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CYANOGENESIS IN *SORGHUM VULGARE*—III.

PARTIAL PURIFICATION AND CHARACTERIZATION OF TWO β-GLUCOSIDASES FROM *SORGHUM* TISSUES*,[1]

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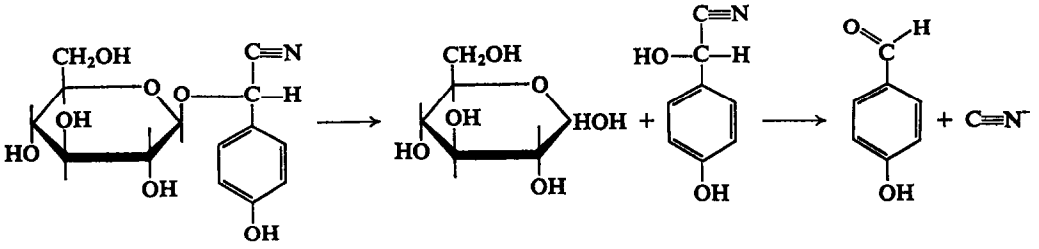
Abstract—Two β-D-glucopyranosidase activities with distinct aglycone specificities were characterized in *Sorghum* vegetative tissues and seeds. Glucosidase I, which was assayed with *p*-nitrophenyl β-D-glucopyranoside, also hydrolyzes salicin, but not dhurrin (*p*-hydroxy-L-mandelonitrile β-D-glucopyranoside). It was concentrated 10-fold and obtained free of β-glucosidase II. Glucosidase II was assayed with dhurrin; it hydrolyzes other mandelonitrile and benzyl β-glucosides as well. Glucosidase II was purified 85-fold from vegetative tissues and 4-fold from seeds, in which it occurs in higher concentrations, but it was not obtained free of glucosidase I. The pH optima and Michaelis constants of the two glucosidases for their respective assay substrates are reported, as well as K_i values for several competitive inhibitors. *Sorghum* oxynitrilase decomposes the L-isomer of *p*-hydroxymandelonitrile.

INTRODUCTION

BECAUSE of the economic importance of *Sorghum vulgare*, particularly because of the wide use of some varieties (Sudangrass) as forage, the cyanogenetic property of the species has been studied extensively. This property depends on the enzymic decomposition of the glucoside dhurrin (1) which occurs, sometimes in substantial amounts, in the stems and leaves of the plant.[2] The decomposition can be formulated as taking place in two steps:



1 2 3

Enzymes corresponding to the two steps have been identified in *Sorghum*—a glucosidase by Dunstan and Henry in the course of their pioneering research on dhurrin,[3] and an oxynitrilase more recently by Bové and Conn.[4] Horikoshi noted the presence, in *Sorghum* seeds, of a

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[1] Part II of this series: C.-H. MAO and L. ANDERSON, *J. Org. Chem.* **30**, 603 (1965).
[2] C.-H. MAO, J. P. BLOCHER, L. ANDERSON and D. C. SMITH, *Phytochem.* **4**, 297 (1965).
[3] W. R. DUNSTAN and T. A. HENRY, *Phil. Trans. Roy. Soc. London, Ser. A* **199**, 399 (1902).
[4] C. BOVÉ and E. E. CONN, *J. Biol. Chem.* **236**, 207 (1961).

glucosidase active against *p*-nitrophenyl β-D-glucoside,[5] but there appears to have been no systematic work on *Sorghum* glucosidases since the original observation of Dunstan and Henry.

The purpose of the present investigation was to obtain more precise information about the glucosidase which hydrolyzes dhurrin in autolyzing *Sorghum* tissue. Such information is desirable because methods for the analysis of "cyanide" in the tissue depend on the presence of dhurrin decomposing enzymes. Also, these enzymes are probably involved in the normal metabolism of dhurrin.

RESULTS

Activity vs. p-*Nitrophenyl* β-D-*Glucopyranoside* (*Glucosidase I*)

Extracts of fresh or dried *Sorghum* vegetative tissues (stems and leaves) contain an enzyme which hydrolyzes the chromogenic substrate *p*-nitrophenyl β-D-glucopyranoside. This activity is designated glucosidase I. A 10-fold purification of the enzyme was achieved by sequential lead acetate, ammonium sulfate, and acetone precipitations, with an ammonium sulfate extract of dried, ground tissue (see Experimental) as the starting material. The resulting preparations had very little activity against dhurrin, suggesting that the plant might contain a separate glucosidase for the hydrolysis of this substrate.

