



Characterization of recombinant human C1 inhibitor secreted in milk of transgenic rabbits

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ABSTRACT

C1 inhibitor (C1INH) is a single-chain glycoprotein that inhibits activation of the contact system of coagulation and the complement system. C1INH isolated from human blood plasma (pd-hC1INH) is used for the management of hereditary angioedema (HAE), a disease caused by heterozygous deficiency of C1INH, and is a promise for treatment of ischemia-reperfusion injuries like acute myocardial or cerebral infarction. To obtain large quantities of C1INH, recombinant human C1INH (rhC1INH) was expressed in the milk of transgenic rabbits (12 g/l) harboring genomic human C1INH sequences fused to 5' bovine α_S1 casein promoter sequences. Recombinant hC1INH was isolated from milk to a specific activity of 6.1 U/mg and a purity of 99%; by size-exclusion chromatography the 1% impurities consisted of multimers and N-terminal cleaved C1INH species. Mass spectrometric analysis of purified rhC1INH revealed a relative molecular mass (M_r) of 67,200. Differences in M_r on SDS PAGE and mass spectrometric analysis between rhC1INH and pd-hC1INH are explained by differential glycosylation (calculated carbohydrate contents of 21% and 28%, respectively), since protein sequencing analysis of rhC1INH revealed intact N- and C-termini. Host-related impurity analysis by ELISA revealed trace amounts of rabbit protein (approximately 10 ppm) in purified batches, but not endogenous rabbit C1INH. The kinetics of inhibition of the target proteases C1s, Factor XIIa, kallikrein and Factor XIa by rhC1INH and pd-hC1INH, indicated comparable inhibitory potency and specificity. Recently, rhC1INH (Ruconest[®]) has been approved by the European Medicines Agency for the treatment of acute attacks of HAE.

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1. Introduction

Human C1 inhibitor (C1INH) is a plasma glycoprotein belonging to the superfamily of serine protease inhibitors (serpins). Serpins adopt a metastable conformation containing an exposed mobile “reactive center” loop that acts as a substrate for the target protease. The process of protease inhibition is accompanied by a large conformational transition where the initial

Michaelis-complex is converted into a stable covalent serpin-protease complex (Huntington et al., 2000).

The C1INH molecule consists of a single polypeptide chain of 478 amino acids (calculated M_r of 52,800) folded in two disulphide-linked domains, a C-terminal or serpin domain showing clear homology to other serpins, and a unique non-serpin mucin-like N-terminal domain of 116 amino acids (Davis et al., 2010; Wagenaar-Bos and Hack, 2006). On SDS-PAGE, human plasma derived C1INH (pd-hC1INH) migrates with M_r of ~104,000 although neutron scattering yields a M_r of ~76,000 (Perkins et al., 1990). The difference in M_r with different analytical techniques is attributed to the presence of six N-linked (three in the N-terminal domain) and at least seven O-linked glycans (all in the N-terminal domain) that account for a 26% carbohydrate content by weight (Bock et al., 1986; Perkins et al., 1990).

The biological activity of C1INH includes inhibition of activation of the contact and complement systems. Target proteases for C1INH are Factor XIIa, kallikrein and Factor XIa of the contact activation system and activated C1s and C1r of the classical pathway as well as MASP1 and MASP2 of the lectin pathway of complement (Davis et al., 2010; Wagenaar-Bos and Hack, 2006). Protease

Abbreviations: C1INH, C1 inhibitor; pd-hC1INH, human plasma-derived C1INH; rhC1INH, recombinant human C1INH; rhC1INH-Thr⁸⁵ and -Thr⁹⁷, rhC1INH lacking Asn¹-Thr⁸⁴ and Asn¹-Thr⁹⁶, respectively; HAE, hereditary angioedema; Ruconest[®], rhC1INH approved in the EU for treatment of angioedema attacks in patients with HAE.

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inhibition is mediated by the C-terminal serpin domain of C1INH since N-terminal removal of residues 1–97 does not affect complex formation with target proteases (Bos et al., 2003b; Coutinho et al., 1994). Although not essential for protease inhibition, the N-terminal domain is involved in stabilization of the serpin domain (Bos et al., 2003b) and binding and neutralization of lipopolysaccharide (endotoxin) activity (Liu et al., 2004, 2005).

Hereditary angioedema (HAE), an autosomal dominant disease with deficiency of C1INH, is characterized by recurrent episodes of angioedema as a result of acute, localized increase in vascular permeability (Frank, 2008). Management of acute angioedema attacks includes administration of pd-hC1INH products (Frank, 2008). In addition to treatment of HAE, C1INH has been studied in protection against ischemia-reperfusion (IR) injuries like acute myocardial or cerebral infarction. The results obtained in various animal models and some clinical studies indicate that treatment with C1INH results in significant protective effects against IR injuries (Davis et al., 2010).

As an alternative for pd-hC1INH, expression of recombinant human C1INH (rhC1INH) in mammalian cell culture, bacterial and yeast-based expression systems has been investigated but as yet no product is commercially available (Bos et al., 2003a; Eldering et al., 1988; Lamark et al., 2001). This may be explained by poor expression levels, inactivation of rhC1INH in culture, and/or non-mammalian glycosylation profiles. Expression of rhC1INH in the milk of transgenic animals provides an alternative to culture-based expression systems since high expression levels (up to 35 g/l) and eukaryotic glycosylation profiles have been reported for various human proteins (Demain and Vaishnav, 2009; Houdebine, 2009). The rabbit as transgenic production platform has the advantage of a relatively short gestation period and the possibility to be maintained in a closed environment to minimize the introduction of adventitious agents. The protein content of rabbit milk is approximately 14% of which about 65% consists of various caseins aggregated in micelles. The remaining proteins in rabbit milk are whey proteins including transferrin, whey acidic protein, immunoglobulins, albumin and α -lactalbumin (Baranyi et al., 1995; Dayal et al., 1982; Grabowski et al., 1991; Jordan and Morgan, 1970).

