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α-Haemolysin from *E. coli*

Purification and self-aggregation properties

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An improved, straightforward purification procedure for *E. coli* α-haemolysin has been developed. The protein exists in the form of large aggregates, held together mainly by hydrophobic forces. In the presence of urea or other chaotropic agents, the size of the aggregates decreases, while the specific activity is increased.

α-Haemolysin; Membrane lysis; Amphitropic protein; *E. coli*

1. INTRODUCTION

E. coli α-haemolysin appears to be related to the pathogenic properties of some strains of this microorganism, being responsible for extraintestinal diseases such as infections of the human genito-urinary tract, meningitis, peritonitis, etc. [1,2]. This toxin is also interesting from the point of view of biotechnology, since it is one of the few proteins extracellularly excreted by *E. coli*; therefore it can be used in the cell export of other gene products in the form of chimeric proteins [3]. α-Haemolysin is produced in an active form during the exponential growth phase of the bacterium [4,5].

α-Haemolysin purification has found a number of difficulties in the past: low haemolysin concentrations in the growth media, protein instability, etc. González-Carreró et al. [6] proposed a purification procedure, and found that 6 M urea greatly increases the stability of α-haemolysin preparations. This paper summarizes a series of experiments aimed at: (i) finding an improved purification procedure, starting from the data by González-Carreró et al. [6]; and (ii) describing some relevant properties of α-haemolysin.

2. MATERIALS AND METHODS

Strain RRIΔM 15 of *E. coli* K 12 was the host for the plasmid used in this work. The recombinant plasmid pSU 124, containing the *hly* genes, was obtained by cloning the *hly* determinant, from the *Eco*RI site at coordinate 9.5 kb to the *bgl*II site at 20.1 kb in the map by Zabala et al. [7], in the *Eco*RI-*Bam*HI sites of vector PUC 8 [8].

E. coli (pSU 124) was grown on LB medium [10] in a shaking incubator, at 37°C, to an $A_{550} \approx 1.0$. Cells were collected by centrifugation (13 000 × *g*, 20 min, 4°C), and the culture supernatants filtered through 0.45 μm Durapore HVP nitrocellulose filters. Solid ammonium sulphate was added to the filtrates to give 55% saturation, with stirring (1 h, 4°C), and the precipitate collected by centrifugation (30 000 × *g*, 15 min, 4°C). It was then redissolved in a small volume of TCU buffer (150 mM NaCl, 6 M urea, 20 mM Tris-HCl, pH 7) [6]. The redissolved pellet was applied to a Sephacryl S-500 column (90 × 2.2 cm), equilibrated and eluted with TCU buffer at 20 ml/h; 6 ml fractions were collected and tested for protein [11], and haemolytic activity [9]. Haemolytic activity (in haemolytic units (HU) · ml^{-1}) is defined as the dilution of α-haemolysin preparation producing 50% lysis of the erythrocyte suspension. Active fractions were pooled, dialyzed when appropriate and concentrated by ultrafiltration through CX-10 Millipore immersible filters. Dialyzed samples contained less than 5 mM urea. SDS-PAGE was carried out with the Pharmacia Phastsystem (Separation File No. 110). The gels (10–15% polyacrylamide gradient) were developed using the silver stain, modified for the Phastsystem procedure.

Proteins were assayed according to Bradford [11] and sugars, by the phenol/sulphuric acid method [12]. Lipids were extracted [13] and lipid phosphorus determined after Bartlett [14]. Fatty acid analysis was performed by gas-liquid chromatography [15]. 2-Keto-3-deoxyoctonate (KDO) was determined colorimetrically by the thiobarbituric acid procedure [16]. Size distributions of particles were determined by photon correlation spectroscopy, also called quasi-elastic light scattering [17], using an autosizer IIC (Malvern Instruments, Malvern, UK), coupled to a Malvern 7032-N multibit correlator. In these measurements, a polydispersity parameter is computed that may vary from 0 (homogeneous sample) to 1 (complete heterogeneity). Steady-state light scattering from haemolysin-containing samples was measured at 90° using a RF 540 Shimadzu spectrofluorimeter with both excitation and emission monochromators adjusted at 500 nm.

