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Further identification of human plasma glycoproteins interacting with the galactose-specific lectin Jacalin

Yannick Pilatte[a,*], Guy Rostoker[a], Karine Violleau[a], Milan Basta[b], Carl H. Hammer[b,1]

[a]*INSERM unité 139, Institut Mondor de Médecine Moléculaire (IM3) CHU H. Mondor, 94010 Créteil Cedex, France*
[b]*LCI/NIAID/NIH, Bethesda, MD 20892, USA*

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Abstract

In this report we show that Jacalin binds the heme-binding protein hemopexin and the C4b-binding protein sgp120 in human plasma. The interaction of Jacalin with hemopexin confirms that a single O-linked oligosaccharide is sufficient to mediate binding of a protein to this lectin. Retention of sgp120 by immobilized Jacalin demonstrated that this protein was O-glycosylated and, therefore, clearly different from another C4b-binding protein, the complement protein C2 which is physicochemically similar but exclusively N-glycosylated. In addition, Jacalin was also shown to bind several proteolytic enzymes which remain to be identified.

1. Introduction

By virtue of their property to specifically bind particular carbohydrate structures, lectins constitute valuable reagents for studying the carbohydrate moiety of glycoproteins [1]. On the one hand when used as soluble probes, they allow detailed structural analyses while, on the other hand, when immobilized on an insoluble matrix, they provide important tools for the purification of many glycoproteins. In this respect, Jacalin, a lectin extracted from Jack fruit (*Artocarpus integrifolia*) seeds and specific for the disaccharide Gal-β1$\rightarrow$3-GalNac, [2,3] has generated a great deal of interest.

Jacalin was first shown to bind IgA selectively along with a limited number of contaminating proteins from human plasma [4]. Later, it was reported that the specific interaction of Jacalin with IgA[2] was restricted to the subclass 1 [5,6]. Jacalin was also reported to bind other human plasma proteins such as C1-Inh [7,8], plasminogen [9] and Factor V [10]. Jacalin is now considered as a useful tool in the identification of proteins bearing O-linked glycan [9,11]. Finally, affinity chromatography on immobilized Jacalin

* Corresponding author.
[1] Current address: LMS/NIAID/NIH, Twinbrook II Facility, 12441 Parklawn Drive, Rockville, MD 20852, USA.

[2] Abbreviations used: C1-inh, C1-inhibitor; EDTA, ethylenediamine tetraacetic acid; IgA, immunoglobulin A; IgG, immunoglobulin G; NPGB, *p*-nitrophenyl-*p'*-guanido benzoate; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; sgp120, 120 kDa sialoglycoprotein characterized by its ability to bind complement component C4b; Tris, tris(hydroxymethyl)amino methane.

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has made the purification of IgA [4] and C1-Inh [8] much easier.

Although, there are still some conflicting results concerning the exact specificity of Jacalin [12], especially its interaction with IgD [13], this lectin has been used in clinical studies. Recently, Monestier et al., taking advantage of the ease of IgA1 purification by affinity chromatography on immobilized Jacalin, investigated the role of this class of immunoglobulin in IgA nephropathy [14]. On the other hand, Jacalin-based assays have been designed for the measurement of IgA1 concentration [15,16] and in some instances these assays have proven to be easier to perform and much more sensitive than classical ELISA methods [16]. However, the accuracy of such assays is limited in the case of plasma or serum, by the fact that, as mentioned above, Jacalin binds several proteins other than IgA [15,16].

Therefore, further identification of the human plasma proteins to which Jacalin binds would allow the use of a Jacalin-based assay in clinical studies requiring the quantitation of serum IgA1. This information would not only improve quantitation procedures but also provide important information about the glycosylation of newly identified ligands of Jacalin. In addition, given the selectivity and the ease of affinity chromatography on immobilized Jacalin there is little doubt that in the near future other plasma proteins will be purified by exploiting their interaction with this lectin. In this context, if we assume that contaminating proteins can be more easily eliminated once they have been identified, a more complete inventory of human plasma proteins interacting with Jacalin might be helpful in the development of new isolation procedures using this lectin.

With that in mind, we reasoned that it might be of interest to identify some of the plasma proteins that we found to interact with Jacalin during the elaboration of a procedure for C1-Inh purification published previously [8] and subsequent attempts to improve this procedure. Our observations are reported in the present paper.

