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COMPARATIVE STUDIES OF AMYLASES IN AN ALIEN GENOME COMBINANT

A THESIS

SUBMITTED TO THE DEPARTMENT OF CHEMISTRY AND SENATE COMMITTEE ON GRADUATE STUDIES OF SIMON FRASER UNIVERSITY

by Weng Yek Lee

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A. INTRODUCTION

Triticale, which is a hybrid of Secale cereale (rye) and Triticum species, had been successfully synthesized by Rimpau in 1888 (96). In spite of the early synthesis of this hybrid species, very few biochemical characterizations of this new genome combinant had been undertaken until the last decade. A few fundamental aspects of such a genome combination have been investigated in some detail recently.

Chromosome doubling may be induced experimentally by treating cells during division with an alkaloid, colchicine. When a tetraploid wheat, Triticum durum (AABB), is crossed with diploid rye, Secale cereale (RR), a zygote with genome constitution of ABR is formed. Upon treatment of this zygote with colchicine, the chromosomes in the zygote are induced to double and consequently a fertile hexaploid synthetic species, Triticale (AABBRR) is produced. The octaploid Triticale has been synthesized in the same way by combining the diploid rye genomes with the genomes of hexaploid wheat, e.g. Triticum vulgare.

Investigations concerning the influence of genome combinations on protein synthesis in cereals by Yong and Unrau (146) revealed that the biosynthetic potential of the alien genomes in the synthetic species, Triticale, was not fully maintained. The question consequently arose whether the biologically active enzymes in alien genome combinants have the same characteristics as those in the parental species. It had earlier been demonstrated that a number of electrophoretic (146, 147) differences in 'storage' proteins existed in the synthesized species when compared to the pattern of the parental species. The possibility therefore existed that 'hybrid' forms of the amylases might be generated in this new genomic

environment. In order to investigate this problem, it was necessary to isolate the requisite enzymes from these species and proceed to compare their physical and chemical properties.

Since cereals are a good source of alpha- and beta-amylases, this was a further factor why they were chosen as the representative enzymes in the present studies. In this investigation, the isolation, purification and characterization of alpha- and beta-amylases as they occured in the hexaploid synthetic species, Triticale (AABBRR), and the two parental species, rye (RR) and durum wheat (AABB) are described in some detail. Certain kinetic aspects and other characteristics of the amylases have also been studied on a comparative basis.

B. LITERATURE REVIEW

The vast amount of basic knowledge stemming from the recent studies of biochemical genetics and biosynthesis is certainly a great scientific accomplishment. Considerable research has been carried out on the synthesis and characterization of new plant species which are comprised of alien genomes. Of the synthetic cereal species, Triticale was the first successfully produced by Rimpau as early as 1888. It is a hybrid of Secale cereale (rye) and Triticum species (wheat). Since then, intensive research has been carried out by cytologists and cytogeneticists with the aim of refining the development of these synthetic species both in octaploid and the hexaploid levels. Biochemical characterization of these synthetic species has been attempted in some detail only in recent years.

A. Kowarski (65) and O. Moritz (79) reported the existence of serological similarities and dissimilarities between proteins extracted from rye, wheat and the synthetic rye-wheat species. O. Hall (43) reported the results of immunoelectrophoresis studies on the octaploid Triticale and the parental species. He found that the integrity of the rye genome was generally maintained in the hybrid species.

Recently, Unrau and Vaisey (135) and Unrau and Jenkins (134) have made comparative studies of the milling, baking and some physical and compositional characteristics between Triticale (rye x wheat) and the parental species. They attributed some observed differences in these characteristics in Triticale to the influence of the rye genomes.

Yong and Unrau (146) have compared the electrophoretic mobility patterns of the proteins extracted from the parental species. They observed many similarities but also some notable dissimilarities between these patterns. They suggested that the parent genomes were unable to maintain their biosynthetic potential fully when in intimate association

in a hybrid species, and the existence of some form of interaction between the two parental genomes in the hybrid species apparently gave rise to new intermediate products.

In the last decade, biochemical genetics and biosynthesis have become scientifically fashionable. The investigations on the genetic control of normal hemoglobins in humans has contributed greatly toward the elucidation of the problem of gene action (51, 52, 53). D. Schwartz (107, 108, 109) reported that a hybrid esterase was found in addition to the two parental types in the heterozygotes of maize. This new hybrid enzyme had an intermediate electrophoretic migration rate between the two parental types. K. Oda et al. (90) synthesized a new koji mold with intermediate characteristics related to enzyme formation. The enzyme levels were classified into two groups: (i) the strains having intermediate activities involving the gene from both parental species, (ii) the strains inheriting the gene from only one of the parents.

P.M. Harney et al. (45) isolated a substance, which was considered the result of gene interaction in the heterozygous individuals, from the flower extracts of interspecific hybrids of Lotus species. More recently, R.E. Alston et al. (2) have studied the hybrid compounds in natural interspecific species and concluded that truely hybrid compounds might be produced in interspecific hybrids.

Amylases are among the earliest known enzymes. Alpha-amylase in wheat extracts was described early in 1811 by Kirchhoff (60) as the factor responsible for the digestion of starch. Amylases are distributed widely, perhaps universally, throughout the animal, plant and microbial kingdoms.

Amylases were found to attack polysaccharides in different manners. By differentiation of their activities, E. Ohlsson (91, 92) called alpha-

amylase the dextrinogenic amylase and beta-amylase the saccharogenic amylase. The methods for separation of alpha- and beta-amylases by heat treatment to destroy beta-amylase and acid treatment to destroy alpha-amylase reported by Kneen et al. (64) became very useful and versatile in the purification steps.

During the last few decades, highly purified and crystallized amylases were obtained from sources such as human saliva (74, 82), human pancreas (28), Bacillus subtilis (27, 42, 75, 125), Pseudomonas saccharophila (71), barley malt (112), wheat (76), sweet potato (6, 7, 23, 24), soybean (34), broad beans (39) etc. The characterizations of amylases from these various sources have been well described.

Alpha-amylases and beta-amylases in wheat and rye in particular have been studied by many investigators, e.g. K.H. Meyer et al. (76), N.M. Naylor (84), E. Ohlsson et al. (93, 125), P.S. Ugrumow (133), E.V. Rowsell (104), T.B. Darkanbaev et al. (17, 18), B.A. Stewart(127). So far only one form of alpha-amylase was found in each source. K.H. Tipples et al. (131, 132) recently isolated three components of beta-amylases from wheat by means of ion-exchange chromatography.

The inheritance of free beta-amylase in 56 barley varieties has recently been investigated by V.M. Bendelow (8). He reported that the level of beta-amylase was controlled by a single gene-pair with incomplete dominance. The level of free (i.e. water soluble) beta-amylase activity is apparently inherited independently of total beta-amylase activity (8).

Gibberellins have been found in plants and their activities have been shown to be hormonal in nature. The effects of gibberellic acid on the production of alpha-amylase in germinating barley and wheat seeds has been rather extensively investigated (20, 32, 122), and the general gibberellic acid-controlled synthesis of alpha-amylase in cereal endosperm had been studied in particular (80, 97, 136, 137, 142).

An amylase-activating substance was extracted from barley green malt by J. Yomo (143). Its chemical properties were found to be similar to those of the hormone gibberellin. Since the amount of gibberellin was found to increase in the germinating grains (101), it was then proposed that gibberellin promoted the activation of alpha-amylase during germination (123, 145). The activity of gibberellic acid had been attributed to neutralization of a growth-inhibitory system which normally limited growth (15). The discovery of gibberellin-like hormones in seeds of several plant species (98), in shoots and roots of pea seedlings (100) and the demonstration of the chemical relationship of these hormones to gibberellic acid by the isolation of gibberellin A_1, which is a dihydro-derivative of gibberellic acid (70), supported the above suggestions. A number of plant physiologists are now investigating this problem and in particular attempting to determine the mechanism of the apparent hormone-mediated development of alpha-amylase during germination of cereals.

C. EXPERIMENTAL METHODS AND RESULTS

I. DEVELOPMENT OF ALPHA-AMYLASES DURING GERMINATION

The seeds of a hexaploid synthetic species, Triticale (AABBRR), and its parental species, a tetraploid wheat (Stewart durum AABB) and Prolific rye (RR) were employed for these studies. All the seeds were harvested in 1964 and stored in a refrigerator at 4°C.

The moisture content of the seeds was found to be 13%. Seeds were sterilized by steeping in 80% ethyl alcohol for two minutes, followed by a few rinses with water. The seeds were consequently steeped in sterilized water for two hours, then placed between wet filter papers in petri dishes in order for germination to proceed at room temperature (25°C). After germinating for a certain period of time, the malted grains were removed and pressed gently between filter papers. The length of the sprouts and roots was measured, followed by air drying at 25°C for two days. The material was subsequently ground into flour in an experiment mill.

S. Hagberg's modified Wohlgemuth method for determination of alpha-amylase activity of wheat and rye (41) was followed with minor alterations. A buffered limit dextrin substrate was prepared according to Cereal Laboratory Methods as follows: A suspension of 5.00 gm (dry weight) soluble starch in distilled water was poured slowly into boiling water (400 ml) with stirring. After boiling for one minute, the suspension was allowed to cool to room temperature. Acetate buffer (25 ml), which contained 3 ml of glacial acetic acid and 4.1 gm of sodium acetate, was added to the starch solution. Beta-amylase (250 mg) was dissolved in 10 ml of distilled water and mixed with the starch solution. The volume was made up to 500 ml and stored at 25°C for not less than 18 hours nor more than72 hours before being used.

A stock iodine solution was made by dissolving 5.50 gm iodine crystals and 11.0 gm potassium iodide in 250 ml distilled water, and stored in darkness. This solution was prepared monthly.

Dilute iodine solution was made daily by dissolving 43 gm potassium iodide in water which contained 4.3 ml of stock solution and the volume was made to 1000 ml.

Flour samples (5 gm) were extracted with 100 ml of 0.2% calcium chloride solution for one hour at 25°C with constant shaking. After centrifuging at 19,000 rpm for 10 minutes at 4°C, the supernatant was diluted suitably with 0.2% calcium chloride solution to give the appropriate alpha-amylase activity. That is, the different amounts of enzyme solutions, namely 30 ml for the ungerminated sample, 5 ml for the sample which had been germinated for one day and two days, 2.5 ml for the sample which had been germinated for three and four days, were diluted to a total volume of 100 ml, respectively.

A blank for zero time (B_o) was prepared by mixing 1 ml of 0.2% calcium chloride, 10 ml of dilute iodine solution and 40 ml of distilled water.

A blank for the starting-point (B_s) was made by mixing 10 ml of dilute iodine solution, 40 ml distilled water and 1 ml of a mixture consisting of one part buffered limit dextrin substrate and three parts of 0.2% calcium chloride solution.

When all the reagents were brought to 25°C, the period of half-life, $t_{\frac{1}{2}}$, was determined as described below. The absorbance of the blank for the starting-point, B_s, was measured at 575 mμ when the blank solution (B_o) gave an absorbance of zero. A solution of buffered limit dextrin (10 ml) was mixed with 30 ml of suitably diluted enzyme extract. After four or more appropriate hydrolysis times (e.g. 5, 10, 20 and 30 minutes),

one ml of the hydrolysis mixture was transferred to a solution containing 10 ml dilute iodine solution and 40 ml distilled water by means of a fast-delivery pipette. The absorbance of the hydrolysate-iodine solution was measured at 575 mμ. The absorbance reading for B_s (E_o = the absorbance after zero time) and the readings after four different time intervals (E_t = the absorbance after t minutes) were plotted on semilogarithmic paper and should constitute a straight line. The half-life (the time required for one-half of the buffered limit dextrin to be digested by alpha-amylase) was evaluated from the intersection of the above line and the horizontal line for half of the absorbance for B_s.

The period of half-life ($t_{\frac{1}{2}}$) in minutes could also be calculated from the formula:

$$t_{\frac{1}{2}} = t \times \frac{\log Eo - \log \frac{Eo}{2}}{\log Eo - \log Et}$$

$$= t \times \frac{0.301}{\log Eo - \log Et}$$

The alpha-amylase unit is defined as number of grams of soluble starch dextrinized by one gram of malt per hour. It can be calculated by using the following formula (105):

$$\text{Alpha-amylase unit} = \frac{\text{weight of starch (gm)} \times 60}{\text{wt. of malt (gm)} \times \text{dextrinization time (min.)}}$$

The results of development of alpha-amylases during germination are shown in Table 1 and graphically in Fig. 1.

The length of sprouts and roots are indicated in Table 2 as average values of ten measurements.

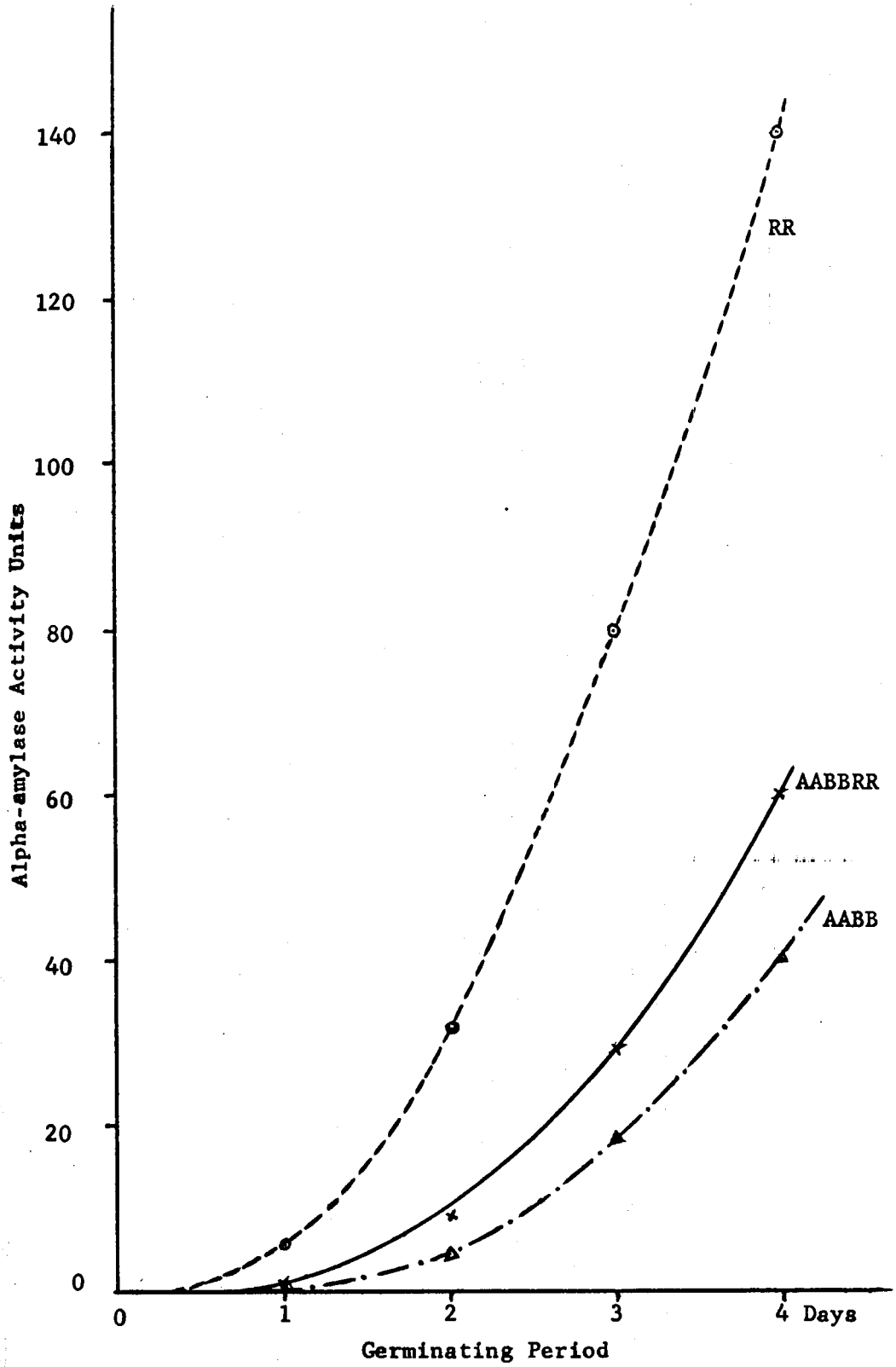
Table 1: Development of alpha-amylases during germination

Sample	$T_{1/2}$			Alpha-amylase units		
	Durum	Triticale	Rye	Durum	Triticale	Rye
Ungerminated	666.5	89.0	60.6	0.02	0.1	0.2
Germinated for one day	259.0	92.5	14.2	0.3	0.8	5.6
Germinated for two days	18.1	8.4	2.4	4.4	8.7	32.0
Germinated for three days	8.6	5.5	2.0	19.0	29.0	80.0
Germinated for four days	4.0	2.7	1.2	40.0	60.0	133.3

Table 2: Growth of sprouts and roots

Sample		Germination Time			
		1 day	2 days	3 days	4 days
Durum	Sprout length, mm.	1	6	14	23
	Root length, mm.	4	14	29	39
Triticale	Sprout length, mm.	2	8	17	28
	Root length, mm.	6	28	37	51
Rye	Sprout length, mm.	4	10	19	31
	Root length, mm.	10	29	42	56

Fig. 1: Development of alpha-amylases during germination



II. EFFECTS OF POTASSIUM GIBBERELLATE ON THE DEVELOPMENT OF ALPHA-AMYLASES DURING GERMINATION

Gibberellic acid (75% potassium salt) was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

The seeds of synthetic species, Triticale, and its parental species were sterilized as described in the previous section. After a few rinses with water, the seeds were steeped in 0.005% potassium gibberellate solution (w/v) for two hours. Then germination was allowed to proceed between wet filter papers in petri dishes at room temperature, 25°C. The 0.005% potassium gibberellate solution was sprayed into the petri dishes occasionally during the germinating period. When germinated for one day, two days, three days and four days in each case, the malted grains were air-dried at room temperature for two days followed by grinding into flour in an experimental mill.

The method for determination of activities of alpha-amylases was exactly the same as described previously in Section I.

The results of effects of potassium gibberellate on the development of alpha-amylases during germination of seeds are shown in Table 3 and Fig. 2.

The length of sprouts and roots are recorded in Table 4 as average values of ten measurements.

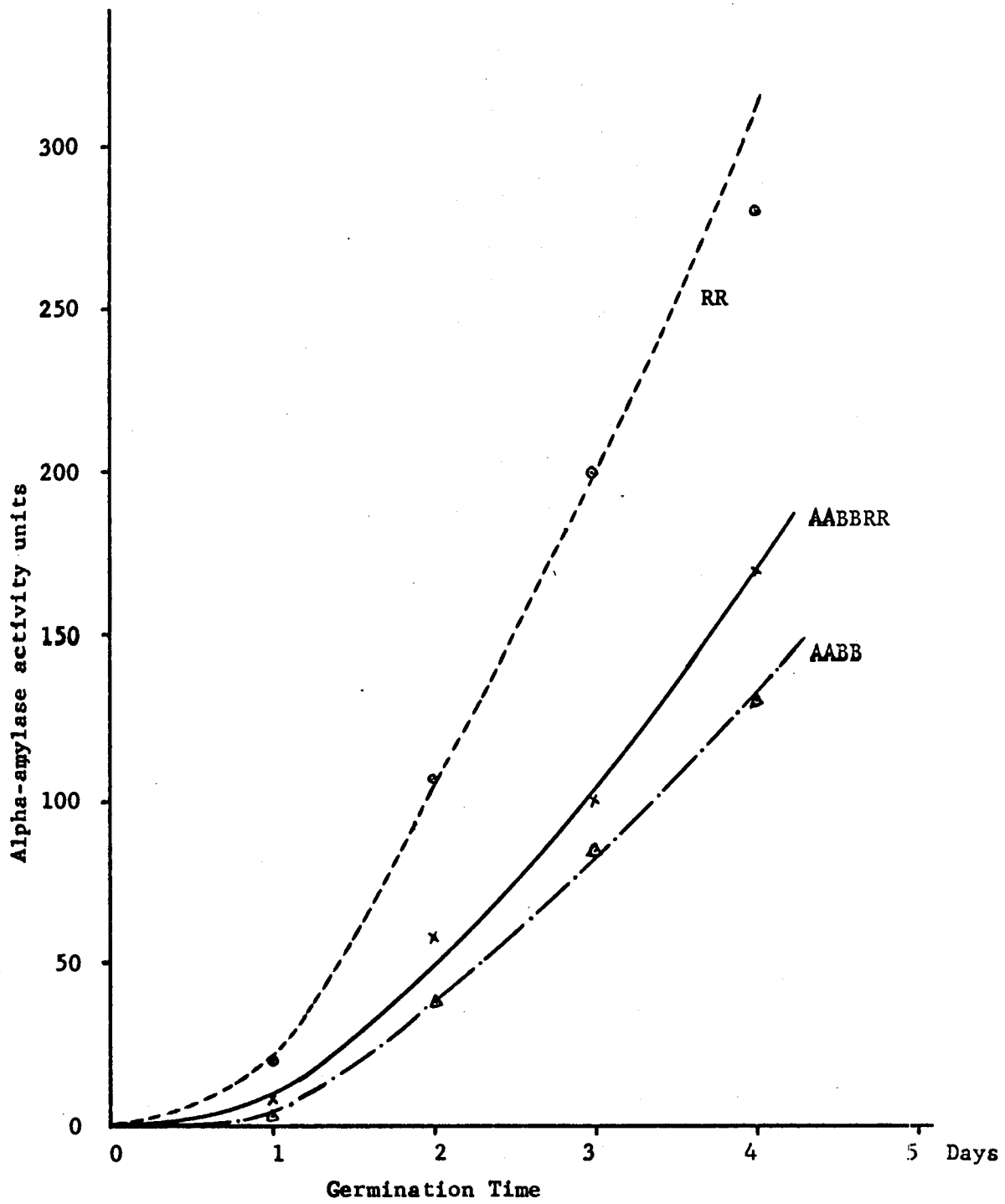
Table 3: Effects of potassium gibberellate on the development of alpha-amylases during germination

	$T_{1/2}$			Alpha-amylase Units		
	Durum	Triticale	Rye	Durum	Triticale	Rye
Immediately after steeping in K-Gibb. for two hours	325.0	30.0	22.2	0.04	0.4	0.6
Germinated for one day	26.0	10.0	4.0	3.1	8.0	20.0
Germinated for two days	2.1	1.4	0.7	38.1	57.1	114.0
Germinated for three days	2.0	1.6	0.8	80.0	100.0	200.0
Germinated for four days	1.2	0.9	0.5	133.3	170.0	266.7

Table 4: Effects of potassium gibberellate on growth of sprouts and roots

Sample		Germination Time			
		1 day	2 days	3 days	4 days
Durum	Sprout length, mm.	2	9	19	31
	Root length, mm.	4	17	32	45
Triticale	Sprout length, mm.	3	6	22	36
	Root length, mm.	6	32	44	61
Rye	Sprout length, mm.	5	13	32	50
	Root length, mm.	10	40	57	72

Fig. 2: Effects of gibberellic acid on production of alpha-amylases



III. ISOLATION AND PURIFICATION OF AMYLASES

(a) ISOLATION AND PURIFICATION OF ALPHA-AMYLASES

The initial steps in the purification of alpha-amylases were carried out according to the procedure developed by S. Schwimmer and A.K. Balls (112) with some modifications. Gel filtration on Sephadex was subsequently employed in the final steps of purification.

