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Purification of a Streptococcal deoxyribonuclease by affinity chromatography based on a DNA–cellulose matrix

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Abstract

An affinity chromatography method, using double stranded DNA, is described for the isolation of a deoxyribonuclease from the medicament Varidase, a wound cleansing preparation produced from the extracellular products of a species of Streptococcus. Using a complex buffer system, which includes EDTA, mercaptoethanol, bovine serum albumin and 50 m*M* NaCl, conditions are produced which allow binding of the nuclease to the chromatographic matrix without apparent degradation of the DNA. Increasing the NaCl concentration to 2 *M* results in the elution and isolation of a relatively non-specific endonuclease with a molecular mass of approximately 38 000 and a p*I* of 4.6. Experiments using ammonium sulphate for partial fractionation of the Varidase proteins, prior to chromatography, improved the yield but were not essential for obtaining a purified product. It is suggested that this technique should be readily applicable for the isolation of deoxyribonucleases from other sources. © 1997 Elsevier Science B.V.

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1. Introduction

Varidase streptodornase–streptokinase is a medicament derived from the refined extra cellular products of a Lancefield group C strain of Streptococcus, *Streptococcus equisimilis* [1] and is marketed in the United Kingdom as a topical agent for the cleansing of purulent wounds. Varidase topical contains in excess of 40 different protein species [2] which constitute 25–30% of the preparation [3]. Of these, the principal therapeutically active components are considered to be streptokinase, a plasminogen activator, and streptodornase, a term encompassing a protein or proteins with deoxyribonuclease activity.

These two active components have separate but mutually enhancing roles. Streptokinase activates plasminogen to form plasmin (which causes the breakdown of fibrin) hence resulting in a rapid dissolution of blood clots and the fibrinous portion of the wound exudate [4]. Streptodornase brings about the degradation of extracellular nucleoprotein found in purulent wounds [3]. The presence of this extracellular nucleoprotein causes leukocytes present in the wound environment to clump and this phenomenon, together with the presence of the fibrinous clot,

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results in poor wound drainage. Degradation of the extracellular DNA, by streptodornase, liquefies the pus allowing the free movement of leukocytes and enhanced phagocytosis, which leads to improved wound healing.

The mechanisms of action of streptokinase are well characterised by many authors [5–8], but little is known of the mechanisms of degradation of DNA by streptodornase or the possible effects of cations and the other constituent proteins within the Varidase preparation on its nuclease activity. As a step towards a greater understanding of the streptodornase activity within Varidase, we have attempted to purify the streptodornase component in order to allow further characterisation.

Affinity chromatography is a technique that finds use in purification regimes for many enzymes. Such protocols usually involve the immobilisation of a substrate analogue onto a stationary matrix using one of the established coupling reactions [9–13]. Whilst synthetic substrate analogues for enzymes that cleave DNA are available, the applicability of these analogues is often dependent on the specificity of the nuclease under investigation. As little was known as to the specificity of streptodornase, the use of such artificial DNA analogues seemed inappropriate and we therefore elected to use a relatively non-specific matrix based on native (double stranded) DNA coupled to a cellulose matrix.

Native DNA–cellulose was originally described independently by Alberts et al. [14] and Litman [15]. It has since been used by many authors for the purification of many diverse proteins including the purification of bacterial RNA polymerase [16], DNA helicases [17], histone proteins [18] and nuclear filament proteins [19]. It has also been employed during the purification of nuclear hormone receptors [20] and anti-DNA antibodies [21]. It has not however been used for the isolation of endonucleases that attack native DNA with little specificity, indeed Alberts and Herrick [22] recommend the use of pancreatic DNase I to remove the DNA and any non-elutable proteins from the cellulose matrix. This communication describes the technique and conditions whereby a DNA–cellulose matrix may be used for the rapid and specific purification of such a type I DNase, streptodornase.

2. Experimental

2.1. Varidase preparation.

The Varidase used in these studies was a sample of Lot G-39, produced by Cyanamid Iberica and kindly donated by Cyanamid of Great Britain (Gosport, Hampshire, UK).

