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|-------|--------------------|--|--|---|-----------------------------------|---|-------------------------------|
| Jan. | 679/1 = B58/1 | 684/1 = M95/1 684/2 = M95/2 | 696/1 = N1/1 | 700/1 = P1/1 700/2 = P1/2 | 710/1 = L58/1 | 714/1 = G96/1 | 720/1 = C1/1 |
| Feb. | 679/2 = B58/2 | 685/1 = M96/1 685/2 = M96/2 685/3 = M96/3 | 696/2 = N1/2 | 701/1 = P2/1 701/2 = P2/2 701/3 = P2/3 | 710/2 = L58/2 | 714/2 = G96/2 714/3 = G96/3 | |
| March | 679/3 = B58/3 | 686/1 = M97/1 686/2 = M97/2 | 696/3 = N1/3 | 702/1 = P3/1 702/2 = P3/2 | 710/3 = L58/3 | 715/1 = G97/1 | 720/2 = C1/2 |
| April | 680/1 = B59/1 | 687/1 = M98/1 687/2 = M98/2 | 697/1 = N2/1 | 703/1 = P4/1 703/2 = P4/2 | 711/1 = L59/1 | 715/2 = G97/2 | |
| May | 680/2 = B59/2 | 688/1 = M99/1 688/2 = M99/2 688/3 = M99/3 | 697/2 = N2/2 | 704/1 = P5/1 704/2 = P5/2 704/3 = P5/3 | 711/2 = L59/2 | 716/1 = G98/1 716/2 = G98/2 716/3 = G98/3 | 720/3 = C1/3 |
| June | 680/3 = B59/3 | 689/1 = M100/1 689/2 = M100/2 689/3 = M100/3 | 697/3 = N2/3 | 705/1 = P6/1 705/2 = P6/2 705/3 = P6/3 | 711/3 = L59/3 | 717/1 = G99/1 717/2 = G99/2 717/3 = G99/3 | 720/4 = C1/4 |
| July | 681/1 = B60/1 | 690/1 = M101/1 690/2 = M101/2 690/3 = M101/3 | 698/1 = N3/1 | 706/1 = P7/1 706/2 = P7/2 | 712/1 = L60/1 | 718/1 = G100/1 | 721/1 = C2/1 |
| Aug. | 681/2 = B60/2 | 691/1 = M102/1 691/2 = M102/2 | 698/2 = N3/2 | 707/1 = P8/1 707/2 = P8/2 | 712/2 = L60/2 | 718/2 = G100/2 | |
| Sept. | 681/3 = B60/3 | 692/1 = M103/1 692/2 = M103/2 692/3 = M103/3 | 698/3 = N3/3 | 708/1 = P9/1 708/2 = P9/2 708/3 = P9/3 | 712/3 = L60/3 | 719/1 = G101/1 719/2 = G101/2 | 721/2 = C2/2 |
| Oct. | 682/1 = B61/1 | 693/1 = M104/1 693/2 = M104/2 | 699/1 = N4/1 | 709/1 = P10/1 709/2 = P10/2 | 713/1 = L61/1 | 719/3 = G101/3 | 721/3 = C2/3 |
| Noy. | 682/2 = B61/2 | | 699/2 = N4/2 | | 713/2 = L61/2 | | 721/4 = C2/4 |
| Dec. | 682/3 = B61/3 | | 699/3 = N4/3 | | 713/3 = L61/3 | | |

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CONTENTS

(Cited in: Current Contents Life Sciences - Biological Abstracts - Chemical Abstracts - Index Chemicus - Index Medicus - Excerpta Medica)