The seeds which had been germinated between wet filter papers in petri dishes for three days were dried in air and ground into flour. The flour sample (60 gm) was added to 300 ml of 0.2% (w/v) calcium chloride solution, pH 5.5, with constant stirring for an hour. The mixture was allowed to stand overnight at room temperature. The residue was removed by centrifugation at 19,000 rpm for 30 minutes at 4°C. After the supernatant was heated at 70°C for 15 minutes to destroy the beta-amylase, the precipitate that formed was removed by centrifugation at 4°C. A light brown solution containing alpha-amylase was obtained. (Assay 1).

Saturated ammonium sulfate solution (pH 5.5), which had been previously cooled to 4°C, was added dropwise from a separatory funnel with constant stirring, until a 50% saturation point was reached. The precipitate was collected by centrifugation and dissolved in a small amount of distilled water. Salts in the crude enzyme solution were removed by dialysing overnight against very dilute acetate buffer (1/1000 N at pH 5.6). (Assay 2).

The volume of the enzyme solution was made up to 100 ml with distilled water. The proteins were fractionated by adding cold acetone (-10°C) to give solutions of various degrees of saturation (v/v). The specific activities of the supernatant at different degrees of saturation

were determined. (Assay 3).

The precipitates obtained from different degrees of acetone saturation were dissolved in distilled water and combined. Cold acetone (-10°C) was again added to retain a certain fraction of precipitate from the solution of the appropriate degree of saturation which contained most of the active amylases, namely the fraction between 25% and 60% saturation for durum wheat, the fraction between 50% and 70% for Triticale and the fraction between 45% and 65% for rye was retained. These fractionated portions of enzymes were dissolved in 20 ml of distilled water and dialyzed overnight against very dilute acetate buffer (1/1000 N at pH 5.2). (Assay 4).

A portion of the crude alpha-amylase extract (4 ml), which contained about 60 mg protein nitrogen, was applied carefully to the top of a Sephadex gel column (3 x 160 cm) which was prepared as described by Gelotte (35). The enzyme was eluted by 0.02 M acetate buffer (pH 5.2) containing 0.001 M calcium acetate. The column effluent was collected in 3-ml fractions by means of an automatic fraction collector. Protein content of the effluent was measured spectrophotometrically at a wavelenght of 280 mμ. Enzymatic activity of the effluent was analyzed by the 3,5-dinitrosalicylic acid method (30) which is described later in Section IV. Final results are shown in Figures 4, 5 and 6. The main fractions which contained active alpha-amylase were combined and dialyzed overnight against very dilute acetate buffer, pH 5.8, at 4°C to remove salts. Pervaporation was subsequently employed to concentrate the enzyme solution. (Assay 5).

Figure 3 reveals the specific activities of alpha-amylases remaining in the supernatant at different degrees of saturation with acetone.

The specific activities of alpha-amylases at each step of purification are shown in Tables 5, 6 and 7. The specific activity of alpha-amylase, in the absence of beta-amylase, was expressed as mg of maltose liberated from a 1% solution of soluble starch per mg of protein nitrogen.

The nitrogen content of the enzyme solution was determined by Nessler's method (5, 56, 78, 130). The Nessler's reagent was prepared by shaking a mixture of 4.0 grams of potassium iodide, 2.94 grams of iodine, 2.9 grams of mercury and 10ml of distilled water, continuously and vigorously in a 125 ml Erlenmeyer flask. While the reaction was taking place, continuous shaking was necessary until the red iodine color had changed to the green color of the double iodide complex. The aqueous solution was decanted from the excess mercury. After washing the mercury with distilled water three times, the washings were combined with the original aqueous solution and subsequently poured into 750 ml of boiling water in which a quantity of gum ghatti (about 1 to 2 gm) had been dissolved. The volume of the solution was made up to one liter when cooled, and shaken vigorously to insure uniformity. The reagent was stored in an amber bottle.

The digestion reagent was prepared as follows: 1.0 gm of hydrated copper sulfate, $CuSO_4 \cdot 5H_2O$, and 1.2 gm of selenous acid (H_2SeO_3) were ground into a powder in a mortar and dissolved in 25 ml of concentrated phosphoric acid, after which 75 ml of concentrated sulphuric acid was added.

Microliter quantities of liquid samples were placed in Pyrex test tubes (10 x 75 mm) and evaporated in an oven at 110°C. The digestion reagent (0.2 ml) was introduced to each tube. The tubes were subsequently placed into drilled holes in a brass heating block. The contents of the

tubes were refluxed at 370°C on a hot plate. After refluxing for 15 to 30 minutes the color of the content changed from dark brown to light yellow. The tubes and contents were removed and allowed to cool. Two drops of 30% hydrogen peroxide were added to each tube. The tubes were replaced in the brass block and heated at 370°C for about 20 minutes. The contents became perfectly clear after oxidizing with hydrogen peroxide. When the tubes were cooled, the contents were transferred quantitatively into 10-ml volumetric flasks and made to volume. A portion of this sample solution (5 ml) was transferred to a test tube and 2.0 ml of Nessler's reagent was added. Sodium hydroxide (3 ml, 4 N) was subsequently introduced. After mixing thoroughly by rotation, the mixture was allowed to stand for 15 minutes and transferred to colorimeter tubes. The absorbance was measured at 515 millimicrons in a spectrophotometer. A standard curve was constructed by determining the absorbance when microquantities, up to a maximum of 80 micrograms, of standard ammonium sulfate were used. A new standard curve was constructed periodically.

(b) DETERMINATION OF ELECTROPHORETIC MOBILITIES OF ALPHA-AMYLASES

When the purified enzymes from gel filtration were subjected to electrophoresis at pH 4.0 in aluminium lactate buffer (refer to Section III c) only a single band was observed in the electropherogram for each alpha-amylase preparation. This indicated the homogeneity and purity of the isolated enzymes.

The electrophoretic mobilities of the alpha-amylases were determined by applying a voltage gradient of 15 V/cm and electrophoresing for six hours. The results are shown in Table 8.

Fig. 3: Fractionation of alpha-amylases

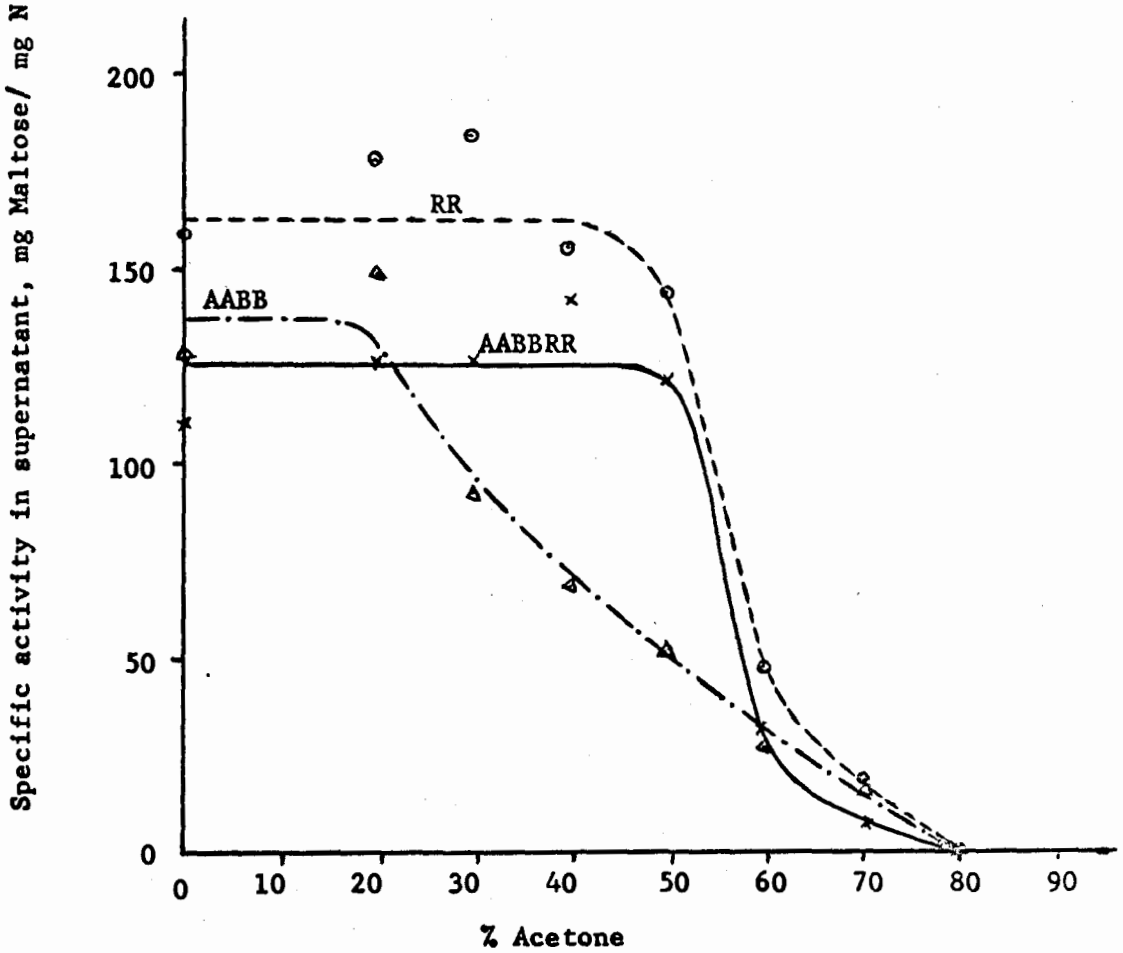
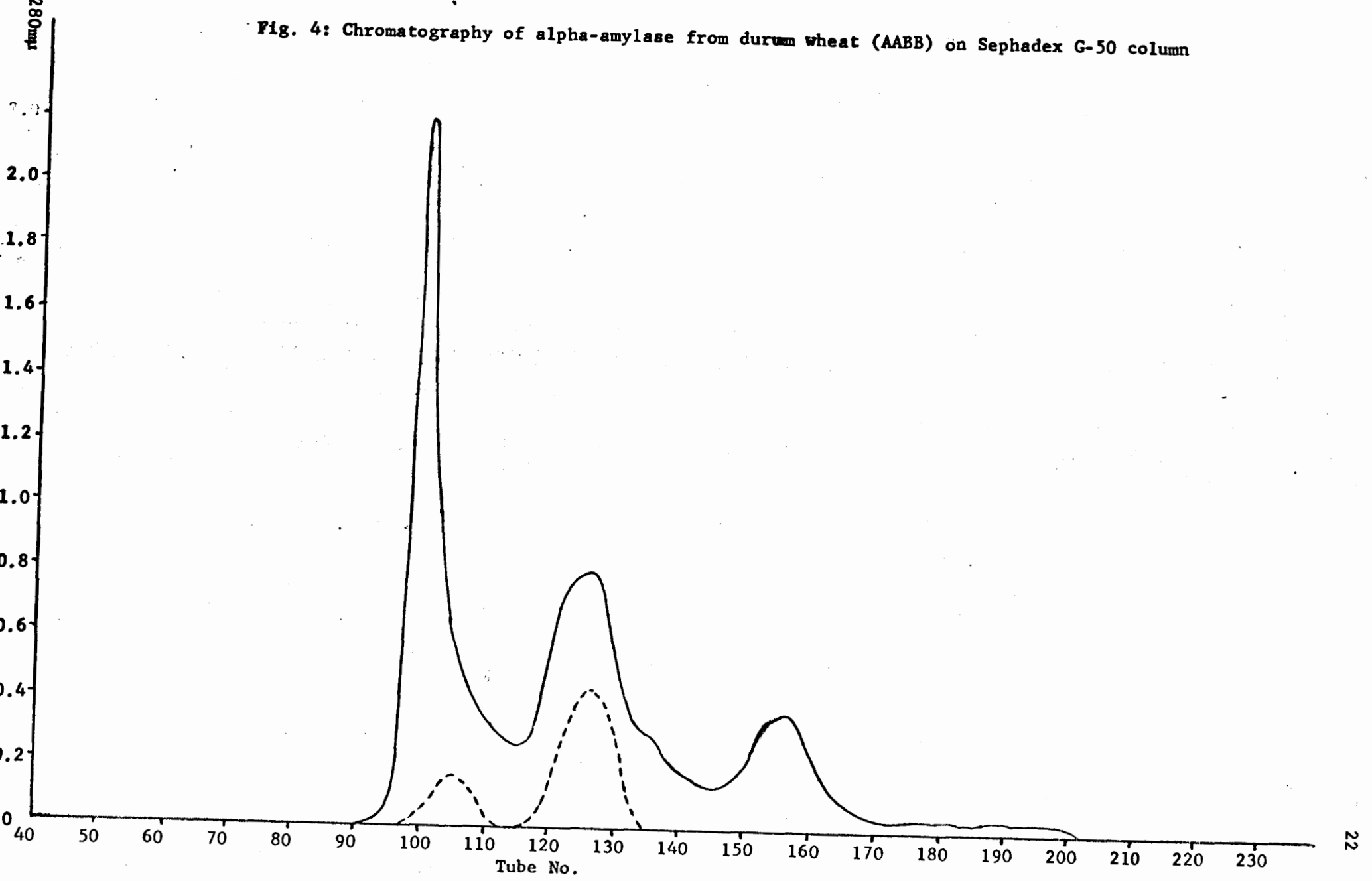


Fig. 4: Chromatography of alpha-amylase from durum wheat (AABB) on Sephadex G-50 column



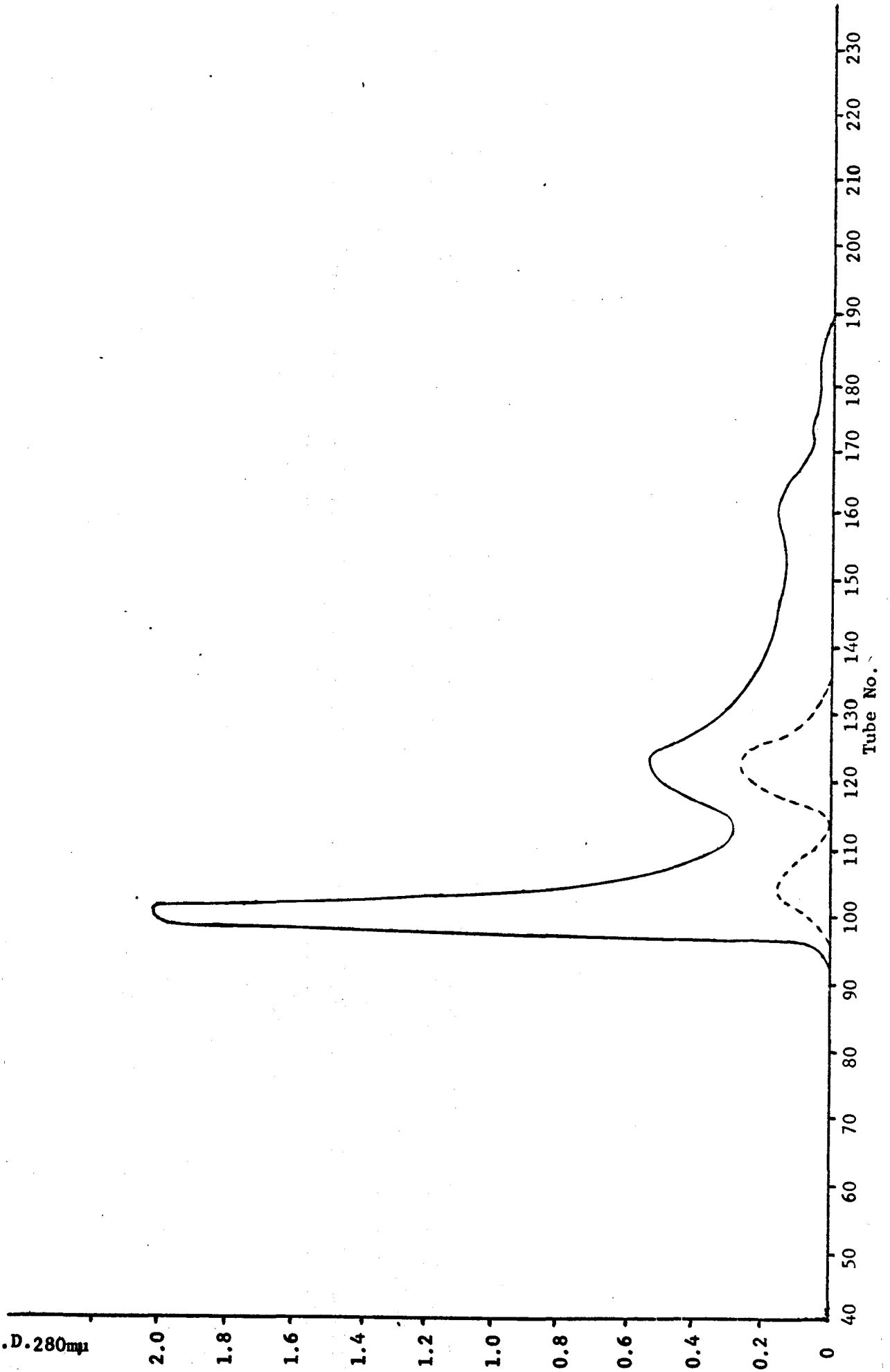


Fig. 5: Chromatography of alpha-amylases from Triticale (AABBRR) on Sephadex G-50 column

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

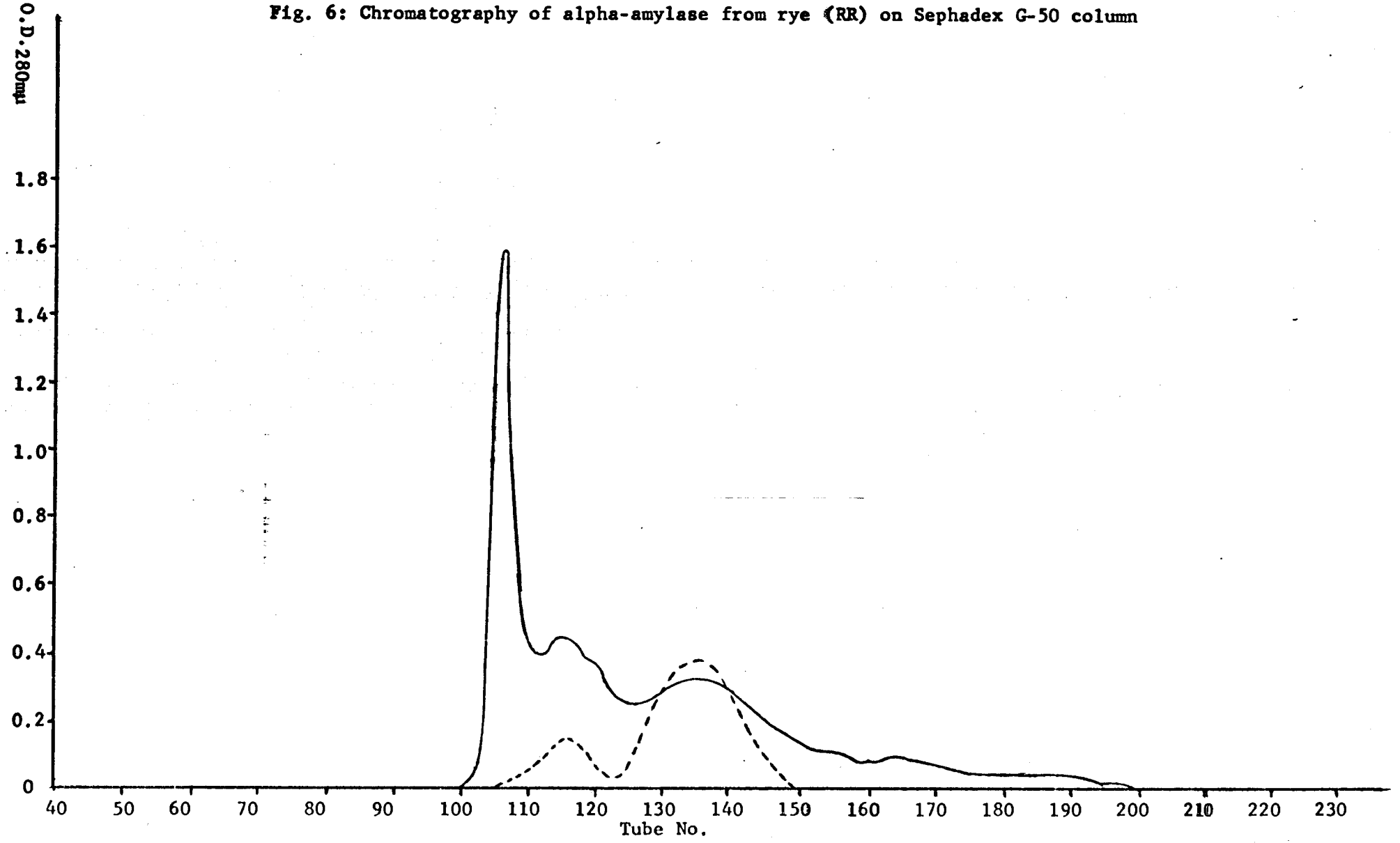


Table 5: Purification of alpha-amylase from durum wheat (AABB)

