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# Evaluation of the chromatographic procedure for the preparation of a high-purity C1-esterase inhibitor concentrate from cryosupernatant plasma

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ABSTRACT

This report is an analysis of the parameters involved in the preparation of a C1-esterase inhibitor concentrate. We investigated ways to improve the purification by finding the optimal pH, ionic strength, and quantity of chromatographic media to use at each step. The optimisation of the process gave a greater than two-fold increase in both yield and purity over published methods. The product retained more than 50% activity upon dry heat-treatment at 80°C for 72 h, and the yield of the heat-treated product was 180 I.U./kg of plasma.

## INTRODUCTION

C1-esterase inhibitor (C1-Ina) is the major regulatory protein of the classical pathway of complement activation and it is also involved in the regulation of several other plasma proteolytic systems including the coagulation, fibrinolytic and contact systems [1]. A deficiency of C1-Ina has been associated with the disorder hereditary angioneurotic edema [2]. Substitution therapy with purified C1-Ina has been used for the treatment of this disorder [3] and has also been suggested for the treatment of sepsis [4].

Wickerhauser *et al.* [5] have reported a method for preparation of heat-treated, high-purity C1-Ina concentrates. Benny and Brimer [6] reported a modification to that procedure by substituting ion-exchange chromatography for ammonium sulphate precipitation as the last purification step. Both methods produced about 450-fold purification of C1-Ina from cryosupernatant. We decided to optimise the process of Benny and Brimer [6] by analysing the parameters involved at each purification step.

## EXPERIMENTAL

### *Cryosupernatant*

Cryosupernatant derived from fresh frozen plasma [7] was used as the starting material for the isolation of C1-Ina. All experiments were conducted with 3-l pools of cryosupernatant and carried out at 20°C unless otherwise stated.

### *General C1-Ina preparation*

The method of purification was according to Benny and Brimer [6] but with modifications in the buffer conditions. Cryosupernatant was batch-stirred with dry DEAE-Sephadex A50

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(Pharmacia, Uppsala, Sweden) for 30 min at different pH values. C1-Ina was eluted with high ionic strength of standard phosphate-buffered saline (PBS) at the same pH. The solution was desalted into 50 m*M* acetate–0.1 *M* NaCl. Polyethylene glycol (PEG-4000; Serva, Heidelberg, Germany) was added to give a concentration of 20% (w/v), the pH adjusted, the solution mixed for 30 min and the supernatant recovered by centrifugation at 4000 *g* for 20 min at 15°C. The supernatant was then diluted 1:1 (v/v) into 50 m*M* acetate buffer and loaded onto a pre-equilibrated CM-Sepharose FF (CM-Seph, Pharmacia) column. After loading, contaminating proteins were eluted with a 50 m*M* acetate–100 m*M* NaCl buffer. C1-Ina was recovered by step-gradient elution

