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PURIFICATION OF PROENZYMIC AND ACTIVATED HUMAN C1s FREE OF C1r

EFFECTS OF CALCIUM AND IONIC STRENGTH ON ACTIVATED C1s

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Summary

1. A rapid method for the purification of the proenzymic and activated forms of C1s is presented. In the case of proenzymic C1s, di-isopropyl phosphorofluoridate (0.5—5 mM) is added at all stages of the purification procedure, which includes euglobulin precipitation followed by DEAE-cellulose chromatography and affinity chromatography on anti-$C\bar{1}r$ IgG-Sepharose 6B. The final step completely removes contaminant traces of $C\bar{1}r$ and/or $C\bar{1}r$, ensuring that the final preparation of C1s is stable in the proenzyme form and suitable for activation studies.

2. The apparent molecular weight of C1s and $C\bar{1}s$ determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis is 85 000 ± 2000. Reduction followed by alkylation of $C\bar{1}s$ gives two fragments of apparent molecular weights 57 000 and 28 000. Results of N-terminal amino acid determination and labelling with di-iso[^{3}H]propyl phosphorofluoridate are consistent with previous reports.

3. The influence of calcium and ionic strength on the structure and activity of $C\bar{1}s$ has been investigated. Calcium leads to a shift of the sedimentation coefficient from 4.3 to 5.6 S, whereas variation in ionic strength has no effect on this parameter. The thermal inactivation curve is profoundly modified both by calcium and ionic strength. In contrast, the esterase activity is only slightly influenced as judged from the absence of gross modification of K_m and V.

Abbreviations: EDTA, ethylene diamine tetracetic acid; Tos-Arg-OMe, *p*-toluene sulfonyl-L-arginine methyl ester; Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; Bz-Arg-OEt, *N*-α-benzoyl-L-arginine ethyl ester. 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 in Enzyme Nomenclature (1973).

Introduction

Complement, one of the enzyme cascade systems of blood, participates in the immune defence of the organism. This system consists of multiple components in their precursor forms which undergo sequential activation after an initial triggering. The complement system can be activated by two pathways. The classical pathway, which includes components C1 to C9, can be triggered by interaction with immune complexes involving IgG or IgM. The alternative pathway which includes five other proteins can be initiated by polysaccharides and various antigen-antibody complexes. The split products of the activated components mediate various important biological effects (e.g. immune adherence, chemotactic activity, histamine release) whereas the terminal components participate in the modification and final lysis of cell membranes [1,2].

The initial step of the classical pathway involves the binding and activation of the multimolecular complex C1 on immune complexes of IgG or IgM. C1 consists of subcomponents C1q, C1r, C1s. A fourth subcomponent, C1t has been described [3] but its functional significance and its contribution to C1 are not clearly established. Two of the subcomponents of C1, C1r and C1s are proenzymes of serine proteases. Recent reports have dealt with the purification of subcomponent C1s and the activation of C1s by $C\bar{1}r$ in the fluid phase or in the reconstructed C1 complex [4—7]. However, the mechanism of activation of C1 on interaction with immune complexes has not been established.

This paper describes an improved method for purifying the proenzymic and activated forms of C1s characterized by the total elimination of any trace contaminant of unactivated or activated C1r. New information on the physicochemical and functional properties of C1s is presented which contribute to the study of the intramolecular processes resulting in the activation of C1s in the C1 complex.

Materials and Methods

Materials. Human citrated plasma was obtained from the Centre de Transfusion Sanguine, Grenoble. It was made 20 mM with $CaCl_2$ and left to clot overnight at 4°C. The clot was removed by centrifugation for 30 min at 12 500 × *g* followed by filtration through gauze. The serum was stored at −80°C prior to use.

Di-isopropyl phosphorofluoridate was purchased from Sigma. It was dissolved in propan-2-ol at 0.5 M and kept at −20°C. Di-iso[^{3}H]propyl phosphorofluoridate was purchased from the Radiochemical Centre, Amersham (U.K.). Antisera were from Behring (France) and used as such or after precipitation of their immunoglobulin fraction by three sodium sulphate steps according to Prahl and Porter [8]. Anti-human C1t immunoglobulin fraction was obtained from Atlantic Antibodies (U.S.A.). Ile-Ile was from Interchim, Montluçon (France). Dimethylsuberimidate and dimethyladipimidate were purchased from Pierce Chemical (U.S.A.). Bz-Arg-OEt, Ac-Tyr-OEt, Tos-Arg-OMe were from Sigma.