| | | | |
|--|-----|---|-----|
| Purification and characterization of extracellular poly-amine oxidase produced by <i>Penicillium</i> sp. No. PO-1 (BBA 31226) by Y. Kobayashi and K. Horikoshi (Saitama, Japan) ... | 133 | Purification and properties of the pyruvate dehydrogenase complex from <i>Salmonella typhimurium</i> and formation of hybrids with the enzyme complex from <i>Escherichia coli</i> (BBA 31239) by R. Seckler, R. Binder and H. Bisswanger (Tübingen, F.R.G.) ... | 210 |
| Charge transfer in peptides. Intramolecular radical transformations involving methionine, tryptophan and tyrosine (BBA 31231) by W.A. Prütz, F. Siebert, J. Butler, E.J. Land, A. Menez and T. Montenay-Garestier (Freiburg, F.R.G., Manchester, U.K. and Gif-sur-Yvette and Paris, France) ... | 139 | Photoaffinity labeling of membrane-bound porcine aminopeptidase N (BBA 31250) by D. Gratecos, L. Varesi, M. Knibiehler and M. Sémériva (Marseilles, France) ... | 218 |
| Charge transfer between tryptophan and tyrosine in proteins (BBA 31233) by J. Butler, E.J. Land, W.A. Prütz and A.J. Swallow (Manchester, U.K. and Freiburg, F.R.G.) ... | 150 | Demonstration that bovine erythrocyte cytochrome b_5 is the hydrophilic segment of liver microsomal cytochrome b_5 (BBA 31240) by S.R. Slaughter, C.H. Williams, Jr. and D.E. Hultquist (Ann Arbor, MI, U.S.A.) ... | 228 |
| Characterization of the proteolytic activity firmly attached to yeast phosphoenolpyruvate carboxykinase (BBA 31227) by I. Beck, M. Möller and H. Holzer (Munich and Freiburg, F.R.G.) ... | 163 | Effect of vanadate on the formation and stability of the phosphoenzyme forms of 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase and of phosphoglucomutase (BBA 31246) by J. Carreras, F. Climent, R. Bartrons and G. Pons (Barcelona, Spain) ... | 238 |
| Isolation and characterization of glycopeptides of human transcortin (BBA 31224) by O.A. Strel'Chyonok, G.V. Avvakumov, I.V. Matveentseva, L.V. Akhrem and A.A. Akhrem (Minsk, U.S.S.R.) ... | 167 | The isoelectric focusing of human thyroglobulin (BBA 31236) by C. Davoli, S. Grimaldi, G. Rusca, M. Andreoli and H. Edelhoch (Rome, Italy) ... | 243 |
| Isolation and purification of rat acute-phase α_2 -macroglobulin (BBA 31229) by D.E. Panrucker and F.L. Lorscheider (Calgary, Canada) ... | 174 | Isolation and partial characterization of prochymosin and chymosin from cat (BBA 31244) by T. Jensen, N.H. Axelsen and B. Foltmann (Copenhagen, Denmark) ... | 249 |
| Radioimmunoassay of rat acute-phase α_2 -macroglobulin (BBA 31230) by D.E. Panrucker and F.L. Lorscheider (Calgary, Canada) ... | 184 | Recombination kinetics following nanosecond laser photolysis of carbonmonoxyhaemoglobin (BBA 31238) by R. Catterall, D.A. Duddell, R.J. Morris and J.T. Richards (Salford, U.K.) ... | 257 |
| Purification and partial characterization of two forms of urinary trypsin inhibitor (BBA 31235) by Y. Tanaka, S. Maehara, H. Sumi, N. Toki, S. Moriyama and K. Sasaki (Tokyo, Shimane, Miyazaki and Hiroshima, Japan) ... | 192 | Circular dichroism studies on α_2 -antiplasmin and its interactions with plasmin and plasminogen (BBA 31264) by T. Nilsson, I. Sjöholm and B. Wiman (Umeå and Uppsala, Sweden) ... | 264 |
| The effect of aging on rat liver phosphoglycerate kinase and comparison with the muscle enzyme (BBA 31237) by L.S. Hiramath and M. Rothstein (Buffalo, NY, U.S.A.) | 200 | Purification and characterization of human CT -esterase inhibitor (BBA 31265) by T. Nilsson and B. Wiman (Umeå, Sweden) ... | 271 |

continued

INFORMATION FOR CONTRIBUTORS TO BIOCHIMICA ET BIOPHYSICA ACTA

The 1982 edition of this booklet, offering detailed guidance to authors, is available free of charge from the BBA Editorial Secretariat, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. It is also published as *Biochim. Biophys. Acta* 715 (1982) 1-23.

