Beta-Amylase of an Alien Genome Combinant

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ABSTRACT

The genome of tetraploid wheat, n=14, genome notation AABB, has been successfully combined with diploid rye, n=7, genome notation RR, to yield a viable synthetic species (alien genome combinant), n=21, AABBRR. A comparative study of the beta-amylase of this alien genome combinant was undertaken. With a few notable exceptions, the hybrid enzyme exhibited intermediate physical properties. In both parental species and the synthetic species, two polymorphic forms of the enzyme were detected by gel electrophoresis. The components of the hybrid species showed rather distinctly lower relative mobilities. The hybrid enzyme contained three free SH groups, whereas one parent (AABB) had four and the other (RR) had two. The sedimentation velocities and apparent molecular weights were identical. The differences in the amino acid composition of the hybrid enzyme strongly indicated the existence of a discrete protein.

A species hybrid (alien genome combination) was successfully synthesized by Rimpau combining (crossing) rye (*Secale cereale*) and wheat (*Triticum*) species as early as 1888 (1). When tetraploid wheat, e.g. *Triticum durum*, with a genomic constitution of AABB is crossed with diploid rye, *Secale cereale*, genomic constitution RR, a triploid zygote is obtained with a necessary genomic constitution of ABR and which would not develop as such. However, treatment of such a zygote with colchicine will induce chromosome duplication and a fertile hexaploid species AABBRR is obtained. This synthetic species is appropriately referred to as *Triticale*. Octaploid alien genome combinants have also been synthesized by combination of the common hexaploid wheat, e.g. *Triticum vulgare*, genome constitution AABBDD, with diploid rye (RR) to eventually give a combinant with genome constitution of AABBDDRR.

The synthesis of species hybrids of the above type adds a further dimension in the study of transcription of genetic information. It is obvious that with a rather complex system such as the alien genome combinants described, it would probably be more difficult to detect small changes. In some comparative studies previously undertaken, a number of discontinuities in electrophoretic patterns and amino acid composition of storage proteins have been observed (2,3). Since changes were evident in the storage-type proteins, comparative investigations concerning characteristics of enzyme proteins in the alien genome combinant and the parental species were pursued. The alpha-amylase of the species hybrid was found to have intermediate characteristics except in the case of electrophoretic mobility in polyacrylamide gel and amino acid composition (4). Schwartz (5,6,7) found a hybrid esterase in the heterozygotes of maize which had intermediate electrophoretic mobility when compared to the parental types which were also present. Alston et al. (8) report the presence of "hybrid" phenolics in natural interspecific hybrids of Lotus.

In the present investigation, a comparative study was undertaken of the beta-amylases of the parent species, AABB and RR, and the alien genome combinant, AABBRR.

TABLE I. SPECIFIC ACTIVITY OF BETA-AMYLASE FRACTIONS

	Specific Activity		
	AABB	AABBRR	RR
Supernatant of $(NH_4)_2SO_4$ soln.			
0.1M	461	342	207
0.2M	408	328	194
0.3M	245	210	153
0.4M	60	92	93
Gel electrophoresis			
Component 1	2,260(2.02)[a]	1,640(2.00)	1,020(2.16)
Component 2	2,120(1.88)	1,610(1.77)	992(2.05)

[a]The figures in parentheses are the electrophoretic mobilities ($cm.^2 v.^{-1} sec.^{-1} \times 10^{-5}$).

EXPERIMENTAL METHODS

Preparation of Flour Samples

Seed of the hexaploid synthetic species, *Triticale* (AABBRR), and those of the two parental species, the tetraploid wheat, *Triticum durum* var. Stewart (AABB) and diploid rye, *Secale cereale* var. Prolific (RR), harvested in 1964, was stored at 40°C. before milling. The moisture content of the seed was adjusted to 14%, and 5- to 10-lb. samples were milled in a Buhler experimental mill. Flour samples obtained were consequently stored at 4°C. until further use.