2. Experimental

2.1. Reagents and materials

Immobilon, 0.45 μm polyvinylidene fluoride (PVDF), blotting membrane was purchased from Millipore Corporation (Bedford, MA, USA). Collodion bags were from Schleicher and Schuell (Keene, NH, USA). Gold-labeled goat anti-rabbit serum and silver enhancement kit were obtained from Jansen Life Sciences (Piscataway, NJ, USA). Jacalin and Jacalin-agarose were purchased from Vector Laboratories (Burningham, CA, USA) and Phenyl-Sepharose from Pharmacia fine Chemicals (Piscataway, NJ, USA). Sialidase from *Vibrio cholerae*, Peptide N-glycohydrolase F (PNGase F) from *Flavobacterium meningosepticum* and endo-α-N-acetylgalactosaminidase (O-glycan-peptide hydrolase or O-glycanase) from *Diplococcus pneumoniae* were from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA). Electrophoresis and Western blotting reagents were from Bio-Rad Laboratories (Richmond, CA, USA). S-2302 substrate was purchased from Helena Laboratories (Beaumont, TX, USA).

2.2. Antisera to isolated proteins

Rabbit IgG antihuman hemopexin was obtained from Nordic immunological labs (Capistrano Beach, CA, USA). Rabbit antiserum specific for human C1-Inh was prepared in our laboratory by injection of the highly purified protein [17] in Freund's complete adjuvant followed by repeated boosting with the antigen in incomplete adjuvant. A similar procedure was used to obtain a specific rabbit antiserum to human sgp120 [18].

2.3. Plasma preparation

Blood from normal healthy volunteers was collected in plastic tubes containing a 20 × stock mixture (3 ml/60 ml of blood) of protease inhibitors: *p*-nitrophenyl-*p'*-guanido-benzoate (NPGB, Sigma, St. Louis, MO, USA), ethylene-

diaminetetraacetic acid (EDTA, Sigma) and soybean trypsin inhibitor (SBTI, Sigma) to achieve concentrations of 25 μM, 10 mM and 50 μM, respectively. Plasmas were obtained by centrifugation of the blood.

2.4. Plasma fractionation

Plasma was fractionated as previously described [8] and the whole procedure was carried out at 4°C. Briefly, inhibitor-treated plasma was brought to a final concentration of polyethylene glycol 3350 (PEG 3350, J.T. Baker Chemical Co., Phillipsburg, NJ, USA) of 21.4% (w/v). The solid powder was added to the plasma with constant stirring and allowed to equilibrate for 1 h. The precipitate was removed by centrifugation in a Sorvall RC2-B centrifuge at 10 000 g for 30 min. The supernatant was then adjusted to a final concentration of 45% PEG 3350 (w/v), equilibrated for 1 h and centrifuged to recover the precipitated proteins.

2.5. Chromatographic procedures

Proteins were purified by using an FPLC system including two P-500 pumps and an MV-7 injection valve monitored by a LCC500 controller. Ion-exchange chromatography was performed on a prepacked HR 5/5 Mono Q column (Pharmacia) and hydrophobic interaction chromatography on a XK 16/20 column (Pharmacia) packed with 10 ml Phenyl-Sepharose. An alternative immobilized Jacalin column usable with the FPLC system was also prepared by coupling Jacalin to a 5-ml NHS-activated HiTrap column. A 30-mg amount of Jacalin was applied to the column as recommended by the manufacturer except that the coupling buffer was supplemented with the haptenic sugar mellibiose (50 mM) in order to protect the binding site of the lectin. Comparison of the optical density at 280 nm of the lectin solution before and after application to the column revealed that about 25 mg of Jacalin were coupled. In preliminary experiments, saturating amounts of 21.4–45% PEG were applied to both the HiTrap-Jacalin column and a 5-ml Jacalin-agarose column as previously described [8]. Protein quantitation and SDS-PAGE analysis of the material eluted from both columns by 0.125 mM mellibiose indicated that they had similar binding properties and capacity.

2.6. Electrophoretic procedures

Proteins were electrophoretically analyzed on 7.5% polyacrylamide discontinuous mini slab gels in a mini Protean II Cell (Bio-Rad) according to the method of Maizel [19].

Electrophoretic transfer of proteins from SDS-PAGE slab gels onto Immobilon membrane was performed in a mini transblot cell (Bio-Rad) for 1 h on ice at 250 mA. Prestained molecular mass markers (Bio-Rad) were used to assess transfer efficiency and determine the size of blotted proteins.

After transfer the Immobilon membranes were blocked overnight at room temperature with 5% (w/v) skimmed milk in 0.05 M Tris buffer pH 7.4 containing 0.15 M NaCl, 0.25 mM thimerosal, 10 mM EDTA and 0.05% Tween 20 (SM-T^{3}EBS) and then incubated with saturating amount of specific rabbit antiserum in SM-T^{3}EBS at 37°C for 2 h. After washing with SM-T^{3}EBS buffer, the membrane was incubated with gold-labeled goat antibodies to rabbit IgG for 2 h at 37°C, washed again and developed with the intense BL silver enhancement kit as described by the manufacturer.