The safety and efficacy of rhC1INH (Ruconest®) as replacement therapy for acute attacks of HAE has been demonstrated in open label (dose of at least 2100 U, ~30 U/kg) and randomized placebo-controlled double-blind (at 50–100 U/kg) studies (Choi et al., 2007; Moldovan et al., 2012; van Doorn et al., 2005; Zuraw et al., 2010). Here, we describe the production and characterization of the rhC1INH protein product.

2. Materials and methods

2.1. Materials

Human plasma-derived C1 inhibitor (Cetor®) was obtained from Sanquin (Amsterdam, The Netherlands). Bovine serum albumin (BSA) and melibiose were from Sigma–Aldrich (St. Louis, MO). Horseradish-peroxidase was obtained from Roche Diagnostics (Mannheim, Germany). Activated human C1s was from Calbiochem (La Jolla, CA). Human FXIa, human Factor α -XIIa and human kallikrein were from Enzyme Research Laboratories (Swansea, U.K.). The chromogenic substrates S-2302 (H-D-Pro-Phe-Arg-pNA), S-2314 (H-D-Val-Ser-Arg-pNA), S-2366 (pyroGlu-Pro-Arg-pNA) were obtained from Chromogenix (Milan, Italy) and Pefachrome C1E-5019 (CH₃CO-Lys(Cbo)-Gly-Arg-pNA) was obtained from Kordia (Leiden, The Netherlands). All Sepharose and Sephacryl media were obtained from GE Healthcare (Uppsala, Sweden) and Jacalin agarose was obtained from Vector Labs (Burlingame, CA). Where applicable, chemicals and methods complied with the monographs described in the pharmacopeias.

2.2. Generation of transgenic rabbits

Transgenic New Zealand White rabbits harboring mammary gland specific expression vectors based on 5' regulatory elements from the bovine α S₁ casein gene fused to genomic human C1INH sequences were obtained by micro-injection. The coding sequence for rhC1INH including the signal sequence is identical to the majority of published human C1INH sequences indicating that the rhC1INH coding sequence represents a normal allele of the human C1 inhibitor gene. A detailed description of the construction of the C1INH expression vector, the selection and genetic characterization of founder and production animals is considered beyond the scope of this article and will be published separately.⁴

2.3. Purification of rhC1INH

Transgenic rabbit milk was defatted by centrifugation (20 min, 1600 × g, 4 °C) and applied to a SP Sepharose column, equilibrated in 20 mM sodium citrate, 20 mM NaCl of pH 7.0. Unbound proteins were removed by washing the column with 5 column volumes (CV) of the same buffer and bound proteins were eluted with 20 mM sodium citrate, 0.2 M NaCl of pH 7.0. The eluate was filtered over 0.22 μ m and incubated with 1% Tween-80 and 0.3% tributyl phosphate (solvent/detergent treatment). Next, the sample was diluted with 2 volumes of 20 mM sodium phosphate pH 7.0 and applied to a Q Sepharose column in 20 mM sodium phosphate, 50 mM NaCl of pH 7.0, to separate rhC1INH from added chemicals and rabbit protein impurities including minute amounts of endogenous C1INH. After washing (5 CV), Q Sepharose-bound rhC1INH was eluted with 20 mM sodium phosphate, 0.22 M NaCl of pH 7.0. Residual protein impurities in the Q Sepharose-eluate were bound to a zinc-charged chelating Sepharose column in 20 mM sodium phosphate, 0.1 M NaCl of pH 7.0. Next, rhC1INH was filtered over Planova 15 N hollow fibers (Asahi, Tokyo, Japan) and formulated in 20 mM sodium citrate, 6.5% sucrose of pH 6.8 by ultrafiltration using Biomax-10 membranes (Millipore, Billerica, MA). The rhC1INH product was obtained after sterile filtration (0.22 μ m), filling at 2100 units per vial and subsequent lyophilization.

2.4. Purification of rabbit C1INH

Three-hundred milliliters of plasma obtained from New Zealand White rabbits was fractionated using polyethylene glycol 4000 at a final concentration of 20% (w/v) and subsequent centrifugation (30 min, 1600 × g, 4 °C) to remove precipitated proteins. The supernatant was loaded on a SP Sepharose column (1.6/15 cm) and unbound proteins were removed by washing with 20 mM sodium phosphate pH 7.0. Bound proteins were eluted with 20 mM sodium phosphate, 0.5 M NaCl of pH 7.0 and subjected to Jacalin agarose column (1.6/5 cm) chromatography as described (Pilatte et al., 1989). The eluate of the Jacalin agarose column was diluted with 20 mM sodium phosphate pH 5.5 and loaded on a Q Sepharose column (0.46/15 cm) equilibrated in 20 mM sodium phosphate, 50 mM NaCl of pH 5.5. Bound proteins were eluted from the Q Sepharose column with a linear gradient from 0.05 to 0.5 M NaCl in 9 CV of 20 mM sodium phosphate pH 5.5. Fractions containing rabbit C1INH were neutralized to pH 7.0 and stored at –70 °C until use.

2.5. Activity of rhC1INH

Fifty microliters of serial dilutions of C1INH standard and test samples in phosphate buffered saline, pH 7.4 (PBS), 0.05%

⁴ M.L.M. Manne, manuscript in preparation.

Tween-20 were incubated with 50 μ l of activated human C1s (1.5 μ g/ml) in microtitre plates (Nunc; Nunc, Roskilde, Denmark) for 1 h at 25 °C. Subsequently, 50 μ l of 1 mM Pefachrome C1E-5019 chromogenic substrate was added and the release of pNA by the remaining C1s activity was followed spectrophotometrically at 405 nm and 37 °C for 45 min using a Power Wave microtitre plate reader (BioTek, Winooski, VT). For each sample, the mean V (Δ mA/min) was calculated and the corresponding C1INH activity was determined by reference to the C1INH standard. One unit of C1INH activity was defined as the C1 esterase inhibiting activity present in 1 ml of pooled normal human plasma.