| | |
|--|-----|
| Tropomyosin structure and intermolecular contacts in Mg paracrystals (BBA 31222) | |
| by S.D. Golladay (Chicago, IL, U.S.A.) | 277 |

BBA Reports

| | |
|--|-----|
| Arrhenius plot characteristics of membrane-bound 3-hydroxy-3-methylglutaryl coenzyme A reductase from the latex of <i>Hevea brasiliensis</i> (BBA 30018) | |
| by A.B. Sipat (Selangor, Malaysia) | 284 |

| | |
|--|-----|
| Inhibitory action of proline-containing peptides on Xaa-Pro-dipeptidylaminopeptidase (BBA 30023) | |
| by M. Harada, K.M. Fukasawa, K. Fukasawa and T. Nagatsu (Shiojiri and Yokohama, Japan) | 288 |
| Titles of related papers in other sections | 291 |

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BBA 31265

PURIFICATION AND CHARACTERIZATION OF HUMAN C $\bar{\text{I}}$ -ESTERASE INHIBITOR

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(Received December 16th, 1981)

Key words: C $\bar{\text{I}}$ -esterase; Disulfide; Esterase inhibitor; Complement; (Human)

A new purification method for C $\bar{\text{I}}$ -esterase inhibitor is described, which is essentially a three-step procedure: precipitation with poly(ethylene glycol), chromatography on DEAE-cellulose and hydrophobic interaction chromatography on hexyl-Sepharose. The final product is a single-chain glycoprotein with a molecular weight of about 100 000 and NH $_2$ -terminal asparagine. The molecule is fully active as judged by complex formation with C $\bar{\text{I}}$ s. Two of its three disulphide bridges can be easily reduced and S-carboxymethylated under non-denaturing conditions without loss of activity. However, at high dithioerythritol concentration the third disulphide bridge is also cleaved and accompanied by loss of the activity, indicating that this disulphide bridge is involved in maintaining the conformation around the reactive site in the inhibitor.

Introduction

Human plasma contains several protease inhibitors of well-known functions. Among these, the C $\bar{\text{I}}$ -esterase inhibitor is important in the regulation of the complement system [1] and possibly also in the kininogen system [2] and the intrinsic fibrinolysis [3]. Several purification methods for the C $\bar{\text{I}}$ -esterase inhibitor have been described [1,4–8], although few seem to give fully satisfactory preparations suitable for functional studies. In order to perform such studies we have therefore developed a simple method for purification of C $\bar{\text{I}}$ -esterase inhibitor which can be operated on a large scale and results in a high yield of pure product, with properties as close to the native molecule as possible.

Materials and Methods

Human plasma. Freshly frozen citrated human plasma was obtained from the Blood Centre of

Umeå University Hospital (courtesy of Dr. B. Cedergren).

C $\bar{\text{I}}$ s. This was prepared essentially according to the method of Chapuis et al. [9]. The final product was a homogeneous protein as established by SDS-polyacrylamide gel electrophoresis, all of which could form a stable complex with C $\bar{\text{I}}$ -esterase inhibitor, if this was added in excess.

Reagents. Hexyl-Sepharose was obtained by coupling 100 ml 1 M hexylamine (Kebo AB, Stockholm, Sweden), adjusted to pH 9 with hydrochloric acid, to 1000 ml CNBr-activated Sepharose 4B [10]. Sepharose 4B® and DEAE-Sephacel® were from Pharmacia Fine Chemicals (Uppsala, Sweden). Polybrene was from Aldrich (Beersel, Belgium) and the chromogenic tri-peptide-*p*-nitroanilide substrate S-2400 was a gift from Kabi peptide Research (Mölnådal, Sweden, courtesy of Dr. Lars Mellstam). All reagents and chemicals used were of analytical grade.

NH $_2$ -terminal amino acid determination. This was performed by manual Edman degradation [11], and the phenylthiohydantoin were identified by thin-layer chromatography as described previously [12].