Isolation and Purification of Enzyme

Flour samples were steeped for 12 hr. in 1% NaCl solution containing 10^{-4}M EDTA. Alpha-amylase was precipitated by acidification to pH 3.7 and storage at 4°C. for 6 to 7 days. The pH of the clear solution obtained after centrifugation (18,000 r.p.m., 20 min., 4°C.) was adjusted to 5.3 with ammonium hydroxide followed by ammonium sulfate fractionation (9,10), and the fraction with highest specific activity was retained for further purification (see Table I).

The crude enzyme, 2% protein, in 0.01M acetate buffer (10-ml. aliquots), pH 4.8, was subjected to gel filtration (Sephadex G-50, 3 X 160-cm. column). The effluent was monitored spectrophotometrically (280 mμ) and specific activity was determined by the 3,5-dinitrosalicyclic acid procedure (11). Enzyme protein which emerged at the activity peak was collected and, after concentration, refiltered through the gel column until no "side bands" remained (Figs. 1, 2, 3). The enzyme was further purified by gel electrophoresis with 6% (w./v.) polyacrylamide (12) which had been equilibrated with aluminum lactate buffer (8.5×10^{-3}M, pH 4.1, ionic strength 0.05) (13,14). Extended electrophoresis of 50 to 200 mg. enzyme protein gave two active isomorphs which were extracted from the gel (Fig. 4 and Table I).

Temperature Effect on Activity

Enzyme solutions were diluted to 0.06 mg. nitrogen per ml. Specific activities were determined at a range of temperature (Fig. 5 and Table II) and are expressed as mg. maltose liberated from a 1% starch solution in 3 min. (15).

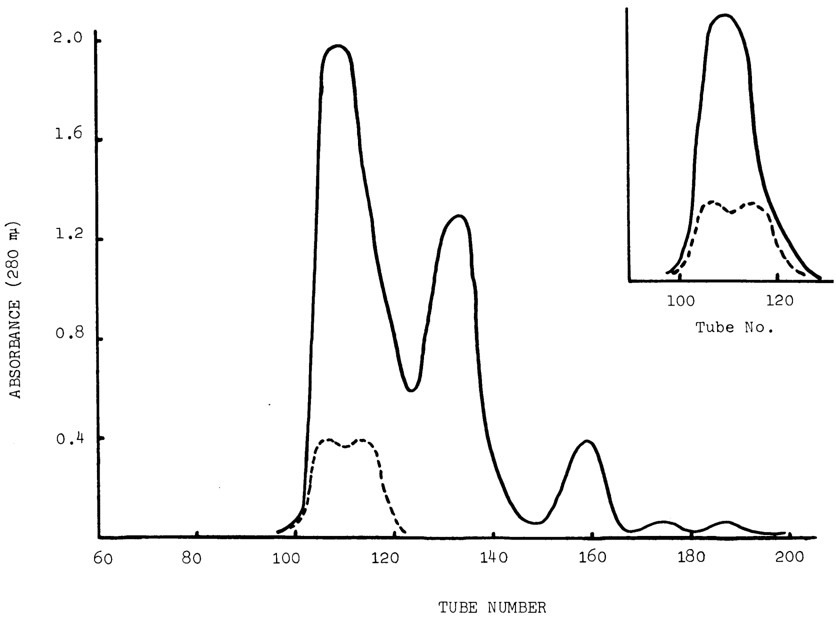

Fig. 1. Gel filtration and refiltration of beta-amylase fraction from AABB. Inset: absorbance and activity curve after third filtration.

