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Purification and physicochemical properties of a -amylase from irradiated wheat

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Abstract. α-Amylases from control and gamma-irradiated (at 0.2 and 2.0 kGy dose levels) wheat seedlings were purified to homogeneity and characterized. The molecular weight of the enzyme from a 2 kGy irradiated sample was slightly lower than that of the control; other general and catalytic properties also showed some differences. α-Amylase from the irradiated (2 kGy) sample had a narrow range of pH optimum and was inactivated faster at alkaline pH and by heat treatment than the enzyme from unirradiated wheat. A high apparent Michaelis constant (K_m) and a low maximal velocity (V_{max}) for the hydrolysis of soluble starch catalyzed by the enzyme from irradiated (2 kGy) wheat, suggested some modifications in the formation of the substrate α-amylase complex. Further, of the total number of amino acid residues lost on irradiation, dicarboxylic amino acids constituted the largest percentage; these structural alterations in the enzyme may be responsible for its partial inactivation. The total sugars liberated upon amylolysis of starch with the 2 kGy irradiated enzyme were lower than control, and there was accumulation of higher maltodextrins in the place of maltose.

Keywords. Wheat; α-amylase; Y-irradiation; purification; physicochemical properties.

Introduction

α-Amylase (EC 3.2.1.1) in wheat (Varner et al., 1965) and barley (Daussant and Corvazier, 1970) has a decisive role in seedling growth. It was shown earlier (Ananthaswamy et al., 1971) that the activity of α-amylase isolated from Y-irradiated wheat seedling was significantly lower than that in the control. This was attributed to a radiation-induced delay in the differentiation of the enzyme into its functional isoenzymic forms (Machaiah and Vakil, 1979). Prior treatment with ionizing radiation reduces the catalytic activities of catalase and lipase in safflower (Singh, 1974) and peroxidase isoenzymes in beans during germination (Habeck and Curtis, 1974). In the present investigation, α-amylases isolated from control and gamma-irradiated wheat seedlings, were partially purified and characterized. An attempt was made to correlate the partial inactivation of the enzyme to the structural modifications due to exposure of wheat to a high dose of radiation.

Materials and methods

Samples of 'CV, Vijay' variety of wheat were obtained from the Agricultural Research Station, Niphad, Maharashtra. The seeds were stabilized at 11%

moisture level, sealed in polyethylene bags and irradiated (at 0.2 and 2.0 kGy dose levels) in ^{60}Co-gamma chamber-900, with an influx of 2500 Ci and overdose ratio of 10%. The samples were stored at 25°C for 4 weeks before the commencement of germination tests. Glycogen (rabbit liver) was purchased from V.P. Chest Institute, New Delhi and Sephadex G-75 from Pharmacia, Uppsala, Sweden. Bio-Gel P-100 was obtained from Bio-Rad Laboratories, Richmond, California, USA. Other chemicals were from Sigma Chemical Co., St. Louis, Missouri, USA.

Germination

Control and irradiated wheat samples (5 g lots) were surface-sterilized with 1% sodium hypochlorite solution and imbibed with distilled water for 16 h. Incubation was carried out for 4 days at 25°C in dark on petri-dishes layered with filter paper, and moistened with streptomycin and penicillin (5 µg/ml), to check microbial growth.