2.2. Ammonium sulphate fractionation.

Prior to performing DNA–cellulose chromatography, ammonium sulphate fractionation was performed on the crude Varidase preparation. This fractionation was carried out by dissolving 500 mg of Varidase in 10 ml of distilled water and sufficient solid ammonium sulphate (AnalaR, BDH, Poole, UK) was added to give a 40% saturated solution. This mixture was then left to stir at 4°C for 6 h and then centrifuged at 15 000 *g* for 20 min at 4°C. Following centrifugation the precipitate was discarded and further ammonium sulphate added to the supernatant to achieve an 80% saturated solution. The resulting precipitate from this concentration of ammonium sulphate was re-suspended in 20 ml of distilled water, dialysed against distilled water to remove the ammonium sulphate, then lyophilised.

2.3. DNA–cellulose chromatography.

A DNA–cellulose matrix was produced using Sigmacell Type 50 cellulose (Sigma, Poole, UK) and calf thymus DNA D1501 (also Sigma). Before binding DNA to the matrix, the cellulose was pretreated in order to prevent non-specific adsorption of proteins to the column matrix during the chromatographic separation. This was achieved by first washing three times in boiling ethanol and then successive washes by filtration and re-suspension with 0.1 *M* NaOH (AnalaR, BDH), 1 m*M* Na_3EDTA (Sigma) and 10 m*M* HCl (AnalaR, BDH). Finally it was rinsed with distilled water until neutrality, or the normal pH of the distilled water was reached. Excess water was then decanted off and the moist cellulose lyophilised.

DNA–cellulose was then prepared according to the method of Alberts and Herrick [22]. Once

prepared, the resin was stored as a frozen slurry in pH 7.4 Tris–HCl buffer (10 m*M*) containing 1 m*M* Na_3EDTA until required. Prior to use, the slurry was allowed to thaw at room temperature and the Tris–EDTA buffer filtered off. The resin was then re-suspended in a running buffer (pH 8.1) composed of 50 m*M* NaCl (AnalaR, BDH), 1 m*M* Na_2EDTA (Sigma), 1 m*M* 2-mercaptoethanol (Sigma), 10% (w/w) glycerol (Evans Medical, Greenford, UK), 100 $\mu g\ ml^{-1}$ Bovine serum albumin (Sigma) and 20 m*M* Tris (AnalaR, BDH), and packed into a column 20 cm$\times$5.3 cm^2 giving a total bed volume of 100 ml. The resin was then equilibrated with several column volumes of the running buffer before use.

Following equilibration, a sample of the 80% ammonium sulphate precipitated material was loaded onto the column dissolved in the affinity running buffer. Any unbound material was then washed through the column with this running buffer at a flow-rate of 4 ml h^{-1}. After completion of the wash phase bound material was eluted using a buffer of the same composition as the running buffer but with a raised NaCl content (2 *M*). The eluate was collected into 1 ml fractions which were then analysed for nuclease activity. Active material was then pooled, lyophilised and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and isoelectric focusing (IEF) analysis (details in figure legends).

Fractions were assayed for nuclease activity by an automated technique previously developed in this laboratory using a schematic developed for the Technicon AutoAnalyser II system. The full details of this assay are published elsewhere [23]. Briefly, a sample of each fraction is blended with a substrate stream consisting of 0.004% (w/v) double-stranded DNA (Sigma) dissolved in pH 5.0 acetate buffer (0.05 *M*), containing 4.2 m*M* $MgSO_4$ (AnalaR, BDH) and 4.2 m*M* $CaCl_2$ (AVS, BDH). Brij-35 (Technicon Chemical Co., ORCQ, Belgium) was added to this solution at a concentration of 0.05% (v/v) and is necessary to promote the establishment of a good hydraulic pattern within the AutoAnalyser system. Following mixing by inversion, the substrate and sample are incubated for 3 min at 30°C after which the absorbance of the solution at 260 nm is measured. Nuclease activity results in an increase in the absorbance of the substrate solution at this wavelength.