2.6. C1INH antigen-specific ELISA

Microtitre plates (high-binding; Corning, Oneonta, NY) were coated with 100 μ l of 3.5 μ g/ml rabbit anti-human C1 inhibitor antibodies (Dako Corp., Copenhagen, Denmark) in PBS for 16 h at ambient temperature. Plates were then washed with PBS containing 0.02% Tween-20 and incubated with serial dilutions of C1INH standard and test samples in PBT (PBS, 0.1% BSA, 0.1% Tween-20). After 1 h, plates were washed and incubated with 0.2 μ g/ml peroxidase-conjugated rabbit anti-human C1 inhibitor antibodies in PBT. After another hour, plates were washed and TMB-substrate solution (Pierce, Rockford, IL) was added. Substrate conversion was stopped by the addition of 2 M H_2SO_4 and the absorption at 450 nm was measured. All incubations were performed with 100 μ l volumes.

2.7. ELISAs for rabbit proteins

Sheep anti-rabbit C1INH and anti-rabbit milk protein antisera were obtained after repeated intramuscular injections with rabbit C1INH or normal rabbit milk. IgG fractions were purified from sera by protein G Sepharose chromatography, and in case of anti-rabbit C1INH adsorbed with pd-hC1INH coupled to CNBr-activated Sepharose to remove cross-reacting antibody. Sandwich-type ELISAs for rabbit milk proteins and rabbit C1INH were performed essentially as described for the C1INH antigen-specific ELISA.

2.8. Protein content

Protein concentrations were determined by absorption measurement at 280 nm (Stoscheck, 1990). The absorption coefficient used for purified rhC1INH fractions was 0.4 ml mg^{-1} cm^{-1} , which is according to the values reported for pd-hC1INH (Perkins et al., 1990).

2.9. Analytical chromatography, protein sequencing and mass spectrometry

Analytical cation-exchange chromatography was performed as described (van Veen et al., 2002). Briefly, purified rhC1INH (50 μ g) was applied to a Mono S HR 5/5 column (GE Healthcare) in 20 mM sodium phosphate pH 7.0. The column was subsequently washed with 5 ml and bound proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in 15 ml buffer at a flow rate of 1.0 ml/min.

Size-exclusion chromatography was performed using a TSK G3000 SW_{XL} column (0.78/30 cm; Tosoh Biosciences, Stuttgart, Germany). Protein (60 μ g) was filtered over the TSK G3000 column in 0.25 M sodium phosphate pH 6.8 at a flow rate of 0.5 ml/min.

Reversed phase HPLC was performed using a Vydac 218 TP C₁₈ column (0.21/25 cm; Alltech Inc., Deerfield, IL). Protein (25 μ g) was applied in 0.05% TFA and eluted with 57% acetonitrile, 0.05% TFA at a flow rate of 0.2 ml/min.

N- and C-terminal sequencing analysis was performed by Eurosequence (Groningen, The Netherlands). The N-terminal

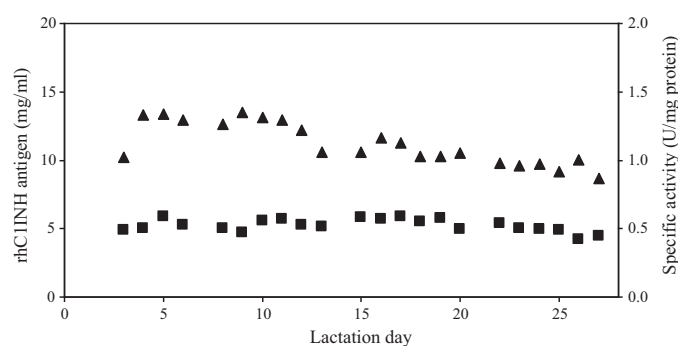


Fig. 1. Expression of rhC1INH in transgenic rabbit milk throughout the lactation. Individual milk samples were tested for rhC1INH antigen concentration (mg/ml; triangles) and specific activity (U/mg total protein; squares).

sequencing was according to the automatic Edman degradation procedure using an Applied Biosystems sequencer (model 494). The C-terminal sequence analysis was performed as described (Boyd et al., 1992), using a modified Applied Biosystems sequencer (model 477A).

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed at M-Scan (Wokingham, UK) using a PerSeptive Biosystems Voyager STR MALDI-TOF mass spectrometer. The samples were diluted to 1 mg/ml in 0.1% TFA and analyzed using a matrix of sinapinic acid in 0.1% TFA and 50% acetonitrile. BSA was used as calibrator.

2.10. Inhibition of target proteases

The pseudo first-order inhibition kinetics of target proteases by C1INH were determined in the presence of excess inhibitor according to the progress curve method as described (Eldering et al., 1992).

3. Results

3.1. Expression of rhC1INH in the milk of transgenic rabbits

Transgenic New Zealand White rabbit lines harboring the genomic C1INH gene under regulatory control of the bovine α S₁ casein promoter were generated by microinjection. The rhC1INH expression levels in milk of approximately 40% of the transgenic rabbit lines were above 10 mg/ml⁴ by the C1INH antigen-specific ELISA. The lactation period of rabbits typically consists of thirty days. The expression of rhC1INH in milk throughout a single lactation period of a rabbit from the line selected for pharmaceutical development is shown in Fig. 1. The level of rhC1INH antigen slightly declines through lactation whereas the specific activity in units per mg total protein is constant. The average values for rhC1INH antigen and specific activity were 12 mg/ml and 0.5 units/mg protein, respectively.

3.2. Purification of rhC1INH

rhC1INH was purified from transgenic rabbit milk by three consecutive purification steps at pH 7.0, i.e. cation exchange chromatography on SP Sepharose, anion exchange chromatography on Q Sepharose and affinity chromatography on zinc chelating Sepharose. The chromatogram of the defatted transgenic milk on SP Sepharose (Fig. 2) shows a distinct protein peak eluting from the column at 0.2 M NaCl that is well separated from the unbound proteins.