Sample	Enzymatic Activity		Nitrogen Content		Specific Activity, mg Maltose/mg N
	$O.D._{560mu}$	mg Maltose/ml	$O.D._{515mu}$	ug/ml	
Assay 1	0.368	8.4	0.140	165	51
Assay 2	0.371	8.5	0.055	66	129
Assay 3: Supernatant of 20% acetone	0.330	7.5	0.045	55	137
Supernatant of 30% acetone	0.368	8.4	0.076	90	94
Supernatant of 40% acetone	0.440	10.0	0.124	148	68
Supernatant of 50% acetone	0.252	5.8	0.095	113	51
Supernatant of 60% acetone	0.066	1.5	0.045	55	27
Supernatant of 70% acetone	0.066	1.5	0.082	100	15
Supernatant of 80% acetone	0	0	0.077	92	0
Assay 4	0.211	4.9	0.028	35	140
Assay 5	0.432	9.9	0.025	32	310

Table 6: Purification of alpha-amylase from Triticale (AABBRR)

Sample	Enzymatic Activity		Nitrogen Content		Specific Activity, mg Maltose/mg N
	$O.D._{560m\mu}$	mg Maltose/ml	$O.D._{515m\mu}$	µg/ml	
Assay 1	0.432	9.9	0.145	170	58
Assay 2	0.371	8.5	0.063	77	110
Assay 3: Supernatant of 20% acetone	0.262	6.0	0.042	48	125
Supernatant of 30% acetone	0.403	9.2	0.060	73	126
Supernatant of 40% acetone	0.298	6.8	0.040	48	142
Supernatant of 50% acetone	0.432	9.9	0.068	82	121
Supernatant of 60% acetone	0.070	1.6	0.046	55	29
Supernatant of 70% acetone	0.019	0.4	0.033	42	9
Supernatant of 80% acetone	0	0	--	--	0
Assay 4	0.280	6.4	0.029	34	190
Assay 5	0.282	6.5	0.015	20	320

Table 7: Purification of alpha-amylase from rye (RR)

Sample	Enzymatic Activity		Nitrogen Content		Specific Activity, mg Maltose/mg N
	O.D.$_{560m\mu}$	mg Maltose/ml	O.D.$_{515m\mu}$	µg/ml	
Assay 1	0.547	12.6	0.156	185	68
Assay 2	0.661	15.2	0.080	95	160
Assay 3: Supernatant of 20% acetone	0.509	11.6	0.054	65	180
Supernatant of 30% acetone	0.590	13.5	0.060	73	185
Supernatant of 40% acetone	0.780	17.8	0.095	114	156
Supernatant of 50% acetone	0.792	18.2	0.106	125	145
Supernatant of 60% acetone	0.168	4.2	0.078	92	46
Supernatant of 70% acetone	0.060	1.4	0.062	75	18
Supernatant of 80% acetone	0	0	--	--	0
Assay 4	0.488	11.2	0.031	38	295
Assay 5	0.478	10.9	0.020	25	436

Table 8: The electrophoretic mobilities of alpha-amylases

Sample	Mobility, $cm^2\ V^{-1}\ sec^{-1} \times 10^{-5}$				
					AVERAGE
Durum	2.36	2.31	2.33	2.36	2.34
Triticale	2.43	2.41	2.40	2.39	2.41
Rye	2.08	2.08	2.05	2.06	2.07

(c) ISOLATION AND PURIFICATION OF BETA-AMYLASES

The initial purification steps employed by K.H. Tipples et al. (13) were followed with some modifications. In the later stages, combination of gel filtration on Sephadex and zone electrophoresis was employed instead of ion-exchange column chromatography. Flour samples (60 gm) were extracted with 180 ml of 1% NaCl solution (w/v) containing 0.0001 M EDTA. The flour was gradually added to the salt solution with care to avoid the formation of large aggregates. The suspension was kept overnight at room temperature. A light brown supernatant was obtained by centrifugation at 18,000 rpm for 20 minutes at 4°C. The insoluble residue was discarded. (Assay 1).

The supernatant was cooled to 4°C and after adjusting the pH to 3.7 with 5 N acetic acid, it was stored in a refrigerator at 4°C for six days to remove traces of alpha-amylase. The precipitate that formed was removed by centrifugation at 4°C for 20 minutes. (Assay 2).

The pH of the supernatant was brought to 5.3 with 4% ammonium hydroxide (v/v) and cooled to 4°C. Saturated ammonium sulfate solution (pH 5.3, at 4°C) was added dropwise from a separatory funnel with constant stirring to 0.66 saturation (v/v). The precipitate was collected and dissolved in distilled water, then kept at 4°C overnight. Some precipitate that formed during this time was removed by centrifugation. (Assay 3).

Ammonia was added to adjust the pH of the supernatant to 5.5. The volume was made up to 50 ml with distilled water. Cold saturated ammonium sulfate solution, pH 5.5, was added dropwise, over a 20 minute period, to give 0.18 saturation (v/v). The precipitate was removed by centrifugation. Saturated ammonium sulfate solution was added again to give 0.5 saturation (v/v). The precipitate was collected and dissolved

in a small amount of distilled water.

The enzyme solution was placed in Visking dialyzing tubing (1 inch in diameter) and dialyzed overnight against very dilute acetate buffer (1/1000 N at pH 4.8). (Assay 4). The specific activities of the supernatant at various degrees of saturation are shown in Fig. 7. The dialyzed enzyme solution was poured cautiously on the top of a Sephadex G-50 column (3 x 160 cm) which had been previously equilibrated with 0.01 M acetate buffer solution at pH 4.8. The same buffer solution was used as eluant. Fractions (3 ml) of the effluent were collected in an automatic collector. Protein content of the effluent was determined by measuring the absorbance at 280 mμ for each tube. Enzymatic activity of the effluent was analyzed by the 3,5-dinitrosalicylic acid method (30). The results are shown in Fig. 8, 9 and 10. Two forms of beta-amylase were found in the effluent from the column in each case. These two forms of beta-amylases had nearly the same chromatographic properties. The effluent which contained the enzymatically active fractions were pooled and dialyzed overnight against very dilute acetate buffer, 1/1000 N at pH 4.8, to remove salts. Pervaporation was employed to concentrate the enzyme solution.

Further separation could be achieved by zone electrophoresis in polyacrylamide gel. Electrophoresis was carried out in 6% (w/v) polyacrylamide gel which had been equilibrated with 0.0085 M aluminium lactate buffer (pH 4.1, ionic strength 0.05) by several changes of the buffer solution. A stock aluminium lactate buffer solution was prepared by activating 5.47 grams aluminium foil with a small amount of saturated mercuric chloride solution and then dissolved in 38.5 ml of lactic acid as described by R.W. Jones et al. (22, 57). The reaction was allowed to proceed overnight. The solution was filtered

through celite and the filtrate was made up to one liter. A portion (100 ml) of the stock solution was added to 1.8 liters of distilled water. After adjusting the pH to 4.1 with concentrated lactic acid, the volume was made up to two liters with distilled water.

The method of preparation of polyacrylamide gel reported by S. Raymond et al, (102) was followed with some modifications. A 6% (w/v) Cyanogum solution was prepared by dissolving 6 grams of Cyanogum in 100 ml distilled water followed by filtration. After bubbling nitrogen gas through the solution for five minutes to expel oxygen which would inhibit the polymerization of acrylamide gel, 0.6 ml of freshly prepared 10% (v/v) solution of dimethylaminopropionitrile (DMAPN) and 0.6 ml of freshly prepared 10% (w/v) solution of ammonium persulfate were added as catalysts. The solution was poured into lucite (perspex) troughs of inner space dimensions 23 x 4 x 0.5 cm. A glass plate was placed carefully to cover the trough so as to avoid the air bubbles being trapped in the gel. The length of time for polymerization could be shortened if the Cyanogum solution was heated to 40°C before pouring into the lucite trough.

A small portion (100 µl) of the protein sample was applied to a small piece of filter paper which was subsequently inserted into the sample application slot in the polyacrylamide gel. Electrophoresis was carried out with six slabs of gel at one time under identical conditions. After electrophoresing for six hours at 600 volts (voltage gradient: 15 v/cm), one of the gel plates was sliced into 2.5 mm thick sections and dyed with 1% amido-black 10 B in a mixture of methanol: acetic acid: water = 5: 1: 5. The slabs were destained by rinsing several times in the solvent. The remaining gel slabs were cut into the different zones which corresponded to the bands observed in the dyed gel. The

protein in various zones was eluted by soaking the gel segments in 0.008 M acetate buffer, pH 4.8, overnight at 4°C followed by centrifugation in basket-like centrifuge tubes. The clear enzyme solution was filtered through a Sephadex G-50 column (1 x 50 cm) to remove monomeric material from the acrylamide gel. After dialyzing overnight against distilled water, the enzyme solution was concentrated by pervaporation. The specific activities of these fractions were determined. (Assay 5).

The specific activities of beta-amylases at different stages of purification are shown in Tables 9, 10 and 11. In assay 5, component 1 refers to the beta-amylase component which had the higher electrophoretic mobility while component 2 refers to the one which had the lower mobility.

(d) DETERMINATION OF ELECTROPHORETIC MOBILITIES OF BETA-AMYLASES

The electrophoretic mobilities of the purified beta-amylases were determined as described in Section III (b). The experiment was repeated four times. The results are shown in Table 12.

Fig. 7: Fractionation of beta-amylases

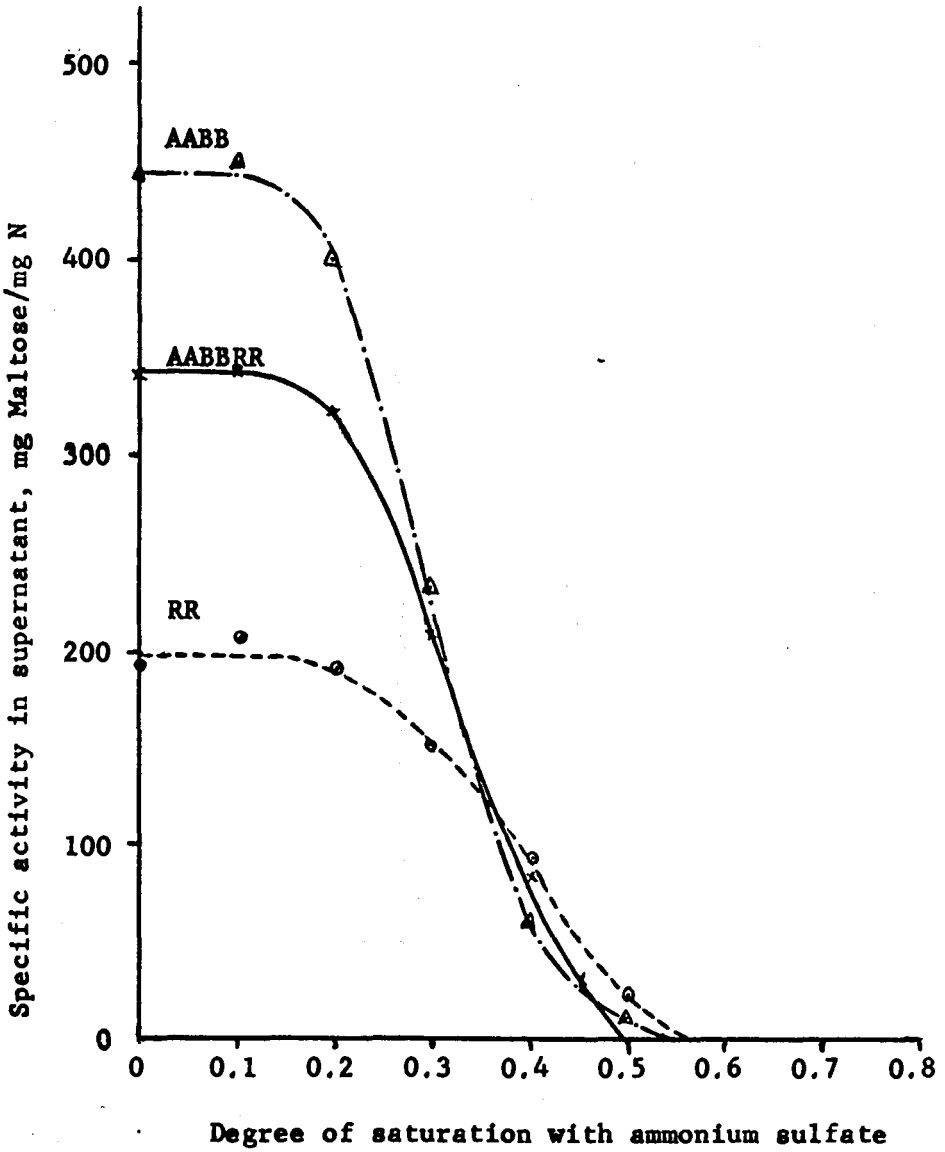


Fig. 8: Chromatography of beta-amylases from Durum (AABB)

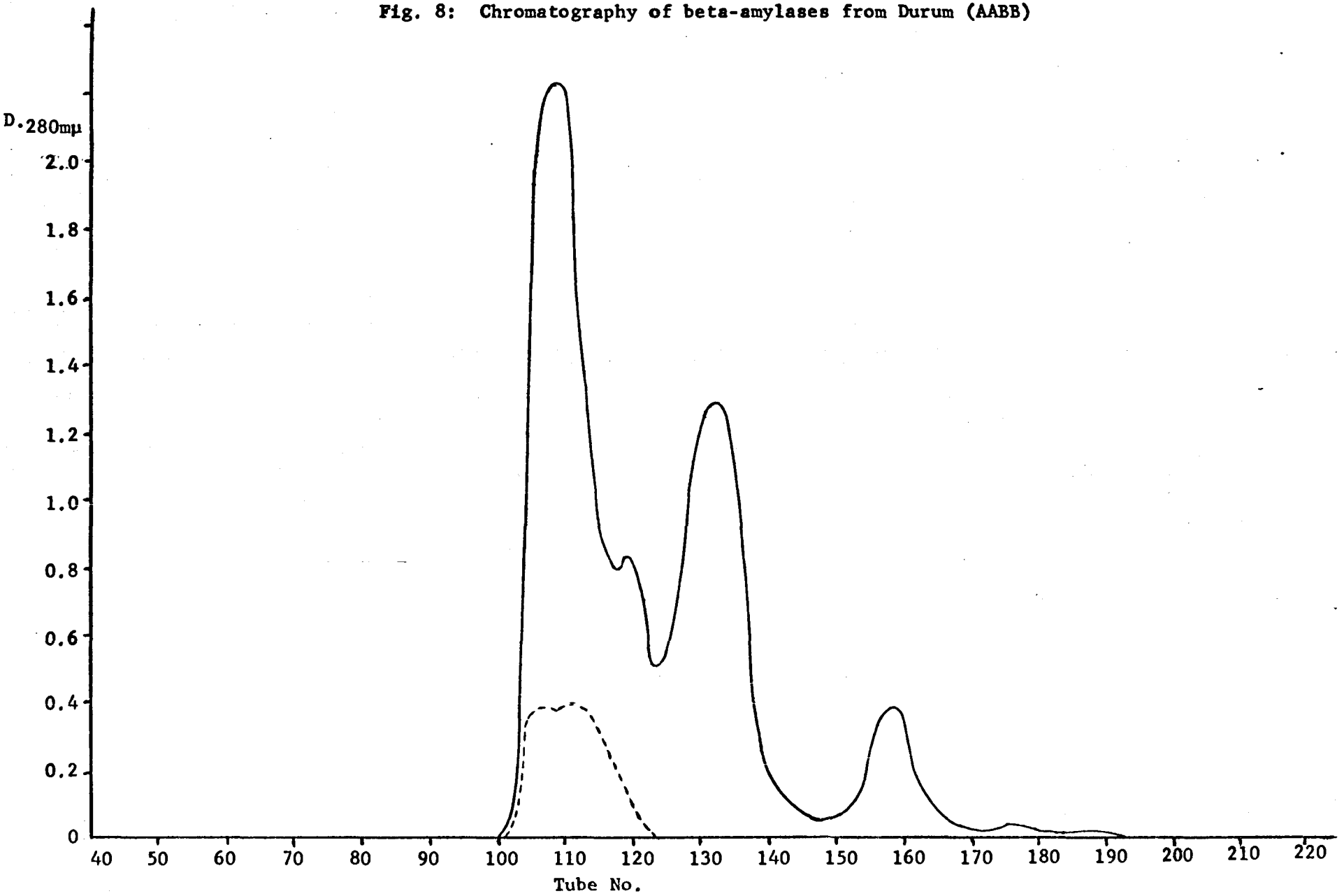


Fig. 9: Chromatography of beta-amylases from Triticale (AABBRR)

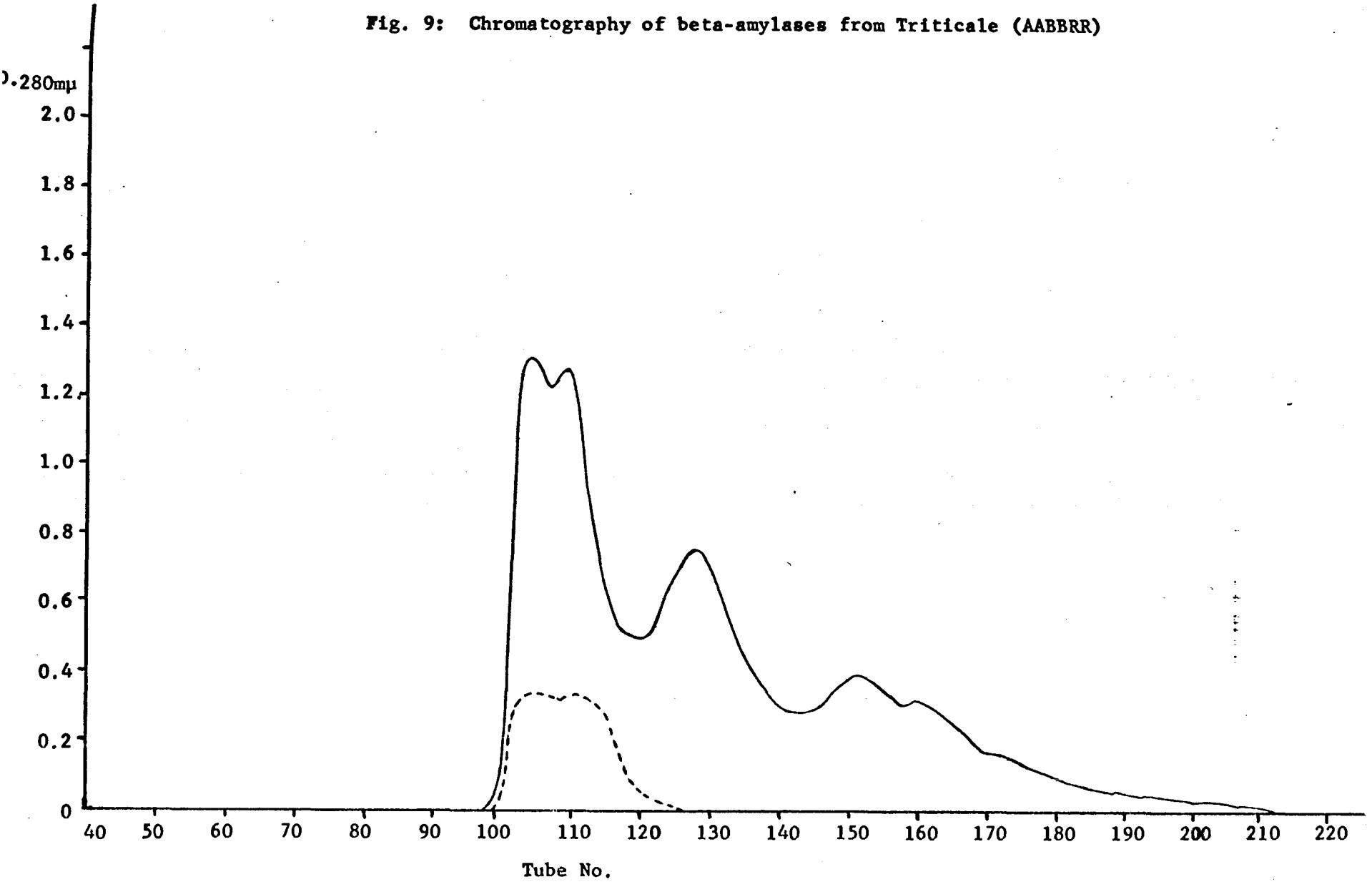


Fig. 10: Chromatography of beta-amylases from Rye (RR)

