C1-inhibitor potentiation by glycosaminoglycans
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Chapter 4

Recombinant human C1-Inhibitor produced in *Pichia pastoris* has the same inhibitory capacity as plasma C1-Inhibitor

Ineke Bos, Eric de Bruin, Yani Karuntu, Piet Modderman, Eric Eldering and Erik Hack

ABSTRACT

Therapeutic application of the serpin C1-Inhibitor (C1-Inh) in inflammatory diseases like sepsis, acute myocardial infarction and vascular leakage syndrome seems promising, but large doses may be required. Therefore, a high yield recombinant expression system for C1-Inh is very interesting. Earlier attempts to produce high levels of C1-Inh resulted in predominantly inactive C1-Inh. We describe the high yield expression of recombinant human C1-Inh (rhC1-Inh) in *Pichia pastoris*, with 180 mg/L active C1-Inh at maximum. On average 30 mg/L of 80-100 % active C1-Inh was obtained. Progress curves were used to study the interaction with C1s, kallikrein, coagulation factor XIIa and XIa, and demonstrated that rhC1-Inh had the same inhibitory capacity as plasma C1-Inh. Structural integrity, as monitored via heat stability, was comparable, despite differences in extent and nature of glycosylation. We conclude that the *P. pastoris* system is capable of high level production of functionally and structurally intact human C1-Inh.
The SERine Protease INhibitor (serpin) C1-Inhibitor (C1-Inh) is a major inhibitor of the classical pathway of complement and of the contact system. Thus C1-Inh is an inhibitor of several parts of the inflammatory reaction. Application of C1-Inh in inflammatory diseases like sepsis, acute myocardial infarction and vascular leakage syndrome seems a promising treatment (reviewed by Caliezi et al., ). However the inhibitory capacity of C1-Inh is weak in comparison with other serpins and thus large doses are required. The current production of C1-Inh purified from human plasma is not sufficient to allow application in the various potential therapeutical settings. An expression system for recombinant C1-Inh that provides active C1-Inh in large quantities is therefore very attractive.

C1-Inh is a plasma glycoprotein of 478 amino acids, and consists of a serpin domain and a unique N-terminal domain of 116 amino acids. Two disulphide bridges linking Cys101 to Cys266 and Cys100 to Cys183, connect the N-terminal and the C-terminal domain. The inhibitory members of the serpin family exist in a labile conformation, which is obligatory for full functional capacity. These structural constraints have hampered efficient production of recombinant serpins, as well as crystallisation studies. Several expression systems for C1-Inh have been described, but the production of active C1-Inh at high levels has not been established yet. This can be due to inefficient disulphide bond formation, inclusion body formation, the metastable active structure of serpins, or to low yield expression systems used in the past. The methylothrophic yeast \( P. \) pastoris can perform the post-translational modifications, including correct disulphide bond formation and glycosylation, required for the production of active C1-Inh, and easily be grown in large quantities. Expression levels between 0.15 until 15 g/L have been reached for expression of several mammalian proteins secreted by this yeast. Here, we report the expression of active C1-Inh in \( P. \) pastoris (strain GS115), providing a basis for large-scale production of recombinant human C1-Inh (rhC1-Inh). The method of progress curves for analysis of the kinetics of the interaction between C1-Inh and its target proteases C1s, kallikrein, factor Xla and factor XIIa facilitates a thorough comparison of plasma and rhC1-Inh. The interaction with kallikrein and factor Xla has not been studied before with progress curves. This method gives information about association as well as dissociation constants. Analysis with progress curves demonstrated full inhibitory capacity of rhC1-Inh. This is the first report that describes production of recombinant C1-Inh, a structurally labile protein, at high levels and in a fully active form.
MATERIALS AND METHODS