The non-reduced SDS-PAGE profiles of non-transgenic milk, transgenic milk and the SP Sepharose column fractions are shown in Fig. 3A. The SDS-PAGE profile of transgenic milk (Fig. 3A, lane 2)

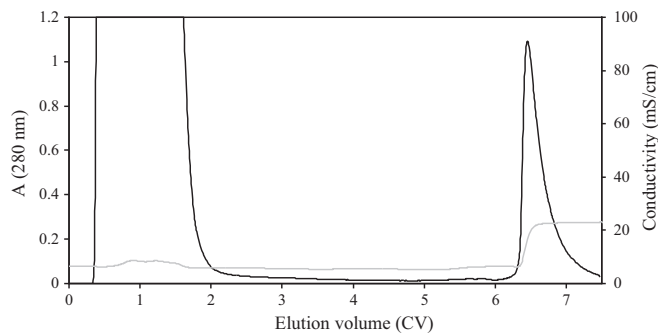


Fig. 2. Purification of rhC1INH from transgenic rabbit milk by chromatography on SP Sepharose. Defatted transgenic rabbit milk was loaded on a SP Sepharose column in 20 mM sodium citrate, 20 mM NaCl of pH 7.0. The column was then washed with the same buffer and eluted with 20 mM sodium citrate, 0.2 M NaCl of pH 7.0. Left and right y-axes indicate the absorbance at 280 nm and the conductivity (mS/cm), respectively.

is similar to that of non-transgenic milk (Fig. 3A, lane 1) except for the presence of a protein band of M_r 92,000. A protein migrating to the same position was detected in transgenic milk (Fig. 3B, lane 2), but not in non-transgenic milk (Fig. 3B, lane 1) by immunoblotting analysis using polyclonal anti-human C1 inhibitor antibodies, i.e. confirming the specificity of the anti-human C1 inhibitor antibodies. Taken together, the results indicate that the protein migrating on non-reduced SDS-PAGE at M_r 92,000 is rhC1INH.

SDS-PAGE analysis of the SP Sepharose column fractions revealed that most rabbit milk proteins including caseins (M_r s ~30,000) do not bind and are removed in the column flow-through and wash (Fig. 3A, lanes 3 and 4). SDS-PAGE analysis of the SP Sepharose eluate and final rhC1INH preparation revealed rhC1INH of M_r 92,000 purified to approximately 95% and homogeneity, respectively (Fig. 3A, lanes 5 and 7).

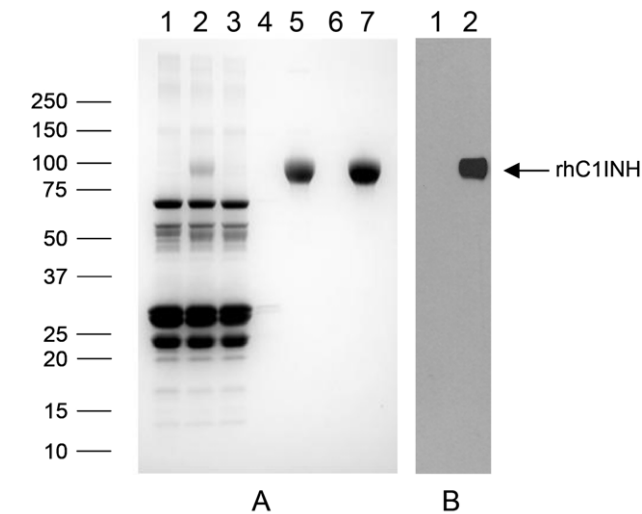


Fig. 3. (Panel A) SDS-PAGE analysis of the purification of rhC1INH. Rabbit milk and column fractions were subjected to non-reduced SDS-PAGE (4–20%) analysis. Lane 1: non-transgenic milk; lane 2: transgenic milk; lanes 3–5: SP Sepharose column flow-through, wash and eluate, respectively; lane 6: PBS; lane 7: flow-through of zinc chelating Sepharose column. Proteins, 20 μ g for milk samples and 10 μ g for purified rhC1INH, were visualized with Coomassie Brilliant Blue. The migration of the standard protein markers is indicated on the left ($10^{-3} \times M_r$). (Panel B) Detection of rhC1INH in transgenic rabbit milk by immunoblotting analysis. Lane 1: non-transgenic milk; lane 2: transgenic milk (0.4 μ l each) were subjected to non-reduced SDS-PAGE (4–20%) and transferred to nitrocellulose. Blots were blocked and subsequently incubated with peroxidase-labeled anti-human C1INH antibodies. C1INH-bound antibodies were detected through chemiluminescence.

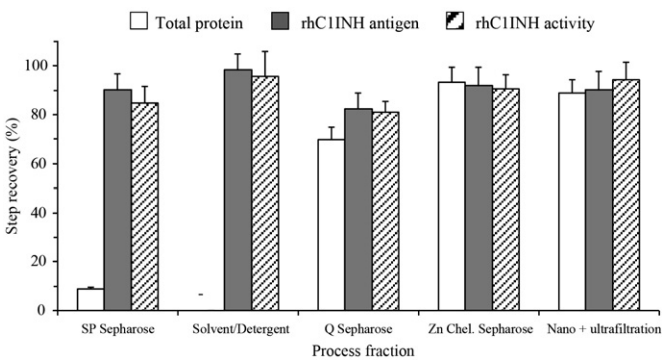


Fig. 4. Recovery of rhC1INH at downstream processing. The recovery of rhC1INH from transgenic rabbit milk was determined for total protein, rhC1INH antigen and activity. Results from 19 purification runs are expressed as mean and SD. The recoveries of the nano and ultrafiltration steps following the chromatographic purifications were combined.

The recoveries of protein and rhC1INH throughout the purification process are shown in Fig. 4. The recovery of both rhC1INH antigen and activity by each process step exceeded 80%. The similar recoveries of rhC1INH antigen and activity indicates that inactivation of rhC1INH had not occurred during purification. The overall recovery of rhC1INH from transgenic milk is approximately 60%. The protein recovery of the SP Sepharose column step is approximately 10% which corresponds with the yield of rhC1INH from the column. The protein recovery of the Q Sepharose column is slightly lower compared to the recovery of rhC1INH indicating that residual protein impurity after SP Sepharose chromatography is removed by this step.

