Phytochemistry, Vol. 20, No. 7, pp. 1493–1496, 1981.
Printed in Great Britain.

0031–9422/81/071493–04 $02.00/0

α-GALACTOSIDASE FROM SWEET CHESTNUT SEEDS

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(*Revised received* 8 *December* 1980)

Key Word Index—*Castanea sativa*; Fagaceae; galactomannan; α-galactosidase; isoenzymes; raffinose; seed; stachyose.

Abstract—The major sugars of fresh seeds of *Castanea sativa* were shown to be raffinose, stachyose and sucrose. Drying seeds at 25° for 14 weeks increased the ratio raffinose:stachyose from 1.1 to 3.5, reduced sucrose content by *ca* 50% and decreased total extractable α-galactosidase. The enzyme activity was resolved into two peaks, a high MW form I (apparent MW 215 000) and a low MW form II (apparent MW 53 000). The latter form was predominant in the extract of fresh seeds whereas the former was the main form in the 14-week dried seeds. An increase in the amount of enzyme I was also observed when a buffered extract (pH 5.5) of fresh seeds was stored at 4°. Enzymes I and II had pH optima of 4.5 and 6, respectively. Both enzymes hydrolysed *p*-nitrophenyl α-D-galactoside at a much greater rate than the natural substrates raffinose, stachyose, locust bean gum and carob gum. However, enzyme I showed preference for stachyose as compared to raffinose; the opposite order was observed for enzyme II.

INTRODUCTION

α-D-Galactosyl derivatives of sucrose are of common occurrence in plant seeds [1]. Raffinose and stachyose which are mono- and digalactosyl sucrose derivatives, occur most abundantly and probably rank next to sucrose in their distribution [1]. Other α-D-galactosyl oligosaccharides and polysaccharides (e.g. galactomannans) have also been detected in seeds [1, 2]. These carbohydrate molecules accumulate in seeds during the process of maturation and serve as storage products in the resting seeds. However, during germination the oligosaccharides are the first to be mobilized followed by the polysaccharides [1–3]. D-Galactose, the initial product of the degradation process, is rapidly transformed and consumed through the glycolytic pathway, thus forming an important source of energy for the growing seedling [1].

The enzyme responsible for the primary attack on the α-D-galactosyl carbohydrates is α-galactosidase (α-D-galactosyl galactohydrolase, EC 3.2.1.22). Multiple molecular forms of this enzyme have been demonstrated and it has been shown that their concentrations change dramatically during maturation as well as during germination of seeds [4–7]. Differences in specificity of the enzyme forms towards their natural substrates have also been shown [1, 8, 9]. In this communication two forms of the enzyme have been shown to occur in sweet chestnut seeds and some of their properties studied.

RESULTS AND DISCUSSION

Oligosaccharides

Sucrose, raffinose and stachyose were the major ethanol-soluble oligosaccharides in mature and fresh seeds of *Castanea sativa*. Results presented in Table 1 show that the amount of raffinose was slighly higher than that of stachyose but sucrose was nearly 4.5-fold higher than the sum of the two oligosaccharides. The contents of sucrose and stachyose fell progressively during the drying period of the seeds but that of raffinose and also the raffinose/stachyose ratio increased in this period. Turnover of sucrose and galactosyl oligosaccharides is known to occur in seeds [1, 10]; however, in sweet chestnuts there was no detectable amount of galactose, glucose or fructose at any stage of drying. It is likely that the seed rapidly utilizes these components. Moreover, sucrose may act as a primer for the synthesis of raffinose and stactyose [1] in the seed, hence the absence of free glucose and fructose in the tissue.

α-Galactosidase

α-Galactosidase activity was present in the seeds at all stages of drying (Table 2) but the total activity and the specific activity of the enzyme decreased during this period. The data presented in Tables 1 and 2 show that α-galactosidase and its natural substrates coexist in the tissue. It is possible that the *in vivo* regulation of the levels

Table 1. Amounts of oligosaccharides in *Castanea sativa* seeds at different stages of drying*

Stage of drying† (weeks)	Amount of oligosaccharide (mg)/10 g seed‡			Ratio of raffinose/ stachyose
	Sucrose	Raffinose	Stachyose	
0	185	21.4	19.5	1.1
3	154	26.3	15.7	1.7
6	122	29.2	12.5	2.3
14	98	35.8	10.3	3.5

* The results represent mean values of experiments done in triplicate. The average variation was ±7%.

