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GLUCOSIDASES AND GALACTOSIDASES IN SOILS

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Summary—An improved method to assay activities of α- and β-glucosidases and α- and β-galactosidases in soils is described. It involves extraction and colorimetric determination of the *p*-nitrophenol released when 1 g of soil is incubated with 5 ml of buffered *p*-nitrophenyl glycoside solution at 37°C for 1 h. The reagents [0.5 M $CaCl_2$ and 0.1 M Tris (hydroxymethyl)aminomethan THAM, pH 12] used for extraction of the *p*-nitrophenol released give quantitative recovery of *p*-nitrophenol added to soils and do not cause chemical hydrolysis of the substrates. Results showed that these enzymes have their optimum activities at buffer pH 6.0. The initial rates of *p*-nitrophenol release obeyed zero-order kinetics. β-Glucosidase activity was the most predominant of the four enzymes. The temperature dependence of the rate constant conformed to the Arrhenius equation up to the point of enzyme inactivation (60°C for α- and β-galactosidases and α-glucosidase and 70°C for β-glucosidase). The average activation energy values of these enzymes in three soils were 43.1, 30.8, 57.0 and 32.6 kJ mol^{-1} for α-glucosidase, β-glucosidase, α-galactosidase and β-galactosidase activities, respectively. By using the Lineweaver–Burk plot, the K_m values were the lowest for β-glucosidase activity. The V_{max} values varied among the four enzymes and soils studied.

INTRODUCTION

The enzymes acting on glycosyl compounds (EC 3.2), including glycoside hydrolases (EC 3.2.1), are among the hydrolases least studied in soils. The general name glycosidases or glycoside hydrolases has been used to describe a group of enzymes that catalyze the hydrolysis of different glycosides. The equation of the reaction is:

$$\text{Glycosides} + H_2O \rightarrow \text{Sugar} + \text{Aglycons}.$$

The terms glycoside and glucoside have been used interchangeably. To avoid ambiguity, it is now customary to designate as glycoside those substances that on acid hydrolysis liberate one or several monosaccharides and an aglycon. The *Commission on Enzymes of the International Union of Biochemistry* has classified all these enzymes into 39 groups (Florkin and Stotz, 1965). These include enzymes such as cellulase and amylase and some important glycosidases that catalyze the hydrolysis of disaccharides. Glycosidases usually have been named according to the types of bond that they hydrolyze. Among the glycosidases, α-glucosidase (obsolete name maltase, EC 3.2.1.20), which catalyzes the hydrolysis of α-D-glucopyranosides and β-glucosidase (obsolete name gentiobiase or cellobiase, EC 3.2.1.21), which catalyzes the hydrolysis of β-D-glucopyranosides, are involved in hydrolysis of maltose and cellobiose, respectively. Other important glycosidases are α-galactosidase (obsolete name melibiase, EC 3.2.1.2) and β-galactosidase (obsolete name lactase, EC 3.2.1.23). These enzymes catalyze the hydrolysis of melibiose and lactose, respectively.

Glucosidases and galactosidases are widely distributed in nature. They have been detected in microorganisms, animals and plants (Bahl and Agrawal, 1972; Dey and Pridham, 1972; Wallenfels and Weil, 1972). Their presence in soils has also been reported (Skujins, 1976). Although considerable attention has been given to the synthesis, role, and properties of these enzymes in microorganisms and plants, relatively little attention is devoted to studies of these hydrolases in soils. These enzymes play a major role in degradation of carbohydrates in soils. The hydrolysis products of these enzymes are believed to be important energy sources for soil microorganisms.

A few methods have been proposed for estimation of the activities of these enzymes in soils. Early work by Hofmann and Hoffmann (1954) involved estimation of the reducing compounds produced when soil was incubated with α- or β-phenyl galactoside or α- or β-phenyl glucoside for 24 h. The enzyme activity was expressed in ml of 0.1 N $Na_2S_2O_3$. Another method used by Hoffmann and Dedeken (1965) involved estimation of the β-glucosidase activity of soils by determining the saligenin (2-oxymethylphenol) released when soil was incubated with salicin (β-glucoside saligenin) in acetate buffer pH. 6.2 in the presence of toluene. Kiss and Peterfi (1960) added lactose to the reaction mixture and estimated the degradation products by paper chromatography. *p*-Nitrophenyl or *o*-nitrophenyl glucosides have been used for estimation of glucosidases and galactosidases activity in soils, but the methods involve either extraction of the *p*-nitrophenol or *o*-nitrophenol released by filtration after addition of ethanol or by centrifugation after addition of dilute H_2SO_4; the yellow color of the *p*-nitrophenol or *o*-nitrophenol extracted is produced by addition of Tris buffer or Na_2CO_3 (Hayano, 1973; Rysavy and Macura, 1972). In addition to incomplete extraction of the *p*-nitrophenol released, ethanol evaporates