Materials
The C1-Inhibitor cDNA from a liver cDNA library has been described elsewhere. This gene is of the allotype with Met48→Val, and Glu165→Gln. Advantage DNA Polymerase was purchased from Clontech (Palo Alto, CA, USA). Restriction enzymes XhoI and NotI, T4 DNA ligase, Calf Intestinal Alkaline Phosphatase (CIAP), custom primers, Taq DNA Polymerase, the expression vectors pPIC9 and pPICZαA, the P. pastoris strain GS115, zeocin, the TA-cloning kit, NuPAGE gels and buffers, yeast extract, and peptone were purchased from Invitrogen Life Technologies (San Diego, CA, USA). Restriction enzymes Pmel and SalI, as well as EndoH were purchased from New England Biolabs (Beverly, MA, USA). Qiagen miniprep spin kit was purchased from Qiagen (Hilden, Germany). Yeast Nitrogen Base was purchased from Difco (Becton Dickinson and Company, Sparks, MD, USA). Biotin, carbenicillin, glycerol, and soy-bean-trypsin-inhibitor were purchased from Sigma Biochemicals (Steinheim, Germany). Methanol was purchased from Merck (Darmstadt, Germany).

Plasma-derived C1-Inh (Cetor) and intravenous immunoglobulins (IvIg) were obtained from Sanquin, division Plasma Products, located at the CLB (Amsterdam, the Netherlands). The proteases human Factor XIIa, XIIa and kallikrein were purchased from Kordia (Leiden, the Netherlands), C1s for kinetic assays was purchased from Calbiochem (La Jolla, CA, USA). Chromogenic substrates S2314, S2302 and S2366 were purchased from Chromogenix (Milano, Italy).

Biotinylated polyclonal rabbit antibodies against human C1-Inh, and the monoclonal antibodies (mAb) RII (against human C1-Inh), and KII (specific for the cleaved form of human C1-Inh) have been described before. C1s for ELISAs was purified and biotinylated (C1s-BT) as described before. Streptavidin coupled to polymerised horseradish peroxidase (poly-HRP) was obtained from the CLB (Dept. Immune Reagents). Streptavidin coupled to monomeric horse radish peroxidase was obtained from Amersham Pharmacia Biotech (Upsala, Sweden) as well as a MonoS HR5/5 column for cation exchange chromatography.

Expression of human C1-Inhibitor in P. pastoris GS115
C1-Inh cDNA was cloned in the expression vector pPICZαA by PCR. The pPICZαA vector is designed for secretion of the target protein into the supernatant by use of the α-factor signal sequence. Primers were designed with the XhoI site followed by the Kex13 cleavage site at the 5' end of the gene and with a NotI site after the stop codon at the 3' end of the gene. PCR was performed with the proof-reading enzyme Advantage DNA polymerase and the PCR product was extended with an overhanging 3' adenosine for cloning in the pCR2.1 vector of the TA-cloning kit according to the instructions of the manufacturer. The C1-Inh gene was cloned from the pCR2.1 vector in the pPICZαA vector and the sequence was verified with sequence analysis (Applied Biosystems, 377XL platform). Plasmid DNA was purified with the Qiagen miniprep spin kit and plasmid DNA was linearised with Pmel. GS115 cells were made electro-competent according to

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the protocol from Invitrogen. Briefly, cells were grown until OD600 between 1.3 and 1.5, centrifuged and washed with water for 4 times and with 1M sorbitol once. Electroporation was performed in 0.2-cm cuvettes with 400 Ω resistance, at 1500 V and a capacitance of 25 µF. Cells were plated on yeast-peptone-sorbitol plates with 100 µg/ml zeocin and grown for 3 days at 30°C. Single colonies were selected and grown overnight until OD600 was between 2 and 6 in buffered medium with glycerol (1% v/v), yeast and peptone (BMGY). An equivalent volume of OD600 of 1.0 was grown in buffered medium with methanol (0.5 % v/v), yeast and peptone (BMMY). The clone with the highest C1-Inh production was grown in batch culture for 3 days in BMMY. The supernatant of P. pastoris GS115 containing C1-Inh was dialysed against Phosphate Buffered Saline (PBS) immediately after harvesting and stored at -20°C.

