Alpha-Amylase of a Synthetic Cereal Species

W. Y. Lee[1] and A. M. Unrau

A comparative investigation of the hybrid alpha-amylase of a synthetic cereal species (alien genome combinant of diploid rye, RR, and tetraploid durum wheat, AABB, giving AABBRR) was conducted. The enzymes were purified by ammonium sulfate and acetone fractionation, gel filtration and refiltration of active fraction followed by preparative electrophoresis on polyacrylamide gel. Ultracentrifugation showed a single component and identical sedimentation for the three enzymes. A mixture of the three enzyme preparations gave one elution peak upon gel filtration, although gel electrophoresis indicated three incompletely resolved bands as analyzed. The hybrid enzyme showed intermediate temperature and pH responses and K_m and V_m values. Significant deviations were observed in the amino acid composition. The results indicate that the AABBRR enzyme is not a mixture of AABB and RR enzymes since this would in all cases result in intermediate values for amino acid composition.

Some general characteristics, particularly gel electrophoretic patterns and amino acid composition of storage type proteins of a synthetic cereal species (alien genome combination) and the parental species, have been reported previously (Yong and Unrau, 1964; Yong and Unrau, 1966) which indicated what may be referred to as hybrid effects. A limited incidence of rather marked discontinuities were, however, also evident. A rather striking feature was the appearance of new, distinct protein bands which were characteristic of one or both parent species. The amino acid compositions of the proteins from the synthetic species were generally intermediate when compared to those for the parent species. Only in relatively few instances was a significantly higher or lower incidence of a particular amino acid observed in protein fractions of the synthetic species (Yong and Unrau, 1966).

A more critical approach to protein biosynthesis in a synthetic species (alien genome combinant) would involve catalytically active (enzyme) proteins. Schwartz (1960, 1962, 1964) reported the isolation of a hybrid esterase in addition to the two parental types in the heterozygotes of maize. The new hybrid enzyme exhibited an intermediate electrophoretic migration rate when compared with the values obtained for the enzymes of the parents. Oda and Iguchi (1963) classified enzymes obtained from a newly synthesized koji mold as having either intermediate activities involving the respective genes from both parents or a more characteristic activity in which case the new species inherited the respective gene from only one parent.

Cereal species are an historic source of alpha-amylase and this enzyme as it occurs in wheat and rye has been extensively investigated (Darkanbaer and Kimbatbekov, 1966; Stewart, 1964). Only one form of alpha-amylase has so far been reported. More recently, a distinct association has been shown to exist between gibberellic acid and alpha-amylase activation (Varner, 1964; Varner and Chandra, 1964; Yomo, 1964). Since amylases can be isolated with relative ease from germinating cereal seed, a comparative study of alpha-amylase of the synthetic species and its parents was undertaken.

Department of Chemistry, Simon Fraser University, Burnaby 2, B.C., Canada.

[1] Present address, Department of Chemistry, McGill University, Montreal, Canada

EXPERIMENTAL METHODS AND RESULTS

The synthetic species, *Triticale*, will be referred to by its historic genome complimentation AABBRR. The parent rye (var. Prolific) will be referred to by its genome compliment RR and the other parent species is tetraploid wheat (var. Stewart durum) with genome compliment AABB.

Alpha-Amylase Development during Germination. Seeds were sterilized by steeping in 80% alcohol for 2 minutes followed by several rinses with water. The treated seeds were steeped in sterilized water for 2 hours, placed between moist filter paper and allowed to germinate (25° C.). The sprouted grains were air dried at 25° for 2 days, and milled. Hagberg's (1961) modified Wohlgemuth method for determination of alpha-amylase activity of wheat and rye was followed with minor alterations.

The alpha-amylase unit is defined as the number of grams of soluble starch dextrinized by 1 gram of malt per hour. The development of alpha-amylase activity during germination is shown in Figure 1.

Effect of Potassium Gibberellate on Alpha-Amylase Development during Germination. Seeds were sterilized as described in the previous section. After several rinses with water, the seeds were steeped in 0.005% potassium gibberellate solution (w./v.) for 2 hours. Germination was allowed to proceed between wet filter papers in petri dishes at 25° C. Further amounts of the 0.005% potassium gibberellate solution were sprayed into the petri dishes during the germinating period. After 1, 2, 3, and 4 days of germination, the malted grains were air-dried (room temp.) for 2 days. The effects of gibberellate on the development of alpha-amylase activity during germination is shown in Figure 2.

