THE LS-ANTIGEN OF VACCINIA

IV. Chemical Analysis of LS and the Effect of Chymotrypsin on LS*

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Recent observations in our laboratories (1, 2) have demonstrated that a single molecular substance carries all of the serological activity of both the L- and S-soluble antigens of vaccinia. Craigie and Wishart, (3) who originally described these two antigens thought that they occurred in the form of a complex in nature, but, from their own observations (3) and from those of Parker (4), they were led to believe that the two serological substances could be dissociated into separate L- and S-fractions under certain conditions (5). On the basis of our experience with degradation products of LS-antigen (1, 2) we formulated a different hypothesis to explain the apparent dissociation of the complex antigen; this dealt with the loss of precipitability of one part of the LS-molecule through degradation procedures which did not affect the other serologically active portion. Such an explanation would stand on a firm foundation if it were possible regularly to prepare a substance from pure LS-antigen which precipitated only with L-antibody.

The purpose of this paper is to report the results of chemical analytical studies on pure LS-antigen which indicate the protein character of the molecule. In addition, a method for the enzymatic digestion of LS which results in a loss of the serological activity of the S-part of the molecule without destruction of the L-portion will be described.

Materials and Methods

LS-antigen was obtained from vaccine dermal filtrate by the method of Shedlovsky and Smadel (2) and was shown to be in a state of electrophoretic and ultracentrifugal homogeneity. Serological methods described in previous papers of this series (1, 2) were used in the present work. The techniques employed for the chemical analysis of LS and in the study of its digestion with chymotrypsin were similar to those used in our investigations of elementary bodies of vaccinia (6, 7). Crystalline chymotrypsin was obtained from Dr. J. S. Fruton of The Rockefeller Institute.

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EXPERIMENTAL

Chemical Analysis of LS-Antigen

LS-antigen in the form of a dry white powder was employed for chemical analyses. 45.8 mg. of this material was obtained by drying from the frozen state a salt-free solution of LS which had previously been shown to contain a single homogeneous component on electrophoresis and ultracentrifugation. On the basis of precipitin titrations and dry weight determinations, approximately one part in 3 million of the antigen was sufficient to give visible precipitation with optimal amounts of L- or S-antibodies. This lot of LS-antigen was found to contain 15.8 per cent N and 50.6 per cent carbon. Tests for lipids, phosphorus, nucleic acid, and glucosamine were made on appropriate amounts of the material (5 to 20 mg.); the results were negative in each case. Analytical data on other pure preparations of LS-antigen were less complete but the results obtained were in apparent agreement with the above findings. The results of these basic analytical studies indicate that the LS-molecule has the properties of an ordinary protein substance.

Effect of Chymotrypsin on LS-Antigen

The chemical evidence which indicated the protein nature of the entire LS-molecule eliminated certain ideas (1) that we had had earlier concerning the structure of this substance, namely, that the S-portion of the antigen consisted of a protein material of the type described by Parker and Rivers (8) to which a labile non-protein L-structure was bound in a chemical linkage. In order to establish more thoroughly the apparent fact that LS was a protein molecule, the effects of several proteolytic enzymes on the antigen were investigated. The fortuitous choice of crystalline chymotrypsin in the initial experiments led us to employ most of our available material in the study of the striking changes produced by this enzyme on LS.

Digestion of LS-Antigen with Crystalline Chymotrypsin.—The effect of crystalline chymotrypsin on LS-antigen was investigated in the following experiment.

4.0 cc. of a solution of pure LS-antigen which contained 0.443 mg. of N per cc. were freed of buffer salts by dialysis and then diluted to 10 cc. with 0.06 μ phosphate buffer solution, pH 7.6. 2.0 cc. of the diluted antigen were treated with 0.08 mg. of active crystalline chymotrypsin and, as a control, an identical quantity of antigen was mixed with 0.08 mg. of heat-inactivated chymotrypsin. Samples of the mixtures were removed immediately and at intervals up to 22 hours for determinations of amino nitrogen and for serological studies. The results of the serological tests are summarized in Table I.

Partial digestion of LS by active chymotrypsin was revealed by the increased value for amino nitrogen in the mixture after incubation and by the failure to detect a similar increase in the mixture containing inactive enzyme. The

serological data presented in Table I indicate that the solution of LS which had been treated with enzyme reacted to approximately its original titer with L-antibody but failed to precipitate with S-antibody. The substance which precipitated with L-antiserum was indeed the heat labile antigen, for warming to 56°C. for ½ hour destroyed its capacity to flocculate with L-antibody.

