

Separation of a material in streptokinase-streptodornase which induces delayed skin reaction

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Streptokinase-streptodornase (SK-SD) is a mixture of proteins elaborated by β -hemolytic streptococci. Previous studies have documented several biologic activities of this mixture including: (1) activation of plasminogen by streptokinase and (2) eliciting delayed skin test reactions in a high percentage of adults with a gross and histologic appearance similar to that seen in the tuberculin reaction. In the present study, fractions of SK-SD responsible for plasminogen activation and for skin test antigenicity were separated by column chromatography on DEAE Sephadex. Peak I fractions elicited strongly positive skin tests but did not activate plasminogen. Peak III fractions activated plasminogen but were devoid of skin test reactivity. Therefore, we concluded that the protein mediating the delayed skin reaction has been separated from that with streptokinase activity.

In past studies employing streptokinase-streptodornase (SK-SD) (Vari-dase) in the treatment of thromboembolic disease, the occurrence of side reactions has been noted. The possibility of hypersensitivity as being a factor in these reactions is supported indirectly by the fact that a large majority of adults have been found to exhibit delayed skin test reactivity to crude SK-SD, with a temporal pattern and histologic appearance of the responses similar to those seen with tuberculin skin testing. Since this skin test reactivity could be transferred with blood leukocytes, most investigators have concluded that skin test reactivity to SK-SD reflects a specific cell-mediated immune response.

One of the key questions arising from these studies was the identity of the material in the SK-SD mixture which mediates this phenomenon. In particular it seemed important to determine: (1) whether it was possible to isolate this active material and (2) whether it was identical to streptokinase (i.e., that material in the SK-SD mixture which activates human plasminogen). This paper confirms that a delayed-reacting substance (DSR) is present in the crude SK-SD preparation and demonstrates that it is separable from SK.

MATERIAL AND METHODS

In order to isolate the DSR from SK-SD it was necessary to establish a procedure for preparing samples and fractions and for assaying delayed skin reactivity as well as capacity to activate plasminogen.

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Supported in part by Research Grant No. 5 RO1 HE 10907 and Training Grant No. 5 TO1 AI 00319, From the National Institutes of Health.

Received for publication Feb. 26, 1971.

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Preparation of materials to be assayed

The concentration of starting material, SK-SD (Varidase), and the various fractions obtained during purification is expressed in terms of units at optical density 280 (OD_{280}). No attempt was made to accurately estimate the extinction coefficient of the starting materials being tested because the extinction coefficient of one protein would vary from that of another. One to 5 ml. aliquots of stock SK-SD and all fractions obtained in purification were dialyzed for 18 hours at 4° C. against 2,000 ml. volumes of H_2O . The OD_{280} of the stock and of each fraction was determined, and each sample to be tested was then stored at -20° C. until such time that the starting material and all fractions could be tested simultaneously. Following this, each sample was thawed; and working from the OD_{280} of these thawed samples each sample was then diluted such that the final OD_{280} would be 1×10^{-2} , 1×10^{-3} , 1×10^{-4} , and 1×10^{-5} . This means of handling the samples gave assurance that equivalent amounts of each fraction were used in the studies in which we were comparing their activities.

We also estimated the amount of protein and units of SK activity in the SK-SD starting material and related it to the OD_{280} , which we used as our principle means for adjusting protein concentrations. Thus, the number of micrograms of protein and SK units in a 1×10^{-3} OD_{280} aliquot of starting material (SK-SD) averaged 0.5 μg of protein per milliliter and 15 SK units per milliliter, respectively. The amount of protein in this case was estimated from the protein nitrogen as determined by the micro-Kjeldahl method and assuming that the protein mixture SK-SD had an average of 14 per cent nitrogen by weight.² Again it should be noted that we are dealing with a mixture of proteins (each with its own extinction coefficient and per cent nitrogen) from which a more purified protein is being prepared. Thus, the application of OD_{280} or per cent nitrogen as an estimate of the amount of protein present across all the steps in purification represents an approximation with an error which could be as high as ± 20 per cent. These aliquots plus the starting material were then sterilized by passing through a Millipore Swinney (0.45 μ) filter and stored in sterile rubber stopper vials at 4° C.

The starting material and fractions were then assayed for their capacity to elicit a delayed skin reaction in a sensitive individual and to activate plasminogen.

Assay of delayed skin reaction

The panel of test subjects consisted of 7 normal male volunteers 32 to 45 years old. When the starting material and the fractions from various steps of the purification procedure had been accumulated, the entire series (up to 8 samples) were tested on the upper and lower arms of 4 to 6 subjects, and the reactions were read and compared at 24 and 48 hours. This was done by the intradermal injection of 0.05 ml. of a given dilution (i.e., 1×10^{-5} OD_{280} units) of the material prepared as described above. The reactions were then read by scoring the diameter of the area of induration after 8, 24, and 48 hours. A reaction had to be 5 mm. or more to be scored as positive. The scores for a given fraction among the subjects were totaled and scored as so many positives of the number of subjects tested (i.e., 6/7 tested, Table I).