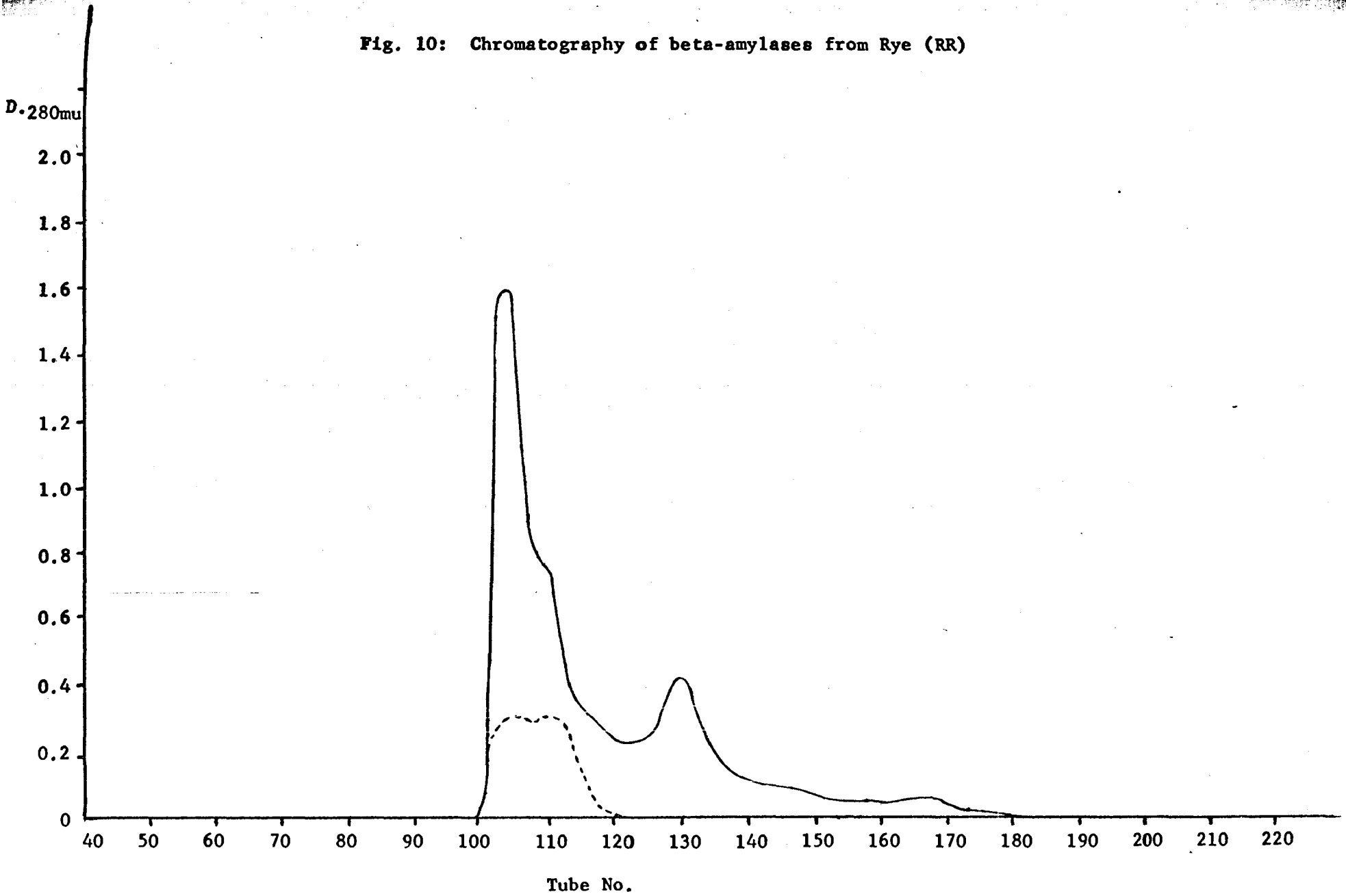


Table 9: Purification of beta-amylases from durum wheat (AABB)

Sample	Enzymatic Activity		Nitrogen Content		Specific Activity, mg Maltose/mg N
	$O.D._{560m\mu}$	mg Maltose/ml	$O.D._{515m\mu}$	µg/ml	
Assay 1	0.260	23.8	0.095	112	212
Assay 2	0.249	22.6	0.060	72	314
Assay 3	0.435	39.8	0.075	90	442
Supernatant of 0.1 saturation	0.363	33.2	0.060	72	461
Supernatant of 0.2 saturation	0.343	31.4	0.064	77	408
Supernatant of 0.3 saturation	0.415	38.0	0.132	155	245
Supernatant of 0.4 saturation	0.033	3.0	0.040	50	60
Supernatant of 0.5 saturation	0.026	2.4	0.160	186	13
Supernatant of 0.55 saturation	0	0	0.042	51	0
Assay 4	0.262	24.0	0.042	51	470
Assay 5: Component 1	0.619	56.5	0.020	25	2260
Component 2	0.532	48.8	0.019	23	2120

Table 10: Purification of beta-amylases from Triticale (AABBRR)

Sample	Enzymatic Activity		Nitrogen Content		Specific Activity,
	$O.D._{560m\mu}$	mg Maltose/ml	$O.D._{515m\mu}$	µg/ml	mg Maltose/mg N
Assay 1	0.102	9.4	0.050	60	157
Assay 2	0.156	14.2	0.055	66	215
Assay 3	0.378	34.4	0.083	100	344
Supernatant of 0.1 saturation	0.374	34.2	0.083	100	342
Supernatant of 0.2 saturation	0.270	24.6	0.062	75	328
Supernatant of 0.3 saturation	0.310	28.4	0.113	135	210
Supernatant of 0.4 saturation	0.080	7.4	0.068	80	92
Supernatant of 0.45 saturation	0.055	5.0	0.144	170	29
Supernatant of 0.5 saturation	0	0	0.354	410	0
Assay 4	0.315	28.8	0.062	75	384
Assay 5: Component 1	0.575	52.5	0.025	32	1640
Component 2	0.618	56.4	0.028	35	1610

Table 11: Purification of beta-amylases from rye (RR)

Sample	Enzymatic Activity		Nitrogen Content		Specific Activity, mg Maltose/mg N
	O.D. 560mμ	mg Maltose/ml	O.D. 515mμ	μg/ml	
Assay 1	0.056	5.2	0.060	72	72
Assay 2	0.073	6.8	0.042	51	133
Assay 3	0.412	37.8	0.167	195	198
Supernatant of 0.1 saturation	0.181	16.6	0.067	80	207
Supernatant of 0.2 saturation	0.272	24.8	0.108	128	194
Supernatant of 0.3 saturation	0.258	23.6	0.130	154	153
Supernatant of 0.35 saturation	0.152	14.0	0.127	150	93
Supernatant of 0.4 saturation	0.092	8.6	0.354	410	21
Supernatant of 0.45 saturation	0.018	1.6	0.465	540	3
Supernatant of 0.5 saturation	0	0	0.370	430	0
Assay 4	0.315	28.8	0.055	66	436
Assay 5:					
Component 1	0.412	37.8	0.030	37	1020
Component 2	0.370	33.8	0.028	34	992

Table 12: The electrophoretic mobilities of beta-amylases

Sample	Beta-amylases	Mobility, $cm^2\ V^{-1}\ sec^{-1} \times 10^{-5}$				
						Average
Durum	Component 1	2.00	2.03	2.03	2.02	2.02
	Component 2	1.87	1.86	1.89	1.90	1.88
Triticale	Component 1	1.99	1.98	2.03	1.99	2.00
	Component 2	1.78	1.76	1.80	1.75	1.77
Rye	Component 1	2.18	2.17	2.14	2.15	2.16
	Component 2	2.05	2.07	2.07	2.02	2.05

IV. EFFECT OF TEMPERATURE ON ACTIVITIES OF AMYLASES

(a) EFFECT OF TEMPERATURE ON ACTIVITIES OF ALPHA-AMYLASES

The enzymatic activity (v) of the isolated enzyme protein was determined colorimetrically as described by P. Bernfeld (10). It is expressed as mg maltose liberated from 1% soluble starch solution in three minutes. The color reagent was prepared by dissolving 1 gm of 3,5-dinitrosalicylic acid in 20 ml of 2 N sodium hydroxide and 50 ml of water. After the addition of 30 gm potassium sodium tartrate, the solution was diluted to 100 ml and the solution was kept in amber bottles. Starch substrate solution (1% w/v) was prepared by stirring one gram of soluble starch into 20 ml of 0.02 M sodium glycerophosphate-HCl buffer at pH 5.9 and adding it to 80 ml of hot but otherwise the same buffer. The starch solution was further heated for one minute with constant stirring. After cooling, the solution was transferred to a 100 ml volumetric flask, and made to volume with distilled water. A fresh solution was frequently prepared.

Chromatographically pure enzyme solutions which had been diluted to about 0.08 mg nitrogen per ml and starch substrate were placed in separate test tubes and equilibrated in a constant temperature water bath until the desired temperature had been attained. One ml of starch solution was introduced into one ml of enzyme solution by means of a fast-delivery pipette. After exactly three minutes, one ml of the reaction mixture was removed and transferred to a test tube containing one ml of the color reagent. The test tube was immersed in boiling water for six minutes. After cooling to room temperature, 10 ml of water was added and the absorbance measured at wavelength of 560 mμ with a spectrophotometer. The experiment was repeated three times.

The reaction for determination of amylase activity with 3,5-dinitro-

salicylic acid (12) was found to obey Beer's Law according to the relation (111): $M = 2.8\ L$

where M = the microequivalents of maltose,

L = the optical density of the solution of reduced reagent.

The dependence of activities of alpha-amylases on temperature is shown in Fig. 11.

(b) EFFECT OF TEMPERATURE ON ACTIVITIES OF BETA-AMYLASES

The general procedure used in these experiments was similar to that described in Section IV (a), except that 0.008 M acetate buffer at pH 4.8 was used instead of sodium glycerophosphate-HCl buffer. The purified enzyme solution from gel filtration was diluted to approximately 0.035 mg nitrogen per ml. In the case of Triticale, the chromatographically pure enzyme was diluted to 0.06 mg nitrogen per ml. The reaction mixture consisted of 1 ml of enzyme solution and 9 ml of starch substrate solution. The results are shown in Fig. 12.

Arrhenius plots of the effects of temperature on the activity of the amylases are shown in Fig. 13 and 14. The graphs may be obtained by plotting the logarithm of activity (v) versus the reciprocal of the absolute temperature (T).

Arrhenius (3, 4) derived an empirical formula to express the influence of temperature upon the rates of chemical reactions as follows:

$$v_2 = v_1 e^{\frac{E}{R}\left(\frac{T_2 - T_1}{T_2 T_1}\right)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(Eq. 1)}$$

where v_1 and v_2 are rates of reaction corresponding to absolute temperature T_1 and T_2,

E is the energy of activation,

R is the universal gas constant (1.98 calories/deg/mole).

The ratio of rates of reaction (v_2/v_1) is referred to as the temperature coefficient. Its value is always between 1 and 2. The values of energies of activation and heat inactivation could be calculated by substituting the values into Eq. 1. They are summerized in Tables 17, and 18.

Table 13: Effect of temperature on activities of alpha-amylases ($O.D._{560m\mu}$)

Temperature °C	Durum	Triticale	Rye
0	0.020	0.051	0.105
10	0.053	0.119	0.251
20	0.128	0.203	0.445
30	0.234	0.290	0.550
40	0.360	0.390	0.652
50	0.449	0.489	0.710
60	0.460	0.515	0.660
70	0.245	0.330	0.454
80	0.045	0.135	0.272
90	0	0	0

Table 14: Effect of temperature on activities of beta-amylases (O.D.560mμ)

Temperature ° C	Durum	Triticale	Rye
0	0.012	0.018	0.006
10	0.018	0.030	0.010
20	0.055	0.090	0.032
30	0.092	0.146	0.059
40	0.135	0.196	0.077
50	0.166	0.259	0.094
55	0.144	0.219	0.070
60	0.102	0.121	0.018
70	0	0	0
80	0	0	0

Table 15: Arrhenius expressions for effect of temperature on activities of alpha-amylases

Temp.°K	1/T x10^4	Durum		Triticale		Rye	
		v x 10	log v	v x 10	log v	v x 10	log v
273	36.62	2.0	0.31	4.6	0.66	10.0	1.00
283	35.33	5.0	0.70	10.8	1.03	23.2	1.36
293	34.11	11.4	1.06	18.6	1.27	46.0	1.66
303	33.01	21.4	1.33	26.4	1.42	50.2	1.70
313	31.95	32.8	1.52	35.6	1.55	60.0	1.78
323	30.95	41.0	1.61	44.4	1.65	65.0	1.81
333	30.03	42.0	1.62	47.2	1.67	60.4	1.78
343	29.15	22.4	1.35	30.2	1.48	43.4	1.64
348	28.73	12.2	1.09	20.6	1.31	33.0	1.52
353	28.33	4.2	0.62	12.4	1.09	23.8	1.38

Table 16: Arrhenius expressions for effect of temperature on activities of beta-amylases

Temp.°K	1/T x10^4	Durum		Triticale		Rye	
		v x 10	log v	v x 10	log v	v x 10	log v
273	36.62	6.0	0.78	8.0	0.90	3.0	0.48
283	35.33	8.0	0.90	19.0	1.28	5.0	0.70
293	34.11	25.0	1.40	41.0	1.61	14.0	1.15
303	33.01	42.0	1.62	64.0	1.81	27.1	1.43
313	31.95	60.0	1.78	90.0	1.95	36.0	1.56
323	30.95	75.2	1.88	120.0	2.08	41.2	1.62
328	30.45	66.1	1.82	100.0	2.00	32.0	1.50
331	30.22	55.0	1.74	74.3	1.87	17.1	1.23
333	30.03	46.0	1.66	55.2	1.74	8.0	0.90
338	29.52	18.4	1.26	19.2	1.28	0	--

Table 17:

Alpha-amylase	Energy of Activation		Energy for Heat Inactivation
	10°C to 20°C	30°C to 40°C	70°C to 80°C
Durum	13,500 cal/mole	8,080 cal/mole	40,600 cal/mole
Triticale	10,300	5,440	17,000
Rye	8,230	3,130	13,700

Table 18:

Beta-amylase	Energy of Activation		Energy for Heat Inactivation
	10°C to 20°C	30°C to 40°C	50°C to 60°C
Durum	11,300 cal/mole	7,440 cal/mole	15,500 cal/mole
Triticale	12,500	7,210	16,200
Rye	12,800	6,040	35,400

Fig. 11: Effect of temperature on activities of alpha-amylases

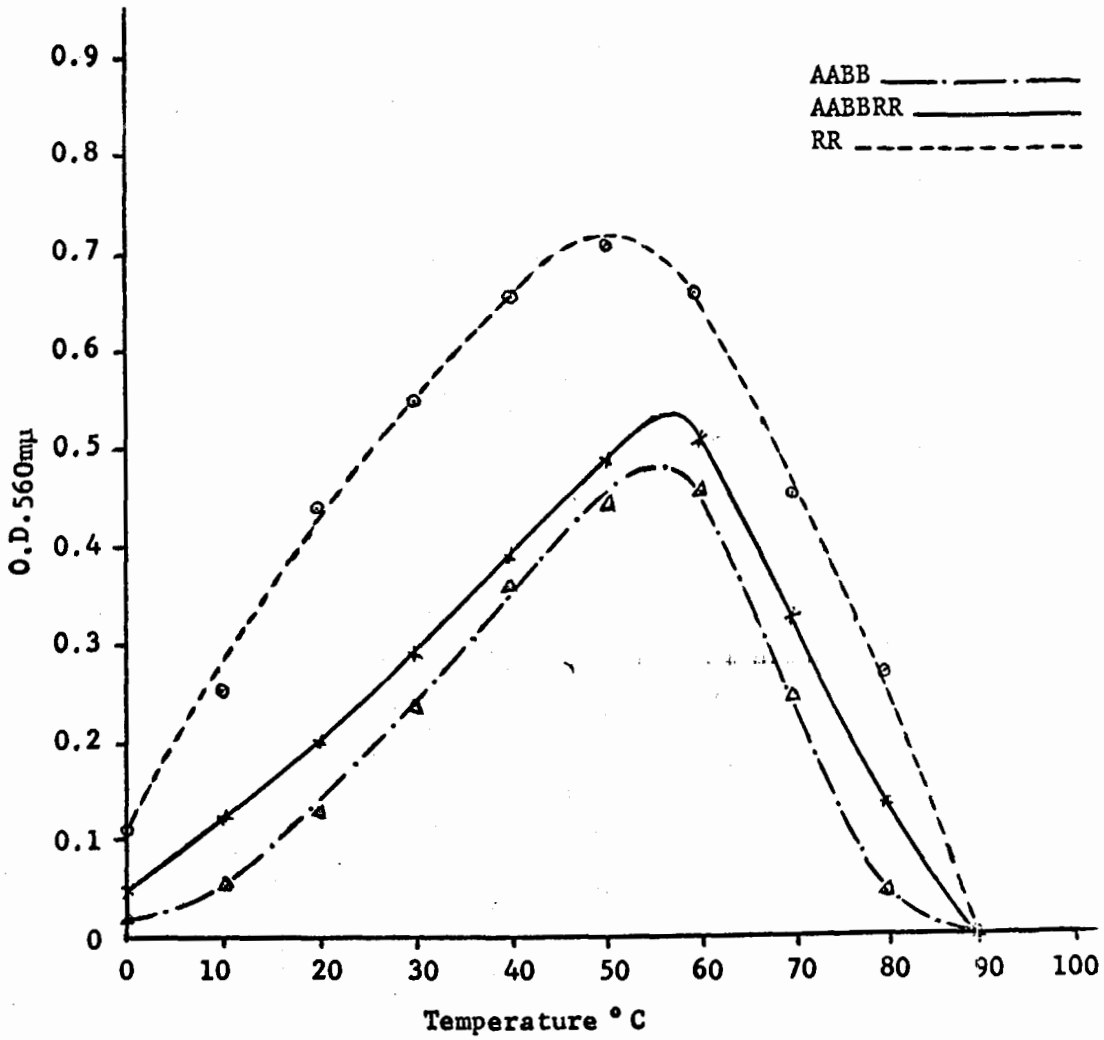
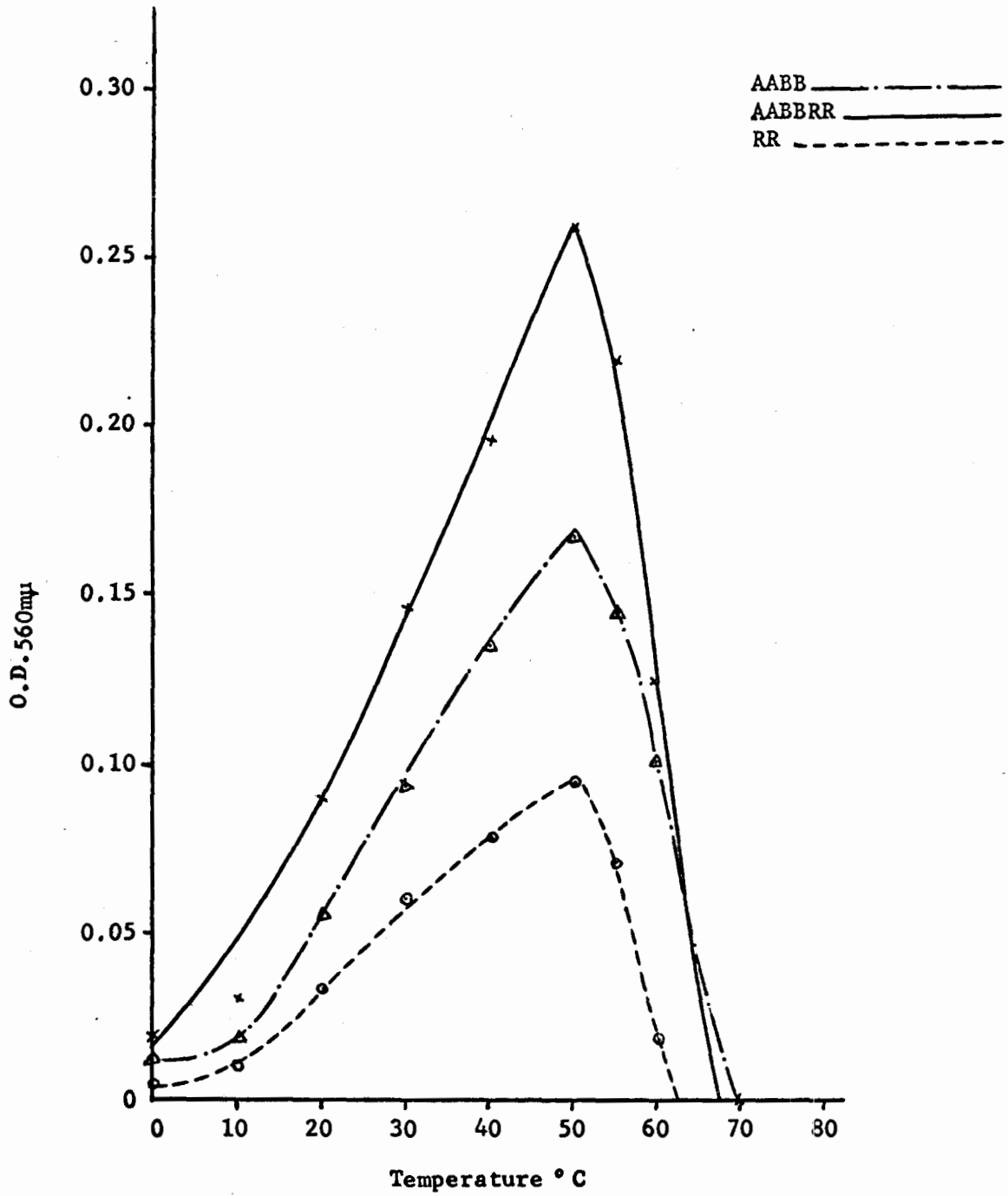