Activity vs. Dhurrin (*Glucosidase II*)

Ammonium sulfate extracts of dried, ground *Sorghum* vegetative tissue showed rather weak activity against dhurrin, but this activity, designated glucosidase II, could be concentrated. Precipitation with ammonium sulfate and acetone, and adsorption on bentonite and calcium phosphate gel proved to be the most effective procedures. Table 1 presents the results of a typical fractionation, in which an 85-fold purification was achieved. Each successive fraction was assayed for both glucosidase I and glucosidase II, and it may be seen that the ratio of the two activities changed markedly during the two adsorption steps. Over 95 per cent of the glucosidase I was eventually removed, but because this glucosidase was initially present in great excess, the final ratio of glucosidase I to glucosidase II activity was still rather high.

Further fractionation of the final ammonium sulfate precipitate was attempted by various chromatographic procedures. None of these gave satisfactory recoveries or purifications, but a partial separation of the two enzymes was achieved with a DEAE-cellulose column. A fraction from this column contained glucosidase I, but no measurable amount of glucosidase II.

Partial Purification of Glucosidase II from Sorghum Seeds

Extracts of seeds from seven strains* of *S. vulgare* were assayed, and all were found to contain both glucosidase activities. In most cases the amounts of glucosidase II were comparable to that in the vegetative tissue used in this work, but the levels of glucosidase I were much lower. The ratio glucosidase I/glucosidase II ranged from 19 down to 1·7. The seed glucosidase II differed from the plant (vegetative tissue) enzyme in that it was not precipitated by ammonium sulfate at 75 per cent saturation, and was not adsorbed on calcium phosphate gel at pH 4·5. However, after acetone precipitation and bentonite adsoption, it could be further purified on a hydroxylapatite column.

* Rox orange; Piper; Sweet Sudan; DeKalb FS-22, FS-1a, and SX-11; Sweet Sudan 372 (F366).

[5] K. HORIKOSHI, *J. Biochem.* (*Tokyo*) **35**, 39 (1942).

TABLE 1. FRACTIONATION OF GLUCOSIDASES FROM *Sorghum* VEGETATIVE TISSUES

Fraction	Protein (mg)	Glucosidase I				Glucosidase II				Ratio G-ase I / G-ase II	Galactosidase*	
		Total activity (mU)†	Sp. act. (mU/mg protein)	Yield (%)	Purification factor	Total activity (mU)†	Sp. act. (mU/mg protein)	Yield (%)	Purification factor		Total activity (mU)†	Sp. act. (mU/mg protein)
P1 5% $(NH_4)_2SO_4$ extract	4100	530,000	130	100	—	4,600‡	1·1‡	100	—	115	62,700	15
P2 30–75% $(NH_4)_2SO_4$ precipitate	1080	470,000	440	90	3·4	4,100	3·8	90	3·5	115	—	—
P3 60% acetone precipitate	238	380,000	1600	81	3·6	3,200	13	78	3·4	119	8,800	37
P4 Bentonite supernate	165	84,000	510	22	0·32	3,000	18	94	1·4	28	4,600	28
P5 Ca phos. gel eluate	40	26,800	670	32	1·3	2,440	61	81	3·4	11	1,640	41
P6 50–80% $(NH_4)_2SO_4$ precipitate	23	24,800	1080	93	1·6	2,140	93	88	1·5	12	1,210	53
Overall				4·7	8·3			47	85			

* Assayed with *o*-nitrophenyl β-D-galactopyranoside.
† mU = milliunits (see Experimental).
‡ Analysis after complete precipitation with ammonium sulfate (see Experimental).

Table 2 gives the results obtained with an extract from a strain of seeds which were some five times richer in glucosidase II than any of the other seeds tested. Although the overall purification was only 4-fold, the specific activity of the final fraction was four times that of the best preparation from vegetative tissue. This fraction also had the lowest glucosidase I/glucosidase II ratio (1·7) of any of our purified preparations.