Protein determination. Purified IgG and C1s or $C\bar{1}s$ were routinely estimated from absorbance measurements at 280 nm using $E^{1\%}_{1cm}$ = 14.0 (IgG) and $E^{1\%}_{1cm}$

= 9.5 (C1s and $C\bar{1}s$) [7]. In other cases, protein concentration was determined by the method of Lowry et al. [9].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. 6% polyacrylamide gels were run at a current of 4 mA per gel as described by Fairbanks et al. [10]. Samples were reduced when necessary by incubation with 130 mM 2-mercaptoethanol in the presence of 4 M urea, 1% (w/v) sodium dodecyl sulphate, 0.1 M Tris · HCl (pH 8.0) for 60 min at 37°C and then alkylated with 140 mM iodoacetamide in the same buffer for a further 20 min at the same temperature. Coomassie Blue was used for protein staining [11]. The apparent molecular weights of proteins were estimated from a standard plot using the following proteins: subunits of phosphorylase *a* (92 000), bovine serum albumin (68 000), IgG heavy chain (52 000), ovalbumin (42 000), IgG light chain (23 000) and soybean trypsin inhibitor (20 000).

Sucrose density gradient ultracentrifugation. Samples were sedimented according to Martin and Ames [12] in 5—20% linear sucrose density gradients at 4°C for 15 h at 38 000 rev./min using a SW 50-1 rotor in a Beckman L2-65B ultracentrifuge. Beef liver catalase (11.6 S), yeast alcohol dehydrogenase (6.7 S) and ovalbumin (3.5 S) were used as standards.

Enzymatic analysis. The esterase activity of $C\bar{1}s$ was measured routinely at 25°C with 1.5 mM Tos-Arg-OMe, 0.9 mM Bz-Arg-OEt or 1.0 mM Ac-Tyr-OEt as substrates using spectrophotometric assays as described previously [5,13]. For most esterase activity measurements, substrate solutions were made in 50 mM Tris · HCl, 150 mM NaCl, 1 mM EDTA (pH 7.6). For optimum pH determination, substrates were dissolved in 30 mM sodium acetate, 20 mM glycine, 50 mM NaCl and pH was adjusted to the desired value by addition of Tris.

N-terminal amino acids. N-termini were identified by the dansyl method [14], the dansyl-amino acids being fractionated on polyamide sheets (5 × 5 cm) as described by Woods and Wang [15].

Labelling of $C\bar{1}s$ with di-iso[^{3}H]propyl phosphorofluoridate. The di-isopropyl phosphorofluoridate-sensitive active site of $C\bar{1}s$ was labeled as follows: $C\bar{1}s$ (1 mg/ml) was incubated with 1 mM di-iso[^{3}H]propyl phosphorofluoridate (0.44 Ci/mmol) in 50 mM Tris · HCl, 150 mM NaCl (pH 7.6) for 30 min at 30°C. After exhaustive dialysis against 50 mM Tris · HCl, 150 mM NaCl (pH 7.6), samples were lyopholized and examined in duplicate by sodium dodecyl sulphate polyacrylamide gel electrophoresis. One gel was stained with Coomassie Blue and the other was cut into 2.0-mm slices which were counted in 10 ml of scintillation fluid.

Double diffusion analysis. Double radial immunodiffusion was performed according to Ouchterlony and Nilsson [16] in 1% (w/v) agarose, 10 mM EDTA, 150 mM NaCl, 0.1 M sodium phosphate (pH 7.0).

Preparation of $C\bar{1}r$ and anti-$C\bar{1}r$ IgG. $C\bar{1}r$ was purified from pooled human citrated plasma by the method of Takahashi et al. [17], including precipitation of $C\bar{1}$ by polyethylene glycol followed by chromatography on IgG-Sepharose 6B and DEAE-cellulose chromatography. $C\bar{1}r$ thus obtained was free of C1s and $C\bar{1}s$. Antiserum was raised in rabbits by immunization with $C\bar{1}r$ according to Porter [18]. IgG was purified from pooled rabbit serum by sodium sulphate precipitation [8] and subsequently treated with 2 mM di-isopropyl phosphorofluoridate for 30 min at 30°C.