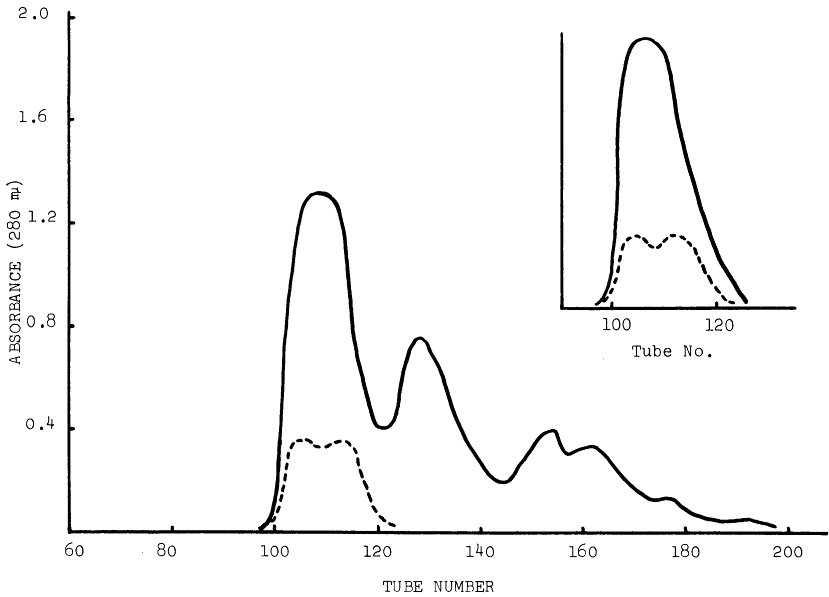

Fig. 2. Gel filtration and refiltration of beta-amylase fraction from AABBRR. Inset: absorbance and activity curve after third filtration.

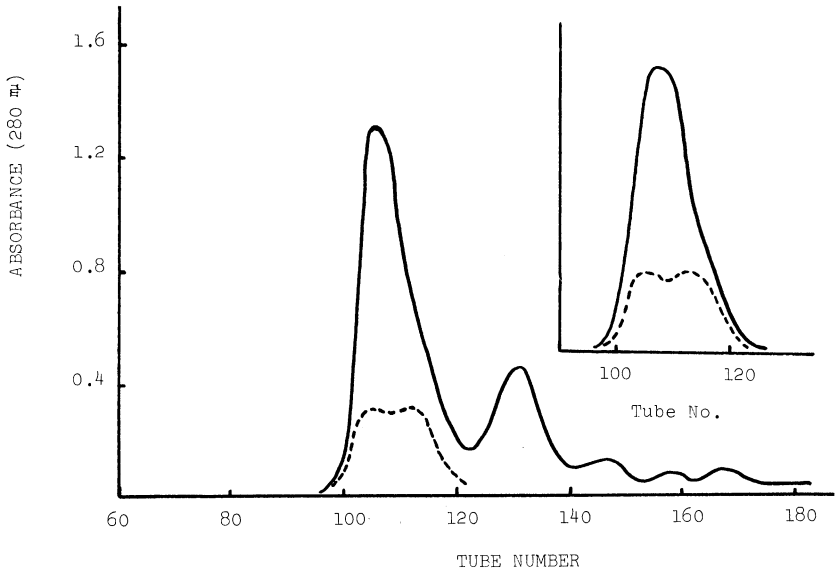

Fig. 3. Gel filtration and refiltration of beta-amylase fraction from RR. Inset: absorbance and activity curve after third filtration.

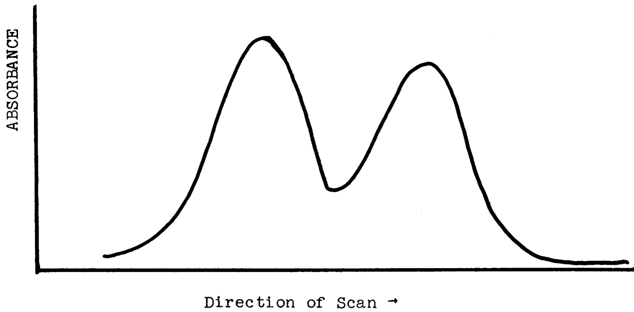

Fig. 4. Densogram trace of gel electropherogram of beta-amylase from AABBRR after third gel filtration. Spectrum trace of polyacrylamide gel slab, 1/8 in. thick, 1 in. wide, and 10 in. long. Incandescent light source, motor-driven stage. Staining procedure of developed gel as described in text.