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during filtration and leads to erroneous results. Centrifugation after addition of H_2SO_4 leads to extraction of colored soil materials that affect the accuracy and precision of method. The objectives of this work were to develop a simple and accurate method for assay of the activities of these enzymes in soils and to study the kinetic parameters and substrate specificity of these soil enzymes.

MATERIALS AND METHODS

The soils used (Table 1) were surface (0–15 cm) samples selected to obtain a wide range in pH, organic C and texture. Before use, each sample was air-dried and crushed (<2 mm). The analyses reported in Table 1 were performed as described by Dick and Tabatabai (1987).

Method for assay of α- and β-glucosidases and galactosidases in soils

Reagents. Toluene was a Fisher certified reagent (Fisher Scientific Co.). Modified universal buffer (MUB) of the desired pH was prepared as described by Tabatabai (1982). *p*-Nitrophenyl-α or β-D-glucoside and *p*-nitrophenyl-α or β-D-galactoside (PNG) (Sigma Chemical Co.) solutions (25 mM) were prepared by dissolving 377 mg of the appropriate compound in 40 ml of MUB, pH 6.0, and adjusting the volume to 50 ml with the same buffer. Calcium chloride (0.5 M) was prepared by dissolving 73.5 g of $CaCl_2 \cdot 2H_2O$ in 700 ml of water and adjusting the volume to 1 l. with water. Tris (hydroxymethyl) aminomethan (0.1 M THAM) (Fisher certified reagent), pH 12, and *p*-nitrophenol (PN) standard solutions were prepared as described by Browman and Tabatabai (1978).

Procedure. A sample of soil (1 g) in a 50-ml Erlenmeyer flask was treated with 0.25 ml of toluene, 4 ml of MUB (pH 6.0) and 1 ml of the appropriate glucoside solution. The flask was swirled for a few seconds to mix the contents, stoppered and incubated at 37°C. After 1 h, the stopper was removed, and 1 ml of 0.5 M $CaCl_2$ added, mixed and treated with 4 ml of 0.1 M THAM, pH 12. The flask was swirled for a few seconds to mix the contents, and the soil suspension was filtered through a Whatman No. 2V folded filter paper. Absorbance of the colored solution was measured by using a Klett–Summerson photoelectric colorimeter fitted with a blue (No. 42) filter (a spectrophotometer that can be adjusted to a wavelength of 400–420 nm can also be used). The maximum absorbance of the color measured is at 400 nm (Bessey *et al.*, 1946). The amount of PN released was calculated from a standard calibration graph prepared as described by Browman and Tabatabai (1978). When the color intensity of the filtrate exceeded that of the highest PN standard, an aliquot of the filtrate was diluted with 0.1 M THAM pH 10 until the colorimeter reading was within the limits of the calibration graph.

Controls were performed with each soil analyzed to allow for yellow color not derived from the PN released by glucosidase or galactosidase activity. For controls, the procedure described was followed, but the addition of 1 ml of PNG solution was made after the addition of 0.5 M $CaCl_2$ and 4 ml of 0.1 M THAM pH 12 (i.e. immediately before filtration of the soil suspension).

Table 1. Analyses of soils

Soil	pH	Org. C	Total N	Clay	Sand
			(%)		
Thurman	7.1	0.47	0.046	5	93
Hagener	6.4	0.92	0.093	13	64
Weller	5.1	1.51	0.131	17	1
Ida	8.0	1.57	0.147	27	3
Clarion	6.4	1.77	0.188	24	39
Webster	5.8	2.54	0.210	23	38
Harps	7.8	3.74	0.305	30	26
Nicollet	6.1	3.32	0.253	30	26
Luton	6.8	4.35	0.388	42	3
Okoboji	7.4	5.45	0.463	34	16

RESULTS AND DISCUSSION

The method developed for assay of the activities of glucosidases and galactosidases in soils is based on quantitative extraction and colorimetric determination of PN in soils and on systematic studies of factors affecting release of PN on incubation of soil with a buffered PNG solution in the presence of toluene. The factors studied included pH and type of buffer, amount of soil and toluene, time of incubation, substrate concentration, and temperature of incubation.

