

# **PRODUCTION OF PLASMA PROTEINS FOR THERAPEUTIC USE**

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## C1-INHIBITOR

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### 17.1 INTRODUCTION

C1-inhibitor (formerly C1-esterase inhibitor or C1-inactivator), a heat-labile plasma protein that inhibits the esterolytic activity of complement factor 1 (C1), was discovered in the 1950s [1]. Soon after, a deficiency of C1-inhibitor was recognized as causing hereditary angioedema (HAE), a disabling and even potentially life-threatening disease that had been described as early as 1888 by Osler [2–4]. C1-inhibitor has now been characterized as a serine protease inhibitor (“serpin”), present in blood plasma, capable of inhibiting a number of proteases that play an important role in several physiological systems. This chapter focuses on a number of aspects of C1-inhibitor and HAE, including the many physiological roles that C1-inhibitor exerts *in vivo*, the production of C1-inhibitor concentrates from human plasma, and the treatment of HAE and potentially other diseases, with C1-inhibitor concentrates.

### 17.2 BIOCHEMISTRY

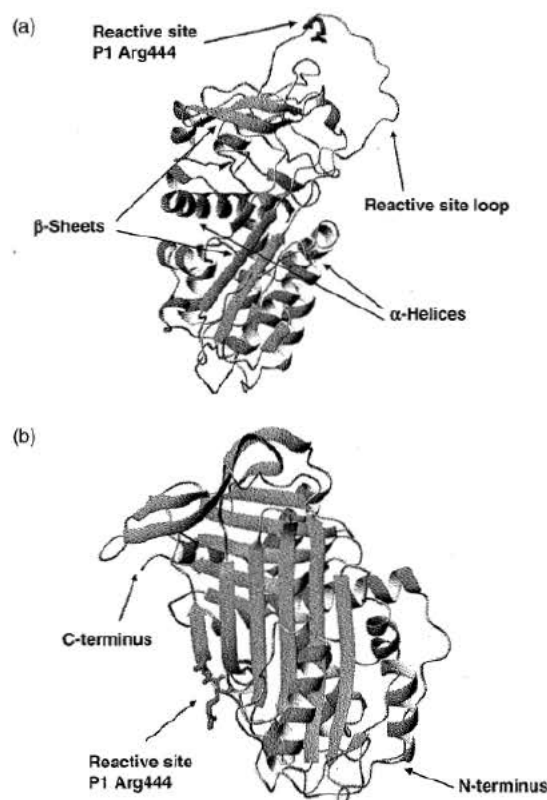
C1-inhibitor is primarily synthesized in the liver but also in other sites such as monocytes, skin fibroblasts and endothelial cells [5–8]. It is a single-chain glycoprotein with an apparent molecular mass of approximately 100–105 kDa as determined by analytical ultracentrifugation and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [9–12]. The MW of the deglycosylated form of the protein as analyzed by SDS-PAGE was reported to be 78 kDa [12]. When based on the amino acid composition (the molecule consists of 478 amino acids), a molecular mass of 53 kDa was calculated without the glycans [13]. The

serpin domain, located on the C-terminus, consists of 366 amino acids while the nonserpin, N-terminal domain comprises of 112 amino acids [14]. C1-inhibitor has 21–27% sequence homology with other serpins, although this homology does not extend to the nonserpin domain. The structure of the amino-terminal part of the C1-inhibitor molecule makes it unique among the serpins as it contains a highly repetitive region comprising 14 tandem repeats of the tetrapeptide Gln-Pro-Thr-Thr and variations thereof [15]. Figure 17.1 shows a theoretical three-dimensional (3D) model of the active form of the serpin domain of C1-inhibitor, and a model of the latent form derived from crystal structure analysis sourced from the Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank [16,17]. The respective access codes to view the structures are 1M6Q and 2OAY.

C1-inhibitor is heavily glycosylated. Ten of the 13 glycosylation sites are present on the N-terminal part of the protein, with the other three on the serpin domain [18]. The molecule contains two disulfide bridges connecting the amino-terminal and the C-terminal parts [13]. There are no free sulfhydryl groups. The isoelectric point was reported to be 2.7–2.8 [9,19]. The molecule is denatured in the presence of methanol or ethanol (e.g., during cold ethanol fractionation of plasma), ether, at 0–2°C in plasma, at low pH, and by heating to 60°C in the absence of stabilizers [1,19–21].

The gene for C1-inhibitor is located on chromosome 11, more specifically on locus 11q11-q13.1 [13] and is denominated *SERPING1*. *SERPING1* is known to be highly susceptible for mutations [22]: to date over 250 different mutations have been described in the special C1-inhibitor gene mutation database [23].





**FIGURE 17.1** 3D-models of the serpin domain of C1-inhibitor (derived from the Protein Database [16]). (a) Theoretical model of the active form [17]. (b) Model of the latent form based on the crystal structure [14]. The glycan structures are not shown.

### 17.3 PHYSIOLOGY

The mean C1-inhibitor concentration in plasma is approximately 0.2 mg/mL [9,10,12,24,25], equivalent to 1 plasma unit (PU) per milliliter, with individual values as low as 0.14 mg/mL [24] and as high as 0.38 mg/mL [25].

Varying values have been reported for the *in vivo* half-life of C1-inhibitor. In normal subjects (and HAE patients under normal physiological circumstances), a half-life of 64–68 h was found [26]. However, a fractional catabolic rate of 2.5%/h has been reported in normal individuals equivalent to a half-life of approximately 28 h [27,28]. In transfused HAE patients a wide range of half-lives for C1-inhibitor clearance have been measured (7–90 h), with a median (or mean) of 30–40 h [29,30]. This variation may represent individual differences in the biotransformation and clearance of the administered C1-inhibitor and a sensitivity to a variation in the administered dose.