3.3. Characterization of purified rhC1INH

Lyophilized rhC1INH product batches were reconstituted and total protein concentration, rhC1INH antigen (mg/ml) and activity (U/ml) as well as residual host-related impurity was determined. The results show that the total protein content matched the rhC1INH antigen content of 25 mg/ml (Table 1). The specific activity of rhC1INH was determined at 6.1 ± 0.2 U/mg total protein which is comparable with the specific activity reported for purified pd-hC1INH (Poulle et al., 1994). The content of rabbit milk protein impurity in purified rhC1INH was 10 ± 3 ppm by the ELISA for rabbit milk protein. Endogenous (rabbit) C1INH was not detected in purified rhC1INH by the rabbit C1INH-specific ELISA, indicating that the level of this protein in rhC1INH is below 0.3 ppm, which is the detection limit of the assay. Bacterial endotoxin levels were below 0.5 EU/ml (Table 1).

N- and C-terminal protein sequencing of rhC1INH revealed single N- and C-terminal sequences (Fig. 5) indicating that the purified protein is intact. The N-terminal sequence was the same as predicted by the DNA sequence indicating correct and complete removal of the C1INH signal sequence in the rabbit mammary

Table 1
Characterization of reconstituted rhC1INH product batches ($n = 19$).

Analysis	Result
Total protein	24 ± 1 mg/ml
rhC1INH antigen	25 ± 3 mg/ml
rhC1INH activity	146 ± 7 U/ml
Specific activity	6.1 ± 0.2 U/mg total protein
rhC1INH purity (SEC-HPLC)	$98.7 \pm 0.3\%$
Total rabbit milk proteins	10 ± 3 ppm ^a
Rabbit C1INH	<0.3 ppm
Endotoxin	<0.5 EU/ml

^a ppm: ng per mg protein.

N-terminal sequence	Position	1	2	3	4	5	6	7	8	9	10	11
	DNA	N	P	N	A	T	S	S	S	S	Q	D
	Protein	N	P	X	A	T	S	S	S	S	Q	D

C-terminal sequence	Position	478	477	476	475
	DNA	A	R	P	D
	Protein	A	X	X	X

Fig. 5. N- and C-terminal sequencing analysis of rhC1INH. The sequencing results obtained for rhC1INH are presented by the standard one-letter code for amino acids and aligned to the predicted sequence based on the DNA sequence of human C1INH. An X represents the absence of detection of an amino acid.

gland. The absence of detection of an asparagine at position 3 in rhC1INH suggests that this amino acid is glycosylated, as has been reported for pd-hC1INH (Bock et al., 1986). No specific amino acid signals were detected above baseline after the first amino acid (Ala⁴⁷⁸) in the C-terminal sequencing analysis (Fig. 5). The absence of detection of amino acids starting at Arg⁴⁷⁷ in rhC1INH is due to the proline residue at position 476 (Fig. 5), which terminates the C-terminal sequencing reaction (Boyd et al., 1992).

Comparison of rhC1INH and pd-hC1INH by non-reduced and reduced SDS-PAGE analysis indicated a difference in M_r between the two molecules. On non-reduced SDS-PAGE, rhC1INH migrated as a diffuse single protein band of M_r 92,000 whereas pd-hC1INH migrated as a major protein band of M_r 106,000 (Fig. 6A). Under reduced conditions M_r s of 90,000 and 100,000 were found for rhC1INH and pd-hC1INH, respectively (Fig. 6B). With MALDI-TOF mass spectrometric analysis, relative molecular masses of 67,200 and 73,700 were determined for rhC1INH and pd-hC1INH, respectively. The M_r found for pd-hC1INH is comparable with the values reported in literature (Perkins et al., 1990). Taking the calculated M_r 52,800 of the complete polypeptide chain of C1INH into account, the mass spectrometry data indicate carbohydrate contents of 21% and 28% for rhC1INH and pd-hC1INH, respectively.

Analytical chromatography of purified rhC1INH on Mono S, Vydac C₁₈ and TSK G3000 columns revealed single protein peaks

eluting at 13.0 min (0.2 M NaCl), 19.6 min (57% acetonitrile) and 15.7 min (7.9 ml), respectively (Fig. 7). Peak surface integration indicated 99% recovery for each analytical chromatographic method. The amount of inactive (reactive center cleaved) rhC1INH as determined by Mono S- and Vydac C₁₈-HPLC was below 0.2% in purified batches (not shown). Batch analysis by TSK G3000 size-exclusion chromatography showed low variation in the purity of rhC1INH ($98.7 \pm 0.3\%$; Table 1). The chromatograms consistently revealed the presence of three minor impurity peaks (peaks 1–3; $0.5 \pm 0.2\%$) eluting in front of the main peak and one peak (peak 4; $0.5 \pm 0.2\%$) eluting after the main peak (Fig. 8A). Comparison of the M_r s of the proteins eluting in peaks 1–3, as determined by reference

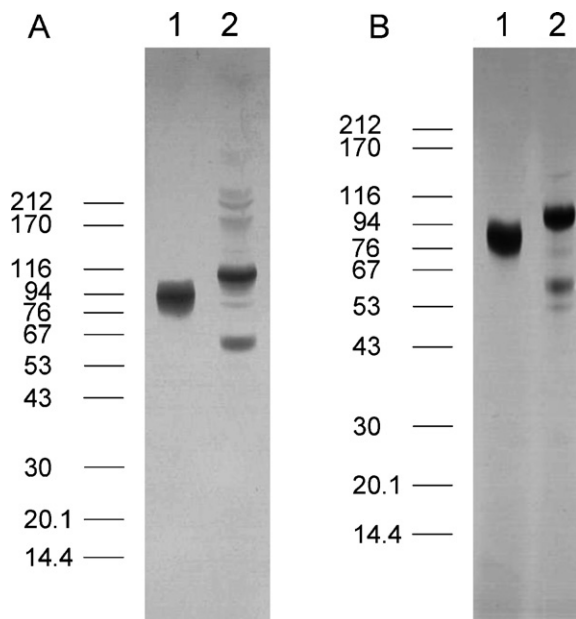


Fig. 6. Non-reduced and reduced SDS-PAGE analysis of rhC1INH and human plasma-derived C1INH. Purified rhC1INH (lane 1) and pd-hC1INH (lane 2) were subjected to non-reduced (A) and reduced (B) SDS-PAGE (8–16%) analysis. Proteins, 100 ng/lane, were visualized with silver. The migration of the standard protein markers is indicated on the left ($10^{-3} \times M_r$).

