Reports

Purification of Retinal S-Antigen to Homogeneity by the Criterion of Gel Electrophoresis Silver Staining

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This report describes a procedure by which high performance liquid chromatographic (HPLC) techniques are used to obtain homogeneous S-antigen. Conventionally prepared S-antigen was purified further by fractionation on the anion exchange Mono-Q column followed by gel filtration on a TSK-3000 column. This procedure produced an S-antigen preparation, which appeared homogeneous by gel electrophoresis using the very sensitive silver staining method. The purified material retained its immunochemical and uveitogenic activity. The availability of homogeneous S-antigen will facilitate studies aimed at elucidating, at the molecular level, the mechanism by which S-antigen induces uveitis. Invest Ophthalmol Vis Sci 25:977-980, 1984

Retinal S-antigen is a soluble protein of about 50,000 daltons, which is associated with photoreceptor outer segments and which has a potent autoantigenic activity. Since its first isolation by Wacker et al, the S-antigen has received much attention because of its ability to induce, in a variety of mammals, an ocular inflammatory condition known as experimental autoimmune uveitis (EAU). The pathogenesis of EAU is of interest clinically as a model system for posterior uveitis; several extensive reviews of the subject are available (eg, ref 2, 3).

S-antigen for induction of EAU generally has been prepared from bovine retinas by a procedure involving ammonium sulfate precipitation and gel filtration followed by ion exchange chromatography or isoelectric focusing. More recently Dorey et al have described a new purification scheme utilizing hydroxyapatite chromatography, which is simpler and faster than the previous methods and which produces samples of similar purity. While the S-antigen purified by these procedures is highly enriched, it can be shown that a number of minor contaminating proteins remain in the preparations. These minor components are of little significance when the S-antigen is being used simply for induction of the disease process in experimental animals. However, it would be essential to have homogeneous preparations of S-antigen for use in studies aimed at determining the identity of S-antigen or elucidating, at the molecular level, the mechanism of its pathogenicity. We report here a procedure in which conventionally isolated S-antigen is further purified to homogeneity using high performance liquid chromatography (HPLC) techniques.

Materials and Methods. Retinal tissue was obtained from fresh bovine eyes. The initial isolation of S-antigen was by the method of Dorey et al. Following ammonium sulfate precipitation at 50% saturation, gel filtration was performed on a 2.6 X 90 cm column of Ultrogel AcA 34 (LKB Instruments, Inc.) followed by adsorption chromatography on HA-Ultrogel (LKB). Dorey et al used either Sephadex G-200 or Ultrogel AcA 34 for the gel filtration step but recommended the latter gel due to its higher flow rate and lower nonspecific adsorption of proteins in the low ionic strength buffer employed. The S-antigen containing fractions were concentrated to about 1 mg protein/ml by negative pressure dialysis at 4°C using a 35% solution of polyethylene glycol compound (Sigma Chemical Co.). Samples to be further purified by HPLC were dialyzed against 10 mM Tris-HCl buffer, pH 8.4 and then applied to a Mono-Q anion exchange column (Pharmacia) fitted to the Pharmacia Fast Protein Liquid Chromatography (FPLC) system. The protein was eluted at room temperature using a gradient of 0 to 0.5 M NaCl in starting buffer. The S-antigen peak, which eluted at about 0.25 M NaCl, was identified by gel electrophoresis and immunodiffusion against polyclonal antiserum to S-antigen. Aliquots of this material were applied subsequently, without dialysis, to a 7.5 X 600 mm Ultropak TSK-G3000 SW gel filtration column (LKB) fitted onto an LKB HPLC system. The column was equilibrated with a phosphate buffer, pH 7.1, which contained 0.1 M KHPO4, 0.1 M KCl, 1 mM dithiothreitol, 1 mM EDTA and 20% glycerol and was eluted at room temperature at a flow rate of 0.5 ml/min. Samples of purified S-antigen to be used for immunization were dialyzed against phosphate-buffered saline at 4°C and concentrated as described above. Immunization of Lewis rats was as previously described. In brief, the tested preparations were emul-
Fig. 1. SDS-polyacrylamide gel patterns obtained for S-antigen at different stages of purification. Lane 1 has protein molecular weight standards (Pharmacia). Lanes 2 and 5 were each loaded with approximately 6 ng of conventionally prepared S-antigen and stained with Coomassie blue R and silver stain, respectively. Lanes 3 and 6 were loaded with equal amounts of S-antigen further purified on the Mono-Q anion exchange column. Lane 3 was stained with Coomassie blue and lane 6 with silver stain. Lanes 4 and 7 were loaded with the S-antigen, which was purified on both the Mono Q and TSK-3000 columns. Lane 4 was Coomassie blue-stained and lane 7 was silver stained. The 1 mM thick gel consisted of a 15% separating gel and a 4.5% stacking gel. Electrophoresis was for 16 hr at 45V. All samples were in 1% SDS and 1% 2-mercaptoethanol and were incubated for 20 sec in a boiling water bath before application to the gel.