C1-inhibitor is the major inhibitor of proteases of the contact system and of the classical and lectin pathways of complement activation. In the contact system these include

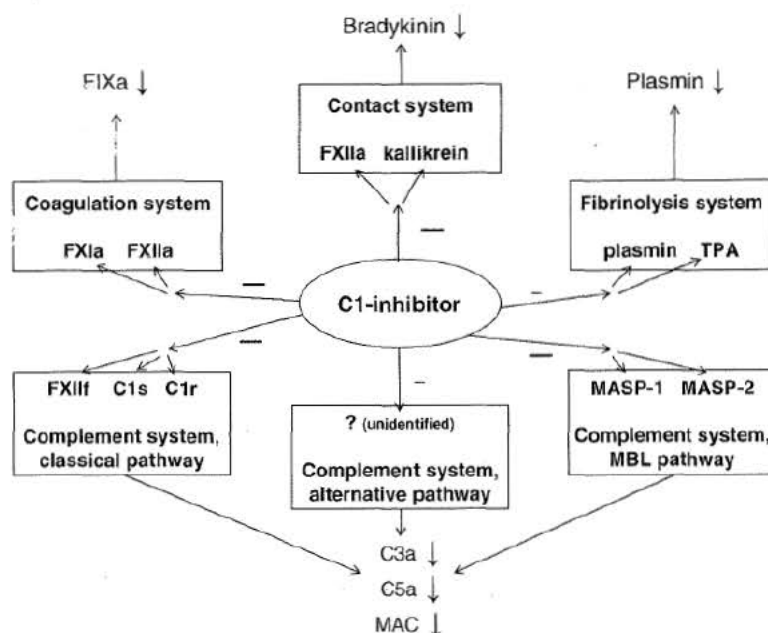
Factor XIIa (FXIIa), the active fragment of Factor XII (FXII), kallikrein, and Factor XIa (FXIa) [31–37]. In the classical complement activation pathway, C1-inhibitor can regulate activated C1s and C1r, while in the lectin pathway for complement activation the mannan binding lectin-associated serine proteases, MASP-1 and MASP-2 are targeted [38–42].

Insufficient inhibition of the contact system by C1-inhibitor results in uncontrolled release of the vasoactive nonapeptide bradykinin [43]. As is discussed fully in Section 17.7, HAE is the best-known clinical condition caused by C1-inhibitor deficiency, leading to uncontrolled bradykinin generation [44]. Treatment is now possible by replacement with plasma-derived concentrates of C1-inhibitor [44–46].

The key regulatory role of C1-inhibitor in preventing unregulated bradykinin production is its action on FXIIa and kallikrein activity [44]. Initiation of Factor XII activation can occur through trauma, infection and other inflammatory responses, and by binding of Factor XII to negatively charged surfaces originating from bacterial lipopolysaccharide, oligosaccharides, connective tissue proteoglycans, damaged basement membranes, extracellular RNA, or platelet polyphosphates [44,47,48]. In the presence of FXIIa, prekallikrein is cleaved to plasma kallikrein that acts upon high molecular weight kininogen (HMWK) to release the vasoactive nonapeptide bradykinin. Plasma kallikrein also converts inactive Factor XII to active FXIIa, generating a positive feedback loop. Amplification of kallikrein production results in augmented bradykinin production and local vasodilatation. The cleavage of bradykinin from HMWK is highly localized since the prekallikrein and the HMWK substrate circulate as a complex. Bradykinin will bind to its bradykinin B2 receptor that is expressed on endothelial cells. This induces an increase in the vascular permeability that allows fluid to move into the interstitial space resulting in edema. C1-inhibitor, through its modulation of FXIIa and kallikrein activity, is a key component of homeostatic mechanisms regulating the function of the contact activation system.

C1-inhibitor also has an important role in the regulation of the complement activation pathway. In fact it is the only plasma protease inhibitor with an identified regulatory role in the classical and lectin pathway of complement activation [49]. In addition, an inhibitory effect on the alternative pathway of complement activation has been shown, albeit the mechanism of action has not yet been elucidated [50]. An *in vitro* study using supraphysiological levels of C1-inhibitor showed that the inhibiting effect was significantly different for the three complement activation pathways and depended upon the applied concentration of the C1-inhibitor [51]. C1-inhibitor also targets other proteases such as plasmin [9,38,52,53] and tissue plasminogen activator [54]. Thus, it may also have a role in the regulation of the fibrinolytic system. Furthermore, C1-inhibitor is an acute phase protein, the concentration of which may rise at least two-fold during an inflammatory reaction [55]. Figure 17.2





**FIGURE 17.2** The central role of C1-inhibitor in the regulation of various physiologic systems: contact system, complement system, coagulation system, and fibrinolytic system. MAC: membrane attack complex; MASP: mannan binding lectin-associated protease.

presents an overview of the central role of C1-inhibitor in various physiological systems. For detailed reviews of the role of C1-inhibitor in the coagulation, complement, and fibrinolytic systems, the reader is referred to Refs [55,56].

The inhibition of classical complement activation by C1-inhibitor can be potentiated *in vitro* by highly sulfated glycosaminoglycans such as heparin and sulfated dextrans through a resultant increased inhibition of C1s and C1r [57,58]. In a rat model, this effect lasted for 60–90 min [59]. Glycosaminoglycans do not have such an effect on the contact system components FXIIa and kallikrein [60,61], but FXIa inhibition was increased more than 100-fold in the presence of dextran sulfate [61]. In principle, this selective potentiation mechanism offers new ways for therapeutically modulating the biological effects of C1-inhibitor on contact activation. However, although recently more insight into the mechanism of heparin potentiation of C1-inhibitor has been gained, it is still unknown whether the potentiation by this route is of physiological significance *in vivo* [14,62].