2.7. Deglycosylation procedure

For desialylation, about 6 μg of protein were treated for 2 h at 37°C with 1 mU of *Vibrio cholerae* sialidase in 0.1 M acetate buffer pH 6.0. O-Glycanase is able to liberate the core structure Gal-β1$\rightarrow$3-GalNac from O-glycans providing that this disaccharide is not substituted by sialic acids [20]. Therefore in enzymatic deglycosylation using O-glycanase, 6 μg of protein were first desialylated as described above and subjected to an overnight incubation at 37°C after addition of 1 mU of O-glycanase to the incubation mixture. PNGase removal of N-linked oligosaccharide

chain requires usually reduction and denaturation of proteins to be complete [21]. Therefore, protein was denatured by boiling in 0.5% SDS and 10 m*M* β-mercaptoethanol, then about 6 μg of denatured protein were diluted 5 times in 0.25 *M* phosphate buffer pH 8.0, containing 25 μ*M* NPGB, 1 m*M* 1,10-phenanthroline, in order to obtain a final SDS concentration of 1 mg/ml. After sequential addition of Nonidet P-40 in a six-fold excess over SDS and 0.1 U of PNGase, the mixture was incubated overnight at 37°C. In experiments combining the three glycosidases, treatment with sialidase and O-glycanase was followed by heat denaturation in the presence of SDS and β-mercaptoethanol and then by overnight incubation with PNGase as described above.

2.8. Protease assay

The peptide *p*-nitroanilide substrate S-2302 (H-D-Pro-L-Phe-L-Arg-*p*NA) was used to detect proteolytic activities. Assays were performed in 0.05 *M* Tris buffer, pH 7.5, using a substrate concentration of 0.67 m*M*. The rate of *p*-nitroanilide hydrolysis was determined from the change in absorbance at 405 nm.

2.9. Protein assay

Proteins were quantified according to Bradford's method [22] using bovine serum albumin as standard.

3. Results

3.1. Jacalin binding to the C4b-binding protein sgp120 and proteolytic activities

Routine SDS-PAGE analysis performed during the course of several C1-Inh purifications consistently revealed the presence of several additional proteins in the material eluted from immobilized Jacalin (Fig. 1, I). Among the contaminating proteins, one displayed the same electrophoretic mobility corresponding to an M_r of 120 kDa as sgp120, a C4b-binding protein previously identified by some of us in human plasma [18]. In order to determine whether this contaminating protein was related to sgp120, several plasmas were subjected to PEG fractionation followed by affinity chromatography on immobilized Jacalin (either HiTrap-Jacalin or Jacalin-agarose) as recommended in the procedure for the isolation of C1-Inh [8]. Eluted

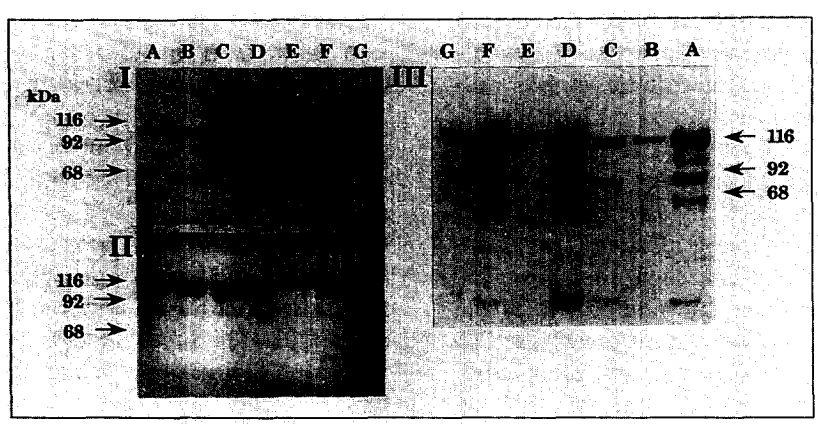

Fig. 1. Electrophoretic analysis of the material eluted from immobilized Jacalin after application of 21.4–45% PEG cut from several plasmas. Proteins were subjected to SDS-PAGE under non-reducing conditions and stained with Coomassie blue (I) or transferred to Immobilon PVDF membrane and probed with a monospecific rabbit anti C1-Inh (II) or a monospecific rabbit anti-sgp120 (III). A–G correspond to different plasmas.

proteins were then subjected to SDS-PAGE, electrophoretically blotted onto Immobilon and probed with rabbit antiserum specific for sgp120. As shown in Fig. 1 III, a band at 120 kDa was found in all tested samples. In addition, proteins with lower M_r were also labeled by the rabbit anti-human sgp120 in certain eluates. The electrophoretic mobilities of these bands were consistent with the pattern of sgp120 fragmentation observed during activation of the intrinsic coagulation pathway or incubation with purified kallikrein (Hammer et al. unpublished observation). Interestingly, sgp120 fragmentation was more pronounced when the material eluted from immobilized Jacalin was not immediately processed (data not shown) suggesting that proteolytic degradation occurred upon storage.