Purification of Enzyme. The initial steps in the purification of alpha-amylases were carried out according to the procedure developed by Schwimmer and Balls (1949) with minor modification. Gel filtration on Sephadex and refiltration of active fractions followed by gel electrophoresis (polyacrylamide) were employed in the final purification steps.

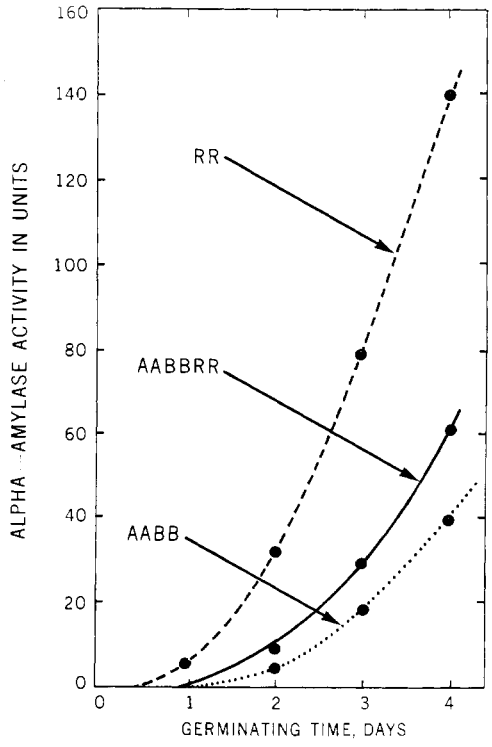

Figure 1. Development of alpha-amylases during germination

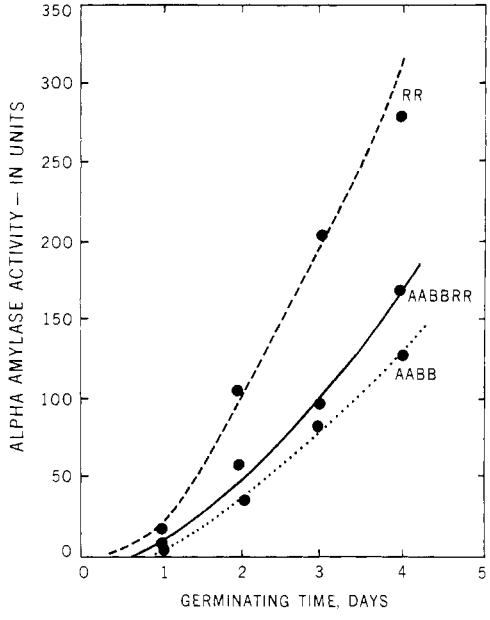

Figure 2. Effects of gibberellic acid on production of alpha-amylases

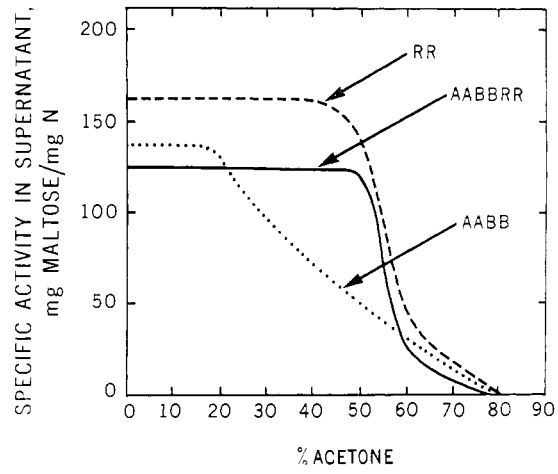

Figure 3. Fractionation of alpha-amylases

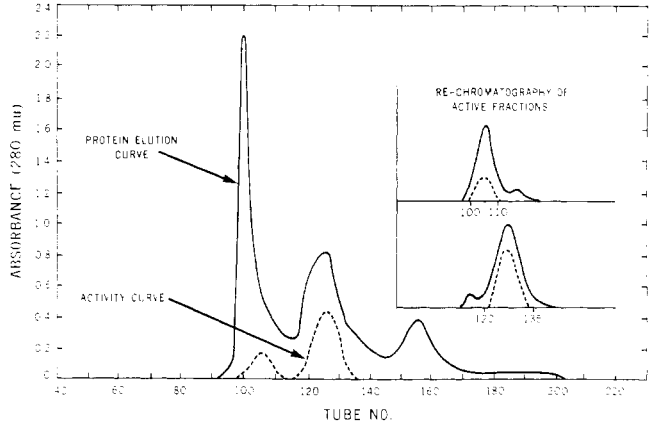

Figure 4. Chromatography of alpha-amylase from durum wheat (AABB) on Sephadex G-50 column

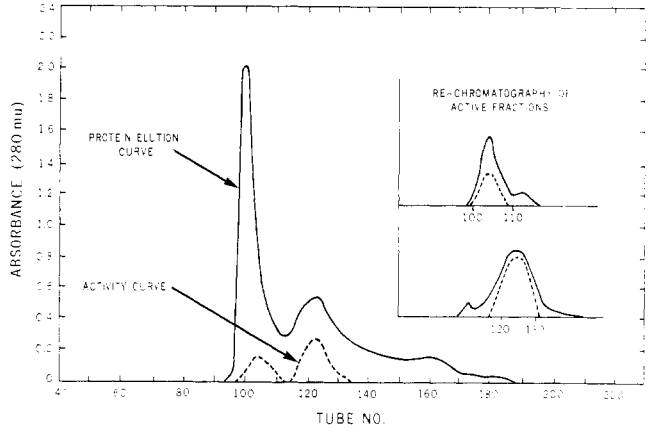

Figure 5. Chromatography of alpha-amylases from Triticale (AABBRR) on Sephadex G-50 column

The precipitates obtained at different degrees of acetone saturation were dissolved in distilled water and the fractions with similar amylase activity combined. A second acetone precipitation was carried out and the fractions were assayed. Most of the active enzyme was found in fractions resulting from 25 to 60% saturation for AABB, 50 to 70% for AABBRR, and 45 to 65% for RR. Figure 3 shows the specific activities of alpha-amylases remaining in the supernatant at different degrees of saturation with acetone. The precipitates were