A SIMPLIFIED PROCEDURE FOR THE PURIFICATION OF C1̄-INACTIVATOR FROM HUMAN PLASMA

Interaction with complement subcomponents C1̄r and C1̄s

A. REBOUL, G. J. ARLAUD, R. B. SIM and M. G. COLOMB

DRF/Biochimie, Centre d'Etudes Nucléaires et Université Scientifique et Médicale, 85X, 38041 Grenoble-Cedex, France

Received 28 March 1977

1. Introduction

C1̄-Inactivator (C1̄ In), a serum α2-glycoprotein, blocks the activity of the subcomponents of C1, C1̄r and C1̄s. It also inhibits Factor XIIa and Factor XIa of the blood clotting system as well as plasmin and plasma kallikrein. In this paper a simplified procedure for the purification of C1̄ In in high yield is presented. It consists of three major steps including PEG-precipitation followed by DEAE-cellulose and Con A–Sepharose chromatography. The protocols described previously by Haupt et al. [1], Nagaki et al. [2], Harpel and Cooper [3], Anderson et al. [4] and Harpel [5] are all characterized by multiple steps not always compatible with the maintenance of maximum functional reactivity during purification of the C1̄ In. C1̄ In obtained by the procedure described here is homogeneous, of high specific activity and is thus suitable for studies of protease–inhibitor interactions. Aspects of the interactions of C1̄ In with C1̄r and C1̄s are reported.

Abbreviations: EDTA, ethylene diamine tetracetic acid; TAME, *p*-toluene sulfonyl-L-arginine methyl ester; DFP, di-isopropyl phosphorofluoridate; SDS, sodium dodecyl sulphate; ZLNE, *N*-α-carbobenzoxy-L-lysine *p*-nitrophenyl ester; PEG, polyethylene glycol. The nomenclature of the components of complement is that recommended by World Health Organization (1968). Activation of a component is indicated by a bar. Enzymatic activities are expressed in nanokatals (nkat) as recommended by the IUPAC-IUB commission on Enzyme Nomenclature (1973).

2. Materials and methods

Human citrated plasma was obtained from the 'Centre de Transfusion Sanguine', Grenoble. C1s and C1̄s were purified as described by Arlaud et al. [6], C1r and C1̄r according to Gigli et al. [7]. C1̄s esterase activity was measured with 1.5 mM TAME in 50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA (pH 7.6) at 25°C, or with 0.1 mM ZLNE in 100 mM Na-phosphate, 100 mM NaCl, 5 mM EDTA (pH 6.0) also at 25°C. Protein was determined by the method of Lowry et al. [8]. Purified C1̄ In, C1̄r and C1̄s were quantified from their absorbance at 280 nm using $E^{1\%}_{1\,cm}$ 4.5, 11.5 and 9.4, respectively [5,7]. In plasma and impure fractions, C1̄ In was specifically estimated by radial immunodiffusion in M-Partigen plates for C1̄ In purchased from Behring.

The inhibitory activity of C1̄ In on C1̄s was calculated from the measurement of the residual TAME esterase activity following the incubation of C1̄ In with C1̄s for 15 min at 37°C. One unit of C1̄ In is defined as the amount of C1̄ In able to inhibit totally one nkat of C1̄s. The inhibition of C1̄r by C1̄ In was measured by monitoring the ability of C1̄r to activate proenzymic C1s. SDS–Polyacrylamide gel electrophoresis was performed as described by Fairbanks et al. [9]. Samples were treated and molecular weights estimated as described elsewhere [6].

Double radial immunodiffusion was performed according to Ouchterlony and Nilsson [10] in 1% agarose, 10 mM EDTA, 150 mM NaCl and 100 mM

Na-phosphate buffer (pH 7.0) using antisera from Behring. Sucrose density gradient ultracentrifugation was performed according to Martin and Ames [11] in a Beckman L2-65B ultracentrifuge at 4°C. Beef liver catalase (11.6 S), yeast alcohol dehydrogenase (6.7 S) and ovalbumin (3.5 S) were used as markers. DEAE-cellulose (DE-52) was purchased from Whatman, Con A–Sepharose from Pharmacia, TAME was from Sigma, methyl-α-D-mannopyranoside from Calbiochem. PEG 6000 from Fluka, polybrene from Aldrich, and ZLNE from Interchim, Montluçon (France).