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Amino acid analysis. The samples were hydrolyzed in constant boiling HCl in vacuo for 24, 48 and 72 h. Analysis was performed on an LKB amino acid analyzer according to the procedures given by the manufacturers.

Immunochemical analysis. Rabbit antiserum to the C \bar{I} -esterase inhibitor was raised by injection of the purified protein mixed with Freund's complete adjuvant. Booster doses were given together with Freund's incomplete adjuvant when the specific antibody titers were decreasing.

Electroimmunoassay was performed according to the method of Laurell [13] and crossed immunoelectrophoresis essentially as described by Clarke and Freeman [14].

Determination of C \bar{I} s and C \bar{I} -esterase inhibitor concentrations. The C \bar{I} s concentration was determined from its absorbance at 280 nm, using an $A_{280\text{nm}}^{1\%}$ of 9.5 [15].

The C \bar{I} s activity was measured using the chromogenic substrate S-2400: a 140 nM solution of C \bar{I} s gave a ΔA_{410} of 0.043/min at a substrate concentration of 0.63 mM in 0.1 M sodium phosphate buffer, pH 7.3, at 25°C.

C \bar{I} -esterase inhibitor concentrations were determined spectrophotometrically using an $A_{280\text{nm}}^{1\%}$ of 5.0 [1], or by titration against purified C \bar{I} s in the following way: 25 μ l of a C \bar{I} -esterase inhibitor sample was incubated for 10 min with 25 μ l C \bar{I} s (final concentration 140 nM) in a cuvette. Then 0.7 ml 0.1 M phosphate buffer, pH 7.3, and 200 μ l 3 mM S-2400 were added and residual C \bar{I} s activity was measured from the $\Delta A_{410\text{nm}}$, as described above. The C \bar{I} -esterase inhibitor could then be calculated from the decrease in $\Delta A_{410\text{nm}}$.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn [16] was performed on non-reduced and reduced samples, as previously described [17].

Reduction and S-carboxymethylation. Reduction of the C \bar{I} -esterase inhibitor was performed using a protein concentration of about 2 mg/ml. Dithioerythritol was added to final concentrations of 0.03–200 mM. For reductions at non-denaturing conditions, a 0.1 M sodium phosphate buffer, pH 7.3, was used. For reductions at denaturing conditions, the same buffer, containing 6 M guanidinium chloride, was used. After incubation for 30 min at

25°C in nitrogen atmosphere, [^3H]ICH $_2$ COONa was added to a final concentration of 80 mM, and incubation was continued in the dark for another 5 min. To the 200 mM dithioerythritol sample, 450 mM ICH $_2$ COONa was used. The labelled protein was isolated by gel filtration on Sephadex G-50 columns (0.8 cm 2 \times 9 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.3. Control samples were similarly treated, except that no dithioerythritol was added. The separated protein samples were analyzed by radioactivity measurement (Packard Tri-carb liquid scintillator) and protein determination by A_{280} or amino acid analysis after acid hydrolysis. As a reference protein, α_2 -antiplasmin (10 mg/ml) was treated in the same way in order to obtain the specific radioactivity of the iodoacetic acid, since it is known that this protein contains three disulphide bridges and no free SH group [18,19].

Results

Purification of C \bar{I} -esterase inhibitor

All procedure were carried out at +5°C. About 1 litre of plasma depleted in plasminogen by treatment with lysine-Sepharose [20] was thawed, made 20 mM in EDTA, and polybrene was added to a final concentration of 0.6 g/l. The plasma was subsequently diluted with an equal volume of PEG 6000 (12%, w/v). After stirring for 30 min, the precipitate was removed by centrifugation, and to the clear supernatant were added about 300 ml of DEAE-Sephacel $^{\text{®}}$, equilibrated with 0.04 M phosphate buffer, pH 7.0.

After stirring for 30 min, the gel was washed on a Büchner funnel with about 15 litres of the same phosphate buffer. The gel was then degassed and packed in a column (5 \times 15 cm), and subsequently eluted employing a linear gradient of NaCl from 0 to 0.3 M (total volume 1800 ml) in the same phosphate buffer (Fig. 1).