**ELISAs**

C1-Inh antigen was detected with an ELISA modified from a radioimmunoassay, as described earlier. Functional human C1-Inh was detected as described before. Briefly, C1-Inh was bound to plates coated with mAb RII against human C1-Inh and detected with biotinylated C1s. Notably, this assay is an end-stage, and not a kinetic assay. In other words, it measures the number of functional C1-Inh molecules and not the kinetics of the interaction between C1s and C1-Inh. Cleaved C1-Inh was measured in an ELISA-system modified from a RIA using the monoclonal antibody KII that has been described before. This mAb reacts with neo-epitopes exposed after cleavage of C1-Inh and is thus specific for inactivated C1-Inh. PBS containing 0.1% (v/v) tween (PT) was used as the buffer for all incubation steps in all the ELISAs.

**SDS-PAGE**

Samples were diluted in Novex sample buffer and run on 4-12 % SDS-PAGE Novex gel in MOPS buffer (50 mM MOPS, 50 mM Tris, 0.1% SDS, 1 mM EDTA, pH 7.7) according to the instructions of the manufacturer (Invitrogen Life Technologies (San Diego, CA, USA)). Proteins were stained with standard Silver or Coomassie Brilliant Blue staining protocols.

**Purification of rhC1-Inh**

The supernatant of P. pastoris GS115 containing C1-Inh was dialysed against PBS, and diluted 1:3 (v/v) in 15 mM trisodiumcitrate. The pH was adjusted to 5.5 with citric acid. A MonoS HR5/5 column was equilibrated with 5 column volumes (cv) binding buffer (10 mM trisodiumcitrate, 50 mM NaCl, pH 5.5), 10 cv elution buffer (buffer10 mM trisodiumcitrate, 1 M NaCl pH 5.5), and 5 cv binding buffer. After sample application the column was washed with 15 cv of binding buffer, and with 10 cv 10 mM trisodiumcitrate, 211 mM NaCl pH 5.5. Elution was started by increasing the gradient from 211 mM NaCl to 715 mM NaCl in 10 cv. Immediately after purification the peak fractions were dialysed against 10 mM trisodiumcitrate, 70 mM NaCl, 2% saccharose, pH 7.0 (±0.2). After dialysis L-valine, L-alanine, and L-threonine were added as stabilisers,
and samples were stored at -80°C. Samples were analysed on SDS-PAGE for purity and in ELISA for quantification of C1-Inh.

**Complex formation of C1-Inhibitor with target proteases**
C1-Inhibitor (7 μM) was incubated with 7 μM target protease (C1s, kallikrein, or XIIa) for 1 hour. Complex formation was visualised on SDS-PAGE as described 29.

**Kinetic analysis of C1-Inhibitor**
The interaction of C1-Inhibitor with various target proteases was determined under pseudo-first order conditions with progress curves, when the rate of the inhibition is determined by the excess concentration of C1-Inh. The method of progress curves for the interaction between C1-Inh and C1s has been described before 30. Progress curves for the interaction with X1a, XIIa and kallikrein were set up in a similar way. In all experiments 5 concentrations of C1-Inh and an excess (at least 100-fold) of chromogenic substrate were added into wells of a 96-well plate. All solutions were prewarmed to 37°C. The reaction was started by addition of a fixed concentration of protease. Changes in absorbance at 405 nm were recorded at 37°C during 9 hours in an Anthos HTIII Titertek Reader (Labtech International Products, Ringer, East Sussex, UK). In pilot experiments it was determined that the wells of the plate had to be pre-incubated with human IgG (100 μl of 50 μg/ml per well) to prevent adsorption of the proteins onto the solid phase. Mineral oil was layered onto the solutions in the wells to prevent evaporation. Data were fitted to the integrated rate equation for slow-binding inhibition $A = v_s \cdot t + (v_0 - v_s) e^{-k_{\text{off}} t} / k' + A_0$ 31 by non-linear regression analysis in GraphPad Prism (GraphPad Prism 2.01 for Windows (GraphPad Software Inc, San Diego CA, www.graphpad.com)). Corrections were made for background hydrolysis when necessary. Fitting generated values for $k'$, $v_0$ and $v_s$. $K'$ was plotted against the inhibitor concentration [I]. The association ($k_{\text{on}}$) and dissociation ($k_{\text{off}}$) constants were calculated based on this plot according to the equation $k' = k_{\text{off}} + k_{\text{on}} [I] / (1 + [S]/k_m)$, where [S] is the substrate concentration and $k_m$ is the Michaelis Menten constant of the substrate with this specific protease. Every experiment was performed in triplicate and the association and dissociation constants were calculated from the plot of $k'$ versus [I] with triplicate values for each $k'$. Data were then analysed by linear regression-analysis of all 15 data-points, as performed with Microsoft Excel. An outlier-test was performed when the square of the Pearson product moment correlation coefficient was below 0.9 until a maximum of three outliers (2/9 ≤ 15). The experiment was discarded when more than three outliers were found.