As expected, there were differences in sensitivity of individuals to the test material. All, however, reacted to the most concentrated aliquot tested (1×10^{-3} OD_{280} units). There appeared to be little or no enhancement or inhibition of individual reactivity by repeated testing.

Assay of streptokinase activity

Again, as in the case of skin testing, the concentrations of all fractions tested were equalized. In this case, 0.2 ml. aliquots of the SK-SD starting material and fractions from column chromatography (i.e., 1×10^{-1} OD_{280} units or 500 SK units per milliliter) were added to 0.5 ml. of 0.08M lysine methyl ester (LMe) and 0.5 ml. (0.4 mg.) of plasminogen.² The specific activity of the plasminogen (Cutter lot No. 660-40) was 10 to 12 casein units per milligram of protein. The samples were incubated at 37° C. for 30 minutes. The reaction was stopped, and the esterase activity was determined as described by Taylor and Botts.²

TABLE I. Assay of the capacity of various fractions of SK-SD eluted from DEAE Sephadex A-50 to induce a delayed skin reaction and to activate plasminogen (lysine methyl esterase activity)

Assay	Subjects	SK-SD starting material	Fraction No.				
			18	36	46	61	77
Delayed skin reaction (mm. induration) induced by 0.05 ml. of 1×10^{-4} OD units/ml. of test material	1 2 3 4 5 6 7	4 0 0 5 0 0 3	20 3 7 10 8 14 10	10 0 4 5 2 5 5	15 0 8 12 7 5 8	10 0 9 15 3 3 5	3 0 0 5 0 0 0
Summary of skin test results		1/7	6/7	4/7	6/7	4/7	1/7
Lysine methyl ester activity Δ OD/30 min.		260	0	0	368	368	373

Chromatographic procedures

Approximately 150 to 200 mg. of SK-SD starting material in 10 ml. of 0.008M Tris-0.15M NaCl, pH 8.5, was dialyzed extensively against 2 changes of 2,000 ml. of the same buffer at 4° C. to remove salts and chromogens. This material was then adsorbed into a 2.5 $\times$ 30 cm. column of DEAE Sephadex A-50 which had been previously equilibrated with 0.008M Tris-0.15M NaCl buffer, pH 8.5. The proteins were then eluted at 4° C. from the column by applying a linear salt gradient (200 ml. of 0.15M NaCl to 200 ml. of 0.40M NaCl). The fractions were collected in 2.4 ml. aliquots. Selected fractions at OD₂₈₀, 1×10^{-3} , 1×10^{-4} , 1×10^{-5} were then examined as described above for: (1) capacity to induce a delayed skin reaction, (2) capacity to activate plasminogen, and (3) heterogeneity of 0.2 ml. quantities of each of the above fractions by electrophoresis on analytic acrylamide gel across a discontinuous pH gradient.³

RESULTS

Fig. 1, A shows the pattern of protein eluted from DEAE Sephadex A-50 together with the capacity of each of the fractions tested to induce a delayed skin response. Maximal skin reactivity was elicited by material from Peak I. Peak III had almost none. The protein patterns as demonstrated on acrylamide gel of each of the fractions tested are also shown at the top of Fig. 1, A. Peak I, which had the maximum reactivity on skin, contained one major slowly migrating band plus several minor bands. Peak II contained a number of bands including those seen in the first peak. No slowly migrating bands were identified on the polyacrylamide gel of Peak III.

Fig. 1, B shows the same protein elution pattern from DEAE-Sephadex A-50 as shown in Fig. 1, A. However, SK activity is plotted in place of skin reactivity. In contrast to the reactivity in skin, the SK activity is very low in Peak I and high in Peaks II and III. Thus, Fig. 1, A and B, demonstrates that the protein responsible for DSR of SK-SD (Varidase) is separable from that responsible for activating plasminogen.

Table I lists in more detail the subjects tested, the plasminogen activator activity (SK), the delayed skin reaction elicited by 1×10^{-4} OD unit per milliliter