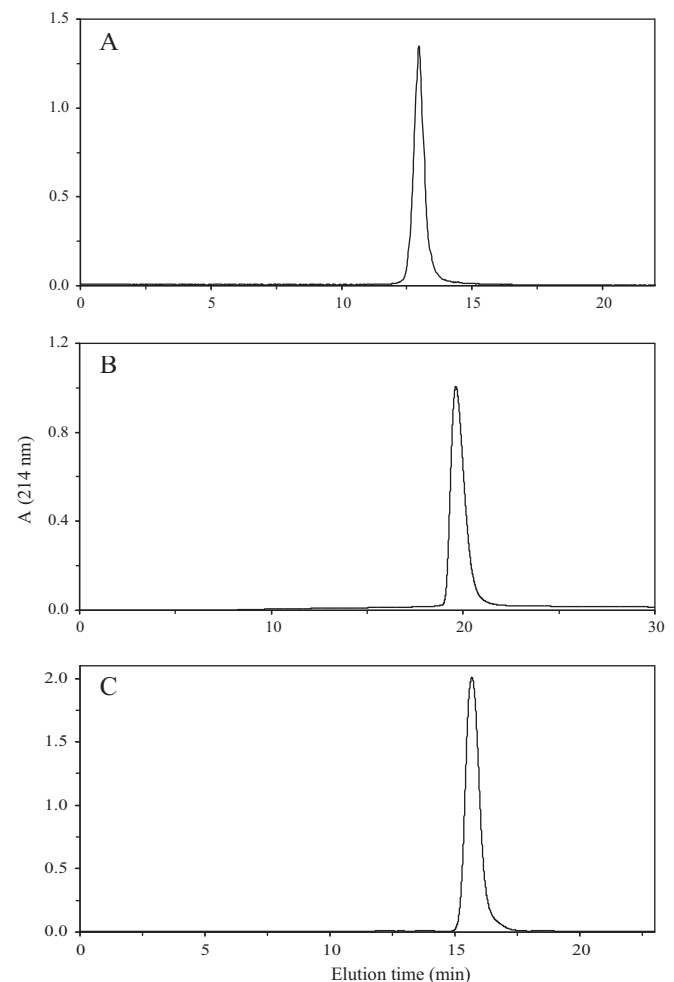


Fig. 7. Analytical chromatography of purified rhC1INH. Mono S cation exchange- (A), Vydac C₁₈ reversed phase- (B) and TSK G3000 size exclusion chromatography (C) of purified rhC1INH was performed as described in Section 2. The left axis indicates the absorbance at 214 nm.

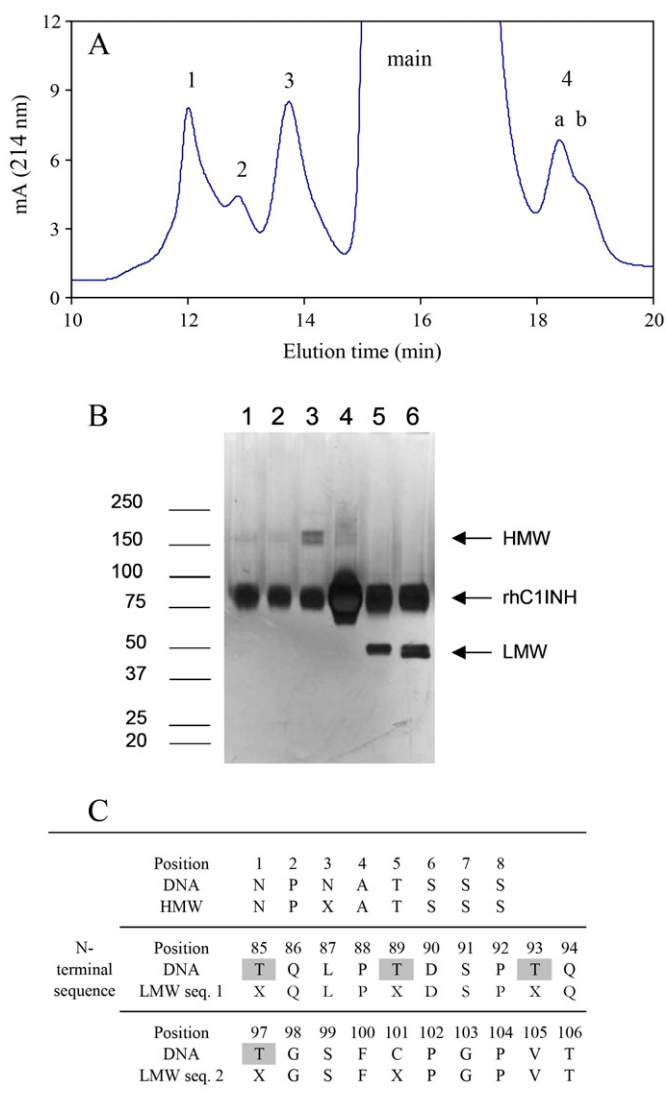


Fig. 8. Characterization of rhC1INH-related impurities. (Panel A) Expanded analytical size exclusion chromatogram of Fig. 7C. (Panel B) Non-reduced SDS-PAGE (4–20%) analysis of peak fractions in Fig. 8A. Lanes 1–3: peaks 1–3, respectively; lane 4: main peak; lane 5: peak 4a; lane 6: peak 4b. Proteins were visualized with silver. HMW and LMW represent high and low molecular weight proteins, respectively. (Panel C) N-terminal sequence analysis of SDS-PAGE gel-isolated HMW and LMW proteins. The results are aligned to the predicted sequence based on the DNA sequence of human C1INH. An X represents the absence of detection of an amino acid. Threonines high-lighted in gray indicate potential O-linked glycosylation sites (Bock et al., 1986).

