Preparation of a Uveitogenic Peptide by Chymotryptic Digestion of Bovine S-Antigen

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Limited proteolysis of bovine S-antigen with α-chymotrypsin resulted in the accumulation of three peptides of MW 24,000, 16,000, and 12,000 daltons, respectively. By ELISA (enzyme-linked immunosorbent assay), MW 24,000 peptide was found to react with anti-S antibodies, but the other two peptides did not react with the antibodies under the assay conditions. The reactive peptide was separated from the smaller peptides by gel filtration on Sephadex G-75 and Sephadex G-50. When the MW 24,000 peptide was injected into Lewis rats, severe to mild uveitis was produced in all injected animals. The results indicate that the pathogenic determinant is on the MW 24,000 peptide.


Experimental allergic uveitis (EAU) is an autoimmune disease induced in various laboratory animals by injection of a soluble retinal antigen (S-antigen). The disease is characterized by inflammation of the uveal tract and loss of retinal photoreceptors.1–3 A bilateral uveitis induced in primates by the antigen shows characteristics resembling those of human uveitic diseases.4 Studies on serum levels of anti-S antibodies in uveitis patients suggest a possible involvement of S-antigen in certain types of human uveitis.5,6 Uveitis patients showed cell mediated responses, but not humoral responses, to S-antigen. With the exception of the pineal gland,7 the retina is the only tissue in which S-antigen is located. In the retina, the antigen has been localized to the visual photoreceptors by immunohistochemical techniques.3,8,9 Using monoclonal antibodies to bovine retinal S-antigen we have demonstrated that S-antigen is associated with rod photoreceptor outer segments.10 S-antigen has been purified to a homogeneous protein of MW about 50,000 daltons from the retina of several species including human retina.3,11,12 A recent paper reports chemical cleavage of bovine S-antigen into several peptide fragments by treatment with cyanogen bromide.13 The fragments possess antigenic reactivity toward anti-S-antigen serum but it is not known whether they are uveitogenic. In the present work, we have digested bovine S-antigen with α-chymotrypsin and separated a peptide (MW 24,000) which retains both antigenic and immunopathogenic activities.

Materials and Methods

Preparation of S-antigen

Bovine retinal S-antigen was prepared as described10 by a modification of Wacker’s method.11 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) of S-antigen and its proteolytic digests was carried out in 10 percent polyacrylamide gels (5.5 × 110 mm) using a continuous buffer system of 0.205 M Tris-acetate, pH 6.6, and 0.1 percent SDS, essentially following Wacker et al.11 For electrophoresis 30–50 μg sample was used per gel. Protein was determined by the method of Lowry et al.14

Digestion of S-antigen with α-Chymotrypsin

Fifty parts (wt) of purified S-antigen were mixed with one part (wt) of TLCK-treated bovine pancreas α-chymotrypsin (Sigma; St. Louis, MO) in 100 mM potassium phosphate buffer, pH 7.6, containing 0.1 percent polyethylene glycol 400 (PEG-400) and 0.02 percent sodium azide and the mixture was incubated at 37°C for a given length of time. The reaction was stopped by addition of phenylmethylsulfonyl fluoride (PMSF) in 2 percent ethanol (to a final concentration of 1 mM). For time-course experiments, the digest was dissolved in SDS buffer and loaded on polyacrylamide gel for electrophoresis. After electrophoresis,
the disk gel was sliced in segments of 4 mm in thickness, placed in a series of small tubes, extracted overnight at 4°C each with 0.5 ml of 50 mM sodium bicarbonate, pH 9.6, containing 0.02 percent NaN₃. The extracts were then subjected to ELISA. For purification of antigenic peptides, proteolysis was stopped after 6 hr of digestion and the digest was dialyzed against 50 mM ammonium bicarbonate for 24 hr at 3°C.