Anti-C1r IgG-Sepharose 6B. Anti-C1r antibodies were linked to Sepharose 6B essentially according to Axen et al. [19]. After activation of Sepharose 6B with CNBr, IgG (2 mg/ml) was coupled overnight at 4°C in the presence of 0.1 M $NaHCO_3$, 0.1 M NaCl (pH 7.9) with a protein to packed Sepharose ratio of 1.7 (w/v). 95% of the protein was bound under these conditions.

Cross-linking experiments. Conditions used were as described by Davies and Stark [20]. C1s or C1s (1 mg/ml) were incubated at room temperature for 1—2 h with 3 mg/ml dimethylsuberimidate or dimethyladipimidate in 0.15 M NaCl, 0.1 M triethanolamine (pH 8.0).

Results

Purification of proenzymic C1s

All manipulations were performed as close to 0°C as possible and di-isopropyl phosphorofluoridate was present at all stages to prevent activation. The results of a typical preparation are summarized in Table I.

Human serum (1 l) was thawed and stirred for 60 min at 2°C in the presence of 50 μg/ml soybean trypsin inhibitor and 5 mM di-isopropyl phosphorofluoridate. Serum was then subjected to euglobulin precipitation as described by Gigli et al. [7] by addition of four volumes of a solution containing 5 mM $CaCl_2$, 2.5 mM iodoacetamide, 0.2 mM *o*-phenanthroline and 0.5 mM di-isopropyl phosphorofluoridate. The pH was adjusted to 7.4 with NaOH and the diluted serum was stirred for 2.5 h at 2°C. The euglobulin precipitate was harvested by centrifugation for 30 min at 12 500 × *g* and suspended in 200 ml of a solution containing 5 mM Tris · HCl, 20 mM L-lysine, 0.5 mM $CaCl_2$ and 5 mM di-isopropyl phosphorofluoridate (pH 7.5, ionic strength adjusted to 0.07 by addition of NaCl). The washed precipitate was centrifuged at 20 000 × *g* for 20 min and suspended in 100 ml of a solution containing 20 mM EDTA, 5 mM L-lysine (pH 7.5) [4]. Di-isopropyl phosphorofluoridate was added to 10 mM and the suspension was stirred for 2 h at 2°C. The extract containing C1s was then centrifuged at 25 000 × *g* for 30 min.

The supernatant was dialysed overnight at 2°C against 0.1 M Tris · HCl, 40 mM NaCl, 1 mM EDTA, 1 mM di-isopropyl phosphorofluoridate (pH 7.5) and

TABLE I

PURIFICATION OF PROENZYMIC C1s

Protein concentration in the dialysed extract was determined by the method of Lowry et al. [9] and in other solutions by absorbance at 280 nm. Tos-Arg-OMe esterase activity was measured as described in Materials and Methods. N.D., not detectable.

Purification step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Tos-Arg-OMe esterase activity
Dialysed extract	120	1.25	150	n.d.
DEAE-cellulose eluate	43	0.23	9.8	n.d.
Anti-C1r IgG-Sepharose eluate	44	0.18	7.9	n.d.
Concentrated C1s	5.2	1.16	6.0	n.d.

applied to a 2.5 × 10 cm column of DEAE-cellulose (Whatman DE-32) equilibrated with the same buffer. The column was thoroughly washed with the starting buffer containing 0.5 mM di-isopropyl phosphorofluoridate until the absorbance at 280 nm of the eluate was less than 0.05. About 80% of the proteins were not retained on the column. Adsorbed proteins were then eluted with a linear NaCl gradient (300 ml of 0.1 M Tris · HCl, 60 mM NaCl, 1 mM EDTA, 0.5 mM di-isopropyl phosphorofluoridate (pH 7.5) and 300 ml of the same buffer containing 180 mM NaCl). C1s was the main protein eluted and was collected as indicated for $C\bar{1}s$ (Fig. 1). A restricted cut was chosen in order to minimize a contamination with C1t which is eluted before C1s.