The C \bar{I} -esterase inhibitor concentration in the eluted fractions was determined by electroimmunoassay. Generally it was eluted in the 0.15–0.18 M NaCl range. The fractions containing antigen were pooled, concentrated to about 30 ml and made 0.4 M in (NH $_4$) $_2$ SO $_4$ by addition of an appropriate amount of the solid substance.

The concentrated DEAE-Sephacel $^{\text{®}}$ pool was

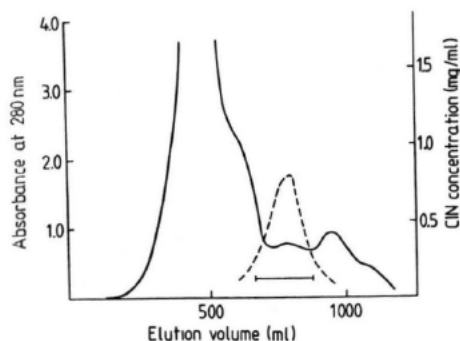


Fig. 1. Elution of the DEAE-Sephacel column by a linear gradient from 0.04 M sodium phosphate buffer, pH 7.0, to the same buffer containing 0.3 M NaCl. A_{280} (—); C \bar{I} -esterase inhibitor (CIN) concentration according to Laurell electrophoresis (-----).

then applied to a column of hexyl-Sepharose (5 \times 20 cm), equilibrated with 0.4 M $(\text{NH}_4)_2\text{SO}_4$ in 0.04 M phosphate buffer, pH 7.0. The C \bar{I} -esterase inhibitor passed through the column unadsorbed, whereas the contaminating proteins were either adsorbed or delayed (Fig. 2).

The first peak containing the C \bar{I} -esterase inhibitor was pooled as indicated, and subsequently dialyzed against 0.1 M sodium phosphate buffer, pH 7.3, in the cold. The solution was thereafter concentrated to give a final concentration of about 3 mg protein/ml and stored frozen at -80°C . Typically about 80–100 mg of the purified C \bar{I} -

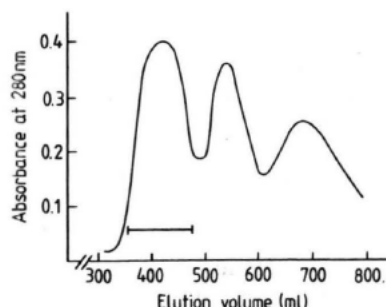


Fig. 2. Hydrophobic interaction chromatography on hexyl-Sepharose. The pooled fraction from the DEAE-Sephacel column was applied to the hexyl-Sepharose column equilibrated with 0.04 M sodium phosphate buffer containing 0.4 M $(\text{NH}_4)_2\text{SO}_4$. The first peak consists of essentially pure C \bar{I} -esterase inhibitor.

esterase inhibitor were obtained from 1 litre plasma.

Characterization of the purified C \bar{I} -esterase inhibitor

Homogeneity. The product obtained after the hexyl-Sepharose step is typically a single-chain protein which is more than 95% pure as shown by SDS-polyacrylamide gel electrophoresis on reduced and non-reduced samples (Fig. 3). On comparison with reference proteins its molecular weight was estimated to about 100000. On crossed immunoelectrophoresis, using a rabbit antiserum raised against the purified protein, only one precipitation arc is observed for the purified C \bar{I} -esterase inhibitor, as well as for human pooled plasma (Fig. 4). Within experimental error the electrophoretic mobility of the pure product is identical or very similar to the native C \bar{I} -esterase inhibitor in plasma. No signs of this inhibitor in complex with proteases such as C \bar{I} s or C \bar{I} r have been detected in normal plasma samples.

The amino acid composition (Table I) is in excellent agreement with that reported by Haupt et al. [4] for most amino acids. The only exception is tyrosine, where our figure is considerably lower. The amino acid composition reported by Harpel [1] is, however, quite different. On NH_2 -terminal amino acid analysis, about 1 mol asparagine per mol protein was found. No other amino acid could be detected.