3. Results

3.1. *Purification of C1 In*

Unless otherwise stated all steps were performed at 4°C. DFP 0.2 M in propan-2-ol was added to citrated human plasma together with polybrene in 0.4 M EDTA (pH 7.0) to final concentration of 2 mM DFP, 1.2 mg/ml polybrene and 20 mM EDTA. The plasma was incubated for 30 min at 25°C.

PEG was then added to a final concentration of 6% (w/v) and the mixture was stirred for 30 min. The precipitate was harvested (15 min, 3000 × *g*) and discarded.

The supernatant was dialysed overnight against 20 mM sodium phosphate, 50 mM NaCl (pH 7.0) and applied to a column of DEAE-cellulose equilibrated in the same buffer. A large amount of protein was not adsorbed and the initial washing of the column was continued until the absorbance at 280 nm dropped below 0.03. The adsorbed proteins were eluted with a linear NaCl gradient from 50 mM to 200 mM in 20 mM sodium phosphate (pH 7.0). C1 In, detected by inhibition of C1s esterase activity was eluted in the buffer containing 120–160 mM NaCl (fig.1).

After concentration on Amicon PM 10 membrane and equilibration in 50 mM Tris–HCl, 100 mM NaCl (pH 8.0) the fraction containing C1 In was applied to a column of Con A–Sepharose equilibrated in the same buffer. The column was washed with the starting buffer until absorbance at 280 nm dropped to zero. This washing removed the last traces of albumin. C1 In was eluted with 2% methyl-α-D-mannopyranoside added to the starting buffer (fig.2) and detected as described for the DEAE-cellulose step. Before storage at −20°C the final eluate was concentrated and

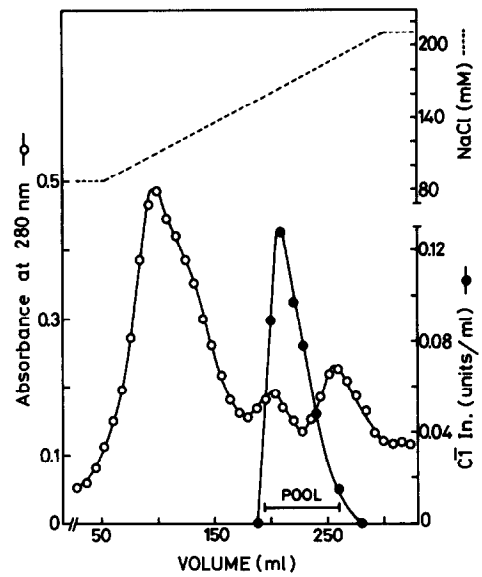

Fig.1. Chromatography on DEAE-cellulose. The dialysed supernatant from PEG precipitation of 110 ml plasma was applied to a DE-52 cellulose column (2.5 × 4 cm) and eluted as described in Results with a 400 ml linear gradient of NaCl. Fractions containing C1 In were pooled as indicated.

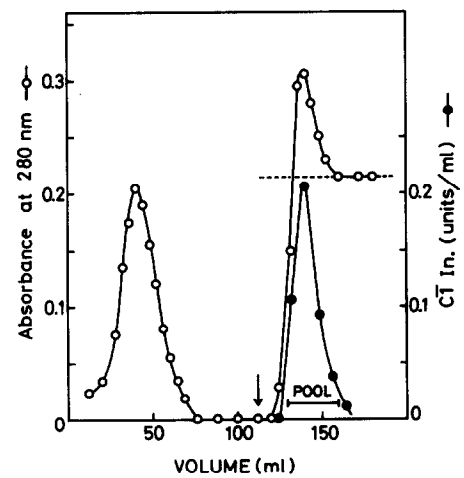

Fig.2. Chromatography on Con A–Sepharose. The pool from DE-52 chromatography was applied to a 2.5 × 4 cm column of Con A–Sepharose. Elution was carried out as described in Results. The arrow indicates the introduction of methyl-α-D-mannopyranoside and corresponds to an increase in A_{280} shown by the broken line.

Table 1
Purification of $C\bar{1}$ In

Steps	Total protein (mg)	$C\bar{1}$ In antigen (mg)	Total activity ($C\bar{1}$ In units)	Spec. act. ($C\bar{1}$ In units/mg protein)	Yield in $C\bar{1}$ In antigen (%)	Purification factor a	Purification factor b
1. Plasma	6.380	26.5	522	0.082	100	–	1
2. PEG Supernatant	3.960	20.6	1030	26	78	1	13
3. DE-52 Eluate	13.1	–	479	36.5	–	140	–
4. Con A–Sepharose eluate after concentration	9.1	9.1	445	49	35	189	243

Total protein was determined by the method of Lowry et al. [8] at the first three steps and by $A_{280\ nm}$ at step 4. $C\bar{1}$ In activity and $C\bar{1}$ In antigen were measured as described in Materials and methods. The purification factors are based (a) on specific activity, (b) on protein content.

equilibrated in 10 mM Tris–HCl, 50 mM NaCl (pH 8.0).

