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Characterization of carbohydrate chains of C1-inhibitor and of desialylated C1-inhibitor

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Carbohydrate chains of C1-inhibitor were identified with a binding assay using different lectins. Lectins from *Sambucus nigra* (SNA) and *Maackia amurensis* (MAA) that are specific for sialic acids bound to C1-inhibitor. Lectin from *Datura stramonium* (DSA) reacted also with the inhibitor indicating complex and hybrid sugar structures. C1-inhibitor was enzymatically desialylated and reexamined for lectin binding. SNA and MAA did not react anymore, but in addition to DSA, peanut agglutinin, which can bind to carbohydrate chains after sialic acids are removed, bound to desialylated C1-inhibitor. C1-inhibitor contains about 30 sialic acid residues per molecule. SDS-polyacrylamide gel electrophoresis showed that desialylated C1-inhibitor had a faster mobility than native C1-inhibitor. The N-terminal sequence of desialylated C1-inhibitor was the same as of native C1-inhibitor and no change in the inhibition of human plasma kallikrein was observed.

C1-inhibitor; Neuramindase treatment; Sialic acid; Lectin; N-terminal sequence

1. INTRODUCTION

The serine proteinase inhibitor superfamily (serpins) consists of more than 40 homologous proteins with different functions. A recent review explains some characteristics of that family based upon the three-dimensional structure of the serpin member α_1-proteinase inhibitor [1]. α_1-proteinase inhibitor, α_1-antichymotrypsin, antithrombin III, and C1-inhibitor from human plasma were among the first purified members of the serpins [2]. These proteins function as inhibitors of serine proteinases from which the name serpin was coined in 1985 by Carrell and Travis [3]. The evolutionary relationship of the superfamily was earlier discovered by sequence comparison of ovalbumin, antithrombin III and α_1-proteinase inhibitor [4].

The C1-inhibitor features some differences when compared to other serpins. Firstly, the homology to other serpins does not begin with the N-terminal end of the inhibitor but rather at residue 120 [5]. Secondly, although all serpins from plasma seem to be glycosylated, some data indicate that the C1-inhibitor has a high carbohydrate moiety. The protein consists of 478 amino acids with a calculated molecular weight of 52,869 Da [5], but molecular weight determination using a SDS- polyacrylamide gel shows an apparent molecular weight of approximately 100,000 Da and using molecular sieve chromatography, C1-inhibitor is eluted at around 200,000 Da, regardless of whether a Sephadex resin [6] or a TSK-HPLC column (unpublished) is used. Furthermore, 13 glycosylation sites have been identified [5] and there are probably more.

In this paper we want to report some characteristics of the carbohydrate chains of the C1-inhibitor and the desialylated C1-inhibitor.

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2. MATERIALS AND METHODS

Human C1-inhibitor was purified according to Harrison [6]. Glycan differentiation kit, labelled antibodies against digoxigenin, and neuraminidase were purchased from Boehringer Mannheim, Germany. Human plasma kallikrein and antibodies against C1-inhibitor were from Medor, Herrsching, Germany. Substrates for kallikrein and neuraminidase were bought from Sigma, Munich, Germany. Chemicals for HPLC runs and for protein sequencing were purchased from Applied Biosystems, Weiterstadt, Germany. Rainbow markers were from Amersham, Braunschweig, Germany. All other reagents were either from Sigma or Merck, Darmstadt, Germany.

The concentration of human C1-inhibitor was calculated using $A_{280}^{1\%} = 3.6$ [6]. The concentration of human plasma kallikrein was estimated from the amount of the protein (1 U = 1/15 mg per vial) and a molecular weight of 98 kDa [7]. One unit human plasma kallikrein (specific activity according to the manufacturer: 15 U/mg kallikrein) was dissolved in 1 ml 0.1 M Tris buffer, pH 8.3 containing 0.005% Triton X-100 and the activity was determined with Bz-Pro-Phe-Arg-pNA (0.1 mM final concentration) as substrate. The activity (mU) was calculated from the absorbance change at 405 nm with $A_{405} = 8{,}800$ $M^{-1} \cdot cm^{-1}$ [8]. Inhibition of kallikrein was measured after preincubating 50 μl 0.1 M Tris buffer containing 0.005% Triton X-100, 50 μl of the dissolved human plasma kallikrein and different amounts of native and desialylated C1-inhibitor for 30 min. The reaction was stopped by diluting the mixture to 1 ml with Tris buffer and the residual human plasma kallikrein activity was immediately measured.