TABLE I

Destruction of S-Portion of LS-Antigen by Digestion with Chymotrypsin

Treatment of LS-antigen	Anti-serum	Dilution of antigen						
		1:10	1:20	1:40	1:80	1:160	1:320	1:640
(1) Untreated	L			++++	++++	++++	++	+
	S			++++	++++	++++	+++	+
(2) Chymotrypsin, no incubation 37°C.	L			++++	++++	++++	+	±
	S			++++	++++	++++	++	±
(3) Chymotrypsin, 8 hrs. at 37°C.	L		++	++++	++++	++++	++	++−
	S	−	−	++	++	+++	++	+
(4) Chymotrypsin, 22 hrs. at 37°C.	L	++++	++++	++++	+++	++	+	−
	S	−*	−*	−*	−*	−‡	−	−
(5) Mother liquor of (4) after removal of insoluble material (6)	L	+++	+++	+++	++++	+++	−	−
	S	−*	−*	−*	−*	−‡	−	−
(6) 5 times reprecipitated material from (4) redissolved in ½ original volume	L		++	+++	++	++	++	+
	S		−‡	−‡	−‡	−‡	−	−

* S-antibody in mixture was inhibited.

‡ S-antibody in mixture was not inhibited.

Thus, it would appear that chymotrypsin under the conditions of this experiment attacked the S-portion of the LS-molecule and left unchanged the L-portion. The selective action of the enzyme is not always so clearly manifest, however, for in other experiments, carried out under almost identical conditions, slightly different results were obtained. For example, in two instances in which L-reacting material was undiminished after digestion some precipitable S-substance remained. In another experiment, L-activity was appreciably reduced by the treatment which also completely destroyed S-activity. This would seem to indicate that a rather delicate adjustment of the proportion of

antigen and enzyme is necessary in order to obtain destruction of S without affecting L, for, if digestion is inadequate some native LS remains and if digestion is carried too far some destruction of the L-part of the molecule occurs.

Inhibition experiments carried out with digested mixtures of chymotrypsin and LS and with fractions obtained from them indicate that the S-portion of the molecule undergoes degradation in a manner analogous to that previously described (1, 2) for the L-portion of the antigenic structure. Both products that appear following digestion are capable of precipitating with L-antibody but not with S-antibody. The more carefully studied material of the two fails to inhibit S-antibody. Therefore, in conformity with the nomenclature introduced to designate the stages of degradation of the L-reacting part of the molecule, this substance has been called LS″. LS″ is highly insoluble at room temperature in ordinary buffer solutions at a pH between 7.0 and 8.0, and this property permits its ready separation from other materials in the digested mixtures.

In the experiment summarized in Table I practically all of the substance in the completely digested mixture that reacted with L-antibody was found in the form of tiny needle-like crystalloids which were easily separated from the mother liquor by centrifugation. This material was collected, washed, and redissolved in buffer solution pH 9.3 and recrystallized from cold buffer solution pH 7.0. The process was repeated four times and the resulting substance was taken up in one-half of its original volume of fluid. A portion of this was diluted with 10 volumes of warm physiological saline, a procedure which dissolves the insoluble material, and serological tests were performed. The titer with L-antibody was equal to that of the original solution of LS before enzyme was added. Furthermore, no inhibition of S-antibody occurred in the precipitin tubes containing dilutions of digested antigen from 1:20 to 1:160, for, following incubation, the S-antibody which had been added for the titration was still capable of precipitating with L′S antigen.

The experimental data just presented were reproduced in all essential respects in the four experiments in which LS was digested with chymotrypsin. They leave no doubt that a degradation product can be prepared regularly from the native molecule which retains is L-activity but possesses no serological properties attributable to the S-portion of the antigen. Therefore, if one had no knowledge of its source he would be justified in regarding the preparation of LS″ as representing pure L-antigen. The experimental evidence suggests that an intermediate stage of degradation of the S-portion may also occur in the course of digestion. This substance, represented by the symbol LS′, was encountered in the experiment summarized in Table I. It remained in the mother liquor after removal of LS″ and was responsible apparently for the moderate amounts of L-activity displayed by that solution and for its inhibiting effect on S-antibody.

LS″ differs markedly in its physical properties from LS, L′S, and L″S′ (2, 9). Crystal-like structures (Figs. 1 and 2) appear during the digestion of concentrated solutions of LS by chymotrypsin in 0.06 μ phosphate buffer at pH 7.6. These crystalloids which dissolve at pH values slightly above 9.0 recrystallized in a matter of hours when the pH was lowered to 7.0 and the solution was

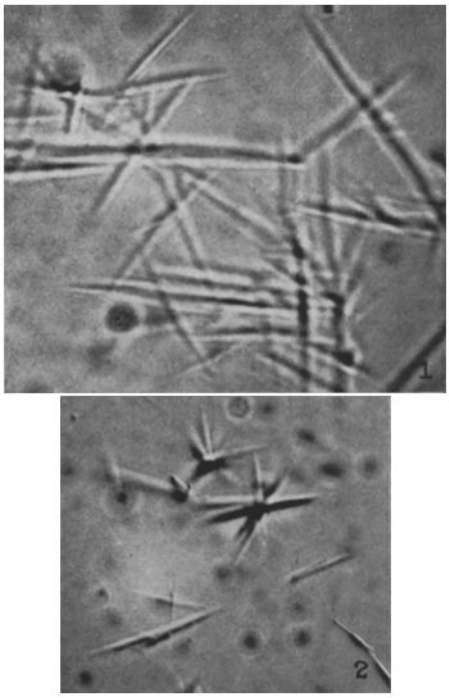

FIG. 1. Needle-shaped, crystal-like structures prepared from LS-antigen lot 22 by digestion with chymotrypsin. This 5 times recrystallized material has L-activity but no S-activity. × 1050.