Purification of α-amylase

The shoot-root axis and scutelum of 4-day old wheat seedlings (from 20 g wheat) were removed and the endosperms homogenized in chilled (3°C) 0.5 M Na-acetate buffer (pH 5.6) containing 0.003 M $CaCl_2$ in a precooled Waring blendor and centrifuged at 5,000 g for 10 min. The supernatant solution was adjusted to pH 8.0 with 1 N NaOH, heated at 70°C to inactivate β-amylase and centrifuged at 5,000 g for 10 min. α-Amylase was precipitated from the crude extract and isolated as enzyme-glycogen complex essentially as described by Loyter and Schramm (1962). Absolute ethanol (final concentration 40%) and 1.6% purified glycogen (0.2 ml/1000 units of the enzyme activity) were added to the cooled supernatant. After two washes with 40% ethanol, the precipitated glycogen- a- amylase complex was dispersed in 0.02 M Tris-HCl buffer (pH 8.2) containing0.002 M $CaCl_2$, incubated for 1 h at 37°C to release the enzyme and centrifuged at 11,000 g for 10 min. Further purification of the enzyme was achieved by passing the supernatant through a Sephadex G-75 column (50X1 cm), pre-equilibrated with 0.02 M Tris-HCl buffer (pH 8.2), containing 0.002 M $CaCl_2$. Three ml fractions were collected and those having α-amylase activity were pooled, and precipitated with $(NH_4)_2SO_4$ (45% saturation). The precipitate, redissolved and dialyzed against the same buffer for 6 h at 25°C, was further subjected to chromatography on Bio-gel P-100 column (65X2.5 cm) and the protein was eluted with the same bufer. The fractions containing α-amylase activity were pooled and the enzyme protein was reprecipitated at 45% saturation of $(NH_4)_2SO_4$. The purified enzyme was dialyzed against the same buffer for 6 h and used for subsequent studies (table 1). The homogeneity of the enzyme was indicated by Polyacrylamide gel electrophoresis. The zymogram showed three protein bands, all having α-amylase activity (data not shown). The same extent of purification and recovery were achieved with irradiated samples.

Protein estimation

Protein content was determined according to the method of Miller (1959) using bovine serum albumin as standard.

Table 1. Purification of α-amylase from germinating wheat.

Purification step	Total proteins (mg/g wheat)	Total activity (units/g wheat)	Sp. act. (units/mg protein)	Purification fold
Crude extract	42.00	5168	123	1
Heat-treated supernatant	17.00	4785	281	2
Glycogen complex	0.97	3970	4085	32
Sephadex G-75 fraction	0.78	3314	4400	37
Bio-gel P-100	0.49	2380	4900	40

α-amylase activity

The enzyme preparation (about 10 µg protein) was incubated with 0.5% starch and 0.01 M acetate buffer (pH 5.6) for 30 min at 37°C. The reaction was stopped by the addition of 5 ml of 0.5 N acetic acid. An aliquot of the reaction mixture was added to 10 ml of iodine reagent and the reduction in the colour was measured at 700 nm in a Bosch and Lomb Spectronic 20 colorimeter. Enzyme unit: The amount of enzyme required to produce a 10% decrease in absorbance at 700 nm in 30 min at 37°C and pH 5.6.

The enzyme (100 µg protein) was incubated in 0.01 M sodium phosphate buffer (pH 7.0) containing 1% sodium dodecyl sulphate, 1% β-mercaptoethanol and 10 mM dithiothreitol at 37°C for 2 h. Electrophoresis was then performed in tubes (0.6 r 10 cm) at 25°C for 5 h in 10% Polyacrylamide gel and 1% sodium dodecylsulphate, essentially as described by Robyt *et al.* (1971). The bands were stained for proteins with Coomassie Blue for 24 h. The molecular weight of a-amylase was determined by comparison of its relative electrophoretic mobility with that of bovine serum albumin (68,000), chymotrypsin (23,000) and myoglobin (17,000). The destained gels were scanned at 600 nm in a Canalco model F microdensitometer (1—control; 2—0.2 kGy; 3— 2.0 kGy) .

Amino acidanalysis

The sample (0.2 to 0.3 mg protein) was hydrolyzed with 6 N HCl in an evacuated sealed tube for 24 h at 110°C. Excess acid was removed in *vacuo* and the residue was taken in 0.2 M sodium citrate buffer (pH 2.2). Amino acid analyses were carried out with a Beckman-Spinco model 120 amino acid analyser (Schramm, 1954). To determine cysteine, an aliquot of the enzyme was oxidized with 1% performic acid prior to acid hydrolysis. Corrections for incomplete hydrolysis and decomposition of amino acids during heating were made (Tkachuk and Kruger, 1974). Tryptophan was estimated separately by the colourimetric method of Spies (1967). Nitrogen was determined by micro Kjeldahl method (Hawk, 1954).