Neuraminidase activity was determined with *N*-acetylneuramine

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lactose as substrate. To 250 μl 0.1 M KP_i buffer, pH 5.0 containing 0.1% N_3^-, 5 μg neuraminidase from *Clostridium perfringens* (1 mg dissolved in 1 ml phosphate buffer) and 10 μl substrate (10 mg *N*-acetylneuraminlactose/ml H_2O) were added. The released sialic acid was determined according to Aminoff [9]. The activity of neuraminidase was 0.9 μg released sialic acid/5 μg neuraminidase per min.

Sialic acids were removed chemically from C1-inhibitor by hydrolysis with 0.1 M sulfuric acid for 90 min at 80°C. Neuraminidase treatment of C1-inhibitor was done by incubating 3–5 mg C1-inhibitor dissolved in 1 ml 0.1 M KP_i, pH 5.0, containing 0.1% N_3^- with 10–20 μl of neuraminidase from *Clostridium perfringens* (1 mg dissolved in 1 ml phosphate buffer) overnight at 37°C. Released sialic acids were quantitatively determined by taking aliquots of the reaction mixture and/or after separation of the sialic acids from C1-inhibitor by gel filtration.

SDS-PAGE was performed according to Laemmli [10] using a Bio-Rad Mini Protean II dual slab cell. Western blots were carried out as described by Towbin [11]. They were developed with digoxigenin-labelled lectins and peroxidase-labelled antibodies raised against digoxigenin [12]. The peroxidasae activity was visualized with H_2O_2/4-chloro-1-naphtol. Reversed-phase HPLC was performed using an Aquapore RP 300 microbore column (2.1 mm × 30 mm) and a 130 A HPLC system (Applied Biosystems). The volume of the injection loop was 2 ml. To elute the C1-inhibitor a linear gradient running from 0.1% TFA to 70% acetonitrile in 0.1% TFA in 45 min was applied immediately after injection. The column temperature was set at 50°C and the flow rate was 200 μl/min (with the 2 ml loop that we used, the gradient actually started 10 min later). Proteins were measured at 214 nm. Protein sequencing was done with an Applied Biosystems 473 A protein sequencer using the program supplied by the manufacturer. C1-inhibitors were desalted using either the above reversed-phase HPLC or extensive dialysis against water followed by lyophilisation.

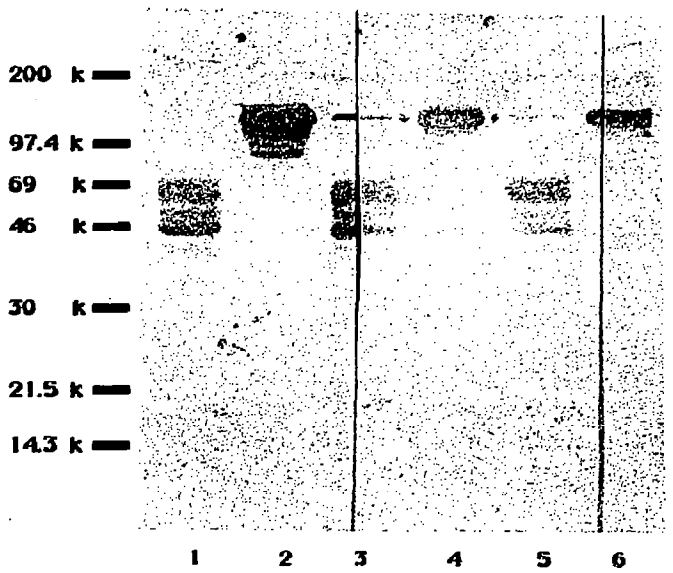

Fig. 1. Western blot analysis of C1-inhibitor with digoxigenin-labelled lectins. Lanes 1, 3 and 5, fetuin; lanes 2, 4 and 6, C1-inhibitor. Lanes 1 and 2 were incubated with digoxigenin-labelled *Sambucus nigra* agglutinin, lanes 3, 4 and 5 with *Maackia amurensis* agglutinin and lane 6 with *Datura stramonium* agglutinin. Molecular mass standards: myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.3 kDa). Fetuin was supplied with the glycan differentiation kit as control protein.