3. Results

The initial ammonium sulphate fractionation serves to increase the relative streptodornase content within the material loaded onto the DNA–cellulose column. Initial precipitation at 40% results in the removal of several major protein species within the Varidase medicament, most notably streptokinase (marked SK in Fig. 2), with little reduction in the streptodornase activity present. The subsequent increase to an 80% saturated solution results in the precipitation of all the streptodornase as no further activity can be detected in the supernatant after this precipitation. This resulting precipitate is rich in the streptodornase protein (marked SD in Fig. 2) hence making it an ideal starting material for the chromatographic separation. The final 80% precipitate exhibits a relative streptodornase activity approximately five times that of the parent Varidase (as calculated by a manual streptodornase assay described elsewhere [23]).

When the lyophilised 80% precipitate is subjected to DNA–cellulose chromatography the majority of the protein species present wash through the column without binding. These proteins account for the first "wash" peak in the chromatogram shown in Fig. 1. This "wash" material does exhibit some streptodornase activity (as denoted by the broken line in Fig. 1) the presence of which is a result of intentional overloading of the column. If the "wash" material is recycled through the column following elution of the bound material then this "wash" activity decreases due to the streptodornase present binding onto the column (see insert in Fig. 1).

After the non-binding proteins have been removed by several washes of the column in the running buffer, this buffer is substituted for the affinity elution buffer (which contains 2 *M* NaCl). After the change to this eluting buffer a second protein peak may be seen and this peak exhibits a high level of relative streptodornase activity. SDS–PAGE (Fig. 2) and IEF (Fig. 3) analyses of this material show that

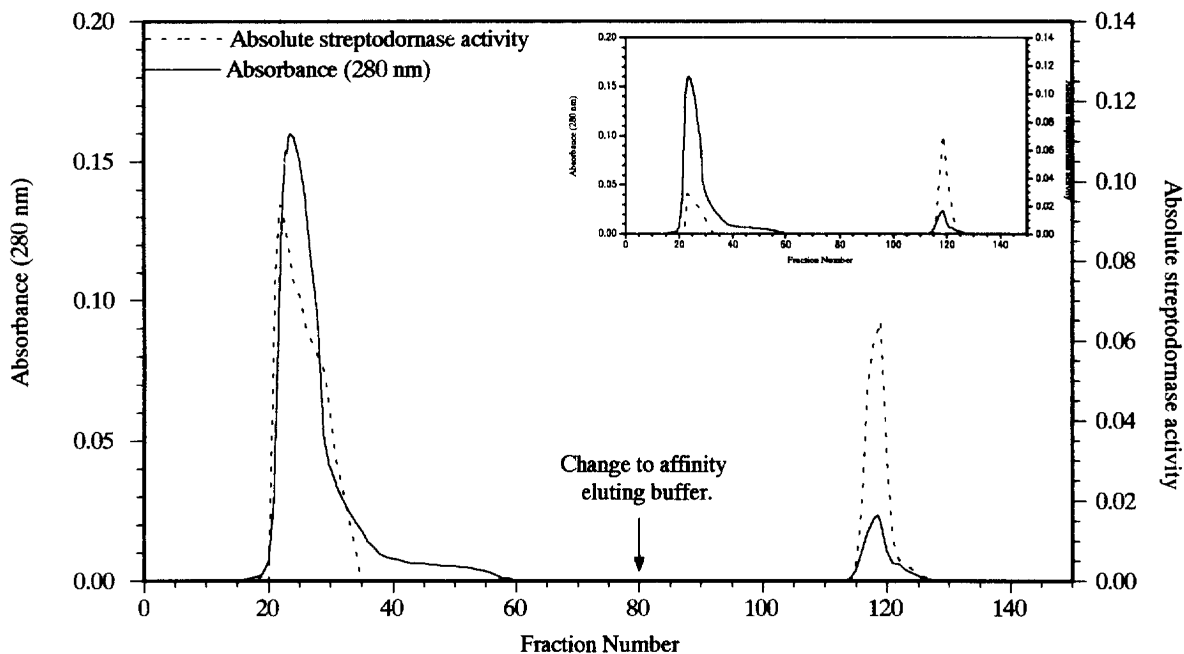

Fig. 1. Column elution profile for the affinity chromatography separation of an 80% ammonium sulphate precipitate of Varidase (Lot G-39), utilising a DNA–cellulose matrix and buffer system detailed in this report.

