Antienzymes to α- and β-Amylase

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Studies on the antigenicity of an α- and a β-amylase have been made. The use of incomplete Freund's adjuvant enhanced the amount of enzyme-neutralizing antibody formed by rabbits to active α-amylase (*Bacillus subtilis*), whereas, under similar conditions, the adjuvant did not enhance antienzyme formation to active β-amylase (barley). The use of this adjuvant prolonged the duration of antibody level produced by both of these enzymes. Acid-inactivated α-amylase was found to produce a circulating antibody in rabbits which will neutralize its homologous active enzyme. It is suggested that the determinant groups on this enzyme, which are responsible for antibody production, are not necessarily the same groupings responsible for activity on substrate.

COMPARATIVELY FEW observations of antibodies to α- and β-amylase have been reported. Sevag (1) has demonstrated that the antienzyme to malt amylase is specific in its enzyme-neutralizing capacity and will not neutralize the activity of an α-amylase. Furthermore, inactivated malt amylase was shown to be nonantigenic in the sense that sera from animals inoculated with this material possessed no antienzyme capacity. Recently, Nomura and Wada (2) have obtained rabbit antiserum against the amylase of *B. subtilis*. Also, Wada (3) has shown that the presence of heat-denatured Taka-α-amylase did not inhibit reaction between this active enzyme and its antibody.

No observations appear to have been made concerning the effect of an adjuvant on the antigenicity of these enzymes, or of any attempt to investigate the serologic relationship of an antienzyme to inactivated α-amylase. It was hoped that an investigation of this nature would help provide more knowledge concerning the molecular groupings of these enzymes which are responsible for activity on substrates and those which are responsible for antigenic activity, as well as the immunospecificity of these antigens when injected into rabbits over a comparatively long period of immunization. Previous investigators (4–9) have shown a diminution in specificity of an antiserum following a prolonged immunization period with various antigens.

MATERIALS AND METHODS

Antigenic Materials.—The α-amylase used in this investigation was obtained from a bacterial source (*Bacillus subtilis*) and the β-amylase was derived from barley; both of these materials were obtained commercially.[1]

Assay for Enzyme Activity.—Glycogen, $[\alpha]_D^{20} = +196$ to 197°, containing no reducing sugars, was the substrate used to demonstrate enzymic activity.[1] The assay method was that of Somogyi (10) for the determination of reducing sugars, as modified by Nelson (11) and Somogyi (12). All measurements for the concentration of reducing sugars, released from the substrate through enzymic activity at 37° for 15 minutes at a pH of 5.3, were made using a Bausch & Lomb Spectronic 20 at a wavelength of 510 mμ (13).

Serologic Tests.—A method for the indirect determination of antienzyme concentration through a modification of the Nelson-Somogyi procedure was performed as described elsewhere (13). The values reported for the reciprocal of serum dilutions represent the dilution of antibody, at the time of sampling recorded, which neutralized the enzymatic activity of the standard enzyme preparation used. It was found that naturally occurring amylase in rabbit sera at dilutions of 1:25 or higher would not affect assay results.

Adjuvant.—Incomplete paraffin-oil adjuvant, described by Freund (14), was prepared to contain 2 parts of paraffin oil (Marcol GX),[2] 1 part of Falba, and 2 parts of the desired concentration of enzyme. The incorporation of the amylases with the adjuvant base and the control tests have been reported elsewhere (13).

Sera.—All rabbits were bled by ear vein puncture, and the sera were preserved at −20°. No preservative was added to these sera.

Immunization.—Those rabbits which were immunized with the enzyme incorporated in the adjuvant base were injected subcutaneously in the scapular region over a period of 78 days. Each group of rabbits was immunized on the basis of equal amounts of total nitrogen. On this basis, 0.027 mg. nitrogen/mg. (± 0.002) of enzymic material promoted the same degree of hydrolysis of substrate, whether it was α- or β-amylase. Where amylase was used as an antigenic agent without the use of incomplete Freund's adjuvant, it was injected in the form of a sterile saline solution every 4 days, either intravenously or subcutaneously, depending on the experiment. The total amount of nitrogen injected was adjusted to be the same at the end of successive

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8-day periods for each group of rabbits during the first 44 days of the immunization period. After this time, further injections were made as shown in Figs. 1 and 2. Blood was collected from the animals at the intervals indicated in the graphs. All assays for antibody content were made at the conclusion of each immunization schedule.