TABLE II. SOME PHYSICAL PROPERTIES OF ISOLATED BETA-AMYLASES

	Temp. Optimum °C.	Energy of Activation 10°–20°C. cal./mol.	Energy of Activation 20°–30°C. cal./mol.	Energy of Heat Inact. 50°–60°C. cal./mol.	pH Optimum	K_m	V_m
AABB	50–55	11,300	7,440	15,500	4.6	10.0	2.50
AABBRR	50–55	12,500	7,210	16,200	4.6	11.1	1.92
RR	50–55	12,800	6,040	35,400	4.6	12.5	1.70

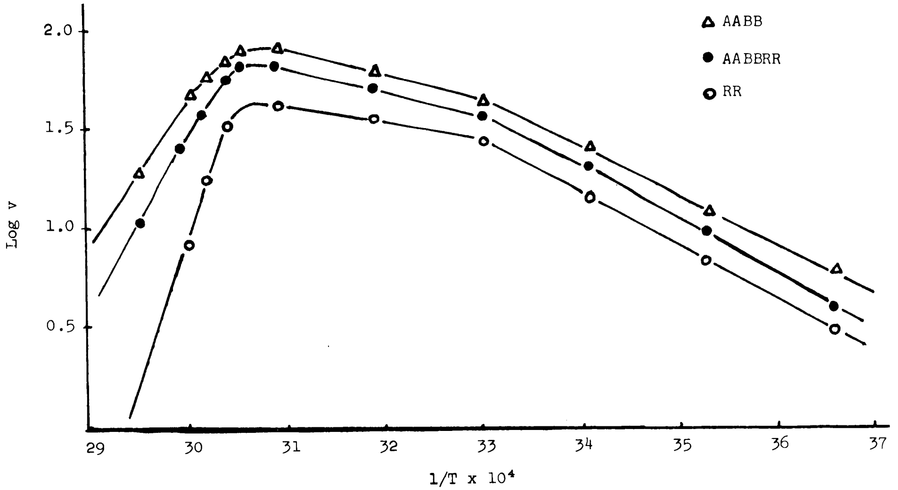

Fig. 5. Effect of temperature on enzyme activity (Arrhenius plot).

Determinations

pH Optimum. The specific activities of the enzyme preparations were determined over a total pH range of 3.0 to 10.6 with acetate buffer (0.1M), pH range 3.0 to 5.6; phosphate buffer (0.1M), pH range 6.0 to 8.0; and sodium bicarbonate-carbonate buffer (0.1M), pH range 9.2 to 10.6 (Fig. 6).

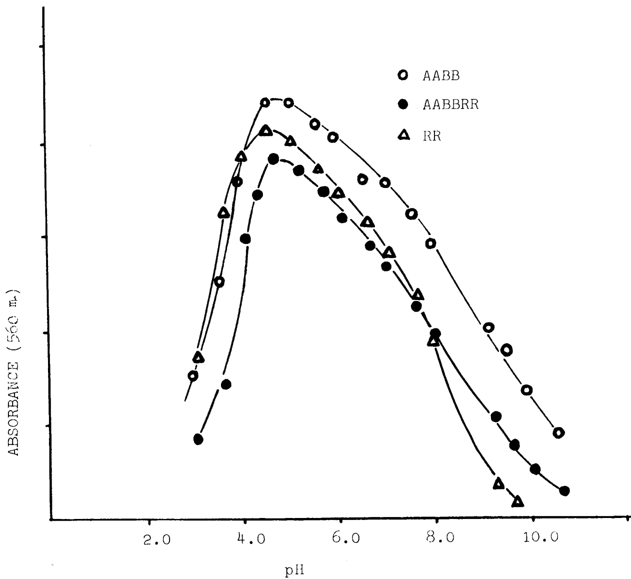

Fig. 6. Influence of pH on activity (reducing power) of beta-amylases from AABB, AABBRR, and RR. The two isomorphous forms from each species, as shown by gel electrophoresis, were not studied independently.