Table 1 summarizes a typical purification. The yield of $C\bar{1}$ In is higher than reported for other purification procedures [1,3] probably due to the small number of preparative steps.

The $C\bar{1}$ In activity of plasma is variable but always less than the activity of the PEG supernatant suggesting that a fraction of $C\bar{1}$ In is not free in plasma, but may exist in loose complexes.

The purification factor calculated either from the increase in specific activity of $C\bar{1}$ In or from the increase in the specific antigen to total protein ratio is close to the theoretical maximum [1]. It may be concluded from the specific activity that the functional integrity of $C\bar{1}$ In is maintained throughout the purification procedure.

The final product is stable for several months at −20°C. Its purity was confirmed by the lack of precipitation in double radial immunodiffusion against antisera to $C\bar{1}$r, $C\bar{1}$s, $C\bar{1}$t, C3, C4, C5, caeruloplasmin, IgG, IgM, albumin and plasminogen.

On SDS–polyacrylamide gels (fig.3) reduction and alkylation of $C\bar{1}$ In did not modify its mobility. The major component had apparent mol. wt 98 000 (± 3000). No splitting of the major band as noted by Harpel and Cooper [3] was observed. However trace quantities of two minor bands of approx. apparent mol. wt 60 000 and 29 000 were detected. These may correspond to a specific split of $C\bar{1}$ In by proteases and their quantity could be diminished by increasing the concentration of protease inhibitors during the purification. Attempts to remove these minor components by chromatography on anti-$C\bar{1}$ In IgG–Sepharose 6B were unsuccessful probably because of the drastic conditions used to elute $C\bar{1}$ In from the immuno-adsorbant (2 M sodium trichloroacetate (pH 7.0) or 1 M propionic acid or 0.2 M glycine–HCl (pH 2.2)). In all cases the functional properties of $C\bar{1}$ In were destroyed by this procedure.

3.2. *Interaction $C\bar{1}$s–$C\bar{1}$ In and $C\bar{1}$r–$C\bar{1}$ In*

C1s was titrated with C1 In as shown in fig.4. Inhibition of $C\bar{1}$s by $C\bar{1}$ In was linear over the whole range. 100% Inhibition corresponded to a 1 : 1 molar ratio of the two proteins and no difference was observed in the presence of EDTA rather than calcium.

In agreement with previous results [2,3,5] it was found that $C\bar{1}$s and $C\bar{1}$ In form a complex which does not dissociate in 1% SDS, 4 M urea (fig.3). The titration of C1s esterase activity showed a 100% inhibition by an equimolar quantity of $C\bar{1}$ In. However, with an excess of $C\bar{1}$s, a band corresponding to residual free $C\bar{1}$ In was always visible when $C\bar{1}$s–$C\bar{1}$ In complexes were separated from excess $C\bar{1}$s by SDS–polyacrylamide gel electrophoresis. The same phenomenon was observed by Harpel and Cooper [3]. Loss of carbohydrates from $C\bar{1}$ In during the purification or sequence microheterogeneity could explain this apparent heterogeneity of $C\bar{1}$ In affinity for $C\bar{1}$s. The two minor bands (60 000 and 29 000 mol. wt) also appeared to be able to form complexes with $C\bar{1}$s.

Sedimentation coefficients measured by sucrose density gradient ultracentrifugation were 3.8 S for $C\bar{1}$ In, 4.8 S for $C\bar{1}$s–$C\bar{1}$ In complex in EDTA and 6.2 S for C1s–$C\bar{1}$ In complex in calcium. No complex