Fig. 12: Effect of temperature on activities of beta-amylases



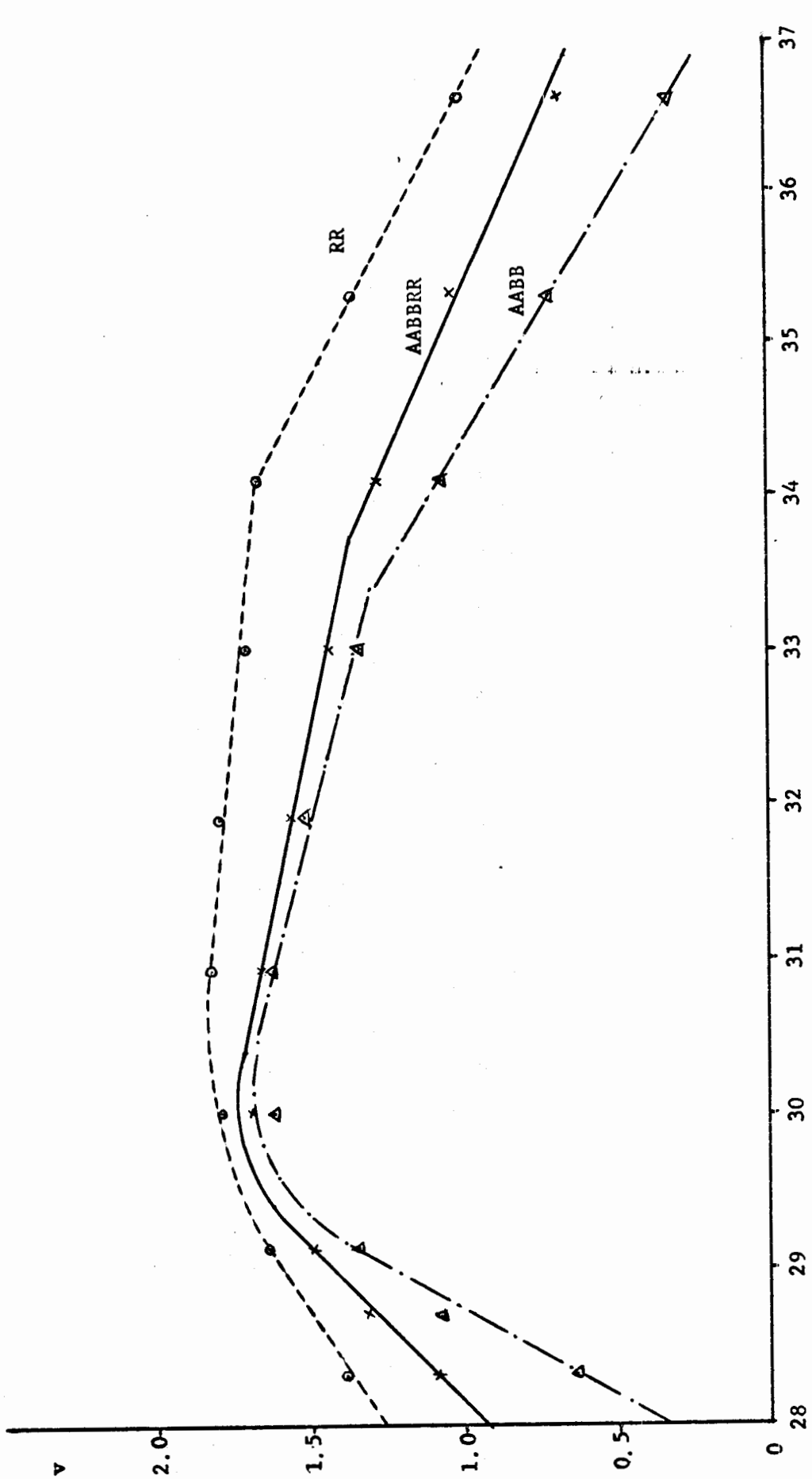
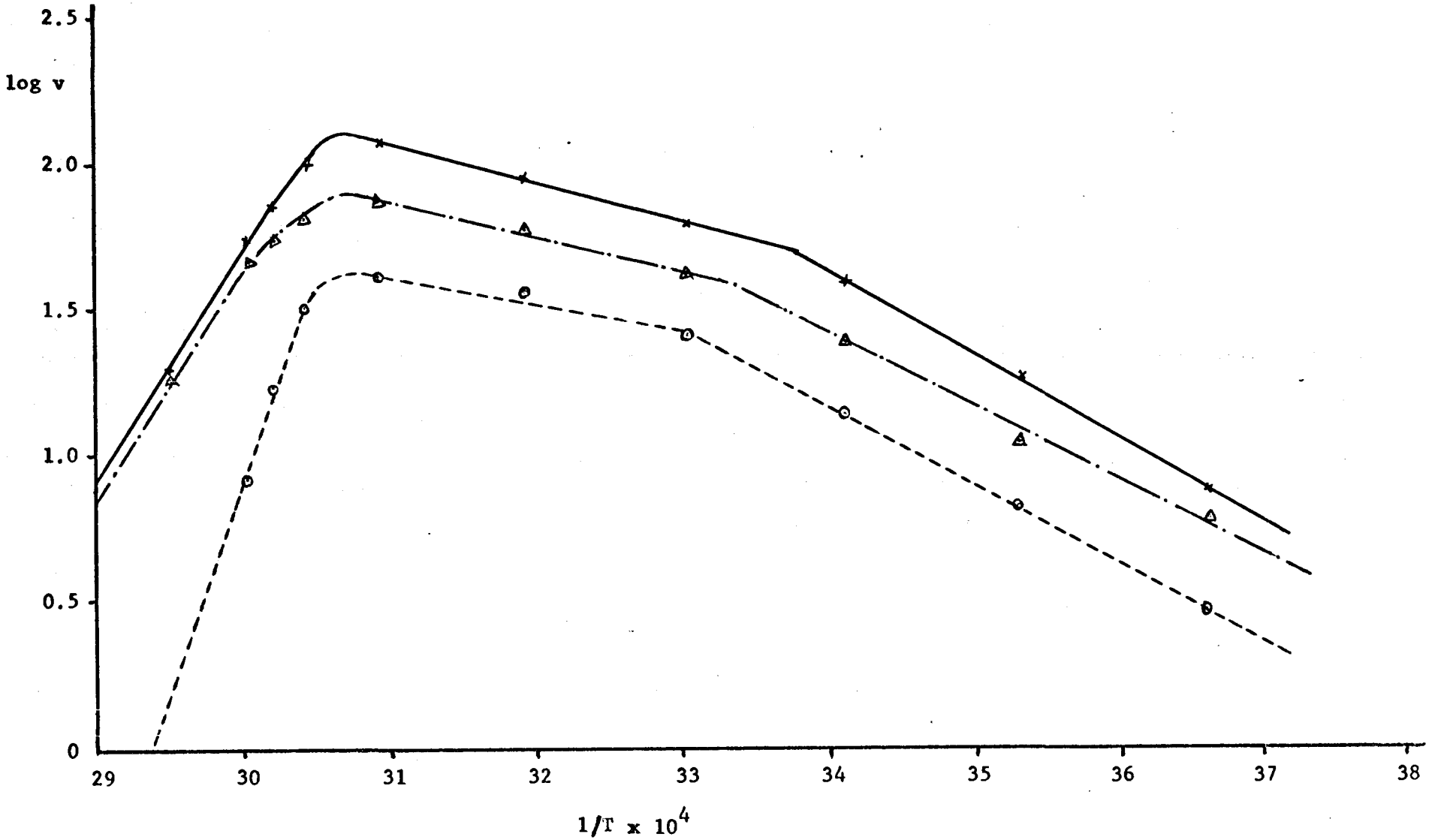


Fig. 13: Arrhenius plots for effects of temperature on activities of alpha-amylases

Fig. 14: Arrhenius plots for effects of temperature on activities of beta-amylases



V. INFLUENCE OF pH ON ACTIVITIES OF AMYLASES

A 2% starch in water solution was prepared as described in Section IV (a) which was subsequently diluted to 1% with an equal volume of buffer solution at various pH values. The buffer solutions were prepared as described by Gomori in Methods in Enzymology Vol. I. Acetate buffer solutions (0.1 M) at a pH range of 3.0 to 5.6, phosphate buffer (0.1 M) at a pH range of 6.0 to 8.0 and sodium bicarbonate-carbonate buffer (0.1 M) at a pH range of 9.2 to 10.6 were prepared. Aliquots (0.1 ml) of the chromatographically pure enzyme were diluted to 1 ml with buffer solutions at the same pH values as the starch solution in a series of test tubes. After standing at 25°C for two hours one ml of 1% starch solution of each pH value was introduced to the enzyme solution of the same pH value, respectively, by means of fast-delivery pipette. After exactly three minutes, one ml of the reaction mixture was transferred to a test tube containing one ml of 1% 3,5-dinitrosalicylic acid solution and followed the procedure described in Section IV (a).

Fig. 15, 16 and 17 show the influence of pH on activities of alpha-amylases which had been incubated in buffer for two hours. The alpha-amylases from all three sources showed optimum activity at a pH at 5.2.

The stability of alpha-amylases were studied by incubating alpha-amylases in buffer solution at various pH values for two hours after which the activities were determined at pH 5.2. This was found to be the most suitable pH value for determining activities of the alpha-amylases. The enzyme solutions at pH values below 3.0 were prepared by adding hydrochloric acid until the desired pH had been attained. The results are shown in Fig. 18, 19 and 20. All alpha-amylases from the three sources were found to be

stable between pH 4 and pH 8.

Beta-amylases from all three sources showed a pH optimum at 4.6 as shown in Fig. 21, 22 and 23.

These experiments were done in duplicate.

Table 19: Influence of pH on activities of alpha-amylases ($O.D._{560m\mu}$)

pH	Durum	Triticale	Rye
3.0	0.170	0.078	0.035
3.6	0.418	0.237	0.160
4.0	0.486	0.418	0.276
4.6	0.515	0.468	0.356
5.0	0.535	0.475	0.419
5.6	0.528	0.472	0.415
6.0	0.495	0.442	0.381
6.6	0.458	0.420	0.300
7.0	0.427	0.380	0.233
7.6	0.376	0.336	0.147
8.0	0.340	0.290	0.102
9.2	0.190	0.119	0.021
9.6	0.161	0.029	0
10.0	0.041	0	0
10.6	0.016	0	0

Table 20: Stability of alpha-amylases at various pH values (O.D.$_{560m\mu}$)

pH	Durum	Triticale	Rye
2.0	0	0	0
2.6	0	0.015	0.042
3.0	0.020	0.060	0.110
3.6	0.230	0.228	0.298
4.0	0.288	0.271	0.332
4.6	0.305	0.280	0.339
5.0	0.312	0.293	0.353
5.6	0.310	0.294	0.348
6.0	0.306	0.294	0.350
6.6	0.318	0.287	0.348
7.0	0.308	0.290	0.352
7.6	0.302	0.278	0.352
8.0	0.300	0.268	0.348
9.2	0.202	0.125	0.328
9.6	0.080	0.031	0.290
10.0	0.021	0	0.110
10.6	0	0	0.012

Table 21: Influence of pH on activities of beta-amylases ($O.D._{560m\mu}$)

pH	Durum	Triticale	Rye
3.0	0.155	0.083	0.086
3.6	0.258	0.142	0.163
4.0	0.360	0.294	0.192
4.6	0.451	0.378	0.206
5.0	0.440	0.362	0.197
5.6	0.418	0.340	0.184
6.0	0.403	0.313	0.170
6.6	0.367	0.284	0.155
7.0	0.350	0.263	0.138
7.6	0.322	0.220	0.116
8.0	0.291	0.191	0.097
9.2	0.202	0.100	0.015
9.6	0.181	0.072	0.006
10.0	0.137	0.045	0
10.6	0.090	0.020	0

Fig. 15: pH-Activity relationship of alpha-amylase from Durum Wheat (AABB)

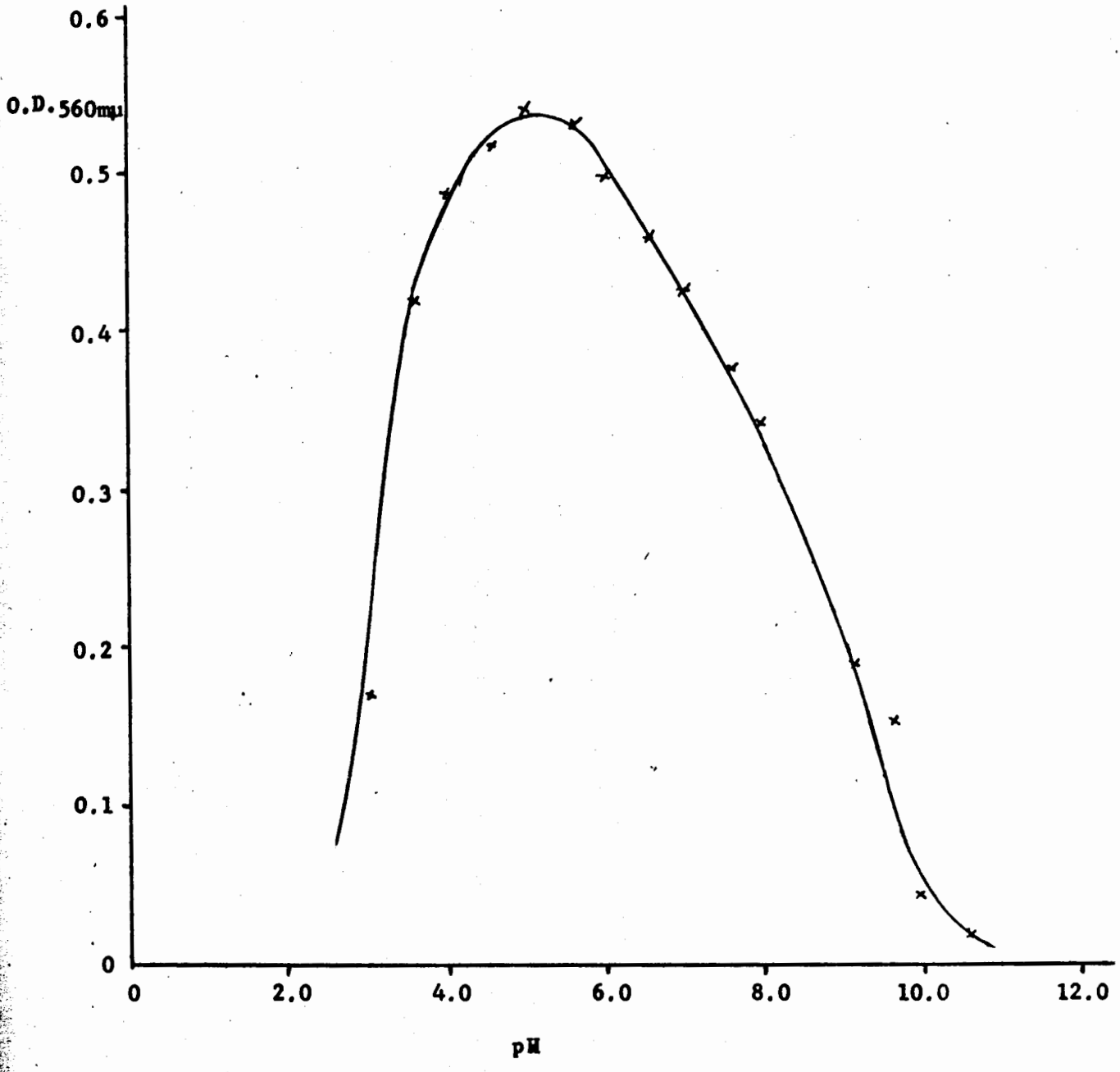


Fig. 16: pH-Activity relationship of alpha-amylase from Triticale (AABBRR)

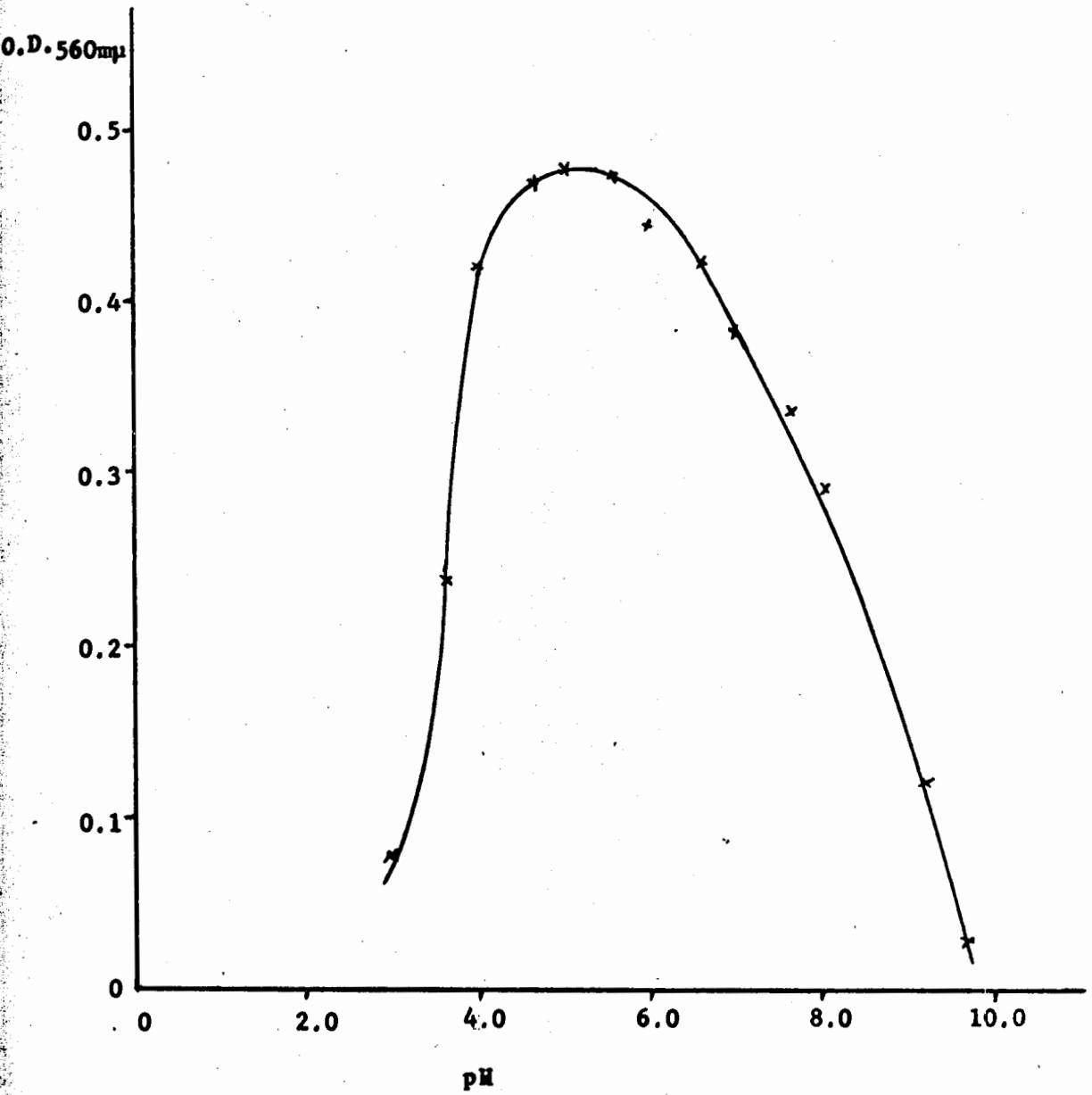


Fig. 17: pH-Activity relationship of alpha-amylase from Rye (RR)

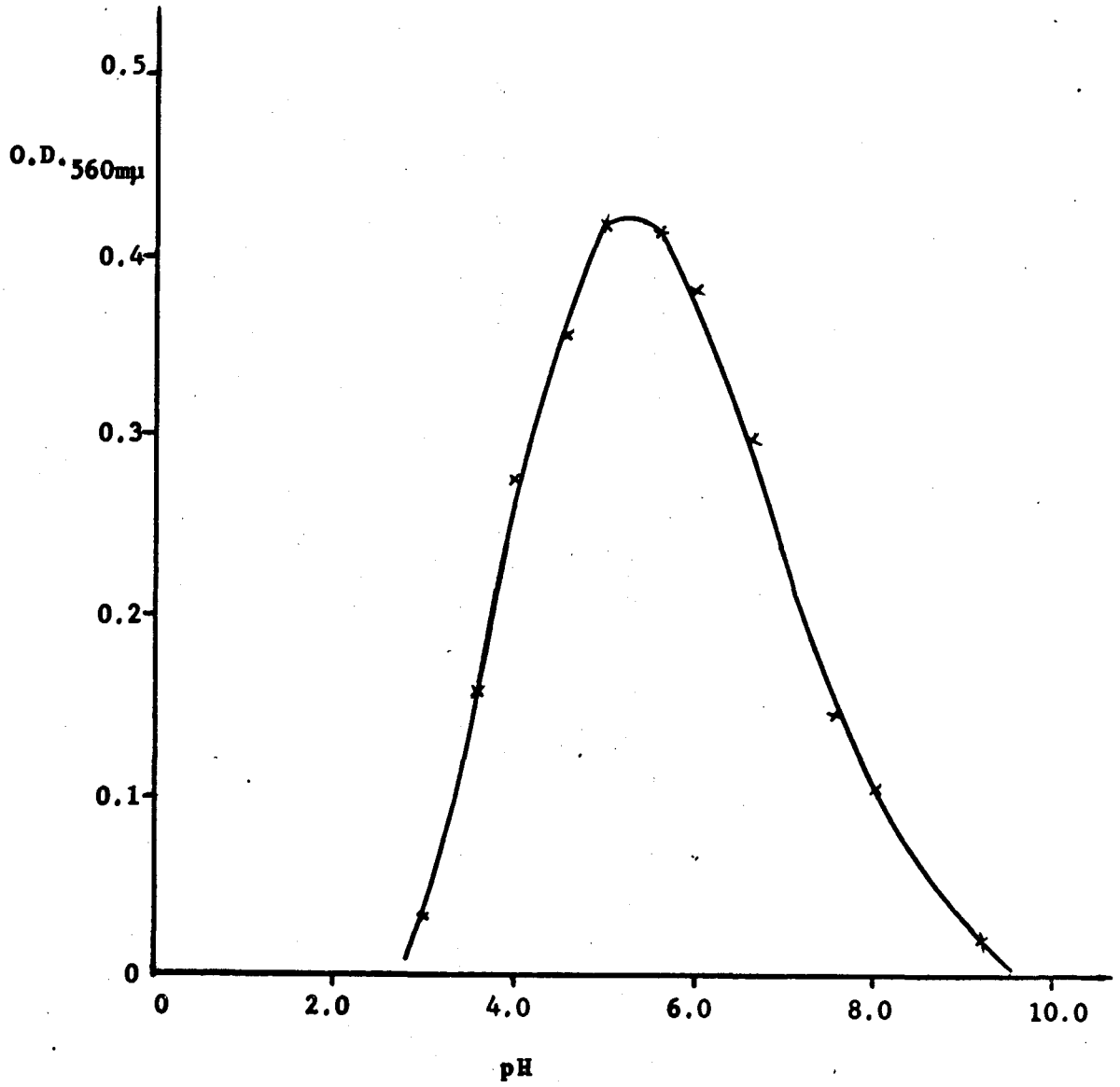


Fig. 18: Stability of alpha-amylase from Durum (AABB)

