*Biochimica et Biophysica Acta*, 356 (1974) 82–90


BBA 76661

# DETERGENT-SOLUBILIZED HL-A ANTIGENS FROM HUMAN PLATELETS· A COMPARATIVE STUDY OF VARIOUS PURIFICATION TECHNIQUES

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(Received December 27th, 1973)

## SUMMARY

HL-A alloantigens, solubilized by the non-ionic detergent NP-40, from human platelets have been purified by 3 different techniques: electrofocusing, preparative polyacrylamide-gel electrophoresis and filtration on Sepharose 6B

In all cases, the purified active fractions presented the following common characteristics active molecules with molecular weights $> 200\,000$, which could be dissociated by analytical polyacrylamide gel electrophoresis, in sodium dodecyl-sulfate-containing systems, into 2 major components, stained by the periodate–Schiff reagent, with molecular weights of about 45 000 and 90 000

Only electrofocusing enabled the crude extracts to be purified in the absence of the detergent, with a good recovery of active molecules

The presence of detergent in the gel and buffer systems was necessary to realize the purification of the crude extracts by preparative polyacrylamide-gel electrophoresis

For filtration on Sepharose 6B, the presence of detergent enabled the active molecules to be eluted in a single peak.

The importance of the detergent/protein ratio for the separation of the different HL-A specificities is discussed

## INTRODUCTION

Little information is so far available concerning the purification of detergent-solubilized HL-A antigens by classical biochemical techniques This can be explained by the difficulty in removing the detergent without a generally great loss of solubility of the active molecules and by the interference of the residual detergent often observed in immunological assays used to detect the antigens

Schwartz et al. [1] overcame these difficulties by using an indirect immune precipitation method to isolate and characterize NP-40 solubilized H-2 alloantigens and Springer and Strominger [2] used the same method for the characterization of detergent-solubilized HL-A antigens Their contribution to the knowledge of the

biochemical properties of histocompatibility antigens must be considered as fundamental, because, by this technique, they could demonstrate that different antigenic determinants were carried by different molecules, which are glycoprotein subunits with molecular weights of about 45 000.

Recently, we reported results concerning HL-A antigens from human platelets solubilized by various amounts of NP-40 and their purification by electrofocusing [3].

The present paper deals with further data on the active fractions purified by electrofocusing and describes the results obtained from two other purification techniques. preparative polyacrylamide-gel electrophoresis and filtration on Sepharose 6B.

## MATERIALS AND METHODS

### *Human platelets*

Platelets were obtained by differential centrifugation under the same conditions and from the same normal donors as previously described [3].

### *Preparation of the solubilized extracts*

The solubilization of the antigens was always realized from crude membrane preparations: $10^{11}$ platelets (recovered from one plasmapheresis), containing about 180–200 mg protein, were suspended in 50 ml of 0.05 M Tris–HCl buffer (pH 7.3)–0 15 M NaCl and homogenized for 15 min in a Teflon homogenizer After centrifugation (35 000 $\times g$ for 30 min), the supernatant, containing about one half of the proteins from the initial preparation, was discarded and the pellet, resuspended in 10 ml of the same buffer, was incubated with 0 5 % NP-40 (v/v) (Compagnie Française des Produits Shell, Paris) for 30 min at 4 °C, with continuous mixing, corresponding to a final concentration of about $0.8 \cdot 10^{-6}$ mole of NP-40 per mg of protein. Insoluble material was then removed by high speed centrifugation (165 000 $\times g$ for 90 min)

### *Elimination of the detergent*

It was realized by extensive dialysis on Diaflo membranes, PM-10 (Amicon).

### *Detection of HL-A specificities*

The antigenic activity was monitored by the inhibition of lymphocytotoxicity tests, using antisera and experimental conditions previously described [3]. The 50 % inhibition dose ($ID_{50}$ or inhibition unit) of an extract was the smallest amount which could diminish the activity of a lymphocytotoxic serum by half. However, the inhibitory activity of various amounts of the extracts was always tested against 1 antibody unit only, while in our previous paper [3] the cytotoxicity percentage was determined against 0.5, 1, 2, and 3 antibody units and the final total toxicity percentage was obtained by adding the four percentages. For these reasons the inhibition unit was roughly 5 times smaller with the present system.

