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THE β-GLUCOSIDASE AND β-GALACTOSIDASE COMPONENTS OF *MEDICAGO SATIVA* L.

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Abstract—The β-galactosidase (EC.3.2.1.23) of alfalfa seed is not readily separated from the β-glucosidase (EC.3.2.1.21) activity. Heat denaturation studies and reactions to specific inhibitors indicate the separate identity of the two enzymes. The β-glucosidase may be fractionated into several active components. A role in germination is suggested.

INTRODUCTION

EXTRACTS of alfalfa seed (*Medicago sativa.* L.) were shown by Hill[1] to contain a number of glycosidases, including α- and β-galactosidases, α-mannosidase and *N*-acetyl-β-glucosaminidase, the preparation differing from almond emulsin in having a high β-galactosidase activity with relatively little β-glucosidase. Both these activities have been shown by Heyworth and Walker[2] to utilize the same active site in almond emulsin, the enzyme being apparently non-specific for carbon-4 of the sugar moiety, but a recent comparative study[3] demonstrated that many sources of these enzymes consist of a complex mixture, with isoenzymes of both components.

Hutson[4] found that alfalfa extracts had marked β-glucosidase activity when cellobiose was used instead of aryl-β-glucosides as the test substrate, and he was able to demonstrate that transglucosylation occurred with the formation of tri-saccharides, but transfer of other sugars was not detected.

In the present investigation we have attempted to establish the separate identity of the two enzymes. The activity towards a number of synthetic glycosides has been compared, as well as cellobiase and lactase activity, using crude seed flour extracts and partially purified enzyme. It was found that although the two enzymes had similar physical properties and had a low aglycone specificity they could be distinguished by their stability to heat, and could be selectively inhibited. The β-glucosidase activity was found to occur in multiple forms.

RESULTS AND DISCUSSION

Attempts to fractionate an aqueous extract of defatted alfalfa flour by ammonium sulphate gave little or no activity in the precipitate obtained at 50 per cent saturation. About 30 per cent of the activity was precipitated between 50 and 66 per cent saturation, and the remainder was still in the solution; however, dialysis and concentration of this soluble enzyme resulted in almost complete loss of activity. Similarly the preparation of acetone powders from the

[1] K. HILL, *Ber. Verhandl. Sachs. Acad. Wiss. Leipzig, Math.-Phys. Kl.* **86**, 115, (1934).
[2] R. HEYWORTH and P. G. WALKER, *Biochem. J.* **83**, 331 (1962).
[3] R. G. PRICE and D. ROBINSON, *Comp. Biochem. Physiol.* **17**, 129–138 (1966).
[4] D. H. HUTSON, *Biochem. J.* **92**, 142 (1964).

aqueous extract yielded only 22 per cent of the original activity as a powder which had only a two-fold increase in specific activity. Precipitation of a 10% w/v solution by the addition of an equal volume of absolute ethanol at −15° produced 70 per cent of the total activity as a highly hygroscopic powder with 2·5× purification.

A nine-fold concentration of β-galactosidase was obtained by coagulation at pH 4·5 and 37°. This left all the original activity in the solution, which could then be freeze-dried to give a readily soluble pale yellow powder.

A higher degree of purification (× 33) was obtained by precipitation with tannin, but this only yielded 30 per cent of the total activity; the rest remained in the solution and appeared to be still active in the presence of large amounts of tannin. Similar yields were obtained if a solution of the material obtained after heat coagulation was precipitated in this way. When incremental amounts of tannin were added the individual precipitates all had the same specific activity, until a point was reached when no further precipitation occurred. The whole of the enzyme activity can therefore not be obtained by this method and the preparation is not necessarily representative of the original material. The effect of tannin on almond β-glucosidase has recently been extensively investigated.[5]

Both the tannin precipitate and heat coagulated enzyme preparations contain a large amount of non-reducing carbohydrate material as detected by the phenol-sulphuric acid method. It has been suggested that glycosidases may be glycoproteins,[6–8] but it is more likely in this case that the bulk of the carbohydrate is extraneous material to which the enzyme is bound, since the more active preparations contained lower amounts of carbohydrate (Table 1) and lucerne endosperm is known to contain galactomannan, as a major structural polysaccharide.[9]

TABLE 1. CHARACTERISTICS OF ALFALFA SEED PREPARATIONS

Preparation	Carbohydrate* (%)	Nitrogen (%)	β-galactosidase† activity/mg N	Yield (%)
Crude flour	—	5·85	1·28	—
Agglutinated flour	33·5	3·0	11·44	100
Tannin precipitate	15·25	5·65	43·10	30

* Estimated as glucose by phenol–sulphuric acid method.