#### 17.4 MECHANISM OF ACTION

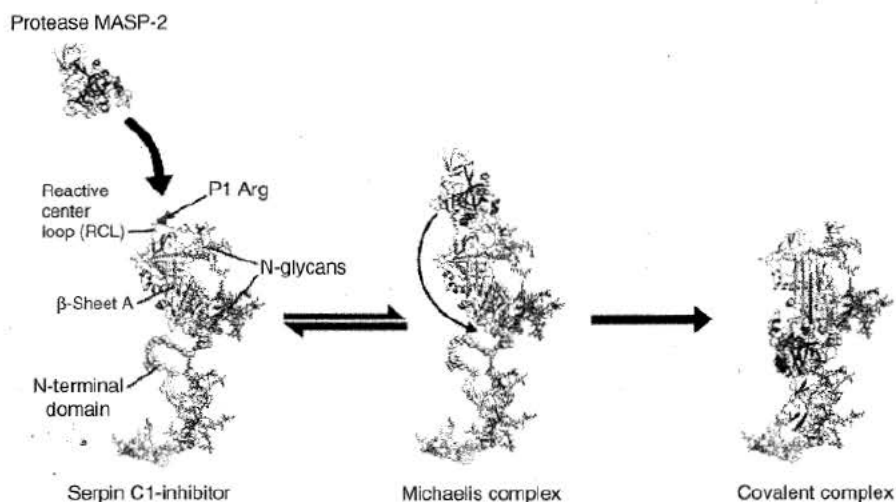
The C1-inhibitor inactivates the target proteases C1s, C1r, FXIIa, FXIa, MASP-1, and MASP-2 by exposing a loop containing the active site P1–P1' peptide bond (Arg444–Thr445) of the inhibitor [13,18,56]. This is recognized as a

substrate by the protease and results in cleavage of the exposed C1-inhibitor reactive site by the protease, leading to a covalent, SDS-stable complex between the protease and C1-inhibitor and a rapid conformational change of the C1-inhibitor protein that inactivates the protease ("mousetrap" effect) [63]. In this process, in addition to the protease, C1-inhibitor is also inactivated due to the covalent binding to the target proteases. Thus, C1-inhibitor is called a suicide inhibitor. The mechanism of action is described in more detail by Wagenaar-Bos and Hack [18]. Following the inactivation reaction, the C1-inhibitor–protease complexes are cleared from the circulation through binding to serpin-specific receptors in the liver (low-density lipoprotein receptor-related protein) [64] or uptake by neutrophils and monocytes [65].

Figure 17.3 shows a 3D model of the binding and inactivation of MASP-2 by C1-inhibitor. The serpin domain of C1-inhibitor was modeled using the crystal structure (2OAY) previously reported [14,16].

#### 17.5 MANUFACTURE

Plasma-derived C1-inhibitor concentrates have been used clinically to treat HAE in Europe, Canada, and Japan for many decades. However, only a few manufacturers have implemented a production process for C1-inhibitor in their



**FIGURE 17.3** Mechanism of inactivation of the target protease MASP-2 by C1-inhibitor. The left and middle model show the binding of MASP-2 to the reactive center loop of C1-inhibitor and the formation of the noncovalently bound complex. Cleavage of the P1-P1' peptide bond by MASP-2 is followed by a rapid conformational change of C1-inhibitor (mousetrap effect) by which the protease is buried in the central  $\beta$ -sheet of C1-inhibitor and is covalently bound to it (right model). Only a portion of MASP-2 is shown. The N-terminal domain and glycans were modeled from neutron diffraction and electron microscopic data. (Modeling and pictures courtesy of Dr. L. Beinrohr, Institute of Enzymology, Budapest, Hungary.)

plasma fractionation plant. One of the first successful attempts to purify C1-inhibitor with relatively high purity and yield was reported by Pensky in 1961. Starting from serum, contaminating proteins were precipitated with 40% saturated ammonium sulfate (SAS) and further purification was achieved by ion-exchange chromatography [21]. In 1970, another procedure was reported from Behringwerke AG (Marburg, Germany) that described the purification of C1-inhibitor for biochemical characterization from 1:2 diluted recalcified acid citrate dextrose (ACD) plasma by the use of chromatography on DEAE-cellulose, followed by fractionated SAS precipitation (50–65% SAS fraction) and a Sephadex® G-150 gel filtration [9]. In 1982, Nilsson and Wiman [11] published a purification procedure consisting of precipitation of C1-inhibitor from plasma by polyethylene glycol (PEG), followed by chromatography on DEAE-cellulose and hydrophobic interaction chromatography on hexyl-Sepharose®. Table 17.1 summarizes these and a number of other published procedures for the purification of C1-inhibitor. Many of these activities were for research purposes and synthetic protease inhibitors were often added to the starting material to prevent degradation of C1-inhibitor. As these substances can be toxic, these purification procedures are not suitable for manufacturing C1-inhibitor for clinical application.

A variety of virus reduction steps have been applied in the published C1-inhibitor manufacturing processes. These have included pasteurization, using appropriate stabilization conditions (3 M potassium or ammonium citrate [83] or 60% sucrose [67]), dry-heat treatment (68–100°C) [71], “vapor heat” treatment [74,84,85], S/D treatment [79], and nanofiltration [79,80,82].

Based on the purification approaches detailed above, a few plasma product manufacturers embarked on developing a large-scale procedure for producing C1-inhibitor for clinical use. One of the first products manufactured involved C1-inhibitor captured from cryoprecipitate-depleted plasma by adsorption to DEAE-Sephadex A-50 equilibrated at a slightly lowered ionic strength. Further purification was achieved by incremental precipitation steps using ammonium sulfate [66]. In later versions of this product, as produced by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (now Sanquin Blood Supply Foundation, Amsterdam, The Netherlands), the safety for transmission of hepatitis B virus was improved by addition of a small amount of anti-hepatitis Bs immunoglobulin and for HIV by the introduction of a dry-heat treatment step (72 h, 60°C) in 1986 [86]. This concentrate was succeeded by a next-generation product, launched in 1997, called Ceter®<sup>®</sup>, and was based on purification principles published by Wickerhauser and Benny [71,72].