TABLE III. DETERMINATION OF SULFHYDRYL GROUPS IN BETA-AMYLASES

Species	Component	SH µM/mg. N	No. SH per Molecule
AABB	1	0.405	4.2
	2	0.395	4.1
AABBRR	1	0.316	3.2
	2	0.305	3.1
RR	1	0.214	2.2
	2	0.209	2.2

Enzyme-Substrate Affinity. The Michaelis-Menten constants for the enzyme preparations were determined with 0.008M acetate buffer, pH 4.8 (Table II).

Sulfhydryl Groups. Sulfhydryl groups were determined by spectrophotometric titration (16) involving mersalyl acid, the anhydride of O-([3-(hydroxy-mercuri-2-methoxypropyl)]-carbonyl)-phenoxyacetic acid and an azopyridine dye (pyridine-2-azo-*p*-dimethylaniline) (Table III).

Ultracentrifugation Analysis

Protein samples eluted from polyacrylamide gel slabs were finally taken up in phosphate buffer (pH 6.5) containing 8M urea. The protein concentration was 0.60% in each run. Each component was run separately and in a 1:1 admixture and, finally, a 1:1 admixture of the whole enzyme complex from each of the parental species (AABB and RR) was run as well. A representative pattern of the total beta-amylase of AABBRR is shown in Fig. 7. Approximate molecular weights were estimated with the use of published constants (10). The sedimentation coefficients

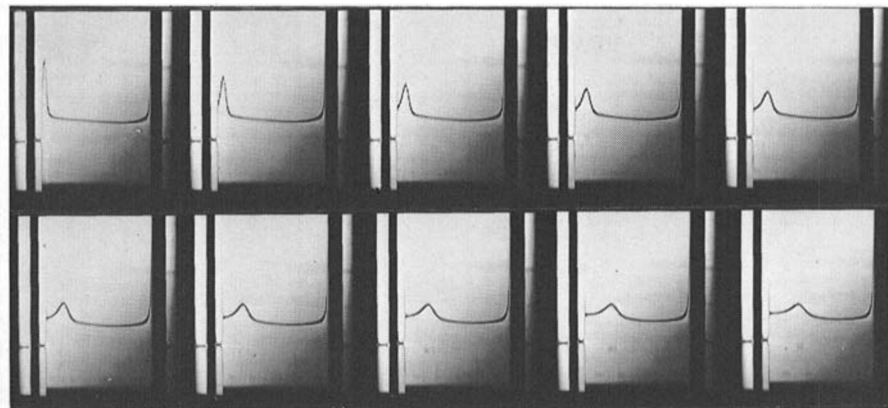

Fig. 7. Ultracentrifuge pattern of combined beta-amylase (components 1 and 2) from AABBRR, 0.6% concentration, phosphate buffer, pH 6.5, containing 8M urea, ionic strength 0.09 (NaCl), rotor speed 52,000 r.p.m., exposure intervals 16 min.

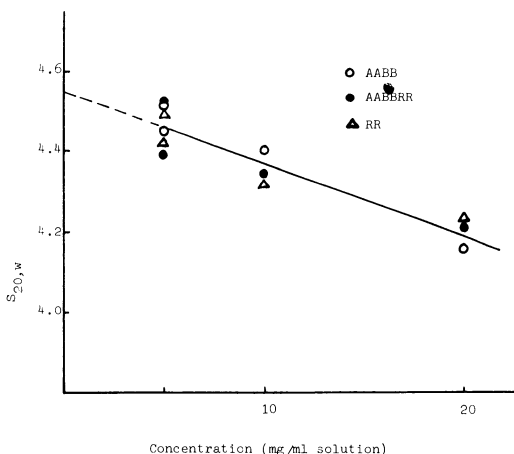

Fig. 8. Dependence of sedimentation coefficients (normalized to viscosity and density of water at 20°C.) on concentration.

