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Production and Purification

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An antibody chemically and immunologically active against bovine testicular hyaluronidase was produced in rabbits by injecting them with purified hyaluronidase in complete Freund's adjuvant. Nonspecific antibodies, reacting with human and bovine proteins which had little or no hyaluronidase activity, were also detected by the double agar immunodiffusion technique using antiserum against these proteins. After absorption of the antiserum with bovine heart and liver and removal of the precipitation bands against human and bovine tissue proteins, the antiserum inhibited the chemical activity of the purified hyaluronidase and exhibited immunologic activity against the purified hyaluronidase as shown by the agar double immunodiffusion technique.

Hyaluronidase activity has been demonstrated in a number of body fluids and organs and has been found to be more abundant in testicular tissue.[2] Localization at a cellular level has been accomplished using antihyaluronidase serum and the immunofluorescent technique of Coons.[4,9] The antihyaluronidase serum, which has been produced by the injection of crude[1,9] or purified[4] hyaluronidase in rabbits and guinea pigs, has not been investigated thoroughly enough to establish its specificity against hyaluronidase. Its use in immunofluorescence studies is therefore questionable.

The purpose of this presentation is to describe the antigenic properties of an antiserum, produced by the injection of chromatographically pure bovine testicular hyaluronidase, and to outline a monitoring system for its purification.

Materials and Methods

Animal Immunization — Seven white rabbits, 2.3 to 3.2 kg (5 to 7 lb.) each, were given intramuscular and intraperitoneal injections of chromatographically pure bovine testicular hyaluronidase (2,400-3,600 TRU/mg)†[8]; 10 mg/ml in equal parts of normal saline and Difco's complete Freund's adjuvant. The total immunizing dose was 35 mg of the hyaluronidase product, 20 mg as an initial dose and 5 mg at weekly intervals starting one month after the initial injection. Forty ml of heart blood were withdrawn 12 days after the last injection. Booster doses for anamnestic responses

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†Purchased from Worthington Biochemical Corp., Freehold, N.J.

were initiated one month later with 10 mg of purified hyaluronidase in 1 ml of the normal saline—Freund's adjuvant mixture. The animals were again bled 12-14 days following the booster injection.

Tests for Activity and Specificity

Antihyaluronidase Activity — Reduction in hyaluronidase activity by each rabbit's serum was determined by mixing 0.5 cc of rabbit serum [1:5 dilution in veronal buffered-saline (VBS), pH 7.4] and 0.5 cc of purified hyaluronidase (2 mg per 6 cc distilled water). These were incubated for one hour at 37C and then in the refrigerator (4-7C) for 16 - 20 hours. The specimens were then centrifuged at 2,500 rpm for 30 minutes and the supernatant was assayed for hyaluronidase activity using the turbidimetric method.[7] For comparison, parallel control studies were conducted with a similarly treated normal rabbit serum (at 1:5 dilution) and purified hyaluronidase. Antiserum was also tested for its antihyaluronidase activity, after it had been absorbed according to procedures described below.

Immunologic Studies — The immunologic activity of pooled rabbit antisera, each capable of reducing hyaluronidase activity, was studied by the agar immunodiffusion slide test.[5] Undiluted antiserum was diffused against undiluted and serial twofold dilutions (1:2 to 1:2048) of various tissue antigens; purified hyaluronidase (17.7 mg per ml), human serum, human heart, human liver, bovine serum, bovine heart, bovine liver and crude bovine testicular hyaluronidase. The tissue antigens, which were obtained by the hyaluronidase extraction procedure[3] and which contained 50-60% protein, were used at a concentration of 333 mg per ml. The hyaluronidase activity of each antigen was determined[7] and found to be 0-0.5 turbidity reducing units (TRU) per mg of extract or per ml of serum for the various antigens. The activity of the crude and purified testicular hyaluronidase was 500 and 2,500 TRU per mg respectively. Each of the various antigens was immunochemically tested with the pooled antiserum. This had been previously absorbed separately with bovine heart, combined bovine heart and liver, human liver, human heart, human serum, bovine serum and crude bovine testicular hyaluronidase. Absorption was accomplished after determining the zone of equivalence for each antigen by quantitative precipitation tests in tubes. Thus maximal removal of nonspecific antibodies was obtained without total loss of antihyaluronidase activity. In the absorption process lyophilized tissue extracts or serum were added to the antiserum. This mixture was incubated at 37C for one hour and in the refrigerator (4-7C) for 16-20 hours prior to centrifugation at 2,500 rpm for 30 minutes. If precipitate remained, antibody titer at the zone of equivalence was again determined for the supernatant fluid and the precipitate removed by additional absorption. This was repeated until no precipitate remained.