TABLE 2. PARTIAL PURIFICATION OF GLUCOSIDASE II FROM SEEDS OF SWEET SUDAN 372 (F366)

		Glucosidase II				
Fraction	Protein (mg)	Total activity (mU)*	Sp. act. (mU/mg protein)	Yield (%)	Purification factor	Ratio G-ase I / G-ase II
S1 60% acetone precipitate	245	24,000	98	100	—	2·9
S2 Bentonite supernate	165	22,500	136	94	1·4	2·6
S3 Hydroxylapatite eluate	38	15,200	400	68	2·9	1·7
Overall				63	4·1	

* mU = milliunits (see Experimental).

Properties of the Sorghum Glucosidases

Stability. In the purified preparations just described both glucosidase I and glucosidase II proved to be very stable. When solutions in buffers of pH 4·6–7·0 were stored several months in the refrigerator, neither activity diminished. Freezing and thawing, lyophilization, dialysis for 18 hr, and dilution to 0·1 mg protein/ml likewise had no effect on either activity.

The stabilities of the seed glucosidases to 15 min incubations at 37° were investigated as a function of pH, with the results shown in Fig. 1. On the acid side of the stable range, both enzymes were denatured, glucosidase II more rapidly than glucosidase I. On the alkaline side, glucosidase I began to show instability at pH 8, whereas glucosidase II was still stable at this pH. This property might provide a basis for the selective removal of glucosidase I from seed enzyme preparations.

pH Optima. pH-activity plots are shown in Fig. 2. The curves for the two preparations (seed and plant) differ considerably in the case of glucosidase I, but are similar for glucosidase II. Part of the decrease in activity below pH 4 must be attributed to denaturation.

Michaelis constants. The initial velocities of the hydrolysis reactions were determined over a range of substrate concentrations ($< K_m$ to $4–10 \times K_m$), in the presence and in the absence of inhibitors. Converted to reciprocal plots (Lineweaver–Burk), the data gave straight lines. The Michaelis constants derived from the plots are listed in Table 3.

Inhibitors. D-Glucose, D-glucono-1,4-lactone and D-glucono-1,5-lactone were tested as inhibitors for both glucosidases. In addition dhurrin was tried as an inhibitor of glucosidase I, and *p*-nitrophenyl glucoside as an inhibitor of glucosidase II. All the lines in the reciprocal plots, including those obtained in the absence of inhibitors, had a common intercept, indicating that all the inhibitions were competitive. (*p*-Nitrophenyl glucoside may be a competing substrate for glucosidase II—see Discussion.) The K_i values are given in Table 3. The figures for the lactones are relative only, since the lactone solutions used varied in age from 1–7 days (they were stored in the refrigerator after the first 24 hr), and were therefore perhaps not of

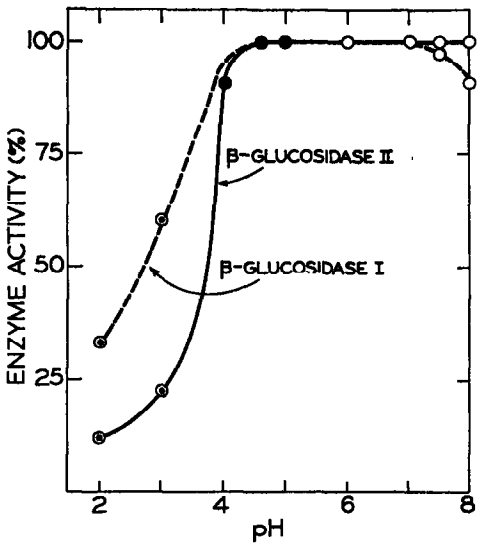

FIG. 1. INCUBATION OF SEED GLUCOSIDASES I AND II AT VARIOUS pH's.

Fraction S3 was used. The mixed enzymes were incubated at 37° in 0·05 M sodium formate (⊙), sodium acetate (●), or sodium phosphate (○) buffers for 15 min, then the activities were assayed in the usual way.