$$MW = \frac{(8.314\times10^{7})\ (293.1)\ (4.57\times10^{-13})}{(6.48\times10^{-7})\ (1-0.733)\ (0.998)} \cong 64{,}000$$

reduced to standard conditions were estimated from the slope of the line from a plot of log (distance of boundary to axis of rotation) vs. time (17). A plot of S vs. concentration of protein and extrapolation to zero concentration gives $S^{o}_{20,w}$. Sedimentation values ($S_{20,w}$) for the three enzyme proteins are shown in Fig. 8. Extrapolation to zero concentration results in an $S^{o}_{20,w}$ value of 4.57. In the calculation for molecular weight, the values used for D (diffusion coefficient), $\bar{v}$ (apparent specific volume), and ρ (density of the medium) were those reported by Tkachuk and Tipples (10). Approximate molecular weights were also estimated by comparing the elution time of the proteins to several proteins of known molecular weight with the use of a Sephadex G-50, 3 X 160-cm. column. The results thus obtained are shown in Fig. 9.

Amino Acid Composition

The amino acid composition (excluding tryptophan) of each component was determined in the usual manner with an amino acid analyzer (Table IV). The protein was hydrolyzed in thick-walled, sealed Pyrex glass tubing (6 to 8 mg., 5 ml. 6N HCl, flushed with oxygen-free nitrogen) at 105°C. for 22 hr. The contents of the tubes were diluted (about 20 ml.) and then evaporated to dryness; citrate buffer (pH 2.2) was then added. Humin was removed by filtration and the filtrate made to volume with buffer. Total nitrogen recovery was greater than 89% in all analyses. The composition of a 1:1 admixture of active protein from the two parental species (AABB and RR) was also determined to illustrate an arithmetic average amino acid composition (Table V).

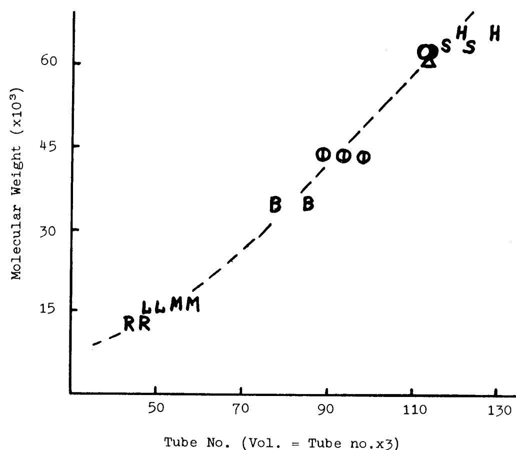

Fig. 9. Plot of elution volume (tube no. X 3 ml.) against MW of various proteins and the three beta-amylases. Gel column described in text. Open circles, AABB; solid circles, AABBRR; triangle, RR; H, hemoglobin (bovine); S, serum albumin; circles with vertical bar, ovalbumin; B, betalactoglobulin; M, myoglobin; L, lysozyme; R, ribonuclease.

TABLE IV. AMINO ACID COMPOSITION OF BETA-AMYLASES[a](mol. % amino acid[b])