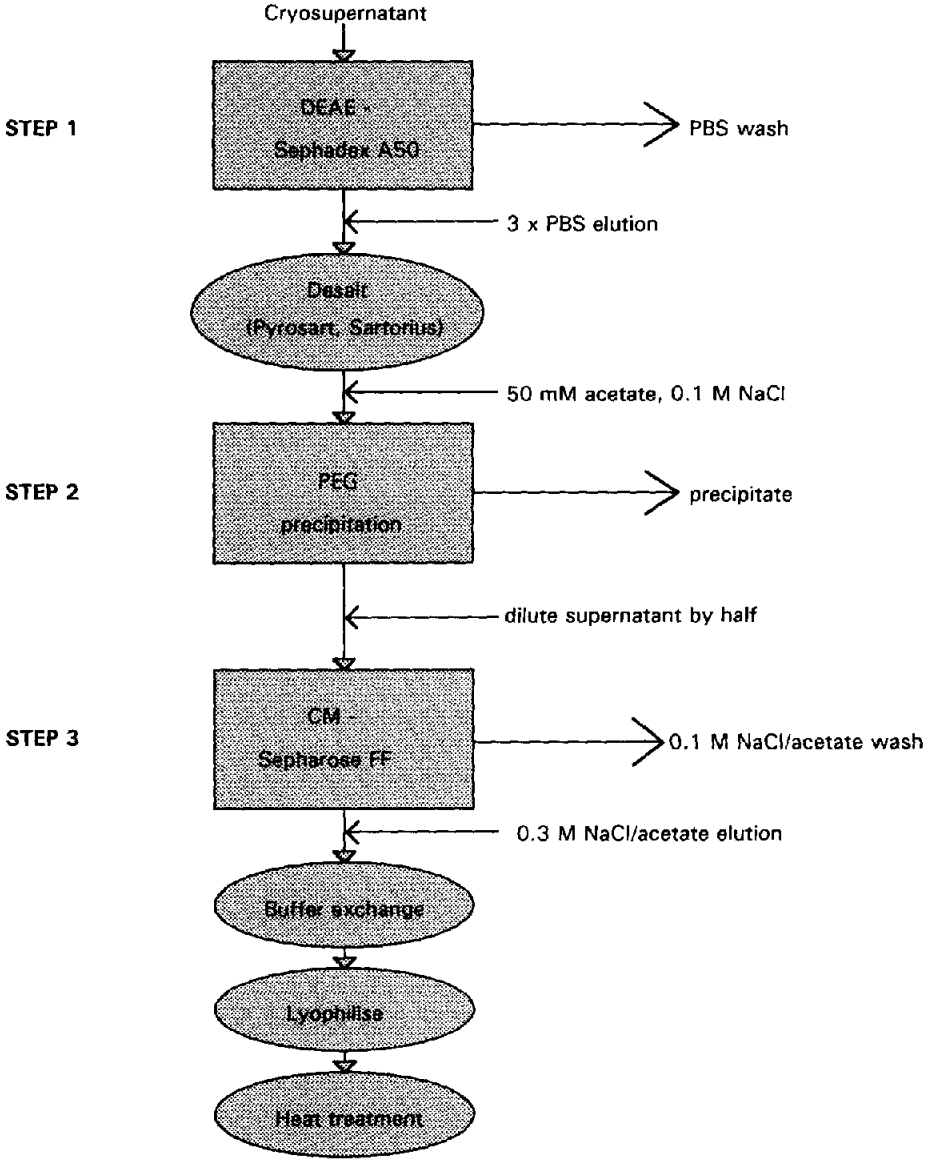


Fig. 1. Schemnatic representation of the purification process of C1-Ina. The boxes refer to the three major steps of purification. The circles refer to other steps in the purification.

containing the same buffer but with 300 m*M* NaCl. The pH was adjusted in each step accordingly for the pH experiments.

The solution was then ultrafiltered into a 10 m*M* sodium citrate–130 m*M* NaCl buffer, pH 7.1, and concentrated using a disposable ultrafiltration unit (Pyrosart, Sartorius, Goettingen, Germany). It was sterile-filtered, dispensed, and lyophilised. The lyophilised product was heat-treated at 80°C for 72 h.

*Analytical methods*

C1-Ina functional activity was determined by a chromogenic peptide substrate assay (Berichrom, Behringwerke, Marburg, Germany), according to the manufacturer's instructions. C1-Ina and complement factors C2 and C4 antigenic activities were determined by rocket immunoelectrophoresis with appropriate antisera [8]. Total protein was determined by either absorption at 280 nm or by Bradford's method [9], both calibrated against a human albumin standard and appropriate buffer. Analytical electrophoresis (sodium dodecyl sulphate polyacrylamide electrophoresis, SDS-PAGE) was carried out in a 9% polyacrylamide gel in the presence of 0.1% SDS [10]. The gels were stained with 0.5% Coomassie blue.

## RESULTS AND DISCUSSION

The method of purification of C1-Ina from cryosupernatant [6] consisted of three purification steps as outlined in Fig. 1. We investigated ways to improve the purification at each step by finding the optimal pH, ionic strength, and quantity of chromatographic media to use.

*Optimisation of step 1*

*Quantity of media.* The first step involved the primary capture of C1-Ina from cryosupernatant using batch adsorption with DEAE-Sephadex A50. Fig. 2 shows that the recovery of C1-Ina from cryosupernatant increases with the quantity of DEAE-Sephadex A50 used. However, this increase was not linearly related to the amount of resin beads used (for example, doubling the beads from 7 to 14 g per litre resulted in only an extra 15% recovery). Increasing the amount of beads led to binding of other proteins while decreasing the amount led to a poor yield of C1-Ina at this step. Gel media at 8 g per litre of cryosupernatant was established to be the most efficient and economical for recovery of C1-Ina. All subsequent experiments used this ratio.