**Heat stability of C1-Inhibitor**
C1-Inh (250 μg/ml) was incubated for 2 hours at various temperatures, centrifuged for 20 minutes at 10,000 g, whereafter C1-Inh antigen was quantified by ELISA. The amount of C1-Inh antigen is expressed as the percentage of the amount of antigen in identical samples that were kept at 4°C for 2 hours.
Deglycosylation of C1-Inhibitor
C1-Inh (1 µg at 20 µg/ml concentration) was denatured and subsequently incubated with 500U EndoH according to the instructions of the manufacturer to remove N-linked glycosylation.

RESULTS

Expression of C1-Inhibitor in P. pastoris GS115
GS115 transformed with pPICZαA-C1-Inh produced up to 180 mg/L of active C1-Inh, upon induction in batch culture for 4 days. After 3 days of yeast culture, the amount of C1-Inh antigen still increased while the amount of active C1-Inh decreased (figure 1). This was probably due to proteolytic cleavage of C1-Inh, as was determined in an ELISA with a mAb specific for cleaved C1-Inh (KII). All C1-Inh antigen was cleaved 7 days after induction. Expression levels were variable; 27 ± 8 mg/L of C1-Inh antigen (with 22 ±4 mg/L active C1-Inh, i.e. 82 ±17 % active) was obtained on average on day 3. Supernatant of the yeast cells grown for 3 days contained only 2 major proteins; C1-Inh and alcohol oxidase (figure 2b, lane 1). In contrast to plasma C1-Inh, rhC1-Inh appeared to be a smear on SDS-PAGE (figure 2b, lane 4 & 5).

Figure 1
Time curve of expression of C1-Inh, secreted in the supernatant of P. pastoris GS115. C1-Inh production was induced in P. pastoris GS115 pPICZαA C1-Inh in buffered medium with methanol, yeast extract and peptone. Samples from various time points were analysed with ELISA as described in material and methods. The experiment was performed 3 times with similar results.
Purification of rhC1-Inh

The supernatant of GS115, containing C1-Inh, was applied on a MonoS HR5/5 column in 10 mM trisodiumcitrate, 50 mM NaCl, pH 5.5, and eluted by increasing the salt concentration (figure 2a). Elution started at 324 mM NaCl. The start material contained C1-Inh and alcohol oxidase (aox); the flow-through and washout of the pichia supernatant contain only aox, as shown in figure 2b (lane 2). The fraction containing C1-Inh and trace amounts of aox was identified (figure 2b, lane 4). The purified preparation was enriched for active C1-Inh: 70% of C1-Inh was active in the starting material, while C1-Inh was for 100% active in the peak fractions and the small peak at 540 mM NaCl contained inactive C1-Inh (data not shown). We demonstrated with plasma C1-Inh that indeed active C1-Inh elutes at lower salt concentration than cleaved C1-Inh (data not shown).

Complex formation with target proteases

RhC1-Inh can form complexes with the target proteases C1s, kallikrein, and coagulation factor Xla (figure 3). RhC1-Inh has the same capacity to form complexes as plasma C1-Inh. We were not able to demonstrate SDS-resistant complexes of C1-Inh with coagulation factor Xla, also not with plasma C1-Inh.
Progress curves for the interaction of plasma C1-Inh and rhC1-Inh with target proteases
Km was determined prior to the pseudo-first order analysis according to the equation V = Vmax * S/ [Km + S] and is shown in table I. The system for analysis of the kinetics of the interaction of C1-Inh was set up with plasma C1-Inh (Cetor) under pseudo-first order conditions. The conditions for the different target proteases are summarised in table I. Substrate consumption was between 1.8 and 12 % in the presence of C1-Inh (table I).