Dialyzed haemolysin preparations were treated with a variety of hydrolytic enzymes in a medium containing 150 mM NaCl, 20 mM Tris-HCl, pH 7. Phospholipase C from *Bacillus cereus* and egg-white lysozyme were purchased from Boehringer; trypsin from bovine pancreas was supplied by Sigma. Haemolysin preparations were incubated for 10 min at 37°C (phospholipase C), 15 min at 25°C (lysozyme), or 15 min at 37°C (trypsin).

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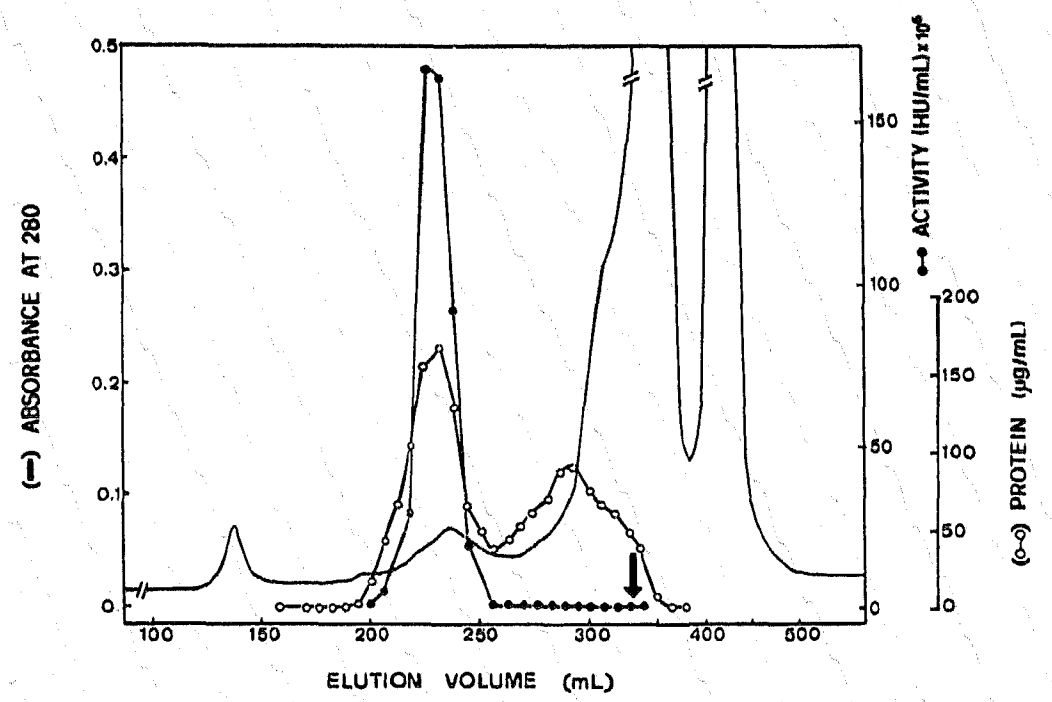

Fig. 1. Elution profile of a representative run in the Sephacryl S-500 column. Continuous line: absorbance at 280 nm; (o) protein. (•) α-haemolysin activity. After elution of about 300 ml (arrow) flow was increased to 0.6 ml/min.

3. RESULTS

The data on α-haemolysin purification are summarized in Figs 1 and 2, and Table I. The various purification steps are shown in Table I; a purification of 251-fold has been achieved. The apparent yield is much higher than 100%, apparently because of activation effects during purification. Fig. 1 shows a representative example of the Sephacryl S-500 column elution; within the limits of the technique, protein contents and haemolytic activity present coincident, symmetric peaks, also corresponding to a peak in A_{280} of the column eluate. (The two large peaks of A_{280} at higher elution volumes correspond to pigments in the culture medium.) A silver-stained SDS gel is represented in Fig. 2: polypeptides from the culture filtrate and the purified protein are shown, together with molecular weight markers. It is apparent that a single polypeptide, corresponding to α-haemolysin activity and with $M_r \approx 110\,000$, has been obtained with a remarkable degree of purity.