† μmoles 4-methylumbelliferone liberated from its β-galactoside in 1 hr at pH 5 and 37°.

During the purification the enzymes retained their activity to all the substrates used, and β-glucosidase showed a degree of concentration similar to that obtained with β-galactosidase. On the other hand it is clear that *N*-acetyl-β-glucosaminidase is not concentrated in the same fashion. (Table 2.)

The physical similarity of glucosidase and galactosidase is not supported when one examines the stability of the enzymes to heat treatment. The preparation was maintained at pH 5 and 55° for varying periods before cooling and assaying for both activities at 37°. There

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[6] K. WALLENFELS and O. M. MALHOTRA, *Advan. Carbohydrate Chem.* **16**, 239 (1961).
[7] E. H. FISCHER, L. KOHTER and J. FELLIG, *Helv. Chim. Acta*, **34**, 1132 (1951).
[8] H. BAUMAN and W. W. PIGMAN, In *The Carbohydrates* (edited by W. W. PIGMAN) p. 536. Academic Press, New York (1957).
[9] E. L. HIRST, J. K. N. JONES, and W. O. WALDER, *J. Chem. Soc.* 1443 (1947).

TABLE 2. HYDROLYSIS OF β-D-GLYCOSIDES BY ALFALFA EMULSIN PREPARATIONS

Substrate	Concentration (mM)	μmoles hydrolysed/hr per mg		Optimum pH
		Crude flour	Tannin ppt.	
Galactosides				
phenyl	5	0·44	7·6	3·5
o-nitrophenyl	2·5	0·18	4·46	4·0
p-nitrophenyl	5	2·47	54	4·0
p-aminophenyl	10	0·67	11·2	4·0
4-methylumbelliferyl	0·5	0·107	2·37	4·0
glucosyl (Lactose)	15	—	0·14	5·0
Glucosides				
p-nitrophenyl	5	0·11	2·88	5·0
4-methylumbelliferyl	0·5	0·0044	0·068	5·0
glucosyl (cellobiose)	15	—	0·0125	5·0
N-acetylglucosaminide				
p-nitrophenyl	3	2·36	8·75	4·0

was a rapid drop in β-glucosidase activity with a loss of 50 per cent of the activity in a few minutes, followed by a slower decline and after 1 hr some 20 per cent of the original activity remained and appeared stable to further incubation periods of 1 hr at 55°. (Fig. 1.) The

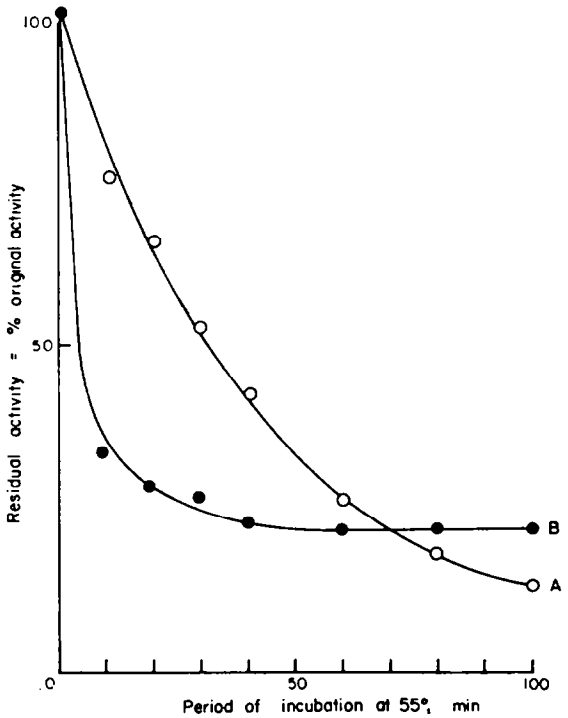

FIG. 1. HEAT DENATURATION AT 55° IN PHOSPHATE–CITRATE BUFFER (0·1 M) pH 5, FOLLOWED BY ASSAY AT 37° WITH 4-METHYLUMBELLIFERYL GLYCOSIDES.
A—β-galactosidase
B—β-glucosidase.