TABLE 17.1 Principles of Published Purification Methods of C1-Inhibitor from Human Plasma or Serum

Source Material and Additives	Purification Steps	Purification <sup>a</sup> Factor from Plasma Purity and/or Specific Activity	Yield (%)	References and Year of Publication
Serum	(1) 40% SAS precipitation	236×, 90 U/mg N	60 (40–90) (activity)	[23] 1961
Recalcified ACD plasma	(2) Dowex <sup>®</sup> 2-X10 anion-exchange chromatography (1) DEAE-cellulose DE32 <sup>®</sup> chromatography (2) 50–65% SAS precipitation (3) Sephadex G-150 gel filtration (4) Zone electrophoresis	248×	34 (activity)	[9]* <sup>b</sup> 1970
Prothrombin complex-depleted citrated cryosupernatant plasma	(1) DEAE-Sephadex A-50 chromatography (2) 50–65% SAS precipitation	100–150×	35 (activity)	[66]* 1974
ACD or CPD plasma + EDTA, polybrene, DFP	(1) 6% PEG 6000 precipitation (2) DEAE-cellulose DE32/DE 52 <sup>®</sup> chromatography (3) Concanavalin A-Sepharose chromatography	300×, >95%	33–42 (activity)	[10] 1981
Plasminogen-depleted EDTA plasma + EDTA, polybrene	(1) 6% PEG 6000 precipitation (2) DEAE-Sephacel <sup>®</sup> chromatography (3) Hexyl-Sepharose chromatography	>95% on SDS-PAGE	50 (activity)	[11]* 1982
Fresh EDTA plasma + benzamidine	(1) 5% PEG 4000 precipitation (2) Lysine-Sepharose chromatography (3) DEAE-Sephadex A-50 chromatography (4) Sephadex G-150 gel filtration (5) Hydroxylapatite chromatography	610× Homogeneous on SDS-PAGE	72 (antigen)	[12] 1983
Prothrombin complex-depleted citrated cryosupernatant plasma	(1) QAE-Sephadex chromatography (2) 60% SAS precipitation (3) Pasteurization (+60% sucrose) (4) 60% SAS precipitation (5) Phenyl-Sepharose chromatography	90%, 40–60 U/mg	20 (activity)	[67,68]* 1986
Serum or plasma +/– heparin, DFP	(1) Monoclonal anti-C1-inhibitor chromatography (2) FPLC Mono Q HR chromatography	Homogeneous on SDS-PAGE	40 (antigen)	[69] 1987
ACD plasma + PMSF, NPGb	(1) 16–45% PEG 4000 precipitation (2) DEAE-Sephacel chromatography (3) Zn <sup>2+</sup> -chelate agarose chromatography (4) Anticontaminant immunoabsorption chromatography	785× Homogeneous on SDS-PAGE	33 (antigen) 61 (activity)	[70] 1987
Cryosupernatant plasma	(1) DEAE-Sephadex A-50 chromatography (2) 20% PEG 4000 precipitation (3) CM-Sephadex C-50 chromatography (4) 50–65% SAS precipitation (5) Dry-heat treatment	6.0 PU/mg (antigen) 6.5 PU/mg (activity)	20 (antigen) 24 (activity)	[71]* 1987
Factor IX-depleted plasma	(1) DEAE-Sephadex A-50 chromatography (2) 20% PEG 4000 precipitation (3) CM-Sepharose FF chromatography	417×, >90% 6.7 PU/mg	16 (activity) (160 PU/kg)	[72]* 1988

(continued)



TABLE 17.1 (Continued)

Source Material and Additives	Purification Steps	Purification <sup>a</sup> Factor from Plasma, Purity and/or Specific Activity	Yield (%)	References and Year of Publication
Cryosupernatant of fresh-frozen plasma	(1) DEAE-Sephadex A-50 chromatography (2) 20% PEG 4000 precipitation (3) CM-Sephacel FF chromatography (4) Dry-heat treatment (80°C, 72 h)	795×, >95% 20 PU/mg	33 (activity) (179 PU/kg plasma)	[73]* 1992
Cryosupernatant plasma	(1) DEAE-Sephadex chromatography (2) PEG precipitation (3) SAS precipitation (4) "Vapor" heating	≥0.8 PU/mg	Not mentioned	[74,75]* 1998
Citrated plasma + EDTA, NPBG, SBTI	(1) 21.4–45% PEG 3350 precipitation (2) Jacalin-agarose chromatography (3) Phenyl-Sepharose chromatography	778× <sup>c</sup> (antigen) 2955× <sup>c</sup> (activity) Homogeneous on SDS-PAGE	39 (antigen and activity)	[76] 1989
	(1) 21.4–45% PEG 3350 precipitation (2) Jacalin-agarose chromatography (3) DEAE-Sepharose FF chromatography (4) Phenyl-Sepharose chromatography	39 U/mg Homogeneous on SDS-PAGE	Not mentioned	[77] 1993
Preadsorbed fresh-frozen plasma + benzamidine, PMSF	(1) Cibacron <sup>®</sup> Blue-Sepharose chromatography (2) Mono Q FPLC chromatography	Homogeneous on SDS-PAGE	23 (activity)	[78] 1990
Citrated plasma, depleted of cryoprecipitate, prothrombin complex and antithrombin	(1) DMAE-Fractogel <sup>®</sup> EMD 650M chromatography (2) S/D treatment (3) SO <sub>3</sub> -Fractogel EMD 650M chromatography (4) 35 and 15 nm filtration	>80% 6.5 PU/mg	45 (antigen) 58 (activity)	[79]* 1994
Citrated plasma	(1) QAE-Sephadex A-50 chromatography (2) 12% PEG 4000 precipitation (3) Tween <sup>®</sup> 80 treatment and QAE-Sephadex adsorption (4) 15 nm filtration	6.0 PU/mg	50 (activity)	[80]* 2001
Prothrombin complex-depleted cryosupernatant citrated plasma	(1) DEAE-Sepharose FF chromatography (2) 20% PEG precipitation (3) CM-Sepharose FF chromatography (4) Pasteurization (10 h, 60°C) (5) 15 nm filtration (two filters in series)	>90% ≥4 PU/mg	35 (activity)	[81,82]* 1999, 2007