to the elution pattern of protein standards, with the M_r of rhC1INH eluting in the main peak suggested that the proteins present in peaks 1–3 are multimeric forms of rhC1INH (result not shown). On SDS-PAGE, the majority of protein present in peaks 1–3 migrated to the same position as rhC1INH (Fig. 8B, lanes 1–3) indicating that these proteins represent multimeric forms of rhC1INH not stable in SDS. Beside the multimeric forms of rhC1INH, the SDS-PAGE profile of peak 3 indicated the presence of a doublet of high molecular weight (HMW) protein bands migrating to $M_r \sim 160,000$ (Fig. 8B, lane 3). N-terminal sequencing of SDS-PAGE gel-isolated HMW proteins revealed only one N-terminal sequence matching the N-terminal sequence of intact human C1INH (Fig. 8C, HMW). Taking the apparent molecular weight of the HMW impurities into consideration, the HMW proteins probably represent dimeric forms of rhC1INH stable to denaturation by SDS. SDS-PAGE analysis

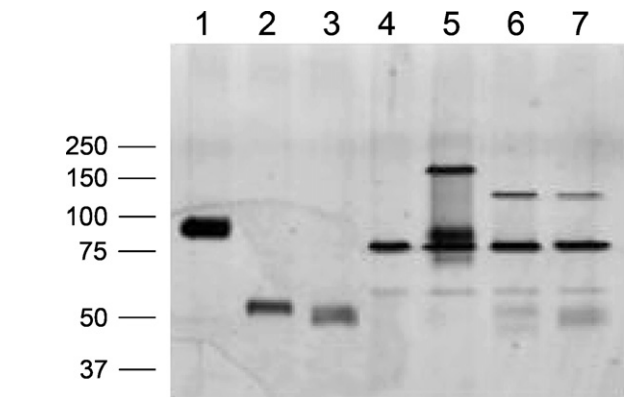


Fig. 9. Inhibition of C1s by rhC1INH and the LMW proteins. Non-reduced SDS-PAGE (4–20%) analysis of rhC1INH proteins incubated without (lanes 1–3) or with human C1s (lanes 5–7). Lanes 1 and 5: rhC1INH; lanes 2 and 6: rhC1INH-Thr⁸⁵; lanes 3 and 7: rhC1INH-Thr⁹⁷; lane 4: human C1s control. Proteins (50 ng) were visualized with silver.

of peak fractions taken from peak 4 revealed the presence of two low molecular weight (LMW) proteins of M_r 54,000 and 49,000 (Fig. 8B, lanes 5 and 6), which by N-terminal sequencing represented two human C1INH related sequences starting at amino acids 85 and 97, respectively (Fig. 8C, LMW sequences 1 and 2). The absence of detection of Thr⁸⁵, Thr⁸⁹, Thr⁹³ and Thr⁹⁷ by N-terminal sequencing indicate that these potential O-linked glycosylation sites (Bock et al., 1986) are glycosylated in rhC1INH. Immunoblotting analysis using anti-human C1 inhibitor antibodies confirmed that both the HMW and LMW proteins relate to rhC1INH (result not shown). Taken together, size-exclusion chromatography revealed that about 1% of rhC1INH consists of multimeric and cleaved rhC1INH species.

3.4. Inhibition of target proteases by rhC1INH

Inhibition of human C1s by pd-hC1INH results in the formation of a 1:1 SDS-stable complex of $M_r \sim 180,000$ on SDS-PAGE analysis (Harpel and Cooper, 1975). Incubation of rhC1INH with human C1s resulted in the presence of an additional protein band of M_r of $\sim 180,000$ (Fig. 9, lane 5) indicating the formation of a 1:1 SDS-stable complex between human C1s and rhC1INH, i.e. absent in either rhC1INH and C1s controls (Fig. 9, lanes 1 and 4). To assess whether the LMW proteins could inhibit target proteases, rhC1INH was fractionated by size exclusion chromatography and isolated LMW proteins (Fig. 9, lanes 2 and 3) were incubated with human C1s and subjected to SDS-PAGE analysis. The results revealed the presence of protein bands migrating at $M_r \sim 140,000$ (Fig. 9, lanes 6 and 7) indicating protease inhibition activity of both LMW proteins, further denoted as rhC1INH-Thr⁸⁵ and rhC1INH-Thr⁹⁷. No complexes were observed on SDS-PAGE after incubation of the HMW proteins with human C1s (result not shown).

The inhibitory potency and specificity of rhC1INH was characterized by analyzing the kinetics of inhibition of the target proteases C1s, Factor XIIa, kallikrein and Factor XIa as described (Eldering et al., 1992). The second-order rate constants of inhibition (k_{on}) for the four target proteases by rhC1INH and pd-hC1INH are shown in Table 2. The results indicate that the k_{on} values for inhibition of the four target proteases are comparable for rhC1INH and pd-hC1INH and in line with the values reported for pd-hC1INH (Table 2). The k_{off} values (not shown) were found to be negligible for both rhC1INH and pd-hC1INH, confirming that the complex formation between target proteases and both C1 inhibitor variants is virtually irreversible.

Table 2

Second-order rate constants for inhibition of target proteases by rhC1INH.

	Second-order rate constant, k_{on} ($M^{-1} s^{-1}$)			
	C1s	Factor XIa	Factor XIIa	Kallikrein
rhC1INH	$6.1 \pm 0.3 \times 10^4$	$9.8 \pm 0.5 \times 10^2$	$6.9 \pm 0.5 \times 10^3$	$9.1 \pm 0.1 \times 10^3$
pd-hC1INH	$5.1 \pm 0.3 \times 10^4$	$9.0 \pm 0.2 \times 10^2$	$5.7 \pm 0.4 \times 10^3$	$7.6 \pm 0.3 \times 10^3$
pd-hC1INH (Eldering et al., 1992)	$4.8 \pm 0.6 \times 10^4$	–	–	–
pd-hC1INH (Wuillemin et al., 1996)	–	1.8×10^3	9×10^3	2.6×10^4
pd-hC1INH (Bos et al., 2003a)	$6.2 \pm 0.4 \times 10^4$	$3.9 \pm 0.3 \times 10^2$	$4.5 \pm 0.3 \times 10^3$	$7.8 \pm 0.4 \times 10^3$

4. Discussion

Human C1INH is a therapeutic modality in prophylaxis and treatment of acute angioedema attacks in patients with HAE, but beneficial effects in ischemia-reperfusion injury need to be confirmed by clinical studies (Gesueti et al., 2009; Horstick, 2002). Testing and application of C1INH in conditions like acute myocardial or cerebral infarction would require very large amounts of the protein. In this paper, we describe the characterization of rhC1INH secreted in milk of transgenic rabbits.