FIG. 2. A similar preparation obtained by digestion of LS-antigen lot 21. Rosette-shaped masses of needle-shaped structures are illustrated. × 700.

chilled. The crystalline-like material does indeed represent LS″ since the small needle-like structures after being washed, dialyzed, and recrystallized repeatedly still retained most of the L-activity of the original solution of LS (see Table I). Until the true crystalline nature of the needles is established it seems preferable to refer to them as crystalloids. The crystalloids are highly insoluble at room temperature in the following solutions: distilled water, physiological saline, pH 7.2, 0.1 μ acetate buffer, pH 5.0, 0.1 μ cacodylate buffer, pH 6.3, and 0.1 μ veronal buffer, pH 8.4, and pH 8.8. Practically all of the LS″ material dissolved at room temperature in saline solutions of 2 to 5 times physiological

concentrations which had pH values of 9.0 to 9.3 and, in low concentration, the crystalloids dissolved in neutral physiological saline warmed to 37°C.

The insolubility of LS″ crystalloids has interfered with the performance of a number of experiments that would be of interest. For example, precipitation titrations were carried out with dilute solutions of the material but certain absorption experiments with concentrated solutions were unsuccessful, due no doubt to the high salt concentrations necessarily present in the mixtures in order to dissolve the antigen.

Chemical examination of 6.2 mg. of recrystallized LS″ showed that this material contained 15.4 per cent nitrogen, and gave positive biuret and Millon's tests. It is evident, therefore, that the nitrogen content of native LS and of the form with only L-activity are essentially the same and that both values are characteristic of protein substances. A similar amount of nitrogen was also found in preparations of purified S-antigen (according to the current nomenclature, L′S) by Parker and Rivers (8).

Evidence of another type indicates that the L- as well as the S-part of the LS-molecule is protein in nature. Digestion with a highly purified preparation of the proteolytic enzyme papain completely destroys the capacity of LS-antigen to be precipitated by either antibody.

Digestion of Heated LS-Antigen (L′S) with Crystalline Chymotrypsin.—Earlier attempts in this laboratory (8) to digest preparations of purified heat-stable antigen of vaccinia with crystalline chymotrypsin did not result in destruction of the serological activity of the material. Therefore, we considered the possibility that heating alone might affect the LS-molecule in such a manner that its susceptibility to chymotrypsin might be altered. This possiblity was investigated in the following experiment.

4.5 cc. of an unbuffered solution of LS-antigen prepared in the usual manner and having a titer of 1:800 with both L- and S-antibody was diluted to 6.0 cc. with a solution of disodium phosphate buffer pH 7.6. Crystalline chymotrypsin was added in the proportion of 0.08 mg. per cc. of antigen. 4.5 cc. of another portion of the same solution of LS which had been heated at 56°C. for 1 hour (thus making L′S) were treated with identical amounts of enzyme and buffer solutions. Both mixtures were incubated at 37°C. for 24 hours. At the end of this period the digested solution of LS contained needle-like crystalloids, as in the experiment described in detail above. In contrast, the digested solution of L′S developed no precipitate of any type. The treated solutions of both antigens contained increased amounts of amino nitrogen after incubation; furthermore, the release of free amino nitrogen groups was about the same for the two samples. Serological studies on the two solutions showed that neither precipitated with S-antibody; that digested L′S failed to precipitate with L-antibody; and finally, that the digested solution of LS had a titer of 1:400 with L-antiserum.

The results of this experiment indicate that heat degraded LS-antigen, like the native substance, is partially digested by crystalline chymotrypsin. Liberation of amino nitrogen and destruction of the serological activity of the S-portion of the molecule occurred during such treatment. It is of interest that degradation of the L-portion of the molecule by heat prior to digestion with chymotrypsin interfered with the formation of crystalloids of the type obtained when native LS was treated with the enzyme.

SUMMARY

Pure LS-antigen of vaccinia contains 15.8 per cent N and 50.6 per cent C. These analytical data together with the absence of lipids, phosphorus, nucleic acid, and glucosamine in preparations of the antigen confirm the protein nature of the substance. The action of proteolytic enzymes on LS offers further confirmation of the protein character of the antigen.

Both the L- and S-activities of the antigen are destroyed by digestion with papain. The effects of crystalline chymotrypsin on LS-antigen are particularly interesting for, under proper conditions, this enzyme destroys the serological activity of the S-portion of the molecule without affecting the L-portion. This newly prepared degradation product of LS, called LS″, contains the same amount of N as the native substance but unlike LS, it forms needle-shaped crystalloids.

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