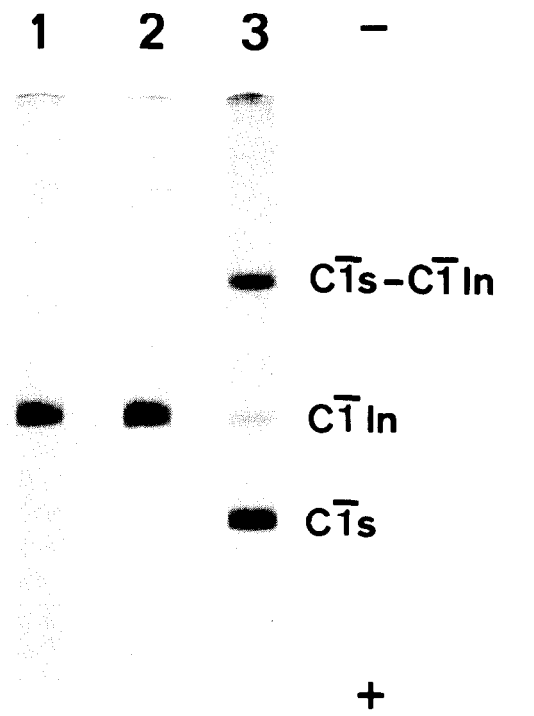

Fig.3. SDS–Polyacrylamide gel electrophoresis of purified $C\bar{1}$ In and $C\bar{1}$s–$C\bar{1}$ In complex. Electrophoresis was carried out as described in Materials and methods. About 11 μg protein was applied to gel 1 (reduced $C\bar{1}$ In) and to gel 2 (unreduced $C\bar{1}$ In). A mixture of 12 μg $C\bar{1}$s and 5.5 μg $C\bar{1}$ In preincubated as described in Materials and methods was applied to gel 3 (unreduced $C\bar{1}$s–$C\bar{1}$ In).

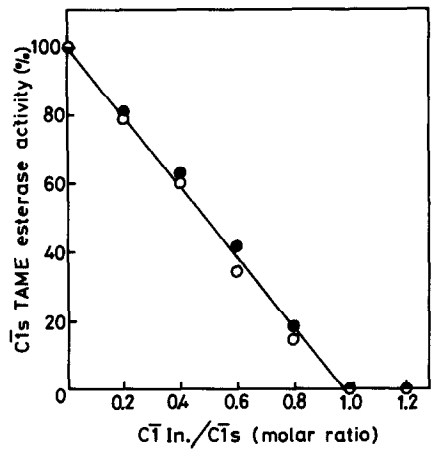

Fig.4. Titration of C1s by C1 In. Effect of calcium. C1s (35 μg) was incubated with increasing amounts of C1 In (0–50 μg) for 10 min at 30°C in 15 mM Tris–HCl, 90 mM NaCl, 2 mM EDTA or $CaCl_2$ (pH 7.6). Samples were cooled on ice and tested for C1s esterase activity as described in Materials and methods in a medium containing respectively 1 mM EDTA or 1 mM $CaCl_2$. (–○–) $CaCl_2$, (–●–) EDTA.

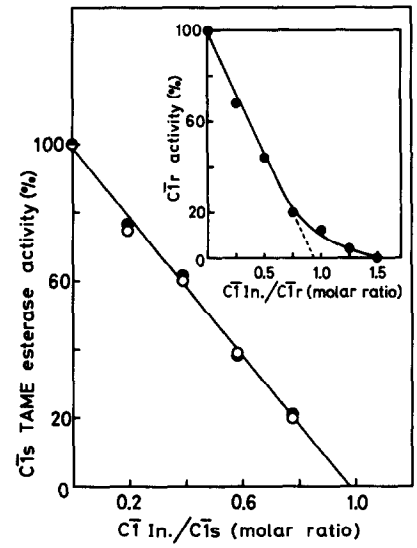

Fig.5. Titration of C1s by C1 In. Effect of C1r. C1s (14.5 μg), or C1s together with C1r (24 μg) were incubated in the presence of increasing amounts of C1 In (0–13.2 μg) for 10 min at 30°C in 20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA (pH 7.6). Samples were cooled in ice and tested for C1s esterase activity as described in Materials and methods. (–●–) C1s alone. (–○–) C1s + C1r. Insert: Titration of C1r by C1 In. C1r (3.4 μg) was preincubated for 15 min at 37°C with increasing amounts of C1 In (0–6.2 μg) in 25 mM Tris–HCl, 125 mM NaCl, 1 mM EDTA (pH 7.5) and cooled at 0°C. C1s (25 μg) was added and the mixture incubated for 15 min at 37°C in the same buffer. C1s generated was estimated with ZLNE as described in Materials and methods. Molar ratios were calculated assuming mol. wt 83 000 for C1r.

between C1 In and proenzymic C1s was detectable by this method.