β-galactosidase on the other hand decayed exponentially, suggesting a single enzyme species with a half life of 32 min at this temperature.

Further evidence for the separate identity of the two activities was obtained by inhibition with D-glucono-(1→5) and D-galactono-(1—4)-lactones as described by Conchie and Levvy,[10]. These have specific inhibitory effects on the two enzymes, and the β-glucosidase can be completely inhibited by D-glucono-(1→5)-lactone at concentrations which have no effect on the β-galactosidase (Table 3). The effect of D-galactono-(1→4)-lactone is less impressive but concentrations which produced 50 per cent inhibition of the β-galactosidase gave only 10 per cent inhibition of the β-glucosidase. The results suggest that the binding sites are independent and specific for the sugar.

TABLE 3. INHIBITION OF ALFALFA β-GLUCOSIDASE AND β-GALACTOSIDASE BY GLYCONOLACTONES

Inhibitor concentration (mM)	Percentage inhibition			
	β-galactosidase		β-glucosidase	
	D-galactono-(1→4)-lactone	D-glucono-(1→5)-lactone	D-galactono-(1→4)-lactone	D-glucono-(1→5)-lactone
0·28	0	0	9	99
1·4	46	9	9	100
2·8	54	11	11	100
14	76	31	25	100
28	83	54	43	100

* Results are expressed as % inhibition compared with controls similarly incubated with 4-methylumbelliferyl glycosides under standard assay conditions.

The response of alfalfa β-glucosidase to heat denaturation may be explained by supposing at least three components, distinguished by a high, medium or low stability. Chromatography on DEAE-cellulose columns confirmed that a number of glucosidase components may occur while most of the β-galactosidase activity was located in a single peak (Fig. 2). A stepwise elution allowed five fractions with β-glucosidase activity to be collected, and the inactivation of these fractions at 55° (Table 4) shows that the components have widely varying stabilities, when half-lives calculated from the logarithmic plot of the decay curves are compared. The significance of multiple forms that are without marked differences in substrate specificity has been critically examined by Jermyn[11] who suggests that they may arise by complexing a single enzyme species with different polysaccharides. In such cases, variations in stability should not be used as the sole criteria of individuality, since this stability and the batch of differences that frequently occur in the proportions of components from a single source may be ascribed to the nature of the complexing carbohydrate. Nevertheless, a selective formation of complexes may play an important part in the localization and distribution of the enzyme in the living cell.

Although in the present case no distinctive properties have so far been observed when aryl glucosides are used as substrates, it was found that fractions 4 and 5 (Table 4) were the only ones that had cellulase activity as shown by a decrease in viscosity when incubated with 1% carboxymethylcellulose solution.

[10] J. CONCHIE and G. A. LEVVY, *Biochem. J.* **65**, 389 (1957).
[11] M. A. JERMYN, *Australian J. Biol. Sci.* **15**, 769 (1962).