The ionic strength of the pooled C1s fractions was adjusted to 0.4 with 5 M NaCl and di-isopropyl phosphorofluoridate was added to 1 mM. The pool was then passed through a 1.5 × 8 cm column of anti-$C\bar{1}r$ IgG-Sepharose 6B equilibrated with 0.1 M Tris · HCl, 150 mM Nacl, 1 mM EDTA, 1 mM di-isopropyl phosphorofuloridate (pH 7.5). The eluate was concentrated by ultrafiltration and 5—8 mg of C1s was finally obtained. Di-isopropyl phosphorofluoridate was eliminated by dialysis against 20 mM Tris · HCl, 150 mM NaCl (pH 7.6). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Fig. 2) showed that C1s was in the proenzyme form and no esterase activity against Tos-Arg-OMe, Bz-Arg-OEt or Ac-Tyr-OEt was found with 150 μg of protein. No reaction was found by double-diffusion analysis using antisera against C1r, C1q, C3, C4, $C\bar{1}$-inhibitor, IgG, IgM and plasminogen.

The final stage of the purification was found to be essential to obtain stable C1s in the proenzyme form: C1s eluted from DEAE-cellulose contains contaminant traces of C1r and/or $C\bar{1}r$ which are not visible on sodium dodecyl sulphate-polyacrylamide gels loaded with 40 μg protein but can be detected by double-diffusion on agarose using diluted anti-$C\bar{1}r$ antiserum. These preparations can be activated by incubation at 37°C, as measured by the appearance of Tos-Arg-OMe esterase activity (Fig. 3). Anti-$C\bar{1}r$ IgG-Sepharose 6B completely removes contaminant C1r and/or $C\bar{1}r$ and C1s thus obtained is stable and remains unactivated after 2 h at 37°C (Fig. 3).

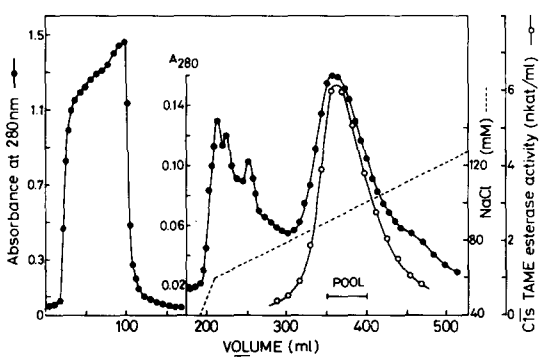


Fig. 1. Purification of $C\bar{1}s$ by DEAE-cellulose chromatography. The dialysed extract containing $C\bar{1}s$ was applied to a 2.5 × 10 cm column of DEAE-cellulose (Whatman DE-32) and eluted by a linear NaCl gradient as described in Materials and Methods. Fractions (3.5 ml) were collected at 2°C and analysed for $C\bar{1}s$ Tos-Arg-OMe esterase activity (○———○), absorbance at 280 nm (●———●) and NaCl concentration (- - - - -) as deduced from conductivity measurements. A pool of active fractions was made as indicated.