Progress curves for plasma and rhC1-Inh with C1s, kallikrein, factor Xlla and factor XIa were determined. The interaction of plasma C1-Inh with kallikrein is shown in figure 4a as an example. Non-linear regression analysis resulted in the values for k', v0 and v00. The plot of k' versus [I] resulted in a straight line (figure 4 b) implying that the inhibitor interacted with the protease according to a simple one-step reversible reaction over the concentration range used in the experiments. Association and dissociation constants calculated from these data, are shown in table II.

Heat stability of C1-Inhibitor
The unfolding of serpins at elevated temperatures is considered a good indicator of the labile tertiary structure characteristic of this class of proteins. Consequently, heat stability of rhC1-Inh was compared with that of plasma C1-Inh. Detectable antigen levels remaining in solution, expressed as a percentage of the amount at 4°C, are depicted in figure 5. Importantly, rhC1-Inh displayed very similar heat stability compared to plasma C1-Inh, although there was a slight trend showing that rhC1-Inh is less stable at 45 and 50 °C. However, this difference was not statistically significant when the data were analysed with a one-way ANOVA with a multiple comparison post-test.
Table I
Conditions for kinetic analysis of the interaction of C1-Inh with the target proteases kallikrein, C1s, Xla and XIIa under pseudo-first-order conditions.

<table>
<thead>
<tr>
<th>Protease</th>
<th>[Protease]</th>
<th>Substrate</th>
<th>$K_m$ (nM)</th>
<th>[Substrate] (mM)</th>
<th>[C1-Inh] (nM)</th>
<th>Substrate consumption (%)</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kallikrein</td>
<td>0.025 nM</td>
<td>S2302 H-D-Pro-Phe-Arg-pNa</td>
<td>0.5</td>
<td>2.5</td>
<td>50 - 70 - 90 - 110 - 130</td>
<td>2.4</td>
<td>Phosphate buffered saline, 0.1 % Tween-20, 0.02% NaN3</td>
</tr>
<tr>
<td>C1s</td>
<td>1 nM</td>
<td>S2314 H-D-Val-Ser-Arg-pNa</td>
<td>1.3</td>
<td>3.5</td>
<td>10 - 15 - 20 - 25 - 30</td>
<td>1.8</td>
<td>10 mM NaPO4, 150 mM NaCl, 50 mM Tris pH 8.0, 0.1 % Tween-20, 0.02% NaN3</td>
</tr>
<tr>
<td>Xla</td>
<td>0.025 nM</td>
<td>S2366 pyroGlu-Pro-Arg-pNa</td>
<td>0.3</td>
<td>0.625</td>
<td>400 - 600 - 800 - 1000 - 1200</td>
<td>12</td>
<td>0.1 M Tris pH 7.4, 140 mM NaCl, 0.1 % Tween-20, 0.02% NaN3</td>
</tr>
<tr>
<td>XIIa</td>
<td>0.5 nM</td>
<td>S2302 H-D-Pro-Phe-Arg-pNa</td>
<td>0.2</td>
<td>1.25</td>
<td>60 - 100 - 140 - 180 - 220</td>
<td>7.1</td>
<td>Phosphate buffered saline, 0.1 % Tween-20, 0.02% NaN3</td>
</tr>
</tbody>
</table>

Table II;
$K_{on}$ and $K_{off}$ of plasma C1-Inh and rhC1-Inh for 4 target proteases

