The Action of Streptococcal Amylase in relation to the Synthesis of an Amylosaccharide by Amyolytic Strains of *Streptococcus pyogenes*

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SUMMARY: Streptococcal amylase behaves like α -amylase, causing branch-chained substrates to lose viscosity with minimal release of reducing groups, and to become achromic with iodine. Reducing values equivalent to 50% of the theoretically obtainable maltose were reached after prolonged hydrolysis.

The enzyme was produced in high concentration by amylase-positive streptococci growing in simplified medium containing substrate. The same strains synthesized a starchy cell substance if grown in plasma-enriched medium containing either maltose or glycogen. When extracted by boiling with weak alkali the new product resembled amylose.

The resemblance of streptococcal amylase to α -amylase was previously noted (Crowley, 1950, 1954). This paper describes a quantitative study of (1) a method of producing potent amyolytic cultures, (2) the amylase action, and (3) the production of a starchy cell substance by the same strains of group A streptococci.

METHODS

Strains. The strains of group A streptococci were SF130, type 1 and type 2/Opaque (both originally isolated by Dr F. Griffith), dried cultures of which were received from Dr Rebecca Lancefield. The strain T2/11020, type 2, had been dried in 1951 when freshly isolated, and was received from the Streptococcal Reference Laboratory.

Culture media. Infusion broth was made by the method of Todd & Hewitt (1932). Peptone dialysate medium was prepared with Benger's peptone (Benger Laboratories Ltd., Holmes Chapel, Cheshire). A suspension of the peptone was dialysed against distilled water to obtain a solution containing *c.* 5% of the dialysable substances; to this were added NaCl 4.5 g./l. and sodium β -glycerophosphate 6 g./l. before sterilizing by filtration. Glutinous corn starch 4%, w/v (Amioca; Brown & Polson Ltd, Wellington House, Strand, London, W.C.2) was dissolved in 0.067 M-phosphate buffer, pH 7.6. This solution was autoclaved and added to the peptone dialysate in the proportion 1 vol. starch solution to 3 vol. peptone solution making a substrate concentration of 1% (v/v) in the final medium.

Measurement of amylase activity. (1) Viscosity reducing power was measured by a modification of the method of Christensen (1945), using Ostwald pipettes with a flow time for distilled water between 11 and 12.5 sec. at 26°. An enzyme sample (0.5 ml.) was mixed with substrate (4.5 ml.), and the flow time

recorded at 2 min. intervals. The substrate, either glutinous corn amylopectin (1.5%, w/v), or non-glutinous corn amylopectin (4%, w/v) was made up in 0.067 M-phosphate buffer and adjusted to an initial flow time of 65–75 sec. at 26°. A fall of 1% in the viscosity (relative to water) of glutinous corn starch at pH 7.0, 26°, during 10 min. was taken as equivalent to 1 unit of amylase. If 0.5 ml. of enzyme reduced the viscosity by 10% then 1 ml. of the sample contained 20 units.

(2) Saccharifying activity was measured by the method of Meyer, Fischer & Bernfeld (1947) as modified by Noelling & Bernfeld (1948). Colorimetric readings were made in a Unicam Spectrophotometer (SP 500) at 480 m μ . Purified corn amylopectin (intrinsic viscosity 1.07) was obtained from the Northern Regional Research Laboratory of the U.S. Department of Agriculture, Peoria, Illinois. Mussel glycogen was from a batch specially prepared by Light and Co. Ltd, Colnbrook, Bucks., England. Purified glutinous corn amylopectin was extracted from commercial Amioca.

pH values were measured by a direct recording pH meter (Electronic Instruments, Richmond, Surrey, England, Model 23). 5 l. or larger volumes of culture were continuously neutralized during incubation by an automatic pH controller made by Dr P. J. L. Sequeira, Pathology Department, Royal Free Hospital, which will be described elsewhere. The cultures were incubated in bottles, immersed to the neck in a temperature-controlled bath and were agitated continuously. Bacterial growth was measured turbidimetrically in the spectrophotometer at 650 m μ .