Extraction and estimation of p-nitrophenol

The colorimetric procedure used for estimation of PN depends upon the fact that alkaline solutions of this phenol are yellow, whereas acid solutions of PN and acid and alkaline solutions of PNG are colorless. Addition of NaOH to the incubated soil–buffer mixture to develop the yellow color of the PN released showed that the substrates, PNG, are hydrolyzed with time in the presence of excess NaOH. Tests indicated, however, that substitution of THAM buffer, pH 12, for NaOH would serve the function of NaOH, but would not lead to hydrolysis of the substrate. The $CaCl_2$–THAM treatment used for extraction of the PN released develops the stable, yellow color of this phenol and tests with several soils showed that it gave quantitative recovery of PN added to soils. Sodium hydroxide solution can be used instead of THAM buffer, pH 12, but the absorbance of the yellow color must be measured immediately after filtration. It is necessary to add $CaCl_2$ before addition of THAM, pH 12, to prevent dispersion of soil colloids and extraction of soil organic matter during treatment with THAM.

The control is so designed that it corrects for the presence of trace amounts of PN in the substrate (PNG) used and for extraction of trace amounts of colored soil material by the $CaCl_2$–THAM treatment. No chemical hydrolysis of the substrates could be detected when the buffered PNG solutions were incubated as described but without soil.

Choice of buffer

Choice of buffer and buffer pH in the assay procedure described was based on studies showing that the amount PN released by incubation of soil with buffered PNG solutions was considerably higher with

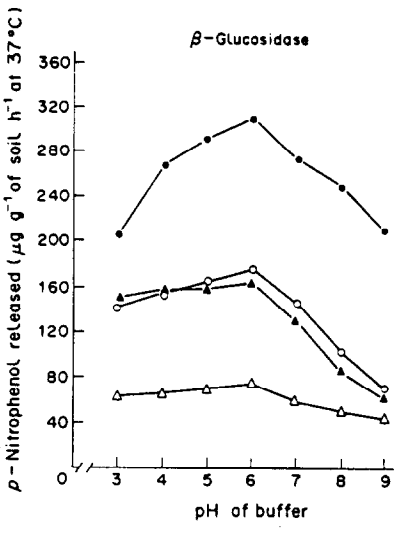

Fig. 1. Effect of buffer pH on release of *p*-nitrophenol in assay of β-glucosidase activity of soils. ●, Luton soil; ○, Okoboji soil; ▲, Webster soil; △, Harps soil.

MUB than with other buffers tested (acetate, citrate and THAM). Choice of pH was based on studies showing that maximum activities occur when using MUB, pH 6.0. Figures 1 and 2 show the effect of buffer pH ranging from 3 to 9 on β-glucosidase and α-galactosidase activities in soils, respectively. The optimum pH values obtained for the other two enzymes (α-glucosidase and β-galactosidase) were also 6.0. The optimum pH values obtained in this study are similar to that (6.2) reported by Galstyan (1965) for β-glucosidase activity of soils by using arbutin as a substrate in the presence of a phosphate buffer. Also, similar pH values for optimum activities of α- and β-galactosidases and glucosidases in soils were reported by Hofmann and Hoffmann (1954).

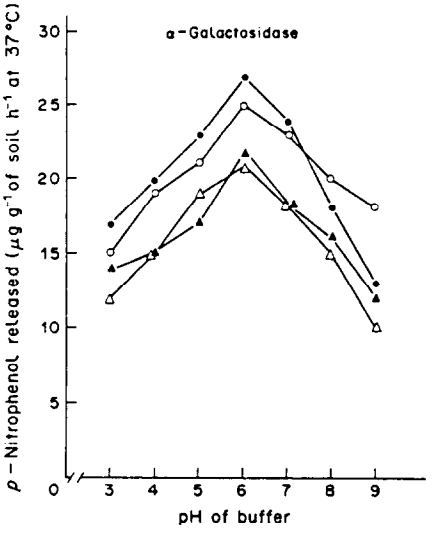

Fig. 2. Effect of buffer pH on release of *p*-nitrophenol in assay of α-galactosidase activity of soils. ●, Webster soil; ○, Okoboji soil; ▲, Harps soil; △, Luton soil.

Studies with glucosidases and galactosidases obtained from other sources showed that the optimal pH for activities of these enzymes to be in the range of 5.0–6.8 (Agrawal and Bahl, 1968; Duerksen and Halvorson, 1958).