ACD: acid citrate dextrose; CPD: citrate phosphate dextrose; DFP: diisopropyl fluorophosphate; NPBG: *p*-nitrophenyl-*p'*-guanidino benzoate; PMSF: phenylmethylsulfonyl fluoride; SBTI: soybean trypsin inhibitor; N: nitrogen; PEG: polyethylene glycol; PU: plasma unit of C1-inhibitor activity; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis. Dowex is a trade mark of Dow Chemical Company; DEAE-cellulose DE32 and DE52 are trade marks of Whatman; Sephadex, Sephacel and Sepharose are trade marks of GE Healthcare Ltd.; Fractogel is a trade mark of Merck Chemicals; Cibacron is a trade mark of Ciba-Geigy Corp.; Tween 80 is a trade mark of ICI Americas, Inc.

<sup>a</sup>Specific activities (U/mg or PU/mg) cannot be compared directly, because of differences in assays and standards. Assuming a plasma C1-inhibitor protein concentration of 0.20–0.25 mg/mL and an activity of 1 PU/mL, 100% pure C1-inhibitor has a specific activity of 4–5 PU/mg. Assuming a total protein concentration in plasma of 60 mg/mL, the theoretical maximally purification factor is 240–300 times.

<sup>b</sup>The asterisk indicates that the method is designed for large-scale production.

<sup>c</sup>Depends on the assay used.

It exhibited a higher purity (>90% homogeneity), a higher concentration (100 U/mL), a higher process yield, and higher safety margins with respect to transmission of viruses [81,82]. The fractionation process for this product in The Netherlands still comprises capturing C1-inhibitor from prothrombin complex-depleted cryosupernatant plasma by anion-exchange chromatography, followed by purification by PEG precipitation, pasteurization (10 h, 60°C), and cation-exchange chromatography. PEG precipitation and pasteurization in this process are validated steps for virus removal [82]. In a later product version, a 15 nm virus removing filtration step (nanofiltration) using two filters in series was implemented [82]. This had no effect on the pharmacokinetics and allowed the anti-hepatitis Bs immunoglobulin [82,87] to be omitted from the product. A similar product produced from US-sourced plasma is now also marketed in the United States by ViroPharma Inc. under the trade name of Cinryze<sup>TM</sup>.

The only other plasma-derived C1-inhibitor product that at present has a marketing license for treating HAE patients is Berinert<sup>®</sup>, produced by CSL Behring (Marburg, Germany). The first-generation product was introduced in Germany in 1979 [88,89]. Since then Berinert has been launched in a large number of countries worldwide, including Japan and recently also the United States. The present generation product, introduced in 1985, incorporates pasteurization and an additional purification step in the manufacturing process. The starting material for the manufacture of the product is cryoprecipitate-depleted plasma the process involves removal of the prothrombin complex proteins with DEAE-Sephadex, the capture of the C1-inhibitor from the flow-through with QAE-Sephadex and further purification by precipitation with 60% SAS followed by hydrophobic interaction chromatography on Phenyl-Sepharose. As a virus inactivation step, pasteurization is performed for 10 h at 60°C in the presence of sucrose and glycine [67,68]. Besides the pasteurization, the hydrophobic chromatography step is also claimed as a virus reduction step [89].

In the past, Immuno AG (Vienna, Austria; now Baxter BioScience) also produced a plasma-derived C1-inhibitor product (C1-esterase inhibitor TIM 3) but marketing of this product ceased in the 2000s. The production process consisted of adsorption of C1-inhibitor onto DEAE-Sephadex and purification by consecutive precipitations using PEG and ammonium sulfate [75]. Virus inactivation was achieved by the so-called "vapor heat" treatment, consisting of heating the moist, lyophilized product at 60°C for 10 h at slightly elevated pressure [74,84,85]. Another Baxter process comprised capturing of C1-inhibitor on QAE-Sephadex, followed by PEG precipitation, treatment of the supernatant solution with a detergent (Tween 80), and subsequent capture of the protein onto an anion-exchange resin. The recovered eluate was then filtered through a 15 nm virus removal filter. Finally, the finished product was treated with dry heat ( $\geq 72$  h,  $\geq 80^\circ\text{C}$ )

[80]. A C1-inhibitor product based on this process has not been launched on the market.

The Centre Régional de Transfusion Sanguine (CRTS) in Lille, now part of LFB (Laboratoire Français du Fractionnement et des Biotechnologies, Paris, France) has also developed a large-scale production scheme for C1-inhibitor but this product was never taken into clinical trials and it was never marketed [79]. The process made use of anion-exchange chromatography on DMAE-Fractogel EMD and adsorption to SO<sub>3</sub>-Fractogel EMD. As virus reduction steps, S/D treatment and sequential 35 and 15 nm filtration were applied.

## 17.6 CHARACTERIZATION

Therapeutic C1-inhibitor products are tested by a variety of analytical methods before release. Because of its limited worldwide use and the small number of manufacturers, currently no pharmacopoeia requirements exist for C1-inhibitor concentrates. Therefore, manufacturers analyze their C1-inhibitor products using their own specific analytical methods. However, the European Pharmacopoeia and the United States Pharmacopoeia monograph requirements for other plasma proteins are used as a basis. Like all pharmaceutical products for human use, the product needs to be tested for sterility, absence of pyrogens and endotoxins, excipients, and osmolality. Biological activity, usually measured in a chromogenic assay as the capacity to inhibit C1s, as well as antigen levels are used as relatively simple means to characterize the product [90]. Prompted by the large variation in assay results between laboratories worldwide reported by Wagenaar-Bos, a program has been run by the National Institute of Biological Standards and Control (NIBSC, London, UK) to raise International Standards for measuring C1-inhibitor in plasma and concentrates [90,91]. These Standards (08/262 for plasma and 08/256 for concentrates) have now been approved by the WHO Expert Committee on Biological Standardization, and represent a great advance in the standardization of testing requirements and for the adoption of measures to enhance the quality of therapeutic C1-inhibitor concentrates.