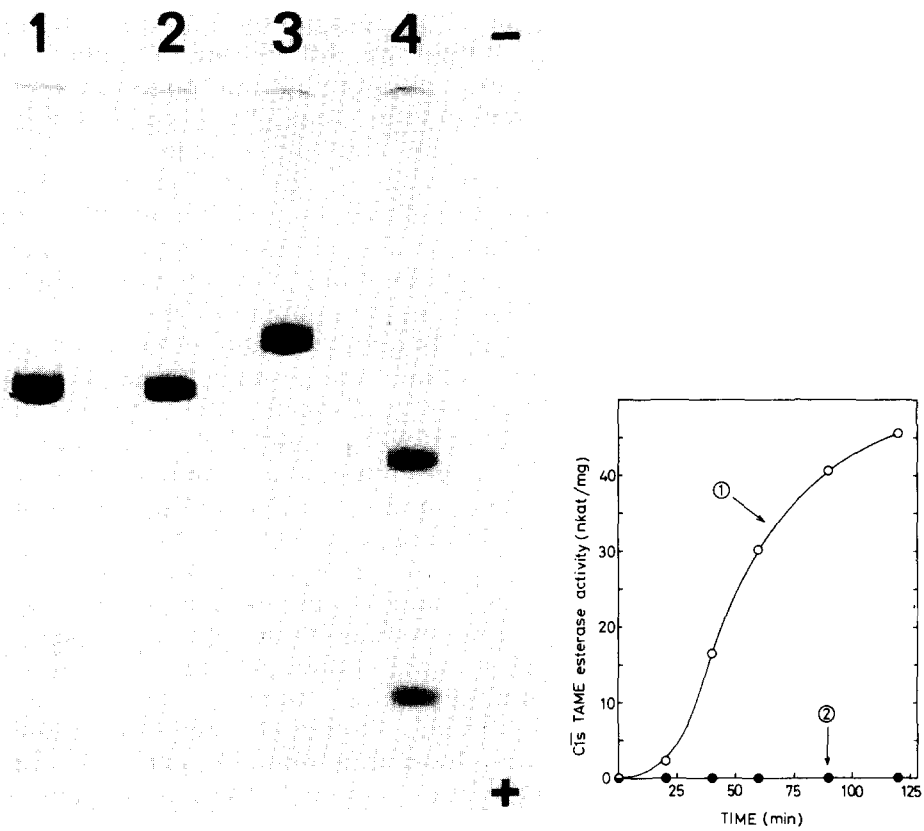


Fig. 2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of purified C1s and $C\bar{1}s$. Electrophoresis was carried out as described in Materials and Methods. Gel 1, unreduced C1s; gel 2, unreduced $C\bar{1}s$; gel 3, reduced and alkylated C1s; gel 4, reduced and alkylated $C\bar{1}s$. About 16 μg of protein was applied to each gel.

Fig. 3. Activation of C1s isolated from DEAE-cellulose. C1s isolated from DEAE-cellulose and C1r-free C1s obtained from anti-C1r IgG-Sepharose 6B (Both 1.10 mg/ml) were incubated for various times at 37°C in 20 mM Tris · HCl, 1 mM EDTA, 150 mM NaCl (pH 7.6). Samples were then cooled in ice and tested for Tos-Arg-OMe esterase activity as described in Materials and Methods. Curve 1, C1s isolated from DEAE-cellulose chromatography (○———○); curve 2, C1r-free C1s (●———●).

Purification of $C\bar{1}s$

The same procedure was performed for euglobulin precipitation, extraction of $C\bar{1}s$ and DEAE-cellulose chromatography, except that di-isopropyl phosphorofluoridate was omitted at all steps. Results of a typical preparation are shown in Table II.

$C\bar{1}s$ was detected on DEAE-cellulose eluate by measurement of Tos-Arg-OMe esterase activity (Fig. 1), pooled as indicated and concentrated by ultrafiltration to about 0.5—0.6 mg/ml. The pooled $C\bar{1}s$ fractions were then incubated at 37°C until esterase activity reached a maximum value. A marked increase in specific activity (46%) occurred at this step (Table II). Fully activated $C\bar{1}s$ was diluted with one volume of 0.1 M Tris · HCl, 150 mM NaCl, 1 mM EDTA (pH 7.5) and contaminant traces of $C\bar{1}r$ were then removed on anti-C1r IgG-Sepharose 6B. The specific Tos-Arg-OMe esterase activity of this material

TABLE II

PURIFICATION OF $C\bar{1}s$

Protein concentration and Tos-Arg-OMe esterase activity was determined as described in Table I. Maximum activation of $C\bar{1}s$ was obtained by incubation of concentrated DEAE-cellulose eluate for 90 min at 37°C.

Purification step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Tos-Arg-OMe esterase activity (nkat)	Specific activity (nkat/mg)
Dialysed extract	105	1.1	116	607	5.2
DEAE-cellulose eluate	52	0.16	8.5	226	26.6
DEAE-cellulose eluate after concentration and activation	11.5	0.63	7.3	356	48.8
Anti-$C\bar{1}r$ IgG-Sepharose eluate	25	0.23	5.7	280	49.1
Concentrated $C\bar{1}s$	5.0	0.96	4.8	237	49.3

ranged from 48 to 50 nkat/mg. Sodium dodecyl sulphate polyacrylamide gels (Fig. 2) showed that $C\bar{1}s$ was fully activated.