3. RESULTS

3.1. *Types of carbohydrate chains in C1-inhibitor*

In order to investigate what types of carbohydrate chains are linked to C1-inhibitor, the binding of C1-inhibitor to lectins was studied in Western blots, where the first antibody was replaced by different lectins that were labelled with digoxigenin. The lectins and their specificity used to differentiate the types of carbohydrate chains in C1-inhibitor are listed in Table I. Screening of C1-inhibitor with these lectins showed (Fig. 1), not unexpectedly, a positive reaction with SNA and MAA (lanes 2 and 4), the lectins specific for sialic acids [13]. Besides these two lectins, the lectin DSA (lane 6) produced also a positive reaction indicating the presence of complex and hybride sugar structures [13]. The other two lectins GNA and PNA did not bind to C1-inhibitor. The negative reaction with GNA suggests the lack of high mannose type sugars [13]. PNA can react with *O*-glycosidic bound carbohydrates, which are present in C1-inhibitor [5], but since sialic acids can prevent the binding of PNA to carbohydrate chains [13], a positive reaction should be expected after sialic acids have been removed from C1-inhibitor.

Table I

Lectins to identify carbohydrate types of glycoproteins

Lectin	Specificity
Galanthus nivalis agglutinin (GNA)	Mannose α(1–3)mannose Mannose α(1–6)mannose Mannose α(1–2)mannose (high mannose or hybrid type)
Sambucus nigra agglutinin (SNA)	Sialic acid α(2–6)galactose
Maackia amurensis agglutinin (MAA)	Sialic acid α(2–3)galactose
Peanut agglutinin (PNA)	Galactose β(1–3) *N*-acetyl-galactoseamine
Datura stramonium agglutinin (DSA)	Galactose β(1–4) *N*-acetyl-galactoseamine (complex and hybrid type)

3.2. *Removal of sialic acids from C1-inhibitor and their quantitative determination*

In an initial experiment, C1-inhibitor was incubated with different amounts of neuraminidase and the digestion was analyzed with the lectins SNA and MAA, which can distinguish between sialic acids linked via α(2–6) or via α(2–3) to galactose (see Table I). We had some indication that neuraminidase preferred the α(2–3) bond, because the lectin SNA still reacted with neuraminidase treated C1-inhibitor while the lectin MAA failed to do so. To remove all sialic acids an overnight incubation as described in section 2 was necessary, so neither MAA nor SNA gave a positive signal. The amount of sialic acid bound to C1-inhibitor was deter-

mined after separation of the released sialic acid using molecular sieve chromatography. 32–34 residues of sialic acids were bound to 1 molecule C1-inhibitor. This number is slightly higher than the 26 residues of sialic acid found after hydrolysis of C1-inhibitor in 0.1 M H_2SO_4 for 90 min.

3.3. *Characterization of desialylated C1-inhibitor*

3.3.1. Binding of lectins to desialylated C1-inhibitor

After sialic acid has been removed the reaction of C1-inhibitor with lectins (Table I) gave a different pattern. MAA (not shown) and SNA did not bind to C1-inhibitor anymore, DSA bound (not shown), but now PNA, which did not bind to the native C1-inhibitor, gave a strong reaction with desialylated C1-inhibitor (Fig. 2) confirming the presence of *O*-glycosidic linked sugars.

3.3.2. Chromatography of desialylated C1-inhibitor

Desialylated C1-inhibitor was applied to a reversed phase HPLC using a microbore column (Fig. 3B). Although negative charges have been removed and the desialylated C1-inhibitor is supposed to be more hydrophobic, it was eluted earlier than the native C1-inhibitor