Recombinant C1INH was secreted as an intact (non-cleaved) active molecule at an average level of 12 g/l in milk of production animals (Fig. 1 and Fig. 3B). Previous reports on production of C1INH by recombinant DNA technology described relatively low expression levels and/or proteolytic inactivation in culture (Bos et al., 2003a; Eldering et al., 1988).

A three step purification procedure of rhC1INH from transgenic milk yielded a virtually pure protein preparation at a final recovery of approximately 60% (Figs. 3 and 4). The use of SP Sepharose allowed for direct processing of milk without prior removal of the caseins, since caseins as well as most other rabbit milk proteins did not bind to the resin at pH 7.0 (Figs. 2 and 3). The low variation in purification recovery (Fig. 4), in rhC1INH antigen and activity levels, in purity as well as residual host-derived impurity (approximately 10 ppm rabbit proteins by ELISA) and the low endotoxin content of reconstituted rhC1INH product (Table 1) indicates that the manufacturing process for rhC1INH is robust and well-controlled.

Protein sequencing of purified rhC1INH yielded intact N- and C-termini (Fig. 5). The intactness and predicted polypeptide sequence of rhC1INH was confirmed by tryptic peptide mapping using RP-HPLC and mass spectrometry (results not shown). From the differences in M_r for rhC1INH and pd-hC1INH (67,200 and 73,700) by MALDI-TOF mass spectrometric analysis, it was inferred that differential glycosylation of these C1INH species (calculated carbohydrate contents of 21% and 28%) accounts for the differences in M_r on non-reduced and reduced SDS-PAGE (Fig. 6). Pd-hC1INH contains disialylated complex-type N-glycans and monosialylated core 1-type O-glycans (Perkins et al., 1990; Strecker et al., 1985), whereas rhC1INH contains an array of complex-, oligomannose- and hybriide-type N-glycan structures and core 1-type O-glycans with relatively low degree of sialylation (Koles et al., 2004b). Differences in glycosylation between numerous human recombinant proteins and their natural counterparts have been observed and are expected since the structures of the attached glycans are species, tissue, cell-type and protein-specific (James et al., 1995; Opdenakker et al., 1993).

Batch analysis revealed consistency in N-linked and O-linked glycosylation of rhC1INH (Germano et al., 2006; Koles et al., 2004a) indicating that the previously reported variation in glycosylation of rhC1INH among rabbits and throughout the rabbit lactation (Koles et al., 2004a) is under control at the level of batch production. The differential glycosylation of rhC1INH compared to pd-hC1INH did not impact the inhibitory potency and specificity of

rhC1INH for its target proteases since the results of the inhibition kinetics were comparable for both molecules (Table 2). Comparable inhibition of the non-target protease plasmin was observed for pd-hC1INH and rhC1INH whereas thrombin was not inhibited (not shown). The inhibition of plasmin by C1INH has been associated to an increased risk for thromboembolic complications in patients receiving pd-hC1INH products (Arzneimittelkommission der deutschen Ärzteschaft, 2000; FDA, 2010). However, the analysis of various coagulation and fibrinolytic parameters in pre- and post-treatment plasma and absence of thromboembolic complications in HAE patients receiving rhC1INH up to 100 U/kg, argues against a prothrombotic effect of rhC1INH in HAE patients and suggests that the in vitro inhibition of plasmin by C1INH is of minor importance in vivo (Choi et al., 2007; Moldovan et al., 2012; Relan et al., 2012; van Doorn et al., 2005; Zuraw et al., 2010).

Size-exclusion chromatography of rhC1INH revealed minute amounts of multimerized- and N-terminal cleaved rhC1INH variants, each representing approximately 0.5% of total protein. The majority of the multimerized rhC1INH appeared to dissociate in SDS (Fig. 8B) indicating that they are formed in a way typical for multimerization of serpins i.e. insertion of the reactive center loop in the β -sheet A of an adjacent molecule (Huntington and Whisstock, 2010; Patston et al., 1995). Multimeric forms of C1INH are inactive and can be induced in C1INH preparations by e.g. heating (Patston et al., 1995). The observed inhibitory activity (Fig. 9) of the two LMW rhC1INH variants lacking N-terminal amino acids 1–84 (rhC1INH-Thr⁸⁵; M_r 54,000) and 1–96 (rhC1INH-Thr⁹⁷; M_r 49,000), confirms literature reports that deletion of N-terminal sequences up to Thr⁹⁷ in C1INH does not lead to a loss in inhibitory activity (Bos et al., 2003b; Coutinho et al., 1994). Formation of rhC1INH-Thr⁸⁵ and rhC1INH-Thr⁹⁷ by alternative splicing is unlikely given the fact that both Thr⁸⁴-Thr⁸⁵ and Thr⁹⁶-Thr⁹⁷ are located within exon 3 (Tosi, 1998). The minute amounts of cleaved rhC1INH-Thr⁸⁵ and rhC1INH-Thr⁹⁷ in purified rhC1INH may be explained by limited proteolysis in the rabbit mammary gland, milk and/or during purification of the molecule. Several proteases have been reported that can cleave C1INH in its N-terminal mucin-like domain although Thr⁸⁴ and Thr⁹⁶ have not been published as proteolytic sites (Pemberton et al., 1989; Reboul et al., 1987; Schoenberger et al., 1989).

Open label and randomized placebo-controlled double-blind studies revealed that rhC1INH (Ruconest®) at 30–100 U/kg is safe, well tolerated and efficacious in treatment of acute angioedema attacks in HAE patients (Choi et al., 2007; Moldovan et al., 2012; van Doorn et al., 2005; Zuraw et al., 2010). Repeated administrations of Ruconest® revealed that the product has a low potential to induce antibodies against C1INH or host-related impurities. The occasional formation of such antibodies had no clinical consequences regarding the safety and efficacy profiles of Ruconest® (Hack et al., 2012; Moldovan et al., 2009). The recent approval of the rhC1INH product by the European Medicines Agency for treatment of acute HAE attacks illustrates the feasibility of large-scale production of biopharmaceuticals in the milk of transgenic animals.

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