### *Purification of the soluble extracts*

*Preparative polyacrylamide-gel electrophoresis.* Preparative polyacrylamide-gel electrophoresis was performed, at pH 8 9 [4], using the Quickfit instrumentation

apparatus with tap water cooling 25 ml of separating gel was formed, using 7 % acrylamide (Kodak) and after 30 min of ultraviolet polymerization, 10 ml of stacking gel was formed using 4 % acrylamide The polymerization of the stacking gel occurred overnight The anode and cathode compartments contained both 0 05 M Tris–HCl, 0.38 M glycine buffer (pH 8 3). The elution buffer was 0 35 M Tris–HCl buffer (pH 8 3). The gels were prerun at 100 V and 100 mA until a sample of bromophenol blue had been eluted 5 ml of the solubilized extract, in 40 % sucrose, were finally applied onto the column The current was increased to 150 V and 150 mA and the elution speed was 12 ml/h Fractions of 3 ml were collected In a second series of experiments, the same gel and buffer systems have been used in the presence of 0 3 % NP-40 The proteins were determined, by their absorption at 280 nm, in the absence, and at 254 nm, in the presence of detergent, with a Beckman Acta III spectrophotometer Cytotoxicity inhibition tests were performed on extensively dialyzed fractions

*Filtration on Sepharose 6B* It was performed on 58 cm × 2 2 cm columns in a 0 05 M Tris–HCl buffer (pH 7 3)–0 15 M NaCl, in the absence or in the presence of 0 3 % NP-40 1-ml fractions were collected

*Analytical polyacrylamide-gel electrophoresis*

Analytical polyacrylamide-gel electrophoresis was performed in the presence of sodium dodecylsulfate [5] Gels contained 6 % acrylamide for the separating gels and 3 % acrylamide for the stacking gels. The sodium dodecylsulfate concentration in the gels was 0.1 % The electrode buffer (0 025 M Tris–HCl, 0 192 M glycine, (pH 8 3) also contained 0 1 % sodium dodecylsulfate The samples after immersion for 3 min in boiling water were applied onto the gels in the following buffer 0 0625 M Tris-HCl buffer (pH 6 8), 2 % sodium dodecylsulfate, 10 % glycerol and 0 001 % bromophenol blue In some cases, we also added 5 % 2-mercaptoethanol to the last buffer Electrophoresis was carried out with a current of 3–5 mA/gel until the bromophenol blue marker reached 0 2 cm from the end of the gels (5 5 cm × 0 5 cm). They were stained for proteins by amido black or by coomassie brilliant blue [6] and for carbohydrates by the periodate–Schiff reagent [7] The gels were scanned with the gel scanner attachment of the Beckmann Acta III spectrophotometer at either 620 nm (protein stain) or 560 nm (carbohydrate stain)

*Extraction and characterization of lipids*

To the previously dried fractions, which were eluted from Sepharose 6B, 1 5 ml of chloroform was added and after 2 min agitation the lipids were recovered, after low-speed centrifugation Characterization of the chloroform extract was carried out by chromatography on silica gel plates (DC-Fertigplatten Kieselgel, Merck, 20 cm × 20 cm, 0 25-mm gel), activated by heating for 30 min at 100 °C, using a chloroform–methanol–water mixture (65 25:4, by vol ) as migrating solvent [8] The chromatograms were developed with the following reagents· anthrone–sulfuric acid for staining lipids and phospholipids (yellow), glycopeptides (blue) and cholesterol (violet), ninhydrin for staining amino groups and $FeCl_3$–sulfosalicylic acid [9] for staining phosphorus

*Protein determination*

Proteins were determined by the method of Lowry et al [10], on aliquots extensively dialyzed on Diaflo membranes, PM-10.

RESULTS

The solubilization of human platelet antigens by NP-40 always produced crude extracts with a high inhibitory activity, in vitro, confirming previous observations [3], but the behaviour of these extracts and the recovery of the activities of the antigens originally tested varied largely with the applied purification techniques

*Purification of the crude extracts by electrofocusing*

This purification technique has already been described in a previous paper. We further indicated that with a low detergent/protein ratio used for the solubilization, the purified active fractions always gave rise to a single protein band stained by amidoblack, after analytical polyacrylamide-gel electrophoresis at pH 8.9 or 7.5 [3]. More recently, however, when submitted to sodium dodecylsulfate–polyacrylamide gel electrophoresis, this single protein band could be dissociated into two major components, which have been characterized as glycoproteins, with molecular weights of about 45 000 and 90 000 (Fig. 1).