Results

That commercial preparations of purified hyaluronidase contain a variety of impurities was readily demonstrated by immunoprecipitin antibodies induced in rabbits. Precipitation bands were shown in the micro double diffusion in agar slide technic employing the unabsorbed rabbit antiserum and various antigens. (Figure 1). The

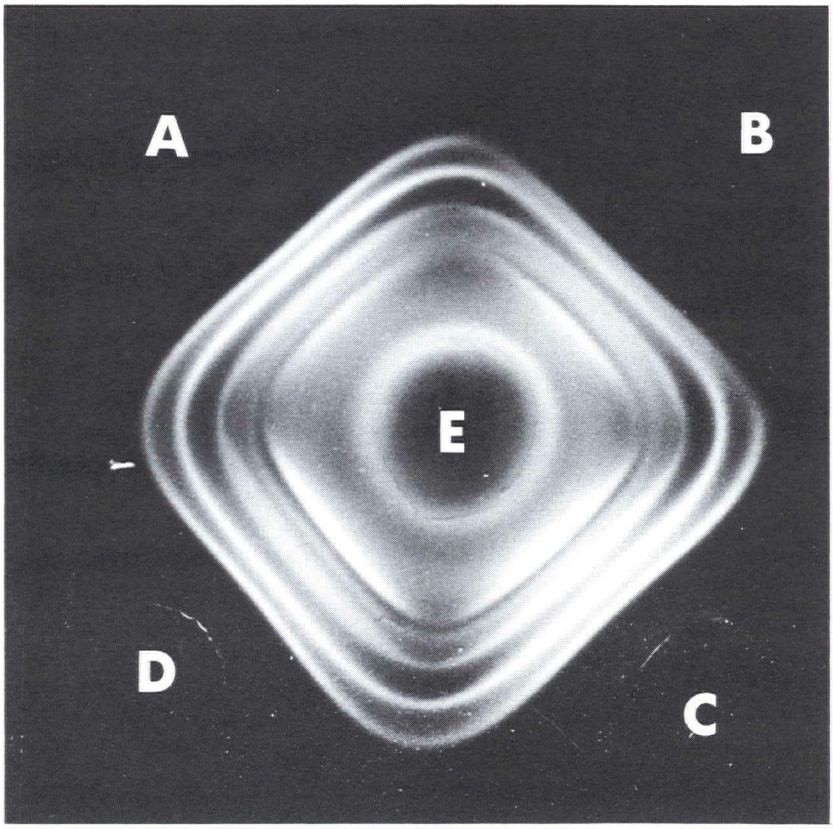

Figure 1

Multiple precipitation bands (graded as 4+ in intensity and number) resulting from the reaction of unabsorbed undiluted rabbit antihyaluronidase in the center well (E) with bovine heart 333 mg per ml in wells A (undiluted), B (1:2 dilution), C (1:4 dilution) and D (1:8 dilution). Similar precipitation bands were observed with other antigens (see Table I).

bands were more intense and more numerous with the bovine tissue antigens than with human protein. Table I gives the results of absorption of the antiserum with the various tissue preparations and sera and indicates the degree of reduction in the number and intensity of the precipitation bands. Removal of the precipitating antibodies from the rabbit antisera against bovine heart and bovine liver antigens was accomplished only by the absorption of the antiserum with combined bovine heart and bovine liver or with crude testicular hyaluronidase. After such absorption of the rabbit antisera, a single precipitation band remained against undiluted and twofold dilutions (1:2-1:512) of purified hyaluronidase but not against tissue antigens (Figure 2 and Table I). The precipitation bands against human tissue and human serum were completely removed from the antisera by absorption with bovine heart, or with human tissue only. Absorption of the rabbit antisera with human tissue, human serum, or bovine serum, however, did not remove the precipitins against the bovine tissue.

TABLE I

SERUM ANTIHYALURONIDASE ACTIVITY*
AGAR IMMUNODIFFUSION AND CHEMICAL INHIBITION STUDY

Antiserum	Antigen								Rabbit Antiserum Antihyaluronidase Activity (%)**
	H. Ht.	H. Liver	H. Serum	Bovine Serum	Bovine Heart	Bovine Liver	C. Hyase +	P. Hyase++	
Unabsorbed	3+	3+	+	2+	4+	4+	4+	4+	82%
Absorbed with:									
Bovine heart	-	-	-	-	1+	2+	3+	3+	58%
Bovine heart & bovine liver	-	-	-	-	-	-	2+(S)	2+(S)	44%
C. Hyase	-	-	-	-	-	-	-	+(S)	39%
Bovine heart & liver, C. Hyase, and P. Hyase	-	-	-	-	-	-	-	-	0
Bovine serum	3+	3+	-	-	4+	4+	4+	4+	ND
Human serum	3+	3+	-	2+	4+	4+	4+	4+	ND
Human liver	-	-	-	ND	3+	3+	3+	3+	ND
Human heart	-	-	-	ND	3+	3+	3+	3+	ND

ND = Not done.
\- = Negative.
* = Numerical grading from 0 to 4 is an arbitrary gradation of the intensity and number of the precipitation bands seen before and after absorption of the antiserum.
** = Reduction in hyaluronidase activity produced by antihyaluronidase antiserum using the normal rabbit serum as representing 0% activity.
+ C. Hyase = Crude bovine testicular hyaluronidase, purchased from Cudahy Laboratories, Omaha, Nebraska.
++ P. Hyase = Purified bovine testicular hyaluronidase.
(S) = Single precipitation band observed. All other bands were multiple.