These observations prompted us to investigate whether the material retained by immobilized Jacalin contained proteolytic activities. Normal plasma (30 ml) was subjected to 21.4–45% PEG fractionation as described above and the last pellet was resolubilized in PBS and applied to the HiTrap-Jacalin column. After extensive washing, the retained proteins were selectively eluted with 0.125 *M* mellibiose in PBS. Fractions corresponding to the peak of eluted proteins were pooled and brought to 45% (w/v) by addition of the appropriate amount of PEG. After centrifugation the resulting pellet was resolubilized in 10 ml PBS and dialyzed extensively against PBS. The dialyzed Jacalin eluate was then assayed for protease activity by using the synthetic substrate S-2302 which is known to be suited for the detection of several enzymes of the intrinsic coagulation pathway [23]. Significant levels of proteolytic activity were found in the Jacalin eluate from all plasmas tested ($n = 7$). To determine whether this proteolytic activity was due to the presence of multiple enzymes, Jacalin eluate was extensively dialyzed against 20 m*M* Tris buffer pH 7.4 and applied to a HR 5/5 Mono Q column equilibrated with the same buffer. After extensive washing a linear gradient of NaCl (0–0.4 *M*) in the same buffer was applied to the column and every fraction was assayed for esterase activity against S-2302. This experiment was performed with three different

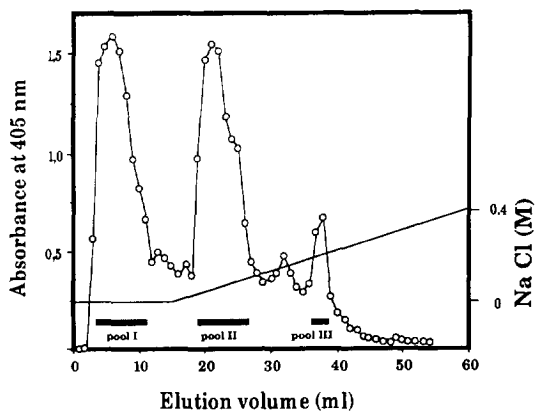

Fig. 2. Elution pattern for the Mono Q HR 5/5 column. The material eluted from the HiTrap-Jacalin column was chromatographed on a Mono Q HR 5/5 column as described in the results section. Fractions were collected at 1.0 ml/tube with a flow-rate of 1.0 ml/min. Proteolytic activity was assayed with S-2302 as described. Fractions shown by the solid bars were pooled.

plasmas and a typical experiment is depicted in Fig. 2. Three peaks of activity were observed, one in the drop-through and two in the gradient. Interestingly, a similar pattern of proteolytic activities was obtained when HiTrap-Jacalin was replaced by Jacalin-agarose (data not shown), indicating that retention of these proteases at this step depends exclusively on the binding specificity of the lectin. Although the same profile of activity was found for the three plasmas, differences in the relative intensity of these peaks was observed. Following regeneration of the HR 5/5 Mono Q column, the material corresponding to the excluded peak was rechromatographed under the same conditions and the activity against S-2302 was again found in the drop-through confirming that this activity did not arise from an initial saturation of the column. Each of the three pools corresponding to the three peaks of activity were prepared as indicated in Fig. 2 and brought to 45% (w/v) PEG. Precipitated proteins were pelleted by centrifugation and resolubilized in a minimum volume of PBS. The three samples were then individually subjected to gel permeation chromatography on a HR 10/16 Superose-12 column equilibrated in

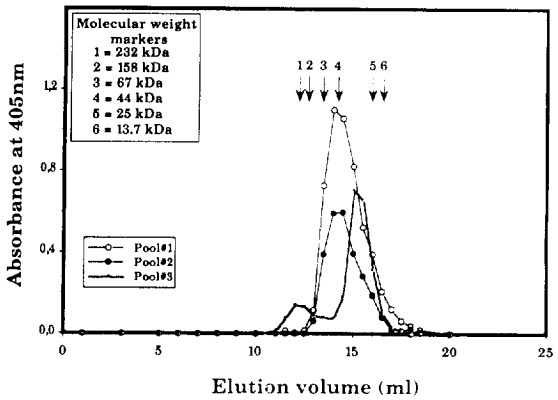

Fig. 3. Elution pattern for the HR 10/16 Superose-12 column. The three pools from the Mono Q column were concentrated by PEG precipitation (45%) followed by resolubilization in a minimal volume of PBS. An aliquot (200 μl) from each pool was subjected to size exclusion chromatography on a prepacked HR 10/16 Superose column equilibrated with the same buffer. Fractions were collected at 1.0 ml/tube with a flow-rate of 0.5 ml/min. Proteolytic activity was assayed with S-2302 as described.