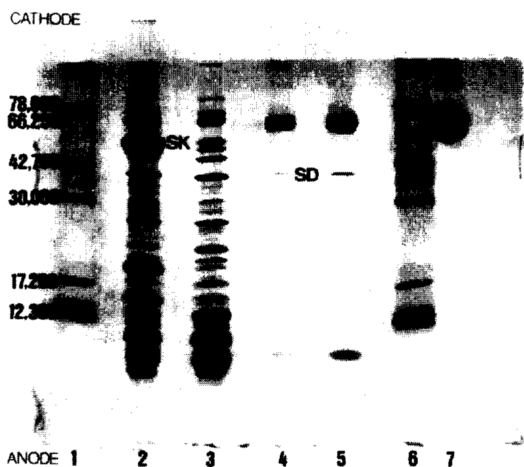

Fig. 2. SDS–PAGE analysis of affinity purified streptodornase isolated using the double-stranded DNA–cellulose matrix. SDS–PAGE gel: pH 8.8; T=15.6%, C=2.6%. Buffer: pH 8.8; 0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS. Running conditions: 2.5 h at 23 mA constant current. Sample loading: Lane 1, molecular mass markers; Lane 2, parent Varidase (Lot G-39); Lane 3, 80% $(NH_4)_2SO_4$ precipitate; Lane 4, affinity purified streptodornase; Lane 5, affinity purified streptodornase; Lane 6, molecular mass markers; Lane 7, bovine serum albumin.

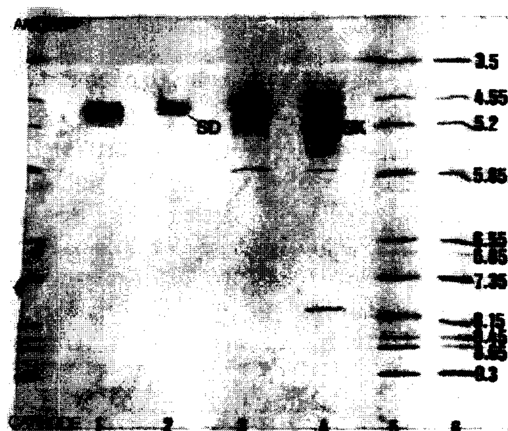

Fig. 3. IEF analysis of affinity purified streptodornase isolated using the double-stranded DNA–cellulose matrix. IEF gel= Ampholine PAG plate (pH 3.5–9.5). Running conditions: 2 h at 1500 V, 50 mA and 30 W. Sample loading: Lane 1, bovine serum albumin; Lane 2, affinity purified streptodornase; Lane 3, 80% $(NH_4)_2SO_4$ precipitate; Lane 4, parent Varidase (Lot G-39); Lane 5, iso-electric point markers; Lane 6, iso-electric point markers.

the streptodornase protein has been purified with a high degree of homogeneity. The SDS–PAGE analysis (Fig. 2) indicates that the purified streptodornase has a molecular mass of approximately 38 000. Using the marker proteins of known iso-electric point to calibrate the IEF gel shown in Fig. 3, we assign a p*I* of approximately 4.6 to the streptodornase protein.

4. Discussion

The process of DNA–cellulose chromatography has been successfully used here for the purification of a relatively non-specific DNA endonuclease in a convenient and rapid procedure. The process was facilitated by an initial ammonium sulphate fractionation as this produced a streptodornase enriched starting material. This stage is not essential and the process can also be carried out directly on the parent Varidase. If the ammonium sulphate fractionation is not performed however, the yield of purified streptodornase is reduced and so this pre-processing is recommended for preparative scale isolation of streptodornase.