Physicochemical properties of C1s and $C\bar{1}s$

The N-terminal amino acid of C1s was identified as Glx. In addition to Glx, the N-terminal determination of $C\bar{1}s$ gave a mixture of isoleucine and Ile-Ile dipeptide. The dipeptide was found to be exceptionally resistant to acid hydrolysis and was not entirely converted to isoleucine even after 72 h in 6 M HCl at 105°C. These results are consistent with previous observations [6,21] that glutamic acid is the N-terminal amino acid of the heavy fragment and Ile-Ile the N-terminal dipeptide of the light fragment of $C\bar{1}s$. The incubation of $C\bar{1}s$ with di-iso[^{3}H]propyl phosphorofluoridate led to a specific labelling. On sodium dodecyl sulphate polyacrylamide gels, the radioactivity migrated, respectively, with the unreduced $C\bar{1}s$ band and the reduced and alkylated light fragment. C1s treated in the same conditions incorporated on a molar basis less than 0.5% of the di-iso[^{3}H]propyl phosphorofluoridate incorporated by $C\bar{1}s$.

Apparent molecular weights for C1s and $C\bar{1}s$ were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis as described in Materials and Methods. A value of 85 000 ± 2000 was found for C1s in both reduced and unreduced samples using appropriate standards. As shown in Fig. 2, a slower migration rate was observed for C1s after reduction and alkylation. As reported by Griffith [22] for other proteins containing cystine, this effect may be explained by the cleavage of disulfide bridges. The same value of 85 000 was found for unreduced $C\bar{1}s$, though reduction and alkylation gave two fragments of apparent molecular weights 57 000 and 28 000. These results are in good agreement with those reported recently [21,23].

The sedimentation coefficient of $C\bar{1}s$ was determined by sucrose density gradient ultracentrifugation. An average value of 4.3 ± 0.1 S was found in the

presence of EDTA, in good agreement with the results reported by Valet and Cooper [5].

Effects of calcium and ionic strength on $C\bar{1}s$

As a preliminary to future studies on the role of calcium and ionic strength in the structural and functional properties of the C1 complex, their effects on the physicochemical properties of isolated $C\bar{1}s$ were investigated.

The sedimentation coefficient of $C\bar{1}s$ was increased from 4.3 S to 5.6 S in the presence of calcium (Fig. 4). Variation in ionic strength between 0.02 and 0.5 had no effect on the sedimentation coefficient in the presence either of calcium or EDTA. These results are in good agreement with those reported by Valet and Cooper [5], suggesting a calcium-dependent conformational change in the $C\bar{1}s$ molecule. However, cross-linking experiments of C1s and $C\bar{1}s$ with dimethylsuberimidate and dimethyladipimidate did not give any evidence of a calcium-dependent dimerization.

The optimal pH of $C\bar{1}s$ was determined with Tos-Arg-OMe and Bz-Arg-OEt as described in Materials and Methods. Maximal activity was found in each case at pH 7.6 ± 0.1 and this value was not affected by the substitution of $CaCl_2$ for EDTA in the assay buffer.

To evaluate the effect of temperature, Tos-Arg-OMe esterase activity of $C\bar{1}s$ was measured between 8 and 32°C at different ionic strengths in the presence of $CaCl_2$ or EDTA. As measured from the Arrhenius plot, the Q_{10} values varied between 1.8 and 2.0 and the activation energy ranged from 4100 to 4600 cal/mol. No significant variations were found in the presence of calcium, and variation in ionic strength between 0.02 and 0.5 had no effect.

The esterase activity of $C\bar{1}s$ was inhibited by incubation with di-isopropyl

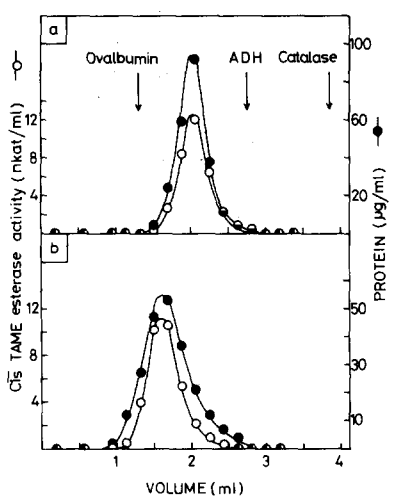


Fig. 4. Sucrose density gradient ultracentrifugation of $C\bar{1}s$. Effect of calcium. Sucrose gradients were prepared in 20 mM Tris · HCl, 50 mM NaCl, 2 mM $CaCl_2$ or EDTA (pH 7.4). $C\bar{1}s$ solutions in the corresponding buffer were layered on the gradients and ultracentrifugation was carried out as described in Materials and Methods. Fractions (180 µl) were collected from the top of the gradients by displacement with 30% sucrose and tested for Tos-Arg-OMe esterase activity (○———○). Protein concentration (●———●) was determined by the method of Lowry et al. [9]. a, $CaCl_2$; b, EDTA; ADH, alcohol dehydrogenase.