Amount of soil and toluene

Studies on the effect of varying the amount of soil on the release of PN by the four enzymes showed that linear relationships were obtained up to 2 g of soil (results are not shown).

Toluene has been used in studies of soil enzymes as an antiseptic or plasmolytic agent. Its role is to stop further synthesis of enzymes by living cells and to prevent assimilation of products of enzyme reactions during incubation. It also liberates the intercellular enzymes (Drobnik, 1961), and it was used in this study for this purpose. Table 2 shows the effect of toluene on the activity of the four glycosidases studied. Addition of toluene to the incubation mixture during the assay procedure increased the activity of these enzymes. The effect of toluene on the activities of these enzymes in soils is similar to its effect on arylsulfatase, urease and phosphatase activities in soils (Tabatabai and Bremner, 1970, 1972; Eivazi and Tabatabai, 1977).

Time and temperature of incubation

Results of studies in which the time of incubation was varied from 1 to 6 h showed that the release of PN was linear with time. This is evidence that the method proposed measures enzymatic hydrolysis of the substrates used and that the glycosidase assay by this method is not complicated by microbial growth or assimilation of the enzymatic reaction products by soil microorganisms. The β-glucosidase activity of the Luton soil was linear with time of incubation up to 2 h (Fig. 3). After this time, the reaction rate started to deviate from linearity, indicating that the substrate concentration used was limiting the reaction rate. No such rate-limiting step was observed with the other soils used or with the other three enzymes studied.

Enzyme reactions normally proceed at a faster rate as the reaction temperature is increased. Then inac-

Table 2. Effect of toluene on glucosidase and galactosidase activities of soils

Soil	Toluene	Glycosidase activity[a]			
		α-Glu	β-Glu	α-Gal	β-Gal
Thurman	+	6	27	26	5
	−	4	14	4	4
Hagener	+	11	56	15	8
	−	7	34	3	4
Weller	+	5	13	10	5
	−	2	10	7	3
Ida	+	14	68	27	15
	−	8	37	6	14
Webster	+	11	168	23	12
	−	7	161	11	10
Harps	+	12	72	22	13
	−	10	55	10	8
Luton	+	16	295	20	30
	−	15	211	16	26
Okoboji	+	14	152	22	29
	−	13	114	19	11

[a] μg *p*-nitrophenol released g^{-1} soil h^{-1} at 37°C.
Glu = glucosidase, Gal = galactosidase.

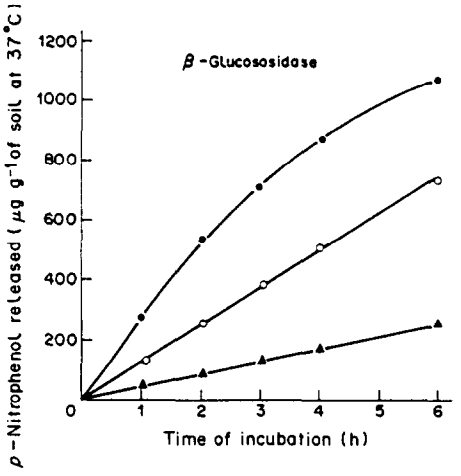

Fig. 3. Effect of time of incubation on release of *p*-nitrophenol in assay of β-glucosidase activity of soils. ●, Luton soil; ○, Webster soil; ▲, Hagener soil.

tivation of the enzyme becomes a more important factor than the increased reaction rate. Studies of the effect of temperature on the four glycosidases showed that α-glucosidase and α- and β-galactosidases are inactivated at 60°C, but β-glucosidase at 70°C (Fig. 4). The β-glucosidase purified from *Phaseolus vulgaris*, however, has been shown to be inactivated at 45°C (Agrawal and Bahl, 1968). The inactivation temperature of glycosidases in soils is similar to that of arylsulfatase and rhodanese activities in soils (Tabatabai and Bremner, 1970; Tabatabai and Singh, 1976). Work on the effect of temperature on enzyme activity has shown that the temperature needed to inactivate enzymes in soils is about 10°C higher than that needed to inactivate enzymes in the absence of soil (Skujins, 1976). Activities of the four glycosidases were assayed at 37°C because this temperature has been used extensively for assay of these and other enzyme activities and because preliminary work with the soils used (Table 1) showed that it is not necessary to use a higher temperature to obtain precise results.