<table>
<thead>
<tr>
<th>Protease</th>
<th>Literature</th>
<th>$K_{on}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$K_{on}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$K_{off}$</th>
<th>$K_{off}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1s</td>
<td>30</td>
<td>4.8 ±(0.6) * 10$^4$</td>
<td>6.2±(0.4)*10$^4$</td>
<td>3.1*10$^{-5}$</td>
<td>6.3±(0.4)*10$^4$</td>
</tr>
<tr>
<td>Kallikrein</td>
<td>39</td>
<td>2.55 * 10$^4$</td>
<td>7.8±(0.4)*10$^3$</td>
<td>6.5*10$^{-6}$</td>
<td>8.2±(1.6)*10$^3$</td>
</tr>
<tr>
<td>Xia</td>
<td>39</td>
<td>1.8 * 10$^3$</td>
<td>3.9±(0.3)*10$^3$</td>
<td>5.7*10$^{-5}$</td>
<td>4.5±(0.3)*10$^2$</td>
</tr>
<tr>
<td>XIIa</td>
<td>39</td>
<td>9 * 10$^3$</td>
<td>4.5±(0.3)*10$^3$</td>
<td>3.0*10$^{-5}$</td>
<td>5.7±(0.2)*10$^3$</td>
</tr>
</tbody>
</table>

- $K_{on}$ is depicted as mean ± standard error
- All experiments were performed three times in triplicate, except factor XIIa and plasma C1-Inh (4 times in triplicate) and kallikrein with rhC1-Inh (2 times in triplicate).
Figure 4
**Pseudo-first order analysis of the interaction of C1-Inh with target proteases;** Progress curves for kallikrein with plasma C1-Inh (a). Plot of $K_{ob}$ versus $[I]$ for C1s, kallikrein, factor Xla, and factor XIIa (b). C1-Inh, substrate and protease were combined in a 96-well plate and substrate conversion was measured at 405 nm. Concentrations of C1-Inh, substrate and protease are shown in table I. These curves are representative for 3 experiments in triplicate.

Figure 5
**Heat stability of rhC1-Inh compared with plasma C1-Inh.** C1-Inh (250 µg/ml) was incubated for 2 hours at various temperatures. Samples were centrifuged at 10000 g for 20 minutes, concentration of C1-Inh antigen was measured in ELISA and expressed as the percentage of antigen concentration in samples kept at 4°C. (For rhC1-Inh and plasma C1-Inh n=4, for cleaved plasma C1-Inh n=1).
Glycosylation of C1-Inh
EndoH digestion of rhC1-Inh resulted in two clear bands in SDS-PAGE analysis (figure 6, lane 1a and 1b). These two bands represented intact and cleaved C1-Inh, as demonstrated with trypsin digested C1-Inh (figure 6 lane 1b and 1c). After EndoH treatment C1-Inh was apparently still glycosylated via its O-linked glycosylation sites, because the sugar groups were stained on a ConA blot (data not shown). Carbohydrate groups from plasma C1-Inh could not be removed with EndoH (lane 2a and 2b). These data indicated that rhC1-Inh, in contrast to plasma C1-Inh which is homogeneously glycosylated, was heterogeneously glycosylated on its N-linked glycosylation sites.

**DISCUSSION AND CONCLUSIONS**
In this study we demonstrated that active recombinant C1-Inh, a serpin in a metastable conformation, can be expressed in *P. pastoris* at levels higher than described for other expression systems 15. RhC1-Inh had the same inhibitory capacity as plasma C1-Inh; comparable association and dissociation constants were found in progress curves. On the other hand, we observed somewhat less complexes of kallikrein-rhC1-Inh compared to kallikrein-plasmaC1-Inh on SDS-PAGE (figure 3). This may be explained by a more rapid denaturation and subsequent dissociation of complexed rhC1-Inh. The functional activity of either preparation towards kallikrein was similar (table II). This suggests that the smaller amount of complexes with rhC1-Inh may have been due to increased sensitivity of rhC1-Inh to denaturation, caused by subtle differences in conformation compared to plasma C1-Inh. A more rapid denaturation of rhC1-Inh under SDS
conditions will lead to release of the protease from the complex, and cleavage of the serpin.

In addition, the tertiary structure of either C1-Inh was comparable as was evident from heat stability experiments. Importantly, this indicates that general folding and structural stability of rhC1-Inh are similar to those of plasma C1-Inh. This is crucial when considering that the recombinant product has to have an extended shelf life.