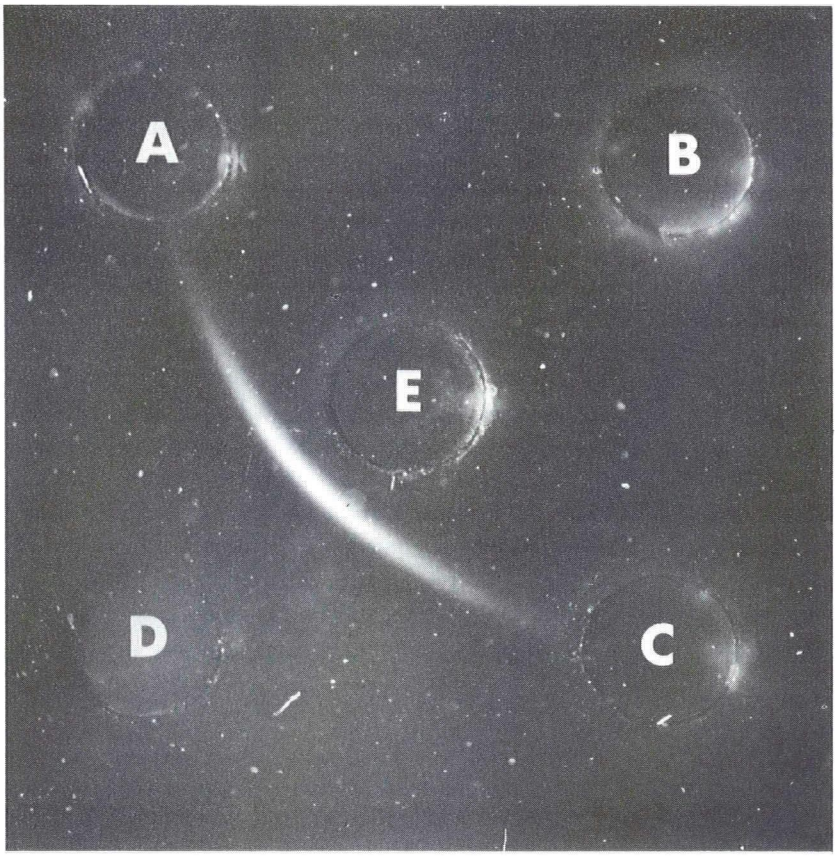

Figure 2

An intense precipitation band remains against purified hyaluronidase 17.7 mg/ml (1:128 dilution—well D) but not against bovine liver 333 mg/ml in wells A (undiluted), B (1:2 dilution) and C (1:4 dilution) after the rabbit anti-hyaluronidase (well E) was repeatedly absorbed with bovine heart and bovine liver.

The antihyaluronidase activity of the *unabsorbed antiserum* and the tissue *absorbed antiserum* is shown on a percentage basis in the last column of Table I. The absorption of the antiserum reduced its antihyaluronidase activity from 82% in the unabsorbed antiserum to 58% (heart), 44% (combined bovine heart and liver) and 39% (crude hyaluronidase) in the absorbed antiserum. Antihyaluronidase activity was reduced to nondetectable quantities only when the antiserum was absorbed with four of the antigens listed in Table I; ie, combined bovine heart and liver and crude and purified hyaluronidase preparations. It was interesting that antiserum absorbed with only the crude hyaluronidase preparation continued to demonstrate a single precipitation band against the purified hyaluronidase preparation. Moreover, the remaining

precipitin demonstrated by double diffusion appears to be associated with a residual 39% antihyaluronidase activity.

Residual antihyaluronidase activity was not determined in antiserum absorbed with human tissue or bovine serum. Such serum continued to demonstrate too many nonspecific precipitation bands by the micro double diffusion agar procedure. Furthermore, such preparations could have little value in attempting to localize intracellular sites of hyaluronidase by immunofluorescent procedures.

Discussion

Purified bovine testicular hyaluronidase preparations contained specific and nonspecific antigenic substances capable of producing precipitin antibodies. Although the antihyaluronidase activity of the antiserum, determined by chemical inhibition studies, was reduced by absorption of the antiserum with bovine tissue proteins, the antiserum continued to form a single precipitin band with purified hyaluronidase but not with bovine or human tissue antigens or bovine or human serum. The absorbed antiserum has been found to be useful in the immunofluorescent cellular localization of hyaluronidase. A monitoring system demonstrating a single precipitin band associated with specific biologic activity affords a more satisfactory method for the production and purification of an antiserum best suited for fluorescent localization of specific substances.

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