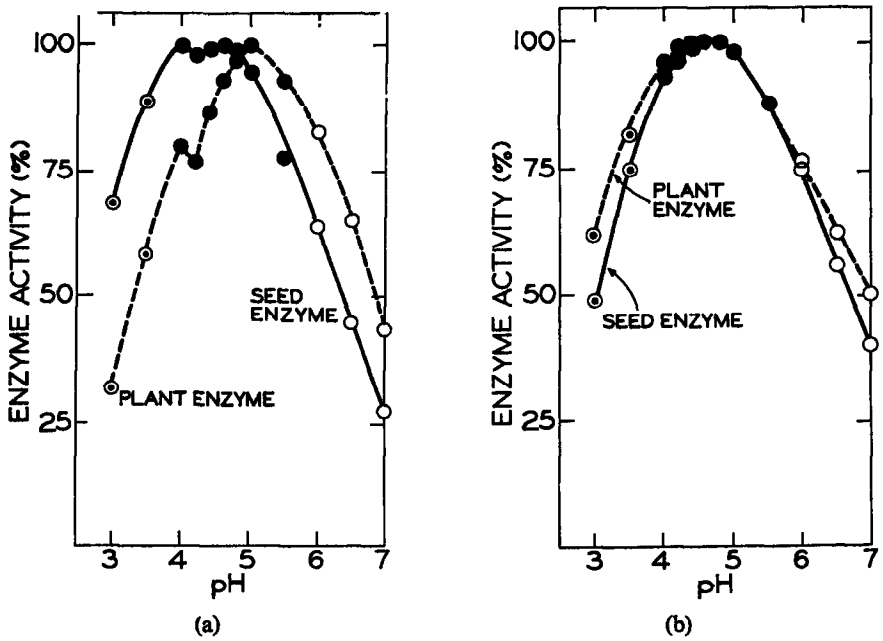

FIG. 2. pH-ACTIVITY CURVES FOR *Sorghum* GLUCOSIDASES.

Activities of the plant enzyme (fraction P6) and seed enzyme (fraction S3) were measured as described for assay, in 0·05 M sodium formate (⊙), sodium acetate (●), and sodium phosphate (○) buffers.

(a) Glucosidase I; (b) glucosidase II.

constant composition. It will be noted that in most instances the K_i values for the two glucosidases differ substantially.

Aglycone specificity .The specificities of the two glucosidases were examined by measuring the rates of hydrolysis of a series of β-glucosides structurally related either to dhurrin or to *p*-nitrophenyl β-glucoside. Since all the enzyme preparations available for use in these

TABLE 3. K_m AND K_i VALUES FOR GLUCOSIDASES I AND II*

	Inhibitor	Glucosidase I (mM)	Glucosidase II (mM)
K_m (plant)	—	0·31	0·55
K_m (seed)	—	0·13	0·31
K_i (plant)	D-Glucose	1·73	0·53
K_i (seed)	D-Glucose	0·22	0·16
K_i (seed)	D-Glucono-1,5-lactone†	0·017	0·032
K_i (seed)	D-Glucono-1,4-lactone†	0·019	0·036
K_i (seed)	Dhurrin	0·34	—
K_i (seed)	*p*-Nitrophenyl glucoside	—	0·055

* Plant enzyme, fraction P6; seed enzyme, fraction S3.
† Calculation based on total gluconate concentration (see text).

TABLE 4. RATES OF HYDROLYSIS OF β-GLUCOSIDES

Substrate (aglycone)	Rates: Enzyme fraction P3 (μmole/min/ml)	Rates: Enzyme fraction P6 (μmole/min/ml)	Ratio $\frac{P6}{P3}$
Dhurrin (*p*-hydroxy-L-mandelonitrile)	0·049	0·282	5·8
p-Nitrophenyl β-D-glucoside	2·55	2·96	1·2
Salicin (*o*-hydroxymethylphenol)*	0·87	1·10	1·3
Taxiphyllin (*p*-hydroxy-D-mandelonitrile)†	0·082	0·46	5·6
Prulaurasin (DL-mandelonitrile)*	0·170	0·97	5·7
p-Hydroxybenzyl β-D-glucoside‡	0·037	0·214	5·8
Benzyl β-D-glucoside*	0·076	0·43	5·7

* 0·9 ml of 5 mM substrate in 0·05 M sodium acetate buffer, pH 4·8; 0·1 ml of enzyme. Released glucose was determined by the Somogyi-Nelson method after 20 min at 37°.
† 0·9 ml of 1·8 mM substrate in 0·05 M sodium phosphate buffer, pH 6·5; 0·1 ml of enzyme; 37°. This pH had to be used to allow the *p*-hydroxy-D-mandelonitrile released to decompose spontaneously (see text on oxynitrilase specificity) to cyanide and *p*-hydroxybenzaldehyde. The latter was read at 284 mμ at intervals of 1 min for 10 min with correction for the fraction ionized (pK_a 7·66).
‡ 0·9 ml of 5 mM substrate in 0·05 M sodium acetate buffer, pH 4·8; 0·1 ml of enzyme; 37°. Released glucose was determined with Glucostat (glucose oxidase) reagent after 20 min.

experiments contained both glucosidases I and II, each glucoside was run with two fractions of the plant enzyme, one of which (P6) was enriched in glucosidase II relative to the other (P3). The substrate concentration in each case was set high enough to give a linear rate over the period of measurement, but rate-concentration relationships were not analyzed in detail.