Activity. Titration of the purified C \bar{I} -esterase inhibitor with C \bar{I} s resulted in an inverse linear relationship between the amount of C \bar{I} -esterase inhibitor added and remaining C \bar{I} s activity. A 10% molar excess of the inhibitor was sufficient to abolish the C \bar{I} s activity.

SDS-polyacrylamide gel electrophoretic analysis of the reaction mixture obtained using C \bar{I} s in



Fig. 3. SDS-polyacrylamide gel electrophoretograms of the purified C \bar{I} -esterase inhibitor: (a), reduced sample; (b), non-reduced sample.

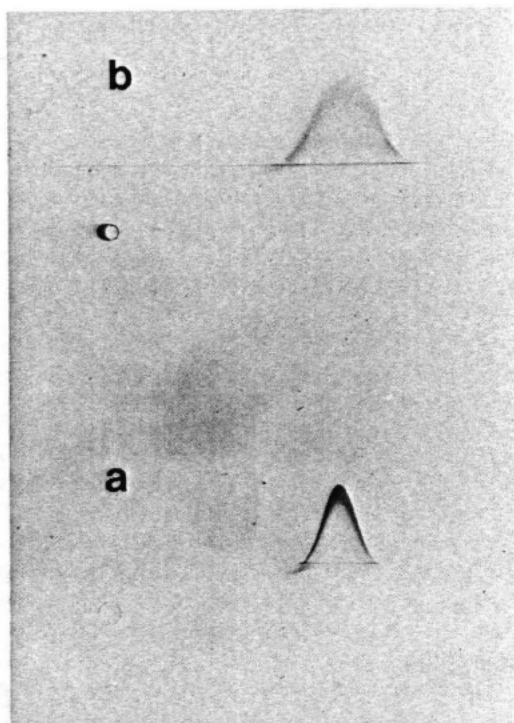


Fig. 4. Crossed immunoelectrophoresis of (a) purified CI-esterase inhibitor, (b) normal human plasma. The second dimension was run into a gel containing 1% (v/v) rabbit anti-CI-esterase inhibitor.

excess revealed that all of the CI-esterase inhibitor reacted to form a complex with the CIs light chain. When the CI-esterase inhibitor was in excess all of the CIs was converted to the enzyme-inhibitor complex, indicating that both the inhibitor and the CIs preparations fully retain their activities.

Stability. A fresh preparation of CI-esterase inhibitor, dialyzed against 0.1 M phosphate buffer, pH 7.3, was divided into aliquots and kept at +5°C, -20°C or -80°C. Sodium azide (0.04%) was added as preservative in the +5°C aliquots. At different times, ranging between 0-50 days, samples were checked for inhibitory activity against CIs. At -80°C or at +5°C no decline in activity could be detected. However, at -20°C a small but steady decline in activity was observed, amounting

TABLE I

AMINO ACID COMPOSITION OF THE PURIFIED PREPARATION OF CI-ESTERASE INHIBITOR

The figures represent the mean value of three determinations.

| Amino acid | $\frac{\mu\text{mol}}{100\text{ mg}}$ | Amino acid | $\frac{\mu\text{mol}}{100\text{ mg}}$ |
|------------------------|---------------------------------------|---------------|---------------------------------------|
| Aspartic acid | 56.5 | Methionine | 14.5 |
| Threonine ^a | 59.9 | Isoleucine | 19.8 |
| Serine ^a | 52.5 | Leucine | 62.7 |
| Glutamic acid | 61.0 | Tyrosine | 8.3 |
| Proline | 42.6 | Phenylalanine | 29.2 |
| Glycine | 17.0 | Histidine | 9.0 |
| Alanine | 38.0 | Lysine | 36.9 |
| Cysteine ^a | 5.5 | Arginine | 16.0 |
| Valine | 37.1 | | |

^a Extrapolated values.

to about 50% during the observation period.