Sugars in the hydrolysate were identified and quantified by descending paper chromatography in butanol-pyridine-water (6:4:3). Reducing sugars on the chromatograms were detected by alkaline silver nitrate spray (Trevelyan *et al.*, 1950).

Results

Total α-amylase activity

The specific activity of the enzyme from 4-day old irradiated (2 kGy) wheat seedlings was 180± 15.3 units and was significantly low ($P<0.001$) compared to the value of 2380±38.3 units in the control. The activity of the enzyme in the seedlings irradiated with a lower dose was 2000 ±17.8.

pH optima

The enzymes isolated from control and 0.2 kGy irradiated samples, exhibited almost identical and broad pH optima, ranging from pH 3.6 to 5.6 (figure 1). The

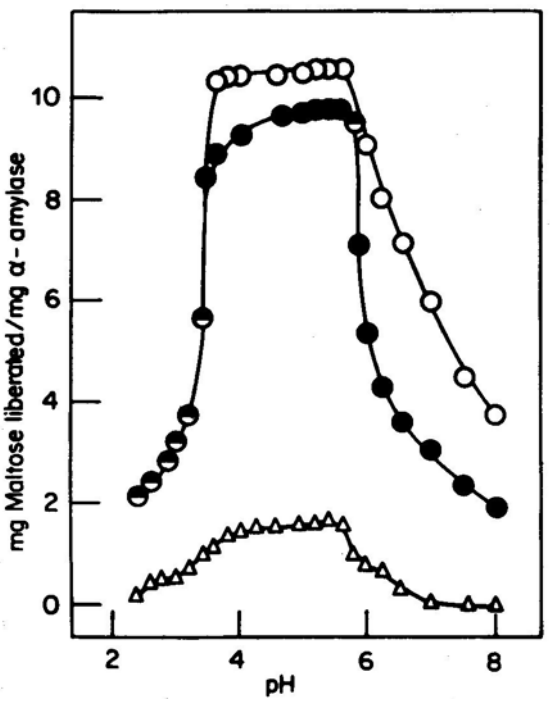

Figure 1. Effect of medium pH on the activity of α-amylase isolated from irradiated wheat seedlings, The pH optimum of α-amylase was determined using 0.02 M HCl-glycine (pH 2.4 to 3.4), 0.02 M Na acetate (pH 3.5 to 5.6) and 0.02 M Na phosphate (pH 5.8 to 8.0) buffers containing 0.001 M $CaCl_2$. The enzyme (10 µg) was added to the reaction mixture containing 2 ml each of the buffer and 0.5% starch and incubated at 37°C. After 10 min, 0.1 ml 5 N HCl was added and the mixture centrifuged (5000 g for 10 min). Reducing sugars in the filtrate were estimated (Somogy, 1952).

Control (O); 0.2 KGy (●) ; 2.0 KGy (Δ).

enzyme from wheat irradiated at higher dose (2 kGy) had a narrower range of pH optima (pH 4.6 to 5.6) and was inactivated at a much faster rate in alkaline medium. Stability of the enzyme expressed as per cent of original activity at the pH optimum of 5.6 was reduced at pH 6.0 by about 60% in the sample irradiated at 2 kGy, compared to only 15% in control. The enzyme was almost completely inactivated at pH 7.0 in the sample irradiated at higher dose, whereas 55% of the activity was retained in the control.