Amino Acid	AABB		AABBRR		RR	
	1 mol. %	2 mol. %	1 mol. %	2 mol. %	1 mol. %	2 mol. %
Lysine	3.40	3.49	3.36	3.31	3.10	2.91
Histidine	2.39	2.48	2.23	2.18	2.76	2.80
Arginine	4.36	4.43	3.98	3.95	3.91	3.89
Aspartic acid	8.18	7.91	6.34	6.40	6.30	6.34
Threonine	4.60	4.78	4.73	4.75	5.16	5.12
Serine	6.10	6.12	6.36	6.50	5.60	5.60
Glutamic acid	14.40	14.52	19.15	18.50	18.00	18.76
Proline	9.94	9.74	10.55	10.55	10.68	10.54
Glycine	9.16	9.27	8.23	8.18	9.76	9.75
Alanine	5.67	5.63	7.05	7.46	7.60	7.64
Half-cystine	2.98	3.03	3.72	3.72	3.72	3.68
Valine	6.83	6.75	6.43	6.31	6.60	6.60
Methionine	1.39	1.40	1.48	1.36	1.29	1.20
Isoleucine	3.38	3.45	3.57	3.62	3.75	3.77
Leucine	8.64	8.50	7.54	7.74	7.38	7.26
Tyrosine	3.23	3.17	2.09	2.23	1.60	1.66
Phenylalanine	4.10	4.09	3.27	3.42	3.14	3.14

[a]Recovery of amino acid nitrogen greater than 89% in all analyses.

[b]Values for ammonia, presumed to arise from glutamine and asparagine, have not been included in calculating the mol. % amino acid composition. The values are presented in mol. % to facilitate easy comparisons between the three enzymes.

TABLE V. AMINO ACID COMPOSITION OF "TOTAL" BETA-AMYLASES AND OF A 1:1 MIXTURE FROM THE TWO PARENT SPECIES (AABB AND RR)

Amino Acid	AABB mol. %	AABBRR[a] mol. %	RR mol. %	1:1 Admixture of AABB and RR mol. %
Lysine	3.44	3.34a	3.00	3.28
Histidine	2.44	2.21b	2.78	2.65
Arginine	4.39	3.97d	3.90	4.63
Aspartic acid	7.90	6.37d	6.32	7.09
Threonine	4.68	4.74e	5.14	4.82
Serine	6.11	6.43a	5.60	5.85
Glutamic acid	14.15	18.82c	18.28	16.22
Proline	9.64	10.55d	10.52	10.02
Glycine	9.06	8.20b	9.66	9.36
Alanine	8.48	7.25b	7.62	8.05
Half-cystine	2.98	3.72d	3.70	3.30
Valine	6.85	6.47e	6.60	6.70
Methionine	1.39	1.42e	1.24	1.32
Isoleucine	3.43	3.60a	3.76	3.58
Leucine	8.56	7.64a	7.32	7.98
Tyrosine	3.19	2.16a	1.63	2.41
Phenylalanine	4.07	3.34d	3.14	3.60

a, Hybrid; b, lower than in either parent genome; c, higher than in either parent genome; d, characteristic of RR genome; e, characteristic of AABB genome.

DISCUSSION OF RESULTS

Both parental species (AABB and RR) have had considerable opportunity to diverge and consequently might have generated enzymes which could have rather distinct physical properties. Two such enzymes which may be studied with some ease because of their common occurrence in both parental species are alpha- and beta-amylase. Beta-amylase activity is dependent on the preservation during isolation of free sulfhydryl groups, and to this end EDTA was employed.

Gel filtration of the active fraction (supernatant from 0.2 to 0.2 $(NH_4)_2SO_4$ saturation) indicated that two equally active components appeared to be present but could not be resolved, presumably because of rather similar molecular size. Fortunately, a significant difference in their electrophoretic mobilities provided a means of resolution and further purification, and each component (isomorph) could be studied individually. Earlier work on beta-amylase indicated that the enzyme was homogeneous, e.g., malt and wheat (18,19), sweet potato (20,21), soybean (22,23). More recently, beta-amylase from cereals has been resolved into a number of active components with the use of ion-exchange chromatography and gel filtration (24,25). Tkachuk and Tipples (10) report resolution of wheat beta-amylases into three components with the use of ion-exchange chromatography.

It will be evident (Table I) that the two components from the hybrid species (AABBRR) did not show an intermediate migration rate; in fact, component 2 showed a significantly lower migration rate than component 2 of AABB. This should be contrasted to the pronounced intermediate specific activity observed for the alien genome combinant. Since specific activity of the beta-amylase has been

associated with the number of free sulfhydryl groups per molecule, it is not surprising to observe the intermediate specific activities for both components from AABBRR.