Titration of C1s esterase activity was used to examine the affinities of C1s, C1r and C1r for the inhibitor relative to that of C1s. As illustrated in fig.5, C1r does not compete effectively with C1s for the inhibitor in the range of concentrations tested. The same absence of competition of C1s and C1r with C1s for binding was also observed. As shown in fig.5 (insert) purified C1r alone is able to interact with C1 In although in this case the inhibition is not linear over the full range.

4. Discussion

C1 In prepared by the procedure described here differs from preparations of C1 In previously described in that only one major component is observed on SDS–polyacrylamide gel electrophoresis. Its functional capacity is close to 100% and, in contrast to the results of Harpel [5], titration of C1s with the inhibitor is linear, demonstrating a very high affinity. An apparent functional heterogeneity of the preparation was observed on SDS–polyacrylamide gel electrophoresis of the C1s–C1 In complex which may be explained by the existence of complexes of high and low affinity, the latter being dissociated by SDS. It is noteworthy that Haupt et al. [1] described several isoelectric forms of C1 In, suggesting structural microheterogeneity. A probable heterogeneity of C1 In in plasma was observed, indicated by the increase in total inhibitory activity after PEG precipitation of the plasma. This suggests the coexistence of free and loosely complexed forms of C1 In in the plasma.

Titrations of C1s in the presence of C1r indicate that the affinity of C1r for the inhibitor is much lower than that of C1s, as has been suggested by Ziccardi and Cooper [12]. This is supported by the observation that inhibition of C1r, unlike C1s, is not linear. Proenzyme C1r and C1s may exhibit a weak

affinity for the inhibitor, but do not compete effectively with $C\bar{1}s$ for binding.

The affinity of $C\bar{1}s$ for the inhibitor is not grossly modified in the presence of calcium. Previous observations [6] show that the access to the $C\bar{1}s$ active site for esters is not modified by calcium. However an increase in the sedimentation coefficient of $C\bar{1}s$ possibly due to a dimerization has been observed in some but not all studies [6,13,14]. In this study an increase in the sedimentation coefficient of the $C\bar{1}s$–$C\bar{1}$ In complex in calcium has been observed, which may support the conclusion that the complex in calcium is a highly asymmetric form (f/f_0 3) containing 2 $C\bar{1}s$ and 2 $C\bar{1}$ In molecules. Further studies of the kinetics and stoichiometry of complex formation between $C\bar{1}s$, $C\bar{1}r$ and $C\bar{1}$ In are in progress.

Acknowledgements

This work was supported partly by the 'Centre National de la Recherche Scientifique' (ERA No. 07.0695), the Délégation Générale à la Recherche Scientifique et Technique (contrats No. 76-71194 and No. 76-7-1195) and the 'Fondation pour la Recherche Médicale'. R. B. Sim is a recipient of an exchange fellowship MRC (UK) – INSERM.

References

[1] Haupt, H., Heimburger, N., Kranz, T. and Schwick, H. G. (1970) Eur. J. Biochem. 17, 254–261.
[2] Nagaki, K., Iida, K. and Inai, S. (1974) Int. Arch. Allergy 46, 935–948.
[3] Harpel, P. C. and Cooper, N. R. (1975) J. Clin. Invest. 55, 593–604.
[4] Anderson, W. H. K., Smith, J. K. and Fothergill, J. E. (1975) Biochem. Soc. Trans. 3, 933–934.
[5] Harpel, P. C. (1976) Methods Enzymol. 45, 751–760.
[6] Arlaud, G. J., Reboul, A., Meyer, C. M. and Colomb, M. G. (1977) submitted.
[7] Gigli, I., Porter, R. R. and Sim, R. B. (1976) Biochem. J. 157, 541–548.
[8] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[9] Fairbanks, G., Stech, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617.
[10] Ouchterlony, Ö. and Nilsson, L. A. (1973) in: Handbook of Experimental Immunology (Weir, D. M. ed) Vol. 1, pp. 19.1–19.39, Blackwell, Oxford.
[11] Martin, R. G. and Ames, B. N. (1961) J. Biol. Chem. 236, 1372–1379.
[12] Ziccardi, R. J. and Cooper, N. R. (1976) J. Immunol. 116, 504–509.
[13] Valet, G. and Cooper, N. R. (1974) J. Immunol. 112, 339–350.
[14] Sim, R. B., Porter, R. R., Reid, K. B. M. and Gigli, I. (1977) Biochem. J. 163, in press.