PBS. As shown in Fig. 3, pools I an II showed similar elution with a single peak corresponding to a molecular mass of approximately 55 kDa. In contrast, pool III showed two peaks of activity which eluted at a volume corresponding to a molecular mass of 215 kDa for the minor one and 35 kDa for the major one. Whether these two peaks correspond to related activities or to different enzymes remains to be determined. However, it should be noted that this minor peak was not observed in the two other analyzed plasmas.

We then tried to determine whether these proteolytic activities were involved in the extensive cleavage of sgp120 that occurred during prolonged storage at 4°C of HiTrap-Jacalin eluates. For this purpose, purified sgp120 [18] and the three concentrated pools were extensively dialyzed against PBS. Then an aliquot of each pool corresponding to 5 μg of protein was incubated at room temperature with or without 5 μg of sgp120 in a final volume of 20 μl. After an overnight incubation, the various mixtures were analyzed by Western blotting using rabbit monospecific anti-sgp120. As shown in Fig. 4, the proteolytic activities contained in the three pools were all capable of cleaving sgp120. Pools I and II both cleaved sgp120 almost completely and yielded fragments of 65, 35 and 25 kDa (see Fig. 8, lanes C and E). A significant amount of intact sgp120 was still detectable after incubation with pool III and an additional fragment of 85 kDa was observed (see Fig. 8, lane G). The sizes of the fragments produced by the three pools were consistent with the cleavage pattern produced by kallikrein which cleaves sgp120 as follows: first a 35 and 85 kDa fragment are generated and then the 85 kDa fragment is further cleaved into a 25 and a 60 kDa fragment. Therefore, the presence of uncleaved sgp120 as well as the accumulation

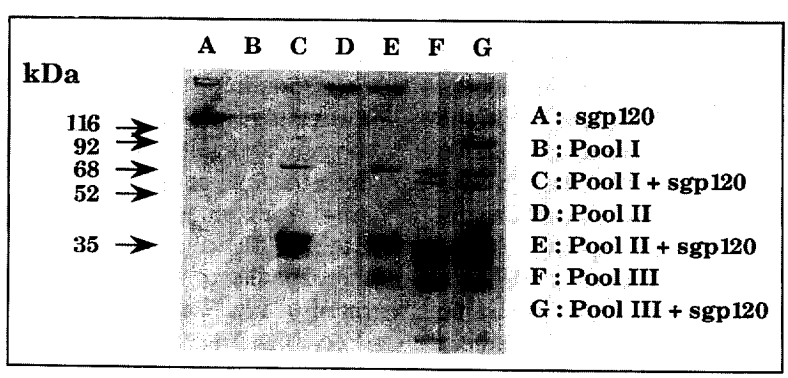

Fig. 4. The three pools containing proteolytic activities capable of cleaving sgp120. For each pool obtained after HiTrap-Jacalin and Mono Q, aliquots corresponding to 5 μg of protein were incubated at room temperature with or without 5 μg of pure sgp120. After an overnight incubation, the mixtures were analyzed by Western blotting using monospecific rabbit anti-sgp120.

of the 85 kDa fragment observed after incubation with pool III indicate that the proteolytic fragmentation of the protein was less efficient than with pools I and II. Moreover it can be seen that a significant amount of sgp120 was detected in a cleaved form in pool III.

3.2. Jacalin binding to hemopexin

In our initial procedure fractions containing C1-Inh were pooled after the two chromatographic steps and concentrated under vacuum using 75 kDa exclusion collodion bags [8]. Due to the limited capacity of the bags, this concentration step appeared to significantly slow down the whole procedure and to limit the possibility to purify C1-Inh simultaneously from several different plasmas. We therefore investigated whether 45% PEG precipitation followed by subsequent resolubilization in a minimal volume of buffer would constitute a suitable alternative procedure.