As shown in Table 4, the activities of the two enzyme fractions against taxiphyllin, prulaurasin, *p*-hydroxybenzyl β-D-glucoside, and benzyl β-D-glucoside were closely proportional to their activities against dhurrin. It could thus be inferred that these glucosides and

dhurrin were being hydrolyzed by the same activity. This means glucosidase II, since glucosidase I was shown to be inactive against dhurrin. The hydrolysis of salicin, on the other hand, paralleled that of *p*-nitrophenyl glucoside.

Specificity for the sugar moiety. The plant extract was tested at each stage of the fractionation with *o*-nitrophenyl β-D-galactopyranoside. Activity was found (Table 1), but the ratio nitrophenyl galactosidase/nitrophenyl glucosidase of the final fraction (P6) was only half that of the original extract. Neither the glucosidase I (in agreement with Horikoshi[5]) nor the glucosidase II activity was inhibited by D-galactose at 5 mM.

Specificity of Sorghum Oxynitrilase

Bové and Conn reported that *Sorghum* oxynitrilase acts on only one of the enantiomorphs of *p*-hydroxymandelonitrile, but did not determine which one.[4] In the experiment with taxiphyllin, the *p*-hydroxy-D-mandelonitrile released from this substrate by glucosidase action was not decomposed by the oxynitrilase in our preparations. *Sorghum* oxynitrilase thus appears to be specific for the L-configuration (2) of the mandelonitrile moiety.

DISCUSSION

Since the presence of cyanogenetic glycosides in plant tissues is normally detected by the fact that the tissues evolve hydrogen cyanide under autolyzing conditions, it is clear that cyanogenetic glycosides and enzymes capable of hydrolyzing them commonly occur together. The question naturally arises whether these enzymes are special enzymes elaborated to deal with the characteristic glycosides of their species, or whether they belong to the more widely distributed types of glycosidases, which are generally thought to have fairly broad aglycone specificity. This question has so far had little study, but an indication that both possibilities may be realized (and, no doubt, a full spectrum of in-between ones) comes from recent work by Butler *et al.*[6] These authors found evidence that in white clover (*Trifolium repens* L.) the glucosidase (linamarase) which hydrolyzes the cyanogenetic glucosides linamarin and lotaustralin is different from the one active against salicin. In linseed, a single enzyme appears to hydrolyze linamarin, lotaustralin and salicin, but this activity is distinct from the one which splits β-glucose disaccharides (cellobiose, amygdalin).

The results of Butler *et al.* parallel those from a number of other investigations indicating that tissues of a given species of plant or microorganism may contain more than one glycosidase of a particular class (*i.e.* β-glucosidase, α-galactosidase, etc.). Examples include barley (separate activities vs. β-glucose disaccharides and simple β-glucosides[7]), *Aspergillus oryzae* (several β-glucosidases of differing specificities[8]), and *Plantago ovata* (two α-galactosidases[9]).

The present work shows that vegetative tissues and seeds of *Sorghum vulgare* contain both a "classical" β-glucosidase and an enzyme active against the characteristic *Sorghum* glucoside.* The conclusion that the two glucosidases are distinct rests primarily on observed variations in the ratio of activities against the substrates *p*-nitrophenyl β-glucoside and

* It is clear from recent work on the β-glucosidase of almond emulsin that the term "glucosidase" as it is used in the present paper and in the current literature has only provisional meaning.[10] In many cases the entities so designated may be families of closely related proteins.