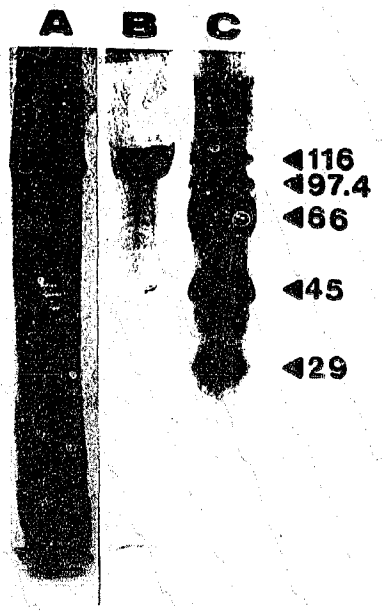

Fig. 2. SDS-PAGE of crude and purified α-haemolysin. (A) Crude filtrate; (B) Purified α-haemolysin; (C) molecular weight markers. Silver stain.

Our α-haemolysin preparation, as obtained from the gel filtration column, consists essentially of sugar (19.6 dry wt%) and protein (78.0 dry wt%). It also contains 0.85 μg lipid P/mg protein, and 2.3 μg 2-keto-3-deoxy-octonate (KDO)/mg protein (average values of two preparations). In accordance with the finding of KDO, a typical component of bacterial lipopolysaccharide [18], qualitative gas-liquid chromatography indicated that the main fatty acid in our preparation had a retention time compatible with 3-hydroxytetradecanoic acid, a fatty acid known to be associated to lipopolysaccharide [19].

The fact that our α-haemolysin preparation is excluded from Sephacryl S-200 solumns, whose exclusion limit for globular proteins corresponds to a $M \approx 500$ kDa, together with the data of SDS-PAGE electrophoresis and chemical composition, clearly suggest

Table I

Purification of α-haemolysin

Purification step	Volume (ml)	Protein (mg/ml) ($\times 10^{-3}$)	Activity (HU/ml)* ($\times 10^6$)	Total activity (HU) ($\times 10^6$)	Specific activity (HU/mg protein) ($\times 10^6$)	Purification (fold)	Yield (%)
13 000 × g supernatant	1300	7.7	0.032	42	4.15	1	100
Filtrate	1300	8.0	0.033	43	4.12	0.99	100
55% A.S.	6.5	1186	147	955	124	30	2273
Sephacryl	11.4	160	167	1904	1044	251	4533

*The haemolytic unit (HU) is defined under Materials and Methods

that this preparation consists of multimeric aggregates. Thus, we examined the particle size by photon correlation spectroscopy. An α-haemolysin preparation was concentrated by ultrafiltration to 0.8 mg protein/ml in TCU buffer; an average particle diameter of 194 nm (polydispersity 0.51) was found. Dialyzing against a urea-free buffer increased the average diameter to 301 nm (polydispersity 0.38) (average values of three measurements). In view of these observations, a more detailed study was carried out on the effect of urea and other chaotropic agents on the size and activity of our preparations. For reasons of convenience, absolute determinations of particle size were not carried out, instead steady-state 90° light-scattering was used to obtain semi-quantitative information on the changes in particle size. The effect of urea is shown in Fig. 3. Starting from a dialyzed, urea-free preparation containing 0.1 mg protein/ml, addition of urea up to 6 M produces an increase in haemolytic activity of more than 100-fold, while the scattering falls gradually to about one-half the original value. Guanidinium chloride, also known as a chaotropic agent, behaves very much like urea (not shown), increasing haemolytic activity and decreasing scattering, the latter suggesting a decrease in aggregate size. When a dialyzed α-haemolysin preparation (0.13 mg protein/ml) is treated with the non-ionic surfactant Triton X-100, which is known to effectively disperse aggregates held together by hydrophobic forces, the observed changes in light scattering suggest that 0.1% (v/v) Triton X-100 (about 1.6 mM) is as effective as 6 M urea in decreasing aggregate size, thus supporting the idea that hydrophobic forces are important in α-haemolysin aggregation.