Fig. 2. Elution pattern, as measured by absorbance at 280 nm, for fractionation of the conventionally prepared S-antigen on the Mono-Q (Pharmacia) anion exchange column. Both the sample and the column were equilibrated with 10 mM Tris-HCl buffer, pH 8.4. The protein was eluted with a gradient of NaCl (0 to 0.5 M) in starting buffer at a flow rate of 0.5 ml/min.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) was performed on 15% slab gels by the method of Laemmlli. Gels were stained with Coomassie blue or with the Bio-Rad Silver Stain. Protein determinations were by the method of Lowry et al. Rocket immunoelectrophoresis was performed according to the method of Laurell using an LKB Multiphor system. The agarose gels contained 1.5% rabbit antiserum against bovine S-antigen. HPLC purified S-antigen was used as standard for quantitation. A concentration range of from 50 to 300 ng of S-antigen per well was found to be suitable for quantitative work.

Results. Figure 1, lane 2, shows the pattern obtained on an SDS-PAGE slab gel for S-antigen prepared by the method of Dorey et al after staining with Coomassie blue. A single major band (~50 kd) is evident with several faint bands visible when the gel is relatively heavily loaded. The presence of these minor contaminants becomes much more evident when an aliquot of the same preparation, run on the same gel, is stained by the more sensitive silver staining technique (Fig. 1, lane 5). It is evident that at least 20–25 minor bands are present ranging in molecular weight from 15,000 to over 100,000. Quantitative analysis of the S-antigen content of this preparation using rocket immunoelectrophoresis indicated that 82% of the protein was S-antigen, which is virtually identical to the value reported by Dorey et al for their preparations.

To further purify the S-antigen, the material described above was dialyzed against 10 mM Tris-HCl, pH 8.4, and applied to a Mono-Q anion exchange column. In general, 2–3 mg of protein was applied in a volume of several milliliters, however, much greater volumes can be applied. The protein was eluted at 0.5 ml/min by a gradient of NaCl (0 to 0.5 M). Figure 2 shows the elution pattern, which was consistently obtained. The major peak eluting at about 14 ml was identified as S-antigen by immunodiffusion against rabbit antiserum against S-antigen. Figure 1, lane 3 shows the Coomassie blue stained electrophoretic pattern for the material from this peak. While it appears homogeneous, staining with silver (lane 6) indicated that traces of several contaminants remained. Components with molecular weights near 26,000, 40,000, and 75,000

*Signified in complete Freund's adjuvant and injected in 0.1-ml aliquots into one hind footpad of the rats. Simultaneously with the immunization, the rats were injected intraperitoneally with Bordetella pertussis bacteria, 10^10 per rat. The rat eyes were examined for clinical and histological changes as described elsewhere. All studies utilizing experimental animals conformed with the ARVO Resolution on the Use of Animals in Research.*
were the primary contaminating species that remained. To remove these components, aliquots of the pooled S-antigen-containing fractions from the Mono-Q column were applied directly to a TSK-3000 column for final purification by gel filtration. It was found that adequate resolution could be obtained by using 0.5 ml samples with a flow rate of 0.5 ml/min. Figure 3 shows the elution pattern obtained. Only one protein peak was evident. That peak, which elutes at about 19 ml contained the S-antigen immunoreactivity. The material now appears homogeneous on SDS-PAGE even when stained with the sensitive silver technique (Fig. 1, lane 7). The fluctuation seen in the TSK-3000 elution pattern at 24–26 ml is at the column volume and results from the fact that the sample was loaded in a buffer different from that with which the column was equilibrated. There was no detectable protein eluting in these fractions.

