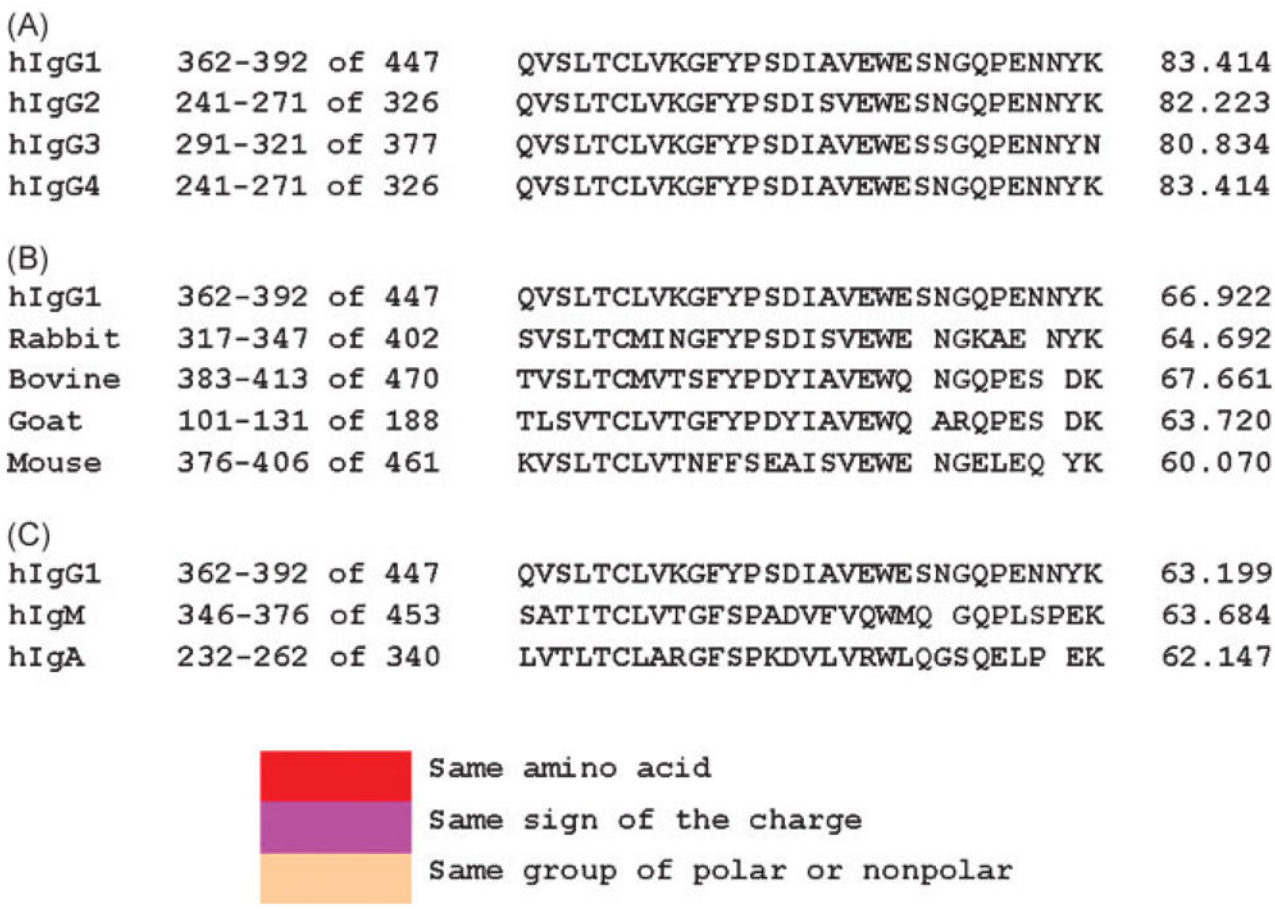

Figure 11.
Amino acid sequence alignments of the predicted HWRGWV binding site on human IgG1 with (A) other subclasses of human IgG, (B) IgGs of different species, and (C) IgM and IgA from human, calculated using NOMAD (http://www.expasy.ch/tools/nomad.html). Red amino acids indicate the loop on hIgG1 predicted to bind HWRGWV. The sequences are from NCBI (http://www.ncbi.nlm.nih.gov/protein): 230581(hIgG1), AAA64252.1 (Rabbit IgG), BAA04205.1 (Mouse IgG), CAA44699.1 (Bovine IgG), ABQ51194.1 (Goat IgG), P01859.2 (hIgG2), P01860.2 (hIgG3), AAB59394.1 (hIgG4), CAA47708.1 (human IgM), and AAB30803.1 (human IgA). All amino acid sequences are of heavy chains except for goat IgG which has only the constant region of the heavy chain.

Table 1

Analysis the HWRGWV-bound fractions of different digestion samples by RP-HPLC, SDS–PAGE, and MS

Enzyme	Pepsin	Pepsin	Lys-C	Glu-C
Digested protein	HIgG	Fc	Fc	Fc
MW of HWRGWV elution by SDS-PAGE, kDa	12, 25, 50	12, 25, 50	12, 25, 45, 50	12, 15, 25, 50
Retention time of dominant peak on RP-HPLC, min	116	120	120	120
MW of dominant RP-HPLC peak by SDS-PAGE, kDa	12	12, 25	12, ≥25	12, 15, ≥25
IgG fragment of dominant RP-HPLC peak speculated according to SDS-PAGE	pFc	pFc, Fc′	pFc, larger than Fc′ fragments	pFc, larger than Fc′ fragments
MS detected fragment	pFc, Leu79–Leu194	pFc	pFc, Gln362–Lys370, Gly371–Lys392	Small fragments
MW of MS detected fragments, kDa	12.698, 11.698	12.730, 11.732	11.228, <3	<3