*Purification of the crude extracts by preparative polyacrylamide-gel electrophoresis*

Attempts to purify the crude extracts by this technique, at pH 8.9, revealed, when most of the detergent has been eliminated by extensive dialysis, that their solu-

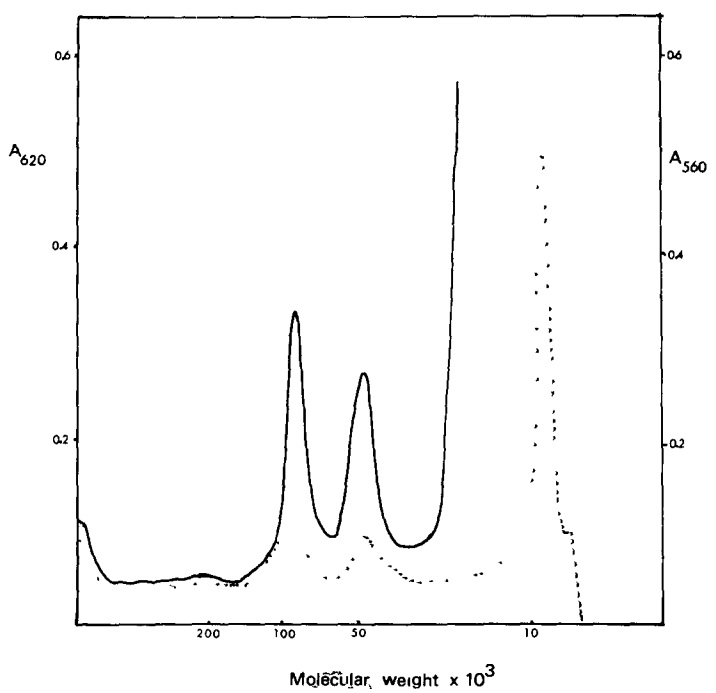


Fig 1. Sodium dodecylsulfate–acrylamide gel scan analysis of an active fraction purified by electrofocusing [3] —, amidoblack-stained gel containing about 50 $\mu$g of proteins, , periodate–Schiff-stained gel containing about 150 $\mu$g of proteins (The strong amidoblack stained zone at the end of the gel is due to ampholines )

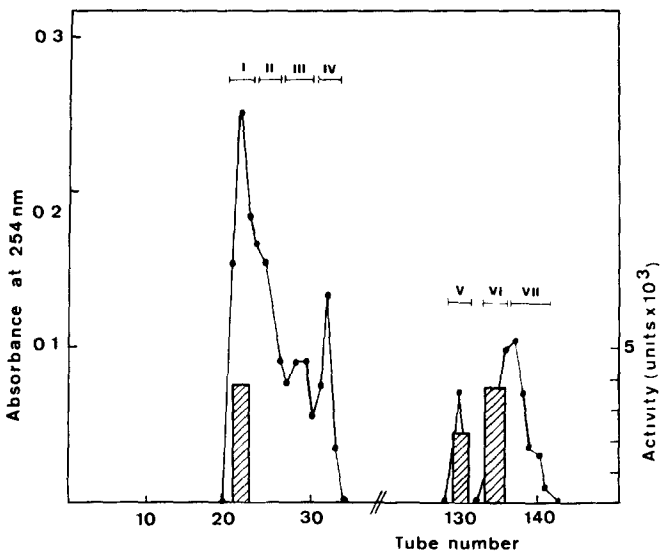


Fig 2 Elution profile of Luthy extract after preparative polyacrylamide-gel electrophoresis, in the presence of 0 3 % NP-40 (Quickfit apparatus) 3-ml fractions, the inhibitory activity of the 3 serologically active peaks (I, V and VI) was tested for HL-A2 and HL-A5

bility was not sufficient enough. indeed, the greatest part of the material precipitated in the gel A second centrifugation (165 000 × *g* for 90 min) was necessary, in order to remove the insolubilized material, leading, at the same time, to the loss of about 80 % of the antigenic activity. After preparative polyacrylamide-gel electrophoresis of the remaining active material only a poor recovery of antigenic active molecules could be obtained.

However, the addition of 0.3 % NP-40 to the preceding gel and buffer systems allowed a good recovery of highly purified active molecules after electrophoresis Fig. 2 represents the elution pattern of such an experiment and indicates the situation of the three serologically active peaks (I, V and VI) By analytical sodium dodecylsulfate–polyacrylamide gel electrophoresis, it was possible to characterize in each of them, periodate–Schiff reagent-stained bands. Peak I contained a major periodic acid–Schiff reagent-stained band with a molecular weight of about 90 000, Peak V included several weak periodic acid–Schiff reagent-stained bands and Peak VI contained 2 major periodic acid–Schiff reagent-stained bands with molecular weights of about 45 000 and 90 000. It was surprising that the active molecules were eluted in three peaks, two of them varying distinctly in their charges. This behaviour might be due to the presence of aggregates including various amounts of lipids, the charges of which can largely modify their electrophoretic mobility.