dissolved in 20 ml. of distilled water and dialyzed overnight against dilute acetate buffer (0.0001*M* at pH 5.2). A portion of this alpha-amylase extract (4 ml.), (about 60 mg. of protein nitrogen) was applied to a Sephadex G-50 gel column (3 × 160 cm.) prepared as described by Gelotte (1964). The enzyme was eluted with 0.02*M* acetate buffer (pH 5.2) containing 0.001*M* calcium acetate and the column effluent collected in 3-ml. fractions. Protein content of the effluent was measured spectrophotometrically at 280 mμ. Enzymic activity of the effluent was determined by the 3,5-dinitrosalicylic acid method (Fischer and Stein, 1961). Results are shown in Figures 4, 5, and 6. The main fractions which contained active alpha-amylase were dialyzed overnight

Table I. Characteristics of Alpha-amylases

Characteristic		AABB	AABBRR	RR
Electrophoretic mobility	Run 1	2.36	2.43	2.08
($cm^2\ sec^{-1}v^{-1} \times 10^{-5}$)	Run 2	2.31	2.41	2.08
	Run 3	2.33	2.40	2.05
	Run 4	2.36	2.39	2.06
	Mean	2.34	2.41	2.07
pH Optimum		4.5–4.6	4.7–4.8	4.9–5.0
pH Stability range		4.0–8.4	3.9–7.9	4.0–9.1
Temperature optimum		54–56° C.	54–56° C.	50–52° C.
Energy of activation				
(a) 10 to 20° C.		13.5 Kcal/mole	10.3 Kcal/mole	8.2 Kcal/mole
(b) 30 to 40° C.		−8.1 Kcal/mole	5.4 Kcal/mole	3.1 Kcal/mole
Energy of heat 70 to 80° C. Activation		40.6 Kcal/mole	17.0 Kcal/mole	13.7 Kcal/mole
K_m(mg. starch/ml.)		7.15	6.25	2.78
V_m(mg. maltose-ml./3 min.)		0.62	1.00	1.10