[6] G. W. Butler, R. W. Bailey and L. D. Kennedy, *Phytochem.* **4**, 369 (1965).
[7] F. B. Anderson, W. L. Cunningham and D. J. Manners, *Biochem. J.* **90**, 30 (1964).
[8] M. A. Jermyn, *Australian J. Sci. Res., Ser. B* **5**, 433 (1952).
[9] J. E. Courtois, F. Petek and To Dong, *Bull. Soc. Chim. Biol.* **45**, 95 (1963).
[10] B. Helferich and T. Kleinschmidt, *Z. Physiol. Chem.* **340**, 31 (1965).

dhurrin. By different manipulations this ratio could be varied from infinity (no activity vs. dhurrin) to 1·7. In particular, the ratio changed 10-fold in the course of the procedure which gave an 85-fold purification of glucosidase II. The two activities also differed in their stabilities at extremes of pH. And finally, certain inhibitors (the gluconolactones with the seed preparations, glucose with the plant preparations) gave substantially different K_i values in the presence of the two different test substrates.

The *Sorghum* glucosidases were not tested with any α-glucopyranosides, or with any glucofuranosides, but in view of the usual sharp specificity of glycosidases for anomeric configuration and ring size, it is presumed that glucosidases I and II may be classified as β-D-glucopyranosidases. If either of the enzymes had β-galactosidase activity, inhibition by galactose would be expected,[11] but the observed lack of such inhibition can hardly be regarded as decisive. It may be concluded, however, from the change in the ratio of nitrophenyl galactosidase to nitrophenyl glucosidase activity during purification, that at least *part* of the β-galactosidase activity of the plant extract is distinct from either glucosidase.

Further delineation, but by no means complete definition, of the aglycone specificities of the two enzymes was achieved by the experiments with additional substrates. The data suggest that not only dhurrin but in fact benzyl glucosides as a class (Table 4) are excluded from the specificity range of glucosidase I. At least this seems to be true at substrate levels which are approximately saturating for glucosidase II; it is of course possible that glucosidase I would show weak activity against the benzyl glucosides at very high concentrations. Glucosidase I may well be an aryl glucosidase (cf. the results of Jermyn with the glucosidases of the mold *Stachybotrys atra*[12]), but tests with a further range of substrates (alkyl glucosides, β-glucose disaccharides) would be required to determine this. Such tests might well reveal the presence of additional β-glucosidases in *Sorghum*.

Glucosidase II readily catalyzed the hydrolysis of β-glucosides differing from dhurrin in having: the opposite configuration at the benzyl carbon (taxiphyllin), no cyano group (*p*-hydroxybenzyl glucoside), no *p*-OH (prulaurasin), and neither cyano group nor *p*-OH (benzyl glucoside). Glucosidase II is thus not a specific dhurrinase. Whether it is completely inactive against aryl glucosides remains to be seen, for although the relative aryl glucosidase activity (here tabulated as glucosidase I) of the fractions declined during purification, no preparations free of such activity were obtained.

While no direct studies were made on the mechanism of action of either enzyme, it may be noted that benzyl glucosides without a para hydroxyl were better substrates for glucosidase II than those with this feature. Thus, the action of the enzyme does not depend on the presence of the para hydroxyl, and the mechanism is presumably the normal one for glucoside hydrolysis. The *alkaline* hydrolysis of *p*- (and *o*-) hydroxybenzyl glucosides goes by a very different mechanism, involving cleavage of the bond between the aglycone and the anomeric oxygen.[1] It would be rather surprising if the enzyme were found to employ this special mechanism.

EXPERIMENTAL

Materials and Methods

Sorghum seeds of the varieties DeKalb FS-1a, FS-22, and SX-11 were gifts from the DeKalb Agricultural Association, Inc., DeKalb, Ill. Other plant materials were supplied by the Department of Agronomy, University of Wisconsin. Previous papers have described the isolation of dhurrin[2] and the enzymic synthesis of *p*-hydroxybenzyl β-D-glucopyranoside.[1]

[11] R. HEYWORTH and P. G. WALKER, *Biochem. J.* **83**, 331 (1962).
[12] M. A. JERMYN, *Rev. Pure Appl. Chem.* **11**, 92 (1961).