*Purification of the crude extracts by filtration on Sepharose 6B*

Previous attempts to fractionate the crude extracts on Sephadex G-200 indicated that most of the antigenic activity was eluted with the void volume of the column [3]

Gel filtration on Sepharose 6B is visualized in Fig. 3 Upon gel filtration in

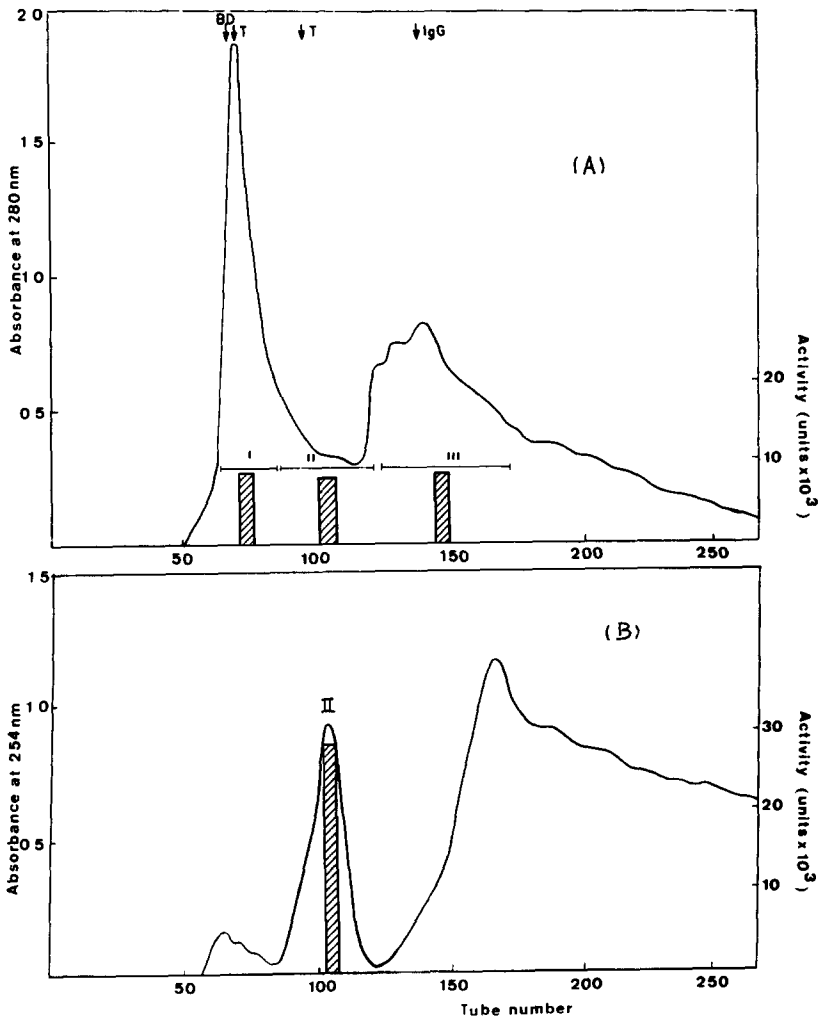


Fig. 3. (A) Gel filtration of Lehideux extract on Sepharose 6B (58 cm×2.2 cm) in 0 05 M Tris–HCl buffer (pH 7.3) 0.15 M NaCl. 1-ml fractions I–III = pooled active fractions. ↓ = test substances used for column calibration, DB = dextran blue (void volume), T = tryptophanase (Sigma) (mol. wt = 500 000 and 280 000), G = complete IgG (mol. wt = 160 000). (B) Gel filtration of extract Lehideux on Sepharose 6B (58 cm×2 2 cm) The column had previously been equilibrated with 0 05 M Tris–HCl buffer (pH 7 3)–0.15 M NaCl containing 0.3 % NP-40 Elution by the same buffer. 1-ml fractions. II = pooled active fraction. The inhibitory activity of the active fractions from both columns was tested for HL-A2 and HL-A5.

the absence of detergent (Fig. 3A), the antigenic active molecules were eluted in a large zone, covering molecular weights from about 100 000 to several millions, while the filtration in the presence of detergent allowed all the antigenic activity to be recovered in a single peak corresponding to an average molecular weight of 200 000–400 000 (Fig. 3B). This observation suggests that the elimination of the detergent