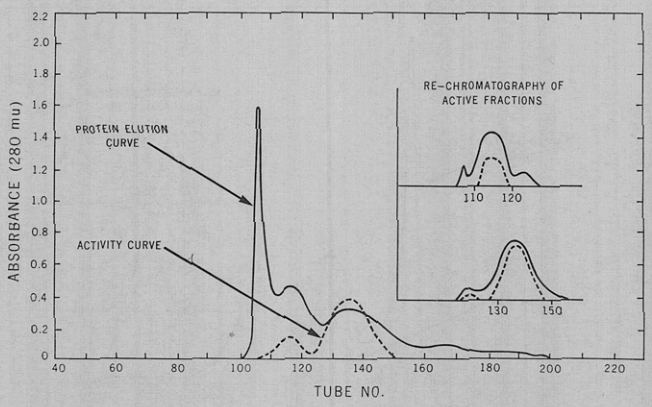

Figure 6. Chromatography of alpha-amylase from rye (RR) on Sephadex G-50 column

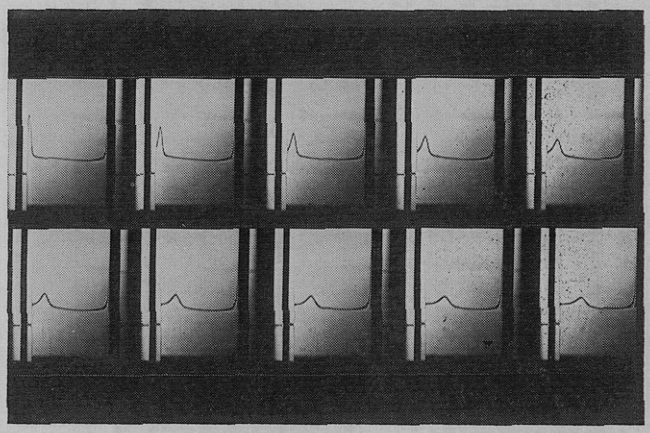

Figure 9. Representative ultracentrifuge pattern of purified enzyme from RR

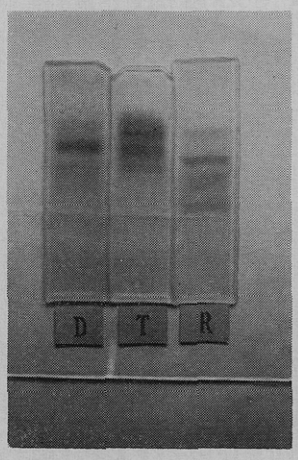

Figure 7. Electropherogram of enzymatically active protein fraction after acetone precipitation (D = AABB, T = AABBRR, R = RR)

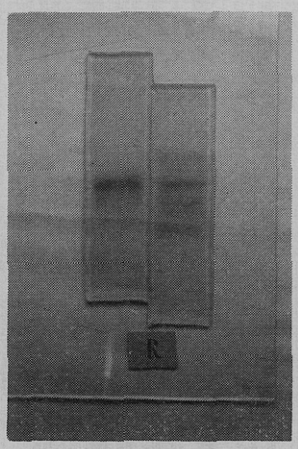

Figure 8. Representative electropherogram of enzyme from RR before and after purification by gel filtration

against dilute acetate buffer, pH 5.8, at 4° C. to remove salts. Pervaporation was subsequently employed to concentrate the enzyme solution. The enzyme protein was rechromatographed and only the material clearly under the activity peaks was collected. Representative electropherograms before and after gel filtration are shown in Figures 7 and 8.

Electrophoretic Mobilities of Alpha-Amylases. The enzyme fractions from gel filtration were subjected to electrophoresis (pH 4.0, aluminum lactate buffer) using polyacrylamide gel strips. The mobilities were determined by applying a constant voltage gradient of 15 v. per cm. and electrophoresing for 6 hours. A single band was observed in the electropherogram for each alpha-amylase preparation. The mobilities are shown in Table I. The enzyme was recovered from the requisite area of the gel by maceration of the gel followed by filtration.

Ultracentrifugation. Enzyme protein shown to be electrophoretically homogeneous was subjected to ultracentrifugation in aluminum lactate buffer, pH 4.0 with and without added urea (2.0*M*). A single sedimenting peak was observed for the three enzyme preparations. The peaks for the three enzymes were superimposable; hence, no apparent difference in their molecular weight could be discerned. A representative ultracentrifuge pattern appears in Figure 9.