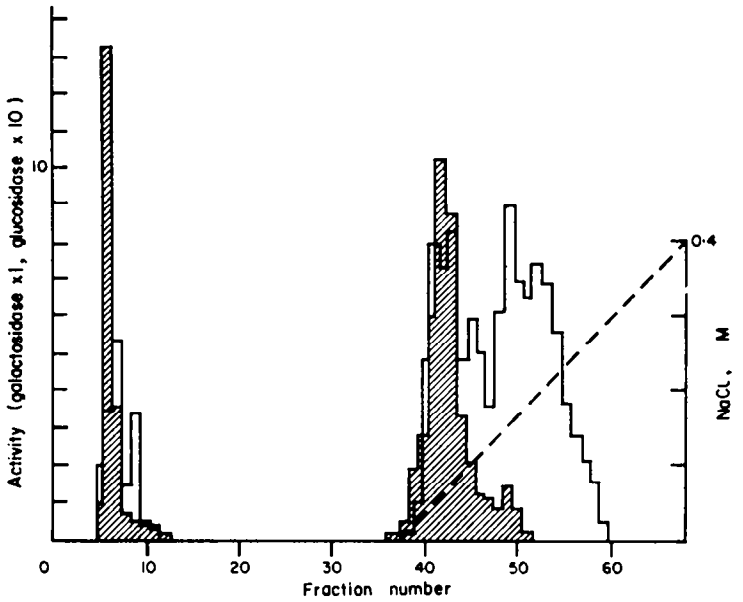

FIG. 2. DEAE-CELLULOSE CHROMATOGRAPHY OF TANNIN PREPARATION FROM ALFALFA FLOUR. The β-galactosidase is represented by the hatched area, β-glucosidase left unshaded. Enzyme activity is expressed as μmoles of 4-methylumbelliferone liberated/hr per ml of eluate.

A number of β-glucosidases occurring in ungerminated barley have been shown to have different substrate specificities.[12] Some hydrolases such as acid phosphatase and amylase are known to increase manyfold during germination, while the level of others remains fairly constant.[13] The rise in activity could be due to net enzyme synthesis, or activation of zymogens already present in the dormant seed.[14] The latent β-amylase of ungerminated wheat is readily released by proteolytic enzymes, or more simply by sulphite or thiol agents which break the disulphide bonds linking it to glutenin.[15] When alfalfa seeds were allowed to

TABLE 4. STEPWISE ELUTION OF β-GLUCOSIDASE COMPONENTS FROM DEAE-CELLULOSE BY CHLORIDE IN 0·01 M PHOSPHATE BUFFER pH 7·3

Peak	Cl concentration for elution (M)	Recovered activity* (%)	Estimated half life at 55°, pH 5·0 (min)
1	—	14·0	37
2	0·05	3·8	<5
3	0·15	24·0	16
4	0·20	27·2	∞
5	0·30	31·0	50

* Activity in that particular peak expressed as % of the total activity of the combined eluates.

[12] F. B. ANDERSON, W. L. CUNNINGHAM and D. J. MANNERS, *Biochem. J.* **90**, 30 (1964).
[13] J. L. YOUNG and J. E. VARNER, *Arch. Biochem. Biophys.* **84**, 71 (1959).
[14] H. J. PRESLEY and L. FOWDEN, *Phytochem.* **4**, 169 (1965).
[15] E. V. ROWSELL and L. J. GOOD, *Biochem. J.* **84**, 73P (1962).

germinate in the dark on wet filter paper at 20°, and groups of 100 seedlings taken for assay at 24-hr intervals, for four days, it was found that the total amount of either enzyme remained the same as that present in the dormant seed. Over this period a net enzyme synthesis is not stimulated, and if pre-formed enzyme is liberated from inactive precursors it must presumably take place in the first few hours. This may be the case, for the β-galactosidase activity of an ice-cold buffer extract (pH 6·5) of alfalfa flour was increased by incubation at 37° for 60 min before diluting for assay. Only 60 per cent of the total activity was available in a similar extract which had been diluted and assayed immediately. The presence of reduced glutathione (0·3% w/v) appeared to accelerate this reaction and maximum activity was reached in 15 min, but when glutathione was added to the pre-incubated extract no further increase in activity was observed, suggesting that the thiol is a releasing agent rather than a specific activator, and that pre-incubation allowed some natural releasing factor to act.

It may be that one function of these glycosidases is the reorganization of storage glycoprotein and structural polysaccharides as an early event in germination, following the uptake of water. In the presence of D-glucono-(1→5)-lactone germination was retarded while β-D-glucurono-(3,6)-lactone which was found not to be an inhibitor of these enzymes at 30 mM concentration had no effect (Table 5), and although galactonolactone did not produce a significantly lower degree of germination after three days, the inhibitory effect on the development of the seedlings was striking.