**Purification of Chymotryptic Peptide on Sephadex**

Dialyzed peptide sample (1–2 mg) was centrifuged at 80,000 × g for 30 min and the supernatant was applied to a Sephadex G-75 (Pharmacia; Piscataway, NJ, particle size = 40–120 μm) column (1.6 × 180 cm). The column was eluted with 50 mM ammonium bicarbonate to collect 2.5 ml fractions at a flow rate of 10 ml/hr. For determination of molecular weight, the column was calibrated with standard proteins [ovalbumin (45,000) carbonic anhydrase (29,000) and cytochrome C (12,400)] purchased from Sigma. ELISA-positive fractions from this column were pooled and frozen slowly at -20°C. The frozen sample was allowed to thaw at room temperature. The early crop of thaw contained enriched peptides. The peptide mixture (1–2 mg) thus concentrated was centrifuged at 80,000 × g for 30 min to remove aggregates and the clear supernatant was placed on a Sephadex G-50 (Pharmacia, particle size = 100–300 μm) column (1.6 × 94 cm). The column was eluted with 50 mM sodium bicarbonate to collect 1.2-ml fractions at a flow rate of 3.6 ml/hr.

**Enzyme-linked Immunosorbent Assay (ELISA)**

A 20-μl aliquot of gel extract was mixed with 180 μl of sodium bicarbonate and ELISA was carried out by a modification of the published procedures. In brief, the well of polystyrene microtitration plate (Dynatech; Alexandria, VA) was coated with sample, washed with 10 mM potassium phosphate, pH 7.0, containing 0.05 percent Tween 20 and 150 mM NaCl, and shaken dry. The protein adsorbed to the well surface was then reacted with rabbit anti S-antigen serum at 37°C for 2 hr, washed with phosphate buffer, and shaken dry. Finally, the well was reacted with peroxidase-labeled goat antirabbit IgG at 37°C for 90 min, washed, and then incubated with a mixture of 0.04 percent o-phenylenediamine and 0.01 percent H₂O₂ in 0.1 M citrate buffer, pH 5.0, at room temperature for 5 min. The reaction was stopped with 50 μl of 8 N H₂SO₄ and the optical density at 490 nm was determined with a Bio-Tek microplate reader Model EL 307 (Burlington, VT). Purified anti-S antibodies were used in some ELISA assays. Rabbit serum was saturated with ammonium sulfate to 40 percent at pH 6.5 and centrifuged. The precipitate was dissolved in 10 mM potassium phosphate, pH 7.5, dialyzed against the buffer, centrifuged and further purified by ion-exchange chromatography on DEAE-agarose. Anti-S antibodies were eluted at 0.02–0.05 M NaCl.

**Induction of Experimental Autoimmune Uveitis (EAU)**

Female albino rats (Lewis, 8–12 wk) were purchased from Harlan-Sprague-Dawley (Walkersville, MD). Two rats were injected with S-antigen and five rats were immunized with peptide. S-antigen (about 80 μg per rat) or MW 24,000 peptide (about 300 μg per rat) was emulsified in complete Freund's adjuvant and injected into the footpads of a rat. Two control animals received saline solution mixed with the adjuvant. Two control animals were injected with the peptide treated for 3 min at 100°C and two animals received S-antigen treated for 3 min at 100°C. The eye was examined with an ophthalmoscope. The animals were killed 15 days after treatment. All procedures involving animals conform to the ARVO Resolution on the Use of Animals in Research.

**Histology of EAU Induced by Chymotryptic Peptide**

Fifteen days post immunization the animals were killed by cervical dislocation and the eyes enucleated. A small incision was made near the optic nerve and the globes were immediately fixed in 10 percent buffered formalin. For histology, the globes were then dehydrated through a graded series of alcohol, cleared in cederwood oil, and embedded in paraffin (Peel-A-Way; Scientific Products; Ocala, FL). Five-micron sections were cut in an AO 820 microtome (American Optical Corp; Buffalo, NY) and mounted on clean microscope slides. The slides were stained with hematoxylin and eosin, examined and photographed in a Nikon Labophot microscope (Nikon Inc.; Garden City, NY) equipped with a UFX camera.