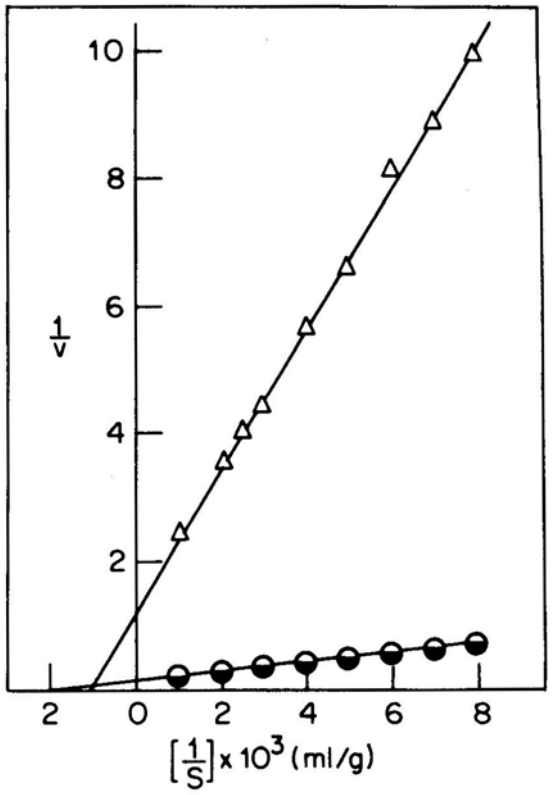

Figure 2. Effect of increasing substrate concentration on α-amylase isolated from control and irradiated wheat seedlings. The enzyme (10 µg) was incubated with 0.1 to 1 mg/ml of soluble starch buffered with 0.1 M Na acetate (pH 5.6, 0.003 M $CaCl_2$) at 37°C for 5 min. Control (O); 0.2 KGy (●) ; 2.0 KGy (Δ).

K_m value for soluble starch for α-amylase

Apparent K_m values for α-amylase, measured at pH 5.6 are shown in figure 2. The Lineweaver-Burk double reciprocal plots yielded straight lines giving comparable apparent K_m values for α-amylase from control (0.555 mg/ml) and 0.2 kGy (0.585 ±0.0048 mg/ml, P<0.1) samples. This value was however about 90% higher (0.95 ±0.026 mg/ml, P < 0.00 1) for the enzyme from 2.0 kGy sample than that of control. Thus V_{max} (calculated as mg maltose released/5 min/mg enzyme at 37°C) was 7.0 and 0.77 for control and 2.0 kGy samples, respectively.

Effect of heat treatment

Although, α-amylase was not inactivated upto 70°C, at pH 8.0, the enzyme loses its activity, when subjected to higher temperature at pH 5.6 (figure 3). However, the rate of heat inactivation was faster with α-amylase isolated from the sample irradiated at 2 kGy compared to those from control and 0.2 kGy irradiated seedlings (figure 3). At 60°C, 76% of the original activity was retained after 30 min of heat treatment in control, whereas only 44% of the activity was recovered from the irradiated (2 kGy) sample.