During the development of the C1-inhibitor products extensive characterization studies are performed using commonly used methods for protein characterization. In addition, specific tests suited for the analysis of glycoproteins are used, including electrophoresis, isoelectric focusing, glycosylation analysis, mass spectrometry, molecular mass analysis, and 2D-fluorescence difference gel electrophoresis (2D-DIGE). This data is used to verify the native state of the molecule. Methods are employed to measure the amount of active C1-inhibitor, through the use of a so-called functional ELISA [59,69,92]. The amount of inactivated C1-inhibitor can also



be measured using a radioimmunoassay with an antibody specific for neoepitopes that arise after cleavage [93].

It is important that the C1-inhibitor molecule in therapeutic preparations has a native conformation. There should be minimal aggregation or degradation of the protein as detected by SDS-PAGE or size exclusion chromatography. This minimizes any possible immunogenicity of the product. The preparations should also have a high purity to avoid side effects that can be caused by copurifying plasma proteins. For instance, although C1-inhibitor products are usually manufactured from prothrombin complex-depleted plasma, it is still necessary to show that any remaining coagulation factors are removed during the manufacturing process to exclude the risk of possible thromboembolic complications following *in vivo* administration.

## 17.7 CLINICAL ISSUES

### 17.7.1 Hereditary Angioedema

HAE is a rare autosomal dominant disorder affecting approximately 1:10,000 up to 1:50,000 people [94,95]. HAE is caused by a significantly reduced capacity to inhibit kallikrein [2] that is caused by a deficiency of C1-inhibitor [3]. This can be either quantitative (HAE type I, 85% of the cases) or qualitative (HAE type II, 15% of the cases). These two types are indistinguishable in clinical presentations.

The disease occurs equally in men and women, and no difference between ethnic groups has been demonstrated. Although HAE is a congenital disease, as many as 25% of the mutations are *de novo* mutations [22,96]. C1-inhibitor mutations that cause HAE type I occur throughout the gene and result in truncated or misfolded proteins that are not efficiently secreted, resulting in decreases in both antigenic and functional levels of C1-inhibitor. Mutations that cause HAE type II usually involve exon 8 at or near the active site, and result in a mutant protein that is secreted, but that is dysfunctional. In addition a type III has been described with similar clinical features as HAE types I and II, but with normal C1-inhibitor functionality. This syndrome is rather heterogeneous, but many cases are caused by a genetic defect in FXII [97,98]. Treatment of attacks is possible by supplying additional C1-inhibitor [98].

Patients are heterozygous for C1-inhibitor deficiency, but typically the functional level of C1-inhibitor is on average 20–30% of the normal level as a result of ongoing consumption and inefficient synthesis. In addition it has been reported that another mechanism for the low concentration of C1-inhibitor may be inhibition of synthesis at the RNA level [99].

The low functional C1-inhibitor levels that have been discussed result in a bradykinin-mediated increase in vascular permeability, leading to recurrent episodes of subcutaneous swelling, involving the skin of extremities, face, trunk, genitalia, gastrointestinal tract, tongue, larynx, and other viscera (Figure 17.4). The frequency of attacks differs



**FIGURE 17.4** Facial swelling in a HAE patient before (a) and during (b) an attack. (Images reproduced with permission from the US Hereditary Angioedema Association (HAEA) [www.haeimages.com](http://www.haeimages.com).) (See insert for color representation of this figure.)



per patient, and may even differ within a patient's life. In the majority of cases first disease symptoms will occur in childhood, with less than 10% of the cases appearing after the second decade of life. They worsen during puberty and then persist throughout life. Patients with earlier onset of the disease tend to have more frequent attacks. The functional C1-inhibitor level does not correspond with the disease severity [100]. Although several factors have been described that can provoke an attack, many attacks seem to occur without any trigger [101]. In fact, the clinical variability of HAE is large and attacks cannot be predicted [102,103].

Swellings can be disfiguring (Figure 17.4), disabling, painful and even life-threatening. Attacks of edema of the gastrointestinal tract may present as an acute abdominal syndrome with severe pain, signs of peritonitis or ileus [100,104]. Laryngeal and upper airway involvement may cause respiratory insufficiency and death from suffocation.

The clinical presentation of the acquired form of angioedema (AAE) resembles that of congenital HAE and is characterized by a decrease in C1-inhibitor activity due to autoantibodies to C1-inhibitor [43]. Treatment of the underlying disease may result in full clearance of AAE [105].

In clinical practice diagnosis of HAE can be troublesome and is often delayed by misinterpretation of the clinical symptoms as urticaria, allergy, abdominal colics, and colics due to nephrolithiasis or appendicitis. Patients with a positive family history of HAE are diagnosed earlier than patients who come from a negative family history. Laboratory testing is needed to confirm the diagnosis of HAE. A diagnostic algorithm has been proposed in an international consensus document for HAE diagnosis [106].

Angioedema is a net result from the uncontrolled generation of vasoactive mediators, such as bradykinin, due to insufficient inhibition of active proteases by C1-inhibitor. Therefore, supplementing C1-inhibitor to a level that is sufficient to inhibit the activation of the contact system is a rational therapeutic approach. After C1-inhibitor administration, relief of symptoms starts within hours and the time for resolution of the symptoms is shortened significantly. A delay in treatment can be fatal in cases of laryngeal attack with the patient suffocating if airway control is not promptly guaranteed. Today treatment involves considering the nature, severity, and frequency of attacks, and quality-of-life issues [95,101].