Based on immunochemical determination, both concentration procedures yielded a similar amount of CI-Inh (data not shown). It should be noted that, as reported previously [8], C1-Inh was significantly cleaved when purified from aged plasma (Fig. 5, lanes A, B and I) while no fragmentation was observed when plasma was processed immediately after collection (Fig. 5, lane II). Although PEG precipitation shortened considerably the whole procedure, the final product was repeatedly contaminated by a significant amount of another protein (Fig. 5). On SDS-PAGE this additional protein migrated with an electrophoretic mobility similar to that of serum albumin with an apparent M_r of about 58 kDa under non-reducing conditions and 68 kDa after reduction. However, unlike albumin, which is not glycosylated, this 58/68 kDa protein appeared to contain both N-linked and probably O-linked oligosaccharide side chains. As shown in Fig. 6, its electrophoretic mobility was significantly altered by enzymatic digestions with glycosidases. The molecular mass of the protein was reduced by 14.4 kDa after N-linked carbohydrate removal by PNGase (Fig. 6, lane D), by 7.4 kDa after sialidase treatment (Fig. 6, lane B) and by 8.6 kDa after combined sialidase and O-glycanase treatment (Fig. 6, lane C). Attempts to remove all carbohydrate side chains linked to the 58/68 kDa protein using the three glycosidases in combination resulted in an M_r reduction of 19.8 kDa (Fig. 6, lane E) which, if we assume that deglycosylation was complete, indicated a molecular mass of 48.2 kDa for the peptide backbone of this glycoprotein. Also note the presence in both samples treated with

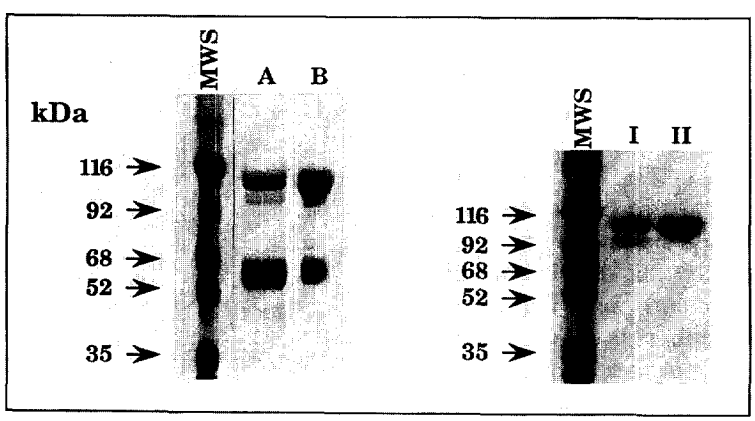

Fig. 5. SDS-PAGE analysis under non-reducing conditions of C1-Inh preparations. Left panel: C1-Inh has been purified as recommended in Ref. [8] except that the pools containing C1-Inh were concentrated by PEG precipitation followed by resolubilization in a minimal volume of buffer. Right panel: For comparison C1-Inh has been prepared according to the initial procedure. Concentration was performed under vacuum using 75 kDa exclusion collodion bags. In lane II, plasma was processed immediately after collection while in lanes A, B and I C1-Inh was purified from aged plasmas.

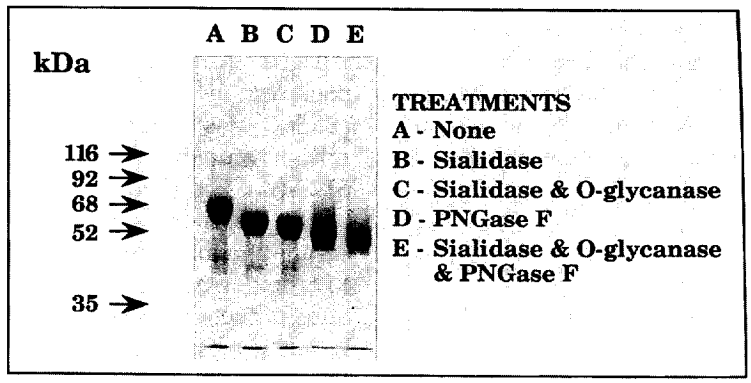

Fig. 6. Alteration of the electrophoretic mobility of the 58/68 kDa protein by treatment with various glycosidases. Equal amounts of the protein partially purified by size exclusion chromatography on Superose-12, were incubated with sialidase, O-glycanase or PNGase alone or in combination as described in the Experimental section and subjected to SDS-PAGE analysis under reducing conditions.

PNGase (Fig. 6, lane D and E) of a minor band which might represent a proteolytic degradation product of the deglycosylated protein, since PNGase preparations are known to be contaminated by trace proteases [21].