RESULTS

The production of amylase in fluid medium

Little or no amylase was found in 16 hr. Todd-Hewitt broth cultures of the strains T 1, T 2/opaque or T 2/11020. Enzyme was found after 24 hr., reached a maximum after 3 or 4 days at 37° and usually decreased thereafter. The maximum amylase activity in the absence of substrate was usually less than three times that at 24 hr. Following the addition of a suitable substrate to the cultures a sharp rise occurred at any time during 7 days incubation at 37°. When the pH was kept at 7.0 for 6–8 hr. after adding substrate, the increase of enzyme was eight- to tenfold or more. During this period there was a three- to fivefold increase of bacterial growth. When the same strains were grown on starch agar and compared with replica cultures on other media, it was found that amylase was produced by certain colony variants (Crowley, 1954). It seemed probable that in selective cultures amylase-producing streptococci would outgrow the rest of the population. This hypothesis was tested by seeding heavy suspensions of streptococci from 24 hr. broth cultures into peptone water containing glutinous corn starch (1%, w/v), when good yields of amylase were obtained. The method was used to produce potent amylolytic cultures and cell-free filtrates which were relatively low in protein nitrogen. In subsequent experiments the medium was prepared with Benger's peptone, which contained dialysable substances supporting better growth than other culture media tested.

Growth and amylase production in simplified medium

The following procedure was adopted. A dried culture was suspended in 2.5 ml. of broth and from this 1 ml. of growing culture was transferred to 10 ml. of broth which was incubated overnight at 37°. Serial subcultures (10% inoculum) were made in 50 ml., 250 ml. and 1 l. of prewarmed broth at 2 hr. intervals, and finally into 5 l. which was incubated overnight at 37°. The streptococci were centrifuged down and the sediment was suspended in 110 ml. phosphate buffer. 100 ml. of the suspension was added to 300 ml. peptone

Fig. 1. The ratio of amylase to bacterial growth in the presence of starch substrate. ●—●, type 1 cell suspensions. A, growth increase of 612.3 mg./ml.; and B, growth increase of 497.6 mg./ml. ○---○, type 2 cell suspensions. C, growth increase of 637 mg./ml.; and D, growth increase of 623.4 mg./ml.

dialysate + starch substrate and incubated in a water bath at 37° for 1.5 hr. The 400 ml. of culture was then added to 1.6 l. of fresh prewarmed medium and incubation continued for 6.5 hr. The pH value was kept between 6.8 and 7.4 by addition of 10% NaOH solution. Culture samples (25 ml.) were removed at hourly intervals, when bacterial growth was measured turbidimetrically and a cell-free filtrate was tested for amylase by the viscosimetric method. Four ml. supernatant were tested for presence of substrate by adding 1 ml. of iodine solution (iodine 5 g., potassium iodide 10 g., in 500 ml. of distilled water).

The ratio of amylase to bacterial growth during incubation of strains T 1 and T 2/opaque is shown in Fig. 1. The cultures did not usually become

completely achromic to iodine for 4.5–5.5 hr. In the later experiments, the substrate was renewed after 4 hr. The bacterial cells were harvested in 110 ml. buffer, and 10 ml. of the suspension was compared with 10 ml. of the initial suspension. The remainder were frozen at -10° and used again. Dilutions of the initial suspension and final suspension were spread on blood agar and starch agar. From the final cultures, 95% or more of the colonies which grew on blood agar also grew on starch agar and were amylase-positive. For the strain T 1 this was an increase of 25–35% in comparison with the initial culture, and for the strain T 2/opaque, an increase of 5–15%.

Production of amylase by the selected population

The thick suspensions of amylase-positive organisms described above yielded amylase in amount equal to or slightly above that of the primary culture when the cells were re-incubated for 5 hr. in fresh peptone + dialysate + substrate medium. When re-incubated a second time the yield was slightly decreased, but after a third re-incubation the yield was halved. It was evident that the amount of amylase obtainable was limited when the organisms were repeatedly subcultured in the simplified medium.