Table 1 summarizes the purification of a typical 1.5 mg sample of S-antigen on the HPLC columns.

The homogeneous preparations of S-antigen were found to be highly uveitogenic: a single dose of as little as 7 μg of such a preparation produced severe clinical and histologic EAU within 13 days after immunization. It is of note that the homogeneous preparations of S-antigen were superior in their uveitogenicity to the conventionally isolated preparations.

**Discussion.** The procedure described above is the first method developed that will produce preparations of retinal S-antigen that are homogeneous by the criterion of the highly sensitive silver stain. Starting with a sample of conventionally isolated S-antigen, both HPLC chromatographic steps can be completed within 2 hours.

Nearly 20% of the protein in the conventionally prepared material is not S-antigen. Since this contaminating material is divided among 20–30 distinct species on SDS gel, most of the individual bands are not readily apparent on Coomassie blue-stained gels. The extent of the contamination only becomes apparent upon silver staining. Elimination of most contaminants is effected by HPLC on the Mono-Q ion exchange column. The S-antigen elutes from this column mainly in a single peak although small amounts of S-antigen also can be detected in the eluate for about 2 ml before the main peak and also for about 2 ml after the main peak. The very sharp peak occurring at about 10 ml contains the ~15,000 dalton component seen on the SDS-PAGE as well as the two bands migrating just above S-antigen (Fig. 1, lane 5), but no S-antigen. The Mono-Q column completely removes the major contaminating band immediately below S-antigen on the SDS gel; this species is not separated from S-antigen by the TSK-3000 column. Thus, to achieve complete purification both columns are needed.

It is of interest to note that following fractionation on either of the HPLC columns, the S-antigen becomes susceptible to aggregation upon heating in SDS. The samples used in Figure 1 were heated at 100°C for only 20 sec to avoid such aggregation, however if samples are heated for longer times, there is a progressive accumulation of apparent dimers and higher oligomers on the SDS gel (not shown).

The S-antigen purified by this procedure gives a strong reaction in immunodiffusion or immunoelectrophoresis with rabbit polyclonal anti-S-antigen serum. Additionally, the material also retains its uveitogenic activity since injection of purified samples into Lewis rats induced uveitis following single doses as low as 7 μg. Thus, for the first time, we can, with

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<th>Table 1. HPLC purification of S-antigen</th>
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<td>Total protein</td>
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Total protein analyses were by the method of Lowry et al. S-antigen was quantitated by rocket immunoelectrophoresis using HPLC purified material as standard. The sample applied to the TSK-3000 column was an aliquot of the pooled S-antigen-containing peak from the Mono-Q fractionation.
VEPs in Humans Reveal High and Low Spatial Contrast Mechanisms

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The effect of contrast on visual evoked potential (VEP) amplitude was examined in nine observers. A 6.0 cycles/deg (cpd) grating was modulated in an “on-off” mode at 7.5 Hz. The VEP response contains significant first and second harmonic components: their growth with contrast is parallel, each function consisting of two limbs. The data are consistent with the hypothesis that the pattern VEP obtained with “on-off” presentation may reflect the contributions of “low” and “high” contrast neuronal populations demonstrated in physiological studies of the primate. Invest Ophthalmol Vis Sci 25:980–983, 1984

Both psychophysical and visual evoked potential (VEP) methods have been used to investigate the spatial contrast properties of the human visual system. There is a good correlation between the VEP and psychophysical contrast sensitivity when VEP threshold is defined by measuring VEP amplitude as a function of grating contrast and extrapolating to zero amplitude.1,2 There is a difference, however, in the shape of the suprathreshold contrast functions described with psychophysical and VEP measurements. Psychophysically, the relationship between physical and perceived contrast is linear.3 Double-slope functions, however, re-

References