No detectable difference was found in the temperature optimum and pH optimum of the enzymes from the three different species. Examination of the values obtained for energies of activation (of the E-S complex) and the energy of heat-inactivation showed again that the enzyme from the hybrid species had intermediate (hybrid) properties. The difference in the E_{act} of AABB and RR at 10° to 20°C. and 20° to 30°C. is only 1.4 to 1.5 kcal. in either case; the values for AABBRR are nevertheless intermediate. The energy of heat-inactivation of the beta-amylase from RR is much larger than for AABB, and in this comparison the value for AABBRR is much more characteristic of that of AABB and structural changes associated with heat-inactivation follow more closely those associated with only one parental species.

The Arrhenius plots for effect of temperature on activity of beta-amylases show a break for each of the individual enzymes, as reported earlier for other enzyme systems (19,26). Plots of the above type have been observed for the urease-urea system (27), invertase-sucrose (28), and amylase-starch (27,29). It has been suggested that the apparent transition may be due to a characteristic change in the configuration of the enzyme molecules (30,31).

The Michaelis-Menten constants of the enzymes from the three species show that the beta-amylase from AABBRR had an intermediate affinity for the substrate. The K_m values obtained for the three enzymes are in good agreement with the specific activities observed for each enzyme. It appears, therefore, that the characteristic affinity of the enzyme from AABBRR was equally affected by both parental genomes.

One of the important characteristics of beta-amylase is that destruction (e.g., oxidation) of the free sulfhydryl groups results in loss of catalytic activity. The results for number of sulfhydryl groups per enzyme molecule (four) for AABB are in good agreement with those reported for wheat beta-amylase (10). Assuming that the beta-amylase of rye has a reasonably similar molecular weight, it is apparent that only half the number of free sulfhydryl groups (two) are present. It is indeed interesting to note that the beta-amylase from the species hybrid shows definite hybridity in that three sulfhydryls are present per molecule. First, it should be noted that no difference in number of sulfhydryls is present between the two beta-amylase components for each species. Second, the number of sulfhydryls is directly proportional to the specific activity of the individual enzymes. Third, all the evidence (gel filtration, gel electrophoresis) indicates that the hybrid enzyme is not an equimolar mixture of the two characteristic parental types. If in fact the hybrid enzyme was constituted of an equimolar mixture of the parental types, then at least four distinct bands should have been evident in the electrophoretic pattern. This was not observed, although a synthetic mixture of the two parental enzymes gave the expected complex pattern with, however, the 1.77 component characteristic of AABBRR missing.

Ultracentrifuge studies indicated that the enzyme components from each species were homogeneous and possessed very similar molecular weights as judged from the superimposability of all individual patterns. An estimated molecular weight from

sedimentation and from gel filtration of about 64,000 is in close agreement with published results (10,31).

A study of the amino acid composition of each component within one species indicated only very minor differences which were not of sufficient magnitude to indicate any significance. A comparison between species of the total amino acid (excluding tryptophan) composition of the beta-amylase protein indicated significant differences. The instances in which the individual amino acid content of the hybrid enzyme from the AABBRR genome was significantly higher or lower than in both parent genomes and where it was definitely characteristic of either one or the other parent genome definitely rule against the existence of a 1:1 molar mixture of AABB and RR enzyme in the synthetic (AABBRR) species. Coupled with the above observation, in a number of instances, some amino acids in the AABBRR enzyme were present at a hybrid (average) level. The nature of the transcription process in the elaboration of the beta-amylase protein and, for that matter, other protein constituents in the hybrid species, must certainly be rather complex. It is known that in the natural genome combinations of which common bread wheat is an example, extraction of one genome results in a tetraploid species which has lost many characteristics common to natural tetraploid wheat, e.g. *Triticum durum*.

Acknowledgment

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