When the purified material is analysed by SDS–PAGE (Fig. 2) three protein species are revealed in the purified material, the streptodornase protein of molecular mass 38 000 and two further species with molecular masses of approximately 63 000 and 8500.

The 63 000 molecular mass protein is also present in the lane corresponding to bovine serum albumin and is therefore present in the purified material as an artefact as it constitutes one of the components of the affinity buffer. This protein is also not present in lanes 2 and 3 which correspond to the parent Varidase and 80% precipitate, respectively. The presence of this albumin component should not be detrimental to the final product and it may serve to stabilise the purified streptodornase, indeed albumin is often added to protein drug preparations to act as a stabiliser. Kabikinase, for example, a streptokinase preparation marketed by Kabi-Pharmacia has albumin added in order to prolong the shelf life of the product. As bovine serum albumin has no endogenous nucleolytic activity, and is effectively inert, it will not affect any activity studies performed.

The relationship between the albumin and the streptodornase is an interesting phenomenon as they appear to be closely associated with each other. We were unable to separate the two species using a Sephacryl S100 gel filtration column (30 cm×0.64 cm^2) with a pH 7.4 phosphate buffer mobile phase (flow-rate 15 ml h^{-1}). This was somewhat unexpected as the two proteins have molecular masses which differ by approximately 25 000 and so would normally separate on such a system [24]. A possible explanation for this could be the formation of a complex between the two proteins which can only be dissociated by the conditions employed in electrophoresis. The presence of albumin does however appear to be necessary for the purification process as the streptodornase enzyme fails to bind to the DNA–cellulose matrix (and is eluted in the wash peak with no loss of activity) if the albumin is omitted from the running buffer.

The 8500 material may be a breakdown product of the streptodornase protein that has arisen during the processing. If the affinity chromatography active product is subjected to gel-filtration on Sephacryl S-100 HR, a small peak is visible after the elution volume of the streptodornase protein (data not shown). The protein present in this smaller peak does not exhibit nuclease activity and corresponds to a protein with a molecular mass of approximately 8500. Whilst this molecule does not exhibit nuclease activity, it is retained by the DNA–cellulose matrix, and as such may equate to a portion of the streptodornase molecule that does not contain the active site in its native conformation, but is still capable of binding to DNA.

IEF analysis (Fig. 3) compliments the SDS–PAGE analysis and shows three bands of very similar iso-electric points. Two of these bands are present in the albumin control lane and so may be attributed to the BSA. The third band is not present in the albumin and it is therefore believed that this band, with a p*I* of 4.6 equates to the streptodornase protein. Apart from the artefactual albumin bands it can be seen that the streptodornase is essentially homogenous with respect to its iso-electric point.

The buffer system employed in this chromatography procedure is formulated to create an environment that is unsuitable for type I DNase nucleolytic activity whilst still allowing the binding of the nuclease to its substrate. The buffer contains 50 m*M*

NaCl, a concentration that has been found to be inhibitory to the activity of streptodornase [2] along with 1 m*M* disodium EDTA which will effectively chelate any divalent cations present in the system, of which magnesium and calcium are activators of streptodornase [2]. 2-Mercaptoethanol is included in the running buffer primarily to prevent the possible formation of di-sulphide bond linked complexes but may also reduce streptodornase enzymatic activity. 20 m*M* Tris maintains the buffer pH at 8.1 which is the pH at which streptodornase exhibits greatest DNase activity [2] and hence is likely to be in the most favourable structural form to bind to DNA. It will be unable to cleave the DNA however due to the inhibitory effects of NaCl and EDTA. The other buffer components, i.e. albumin and glycerol, have also been found to maximise binding of streptodornase to the DNA–cellulose, as the yield of the system is much reduced without their presence. The high salt content in the elution buffer serves to disrupt binding of the streptodornase to the DNA as a result of high ionic strength.

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