† Freshly picked mature seeds were allowed to dry at 25°.

‡ As fr. wt; see Experimental.

Table 2. α-Galactosidase activity from *Castanea sativa* seeds at different stages of drying*

Stage of drying (weeks)	Seed wt† (g)	Total α-galactosidase activity (units)	Specific activity (units/mg protein)
0	20.0	18.2	1.80
3	9.0	14.0	1.32
6	6.7	10.0	0.95
14	6.6	9.3	0.87

*The results represent mean values of experiments done in triplicate. The average variation was ±5%.

† Freshly picked mature seeds (20 g) were allowed to dry at 25°.

of α-galactosyl sugars is affected through compartmentation of the enzyme and/or its substrates. Some α-galactosidases have been shown to be glycoproteins that display lectin activity [11, 12]. One can thus envisage their specific binding to an appropriate site in the cell. On the other hand, binding with a specific sugar molecule may produce an inactive form of the enzyme and the activity may be modulated *in situ* by the process of association/dissociation of the complex. Isoenzyme forms that show specificity in their action on natural substrates may also play a role in determining the relative levels of substrates present in the seed (cf. refs. [3, 7]). Multiple forms of α-galactosidase were first reported by Petek and Dong [13] in coffee beans. Dey and Pridham later showed that the dormant seeds of several species possessed two forms of this enzyme [8, 14]. Gel filtration of the buffered extract of mature fresh seeds of *C. sativa* showed that the α-galactosidase activity could be resolved into two molecular forms, I and II. The low MW form II was the major fraction. The concentration of the high MW form I increases during the drying period of the seeds (Fig. 1). The ratios of II/I were 3.31, 1.62, 0.19 and 0.11 in the extracts of fresh seeds, 3-, 6- and 14-week dried mature seeds, respectively. A similar phenomenon was observed in *Vicia faba* seeds [7]. It is not certain whether enzyme II is being converted to I; the appearance of I and disappearance of II could be explained in a number of ways including *de novo* synthesis of I and proteolytic degradation of II. An interesting example of *in vitro* conversion of α-galactosidase II to I is that from coconut kernel [15, 16]. In this case an inactive protein fraction and K^+ ions were found necessary for the conversion to take place.

Gel filtration experiments further showed that if a cell-free extract of fresh mature seeds was stored at 4°, the amount of enzyme I increased with a concomitant decrease of II. From the elution patterns of the enzyme forms, the ratios of II/I were 3.31 for the fresh extract and 2.5, 1.9 and 1.0 for the 3-, 6- and 10-week-old extracts, respectively. This *in vitro* change in the ratio was also examined using extracts from 3- and 14-week dried seeds. In the former, the ratio changed from 1.6 to 1.0 on storage for 7 weeks, whereas in the latter there was no apparent change. It therefore seems that the relative proportions of the two forms of the enzyme not only depends on the length of storage time of the extract but also on the period of drying the seeds.