TABLE I

CHARACTERISTICS OF TWO EXTRACTS AFTER FILTRATION ON SEPHAROSE 6B

Extract 1 was filtered in the absence and Extract 2 in the presence of NP-40

| Extracts | | Crude extracts | | Active fractions after filtration on Sepharose 6B | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No | Donor | Proteins | Activity★ | Proteins (mg) | | | | Activity★ (I U ) | | | | Lipids | | | Glyco proteins | | |
| | | (mg) | (I U ) | I | II | III | Total | I | II | III | Total | I | II | III | I | II | III |
| 1 | Lehideux | 10 4 | 160 000 | 1 06 | 1 42 | 2 03 | 4 51 | 8 000 | 7 000 | 7 000 | 22 000 | + | − | + | − | + | − |
| 2 | Lehideux | 10 2 | 180 000 | | 3 40 | | 3 40 | 0 | 26 000 | 0 | 26 000 | + | | + | − | (+) | − |

★ The crude extracts and the purified active fractions were tested for their inhibitory activity against anti-HL-A2 and anti-HL-A5 sera

provokes an important reaggregation of the solubilized molecules and explains the partial elution of the active molecules with the void volume of the column in the absence of detergent.

Chloroform extraction of the active fractions eluted from Sepharose 6B in the presence or absence of detergent allowed lipids, phospholipids and cholesterol to be characterized in all of them. These components might be considered as "free" lipids, because their extraction time never exceeded 2 min. It should be noted that the lipids extracted from the three active fractions eluted in the absence of detergent are the same as those present in the active fraction eluted from Sepharose 6B in the presence of detergent, while the peak eluted with the void volume of this last column contained cholesterol only.

Analytical sodium dodecylsulfate–polyacrylamide gels of the active fractions eluted from Sepharose 6B, always contained several constituants, but in all of them 2 major periodate–Schiff reagent-stained bands, with molecular weights of about 45 000 and 90 000, could be characterized.

Table I summarizes the characteristics of the 2 extracts filtered on Sepharose 6B and Table II indicates the recovery of active molecules with the three purification techniques used.

TABLE II

COMPARISON OF THE RECOVERIES OF ACTIVE MOLECULES FOLLOWING THE PURIFICATION TECHNIQUES USED

| Purification technique | 0 3 % NP-40 | Inhibitory activity of the purified fractions (I.U /mg of protein) |
|---|---|---|
| Electrofocusing | – | 16 000 |
| Preparative polyacrylamide-gel electrophoresis | – | 2 800 |
| Preparative polyacrylamide-gel electrophoresis | + | 15 000 |
| Filtration on Sepharose 6B | – | 4 300 |
| Filtration on Sepharose 6B | + | 7 200 |

## DISCUSSION

The results reported in this paper point out that a good preliminary purification of NP-40 solubilized platelet antigens can be obtained by classical techniques. We could characterize in all our active fractions, after analytical sodium dodecylsulfate–polyacrylamide-gel electrophoresis, the presence of two major bands with molecular weights of about 45 000 and 90 000 which, by their staining with the periodate–Schiff reagent, indicate the presence of glycoproteins. These two components can be compared to the "monomeric" and the "dimeric" forms of antigens described by Schwartz et al. [1]. However, after reduction with 2-mercaptoethanol, the larger band could never be entirely eliminated, thus indicating the presence of other non-active proteins.

No separation of the different HL-A specificities could be obtained by any

one of the purification techniques described here, because the detergent/protein ratio used for the solubilization of the active molecules was relatively low (0 8 $10^{-6}$ mole of NP-40 per mg of protein). We have already reported that only a higher detergent/protein ratio ($\geqslant$ 4 $10^{-6}$ mole of NP-40 per mg of protein) allowed the separation of the HL-A specificities by electrofocusing [3], and furthermore, using the indirect immune precipitation method described by Schwartz et al [1], we could separate the different HL-A specificities, when the crude extracts were obtained by a high detergent concentration [11]

Nevertheless, we decided to use in the present study a low detergent/protein ratio for the preparation of the soluble crude extracts. Under these conditions the characterization of the active molecules, during the different purification steps, by the inhibition of lymphocytotoxicity tests, was much easier. Also the chief object of the present work was to compare the recovery of antigenic active molecules using different techniques for the purification of detergent solubilized alloantigens

ACKNOWLEDGEMENTS

This research was supported in part by the I N S E R M (Research Groups U93 and U116), the C N R S (ER102) and the D G R S T (Action concertée "Immunologie de la Transplantation")

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