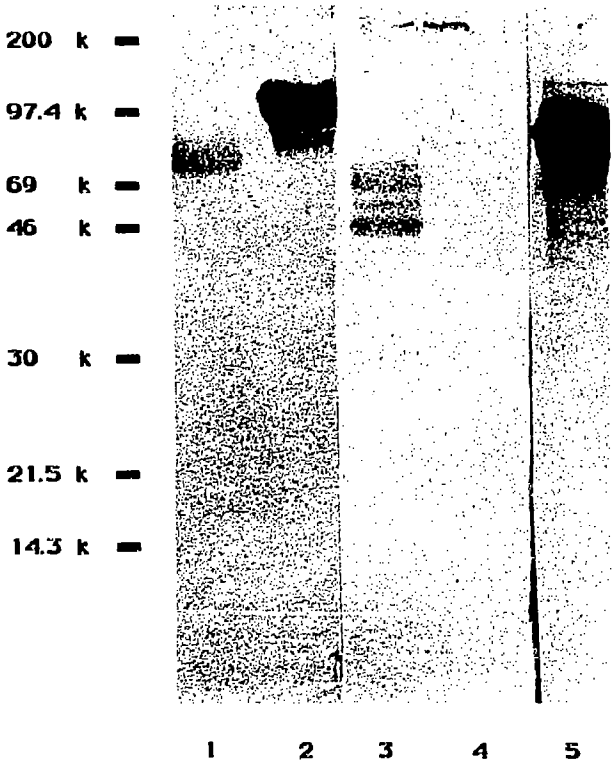

Fig. 2. Western blot analysis of desialylated C1-inhibitor with digoxigenin labelled lectins. Lane 1, transferrin; lane 2, native C1-inhibitor; lane 3, fetuin; lanes 4 and 5, desialylated C1-inhibitor. Lanes 1–4 were incubated with digoxigenin-labelled *Sambucus nigra* agglutinin, lane 5 with peanut agglutin. Transferrin and fetuin were supplied with the glycan differentiation kit as control proteins.

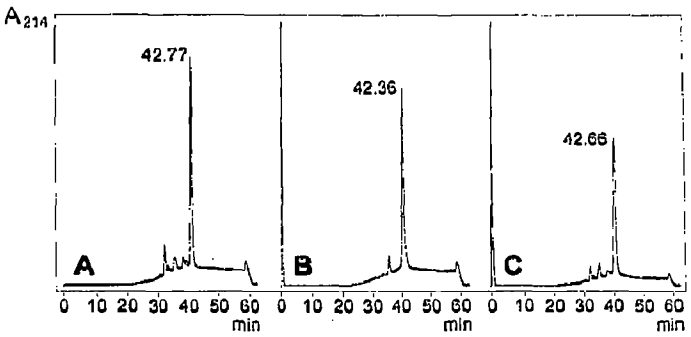

Fig. 3. Analysis of C1-inhibitor by reversed-phase HPLC. A, native C1-inhibitor; B, desialylated C1-inhibitor; C, mixture of native and desialylated C1-inhibitor.

(Fig. 3A). The retention time is only marginally faster, but this slight change was observed repeatedly with different preparations of C1-inhibitor after sialic acid had been removed. Under the experimental conditions used, the injection of both inhibitors together into the column did not result in any separation (Fig. 3C).

3.3.3. N-terminal characterization of desialylated C1-inhibitor

Comparison of lanes 2 and 5 in Fig. 2 shows that desialylated C1-inhibitor runs faster than native. This behaviour was also seen when C1-inhibitor was digested with various proteinases from different sources [14–17]. In the case of digestion with human skin chymase, a new N terminus was detected [14]. To show that no proteolytic digestion had occurred, the N-terminal sequences of native and desialylated C1-inhibitor, respectively, were determined. In both cases, we obtained the known N-terminal amino acid sequence of C1-inhibitor.

3.3.4. Inhibition of plasma kallikrein with desialylated C1-inhibitor

The inhibition of human plasma kallikrein with desialylated and with native C1-inhibitor are shown in Fig. 4. No difference in the inhibition ability of desialylated C1-inhibitor was observed, but an exact kinetic analysis has not yet been performed (see section 4).

4. DISCUSSION

From the serpins of human plasma, only the three-dimensional structures of α_1-proteinase inhibitor and α_1-antichymotrypsin have been described [18,19]. In both cases the P1–P'1 bond (Schechter and Berger notation [20]) of the inhibitors was cleaved which seems to facilitate the crystallisation of serpins. To have crystals of C1-inhibitor would be of considerable interest, because of the differences with other serpins. However, attempts to crystallize native C1-inhibitor have failed so far (M. Bauer and W. Bode, personal communication). Bruch et al. [21] treated C1-inhibitor with thermolysin