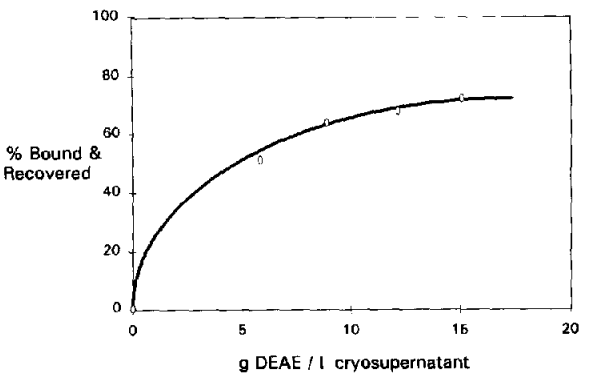


Fig. 2. Recovery of C1-Ina *versus* the quantity of DEAE-Sephadex A50 used. Different quantities of DEAE-Sephadex A50 were added to cryosupernatant at pH 7.3. The recovery of C1-Ina was expressed as a percentage of the amount in starting cryosupernatant.

*Incubation and wash conditions.* Extensive washing of the bound beads (up to 30 times bed volume) with PBS removed albumin and improved the purity of C1-Ina recovered at this stage, as reported previously [11]. There was no significant loss of C1-Ina due to this extensive washing.

Fig. 3 shows the purity and yield of C1-Ina obtained at different pH of incubation and wash. The absorption of C1-Ina to the beads was strongly dependent on pH. The major contaminant in this step, albumin, did not bind strongly to the beads at lower pH values. C1-Ina was eluted from the beads at a 4 × strength PBS buffer. Higher-strength PBS buffer did not improve the yield but contributed to a decrease in the purity of the eluent. The yield was relatively constant from pH 5.5 to 7.5. However, incubation at pH 5.5 gave a purer C1-Ina product without affecting this yield. Pensky *et al.* [12] reported that isolated C1-Ina lost activity on exposure to pH values below 5.5. We found that C1-Ina showed no loss of

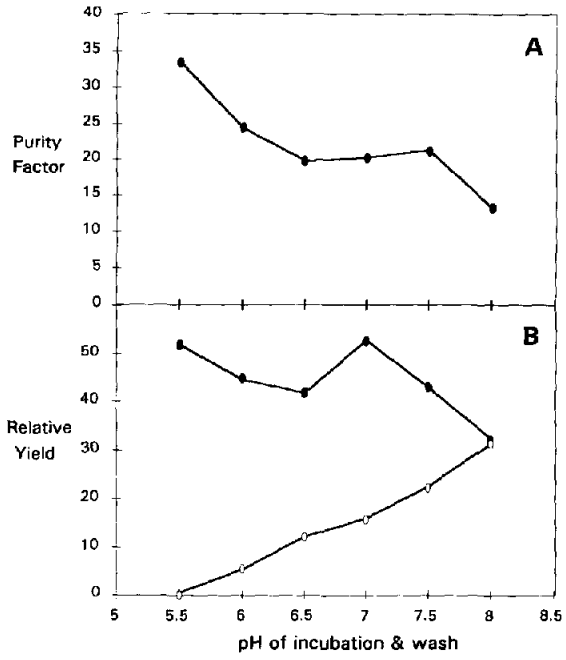


Fig. 3. Yield and purity of C1-Ina obtained in step 1 at different pH values of incubation. The eluent was assayed for functional C1-Ina activity. The purity (A) and yield (B) were plotted against pH. In (B), closed circles refer to C1-Ina activity in the eluent and open circles refer to C-Ina remaining in the depleted cryosupernatant.