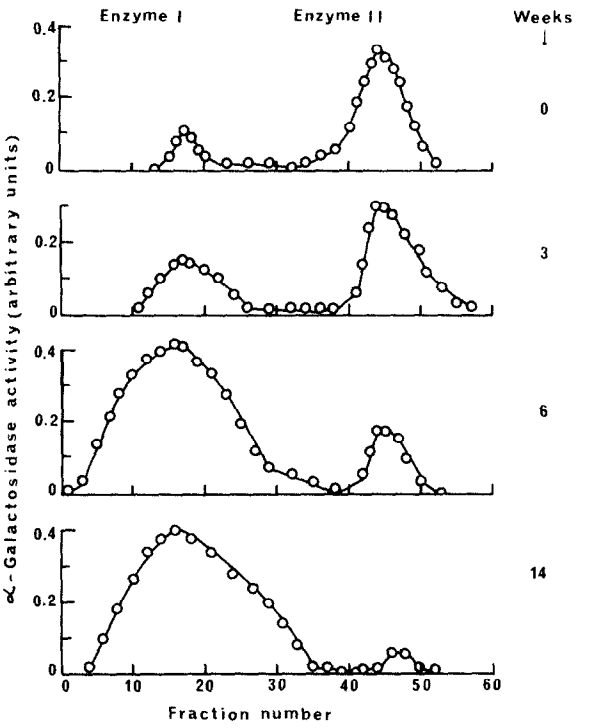

Fig. 1. Gel filtration on Sephadex G-200 of enzyme extracts from *Castanea sativa* seeds at different stages of drying (0, 3, 6 and 14 weeks). α-Galactosidase activity is resolved into two forms (I and II).

Enzyme properties

It was demonstrated in preliminary experiments that when an enzyme extract from the fresh seeds (high concentration of II) was incubated for 1 hr at 30° with a solution containing equimolar concentrations (0.05 M) of raffinose and stachyose, the former sugar was no longer present while the latter could still be detected in the digest. The reverse was observed when the enzyme extract from 14-week dried seeds were used (high concentration of I). However, the results should be considered with caution as one form of the enzyme could be inhibited by the second substrate which is present in the incubation mixture. Enzymes I and II were therefore separated by gel filtration and the relative rates of hydrolysis of some natural substrates were determined (Table 3). Results show that for both enzymes *p*-nitrophenyl α-D-galactoside is hydrolysed at a faster rate than any of the other substrates shown in Table 3. Enzyme I hydrolyses stachyose faster than raffinose, whereas the order was reversed for enzyme II. The lucerne and guar galactomannans which have higher galactose contents were resistant to the action of both enzymes. Such resistance is generally attributed to the structure of the galactomannan; the pattern of distribution of the α-D-galactosyl units along the mannan back-bone plays an important role [1, 2].

The pH profiles of α-galactosidase I and II are shown in Fig. 2. Whereas enzyme I showed a distinct pH optimum at 4.5, enzyme II displayed a broader range with slightly higher activity at pH 6. The relative activity for enzyme I at pH 7 was 64.3% compared to the value at its optimal pH, whereas it was 92.7% for enzyme II. The activity of

Table 3. Substrate specificity of α-galactosidase I and II of *Castanea sativa*

Substrates	Relative rates of substrate hydrolysis (%)*	
	α-Galactosidase I	α-Galactosidase II
p-Nitrophenyl α-D-galactoside	100	100
Raffinose	35	52
Stachyose	47	32
Lucerne gum (M/G, 1.0)†	Not hydrolysed	Not hydrolysed
Guar gum (M/G, 1.5)†	Not hydrolysed	Not hydrolysed
Locust bean gum (M/G, 3.1)†	8.5	3.6
Carob gum (M/G, 4.0)†	15.6	7.4

* Enzyme preparations of I and II were used which had the same specific activity when assayed with *p*-nitrophenyl α-D-galactoside as the substrate.

† M/G represents mannose/galactose ratios of the galactomannans used.

enzyme II showed only a small change in the entire pH range of 3.5–7.0. Several α-galactosidase preparations have been shown to display double pH optima [7], however, it is not known whether it is due to the presence of multiple forms of the enzyme in the preparation.

The Sephadex G-200 column that was used for separating the two forms of α-galactosidase from *C. sativa* extracts was calibrated using proteins of known MWs [17–19]. From the data the apparent MWs of enzymes I and II were 215000 and 53000, respectively. The estimations hold true only with the supposition that the enzyme proteins are globular in nature.

The activities of enzymes I and II remained intact for 3 months when they were stored at 4° in McIlvaine buffer containing thymol. However, there was total loss of activity on freezing and thawing the enzyme samples.