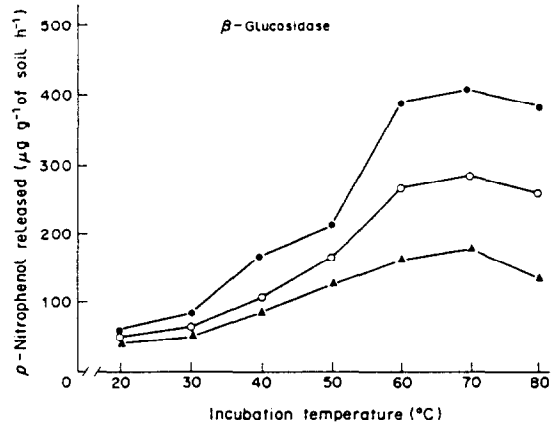

Fig. 4. Effect of temperature of incubation on release of *p*-nitrophenol in assay of β-glucosidase activity of soils. ●, Webster soil; ○, Nicollet soil; ▲, Harps soil.

Table 3. Effect of type of substrates on glucosidase and galactosidase activities of soils

Substrate[b]	Glycosidase activity on soil specified[a]		
	Clarion	Webster	Luton
p-β-Glu	148	169	295
o-β-Glu	36	64	182
p-α-Gal	17	26	20
o-α-Gal	8	11	12
p-β-Gal	20	14	25
o-β-Gal	10	12	20

[a]μg *p*-nitrophenol released g^{-1} soil h^{-1} at 37°C.
[b]Glu = glucosidase, Gal = galactosidase.

Type and concentration of substrate

In addition to using PNG as a substrate, we used ONG (*o*-nitrophenyl glycosides). Results showed that, with three of the four enzymes studied, the activity was higher with PNG than with ONG as a substrate (Table 3). The substrate *o*-nitrophenyl-α-D-glucoside was not commercially available; therefore, the rate of hydrolysis of this substrate could not be compared with that of *p*-nitrophenyl-α-D-glucoside.

Precision

The precision of the method is illustrated by Table 4, which gives the results of replicated analyses of five soils. The four glycosidase activities of the soils, expressed as μg of PN released g^{-1} soil h^{-1} at 37°C, ranged from 5 to 15 for α-glucosidase, from 27 to 296 for β-glucosidase, from 14 to 28 for α-galactosidase and from 7 to 30 for β-galactosidase. The SD of the activity measurements ranged from 0.4 to 2.4. The precision of the method proposed probably owes largely to the fact that the technique used to determine the release of PN is quantitative and that the assay procedure is simple and readily standardized.

Kinetic parameters (K_m and V_{max})

Figure 5 shows Lineweaver–Burk plots of the results obtained in studies of the effect of substrate

Table 4. Precision of method

Soil	Glycosidase[b]	Glycosidase activity[a]		
		Range	Mean	SD
Thurman	α-Glu	4–6	5.3	0.8
	β-Glu	26–28	26.8	0.8
	α-Gal	27–30	27.9	0.8
	β-Gal	6–8	7.3	0.8
Hagener	α-Glu	9–11	9.8	0.8
	β-Glu	56–58	57.0	0.8
	α-Gal	14–15	14.4	0.4
	β-Gal	8–10	9.2	0.8
Ida	α-Glu	11–12	11.8	0.8
	β-Glu	67–73	70.8	2.4
	α-Gal	26–28	27.2	0.8
	β-Gal	14–15	14.8	0.4
Webster	α-Glu	7–10	9.5	1.2
	β-Glu	168–174	171.3	2.4
	α-Gal	25–29	26.7	1.6
	β-Gal	11–13	12.5	0.8
Luton	α-Glu	14–16	14.9	0.8
	β-Glu	294–300	296.0	2.4
	α-Gal	20–22	20.9	0.8
	β-Gal	27–30	29.5	1.2

[a]*p*-Nitrophenol (μg g^{-1} soil h^{-1} at 37°C). Mean of 6 assays; SD = standard deviation.
[b]Glu = glucosidase, Gal = galactosidase.