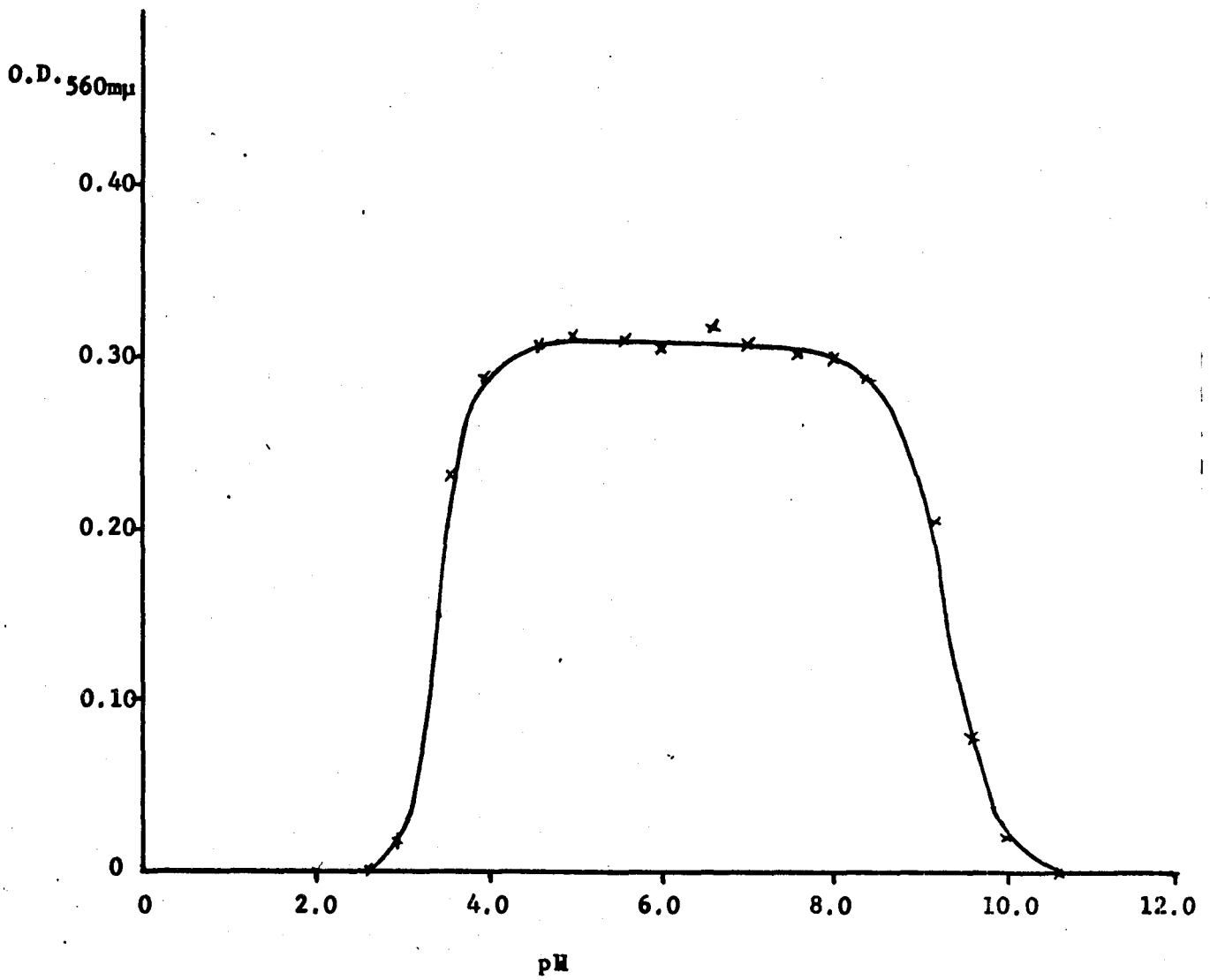


Fig. 19: Stability of alpha-amylase from Triticale (AABBRR)

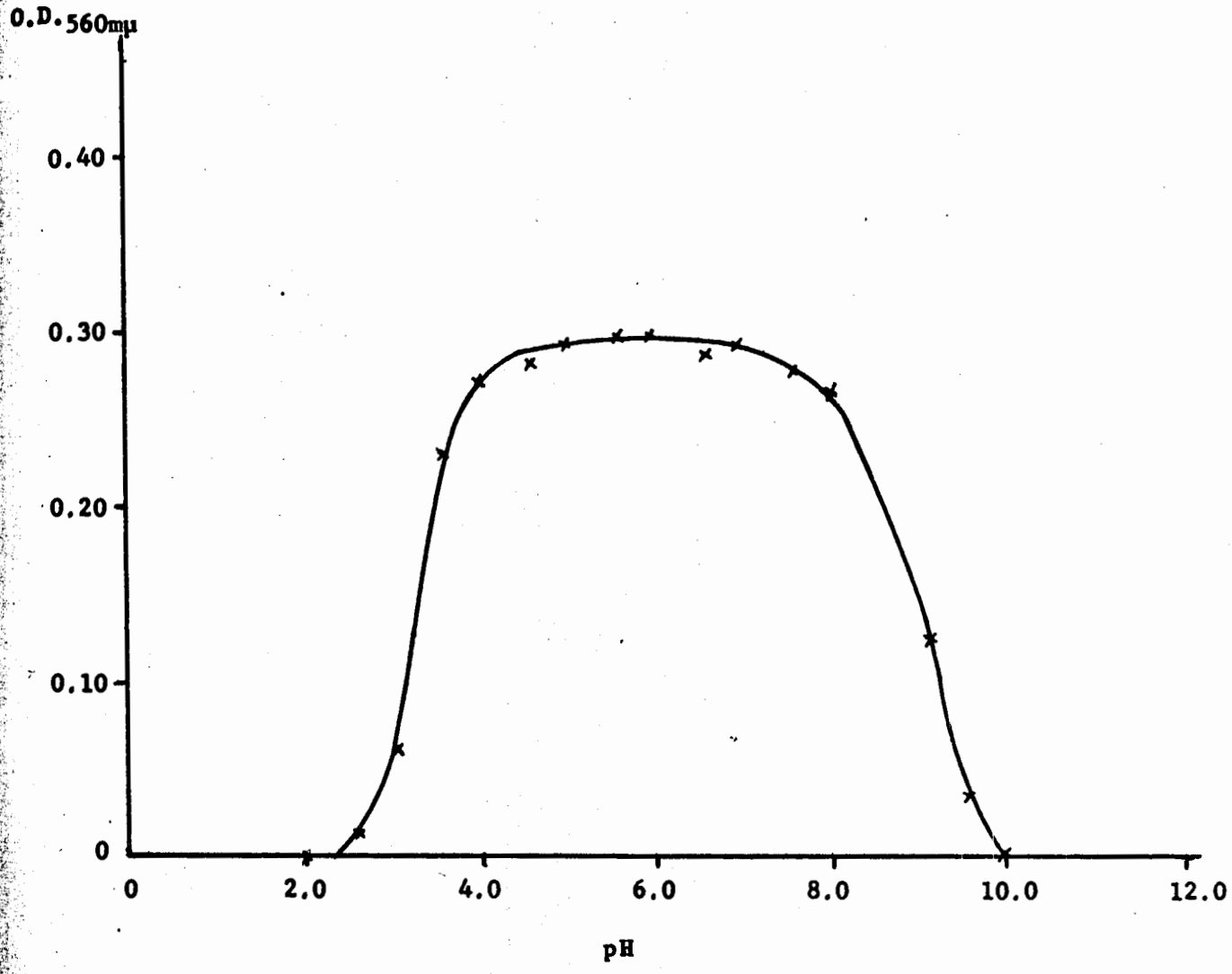


Fig. 20: Stability of alpha-amylase from Rye (RR)

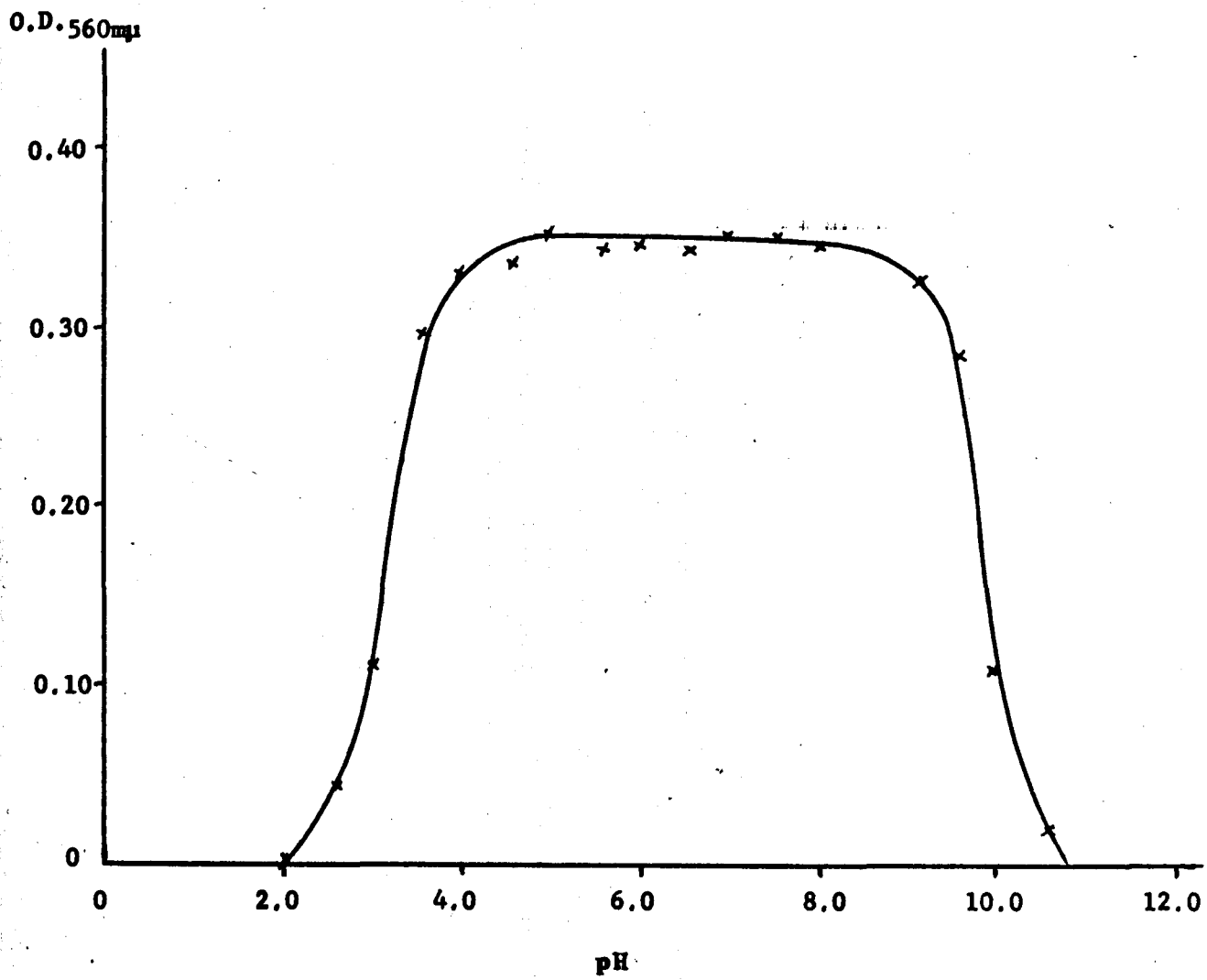


Fig. 21: Influence of pH on activity of beta-amylase from Durum (AABB)

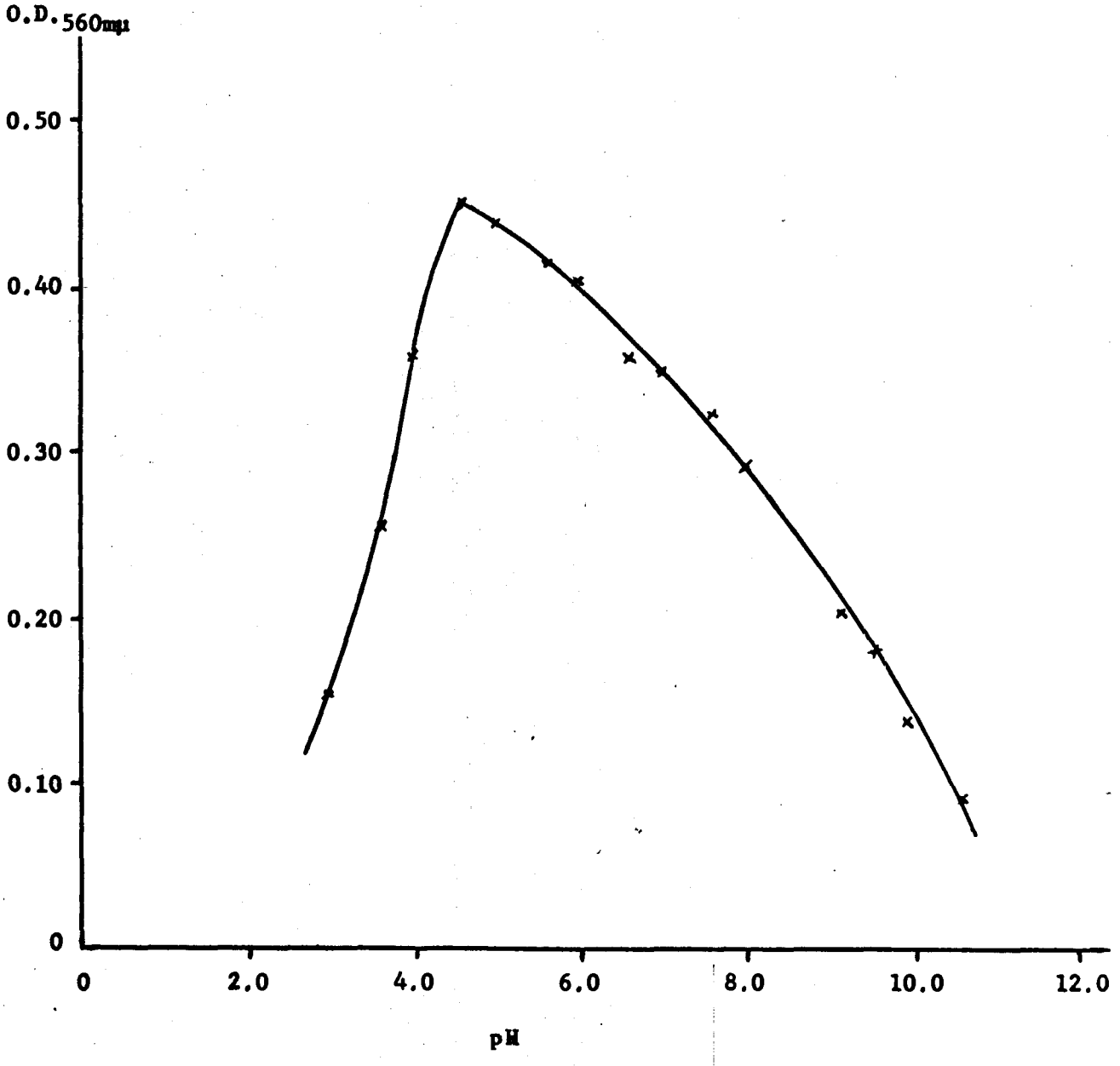


Fig. 22: Influence of pH on activity of beta-amylase from Triticale (AABBRR)

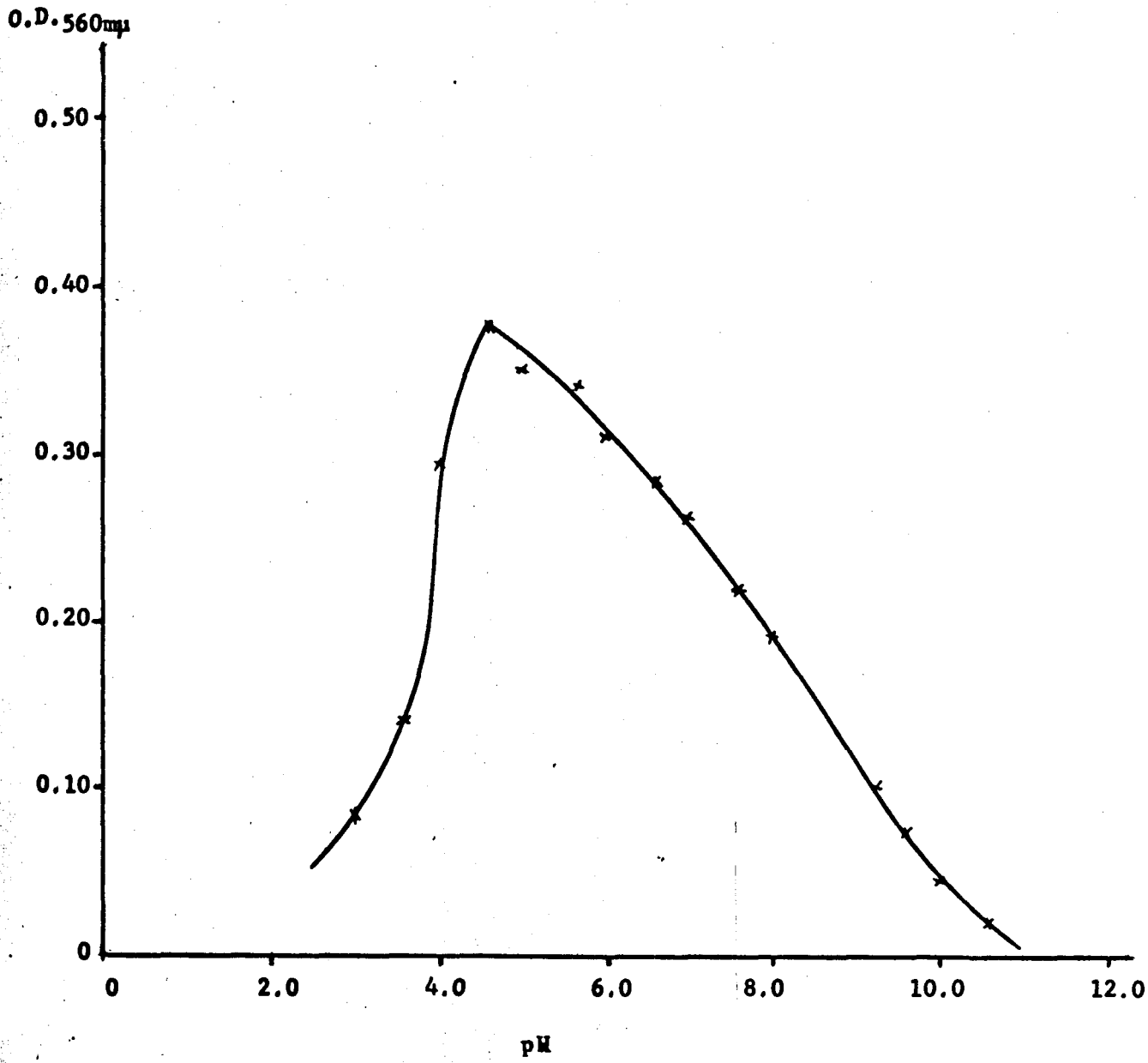
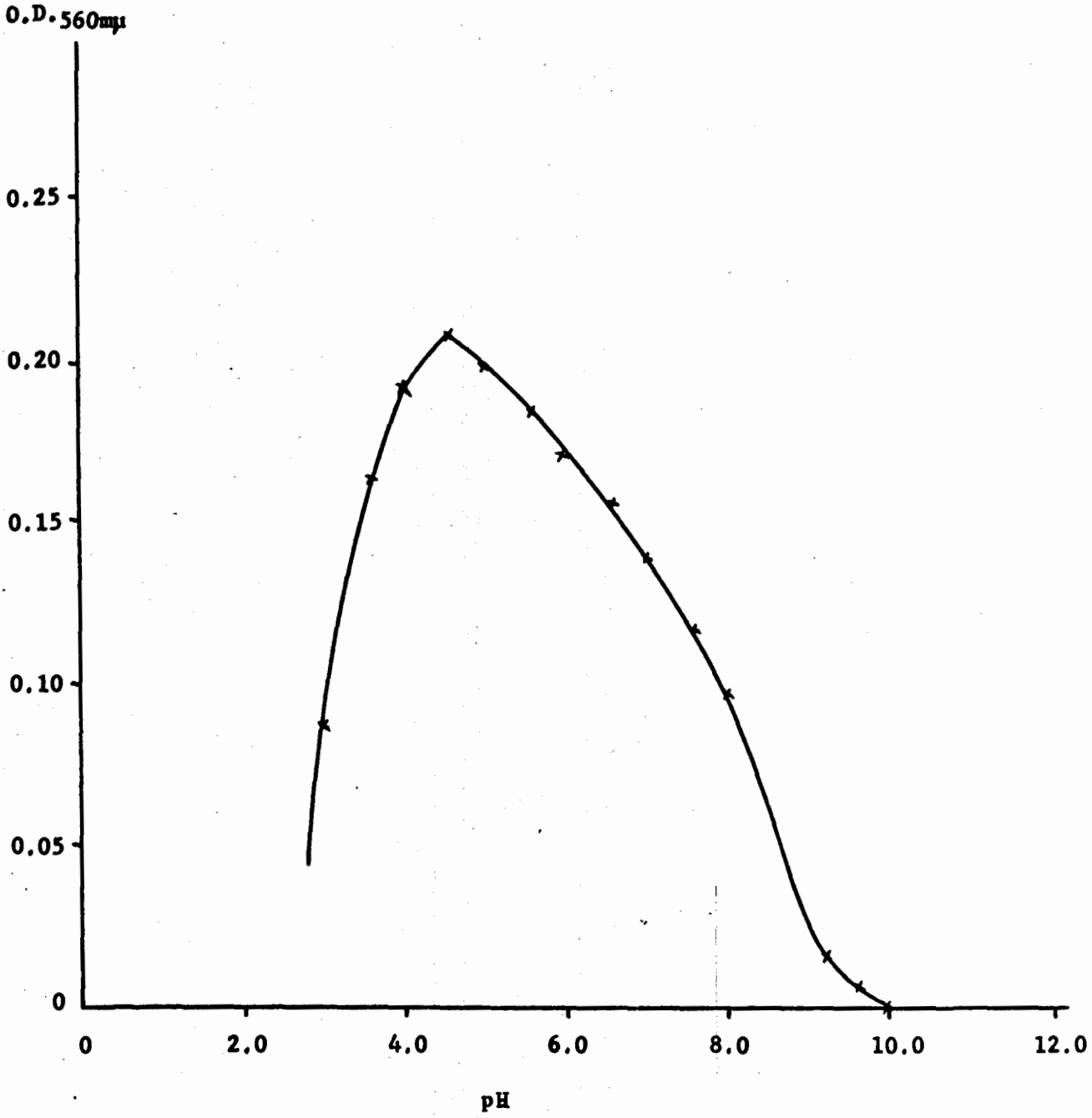


Fig. 23: Influence of pH on activity of beta-amylase from Rye (RR)



VI. DETERMINATION OF MICHAELIS-MENTEN CONSTANTS FOR AMYLASES

(a) DETERMINATION OF MICHAELIS-MENTEN CONSTANTS FOR ALPHA-AMYLASES

The Michaelis-Menten constant, which is the substrate concentration at half maximum velocity of an enzyme-catalysed reaction, can be determined by studying the effect of variation of substrate concentration on the rate of enzyme action.