The effect of cell-free filtrates on branched chain amylosaccharides

Action on amylopectin. The viscosity reducing power and saccharifying activity of cell-free filtrates was measured using glutinous corn amylopectin and highly purified non-glutinous corn amylopectin as substrates.

Fig. 2 shows the fall in relative viscosity of glutinous and non-glutinous amylopectin in mixtures incubated at 26° , in which 1 vol. culture filtrate was incubated with 9 vol. substrate. The rate of attack on glutinous amylopectin was greater than on the non-glutinous, though the former was the more viscous substrate. At 37° reduction in viscosity was too rapid to make useful measurements. Glutinous amylopectin showed increased turbidity during the initial stages of the reaction. This was followed by a progressive decrease of turbidity until the substrate became water-clear. The mixtures became achromic to iodine after the viscosity of the substrate was reduced by approximately 50%.

Saccharifying activity. 1 vol. culture filtrate + 9 vol. substrate were incubated and samples removed at 10 min. intervals during 1 hr. and thereafter at 2, 4, 8 and 18 hr. Reducing values were expressed as percentages of the theoretically obtainable maltose, assuming that the maximum amount of maltose produced by α -amylase from amylopectin was 80%. Maltose production, initially small, showed a slow linear increase which continued after viscosity reduction had slowed down or ceased. Fig. 3 shows the ratio of maltose liberating activity to viscosity reducing activity when enzyme + substrate mixtures were incubated at 26° .

Action on glycogen. Glycogen (from mussels) when made up in 8% solution had only about one-third of the viscosity of 4% non-glutinous amylopectin. The action on glycogen was measured in terms of reducing power, dextrinizing

power and decreased turbidity. At substrate concentrations of 4% or lower, reducing values were of the same order as those obtained with glutinous amylopectin. There was a transient increase of turbidity followed by progressive decrease. The preparation was stained reddish brown by iodine: the mixtures became achromic to iodine during the initial stage of increased turbidity.

Fig. 2.

Fig. 3.

Fig. 2. The action of streptococcal amylase on amylopectin. ●—●, the substrate was glutinous corn starch (2%, w/v) diluted in buffer to have an initial flow time of 70 sec. at 26° at pH 7.0. ○-----○, the substrate was purified corn amylopectin (4%, w/v) in buffer which had an initial flow time of 63 sec. at 26° at pH 7.0. The ratio of enzyme (type 2 filtrate) to substrate was 1:9.

Fig. 3. The ratio of saccharifying activity to liquefying activity during hydrolysis of amylopectin by streptococcal amylase.

Ratio: $\frac{\text{percentage of theoretical maltose released}}{\text{percentage fall in viscosity relative to water}}$.

●—●, with enzyme:substrate::1:19.

○-----○, as above, with enzyme:substrate::1:1.

The substrate was glutinous corn amylopectin with same type 2 filtrate as in Fig. 2.

Thus the amylase action on branched chain amylosaccharides was characterized by rapid decrease in viscosity, rapid dextrinization and minimal reducing values during the initial stages of the reaction. Reducing sugars increased steadily after the initial reaction had ceased. These properties are typical of α -amylase, but the culture filtrates may have contained other enzymes, and two or more acting together may produce synergic or abnormal effects.

The synthesis of a new amylosaccharide

The amylase action which has been described appeared to be relevant to another finding, namely the production of a starch-like substance by the same strains growing in different cultural conditions. If heavy suspensions of

amylolytic organisms were incubated in peptone dialysate medium containing 4% human plasma, and 0.4% of either glycogen or starch, little or no amylase was detected compared with the amount produced in the absence of plasma. Centrifuged sediments from 2 hr. culture samples were, however, stained pale blue by iodine solution, and later samples became dark blue or blue-black. This was due to a starch-like substance produced from maltose, in the absence of starch or glycogen, and the reaction did not therefore require an amylosaccharide as primer. Neither glucose-1-phosphate, glucose, cellobiose, melibiose, saccharose nor lactose was an alternative receptor for maltose.