Inactivation of α- and β-Amylase.—α-Amylase was inactivated according to a modification of the method used by Kneen, Sandstedt, and Hollenbeck (15) and as previously reported (13). Irreversible inactivation of this enzyme took place at a pH of 3.0 when held at 37° for 10 minutes in a 0.02 *M* biphthalate-hydrochloric acid buffer. According to previous findings (13), this method of acid inactivation could not be employed with β-amylase. Therefore, β-amylase in 0.02 *M* phosphate buffer of pH 7.2 was inactivated at a temperature of 55° after 30 minutes.

EXPERIMENTAL AND RESULTS

Route of Antigen Injection.—Using active β-amylase without adjuvant and on the basis of equal amounts of total nitrogen injected, the route of injection, whether it be subcutaneous or intravenous, does not appear to influence antienzyme formation in rabbits. These observations were made using a total of 11 animals.

Effect of Incomplete Freund's Adjuvant on the Antigenicity of α- and β-Amylase.—The rate of appearance of antibodies in the circulation was determined at various times during the immunization schedule and the findings are reported in Figs. 1 and 2. No enhancement in antienzyme formation to β-amylase was found using this adjuvant. Several rabbits from two distinct sources of albino New Zealand breeding stock of both sexes and weighing approximately 2.5 Kg. were used without success in attempts to obtain higher antienzyme titers. On the other hand a potentiation of approximately 10 times in antibody production was observed when α-amylase was used with the adjuvant. Antienzyme production to both antigens was maintained for a prolonged duration with the use of this adjuvant. (See Figs. 1 and 2 at the times of 54 and 100 days.)

Antigenicity of Active and Inactive Amylases.—As illustrated in Figs. 1 and 2, the α-amylase used is a comparatively good antigenic agent as compared to β-amylase derived from barley. β-Amylase inactivated by a mild heating, and using incomplete Freund's adjuvant does not produce detectable circulating antienzymes in rabbits. On the basis of total nitrogen injected, inactivated α-amylase has been shown to produce antienzymes in as high a titer as does the active form of the enzyme when incorporated into this adjuvant. This acid-inactivated α-amylase produced antienzymes which neutralized the activity of its homologous active enzyme on glycogen. Therefore, it appears that the determinant groups on this enzyme responsible for antibody production may not be the same groupings which are responsible for activity on substrate. Cross-neutralization tests with antienzymes to all antigenic agents used showed no apparent inhibition of activity on substrate, even though these antibodies had been produced after a comparatively intensive and prolonged schedule of immunization.

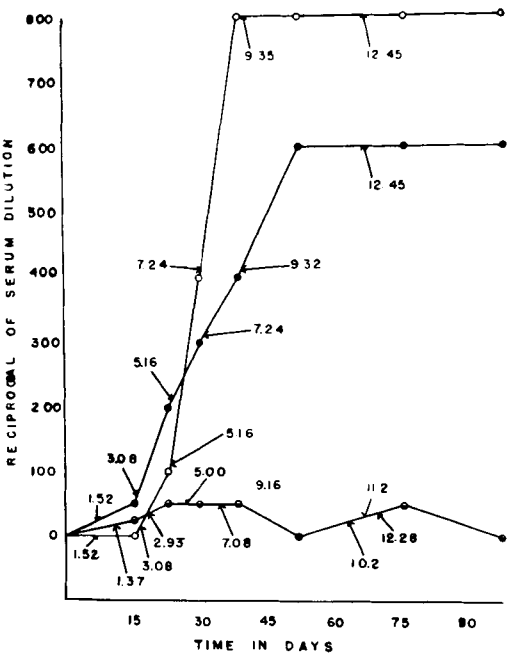

Fig. 1.—Inhibition of α-amylase by homologous antisera. The reciprocal of the antisera dilutions to α-amylase represents the median of the values obtained for each group of animals. Test bleedings were made just prior to injections as designated by points on the graphs. Numbers record cumulative amounts of total N injected at designated times. Despite the comparatively small number of animals used here, there was a consistent difference in the response of each group, and this was significant. O——O, Inactive α-amylase in adjuvant, 4 animals; ●——●, active α-amylase in adjuvant, 3 animals; ◒——◒, active α-amylase in saline, 2 animals.