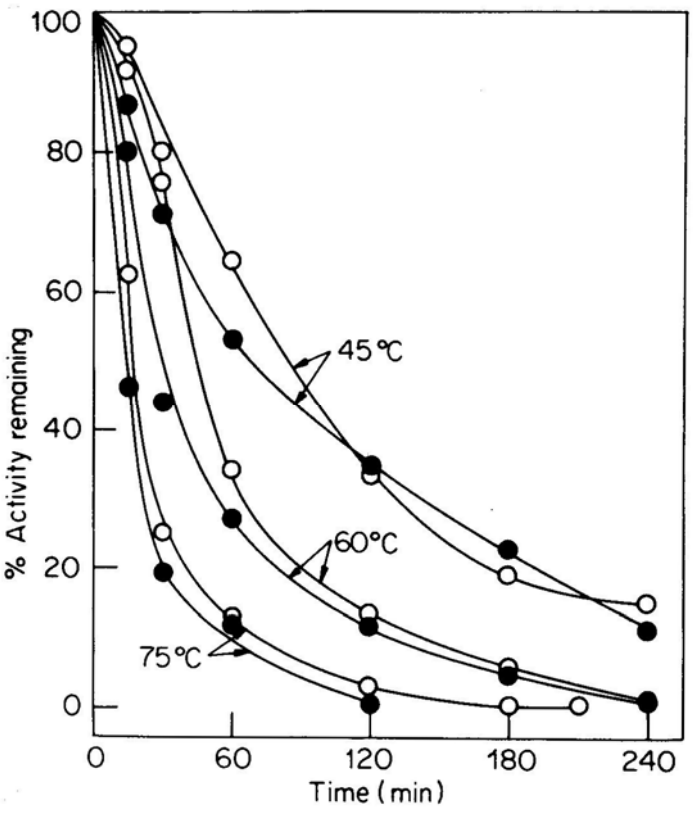

Figure 3. Effect of temperature on the activity of α-amylase. To measure the heat lability, the enzyme (10 µg) was incubated at temperatures ranging from 45 to 75°C for 0 to 4 h in 0.01 M Na acetate buffer (pH 5.6) containing 0.001 M $CaCl_2$.

The solutions were rapidly cooled and α-amylase activity was measured at 37°C. control (O); 2.0 kGy (●).

Isoelectric point

Isoelectric focusing (figure 4) of α-amylase on a gradient of pH from 4.0 to 7.0 resulted in the enzyme from the control sample eluting as a single peak in the range of 5. 4 to 6.3 with a maximum at pH 5.8. Comparable results were obtained with the

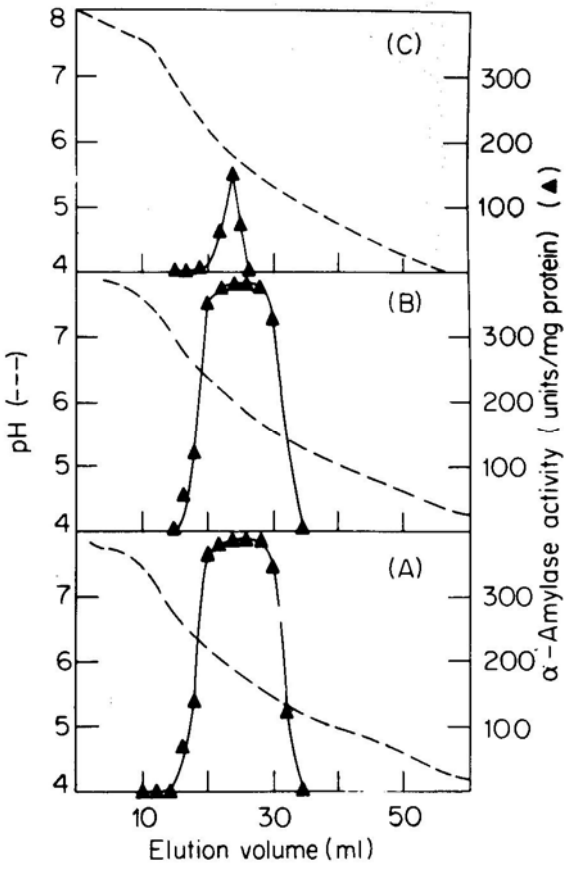

Figure 4. Isoelectric focusing of α-amylases, purified from control and irradiated wheat seedlings, α-amylase (200 µg) was electrofocused on an LKB 8101 column (110 ml) using 5 to 50% sucrose gradient and ampholines of pH from 4 to 7 as described by MacGregor (1978). After 66 h run, 1 ml fractions were collected from the column, and the enzyme activity and PH were determined in each fraction. A, control; B. 0.2 kGy; C, 2.0 kGy.

0.2 kGy irradiated sample. However, the enzyme isolated from wheat irradiated at 2 kGy eluted as a very narrow peak, in the range of pH 5.65 to 6.05 with a maximum at pH 5.8. This may be due to the total absence of the α-isoenzyme, having higher anodic mobility in the irradiated sample (Machaiah and Vakil, 1979).

The molecular weight of α-amylase was determined by gel filtration on a pre-equilibrated Bio-Gel Pl00 column (65X2.5 cm) using 0.02 M Tris-HCl buffer, pH 8.2 and eluted with the same buffer at a flow rate of 22 ml/h. Proteins of known molecular weights (given in paranthesis) albumin (68,000), pepsin (35,000) chymotrypsin (23,000) and myoglobin (17,000) were used to calibrate the column. The enzyme activity and A_{280}absorption were measured in each fraction (5 ml). The enzymes from control, 0.2 kGy and 2.0 kGy samples have molecular weights of 38,500, 38,000 and 37,000 respectively.