Heavy suspensions of washed streptococci (strain T 2/11020) were incubated in a maltose-free medium, namely sodium β -glycerophosphate (0.6%, w/v) glycogen (0.2%, v/v) and human plasma (8%, v/v). After incubation for 30 min. at 37°, blue-staining cocci were present in wet films treated with iodine. The starch-like substance was detectable macroscopically after 2–3 hr. incubation, the cultures staining with increasing intensity thereafter. Synthesis therefore took place in the presence either of maltose alone, or of glycogen alone with the help of something in human plasma. The cultures tended to become very acid during growth, and if the pH value was not continuously adjusted to about 7.5 the yield of starchy organisms was poor. The findings suggested that maltose, produced through the amylolytic breakdown of glycogen, was being used to build up a new amylosaccharide when cultural conditions were favourable for the synthesis.

Extraction of the new amylosaccharide

Heavy growth of starch-filled streptococci was obtained by growing the strain T 2/11020 in 5 l. or 8 l. cultures containing β -maltose, 10 g./l. sodium β -glycerophosphate, 25 g./l., Casamino acids, 10 g./l. and human plasma, 10%, v/v. The pH value was kept at 7.5 during incubation for 18 hr. More maltose (as 10% solution) was added after 5–6 hr. The streptococci were harvested and washed four times in phosphate buffer (pH 8). One vol. thick cell suspension in buffer was added to 3 vol. 0.1 N-NaOH held at 100°, at the rate of 50 drops/min., and the mixture was mechanically stirred for 10 min. after adding all the suspension, at a temperature just below boiling-point. After several hours at bench temperature the extract was centrifuged at 3000 r.p.m. The supernatant was adjusted to pH 7.2, any precipitate which formed being removed by centrifugation, and was then chilled for 30 min. and stirred into 4 vol. of cold ethanol. A heavy precipitate separated after standing overnight at 0°. This was washed in ethanol and acetone and dried in air, yielding a white powder which was stained blue by iodine. In addition to the iodine-staining material the crude substance contained peptides, protein and nucleic acid.

Properties of the starch-like substance. The powder was soluble in water at pH 8, being more readily dissolved at 50° or higher temperatures. The iodine-staining material did not diffuse through cellophane; it was precipitated by acetone, methanol, isopropanol, butanol, thymol and cyclohexanol thus

resembling the amylose fraction of starch. The complex formed with iodine in neutral solution had a true blue tone; the colour either failed to develop, or faded rapidly, in weakly alkaline solution. Red tones appeared during hydrolysis in acid solution. Aqueous solutions contained occasional round or oval granules like starch granules. The alcoholic precipitates were crystalline, like the addition compounds formed by the amylose fraction of starch with alcohols.

After acid hydrolysis the crude dried substance which contained about 60 % of organic material yielded reducing sugars including glucose. Samples of the amylosaccharide partially purified by precipitation with butanol contained another substance which had a maximum absorption at 260 m μ and was probably nucleic acid. The properties of the amylosaccharide are being further studied.

DISCUSSION

By the degradation of branched chain amylosaccharides like glycogen, amylolytic strains of group A streptococci possess a mechanism for producing maltose which is not a normal constituent of animal tissues. In the presence of something provided by animal plasma, such strains could therefore utilize maltose obtained from glycogen to build up the new amylosaccharide. It has been shown that the synthesis begins within half an hour when amylolytic strains are placed in the appropriate environment. It is clearly possible that such strains may synthesize the starchy substance during passage in the host. Since amyloses readily form all kinds of addition compounds, the presence of the starch might alter the properties of streptococcal products which contribute to pathogenicity. This seems worth consideration with regard to the special types of inflammatory response associated with streptococcal infection in a minority of hosts.

Certain concomitant phenomena in amylolytic strains were previously noted (Crowley, 1954). Many strains freshly isolated from acute infections were untypable by M precipitin tests, and the serotypes most often associated with amylase production were antigenically exceptionally poor for preparation of type specific anti-M sera.

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