The 58/68 kDa protein was then separated from C1-Inh and obtained in a pure form by ion-exchange chromatography as follows. The product of the modified procedure for C1-Inh purification was dialyzed against 25 m*M* Tris buffer pH 7.4 and applied to a HR 5/5 Mono Q column equilibrated with the same buffer. Following application of the sample, the column was washed extensively and eluted with a linear salt gradient (0–0.4 *M* NaCl). SDS-PAGE analysis of the gradient fractions indicated that the 58/68 kDa protein was eluted at the beginning of the gradient and C1-Inh at the end with almost no overlapping. As shown in Fig. 7, SDS-PAGE analysis demonstrated that two pools containing the purified proteins could be prepared.

In an attempt to identify this purified protein, double diffusion experiments were carried out with specific antisera either prepared in our laboratory or obtained from various commercial sources. A total of 48 different human plasma proteins including most complement proteins and many acute phase reagents were tested. Among the antisera used in this study only one, specific for the heme-binding protein hemopexin, precipitated the purified 58/68 kDa protein. N-Terminal sequence analysis following Western blotting onto Immobilon PVDF membranes, which showed that the sequence of the first fifteen amino acids (data not shown) was the same as that reported for human hemopexin [24], definitively established the identity of the purified 58/68 kDa protein. The presence of hemopexin in Jacalin eluates from 21.4–45% PEG cut was confirmed for four different plas-

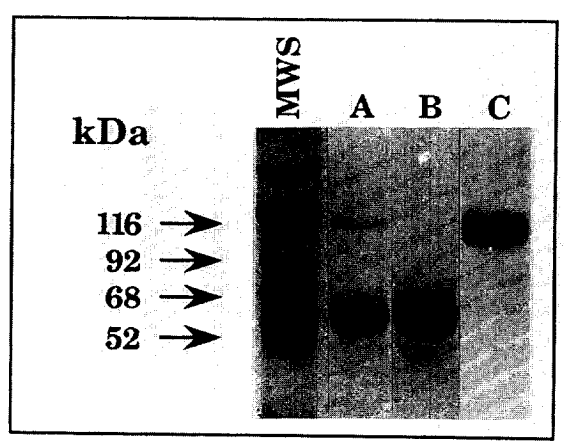

Fig. 7. Separation of the 58/68 kDa protein and C1-Inh by ion-exchange chromatography on Mono Q. The pools prepared with the two proteins after ion-exchange chromatography on Mono Q were analyzed by SDS-PAGE under reducing conditions. Lane A: C1-Inh prepared by the modified procedure (PEG precipitation). Lane B: 58/68 protein pool. Lane C: C1-Inh pool. MWS: Molecular mass standards.

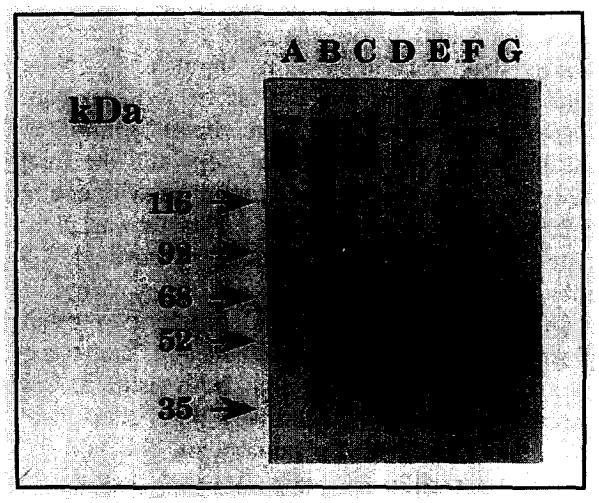

Fig. 8. Further confirmation that the 58/68 kDa protein was hemopexin. Normal plasmas (lanes A–C) and Jacalin eluates from 4 different 21.4–45% PEG cut (lanes D–G) were analyzed by Western blotting using monospecific rabbit anti hemopexin. The high-molecular-mass bands observed in plasmas are likely to be due to cross-reactivity with human IgG.

mas by Western blotting experiments using rabbit antisera specific for hemopexin (Fig. 8).

4. Discussion

In this study we have identified two other human plasma proteins which bind to Jacalin. One of these was a contaminant which appeared in the final product together with C1-Inh after the concentration protocol in the procedure for C1-Inh purification had been changed [8]. This protein, which exhibited an electrophoretic mobility in SDS-PAGE similar to that of albumin, was in fact a glycoprotein as assessed by its sensitivity to various glycosidases. We demonstrated that this protein was the heme-binding protein hemopexin by the Ouchterlony technique and this was definitively established by N-terminal sequence analysis. Retrospective analysis of the deglycosylation experiments also confirmed that the 58/68 kDa protein was hemopexin. As hemopexin, the 58/68 kDa protein contained N- and O-linked carbohydrates and the molecular masses determined for this protein after various deglycosylation treatments were consistent with the hemopexin carbohydrate composition [24]. In addition, a treatment combining sialidase, O-glycanase and PNGase F, which can remove both O- and N-linked carbohydrates, yielded a band with an approximate M_r of 48.2 kDa, a value similar to the molecular mass of 49.3 kDa calculated for the hemopexin peptide backbone on the basis of its amino acid composition [24]. Furthermore, our findings support the concept that Jacalin binds O-glycosylated proteins and that a single O-linked chain is sufficient to mediate binding [9,11] since hemopexin carries only one O-linked oligosaccharide chain [24].