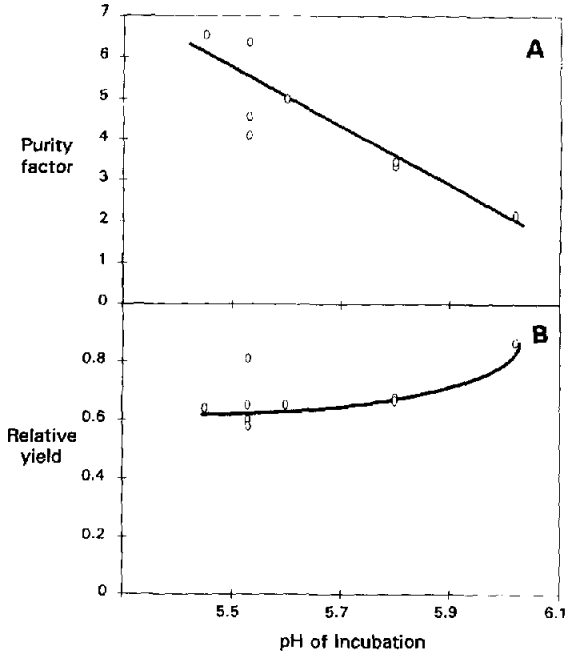


Fig. 4. Yield and purity of C1-Ina obtained at different pH values during PEG precipitation (step 2). The 20% PEG supernatant was assayed for C1-Ina activity and the purity (A) and relative yield (B) were plotted against pH.

functional activity after an overnight incubation at pH 5.5. Hence, the batch trials were conducted at that pH without any fear of inactivation.

*Optimisation of step 2*

The DEAE eluent was first desalted into a buffer containing 0.1 *M* NaCl before the second purification step using PEG precipitation. There was some loss of C1-Ina from non-specific precipitation if less than 0.1 *M* NaCl was present (personal observations). Different concentrations of PEG were investigated. A final concentration of 20% as found to be optimal. At this concentration, complete precipitation of complement factors (C3 and C4) occurred, leaving C1-Ina in the supernatant.

Fig. 4 shows the recovery and purity of C1-Ina in 20% PEG supernatant at different pH values. Lower pH gave better selectivity towards the C1-Ina molecule. The increase in yield with a higher pH was not significantly different. For the batch trials, a pH of 5.5 at 20% PEG was chosen for this step.

*Optimisation of step 3*

This step involved the adsorption and step elution using CM-Seph. The PEG supernatant was diluted before loading to reduce its high viscosity.

*Elution conditions.* Alterations in the eluent salt concentrations or the pH of the wash and eluting buffers affected the yields and purity levels of C1-Ina recovered at this step. Increasing the eluent salt concentration from 0.2 to 0.3 *M* NaCl gave a better recovery but not a significant change in the purity of the product, presumably due to the low levels of impurities at this stage. However, varying the pH affected the purity but not significantly the yield of the recovered C1-Ina product as shown in Fig. 5. For the batch trials, the lower pH of 5.5 was used to have a higher purity.

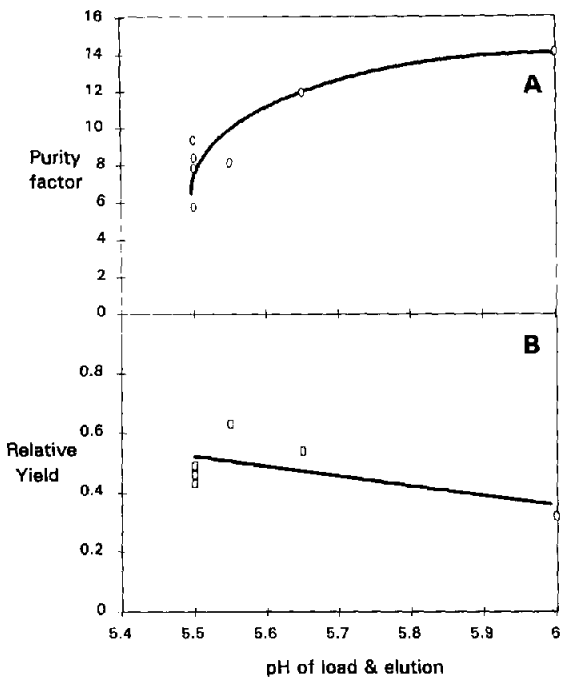


Fig. 5. Yield and purity of C1-Ina obtained at different pH values of absorption to CM-Sepharose FF (step 3). The eluent was assayed for C1-Ina activity and the purity (A) and relative yield (B) were plotted against pH.