Benzyl β-D-glucopyranoside was synthesized according to Slotta and Heller.[13] Taxiphyllin and prunasin tetraacetate (D-mandelonitrile β-D-glucopyranoside tetraacetate) were gifts from Dr. G. H. N. Towers. The prunasin tetraacetate was deacetylated to give prulaurasin.[14] All other chemicals were obtained from commercial sources. Oxynitrilase was purified from *Sorghum* as described by Bové and Conn.[4]

Protein was determined by the biuret method,[15] after precipitation with trichloracetic acid at 5 per cent final concentration. Crystalline bovine serum albumin was used as the standard. Glucose was determined by the Somogyi–Nelson method[16] or, in the presence of alkali-labile glucosides, by the glucose oxidase method.[17]

Glucosidase units. The basic unit is that amount of activity which will catalyze the transformation of 1 μmole of substrate per minute under the assay conditions.[18] Since the use of this unit as such in the present work would give inconveniently small numbers, activities have actually been expressed in milliunits (mU), and specific activities in mU/mg protein.

Assay for glucosidase I. At zero time 0·1 ml of properly diluted enzyme (3–15 mU) was pipetted into 0·9 ml of 1·1 mM *p*-nitrophenyl glucoside in 0·05 M sodium acetate buffer, pH 4·8. After incubation for 10 min at 37°, the reaction was stopped by adding 2 ml of 0·2 M sodium borate buffer, pH 9·8,[19] and the liberated *p*-nitrophenol was read at 400 nm ($a_M = 1{\cdot}84 \times 10^4$).

Assay for glucosidase II was carried out at 37° in a 1-cm cuvette, final volume 1 ml. The substrate was 0·8 or 0·9 ml of dhurrin, 2·0 mM in 0·05 M sodium acetate buffer, pH 4·6. For the seed enzyme fractions 0·1 ml of oxynitrilase (1000 of Bové and Conn's units) was added; for the plant enzyme fractions this was not required. After the addition of 0·1 ml of suitably diluted enzyme (1–6 mU), the absorbancy of the liberated *p*-hydroxybenzaldehyde (3) was measured at 284 nm ($a_M = 1{\cdot}53 \times 10^4$). Readings were made at 1 min intervals for 5 or 10 min to verify linearity of rate. A lag period of *ca.* 1 min was observed when oxynitrilase had to be furnished. The maximal hydrolysis of substrate in any determination was less than 5 per cent.

Glucosidases From Vegetative Tissue

Sorghum plants, var. Leoti, Rox orange, and Sweet Sudan, harvested when about 1 ft high, were dried at room temperature. The dry plants were ground through the coarse screen on a Wiley mill, dried again in vacuum over calcium chloride, then ground through the fine screen. The resulting powder was stored at −25°. It fully retained its glucosidase activities for over 2 yr.

Crude extract was made at room temperature. The dried grass powder was suspended in 10 volumes of 5 per cent (w/v) ammonium sulfate adjusted to pH 7·5 with sodium hydroxide, and the suspension was stirred slowly for 2 hr, then let stand overnight. The solids were collected on 2 layers of cheesecloth and squeezed. The filtrate (P1), 8·5 volumes, was at pH 6·2. The proteins of P1 were precipitated by saturation with ammonium sulfate and redissolved for assay for glucosidase II, in order to circumvent interference from the pigments present in the extract.

[13] K. H. Slotta and H. Heller, *Ber. Deut. Chem. Ges.* **63**, 1024 (1930).
[14] E. Fischer and M. Bergmann, *Ber. Deut. Chem. Ges.* **50**, 1047 (1917).
[15] E. Layne, In *Methods in Enzymology* (Edited by S. P. Colowick and N. O. Kaplan), Vol. 3, p. 450. Academic Press, New York (1957).
[16] J. E. Hodge and B. T. Hofreiter, In *Methods in Carbohydrate Chemistry* (Edited by R. L. Whistler and M. L. Wolfrom), Vol. 1, p. 380. Academic Press, New York (1962).
[17] M. E. Washko and E. W. Rice, *Clin. Chem.* **7**, 542 (1961).
[18] International Union of Biochemistry, *Enzyme Nomenclature*, p. 7. Elsevier, Amsterdam (1965).
[19] J. W. Woollen, R. Heyworth and P. G. Walker, *Biochem. J.* **78**, 111 (1961).

All subsequent operations were conducted in an ice bath or a cold room. Precipitates were separated by centrifugation.