Hemopexin could be easily separated from C1-Inh by ion-exchange chromatography and several milligrams of this protein were obtained in a pure form. Therefore one can expect that affinity chromatography on Jacalin-agarose would considerably improve available procedures for hemopexin isolation. Interestingly, the pink/red color characteristic for the presence of hemopexin–heme complexes was never observed in material eluted from HiTrap-Jacalin suggesting that the interaction of Jacalin with this protein may be restricted to its free form. Supporting this assumption is a recent report showing that binding of Fe by human apolactoferrin results in altered reactivity of this glycoprotein with plant lectins [25].

The other protein that we identified was repeatedly found in the material specifically eluted from the HiTrap-Jacalin column after application of plasma 21.4–45% PEG cut. This protein had an apparent M_r of 120 kDa and its electrophoretic mobility was not altered under reducing conditions. The similarity of this protein with sgp120, a C4b-binding protein previously isolated and characterized [18], prompted us to test the hypothesis that these two proteins might be the same molecule. Western blotting experiments and N-terminal sequence analysis revealed unambiguously that the 120 kDa protein retained by the HiTrap-Jacalin column was effectively sgp120. This information was of importance at two levels. First, it indicated that sgp120 was

O-glycosylated and, therefore, clearly different from another C4b-binding protein, the complement protein C2 which is physicochemically similar to sgp120 [18] but which is exclusively N-glycosylated [26]. Second, it allowed the development of a new isolation procedure for sgp120 giving a significantly better yield than the initial procedure (Y. Pilatte et al., in preparation).

We also demonstrated that the material specifically eluted from the HiTrap-Jacalin column contained three proteolytic activities capable of cleaving sgp120. The presence of proteolytic enzymes in Jacalin eluates was not surprising. This lectin was previously shown to bind plasma zymogens of the intrinsic coagulation pathway and the fibrinolytic system [9] which can be proteolytically converted into active enzymes capable of hydrolyzing S-2302 [23]. These proteolytic activities were probably responsible for the extensive fragmentation of sgp120 observed during prolonged storage of the eluates at 4°C as they generated fragment patterns similar to that produced by kallikrein (Hammer et al. unpublished observation). However, unlike this enzyme [27] they were not inhibited by C1-Inh which is present in high concentration in HiTrap-Jacalin eluates. Rather, they are likely to be responsible for the cleavage of C1-Inh occurring during storage observed by Donaldson and Falconieri [28] when they purified this molecule by the Jacalin-agarose affinity procedure [8]. In our hands, C1-Inh purified by this method was stable and highly active for months when stored at 4°C, with no evidence of degradation (see Ref. [8] and Fig. 5, lane II and Fig. 7). The inhibitor was purified in cleaved form only when plasmas were not processed immediately after collection (see Ref. [8] and Fig. 5, lanes A, B and I). This discrepancy might be related to the fact that these authors did not concentrate the pooled fractions containing C1-Inh after each of the two chromatographic steps. In our initial procedure, concentration was achieved under vacuum using 75 kDa exclusion collodion bags. At the end of the second concentration step, the purified C1-Inh was kept in the same collodion bag and extensively dialyzed against PBS. Given the relatively low molecular mass of the proteolytic activities found in the material eluted from the immobilized Jacalin column, significantly lower than the collodion bag cut-off, it is likely that these proteases might have been eliminated during the two concentration steps and the final dialysis. Along this line, we found no evidence of S-2302 hydrolysis when this substrate was incubated with several preparations of purified C1-Inh.

In conclusion, we have identified two other plasma proteins which bind to Jacalin: hemopexin and sgp120. The fact that both proteins are present in plasma at relatively high concentration, 0.4–1.5 mg/ml for hemopexin [24] and 0.3 mg/ml for sgp120 [18], suggests that they might significantly interfere in the Jacalin-based assay for the measurement of plasma IgA1. Furthermore, the confirmation that Jacalin binds proteolytic enzymes, which remain to be identified, indicates that biological reagents purified according to procedures including affinity chromatography on immobilized Jacalin must be used cautiously.

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