EXPERIMENTAL

Materials. All chemicals used were of analytical grade. Galactomannans were generously supplied by Dr. C. H. Hitchcock of Unilever Research (Bedford) and *p*-nitrophenyl α-D-galactoside was from Koch-Light (Bucks). Sephadex G-200 and the column were from Pharmacia (Uppsala). Sweet chestnut seeds were obtained locally and testa-free seeds were used.

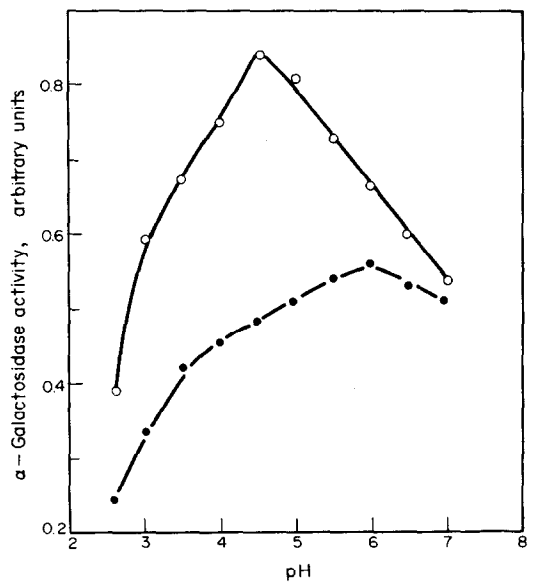

Fig. 2. The pH profiles of α-galactosidase I (○) and II (●) from *Castanea sativa* using *p*-nitrophenyl α-D-galactoside as the substrate.

Sugar extraction. The oligosaccharides were extracted by homogenizing the tissue sample in boiling 80% EtOH by the method described earlier [20, 21]. The sugars were separated by descending PC using H_2O-washed Whatman No. 3 paper and the solvent system *n*-PrOH–EtOAc–H_2O (7:1:2). *p*-Aminobenzoic acid reagent [22] was used for visualizing the sugars. Areas corresponding to standard sucrose, raffinose and stachyose were cut out of the developed chromatograms, eluted with H_2O and the sugars estimated by the PhOH–H_2SO_4 method [23]. The purity and the identity of the samples were also checked by HPLC; experimental conditions were as described earlier [21].

Enzyme isolation and assay. A weighed amount of tissue was finely macerated with pre-cooled McIlvaine buffer [24], pH 5.5 (ratio 1:2, w/v), the slurry was stirred for 1 hr at 4° and then centrifuged for 30 min at 10000 *g*. The supernatant was used as the enzyme extract. The enzyme activity was assayed by adding appropriately diluted enzyme soln to a mixture (final vol., 1 ml) containing McIlvaine buffer, pH 5.5 and 1 mM *p*-nitrophenyl α-D-galactoside held at 30°. The enzymic reaction was stopped after 15 min by adding 0.1 M Na_2CO_3 (5 ml) and the liberated *p*-nitrophenol was quantitated by measuring absorbance at 405 nm ($\varepsilon_{405} = 1.8 \times 10^4$ l./mol/cm). The reaction rate was linear as long as E_{405} did not exceed 0.6 (light path, 1 cm) [25]. The substrate concn used in the assay was not inhibitory to the enzyme activity; higher substrate concns are known to inhibit some α-galactosidases [26, 27]. A unit of enzyme was expressed as the amount of enzyme that liberates 1 μmol *p*-nitrophenol/min.

The hydrolyses of raffinose and stachyose were followed by measuring the reducing power [28] of the reaction mixture (1 ml) in McIlvaine buffer (at pH optimum) containing the substrate (5 mM) and appropriately diluted enzyme soln. Assay using galactomannan was carried out in a similar manner using a final substrate concn of 2.5%.

Protein was determined by the method of ref. [29] using BSA as standard.