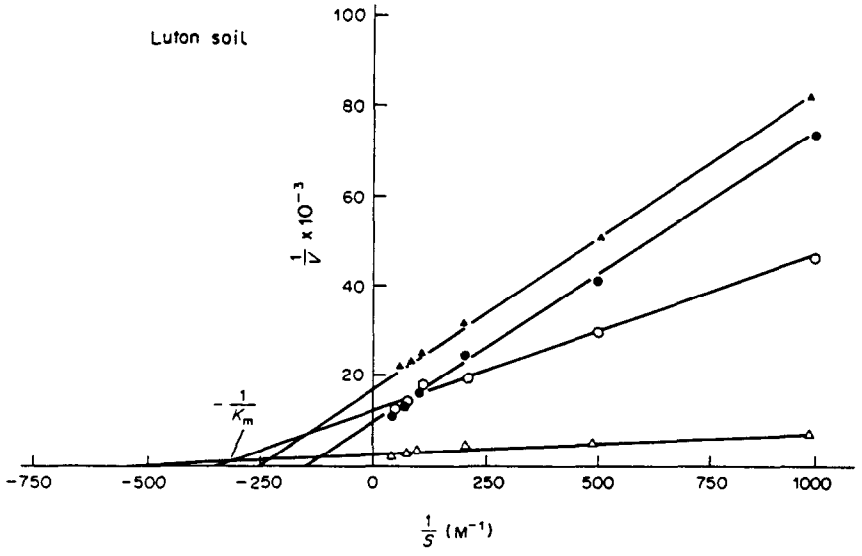

Fig. 5. Lineweaver–Burk plots for glycosidase activities of Luton soil; velocity (V) is expressed as μg of p-nitrophenol released g^{-1} soil h^{-1} at 37°C. ●, α-Galactosidase; ○, β-galactosidase; ▲, α-glucosidase; △, β-glucosidase.

Table 5. K_m and V_{max} values of glycosidase activities of soils

Soil	K_m (mM)				V_{max}*			
	α-Glu	β-Glu	α-Gal	β-Gal	α-Glu	β-Glu	α-Gal	β-Gal
Thurman	—	2.4	8.3	—	—	58	125	—
Hagener	—	1.5	—	—	—	83	—	—
Ida	6.1	1.6	10.0	3.1	37	111	153	48
Webster	10.1	1.4	16.1	14.3	76	256	125	83
Harps	—	1.6	—	—	—	126	—	—
Luton	2.9	1.3	5.6	2.7	53	526	95	85
Okoboji	14.0	1.6	—	7.4	91	344	—	83

*μg p-Nitrophenol released g^{-1} soil h^{-1} at 37°C; Glu = glucosidase; Gal = galactosidase; (—) indicates not determined.

concentration on the release of PN for the four glycosides. The results obtained were plotted as $1/V$ vs $1/S$ to determine the intercept ($1/V_{max}$) and the slope (K_m/V_{max}) of the linear transformation of the Michaelis–Menten equation and thereby calculate the K_m and V_{max} for the activities of the four glycosidases in soils. Similar types of plots were obtained with the other soils. The validity of the method used for assay of activities of these glycosidases in soils is evident from the finding that, with all soils used, the values obtained for the activities of these enzymes at each substrate concentration tested fitted the linear transformation of the Michaelis–Menten equation.

In general, the K_m values of β-glucosidase activity were lower and more uniform than those of the other glycosidases studied (Table 5). Studies with glycosidases purified from *Phaseolus vulgaris* have shown K_m values of 83 μM for β-glucosidase, 657 μM for α-galactosidase and 918 μM for β-galactosidases (Agrawal and Bahl, 1968). The K_m value of β-glucosidase purified from the fungus *Aspergillus terreus* was 780 μM PNG (Workman and Day, 1982). Depending on the soil, the V_{max} values varied among the four glycosidases.

Energy of activation

Application of the Arrhenius equation on the results obtained for the effect of incubation temperature on the activity of the four enzymes showed that the activation energy (E_a) values of the reactions catalyzed by these enzymes in three soils to vary among the enzymes (Table 6). Expressed in kJ mol^{-1}, the average E_a values were 43.1, 30.8, 57.9 and 32.6 for α-glucosidase, β-glucosidase, α-galactosidase and β-galactosidase activities, respectively. The average E_a value of α-galactosidase activity found for the three soils agrees well with that reported for the same enzyme purified from *Phaseolus vulgaris*, but the

Table 6. Activation energy values of glycosidase activities of soils

Soil	Activation energy (kJ mol^{-1})*			
	α-Glu	β-Glu	α-Gal	β-Gal
Webster	41.0	34.7	60.3	24.1
Harps	43.4	27.0	57.9	38.6
Nicollet	44.9	30.5	55.3	35.2
Av.	43.1	30.8	57.9	32.6

*Glu = glucosidase, Gal = galactosidase.

average E_a values for the other three glycosidase activities are much lower than those reported by Agrawal and Bahl (1968).

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