Partial purification of glucosidase I. One hundred ml of extract P1 was adjusted to pH 7·2. The precipitate formed by treatment with 2 ml of 0·33 M neutral lead acetate, pH 7·2, was discarded and the supernate was brought to pH 4·5. Further treatment with 15 ml of 0·5 M neutral lead acetate gave a precipitate, which was collected and extracted twice with 20 ml of sodium pyrophosphate, 0·23 M, pH 9·0, for a ½ hr each time. The combined extract was fractionated with ammonium sulfate after adjustment to pH 7·6. The precipitate formed between 40 and 75 per cent saturation was dissolved by dialysis against deionized water overnight, and the solution treated with acetone at $-10°$ to 60 per cent by volume. The overall yield of glucosidase I activity in the precipitate was about 60 per cent, purification factor 10.

Fractionation with enrichment in glucosidase II. The numerical data are given in Table 1. Extract P1 (1700 ml) was brought to 30 per cent saturation with solid ammonium sulfate, the precipitate was discarded, and the supernate adjusted to pH 4·5 with 1·8 N hydrochloric acid in 30 per cent saturated ammonium sulfate. It was then brought to 75 per cent saturation with ammonium sulfate, and the resulting precipitate was suspended in 300 ml of water. Dialysis against deionized water for 12 hr, then centrifugation, gave 300 ml of clear solution (P2).

To fraction P2, chilled to 0°, acetone at $-10°$ was slowly added with stirring to 60 per cent by volume. After 10 min the precipitate was collected at $-10°$, the pellet was extracted with 100 ml of water, and the extract (P3) was centrifuged to remove insoluble protein.

Fraction P3 was diluted to a protein concentration of 2·4 mg/ml (the optimal range is 2–4 mg/ml), and adjusted to pH 4·6 with 0·1 N HCl. Dry bentonite, five times the weight of the protein in the solution, was added with stirring. After 25 min, the suspension was centrifuged at 15,000*g* for 20 min, and the supernate (P4) was retained.

Fraction P4 was brought to pH 7·4 with 0·1 N sodium hydroxide. Inactive proteins were removed by treatment for 25 min with calcium phosphate gel,[20] approximately three times the weight of proteins in solution. The supernate was adjusted to pH 4·5 with 0·1 N hydrochloric acid and treated a further 25 min with twice the previous amount of calcium phosphate gel. After collection of the gel by centrifugation the enzymes were eluted by extractions (1 hr each) with first 15 ml, then 10 ml. of 30 per cent saturated ammonium sulfate (combined eluate, P5).

After adjustment to pH 5·6 fraction P5 was brought to 50 per cent saturation with ammonium sulfate. A small amount of turbidity which formed during 1 hr was removed by centrifugation at 15,000*g*, and the solution was brought to pH 4·5. Adding ammonium sulfate to 80 per cent saturation and stirring for 1 hr gave a precipitate, which was collected at 15,000*g* and dissolved in 5 ml of 0·01 M sodium phosphate buffer, pH 6·0 (P6).

Partial Purification and Fractionation of the Glucosidases from Sorghum Seed

Seeds of Sweet Sudan 372 (F366), 232 g, were ground through the fine screen on a Wiley mill. The resulting powder was extracted with 750 ml of 0·01 M sodium phosphate buffer, pH 7·5, at room temperature overnight, and the mixture was squeezed through two layers of cheesecloth. The numerical data pertaining to the fractionation of this extract are given in Table 2. Acetone precipitation (to give fraction S1), and bentonite adsorption (to give S2) were performed exactly as described above for the plant extract.

[20] D. KEILIN and E. F. HARTREE, *Proc. Roy. Soc. (London), Ser. B* **124**, 397 (1938).

The supernate from the bentonite adsorption (S2) was dialyzed against deionized water for 12 hr, concentrated by lyophilization to half its original volume, dialyzed again for 12 hr, and finally lyophilized to dryness. The dry residue was then dissolved in 15 ml of 0·025 M sodium phosphate buffer, pH 6·8, and dialyzed against this buffer 12 hr. The hydroxylapatite column (1·4 × 28 cm) was prepared according to Levin[21] and equilibrated with the buffer. 2 ml of enzyme solution containing 22 mg of protein (3000 mU of glucosidase II) were put on the column and it was developed first with the 0·025 M phosphate buffer (100 ml), then with 0·05 M sodium phosphate of the same pH (6·8). Glucosidases I and II were eluted together as a well-defined peak shortly after the buffer molarity was increased.

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[21] O. Levin, In *Methods in Enzymology* (Edited by S. P. Colowick and N. O. Kaplan), Vol. 5, p. 27. Academi Press, New York (1962).