The disulphide bridges in CI-esterase inhibitor. Amino acid analysis has indicated six cysteines in the molecule (Table I), thus forming three disulphide bridges. This has been investigated further by reduction of the CI-esterase inhibitor with dithioerythritol in the presence of 6 M guanidinium chloride, followed by S-carboxymethylation with [³H]CH₂COONa. Using α₂-antiplasmin as a standard, known to possess three disulphide bridges [19], we found 5.9 cysteines in the CI-esterase inhibitor. The small radioactivity incorporated nonspecifically prior to reduction, corresponding to about 0.8 residues, is then already subtracted. Conclusively, the CI-esterase inhibitor contains three disulphide bridges.

Partial reduction of the CI-esterase inhibitor under non-denaturing conditions using different dithioerythritol concentrations followed by S-carboxymethylation with [³H]CH₂COONa is shown in Fig. 5. Two of the three disulphide bridges seem to be easily cleaved even at 3 mM dithioerythritol under non-denaturing conditions. However, to cleave the third disulphide bridge the dithioerythritol concentration has to be increased to 200 mM. Included in Fig. 5 are also the results from activity measurements of the reduced CI-esterase inhibitor samples. Reduction and S-carboxymethylation of the two easily reduced disulphide bridges are performed without loss of

activity. However, cleavage of the third disulphide bridge results in almost complete loss of activity (Fig. 5).

Discussion

Many of the previously published purification procedures for C_I-esterase inhibitor are tedious and result in low yields and varying amounts of degraded forms of the inhibitor. The method recently published by Reboul et al. [5] is simple, but in our hands it only gave a 50%-pure material with several contaminants at low concentrations. Therefore the present method seems to be superior in its simplicity, high yield and quality of the final product. A yield of 80–100 mg/litre plasma represents about 45–55%, assuming a concentration of 180 mg/l in normal plasma [1]. The present method can, furthermore, easily be operated on a large scale.

The purified protein is a single-chain glycoprotein with a molecular weight of about 100000, and asparagine is found to be its NH₂-terminal amino acid. The purified preparation has electrophoretic mobility in the α_2 region identical to that of the C_I-esterase inhibitor in plasma, as judged by crossed immunoelectrophoresis. It is also fully active, as judged by titration against C_Is or analysis of a mixture of C_I-esterase inhibitor and C_Is in molar excess by SDS-polyacrylamide gel electro-

phoresis. If kept frozen at -80°C the material is stable for months, but not if frozen at -20°C . Lyophilization always results in loss of appreciable amounts of activity.

Recently we reported [19,21] that two of the three disulphide bridges in α_2 -antiplasmin could be reduced and *S*-carboxymethylated under non-denaturing conditions without affecting the inhibitory activity. Cleavage of the third disulphide bridge could only be performed in the presence of denaturing agents and was accompanied by complete loss of activity [19,21]. In the present study, similar results are obtained with C_I-esterase inhibitor, although the third disulphide bridge in this case can be cleaved if a high concentration of dithioerythritol is used. This still leads to inactivation of the molecule, indicating that the 'stable' disulphide bridge is indeed important in maintaining the crucial conformation around the reactive site of the inhibitor. The similar behaviour of the disulphide bridges in α_2 -antiplasmin and C_I-esterase inhibitor may indicate a structural relationship between these molecules. This will be further elucidated by amino acid sequence analyses, which are already in progress.

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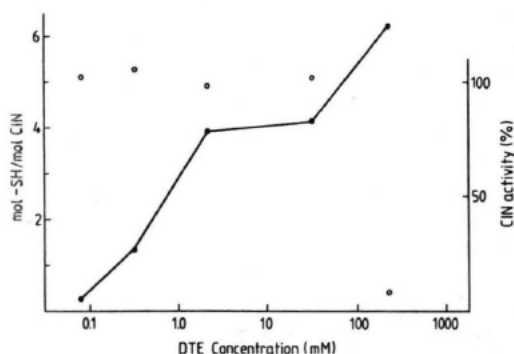


Fig. 5. Reduction of C_I-esterase inhibitor (CIN) by different concentrations of dithioerythritol (DTE) followed by *S*-carboxymethylation with [³H]ICH₂COONa. The number of *S*-carboxymethylcysteines were calculated from the specific radioactivity (●—●). Included in the figure are also the results from activity measurements (○).

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