**Results**

**Chymotryptic Digestion of S-antigen**

Time-dependent changes in the profile of chymotryptic digestion of bovine S-antigen are shown in Figure 1. Coomassie-blue stained protein bands indicate that part of S-antigen was cleaved to two major peptides of MW 42,000 and 26,000 within an hour of digestion. In 2 to 4 hr, both peptides were further degraded and three peptides of MW 24,000, 16,000, and 12,000 became preeminent. The three peptide bands faded gradually during prolonged digestion, indicating further breakdown into smaller fragments which probably electrophoresed out of the gel.
To determine which peptide contains antigenic determinants, the polyacrylamide gels were sliced in segments after electrophoresis. Peptides extracted from the segments were then tested by the enzyme-linked immunosorbent assay (ELISA) using anti-S rabbit serum. As demonstrated in Figure 2, the antigenic activity associated with S-antigen was markedly reduced after 1 hr of chymotryptic digestion, although the newly formed peptides of MW 42,000 and 26,000 showed considerable activity. The activity then shifted from MW 26,000 peptide to MW 24,000 peptide in 4 to 6 hr of digestion and slowly decreased thereafter. The rabbit anti S-antigen used was not absorbed before utilization. Therefore, part of the activity in the ELISA could be attributed to contaminants (eg, autolyzed chymotrypsin fragments) which showed nonspecific reactivity toward the anti S-antigen serum. However, the amount of such contaminants was probably small.

We have also tried other proteases for digestion of S-antigen. Thermolysin (S:enzyme = 50:1, w/w) converted S-antigen to MW 47,000 peptide (major) and MW 42,000 peptide in 24 hr. Subtilisin (S:enzyme = 50:1, w/w) produced peptides of MW 36,000 and 26,000 in an hour and quickly degraded these peptides into smaller fragments during the subsequent incubation period. Digestion of S-antigen with papain (S:enzyme = 20:1, w/w) gave rise to a peptide of MW 47,000 in an hour and no accumulation of smaller peptides was observed. Thus, α-chymotrypsin seemed to be more useful than these proteases for preparation of smaller peptides with antigenic activity in sufficient quantities for further studies.

Separation of ELISA-positive Peptide by Gel Filtration on Sephadex Columns

From the results of Figure 1 and Figure 2, it is evident that a peptide of MW 24,000 with antigenic activity accumulates transiently during chymotryptic digestion of bovine S-antigen. To isolate this peptide, we digested S-antigen with α-chymotrypsin for 6 hr and purified the digest (ie, a mixture of peptides) on a Sephadex G-75 column. The first peak collected in the void volume contained a trace of S-antigen and MW 42,000 peptide as determined by SDS-polyacrylamide gel electrophoresis (data not shown). In a separate experiment, S-antigen (2 mg) was loaded on the G-75 column. S-antigen was recovered in fractions 150 to 176. No trace of S-antigen was detected by ELISA in the fractions collected after fraction 177. Therefore, the second peak was considered to be completely free of undigested S-antigen. The second peak (indicated by two connected arrows in Figure 3) showed positive reactivity toward anti-S antibodies as determined by ELISA (Fig. 3, top) and contained three major peptides of MW 24,000, 16,000 and 12,000, respectively (Fig. 3, top).
Fig. 2. Antigenic activity of chymotryptic peptides as determined by ELISA after electrophoresis. Time of digestion is indicated on right.

bottom). The fractions of peak 2 were pooled and further purified on a Sephadex G-50 column. Of the three peaks separated on the column, only the largest peptide (ie, MW 24,000) was found to possess antigenic reactivity under the assay conditions (Fig. 4). The largest peptide showed positive reactivity toward purified anti-S antibodies. Sufficient quantities of the MW 24,000 peptide were pooled from several runs of G-50 gel filtration and used for immunization of animals.

The result indicates that the peptide of MW 24,000, though less than a half in size of S-antigen, still retains the antigenic determinants.

Production of Experimental Autoimmune Uveitis in Lewis Rats with Chymotryptic Peptides of S-antigen

The pooled fractions of antigenic peptide were concentrated by lyophilization, emulsified with complete Freund's adjuvant and injected into Lewis rats. The eyes were examined from 10 days after treatment. None of the six control rats showed any indication of ocular inflammation. However, all of the five rats which had received an injection of peptide exhibited in both eyes signs of ocular inflammation indistinguishable from those observed in the S-antigen-induced uveitic eye. The general description of pathology was as follows. The diseased eyes showed marked panuveitis, vasculitis, and focal and diffuse retinitis. All affected specimens showed accumulation of subretinal fluid. Many lymphocytic cells, histiocytes and plasma cells were found throughout the uvea and were especially numerous in the exudative fluid beneath the retina. Figures 5C and 5D show the affected areas in the anterior uvea and the retina, respectively. Cellular infiltration in the aqueous is evident and photoreceptor outer segments are entirely lost. Figures 5A and 5B are photographs of sections from control eye.