Amino Acid Composition. Total amino acid composition (excluding tryptophan) was determined on an acid hydrolysate of samples of the enzyme protein using an amino acid analyser. The composition is given in Table II and expressed in mole per cent.

Temperature Effect on Enzyme Activities. The effect of varying temperature on the enzymatic activity (v) of the isolated enzyme protein was determined colorimetrically as described by Bernfeld (1955) and the results are shown in Table I.

The Arrhenius plot of effect of temperature on enzyme activity is shown in Figure 10. By determination of the temperature coefficient (v_2/v_1) followed by substitution into the empirical Arrhenius formula to express effect of heat on a

Table II. Amino Acid Composition of α-Amylases

Amino Acid	Mole % Amino Acid AABB	AABBRR	RR
Lysine	4.92	3.50[a]	4.62
Histidine	2.06	1.08[a]	2.68
Arginine	4.95	5.58[b]	4.82
Aspartic acid	10.37	7.65[a]	11.34
Threonine	3.85	3.33[a]	4.06
Serine	5.14	5.66[b]	4.08
Glutamic acid	11.82	11.40	10.23
Proline	8.25	7.78	6.36
Glycine	11.51	8.55[a]	11.12
Alanine	11.94	11.06	8.93
Half cystine	5.31	7.89[b]	3.39
Valine	8.05	7.72	6.81
Methionine	2.46	2.54	2.19
Isoleucine	4.71	2.59[a]	5.13
Leucine	7.84	7.72	6.72
Tyrosine	4.70	4.00	3.62
Phenylalanine	3.23	1.99[a]	3.66

[a] Significantly lower than either parental genome.
[b] Significantly higher than either parental genome.

chemical reaction, values of activation and heat inactivation were calculated (Table I).

$$v_2 = v_1 e^{\frac{E}{R}\left(\frac{T_2 - T_1}{T_2 T_1}\right)}$$

Influence of pH on Enzyme Activity. A 2% starch in water solution was diluted to 1% with an equal volume of buffer solution at various pH values. The buffer solutions (acetate, phosphate, carbonate-bicarbonate) were prepared as described by Gomori (1955). Table I shows the influence of pH on activities (pH optima) of alpha-amylases which had been incubated in buffer for 2 hours.

The pH stability of alpha-amylases was investigated by incubating alpha-amylases in buffer solution at various pH values for two hours after which the activities were determined at pH 5.2. The results are shown in Table I.

Determination of Michaelis-Menten Constants. The alpha-amylases from AABB, AABBRR, and RR were diluted with 0.02*M* acetate buffer containing 0.001*M* calcium acetate at pH 5.2 to a final concentration of 0.06 mg. of nitrogen per ml. Starch solutions of various concentration in the range of 0.5 to 50 mg. per ml. were prepared in 0.02*N* acetate buffer, pH 5.2. The temperature of all enzyme and starch solutions were brought to 25° C. One milliliter of starch solution of each concentration was transferred into a series of test tubes and 1 ml. of enzyme solution was introduced into each tube by means of a fast-delivery pipet. After exactly 3 minutes, 1 ml. of the reaction mixture was analysed for reducing sugar with 3,5-dinitrosalicylic acid reagent. Michaelis-Menten constants were determined graphically by plotting the reciprocal values of starch concentrations, *S*, against the reciprocal values of reaction velocities, *v* (Figure 11). The values obtained are summarized in Table I.

DISCUSSION

Beta-amylase appears in fairly high concentrations in ungerminated grains of cereals and its activity increases only to a limited extent during sprouting. In contrast, the concentration of alpha-amylase in ripe grains is very low before germination while during the germination process, the enzyme develops rapidly (Kansugai, 1963; Kneen *et al.*, 1942, 1944; Schwimmer, 1947). Investigations on development