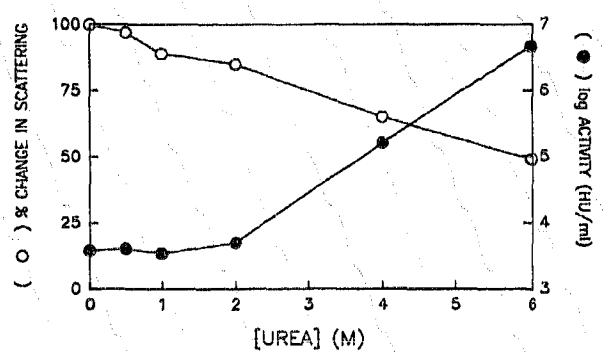

Fig. 3. The effect of urea on the size and activity of α-haemolysin aggregates. (○) Light-scattering from the protein suspension; 100% is the scattering in the absence of chaotropic agents. (●) α-Haemolysin activity.

Both the purified α-haemolysin preparation and the crude filtrate were treated with a series of hydrolytic enzymes, and their effect on haemolytic activity tested. Phospholipase C, at concentrations up to 150 units/ml had no effect whatsoever, and the same was true of lysozyme, at concentrations up to 500 units/ml. It should be stressed that those enzymes were tested with appropriate substrates under the same conditions of our experiment, and the expected activities were found. On the other hand, the pure enzymes, after being diluted under the same conditions as α-haemolysin, had no detectable haemolytic effect by themselves. Trypsin, however, was highly deleterious for α-haemolysin activity: incubation with 0.25% (w/w) for 15 min at 37° completely destroyed the activity, with a concomitant loss of the 110 kDa polypeptide.

4. DISCUSSION

Our α-haemolysin preparation is among the purest described up to now, judging from the silver-stained gels in Fig. 2. Only Wagner et al. [20] have published comparable gels. This demonstrates the important point that a single 110 kDa polypeptide makes up for the protein moiety of haemolysin, as suggested by previous studies [6,21] but not by the data of Bohach and Snyder [19]. With respect to the purification method by González-Carreró et al. [6], our procedure has the advantage of using a more efficient plasmid; α-haemolysin titres in supernatants from cultures containing the pSU 157 plasmid are of about 800 HU/ml, while, with the pSU 124 plasmid, the corresponding values are around 20 000 HU/ml. Our preparation has a considerably higher specific activity (about 10^9 HU/mg protein vs 5.4×10^6 HU/mg protein in their paper), and the protein concentration in the final eluate is four times higher in our case. Also, the use of a Sephacryl S-500 column, in which α-haemolysin aggregates penetrate, instead of the one used by González-Carreró et al. [6], from which the aggregates were excluded, improves the purity of our preparations. The yield of the purification procedure is much higher than 100%. Although the phenomenon remains unexplained

at present, it could be suggested that, in the last two steps of purification, either a conformational change occurs or an inhibitory component is lost, giving rise to the observed activation. Further experimentation should clarify this point.

There have been various suggestions that α-haemolysin is multimeric, or occurs in the form of aggregates [1,6,22]. Bohach and Snyder [19] propose that the aggregate size is greater than 60 nm, and that considerable heterogeneity in size occurs. Our photon correlation spectroscopic measurements provide support to both assumptions, with the additional significant observation that the average size changes with the presence or absence of urea. The possible relationship between activity and degree of aggregation was mentioned already by González-Carreró et al. [6] but specific experiments on this matter have not been presented until now. Our data (Fig. 3 and text) indicate that factors favouring disaggregation also increase the specific activity of α-haemolysin. These observations may explain the data of Wagner et al. [3] showing a lack of correlation between total extracellular α-haemolysin and haemolytic activity of the medium, and the suggestion by Bhakdi et al. [2,23] that α-haemolysin binds erythrocytes in monomeric form.

Various authors have pointed out that, since α-haemolysin activity is destroyed by trypsin, the protein moiety of the aggregates must be essential for haemolytic activity [1]. However, this paper describes for the first time the inhibitory action of trypsin on an α-haemolysin preparation whose protein component is only the 110 kDa polypeptide, thus relating directly this particular polypeptide to α-haemolysin activity. The role of non-protein components in haemolytic activity, suggested by several workers [19,20] is not sufficiently documented as yet.

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