When subjected to Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate and β-mercapioethanol the enzyme could be resolved into two distinct protein bands (figure 5) of apparent molecular weights of 40,000 (A_1) and 83,000 (A_2) daltons. Since both these bands had enzymic activity, though reduced considerably, it was presumed that wheat α-amylase could dimerise. Similar dimerisation of the enzyme from malted wheat on prolonged storage has been reported (Tkachuk and Kruger, 1974). The distribution pattern of the enzyme in these bands indicated that in control, about 75% of the total activity was localized in the A_1 band and the rest in the A_2 band. In contrast, only 20% of the total *a*-amylase was in the A_2 band of the 2 kGy sample.

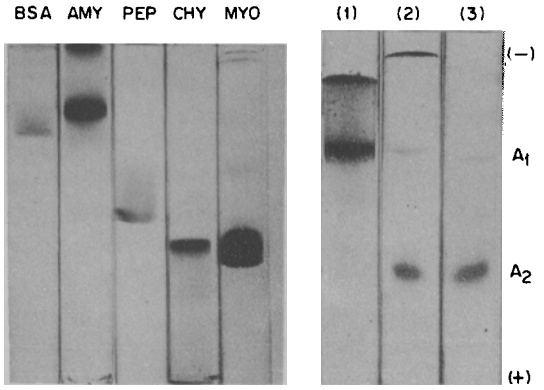

Figure 5. Determination of molecular weight of α-amylase by sodium dodecyl sulphate Polyacrylamide gel electrophoresis. The enzyme (100 µg) was incubated in 0.01 M sodium phosphate buffer (pH 7.0) containing 1% sodium dodecyl sulphate 1% β-mercaptoethanol and 10 mM dithiothretol at 37°C for 2 h. Electrophoresis was then performed in tubes (0.6 X 10cm) at 25°C for 5 h in 10% Polyacrylamide gel and 1% sodium dodecyl sulphate, essentially as described by Robyt *et al*. (1971). The bands were stained for proteins with Coomassie Blue for 24 h. The molecular weight of α-amylase was determined by comparison of its relative electrophoretic mobility with that of bovine serum albumin (68,000), chymotrypsin (23,000) and myoglobin (17,000). The destained gels were scanned at 600 nm in a Canalco model F microdensitometer. 1—control; 2—0.2 kGy; 3—2.0 kGy.

Amino acid composition of α-amylase

The amino acid composition (table 2) shows that about 398, 386 and 373 amino acid residues are present per molecule of α-amylase isolated from control, 0.2 and 2.0 kGy samples, respectively. In the control, aspartic and glutamic acids constituted the 91 acidic amino acid residues, whereas lysine, hisitidine and arginine constituted the 62 basic ones forming an excess of 29 cationic groups. For accurate calculation of the number of acidic and basic amino acid residues, the NH_3 content is not taken into consideration. In α-amylase isolated from irradiated (2 kGy) wheat seedlings the number of basic and acidic amino acid residues were

Table 2. Amino acid composition of purified α-amylase from control and irradiated wheat.

Amino acid	Control	0.2 kGy	2.0 kGy
	amino acid residues		
Lysine	19	17	14
Histidine	17	15	7
Arginine	26	25	10
Aspartic	31	30	22
Glutamic	60	56	40
Threonine	16	16	24
Serine	17	16	20
Tyrosine	10	10	13
Glycine	39	39	36
Alanine	26	26	32
Valine	26	27	28
Isoleucine	19	20	22
Leucine	36	36	38
Proline	26	24	35
Phenylalanine	16	15	16
Tryptophan	10	10	10
Methionine	3	3	5
Half cystine	1	1	1
Total number	398	386	373
Basic	62	57	31
Acidic	91	86	62
NH_3	52	46	33

Amino acid content was calculated as μmol per 100 g of protein and converted to the nearest integral number of residues. Each value represents the average of two estimations in duplicate.

reduced by 50 and 30% respectively. Though histidine residues were less by 40%, no significant differences in tyrosine and tryptophan contents were observed Proline, alanine and serine contents were slightly higher in irradiated samples.