Sephadex G-200 column (2.5 × 80 cm) was prepared according to ref. [17] and equilibrated with McIlvaine buffer, pH 5.5, containing 0.1 M KCl. It was eluted with a downward flow of 30 ml/hr and 3 ml fractions were collected. Enzyme prepn (3 ml) was passed through the column and the fractions collected, after the void vol. (185 ml), were assayed for α-galactosidase activity. The active peaks of enzymes I and II were pooled separately and concd by ultrafiltration (Diaflo PM10 membrane; Amicon, Lexington, U.S.A.). The ratio of II/I was calculated from the total

enzyme activities of the concentrates. Gel filtration gave *ca* 80% recovery of enzyme activity.

Acknowledgements—The author is grateful to Professors J. B. Pridham and H. Kauss for their support and help. Thanks are also due to Miss N. Bandali, Mr. G. D. Key and Miss S. M. Walters for assistance and cooperation. Part of this work was carried out in the Department of Biology, Kaiserslautern University, 6750 Kaiserslautern, West Germany, and was supported by a fellowship from Alexander von Humboldt-Stiftung.

REFERENCES

1. Dey, P. M. (1980) *Adv. Carbohydr. Chem. Biochem.* **37**, 283.
2. Dey, P. M. (1978) *Adv. Carbohydr. Chem. Biochem.* **35**, 341.
3. Pridham, J. B., Walter, M. W. and Worth, H. G. J. (1969) *J. Exp. Botany* **20**, 317.
4. Dey, P. M., Khaleque, A. and Pridham, J. B. (1971) *Biochem. J.* **124**, 27P.
5. Dey, P. M., Khaleque, A., Palan, P. R. and Pridham, J. B. (1973) *Biochem. Soc. Trans.* **3**, 661.
6. Desai, N. N., Dey, P. M. and Pridham, J. B. (1974) *Biochem. Soc. Trans.* **2**, 1132.
7. Pridham, J. B. and Dey, P. M. (1974) *Plant Carbohydrate Biochemistry* (Pridham, J. B., ed.) p. 83. Academic Press, London.
8. Dey, P. M. and Pridham, J. B. (1972) *Adv. Enzymol.* **36**, 91.
9. Williams, J., Villarroya, H. and Petek, F. (1977) *Biochem. J.* **161**, 509.
10. Bewley, J. D. and Black, M. (1978) *Physiology and Biochemistry of Seeds*, Vol. 1. Springer, Berlin.
11. Hankins, C. N. and Shannon, L. M. (1978) *J. Biol. Chem.* **253**, 7791.
12. Sumar, N., Dey, P. M. and Pridham, J. B. Unpublished results.
13. Petek, F. and Dong, T. (1961) *Enzymologia* **23**, 133.
14. Barham, D., Dey, P. M., Griffiths, D. and Pridham, J. B. (1971) *Phytochemistry* **10**, 1759.
15. Balasubramamiam, K., Dey, P. M. and Pridham, J. B. (1974) *Biochem. Soc. Trans.* **2**, 1128.
16. Balasubramamiam, K., Dey, P. M. and Pridham, J. B. (1976) *Phytochemistry* **15**, 1445.
17. Andrews, P. (1964) *Biochem. J.* **91**, 222.
18. Malhotra, O. P. and Dey, P. M. (1967) *Biochem. J.* **103**, 508.
19. Dey, P. M. and Pridham, J. B. (1969) *Biochem. J.* **113**, 49.
20. Dey, P. M. (1980) *FEBS Letters* **114**, 153.
21. Dey, P. M. and Kauss, H. (1981) *Phytochemistry* **20**, 45.
22. Menzies, I. S., Mount, J. N. and Wheeler, M. J. (1978) *Ann. Clin. Biochem.* **15**, 65.
23. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, D. A. and Smith, F. (1965) *Analyt. Chem.* **28**, 350.
24. McIlvaine, T. C. (1921) *J. Biol. Chem.* **49**, 183.
25. Dey, P. M. and Wallenfels, K. (1974) *Eur. J. Biochem.* **50**, 107.
26. Malhotra, O. P. and Dey, P. M. (1967) *Biochem. J.* **103**, 739.
27. Dey, P. M. and Pridham, J. B. (1969) *Biochem. J.* **115**, 47.
28. Nelson, N. (1944) *J. Biol. Chem.* **153**, 375.
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.