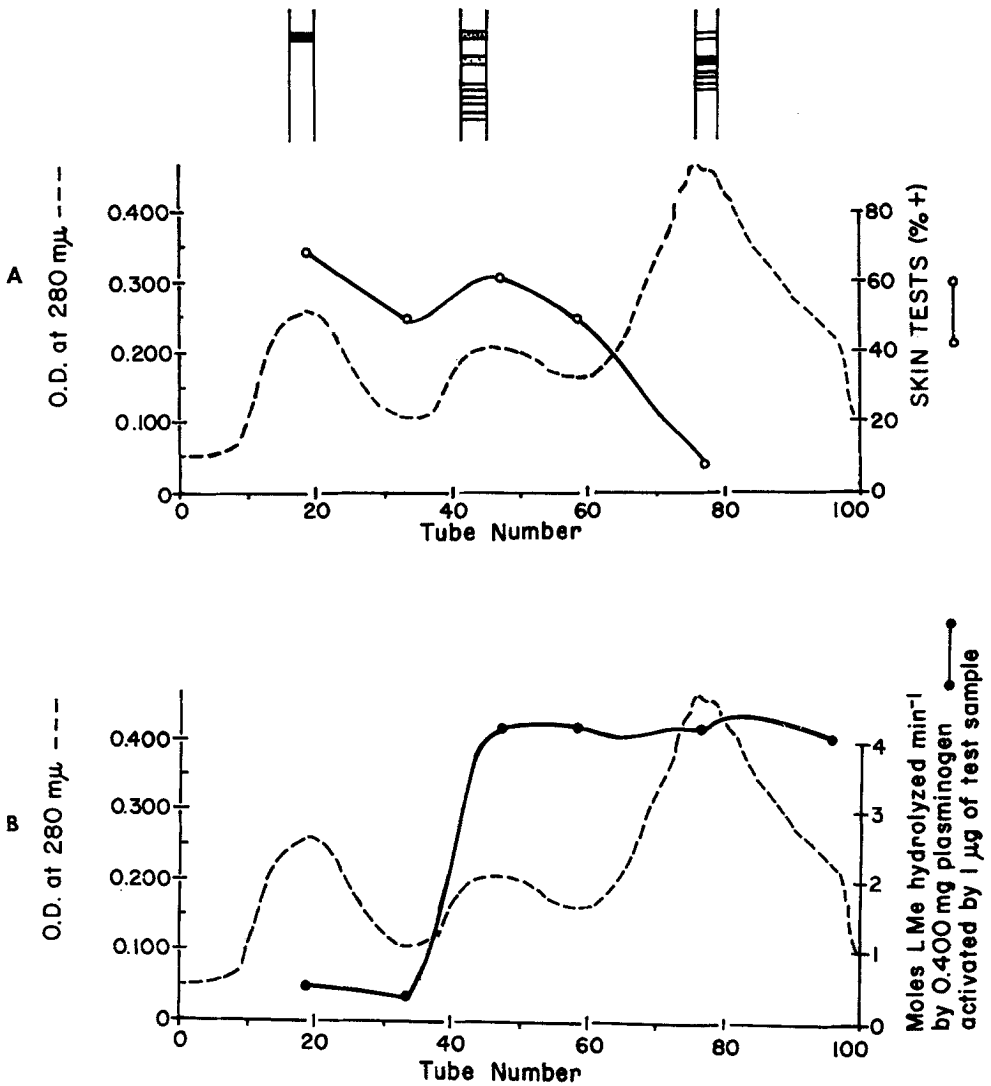


FIG. 1. A, Protein elution pattern of SK-SD eluted from DEAE Sephadex A-50 (----) together with the pattern of the delayed skin reactivity of certain fractions and of the starting material (SK-SD) (—). The protein patterns as observed following electrophoresis on acrylamide gel are shown above each peak. **B,** Protein elution pattern of SK-SD eluted from DEAE Sephadex A-50 (----) together with the pattern of activity (lysine methyl ester, **LMe**, activity) on human plasminogen of certain fractions and of the starting material (—).

of concentrations of the SK-SD starting material, and, by the same concentration, each of the various fractions.

DISCUSSION

SK-SD has been accepted as an antigen which elicits a specific cell-mediated response by most investigators and as such is used to help assess cellular im-

immune function in clinical practice. This acceptance has been based on the work of Lawrence,¹ who demonstrated that skin reactivity of this material could be transferred by blood leukocytes and that an increasing number of children become sensitive to SK-SD with increasing age. The nature of the active material in SK-SD has heretofore not been described.

This communication clearly shows that this active material (DSR) can be separated from SK and that streptokinase does not elicit delayed skin reactions. Thus, it may be feasible to prepare a streptokinase product with no DSR and thereby possibly reduce the side effects frequently associated with studies in which the efficacy of SK as a thrombolytic agent is being investigated.

At the same time, based on the work of Lawrence and on the above studies, it is believed that the active material (DSR) which we have separated from SK is acting through a cellular immune mechanism. Therefore, it should be possible to further purify and characterize DSR and use it both as a laboratory and clinical test in further studies of cellular immune reactions. These studies might also reveal a useful approach to studies of other organisms to which delayed hypersensitivity in human beings is thought to exist.

We wish to acknowledge the excellent technical assistance of Mrs. Helen Ingham, Gloria Heym, and Joanne Beisswenger.

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