TABLE 5. GERMINATION IN THE PRESENCE OF GLYCOSIDASE INHIBITORS*

Time (hr)	Percentage germination			
	Water	0·5% Glucuronolactone	0·5% Galactonlactone	0·5% Gluconolactone
24	32	38	30	7
48	54	69	55	44
72	57	62	59	50

* 100 seeds germinated in the dark at 20° and treated daily with 1 ml water or inhibitor solution.

EXPERIMENTAL

Enzyme Preparations. Alfalfa seeds var. De Puits (Lidstones, Seeds Merchants, Slough) were pulverized in a ball mill, passed through a 30 mesh sieve and the flour de-fatted with diethyl ether in a soxhlet apparatus for 2 hr. The enzymes appeared to be stable under these conditions and the extraction produced a proportionate rise in the activity of the material by removing 10 per cent of the weight as fat.

(*a*) The dried de-fatted powder (30 g) was stirred in 300 ml 0·005 M disodium phosphate–citric acid buffer pH 7·0 for 1·5 hr at room temperature and centrifuged for 10 min at 1000*g*. The sediment was resuspended and extracted with a further 300 ml of buffer in the same way. The supernatants were combined (500 ml) and adjusted to pH 4·5 with 0·1 M sodium phosphate buffer, then warmed to 37° until precipitation occurred (3–5 min). The precipitate was centrifuged off, and the clear supernatant freeze-dried to yield 8·4 g of a golden yellow powder, which accounted for all the β-galactosidase activity of the original flour.

(*b*) Fractional precipitation with tannin gave a more active preparation. Alfalfa flour (100 g) was suspended in 450 ml distilled water containing 5 g $ZnSO_4$, and kept at 2° for 4 hr.

It was then filtered through muslin. Addition of 0·14 g tannin in 5 ml water gave a bulky precipitate with very low enzyme activity and this was centrifuged off. The clear supernatant was treated with a further 1·8 g tannin in 50 ml water, and the precipitate centrifuged. Further additions of tannin produced no more precipitate although much activity remained in solution.

The insoluble residue was shaken with several aliquots of cold acetone (4°) until the solution was no longer coloured and the residue gave no reaction to ferric chloride. It was then taken up in 100 ml of 0·02 M phosphate buffer pH 7·0 and kept for 1 hr at 37°, centrifuged, and the residue extracted twice more in a similar fashion. The combined extracts were freeze-dried to a pale yellow powder (1·20 g) containing 23 per cent of the original activity.

Further experiments showed that after the initial precipitation of inert material the quantity of tannin was not critical, serial additions producing small amounts of precipitate with the same specific activity, and in each case the maximum recovery was 25–30 per cent of the total activity.

Enzyme Assays. The activity towards phenyl, *p*-nitrophenyl, *o*-nitrophenyl, *p*-aminophenyl, and 4-methylumbelliferyl β-D-galactosides was measured as described by Furth and Robinson.[16] Hydrolysis of cellobiose and lactose was measured by the glucose oxidase method.[17] β-Glucosidase activity was measured with *p*-nitrophenyl β-D-glucoside[18] and 4-methylumbelliferyl β-D-glucoside[19] and *N*-acetyl-glucosaminidase by the *p*-nitrophenyl method.[20]

DEAE-Cellulose Chromatography. Whatman DEAE-cellulose powder DE 50 was suspended in 0·01 M phosphate buffer pH 7·3 and packed in a column (10 × 1 cm) under gravity. The sample (10–30 mg) was applied in the same buffer and eluted with 90 ml before introducing a sodium chloride gradient in buffer up to a final chloride concentration of 0·4 M. A constant head device gave a flow rate of 50–60 ml/hr and 3 ml fractions were collected for β-glucosidase and β-galactosidase assay. Similar conditions were used for stepwise elutions, the eluate from each step being assayed to ensure that it was substantially free from activity before the subsequent chloride solution was applied.

Acknowledgement—I wish to thank Mrs. Dagmar Vale for her valuable technical assistance.

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[17] A. Dahlqvist, *Acta Chem. Scand.* **14**, 1797 (1960).
[18] J. Conchie and G. A. Levvy, *Biochem. J.* **65**, 389 (1957).
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