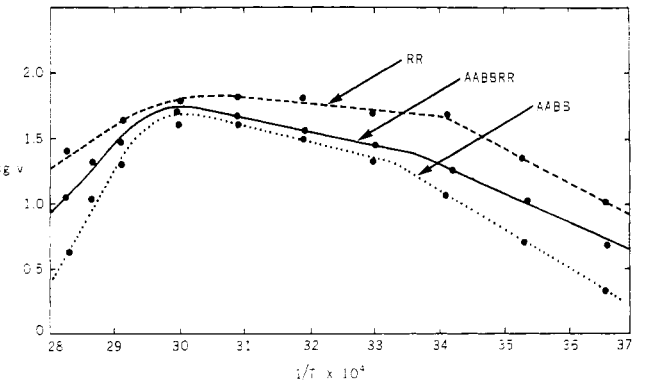

Figure 10. Arrhenius plots for effects of temperature on activities of alpha-amylases

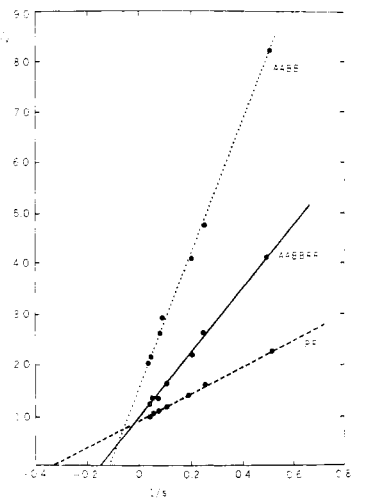

Figure 11. Determination of Michaelis-Menten constants, K_m for alpha-amylases

of alpha-amylase specifically in wheat (Graham, 1963; Olered, 1964; Ugrumow, 1935), rye (Ohlsson and Uddenberg, 1933), and barley (Luers and Rummler, 1933) clearly showed a rapid increase of the enzyme as soon as germination was initiated.

As seen in Figure 1, the activities of alpha-amylases were very low in the ungerminated grains of AABB, RR, as well as the synthetic species, AABBRR. Once the germination process had started, the activities of alpha-amylases increased rapidly and logarithmically as observed in other cereals. The amount of alpha-amylase that developed in the synthetic species was intermediate between that of its parental species, the ratio being 1:1.5:3.5 for AABB, AABBRR, and RR in that order. The same phenomenon was observed in the ungerminated grains.

Gibberellins affect activation of alpha-amylases in cereals during germination (Brian, 1958) as well as the total amount of enzyme produced (Yomo and Iinuma, 1963). The same general effect was noted in our experiments in that α-amylase development was two to three times faster in the treated seed. The gibberellic acid response of alpha-amylase production was species dependent and in agreement with the results of Fleming and Johnson (1961) and Griffiths *et al.* (1964). The relative amount of alpha-amylases present in the gibberellic acid-treated seed of the three species after germination for 4 days was in the ratio of 1:1.3:2.2 for AABB, AABBRR, and RR in that order. Alpha-amylase production in AABB-RR was intermediate between that found in the parental

species further indicating that the biosynthesis of the enzyme appears to follow a hybrid pattern.

Calcium chloride solution (0.2%) was used in the extraction of alpha-amylases because calcium ion has been shown to stabilize and activate the enzyme (Fischer and Hasebach, 1951). At least a ten-fold increase in specific activity was attained using a combination of conventional precipitation together with gel filtration and electrophoresis (Gelotte *et al.*, 1964; Shulman and Apattseva, 1965). The electrophoretic mobilities using aluminum lactate buffers, of alpha-amylases from the three species were different from one another and the electrophoretic mobilities of the enzymes were pH dependent. Electrophoresis of a mixture of the three enzymes resulted in a wide band with no clear separation of the three proteins. Differences in electrophoretic mobilities of the individual enzymes indicated that the alpha-amylase of the synthetic species was intrinsically different from the parental types. This is one of the few departures from the normal hybrid pattern observed in the alien genome combinant. The apparent difference in the charge could be a reflection of an altered amino acid composition and molecular size.

The greatest discontinuity in the comparisons between the enzyme from the hybrid species and the parental species appears in the amino acid composition of the three enzyme proteins. In seven instances, the mole per cent of amino acids in the hybrid enzyme is significantly lower than in either of the parental type enzymes (lysine, histidine, aspartic acid, threonine, glycine, isoleucine, and phenylalanine). The mole per cent of arginine, serine and "half"-cystine was significantly higher. The values for the remaining amino acids analyzed were intermediate or characteristic of one or the other parent.