The relative yield and purity of C1-Ina obtained from step 3 was found to be independent of the initial purity of C1-Ina from step 2. This suggests that both purification steps remove different contaminating proteins.

*Quantity of media.* The loading capacity of CM-Seph for C1-Ina from the PEG supernatant was tested. Approximately 10 ml of CM-Seph per litre of starting cryosupernatant were needed to absorb all the C1-Ina from the PEG supernatant (data not shown). Hence, for a production batch of 30 l of cryosupernatant, a bed volume of 300 ml of CM-Seph is sufficient for the process.

*Trial batches*

Preliminary trial batches were conducted using the optimised conditions for each of the three purification steps. Table I shows the mean recovery and specific activity of C1-Ina obtained at each step of purification starting from cryosupernatant. The overall optimised process resulted in about 30% recovery of C1-Ina with about an 800-fold purificaton over starting cryosupernatant. This represents a two-fold increase in both the yield and purity over those obtained by either Benny and Brimer [6] or Wickerhauser *et al.* [5].

Table II shows the stability of the final lyophilised product upon high heat-treatment for up to 72 h. Although the conditions for lyophilization and formulation were not optimised at the time of the trial, a recovery of more than 50% of inhibitory activity was obtained. Wickerhauser *et al.* [5] reported that after 10 h heat-treatment there was no loss in activity for their product. This compares to a 10% loss for our preparation after that time.

Table III shows the characteristics of the final lyophilised and heat-treated product. This product yielded about 180 I.U. of C1-Ina activity per litre of starting plasma. There were not significant levels of ceruloplasmin or the complement factors, C2 and C4. SDS-PAGE of the final C1-

TABLE I

PURIFICATION OF C1-Ina AT DIFFERENT STAGES OF PURIFICATION USING OPTIMISED CONDITIONS

Each step refers to the step in Fig. 1. The values shown are the mean of eight batches. The values in parentheses are from the non-optimized process, reported in Benny and Brimer [1].

| Fraction | Relative purification | Total purification | Relative yield | Total yield (%) |
|---|---|---|---|---|
| Start cryosupernatant | 1 | 1 | 100 | 100 |
| Step 1 | 40.9 ± 5.9 | 40.9 (9) | 80.0 ± 9.0 | 80.0 (38) |
| Step 2 | 5.6 ± 1.2 | 227 (13) | 64.7 ± 6.6 | 51.7 (28) |
| Step 3 | 3.5 ± 1.2 | 795 (272) | 70.5 ± 7.2 | 32.6 (19) |

TABLE II

HEAT STABILITY OF FINAL PRODUCT

| Sample | Activity remaining (%) |
|---|---|
| Final product, frozen, before lyophilization | 100 |
| Lyophilized product | 95 |
| Lyophilized final product: | |
| heated 80°C, 10 h | 86 |
| heated 80°C, 22 h | 75 |
| heated 80°C, 50 h | 68 |
| heated 80°C, 72 h | 55 |

TABLE III

CHARACTERISTICS OF FINAL HEAT-TREATED PRODUCT OF THE OPTIMIZED METHOD *VERSUS* THE NON-OPTIMIZED PROCESS

| | This method | Non-optimized |
|---|---|---|
| Specific activity (I.U./mg) | 20 | 6.8 |
| Final yield (I.U./kg plasma) | 179 | 160[a] |
| Percent of total protein | >95% | >90% |
| C2 level (I.U./ml) | Not detected | Not detected |
| C4 level (I.U./ml) | Not detected | Not detected |
| Ceruloplasmin (I.U./ml) | Not detected | Not detected |

[a] Heat-treated only for 24 h at 80°C.

Ina product gave a single protein band in the gel indicating a high degree of purity.

The final lyophilised and heat-treated product will be presented for clinical evaluation in this country.

Hence, from this investigation, a two-fold improvement in both the yield and purity over published reports can be obtained. Further improvement are envisaged through optimisation of the other steps, namely desalting, concentrating, formulating, and freeze-drying conditions.

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