Serpins require a metastable conformation for activity and are hence susceptible to proteolytic cleavage. In the *P. pastoris* system the production levels of rhC1-Inh are high, but the moment of harvesting is critical to obtain active C1-Inh. To obtain at least 80 - 100 % active C1-Inh it was crucial to harvest after 3 days of induction (30 mg/L). C1-Inh production in fermentation culture (in GS115 with pPIC9 C1Inh, data not shown) did increase the amount of antigen up to 2 g / L but most C1-Inh thus produced was also in the inactivated, cleaved form. The endogenous protease responsible for cleavage of rhC1-Inh during production is the subject of current investigation.

RhC1-Inh could easily be purified from the culture supernatant by cation exchange chromatography. This allowed efficient separation from the main contaminant in the supernatant, pichia-derived alcohol oxidase. The purified preparation was enriched for active C1-Inh, probably due to the different conformations of active and inactive C1-Inh. A serpin undergoes a dramatic conformational change upon protease binding, which probably explains its different affinity for the cation exchange matrix.

The major difference between plasma C1-Inh and rhC1-Inh was clearly visible on SDS-PAGE: rhC1-Inh formed a smear, whereas plasma C1-Inh migrated as a single band. Plasma C1-Inh, in spite of having 10 N-linked and 3 O-linked glycosylation sites, shows a homogeneous glycosylation pattern. *P. pastoris* is known for its property to add high-mannose groups heterogeneously to glycosylation sites. RhC1-Inh showed 2 clear bands after deglycosylation, representing cleaved and intact C1-Inh. EndoH treatment of plasma C1-Inh did not result in two bands. There are two possible explanations for this difference. The first possibility is that a small fraction of rhC1-Inh is already cleaved but can only be distinguished after EndoH treatment because of the smear in the glycosylated protein. This is, however, unlikely because the percentage of active C1-Inh was similar in both rhC1-Inh and in plasma C1-Inh. The second, more likely, explanation is that the deglycosylation of rhC1-Inh by EndoH led to enhanced susceptibility for proteolytic cleavage. Plasma C1-Inh was not deglycosylated by EndoH and thus less sensitive for proteolytic cleavage.

EndoH-treated C1-Inh was still glycosylated on its O-linked glycosylation sites, which also occurs in *P. pastoris*. Hence, the most likely explanation for the smear of rhC1-Inh on SDS-PAGE is heterogeneous glycosylation on the N-linked glycosylation sites, which is different from plasma C1-Inh because only rhC1-Inh is sensitive for EndoH.

Progress curves have been set up before for the interaction between C1s or factor XIIa and C1-Inh. In a similar way we developed progress curves for the target proteases kallikrein, and coagulation factor XIa to make a comparison between plasma and rhC1-Inh. It can be concluded from table II that the inhibitory capacities of rhC1-Inh are comparable to those of plasma C1-Inh, and that for both C1-Inh variants the dissociation constants are very low. The association constants found with our method are
comparable to values found in literature (table II) \(^{20,39}\), which indicates that this is a suitable analysis. Because plasma C1-Inh and rhC1-Inh are differently glycosylated it can be concluded that the extent and the kind of glycosylation are not crucial for full inhibitory capacity.

Recombinant production of C1-Inh in the \(P.\) pastoris system may be an interesting approach for therapeutic application. The major obstacles remaining are presumably the different glycosylation pattern and the presence of an inactivating protease in \(P.\) pastoris. The former may lead to immunogenicity and decreased half-life of C1-Inh in circulation \(^{40}\), whereas the latter may limit the production of active C1-Inh in large-scale fermentation. The clearance and immunogenicity of C1-Inh is dependent on the glycosylation \(^{40}\). It is well known that yeast glycosylation is different from human glycosylation \(^{36}\). Indeed we observed in pilot experiments that the half-life in rats of rhC1-Inh was about 20 minutes in contrast to a half-life of 4 \(\frac{1}{2}\) hours for plasma C1-Inh \(^{27}\). Currently we are investigating the effect of deletion of glycosylation sites on the clearance. Alternatively, there are increasing possibilities to modify the yeast glycosylation to a human glycosylation. We suggest that solving these obstacles may yield a promising system to produce fully active recombinant C1-Inh or C1-Inh mutants, at very high levels.

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REFERENCE LIST