The sedimentation patterns of the three purified enzymes were superimposable using aluminum lactate buffer with or without added urea. This indicated that the three enzymes possessed identical molecular weights. Since gel filtration has also been used in determining molecular size, the elution of AABB and AABBRR also occurred at similar tube numbers (Figures 4 and 5) while for RR, a slightly longer elution was necessary.

Amylases have been found to possess activity optima at temperatures between 50° and 65° C. The reaction rate decreased rapidly as the temperature was elevated above the optimum temperature. This is mainly due to denaturation of the enzyme protein through disruption of intramolecular hydrogen bonds, which are essential for maintenance of the secondary and tertiary structure. The energy of heat inactivation of the enzyme is therefore necessarily always higher than the apparent energy of activation. Ernstrom (1922) found the energy of activation of malt amylases to be 12,300 calories per mole at pH 5.5 at a temperature range between 10° and 30° C. Eyring and Stearn (1939) determined the energy of inactivation of malt amylases to be 41,630 calories per mole. Luers and Wasmund (1921–22) have reported a temperature coefficient for malt amylase of 1.96 between 20° and 30° C. and an energy of inactivation of 42,500 calories per mole. These values are in good agreement with those of the alpha-amylase from AABB. The energy of activation and energy of heat inactivation of the amylase-starch system of the synthetic species, AABBRR, were of intermediate numerical value, thus further indicating hybrid nature of the enzyme.

The Arrhenius plots (Figure 7) show straight lines with a discontinuity as described by Meyer *et al.* (1953) and Markovitz *et al.* (1956). The break has been observed in many cases, *e.g.*, urease-urea system, invertase-sucrose system, amylase-starch system, etc., but no adequate explanation has been offered.

In certain instances, several factors may alter the pH optimum of an enzyme, such as types of buffer, purity of enzyme, length of reaction time, and temperature, etc. Alpha-amylases from the three sources were found to be stable over a pH range between 4.0 and 9.0 (Table I). When the amylases were incubated in buffer for two hours before determining the activity, an optimum pH between 4.5 to 5.0 was observed; AABB, 4.5 to 4.6; AABBRR, 4.7 to 4.8; and RR, 4.9 to 5.0.

The rate of an enzyme-catalyzed reaction ordinarily increases with increasing substrate concentration (except where the reaction product may act as an enzyme inhibitor) and a typical rate curve may be plotted. The values of the Michaelis-Menten constant (K_m) and maximum velocity (V_m) for the alpha-amylase from the synthetic species was intermediate between those of the parental species (Table I). The values of the Michaelis-Menten constants for alpha-amylases are much smaller than those found for beta-amylases. Since a high K_m value indicates a low enzyme-substrate affinity and vice versa, alpha-amylases must therefore have a higher affinity toward the starch substrate than beta-amylases. These results are in agreement with the reports of Hopkins (1946). This can be explained by the fact that alpha-amylases are endoamylases which act randomly on the α-1,4-glucopyranoside linkage of amylose and amylopectin molecules, whereas beta-amylases are exoamylases which are capable of attacking the polysaccharides only from the nonreducing outer chain ends and breaking every alternate bond to produce maltose. Consequently, the frequency factor for the attachment of alpha-amylases to these polysaccharides is considerably greater than those of beta-amylases.

The values of K_m for alpha-amylase from RR is lower than those from AABB and AABBRR. Recalling the results which indicated that the purified alpha-amylase from rye had the highest specific activity of the three, these results are therefore in agreement. Having a low value of K_m, alpha-amylase from rye acts on polysaccharides faster than the other two alpha-amylases; hence, a higher specific activity is observed. The results show that alpha-amylase of the synthetic species, AABBRR, has an intermediate affinity toward the starch substrate. These observations indicated that the intermediate properties of the biologically active alpha-amylase in the hybrid species is largely inherited from both parental species as a direct result of the interaction between the two parent genomes in the hybrid species.

Although a number of characteristics of the AABBRR enzyme may be described as hybrid in character and could be explained by two enzymes, AABB and RR, acting in admixture, the distinct deviations from such an averaging effect in the amino acid composition rule against this possibility. Precisely how the hybrid enzyme is generated is as yet difficult to explain.

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