Attenuated androgens (danazol, stanazolol, oxandrolone, methyltestosterone) are widely used as prophylactic treatment in HAE. They act by increasing the biosynthesis of many proteins, including the hepatic production of C1-inhibitor protein. They also stimulate the generation of aminopeptidase P that breaks down bradykinin [107]. However, use of attenuated androgens in HAE is associated with a variety of side effects—some of which are severe [95,101]. Therefore, high dosages should be avoided, in particular in women, because of the risk of masculinization. Androgens

are contraindicated in children and in women during pregnancy.

Antifibrinolytic agents (tranexamic acid,  $\epsilon$ -aminocaproic acid) inhibit plasminogen activation with consequent "sparing" of C1-inhibitor usage. They decrease the number and the severity of attacks, but are not as effective as the attenuated androgens and side effects are generally mild [95,101].

In countries where C1-inhibitor concentrates are not available plasma can be used as a source of C1-inhibitor in emergency cases [44,95,108,109]. However, as 1 mL of plasma contains only 1 U (PU) of C1-inhibitor, the required volume of the plasma and the duration of administration can be a limitation. Also, it is questionable whether the administration of plasma, due to the presence of complement components, would be successful. Data suggests that the administration of plasma may even exacerbate HAE attacks [44,95,100].

For these reasons a purified concentrate of C1-inhibitor is the product of choice for treating an attack in a patient with HAE [24,74,85,95,110]. In most cases of acute attack, administration of C1-inhibitor induces relief of symptoms within 2 h, with a concomitant reduction of FXIIa, kallikrein, bradykinin, and plasmin [111]. C1-inhibitor can be used effectively for long-term prophylaxis [112–114] as well as for short-term prophylaxis for an event such as elective surgery, which may trigger an edema attack [115–118].

### 17.7.2 Other Potential Clinical Indications

Interest is increasing in exploring the clinical use of C1-inhibitor in other clinical settings [55,119,120]. This is not only based on the fact that C1-inhibitor downregulates the complement and contact activation systems but also on its strong anti-inflammatory properties that appear to be independent of its protease targeting function [49,55,121–123]. The anti-inflammatory activity seems to be exerted by the heavily glycosylated N-terminal domain of the molecule [124,125] and involves noncovalent interactions with proteins (extracellular matrices), cells, bacteria, and endotoxins. The following is a brief and nonexhaustive summary of recent findings from animal models and preliminary studies in man.

- (1) *Sepsis and Endotoxemia*. These clinical conditions lead to activation of both the complement and contact system, which, when excessive, may result in pathologic inflammation and shock. Sepsis has also been shown to generate inactive C1-inhibitor in the circulation [25]. Therefore, it has been speculated that administration of C1-inhibitor in these conditions could have a beneficial effect.

In sepsis, positive outcomes have been reported in animal models, including baboons [126] and mice and rats [127,128]. This has prompted a number of



preliminary trials in humans [129–133] that have shown favorable results.

In endotoxemia, studies on endotoxin-induced shock displayed encouraging results in dogs [134], mice [135], and man [136]. In addition to inhibiting the inflammatory activated complement, the anti-inflammatory effect is also ascribed to binding of endotoxin to the N-terminal portion of the C1-inhibitor protein [124,125,135].

- (2) Treatment of *Capillary Leak Syndrome* (CLS) with C1-inhibitor has also been tested. It has been reported that administration of C1-inhibitor, in a pilot study of six patients, markedly decreased the side effects of treatment with interleukin 2 [137]. Promising results were observed in a group of 15 patients with CLS following bone marrow transplantation and in two patients with CLS after lung transplantation when transfused with C1-inhibitor. A similar result was observed in a case of severe CLS after stem cell transplantation that was treated with a combination of C1-inhibitor and plasminogen activator [138–140]. Furthermore, in a randomized double-blind trial in 24 neonates undergoing cardiac surgery with cardiopulmonary bypass, results indicated that prophylactic therapy with C1-inhibitor resulted in less inflammatory responses and capillary leak than in the placebo group [141]. It should be noted that a year earlier fatal thromboembolic complications had been observed in a similar patient group, following use of very high dosages of C1-inhibitor (500 PU/kg) in an attempt to prevent CLS [142]. In the above study, the C1-inhibitor was administered at a dose of 100 PU/kg.
- (3) *Ischemia and Reperfusion Injury*. Acute myocardial infarction is associated with local inflammatory reactions, due to activation of the contact system and complement system. In animal models of myocardial infarction beneficial effects have been shown in rats [143], pigs [144], cats [145], and mice [56]. Studies of myocardial and brain infarction in humans have also shown favorable results [146–152]. Other studies involving the examination of the effect of C1-inhibitor in liver ischemia in the rat [153], *ex vivo* reperfusion of pig liver [154], ischemia and reperfusion injury of skeletal muscles in mice [155], intestinal ischemia in a mouse model [156], and a number of animal models of transplantation [157–160] have generated promising results with respect to the possible application in human clinical conditions. There are also indications that C1-inhibitor therapy is capable of inhibiting antibody-mediated organ transplant rejection, as studied in primates [161,162].

- (4) *Pancreatitis*. Several publications describe a beneficial effect of administering C1-inhibitor in animal models of acute pancreatitis [163,164] and in humans [165–167], although in some other animal studies this effect was not confirmed [168].

- (5) Positive outcomes have been reported for a number of other animal models relating to burns [169,170], thermal injury [171], and trauma [172].

A relatively new development is the potential use of C1-inhibitor in fibrinolytic treatment, where it was shown *in vitro* and in a dog model that nonspecific plasminogen activation could be counteracted by C1-inhibitor, allowing higher and hence more effective doses of a recombinant prourokinase mutant [173,174]. Also, positive results of C1-inhibitor use in a mouse model for malaria were announced recently [56]. A comprehensive review of possible novel applications of C1-inhibitor has recently been published [175].