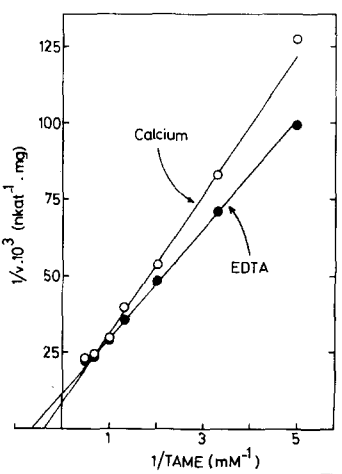
TABLE III

EFFECTS OF CALCIUM AND IONIC STRENGTH ON THE KINETIC PARAMETERS OF $C\bar{1}s$ ESTERASE ACTIVITY MEASURED ON Tos-Arg-OMe AND Bz-Arg-OEt

Substrate solutions were prepared in 50 mM Tris · HCl, 2 mM EDTA or $CaCl_2$, pH 7.6 (low ionic strength) or in 50 mM Tris · HCl, 250 mM NaCl, 2 mM EDTA or $CaCl_2$, pH 7.6 (high ionic strength). $C\bar{1}s$ esterase activity was measured at 25°C and kinetic constants were determined from Lineweaver-Burk plots.

	Tos-Arg-OMe			Bz-Arg-OEt		
	K_m (mM)	V (nkat/mg)	$10^3 \cdot \frac{K_m}{V}$	K_m (mM)	V (nkat/mg)	$10^3 \cdot \frac{K_m}{V}$
EDTA						
High or low ionic strength	1.7	89	18.7	2.5	48	53
Calcium						
High ionic strength	2.0	100	20.0	3.2	53	60
Low ionic strength	2.8	120	23.0	5.0	69	72

phosphorofluoridate. The inhibition was strongly dependent on the concentration of di-isopropyl phosphorofluoridate: after 40 min of incubation at 30°C with 1 mM di-isopropyl phosphorofluoridate a decrease of 80% was observed whereas a decrease of the same order required an incubation of 100 min with 0.3 mM di-isopropyl phosphorofluoridate. The decrease in activity obeyed first-order kinetics and was not affected by calcium.



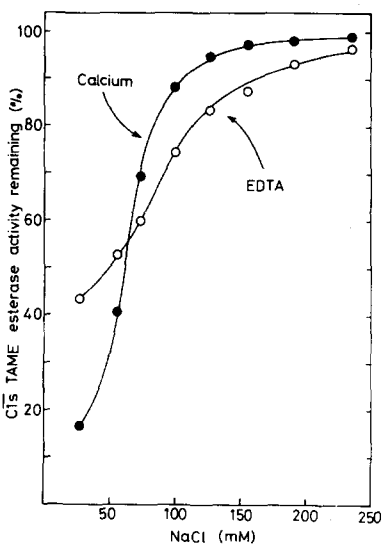


Fig. 5. Lineweaver-Burk plot of $C\bar{1}s$ Tos-Arg-OMe esterase activity. Effect of calcium. Solutions of Tos-Arg-OMe (0.2—2.0 mM) were made in 50 mM Tris · HCl, 2 mM EDTA or $CaCl_2$ (pH 7.6) and the esterase activity of $C\bar{1}s$ was measured at 25°C as described in Materials and Methods. ○———○, $CaCl_2$; ●———●, EDTA.

Fig. 6. Thermal inactivation of $C\bar{1}s$. Effect of calcium and ionic strength. $C\bar{1}s$ (0.32 mg/ml) was incubated at 49°C for 10 min in 20 mM Tris · HCl, 1 mM $CaCl_2$ or EDTA, 25—240 mM NaCl (pH 7.6). Samples were cooled in ice and $C\bar{1}s$ Tos-Arg-OMe esterase activity remaining was measured as described in Materials and Methods. ●———●, $CaCl_2$; ○———○, EDTA.