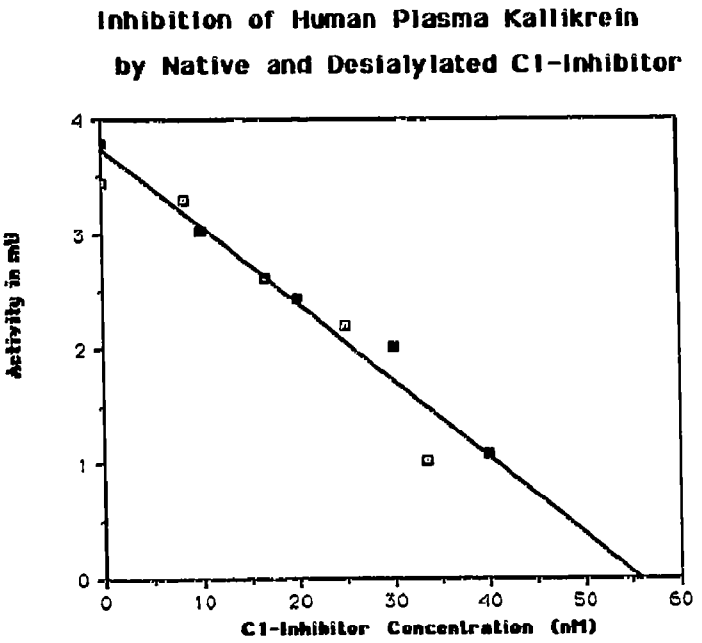

Fig. 4. Human plasma kallikrein inhibition by native (■) and desialylated (□) C1-inhibitor.

and showed with CD measurements that C1-inhibitor changed its structure to a more stable cleaved form. We also tried to obtain modified C1-inhibitor by thermolysin digestion. Although the SDS gel only showed two bands that were close together, N-terminal sequence analysis of the thermolysin-treated C1-inhibitor did not give an unambiguous result (unpublished).

Another reason for having difficulties to grow crystals of C1-inhibitor might be due to its high carbohydrate moiety. It is obvious that the method of choice for obtaining C1-inhibitor without carbohydrate chains would be to produce recombinant C1-inhibitor expressed in *E. coli*. Earlier we used an expression system that was successful in the case of α_1-antichymotrypsin [22]. C1-inhibitor was also expressed as indicated by Western blot analyses, but we could not detect any inhibitory activity against human plasma kallikrein (Rubin, H., Schoenberger, O.L., Wang, M., McLarney, S. and Cooperman, B.S., unpublished results). Thus as an alternative we considered removing the carbohydrate chains enzymatically.

We first screened C1-inhibitor with lectins and showed that sialic acid and complex and hybrid-type *N*-linked carbohydrate chains are present, whereas high-mannose type carbohydrates appeared to be absent. Neuraminidase treatment of C1-inhibitor removed all sialic acids, but we were not successful using endoglycosidase H and F digestion to remove the carbohydrate chains. In a recent report [23] other endoglycosidases, namely peptid-*N*-glycosidase F and *O*-glycosidase, were used. These enzymes seemed to have removed a large portion of the carbohydrate chains of C1-inhibitor as indicated by SDS-gel electrophoresis. However the authors did not further analyze to what extent the inhibitor was deglycosylated (they were only able to lower the molecular weight to 65,000 Da instead of the expected 53,000 Da).

Desialylated C1-inhibitor did not differ from native C1-inhibitor in that the N-terminal sequence was identical. This showed that the faster mobility of desialylated C1-inhibitor in the SDS gel was not caused by proteolytic digestion. In addition to the same sequence desialylated C1-inhibitor retained the inhibitory activity. Although the curves in Fig. 4 are superimposable, a small change in the association rate constant for the reaction of kallikrein with desialylated C1-inhibitor might be possible. The second-order rate constant for the inhibition of different proteinases with C1-inhibitor lies between 10^3 and 10^5 $M^{-1} \cdot s^{-1}$ [24–27] making the exact determination a complicated task, because usually only low amounts of the target enzymes are available. In addition to that, there is mounting evidence that serpins not only form an SDS-stable complex with their target enzymes, but that a relatively stable enzyme–inhibitor complex can occur [22,28–30] from which the reaction can branch leading to a stable SDS complex and to inactive inhibitor [22]. It was even discussed [31] that the protease–serpin complex could be reversible as it is known for Kunitz-and-Kazal type proteinase inhibitors [32–34]. Interestingly, this mechanism was considered for mutant C1-inhibitor expressed in eucaryotic COS cells [31]. Whether the carbohydrate part of C1-inhibitor influences the inhibition mechanism and the rate constant will be analyzed when, in addition to desialylated C1-inhibitor, deglycosylated C1-inhibitor is available.

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