Action pattern of α-amylase on soluble starch

Resolution of the products of α-amylolysis of soluble starch by paper chromatography revealed that the hydrolysis took place in two distinct stages. In the initial stage (figure 6) a series of maltodextrins with low Rf values (G_5 to G_{10}) were predominant, whereas in the second stage (figure 6B), maltose (G_2) and maltotriose (G_3) appeared in appreciable quantities and higher maltodextrins (G_8 to G_{10}) disappeared. The Rf value of glucose was taken as 1.0 and those of other sugars were calculated as: G_2=0.65; G_3=0.42; G_4=0.25; $G_5$0.16; G_6=0.10 and G_7 = 0.06. Achroic point with iodine was reached at 5 h of hydrolysis. The dextrin pattern remained stable up to 24 h of hydrolysis, but only traces of glucose were found after 3 h of hydrolysis. These observations are characteristic of cereal α-amylase (MacGregor et al., 1971).

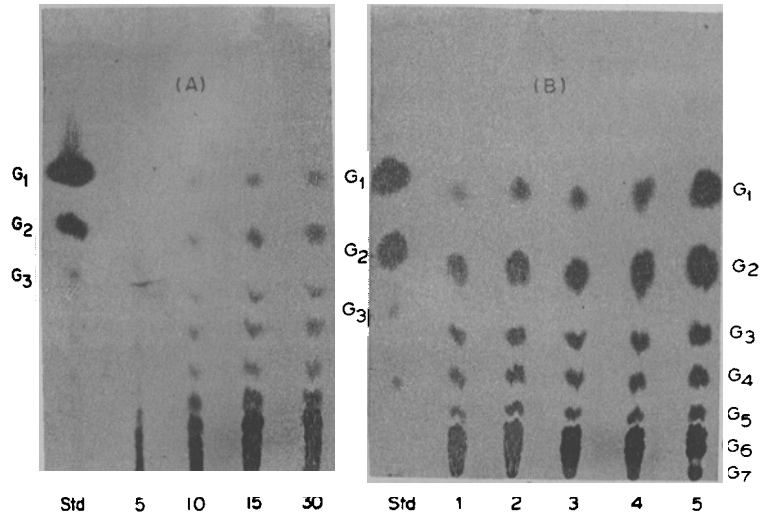

Figure 6. Action pattern of α-amylase (control A) on soluble starch, The purified enzyme (10 µg)isolated from control and irradiated wheat seedlings, was disolved in 0.1 M acetate buffer, pH 5.6 containing 1 ml of 2% starch and incubated at 25°C. Products of hydrolysis in the filtrate were separated by descending paper chromatography. G_1—glucose, G_2—maltose, G_3—maltotriose; G_4—maltotetrose; G_5—maltopentose; G_6—maltohexose; G_7—maltoheptose. The reaction was stopped by immersing the tube in boiling water for 2-3 min, cooled, and centrifuged (5000 g for 10 min). Total sugar in the filtrate was determined by the phenol — H_2SO_4 method as described by Dubois *et al*. (1956) for 5,10,15 and 30 min P and A; for 1,2,3,4 and 5 h, P and B.

Quantitative yield of hydrolytic products of a - amylolysis

The rates of hydrolysis of starch, catalyzed by the α-amylases isolated from control and irradiated wheat seedlings, were measured and compared at constant substrate: enzyme ratio and at achroic point (5 h at 25°C). Maltodextrins produced were analyzed quantitatively. It is seen (table 3) that the amount of reducing sugars produced by α-amylase from the 2 kGy irradiated sample was significantly low (P<0.001), indicating that its hydrolyzing capacity was reduced. After 5 h of hydrolysis, glucose and the lower maltodextrins (G_2 to G_4) constituted only 27% of the total hydrolytic products produced by the irradiated sample compared to 44% by the control α-amylase. Thus, initial cleavage products representing G_6 and G_7 were predominantly present in the irradiated sample (2 kGy).