DISCUSSION

The objective of this investigation was not a comparison of the antigenicity of α-amylase with that of β-amylase, as the origin of the materials used was from divergent sources. Also, we have made no assumption that these materials are to be considered as representative of this class of enzymes as our findings may not be similar to those which may be obtained with the use of other preparations. According to our findings, Sevag's observations concerning the immunospecificity of the antienzyme to β-amylase and its inability to neutralize the activity of an α-amylase, and the lack of antigenicity of inactivated β-amylase have been confirmed.

In this investigation we have shown that α-amylase from *Bacillus subtilis* is a better antigenic agent than β-amylase (barley) in the production of circulating antibodies in rabbits. Also, the enzymatically inactive form of this amylase is as good an antigen as the active α-amylase and will neutralize its activity.

Little and Caldwell (16, 17) have shown that the activity of α-amylase on a substrate requires that the primary amino groups be free, that tyrosine groups are not essential, and that sulfhydryl groups may be lacking entirely. Weill and Caldwell (18) have

reported that with β-amylase, primary amino groups are not essential for activity, whereas tyrosine and sulfhydryl groups are necessary. It may be surmised that these differences between the molecular structures of the two enzymes or the presence or absence of certain groups on the molecule of active β-amylase may help contribute to its comparatively poor antigenicity. According to our observations, the factors which lend themselves to acidic inactivation of α-amylase and its heat stability are not the same with β-amylase, as this enzyme is comparatively thermolabile (13).

Despite the fact that Little and Caldwell (16, 17) carried out their analyses on an α-amylase originating from pancreas, and the α-amylase used in this work was from a bacterial source, and that α-amylases from different sources may vary in characteristics such as stability, pH optima, etc., it is accepted generally that α-amylases do not differ with respect to their specific action on substrate, but only in their affinities for substrate (19).

McGeachin and Reynolds (20) have demonstrated that hog pancreatic and human salivary amylases are antigenic and that their respective antibodies have amylase-inhibiting properties. They have reported that the enzymatic site of these amylases is part of or located close to the antigenic site and is blocked or covered by the large antibody molecules. Using amylase of *B. subtilis*, Wada and Nomura (21) found that the neutralization reaction by antibody was inhibited by the presence of substrate or certain of its degradation products. They suggested that there may be a competitive relationship between substrate and antibody for combination at the same portion of the enzyme surface. Although the antigen-antibody interaction may mask enzymatically active sites, according to our observations, the activity site of α-amylase (*B. subtilis*) for substrate may be destroyed, but the antigenic site remains unaltered.

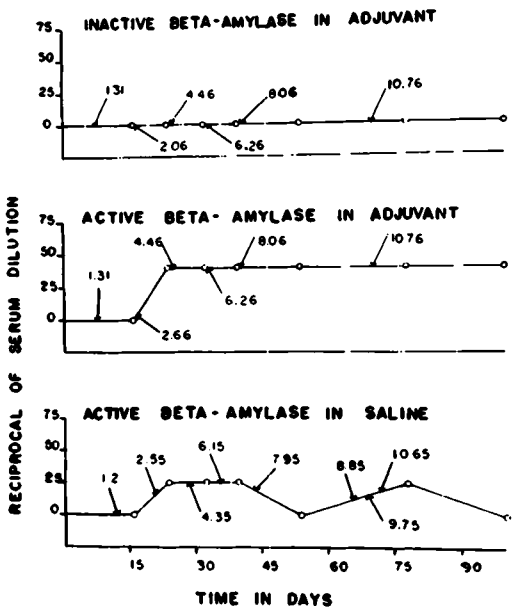

Fig. 2.—Inhibition of β-amylase by homologous antisera. The reciprocal of the dilutions of antisera to β-amylase represents the median of the values obtained for each group of animals. Test bleedings were made just prior to injections as designated by points on the graphs. Numbers record cumulative amounts of total N injected at designated times. Inactive β-amylase in adjuvant, 12 animals; active β-amylase in adjuvant, 7 animals; active β-amylase in saline, 11 animals.