The kinetic parameters of $C\bar{1}s$ esterase activity were also measured with Tos-Arg-OMe and Ba-Arg-OEt at low and high ionic strength, in the presence of calcium or EDTA. As shown in Table III, calcium produces a slight increase of K_m, V and K_m/V. This effect is emphasized at low ionic strength (Fig. 5) and is found for both substrates (Table III). Similar results were obtained with Ac-Tyr-OEt.

The thermal inactivation rate of $C\bar{1}s$ was shown to be critically dependent on the ionic strength (Fig. 6). After 10 min of incubation at 49°C, a maximal stability was found at high ionic strength (I = 0.5) whereas the esterase activity rapidly decreased at low ionic strength (I = 0.05). This effect is emphasized in the presence of calcium (Fig. 6) and may suggest a conformational change in the protein.

Discussion

We have presented here a rapid and convenient procedure for the purification of proenzymic and activated C1s, which appears well adapted to the study of proteases and their zymogens. Assuming a concentration of C1s of 5 mg/100 ml serum, estimated by radial immunodiffusion, the final yield averages 10—20% and can be compared favourably with the yield of the most recent protocols described [4,5,7]. The major advantage of this technique is the absence of any trace of C1r or $C\bar{1}r$ in the final products. This is achieved by the use of a final step of affinity chromatography with anti-$C\bar{1}r$ IgG-Sepharose 6B able to bind both unactivated and activated forms of C1r. A prolonged incubation of C1s at 37°C shows that this C1s does not undergo any spontaneous activation. Morgan and Nair [24] have described an autocatalytic activation of C1s prepared according to Lepow et al. [25]. Sakai and Stroud [4,26] have reported that they obtained preparations of purified C1s able to be activated without the prior addition of $C\bar{1}r$. In a different approach Gigli et al. [7] have found that a mixture of purified C1q and C1s in molar ratio 1 : 4 gave 15—20% of the hemolytic activity of a mixture of purified C1q + C1r + C1s in molar ratios 1 : 4 : 4. It appears that a contaminant C1r and/or $C\bar{1}r$ may be responsible for all the above effects. The presence of this small amount of C1r intimately associated with C1s suggests there may be a very strong affinity which cannot be completely overcome by DEAE-cellulose chromatography and EDTA.

We would like also to emphasize that the use of di-isopropyl phosphorofluoridate in the preparation of proenzymic C1s can lead to erroneous results. At low temperature (0—4°C) the hydrolysis of di-isopropyl phosphorofluoridate appears to be very slow and the residual inhibitor may mask the proteolysis which could proceed when the sample is dialysed.

C1s prepared according to this method compares well with other C1s preparations described previously. The absence of any significant proteolytic degradation of the molecule can be assessed from sodium dodecyl sulphate-polyacrylamide gel electrophoresis after protein staining and from the analysis of the dansyl-N-terminal derivatives.

The amount of $C\bar{1}s$ in preparations of proenzymic C1s was estimated from di-iso[^{3}H]propyl phosphorofluoridate labelling to a maximum of 0.5%. For the

preparations of $C\bar{1}s$, with the same conditions of labelling, the total radioactivity was found in the light chain.

Some of our results show clearly that calcium and ionic strength affect the properties of $C\bar{1}s$. The effects seem to be restricted only to a few parameters. From the influence of calcium on the sedimentation coefficient and from the modifications brought about by calcium and ionic strength in the thermal inactivation of $C\bar{1}s$, it appears that large structural modifications may be involved.

The kinetic constants, however, are only slightly affected by calcium and ionic strength, and calcium does not modify the binding of di-isopropyl phosphorofluoridate to $C\bar{1}s$. These results are confirmed by other experiments which show that calcium and ionic strength do not alter the stoicheiometry of the interaction of purified $C\bar{1}$-inactivator with $C\bar{1}s$ [27]. It can therefore be concluded that the structural modifications of $C\bar{1}s$ do not involve to a significant extent the active site which retains its accessibility.

It has been suggested [5] that C1s forms a calcium-dependent dimer. However, attempts to gain evidence for dimerization by cross-linking have been unsuccessful. The problem may be solved by the study of the associations between C1r and C1s.

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