Discussion

It is clear from these studies that the irradiated ion at low dose (0.2 kGy) had no appreciable effect on some physicochemical properties of α-amylase. However, the narrow range of pH optima, rapid inactivation in alkaline pH and higher heat lability of the enzyme isolated from the 2 kGy irradiated sample suggested that its stability was severely affected by a high dose of radiation. The very low biological activity of α-amylase from the 2 kGy irradiated sample can be attributed to the absence of the α_1-isoenzyme, the most ionized isoenzyme at alkaline pH.

Table 3. Quantitative yields of maltodextrins by action of α-amylase from irradiated wheat seedlings.

		Control		0.2 kGy		2.0 kGy	
Sugar spots	Rf value of sugar	Reducing sugar (μg)	% of total	Reducing sugar (μg)	% of total	Reducing sugar (μg)	% of total
G_1	1.00	20.0	3.90	17.5	3.54	8.0	4.06
G_2	0.65	131.2	25.68	124.0	25.05	35.0	17.77
G_3	0.42	38.0	7.44	30.0	6.10	7.2	3.68
G_4	0.25	37.2	7.28	22.0	4.44	4.2	2.16
G_5	0.16	20.0	3.90	13.0	2.63	1.0	0.05
G_6	0.10	128.0	25.05	145.0	29.29	38.7	19.67
G_7	0.06	84.6	16.56	94.0	18.99	71.7	36.42
Origin	0.00	51.6	10.10	51.0	10.30	31.0	15.74
Total	—	510.6 ±10.82	100.0	496.5 ±5.8[a]	100.0	196.8 ±4.09[b]	100.0

The purified enzyme (10 μg protein), isolated from control or irradiated wheat seedlings, was incubated with 1% starch for 5 h. The spots corresponding to glucose and each maltodextrin (G_2 to G_2) were cut from the undeveloped chromatogram (figure 6), eluted with distilled water and estimated for reducing sugars. Results are expressed as % of total and the values are average of four experiments, in duplicate. [a]$P<0.01$, [b]$P<0.001$.

Nishikawa and Nobuhara (1971) have shown that at least two triplicate genetic systems control different α-amylase isoenzymes in wheat, and these are in very close functional relationship with one another. Rädiosensitivity of α-amylase synthesis depends upon the genome functional state (Bulakh and Grodzinky, 1974). Thus, the low enzyme activity in irradiated sample may be due to more radiation damage of D genome, controlling the synthesis of a_1-isoenzyme (Nishikawa *et al.,* 1975). The elution of α-amylase as a narrow peak during isoelectric focussing (figure 4) also indicates some modifications in the polypeptide chain of the enzyme in the 2 kGy irradiated sample. The high K_m value and change in V_{max} in the irradiated sample (figure 2) also indicate a much different protein.

Further, the catalytic abilities of α-amylases were determined in analyzing the hydrolytic products formed. The results (table 3) indicate that the enzyme isolated from irradiated (2 kGy) wheat was less effective in attacking starch molecules resulting in the accumulation of sugars higher than maltoheptose (G_7). In all the groups, the enzyme hydrolyzed maltose to glucose at a much slower rate (table 3). The G_2/G_1 ratio has been calculated as 6.5 and 4.3 in control and irradiated samples, respectively. Thus, the enzyme isolated from irradiated wheat (2 kGy) was less efficient in attacking higher molecular weight maltodextrins. However, it should be stressed that the enzyme activity was not completely destroyed and the characteristic fractionation pattern of starch to oligosaccharides, was not altered by radiation treatment. Nishikawa *et al.* (1978) have presumed that the functional differentiation of starch degrading activity of the two groups of α-amylases, can be attributed to the differences in the content of their catalytically active isoenzymes. The foregoing results suggest that treatment with high dose of

radiation affects the general and catalytic properties of α-amylase isolated from germinating wheat.

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