## 17.8 FUTURE TRENDS

A clear trend over the past few years in the treatment of HAE has been the use of C1-inhibitor in prophylactic regimens, and even treatment at home. Clinical experience has clearly shown that C1-inhibitor is safe and effective when used prophylactically and this represents a major step forward in the treatment of this potentially life-threatening disease [113,114,176,177]. As with almost every other human plasma-derived therapeutic there are some developments that will have an impact on the production and clinical usage of plasma-derived C1-inhibitor. First of all, C1-inhibitor can be produced via recombinant-DNA technology, such as in transformed COS cells, Chinese hamster ovary (CHO) cells, and yeast (*Pichia pastoris*) [178–180]. At this point, the only recombinant C1-inhibitor product that has been investigated in HAE patients has been produced transgenically [181,182]. This product, called conestat alpha, is extracted from the milk of transgenic rabbits and is produced by Pharming NV, The Netherlands (trade names in Europe and United States are Ruconest® and Rhucin®, respectively). Its glycosylation differs from the human plasma-derived protein [183,184] resulting in a shorter half-life of 3 h [185]. Despite this characteristic, Ruconest is efficacious in treating acute attacks in HAE patients [186,187]. Ruconest was approved in 2010 in the European Union by the European Medicines Agency. At the time of writing of this chapter, a product license application for Rhucin was being assessed by the FDA in the United States.

Other products for treatment of HAE that are based on different principles to that of replacing C1-inhibitor are also



coming on to the market. Among these are Firazyr<sup>®</sup> (icatibant, marketed by Shire) and Kalbitor<sup>®</sup> (ecallantide, marketed by Dyax). Firazyr is a bradykinin B2 receptor antagonist, which can be administered subcutaneously, and is indicated for symptomatic treatment of acute attacks of HAE in adults. In two double-blind randomized trials, efficacy has been demonstrated [188,189]. Adverse reactions were generally mild in severity, transient, and resolved without further intervention. In approximately 10% of the patients, a second dosage was required [189]. Kalbitor, a recombinant version of the first Kunitz domain of tissue factor pathway inhibitor (TFPI), can be used for acute attacks of HAE in patients 16 years of age and older [190]. It is also intended for subcutaneous use and has a half-life of approximately 2 h. Side effects are not uncommon, including anaphylactic reactions that were reported in 2.7% of the patients [191]. The formation of antibodies has also been described in the literature [192].

Firazyr and Kalbitor are alternatives to C1-inhibitor for HAE treatment as they both possess protease inhibiting activity. C1-inhibitor, however, also possesses anti-inflammatory properties independent of its protease inhibiting activity but instead determined by its glycosylation profile (see Section 17.7.2). It is unlikely that Firazyr or Kalbitor could mimic this effect. A recombinant product could mimic this property if it had the complete, "wild type" glycosylation profile. The present transgenic product Ruconest/Rhucin, however, exhibits some differences in glycosylation pattern when compared to the native C1-inhibitor molecule. Thus, it remains to be seen whether this particular product possesses physiological functions of native C1-inhibitor other than just those of protease inhibition. In a mouse brain ischemic injury model Ruconest/Rhucin was shown to be superior to plasma-derived C1-inhibitor due to an enhanced capability to modulate the activity of mannose binding lectin [152].

With respect to pathogen safety there is no longer a significant point of differentiation between plasma-derived and recombinant products. Theoretically, plasma-derived products carry a risk of transmitting pathogens like blood-borne viruses and prions. However, with the present-day technologies for manufacturing plasma-derived products, this risk can be considered as very remote. Similarly, recombinant products have to deal with the potential presence of animal pathogens, but effective measures are also in place. Finally, both types of products always bear a potential risk of immunogenicity, dependent on the presence of denatured protein, nonhuman glycosylation, or nonhuman protein. Only time will reveal the magnitude of this risk.

Another trend that needs to be critically assessed is further potential clinical application of C1-inhibitor. For almost two decades now researchers have speculated whether C1-inhibitor could be used successfully in a variety of clinical settings other than HAE (see Section 17.7.2). Despite a number of positive reports and explorative clinical

trials real breakthroughs in the areas of sepsis, ischemic injury, transplantation, pancreatitis, and so on, have not yet occurred. One explanation for this may be the complexity of these clinical conditions. It could be that additional components other than just C1-inhibitor alone could influence the course of the disease. Other reasons may be that the still limited availability of plasma-derived C1-inhibitor has restricted the opportunities to pursue investigations into alternative uses for C1-inhibitor. In addition, it is possible that many alternative clinical uses may require relatively high doses of C1-inhibitor and this could make the new therapy costly. With the advent of recombinant products, which in principle means an unlimited supply, and also the possibility of engineering the molecule in such a way that it is most effective for treating a targeted disease, the situation may change in the not-too-distant future [193]. Whatever the origin of the C1-inhibitor product, it is clear that clinical trials of proper design are needed to demonstrate whether C1-inhibitor will be a safe and efficacious new asset in the treatment of diseases other than HAE.

## 17.9 CONCLUSION

Present-day plasma-derived C1-inhibitor concentrates are of high quality and are both safe and effective for the treatment of HAE. For effective prophylaxis and the rapid treatment of attacks the preferred procedure consists of administering a concentrated C1-inhibitor product derived from plasma. Whether the recombinant version will also be sufficiently effective in prophylactic treatment of HAE patients still remains to be demonstrated. C1-inhibitor concentrates also have the potential to be used in clinical settings other than HAE, and this represents an opportunity to address unmet medical needs today. The generation of sufficient amounts of material for such studies would most likely depend upon the production of a recombinant C1-inhibitor with a comparable protein sequence, conformation, and glycosylation profile to the native C1-inhibitor.

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