The chromatographically pure alpha-amylases from durum, Triticale and rye were employed for this study. The enzymes were diluted with 0.02 M acetate buffer containing 0.001 M calcium acetate at pH 5.2 to a final concentration of about 0.06 mg nitrogen per ml. Starch solutions of various concentration in the range of 0.5 to 50 mg per ml were prepared in 0.02 M acetate buffer, pH 5.2 as described in Section IV (a). The temperature of all enzyme and starch solutions was brought to 25°C. One ml of starch solution of each concentration was transferred into a series of test tubes. One ml of enzyme solution was introduced into each tube by means of a fast-delivery pipette. After exactly three minutes, one ml of the reaction mixture was added to a test tube and the reducing sugar produced was determined by the 3,5-dinitrosalicylic acid method as described in Section IV (a).

(b) DETERMINATION OF MICHAELIS-MENTEN CONSTANTS FOR BETA-AMYLASES

The procedure was the same as described above except that 0.008 M acetate buffer at pH 4.8 was used instead of 0.02 M acetate buffer at pH 5.2. Addition of calcium acetate was omitted since it is not essential for beta-amylases.

The purified beta-amylases were diluted to about 0.04 mg nitrogen per ml with 0.008 M acetate buffer at pH 4.8.

The results are shown in Table 22 and 23, in which S represents the starch concentration in mg per milliliter and v represents the velocity of reaction as mg maltose produced/ml/3 min.

The Michaelis-Menten equation can be written in a linear form in the following three ways (21, 44, 47, 48, 67):

$$\text{(i)}\quad \frac{1}{v} = \frac{K_m}{V_m} \cdot \frac{1}{S} + \frac{1}{V_m} \qquad \text{..... (Eq. 2)}$$

$$\text{(ii)}\quad \frac{S}{v} = \frac{1}{V_m} \cdot S + \frac{K_m}{V_m} \qquad \text{..... (Eq. 3)}$$

$$\text{(iii)}\quad V_m = v + \frac{v}{S} \cdot K_m \qquad \text{..... (Eq. 4)}$$

These linear equations have many advantages in the graphical evalution of the Michaelis-Menten constant. The double reciprocal plot obtained from Eq. 2 is most widely used (19).

In the present studies, the Michaelis-Menten constants were determined graphically by the double reciprocal plot method (67). Straight lines were obtained when the reciprocal values of starch concentrations (S) were plotted against the reciprocal values of reaction velocities (v). The straight lines intersected the base line at a point giving $-1/K_m$ and intersected the vertical axis at a point which gave $1/V_m$, where V_m represents the maximum velocity. The straight line had a slope of K_m/V_m. The values of Michaelis-Menten constant (K_m) and maximum velocities (V_m) were calculated from Fig. 24 and Fig. 25, and were summarized in Table 24 and Table 25. Each experiment was repeated twice.

Table 22: Effects of substrate concentration on activities of alpha-amylases

Starch conc.		Durum		Triticale		Rye	
S	1/S	v	1/v	v	1/v	v	1/v
0.25	4.00	0	0	0	0	0	0
0.50	2.00	0	0	0.04	25.00	0.20	5.00
2.00	0.50	0.12	8.06	0.24	4.17	0.45	2.22
4.00	0.25	0.21	4.80	0.38	2.63	0.64	1.56
5.00	0.20	0.24	4.11	0.44	2.27	0.70	1.43
10.00	0.10	0.33	3.00	0.62	1.61	0.84	1.19
15.00	0.06	0.40	2.50	0.74	1.35	0.92	1.09
20.00	0.05	0.46	2.18	0.77	1.30	0.96	1.04
25.00	0.04	0.47	2.10	0.80	1.25	0.98	1.02

Table 23: Effects of substrate concentration on activities of beta-amylases

Starch conc.		Durum		Triticale		Rye	
S	1/S	v	1/v	v	1/v	v	1/v
0.25	4.00	0	0	0	0	0	0
0.50	2.00	0	0	0	0	0	0
2.00	0.50	0.42	2.37	0.27	3.71	0.26	3.85
4.00	0.25	0.77	1.30	0.48	2.08	0.44	2.27
5.00	0.20	0.85	1.18	0.58	1.73	0.51	1.96
10.00	0.10	1.27	0.79	0.88	1.14	0.83	1.22
15.00	0.06	1.58	0.63	1.06	0.95	1.01	0.99
20.00	0.05	1.78	0.56	1.22	0.82	1.13	0.88
25.00	0.04	1.88	0.53	1.34	0.75	1.20	0.83

Fig. 24: Determination of Michaelis-Menten constants, K_m, for alpha-amylases

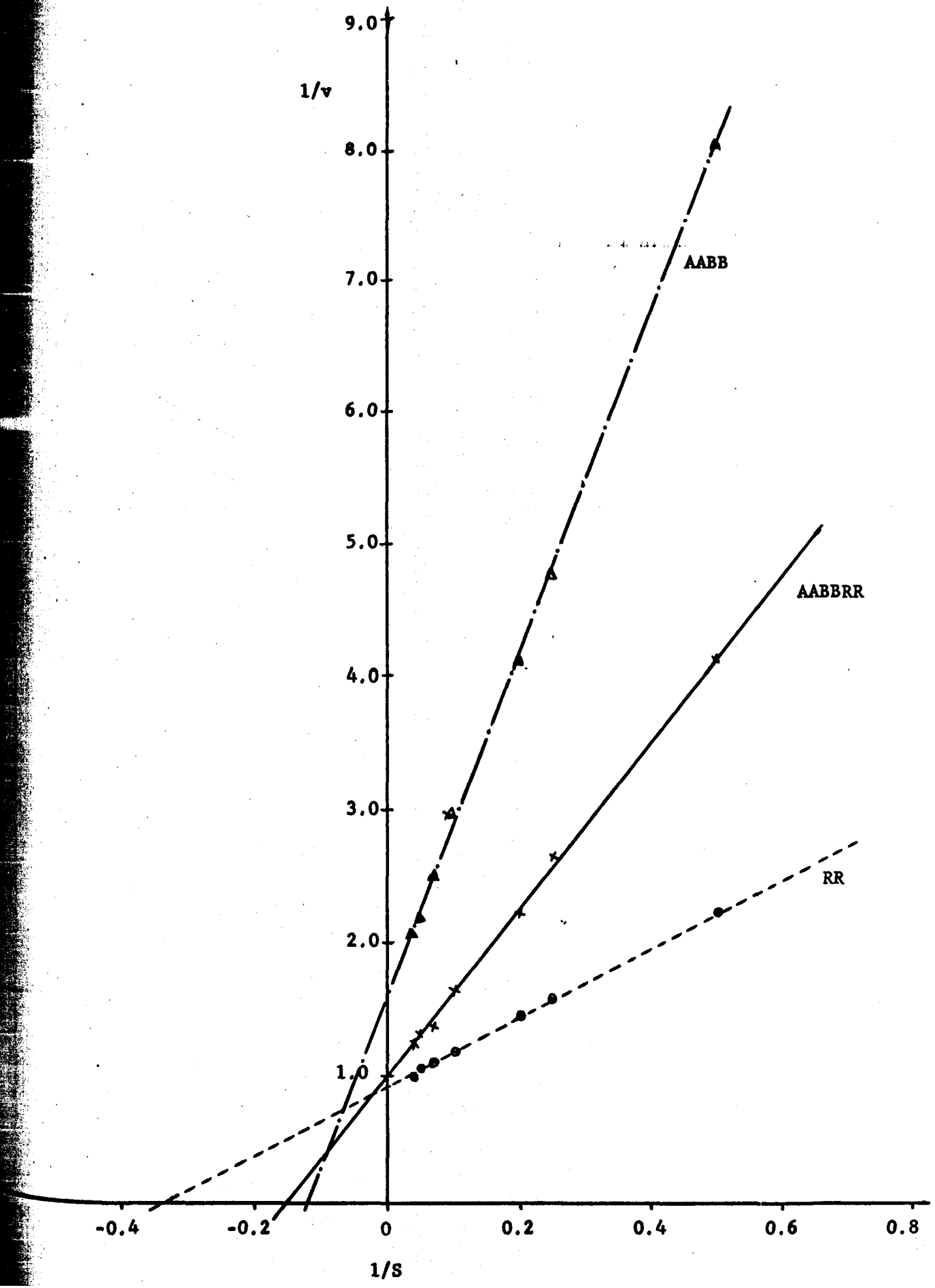


Fig. 25: Determination of Michaelis-Menten Constants for beta-amylases

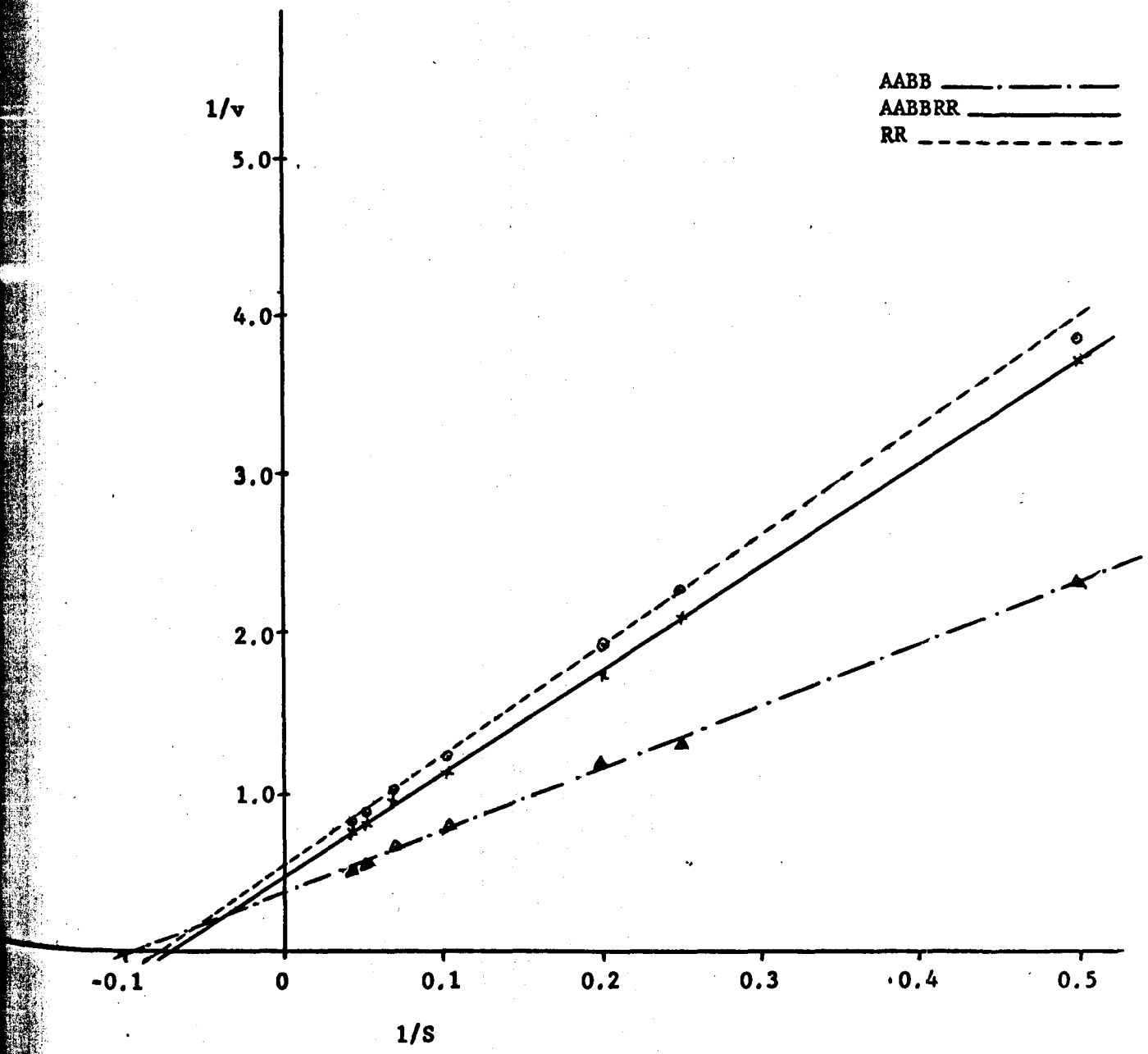


Table 24: K_m and V_m values for alpha-amylases

Sample	K_m, mg starch/ml	V_m, mg maltose/ml/3min.
Durum	7.15	0.62
Triticale	6.25	1.00
Rye	2.78	1.10

Table 25: K_m and V_m values for beta-amylases

Sample	K_m, mg starch/ml	V_m, mg maltose/ml/3 min.
Durum	10.0	2.50
Triticale	11.1	1.92
Rye	12.5	1.70

VII. STUDIES OF SULFHYDRYL GROUPS IN BETA-AMYLASES

The spectrophotometric titration for quantitative estimation of sulfhydryl groups reported by I.M. Klotz et al. (61) was followed with minor alteration. Mersalyl acid, the anhydride of o-{[3-(hydroxymercuri)-2-methoxypropyl]carbamyl}-phenoxyacetic acid, was purchased from Winthrop Laboratories (New York 18, N. Y.). The pyridine-2-azo-p-dimethylaniline was purchased from Sigma Chemical Company, St. Louis 18, Missouri.

Acetate buffer (0.1 M) at pH 5.8 was prepared as the solvent for all reagents. A stock solution of azopyridine dye at approximately 2×10^{-4} M concentration was prepared in acetate buffer. This dye solution was stable for at least one month. The mersalyl acid was weighed accurately to give a stock solution of 1×10^{-3} M in acetate buffer containing 5×10^{-3} M sodium chloride to stabilize the acid. For most reliable results, this solution should not be kept over one week.

The stock solution of azopyridine dye was diluted with acetate buffer in the titration vessel to a final concentration of about 8×10^{-5} M and a total volume of 9 ml. Buffer solution alone was placed in the reference absorption tube. After the solution was deoxygenated by bubbling nitrogen gas through the solution for three minutes, exactly one milliliter of sample solution was introduced to the titration vessel. The absorbance was measured at 550 millimicrons in a spectrophotometer. Standard mersalyl acid solution at a concentration of 1×10^{-4} M was then added from a microburet, several absorbance readings (at 550 mμ) were recorded to establish a base line before the equivalence point was approached. Small increments of mersalyl acid solution were added near and beyond the equivalence point. After each addition of mersalyl acid solution, nitrogen was directed into the cell to mix the solution. When absorbancy values

were plotted against milliliters of mersalyl acid solution added, the equivalence point was indicated by the intersection of the two straight lines.

The results of quantitative titrations of sulfhydryl groups in beta-amylases of the synthetic species, Triticale (AABBRR), and the parental species, Stewart durum (AABB) and Prolific rye (RR) are shown in Table 26. Two components of beta-amylases were isolated from each source as reported in Section III (B). Component 1 of beta-amylases refers to the component which had the higher electrophoretic mobility, whereas component 2 had the lower electrophoretic mobility.

The quantitative titrations of sulfhydryl groups in alpha-amylases were carried out under the same conditions as above. Results showed that no free sulfhydryl groups were present in alpha-amylases of the three species.

Table 26: Determination of sulfhydryl groups in beta-amylases

Sample	beta-amylases	mg enzyme nitrogen	µM mersalyl acid added	µM sulfhydryl groups per mg nitrogen
Durum	Component 1	0.538	0.218	0.405
	Component 2	0.468	0.185	0.395
Triticale	Component 1	0.228	0.072	0.316
	Component 2	0.252	0.077	0.305
Rye	Component 1	0.313	0.067	0.214
	Component 2	0.474	0.099	0.209

D. DISCUSSION

I. DEVELOPMENT OF ALPHA-AMYLASES DURING GERMINATION

It had long been observed that beta-amylase appeared in a fairly high concentration in the ungerminated grains of cereals and its activity increased only to a limited extent during sprouting. In contrast, the concentration of alpha-amylase in ripe grains was very low before germination started while during the germination process, alpha-amylase developed rapidly (59, 62, 63, 68, 110). Many investigators have studied the development of alpha-amylases in wheat (38, 95, 133), rye (93), barley (68) etc. during germination and in all cases, they found that alpha-amylase increased rapidly as soon as sprouting started.

As seen in Fig. I, the activities of alpha-amylases were very low in the ungerminated grains of durum wheat (AABB), rye (RR) as well as the synthetic species, Triticale (AABBRR). Once the germination process had started, the activities of alpha-amylases increased rapidly and logarithmically just as observed in other cereals. It is interesting to observe that the amount of alpha-amylase that developed in the synthetic species, Triticale, was between that of its parental species. The same phenomenon was observed in the ungerminated grains. The amount of alpha-amylases present in the grains of the synthetic species and the parental species after germinating for four days was found to be in the ratio of Durum : Triticale : Rye = 1 : 1.5 : 3.5. From these results it is apparent that the biosynthesis of alpha-amylase of the synthetic species, Triticale, is influenced by both of the parental species.

S. Hagberg's modified Wohlgemuth method for determination of alpha-amylase activity was found useful since it could be employed even in the presence of beta-amylase. The treatment of substrate with excess beta-

amylase for 24 hours before usage tends to eliminate the interference of beta-amylase present in the malt.

As shown in Table 2, the sprout lengths, which indicated the vegetative growth rate, were fairly well correlated with the development of alpha-amylases. It was observed that the higher the growth rate, the faster the development of alpha-amylases. This is what would normally be expected in amylase elaboration in germinating cereal seeds.

II. EFFECTS OF POTASSIUM GIBBERELLATE ON THE DEVELOPMENT OF ALPHA-AMYLASES DURING GERMINATION

Gibberellins have been found to be able to activate the alpha-amylases in cereals during germination (14, 123, 143). Gibberellic acid has also been found to be able to stimulate the production of amylases in ungerminated grains of cereals (32, 144).

In the present studies, the effects of potassium gibberellate on the production of alpha-amylases in the synthetic species, Triticale (AABBRR), and its parental species, durum (AABB) and rye (RR), have been examined. A logarithmic increase in activities of alpha-amylases in the germinating grains of these three species was evident. The development of alpha-amylases in gibberellic acid treated grains is about two to three times faster than in the untreated grains. It was found that the response of production of alpha-amylases to gibberellic acid was variety dependent. This is in agreement with the results reported by Fleming et al. (32) and Griffiths et al. (40).

The ratio of the amount of alpha-amylases present in the gibberellic treated grains of the three species after germinated for four days was found to be: Durum : Triticale : Rye = 1 : 1.3 : 2.2. An interesting observation was made in that the response of alpha-amylase production in Triticale (AABBRR) is intermediate between that found in the parental species. This indicated that the biosynthesis of enzymatically active amylases in the particular alien genome combinant probably follows a normal hybrid pattern.

When the ungerminated grains of durum wheat were steeped in 0.005% potassium gibberellate at 25°C for two hours, the activity of alpha-amylase increased two fold above that observed in the untreated grains. In the

cases of Triticale and rye, the alpha-amylase activities increased three times over those in the untreated grains. Yomo et al. (144) reported that one p.p.m. gibberellin stimulated the amylase activity in ungerminated barley endosperm about 100-fold after 16 hours of incubation at 30°C. Since 0.005% potassium gibberellate solution had been found to be the most effective concentration for activation of alpha-amylases in germinating grains of various varieties of wheat by Fleming (32), this concentration was employed throughout the experiments described in this investigation.

As may be observed in Table 4, the length of sprouts and roots were fairly well correlated with the development of alpha-amylases in the gibberellin-treated seeds just as was found to be the case in the non-gibberellin treated seeds. Again, the above is not an unexpected observation since the vegetative growth rate and rate of enzyme elaboration have been found to exhibit parallel development in numerous biological systems.