Both MW 16,000 peptide and MW 12,000 peptide, when injected with complete Freund's adjuvant (200-
Fig. 3. Gel filtration on Sephadex G-75 of peptides produced by limited chymotrypsinolysis of bovine S-antigen. The lower figure is a profile of gel filtration. The fractions indicated by two connected arrows were pooled for subsequent purification on Sephadex G-50. The presence of three peptides of MW 24,000, 16,000 and 12,000 in the pooled sample is shown by inset gel. The upper figure is the antigenic activity of fractions determined by ELISA.

300 μg per rat) into Lewis rats, did not induce experimental uveitis.

Discussion

By limited proteolysis of bovine S-antigen with α-chymotrypsin a mixture of three major peptides (MW 24,000, 16,000, and 12,000) accumulated, of which MW 24,000 peptide was found to be ELISA-positive. It is not known whether the smaller peptides were produced by further degradation of MW 24,000 peptide or they derived from the other fragment remaining after the 24,000 peptide was generated from S-antigen. Whatever the origin of the smaller peptides is, these peptides were not ELISA-positive under the assay conditions. However, the present result does not rule out the possibility that the smaller peptides also contain antigenic determinants. After cyanogen bromide cleavage of bovine S-antigen a peptide of 25 amino acid residues was isolated. When tested against anti-S antibodies, the peptide demonstrated antigenic reactivity only after conjugation to poly-L-glu-ala-tyr of MW 46,000. It is possible therefore that the peptides of MW 16,000 and 12,000 may show immunoreactivity toward anti-S antibodies if their size is increased by conjugation to a carrier polypeptide. Since evidence suggests that S-antigen has several antigenic determinants, the antigenic determinant of the smaller peptides, if present, may well be different.
from the antigenic determinant of MW 24,000 peptide.

The present finding on the production of EAU in rats by an injection of the MW 24,000 peptide indicates that more than half of the polypeptide is not essential for the pathogenicity of bovine S-antigen. Pathologic features of EAU produced by S-antigen and peptide are indistinguishable. Further studies are

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**Fig. 4.** Gel filtration of chymotryptic peptides on Sephadex G-50. Open circles: profiles of gel filtration; filled circles: antigenic activities determined by ELISA. Note that the antigenic activity is associated only with MW 24,000 peptide fraction.

**Fig. 5.** Histopathologic changes of anterior and posterior parts of eyes of control and peptide-induced EAU Lewis rats. A, Ciliary body and iris, control (original magnification, X93); B, retina and choroid, control (original magnification, X370); C, ciliary body and iris, peptide injected (original magnification, X93); D, retina and choroid, peptide injected (original magnification, X370).
needed to compare quantitatively the pathogenic potency of S-antigen and its chymotryptic peptide. Pathogenicity may decline as the antigen molecule becomes smaller. However, it should be possible to determine the minimum sequence requirement for induction of EAU by further cleavage of the MW 24,000 protein. For induction of allergic encephalomyelitis in Lewis rats, the minimum sequence requirement of myelin basic protein is eight amino acid residues. Stein presented the results of preliminary experiments at the 1984 ARVO meeting in which he prepared a mixture of peptides (MW 10,000–30,000) by Staphylococcus aureus V8 protease digestion of bovine S-antigen and produced EAU in Lewis rats with the peptides. However, it is not clear whether a small amount of undigested S-antigen was present in the peptides. Gregerson and Putterman prepared seven major peptides by cyanogen bromide cleavage of bovine S-antigen. It awaits further studies to determine whether these peptides, when injected into animals, induce EAU.

**Key words**: S-antigen, chymotryptic peptide, purification, experimental autoimmune uveitis, bovine retina

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**References**