In this work, we have shown that the inactivation of α-amylase by acidification does not decrease its antigenicity for rabbits, and according to Little and Caldwell's observations (16, 17), it appears that free primary amino groups on the α-amylase molecule acting as an antigen are not essential to its ability to produce antibody. To the authors' knowledge, the exact mechanism of acid inactivation for this molecule is not known, however, the addition of acid to a protein may alter its structure. Thus, the active site of an enzyme could be destroyed but its antigenic groups may remain unaltered. Since α-amylase activity is lost irreversibly by this method of inactivation, it is suggested that the amino groups are not free, and that this altered molecule either now possesses steric hindrance with substrate or the charges on groups active with substrate have been neutralized.

Although a comparatively intensive and prolonged immunization schedule with all antigenic materials was used, no demonstration of alteration in antienzyme specificity was observed. Recent work on the serology of various mammalian amylase antigens incorporated in incomplete Freund's adjuvant employed as few injections as necessary to avoid the possibility of decreased antibody specificity (20). The inactivated α-amylase used showed no ability to produce a neutralizing antibody against β-amylase (barley). Therefore, alteration of this α-amylase through acid inactivation apparently does not alter its structure sufficiently to promote this modification in its antigenic specificity.

REFERENCES

(1) Sevag, M. G., "Immuno-Catalysis," Charles C Thomas, Springfield, Ill., 1951, p. 222.
(2) Nomura, M., and Wada, T., *J. Biochem. Tokyo*, **45**, 629(1958).
(3) Wada, T., *ibid.*, **46**, 329(1959).
(4) Heidelberger, M., and Kendall, F. E., *J. Exptl. Med.*, **62**, 697(1935).
(5) Kabat, E. A., *ibid.*, **69**, 103(1939).
(6) Sharp, W. B., *Texas Repts. Biol. and Med.*, **3**, 159 (1945).
(7) Tiselius, A., and Kabat, E. A., *J. Exptl. Med.*, **69**, 119 (1939).
(8) Treffers, H. P., Heidelberger, M., and Freund, J.: *ibid.*, **86**, 83(1947).
(9) Hooker, S. B., and Boyd, W. C., *J. Immunol.*, **26**, 469 (1934).
(10) Somogyi, M. J., *J. Biol. Chem.*, **125**, 399(1938).
(11) Nelson, N., *ibid.*, **153**, 375(1944).
(12) Somogyi, M. J., *ibid.*, **195**, 19(1952).
(13) Sanders, H. D., and Stock, J. J., *Can. Pharm. J.*, **93**, 67(April 1960).
(14) Freund, J., *Ann. Rev. Microbiol.*, **1**, 291(1947).
(15) Kneen, E., Sandstedt, R. M., and Hollenbeck, C. M., *Cereal Chem.*, **20**, 399(1943).
(16) Little, J. E., and Caldwell, M. L., *J. Biol. Chem.*, **142**, 585(1942).
(17) Little, J. E., and Caldwell, M. L., *ibid.*, **147**, 229 (1943).
(18) Weill, C. E., and Caldwell, M. L., *J. Am. Chem. Soc.*, **67**, 212(1945).
(19) Hopkins, R. H., *Advances in Enzymol.*, **6**, 389(1946).
(20) McGeachin, R. L., and Reynolds, J. M., *Ann. N. Y. Acad. Sci.*, **94**, 996(1961).
(21) Wada, T., and Nomura, M., *J. Biochem. Tokyo*, **45**, 639(1958).