III. ISOLATION AND PURIFICATION OF AMYLASES

(a) ISOLATION AND PURIFICATION OF ALPHA-AMYLASES

Calcium chloride solution (0.2%) was used to extract alpha-amylases in the isolation process because calcium ion stabilized and activated the enzyme (29, 31, 141). It can be seen in Tables 5,6 and 7 that the specific activity of alpha-amylase using the conventional precipitation method could be increased three to four fold over that of the original extract, and subsequently increased five to six times after gel filtration. In comparison, the specific activity of crystalline alpha-amylase from human saliva obtained by Fischer was also six times higher than that found in the original extract (30).

It was found that two fractions from the gel column effluent were able to liberate reducing suger from soluble starch. These could be alpha-amylase, beta-amylase or 1,6-glucosidase. In order to differentiate the enzymes which emerged from the column, a few definitive tests were carried out. When a 1% amylose solution was used as substrate, both fractions were found capable of degrading the amylose and produced reducing sugar. This showed that both fractions were not or did not contain a 1,6-glucosidase. Further indentification by studying the abilities of the enzymes to decolorise the starch-iodine complex (105) showed that the first fraction which emerged from the column had a very low dextrinizing power, however, the fraction which emerged later had very high dextrinizing power. This property indicated that only the later fraction was alpha-amylase. The first enzymatically active fraction may have been a contamination of beta-amylase which had probably not been completely destroyed during the heat treatment. According to the experiments by E. Kneen et al., barley

malt beta-amylase was destroyed by keeping it in solution at 70°C for fifteen minutes while this treatment did not destroy the alpha-amylase (64). Further investigations regarding this aspect are necessary. Further studies involving gel filtration of beta-amylases (refer to next section) on the same column of Sephadex G-50 revealed that beta-amylases emerged from the column at tube numbers between 100 and 120, whereas alpha-amylases emerged at tube numbers between 120 and 130. From a comparison of these chromatograms of alpha-amylases and beta-amylases, it is evident enough that the first enzymatically active fraction from the column (see Fig. 4, 5 and 6) was undoubtedly a small portion of beta-amylases which survived the heat treatment.

Highly cross-linked dextrans which are available commercially under the name Sephadex have been found very useful in the purification of enzymes, proteins, peptides, amino acids as well as in the desalting process. It has many advantages over silica gel or other supporting media in column chromatography. The wide ranges in particle sizes of cross-linked dextrans enabled the separation of enzymes and proteins of different molecular sizes (35, 99). In the present studies, various grades of Sephadex, including Sephadex G-50, G-100, G-150 and G-200 were employed for the purification of alpha-amylases from durum, Triticale and rye. It was found that Sephadex G-50 gave the best results for separation of alpha-amylases from other associated proteins. This is in agreement with the results reported by Shulman et al. (116).

The Kjeldahl method for the determination of total nitrogen and ammonia in biological materials and enzymatic reaction mixture has been found very useful and possesses reasonably high precision (5). However, when working with large numbers of analyses, Nessler's method is more

convenient and rapid. For best results, Nessler's method was employed to determine a range of approximately 10 to 50 micrograms of nitrogen per sample. It suffers the disadvantage that the stability of Nessler's reagent may change from time to time. This can be overcome by constructing a standard curve periodically.

(b) ELECTROPHORETIC MOBILITIES OF ALPHA-AMYLASES

The electrophoretic mobilities of alpha-amylases from the three sources were found different from one another. Aluminium lactate buffer solutions at various pH values were employed for the electrophoretic studies. It was found that the electrophoretic mobilities of alpha-amylases were pH dependent. The differences in electrophoretic mobilities of alpha-amylases from the three sources show that the alpha-amylase of the synthetic species may be intrinsically different from the parental types. There are a number of factors, e.g. interaction between enzyme proteins, pH of the buffer medium, and possible existance of polymorphic forms of enzyme molecules etc., which affect the electrophoretic properties of enzymes. However, the above suggestion cannot be confirmed unless some other studies, e.g. immunochemical methods etc., are employed to further characterize these particular enzymes.

(c) ISOLATION AND PURIFICATION OF BETA-AMYLASES

Sodium chloride solution (1%) was used as solvent to extract beta-amylases because it had been shown to extract twice as much free amylase as water alone (138). Since free sulfhydryl group was found to be essential in the enzymatically active beta-amylase (36, 37, 59, 139), the oxidation

of free sulfhydryl groups should be prevented in the extraction steps. Disodium ethylene diaminetetraacetate (EDTA) was used in the extractant to prevent oxidation of sulfhydryl groups. Traces of alpha-amylase present in ungerminated seeds of wheat and rye (133) were found to be largely destroyed by acid treatment (pH 3.3 to 4) at low temperature, without much damage to the beta-amylase (13, 92).

Two components of enzymatically active beta-amylases were found in each species. Separation of these two components could not be achieved by using gel filtration on Sephadex because of the similarity in their chromatographic properties. This indicated that their molecular sizes and molecular weights were rather similar. Fortunately, their electrophoretic mobilities were different so that electrophoresis could be employed successfully for the separation of these two components. Electrophoresis was carried out in aluminium lactate buffer which had been found to be suitable for proteins extracted from wheat and other cereals (22, 38). Polyacrylamide gel (102) was used as a supporting medium for zone electrophoresis instead of starch-gel since amylases would hydrolyse the starch-gel.

It has been found that the preparation of polyacrylamide gel in acidic medium was unsuccessful in presence of air (81), consequently the polymer was cast in distilled water alone and equilibrated with aluminium lactate buffer with the necessary changes of this solution (86). The exclusion of oxygen from the gel by bubbling nitrogen gas through the solution was found necessary for satisfactory casting of gel. The time of polymerization could be shortened by heating the solution to 40°C before the catalysts were added. Polyacrylamide gel was found to have many advantages over other supporting media for zone electrophoresis. Its

transparency, flexibility and stability render great usefulness to the particular gel. Furthermore, the gel can be prepared in acid or alkaline buffers and in a wide range of gel concentrations (103).

In the final steps of purification, the enzyme was eluted from the gel after electrophoresis, but monomeric species of the gelling agent were also recovered with the enzyme in the extracted solution. Removal of acrylamide could be achieved by filtration through a column of Sephadex G-50 or G-25.

When each component of the purified beta-amylases obtained from the final step of purification was rechromatographed on a Sephadex G-50 column, it emerged as a single peak. It was therefore considered that the beta-amylases thus obtained were chromatographically and electrophoretically pure.

Formerly beta-amylase was found to be a homogeneous component when extracted from cereals like malt (73), wheat (76), sweet potato (6, 24), soybeans (34) etc. In the past few years beta-amylases from some cereals were resolved into a few components of different molecular sizes by making use of ion-exchange chromatography and gel filtration on Sephadex. Nummi et al. (88, 89) found that there were four immunochemically identical beta-amylases of different molecular sizes in barley by using exclusion chromatography on Sephadex G-100 and G-75 columns. Tkachuk et al. (132) resolved wheat beta-amylases into three components by ion-exchange chromatography on DEAE-cellulose column.

In the present studies, two components of enzymatically active beta-amylases were isolated from the synthetic species, Triticale, and its parental species as well. The specific activities of the purified beta-amylases were found about ten times higher than the original crude extracts. Meyer et al. (76) reported that the specific activity of

purified beta-amylase from wheat was 1,450 mg maltose liberated in 3 minutes per mg of nitrogen and Tkachuk (132) reported a value of 1,370 mg maltose per mg nitrogen. These values were similar to the specific activities of beta-amylase components from the synthetic species, Triticale. The specific activities of beta-amylases from durum were found higher than these, whereas beta-amylases from rye had the lowest specific activities.

The beta-amylases from all three sources were found to have the same chromatographic properties. They emerged from the column between tube numbers 100 and 120 in all three cases.

The purified beta-amylases from the last step of purification were found free from alpha-amylase. This can be verified by mixing the purified enzyme with 1% starch solution at pH 5.2. One ml of the mixture was transferred into a flask containing a mixture of 10 ml of dilute iodine solution and 40 ml distilled water. The absorbance was measured at 575 mμ (refer to Section I). After two hours of incubation, one ml of hydrolysate was again transferred to another flask containing dilute iodine solution. The absorbance of this solution of starch-iodine complex was found unchanged. This revealed that the purified beta-amylases were not contaminated by alpha-amylase.

(d) ELECTROPHORETIC MOBILITIES OF BETA-AMYLASES

The electrophoretic mobilities of the components of beta-amylases in the synthetic species were found lower than those in the parental species. This indicates presumably some alterations existed in the genomes of the synthetic species and gave rise to the beta-amylases with different electrophoretic mobilities. This result cannot be easily reconciled with the hypothesis that the intermediate characteristics apart from electrophoretic

mobility were probably due to the presence of both parental type enzymes and that an arithmetic mean resulted. It may, with some justification, be assumed that the transcription of the genetic code on the respective chromosomal DNA is not altered. The electrophoresis results cannot, however, be rationalized on this basis and it indeed appears that a new 'hybrid' type enzyme molecule was synthesized. It is difficult to visualize how this would be transcribed from the chromosomal DNA present in the alien genome combinant.

IV. EFFECT OF TEMPERATURE ON ACTIVITIES OF AMYLASES

Amylases, like other plant enzymes (128), have been found to possess activity optima at temperatures between 50°C and 60°C. As seen in Fig. 12, the beta-amylases from three sources had the same activity optimum at 50°C. The optimum temperatures of alpha-amylases from the three sources were slightly different from one another, however, they were restricted within the range of 50°C and 56°C as shown in Fig. 11. These slight differences in optimum temperatures of alpha-amylases may not be significant because there is always slight fluctuation in the values of optical density during measurement.

The reaction rate decreased rapidly as the temperature was elevated above the optimum temperature. This is mainly due to the denaturation of the enzyme protein. The intramolecular hydrogen bonds, which are essential for maintenance of the secondary and tertiary structure of the enzyme, break down quickly as the temperature increases and this consequently causes denaturation of the enzyme. Thus the energy of heat inactivation of enzyme is necessarily always higher than the energy of activation.

E. Ernstrom (25) found the energy of activation of malt amylase to be 12,300 cal./mole at pH 5.5 at a temperature range between 10°C and 30°C. Eyring et al. (26) determined the energy of inactivation of malt amylase to be 41,630 cal./mole. H. Luers et al. (69) reported that malt amylase had a temperature coefficient of 1.96 between 20°C and 30°C and the energy of inactivation of 42,500 cal./mole. These values are in good agreement with those of the alpha-amylase from durum wheat.

It should be emphasized that the values of activation energies calculated by Arrhenius equation correspond only to the apparent energies of activation (121, 124). Furthermore it should be pointed out that by

activation energy of an enzyme, it is implied or understood that it is not the enzyme per se which is considered but it is assumed that the enzyme-substrate system is considered.

In general, the activation energy of an enzyme system was found to be independent of different enviromental changes unless these factors altered the catalytic surface of the enzyme (121). A number of workers, J.M. Nelson (85), I.W. Sizer (117, 118, 119, 120), G. Senter (113) and E. Nordfeldt (87) etc. reported that the purity of an enzyme preparation and its source should not affect the energy of activation in most cases.

The values of activation as well as heat inactivation energies of amylase-starch system for synthetic species, Triticale, were found to be between those of the parental species. This indicated that amylases of the synthetic species had intermediate properties between those of the parental species.

The Arrhenius plots in Figures 13 and 14 show straight lines with a break as described by K.H. Meyer et al. (76) and A. Markovitz (71). The break has been observed in many cases, e.g. urease-urea system (119), invertase-sucrose system (124), amylase-starch system (25, 80) etc., but no adequate explanation has been offered. Sizer (121) suggested that the transition might be due to a shift in the configuration of enzyme molecules.

V. INFLUENCE OF pH ON ACTIVITY

It had been found that in some cases several factors would alter the optimum pH value, such as types of buffer, purity of enzyme, length of reaction time and temperature, etc. (50, 106, 140). Therefore optimum pH could not be regarded as a quantitative charateristic of an enzyme (66). However, the values of optimum pH of amylases in this experiment are comparable with one another, since they were determined under identical conditions.

Beta-amylases from the three sources showed optimum pH at 4.6 as shown in Fig. 21, 22 and 23. This is practically the same as malt amylases which has an optimum pH at 4.4 to pH 4.5 determined by Sherman and Thomas (115). The pH-activity curves for beta-amylases are very similar to those published by Meyer et al. (87).

Alpha-amylases from the three sources were found stable over a pH range between 4.0 and 8.0 as shown in Fig. 18, 19 and 20. When amylases were incubated in buffer for two hours before determining the activity, an optimum pH at 5.2 was observed in each case as shown in Fig. 15, 16 and 17. Bernfeld et al. (11) and Muus et al. (82, 83) reported that alpha-amylases from human saliva had an optimum zone between pH 4.5 and 10.5. Alpha-amylase from B. subtilis was found to have optimum pH between 5.0 and 10.0 (72). Alpha-amylase from barley malt showed optimum pH at 5 to 6 (9).

The influence of pH on activities of amylases had been tried in 0.02 M sodium glycerophosphate-HCl at a pH range from 2 to 13. Beta-amylases from the three sources were found to have the same optimum pH at 4.6. However, in the case of alpha-amylases, a higher peak which appeared at pH 4.5 to 5.5 and a lower peak which appeared at pH 9to 10 were observed in the pH-activity curves. It was found that the buffer capacity of the sodium

glycerophosphate system at a high pH value (above 8) was very low, consequently a pH change to a lower value was evident when the buffer solution was mixed with an equal volume of starch solution. The experimental results, which showed two pH optima, were therefore unreliable and the experiments were therefore repeated using the buffer systems which are described in Section C (V).

VI. MICHAELIS-MENTEN CONSTANTS FOR AMYLASES

In most cases, the rate of an enzyme-catalysed reaction increases with increasing substrate concentration except where the product is inhibitory to the enzyme. Ordinarily the rate increases until a maximum is reached.

In 1913 Michaelis and Menten (77) sought to extend Henri's work. (46) and suggested that the enzyme first formed a complex with its substrate and it subsequently broke down to give the free enzyme and the products of the reaction.

The Michaelis-Menten constant (K_m) refers to the substrate concentration at half-maximum reaction rate. That is, $K_m = S$ when $V_m = 2\ v$. The constant (K_m) can be expressed as follows:

$$K_m = \frac{(V_m - v)\ S}{v}$$

where V_m is the maximum velocity,

v is the velocity of reaction,

S is the concentration of substrate.

In the present studies, the rate of hydrolysis of soluble starch by alpha- and beta-amylases has been examined. It was found that the experimental data gave a better fit when the double reciprocal plot was employed.

The values of the Michaelis-Menten constant (K_m) and maximum velocity (V_m) for both alpha- and beta-amylases from the synthetic species, Triticale, were found to be between those of its parental species, durum and rye. The values of K_m and V_m for beta-amylases from these three sources were, however, close to one another.

The values of the Michaelis-Menten constants for alpha-amylases are much smaller than those 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 towards the starch substrate than beta-amylases. In other words, alpha-amylases act on starch much faster than beta-amylases do. These results are in agreement with the reports of Hopkins (49). This can be explained by the fact that alpha-amylases are endoamylases which act randomly on the α-1,4 linkage of amylose and amylopectin molecules, whereas beta-amylases are exoamylases which are capable of attacking the polysaccharides only from the non-reducing 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.

Another interesting point is that the value of K_m for alpha-amylase from rye is lower than those from durum and Triticale. Recalling the results in Section I which indicated that the purified alpha-amylase from rye had the highest specific activity of the three, these results are therefore in agreement. It is evident that having a low valus of K_m, alpha-amylase from rye acts on polysaccharides faster than the other two alpha-amylases, hence a higher specific activity is observed. In the case of beta-amylases, the same phenomenon occured.

These results showed both alpha- and beta-amylases of the synthetic species, Triticale (AABBRR), have an intermediate affinity towards the starch substrate. These observations indicated that the intermediate properties of the biologically active amylases in the hybrid species, Triticale, may in part be inherited from both parental species as a direct result of the interaction between the two parent genomes in the hybrid species.

VII. SULFHYDRYL GROUPS IN BETA-AMYLASES

Weill et al. (139) and Englard et al. (24) reported that the activity of beta-amylase was destroyed by SH-reagents, such as phenylmercuric chloride, p-chloromercuric benzoic acid, iodoacetamide etc. A conclusion was reached that sulfhydryl groups were involved in the active center of beta-amylase and the inactivation was apparently due to intramolecular oxidation of sulfhydryl groups to the disulfide form. M. Ito et al. (54), T. Ito et al. (55), A. Gertler et al. (28, 37) and J.A. Thoma et al. (129) reported that free sulfhydryl groups were essential in the enzymatically active beta-amylases.

The results of the present studies showed that beta-amylases from durum had the highest free sulfhydryl contents of the three species. The free sulfhydryl contents in beta-amylases from Triticale (AABBRR) were again found to be between those of the parental species, durum (AABB) and rye (RR). It is apparent that those beta-amylases with higher specific activities, i.e. beta-amylases from durum, would have the higher free sulfhydryl contents. The two beta-amylase components from the same species had practically the same free sulfhydryl contents.

In order to compare these values with those listed in the literature, it is necessary to express the sulfhydryl contents in terms of numbers of sulfhydryl groups per enzyme molecule. Assuming the molecular weight of wheat beta-amylase was 64,200 as reported by Tkachuk (132), and introducing the protein nitrogen factor of 6.25 (33), the numbers of sulfhydryl groups per enzyme molecule were calculated and summarized in the following table:

sample	Beta-amylases	No. of SH groups/ molecule
Durum (AABB)	Component 1	4.16
	Component 2	4.06
Triticale (AABBRR)	Component 1	3.24
	Component 2	3.13
Rye (RR)	Component 1	2.20
	Component 2	2.15

The results in the above table showed that beta-amylases from durum wheat had four free sulfhydryl groups per molecule. This is in good agreement with the report of R. Tkachuk et al. (132) who found that wheat beta-amylases possessed four sulfhydryl groups per molecule.

When the titrations were carried out in the presence of 8M urea, the results were practically the same within experimental errors. This revealed that the sulfhydryl groups in the native beta-amylase were not masked.

The results concerning the numbers of free sulfhydryl groups per enzyme molecule show that beta-amylases of the synthetic species, Triticale, have an average value of those in the parental species. This observation gives rise to a suggestion that amylases in the synthetic species were probably composed of an equal amount of amylases which were produced by the parental genomes AABB and RR, respectively. This does not, however, rule out the possibility that a new enzyme molecule with a hybrid character (structure, composition etc.) had been produced as a result of an alien genome interaction. Since the genomes of the synthetic species are an arithmetic addition or composite of both of the parental genomes, it seems probable that the genomes from each parental species direct the biosynthesis

of their particular type of amylases.

The determination of disulfide linkages in alpha- and beta-amylases was attempted, but it was not successful employing this spectrophotometric method. The reduction of disulfide bonds in amylases with sodium borohydride reported by B.K. Seon et al. (114) was followed. The reduced enzyme was then titrated with mersalyl acid. Unfortunately, the end point could not be observed. This was because the components of the reduction mixture interferred with the titration. Further, the excess sodium borohydride may reduce the color of dye solution as well.

The result that no free sulfhydryl groups existed in alpha-amylases of Triticale as well as the parental species, is coincident with the findings that no free sulfhydryl groups have been found in all alpha-amylases so far investigated (1, 16, 58, 94).

E. CONCLUSIONS

The characterization of alpha- and beta-amylases of the hexaploid synthetic species, Triticale (AABBRR), and the parental species, tetraploid durum wheat (AABB) and diploid rye (RR), showed that similarities and some dissimilarities existed in their physical and chemical properties. In most cases, amylases of the hybrid species, Triticale, were shown to have characteristics intermediate between those of the parental species.

The chromatographic properties of both alpha- and beta-amylases of the synthetic species, Triticale, and the parental species were essentially the same; further, the beta-amylases of these three sources had identical temperature and pH optima. Alpha-amylases of the three sources had identical pH optima while the temperature optima were slightly different but not significantly so.

These results indicated two possibilities for the explanation of biosynthesis of the active amylases in the particular alien genome combinant. First, the genomes in the synthetic species, Triticale (AABBRR), direct the synthesis of two kinds of active amylases which are identical with those in the parental species and were produced in more or less equal amounts, and the mixture of these amylases gave rise to the observed intermediate properties. Secondly, the biosynthesis of active amylases in the particular alien genome combinant was inherited from the genomes of both of the parental species, and there presumably exists some form of interaction between the two parental genomes in the hybrid species to give rise to new intermediate or 'hybrid' product.

The greater part of the results in the present studies indicated that both alpha- and beta-amylases of the synthetic species had values of specific activities, apparent energies of activation and heat

inactivation, Michaelis-Menten constants and numbers of sulfhydryl groups per enzyme molecule which were between those of the parent species. This again revealed that some of the characteristics of amylases in the synthetic species, i.e. the mode of action, the catalytic power, the affinity towards starch substrate, were influenced by both of the parental species or alternatively were an arithmetic average of the mixture of two amylases which were identical with those in the respective parental species. From the results of present studies, it is difficult to confirm which possibility is true.

As far as beta-amylases were concerned, intermediate characteristics between the parental species were generally observed in the synthetic species except in the case of electrophoretic mobilities. The differences in the electrophoretic mobilities of alpha- and beta-amylases indicated that the amylases in the synthetic species, Triticale, were presumably a hybrid form. This also indicated that the biosynthetic capability of the alien genome combinant was probably altered in some intrinsic fashion as suggested by Yong and Unrau (146). Considering the relative genetic complexity of the plant species used compared to the 'relative simplicity' of lower living forms such as bacteriophage, viruses and even bacteria and molds, it can readily be appreciated that even a very modest approach to the elucidation of the mechanism of the so-called 'hybrid' effects in synthetic plant species such as were studied in this investigation necessarily becomes extremely involved and the present efforts may as a consequence appear, on the surface, as relatively emperical. Such a relative state of knowledge finds a very appropriate analogy in the comparison of the amount of genetic information available on the common fruit fly (Drosophilla) compared to that available for higher plants (e.g. maize, orchids) and higher animals for that matter.

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