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STUDIES ON THE GLUCOSIDASE "LINAMARASE"*

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Abstract—From infra-red spectra it was confirmed that the cyanogenic glucosides linamarin and lotaustralin are β-linked. A survey of the β-glucosidase activity of enzyme extracts from a variety of higher plants revealed that the ability to hydrolyse linamarin and lotaustralin was very limited. In one species, white clover, genotypes segregating for linamarase (linamarin glucohydrolase) showed a clear-cut difference in their complement of β-glucosidases. Extracts from linseed were fractionated for β-glucosidase using DEAE-chromatography, gel diffusion and column electrophoresis techniques. Column electrophoresis proved particularly successful, allowing 105-fold purification of linamarase in one step. The substrate specificity of purified linseed linamarase was examined for a number of glycosides; a moderate degree of specificity was observed for the aglycone moiety. It was confirmed that hydrolysis of linamarin and lotaustralin by emulsin proceeded at a very slow rate.

INTRODUCTION

A VARIETY of plant species, including linseed flax *Linum usitatissimum* L. and white clover *Trifolium repens* L., contain two cyanogenic glucosides linamarin and lotaustralin, the aglycones of which are the cyanohydrins of acetone and methyl ethyl ketone respectively.[1–3] Crude extracts of tissues containing these glucosides readily hydrolyse both glucosides and the term linamarase (linamarin glucohydrolase) has been applied to the enzyme responsible for the hydrolysis.[4,5]

Conflicting results have been obtained in studies of the substrate specificity of linamarase. Marais and Rimington[4] reported that a crude linamarase preparation from *Dimorphotheca cuneata* Less. did not hydrolyse salicin but slowly hydrolysed amygdalin. On the other hand, Coop[5] reported that preparations of linamarase from both linseed and white clover brought about rapid hydrolysis of salicin and slight hydrolysis of amygdalin. It is also of interest that both Finnemore and Cooper,[6] and Coop[5] observed that almond emulsin, the classical source of β-glucosidase (β-D-glucoside glucohydrolase), possessed very weak hydrolytic action against both linamarin and lotaustralin.

One possible explanation of these results is that linamarase is highly specific for linamarin and lotaustralin, at least in some species, and that action on other glucosides is due to the presence of other β-glucosidases. A second, less likely, hypothesis is that linamarin and lotaustralin are α-glycosides. We have re-examined the question in the following ways: (1) The configuration of linamarin and lotaustralin has been investigated by infra-red studies.

[1] G. DILLEMAN, *Encyclopedia of Plant Physiology* **8**, 1050, Springer Verlag, Berlin (1958).
[2] W. KARRER, *Konstitution und Vorkommen der organischen Pflanzenstoffe*, Birkhauser Verlag, Basle (1958).
[3] G. W. BUTLER, *Phytochem.* **4**, 127 (1965).
[4] J. S. C. MARAIS and C. RIMINGTON, *Onderstepoort J. Vet. Sci.* **3**, 111 (1934).
[5] I. E. COOP, *N.Z. J. Sci. Tech.* **22B**, 71 (1940).
[6] H. FINNEMORE and J. M. COOPER, *J. Soc. Chem. Ind.* **57**, 162 (1938).

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(2) The β-glucosidase activities of a selection of unpurified enzyme extracts from plants and other sources have been compared against lotaustralin, linamarin and various other plant glucosides, using paper chromatographic techniques for the detection of liberated glucose. Variation in β-glucosidases in white clover genotypes segregating for linamarase was also examined. (3) Linamarase from linseed has been purified by a factor of 500 over the activity in the initial extract and tests have been made of substrate specificity. (4) The action of almond emulsin on linamarin and lotaustralin has been reinvestigated.

RESULTS

Infra-red spectra of linamarin and lotaustralin

The infra-red spectrum over the range 800–950 cm^{-1} of a sample of mixed linamarin-lotaustralin, isolated from white clover, showed no sign of a peak in the region 830–855 cm^{-1} but a definite peak at 880–890 cm^{-1}. The spectrum of a specimen of pure synthetic linamarin likewise showed no sign of a peak in the region 830–855 cm^{-1} but two definite peaks were observed at 870 and 885–890 cm^{-1} respectively. A control spectrum of α-methyl-D-glucopyranoside showed strong peaks at 845 and 900 cm^{-1}, while a similar control spectrum of β-methyl-D-glucopyranoside showed no peak in the region 830–855 cm^{-1} but a strong peak at 885 cm^{-1}.

β-Glucosidase activity of enzyme extracts

A survey of β-glucosidase activities of unpurified enzyme extracts from various sources against a variety of β-glucosides is presented in Table 1. It was possible to divide the enzyme digests into two classes: (a) those in which no liberation of glucose could be detected, and (b) those in which there was strong liberation of glucose and most of the substrate was hydrolysed.

Appropriate controls showed that none of the preparations liberated any glucose when incubated alone. Within the group of substrates salicin, arbutin, amygdalin and cellobiose, inspection of the intensities of the liberated glucose spots suggested some variations in the rates of hydrolysis of these compounds. In no case was there any sign of intermediate gentiobiose in the amygdalin digests.

β-Glucosidase activity of white clover genotypes

The inheritance of cyanogenesis in white clover has been shown[7,8] to be governed by the interaction of simple dominant genes for the formation of the enzyme linamarase and for the cyanoglucosides linamarin and lotaustralin. These genes are not linked. The presence or absence of linamarase was assessed in previous studies by a modification of the Guignard picrate-paper method.[7] In the present work the more sensitive glucose oxidase assay procedure was used to examine the β-glucosidase activity against salicin and linamarin of unpurified extracts prepared from white clover genotypes which had been assessed to contain or to lack linamarase by the picrate-paper method. (L^+, L^-).

It was found that whereas an extract prepared from leaves of four L^+ genotypes gave a linamarin/salicin activity ratio of 1·24, the corresponding ratio for an extract from herbage of four L^- genotypes was 0·24. In other experiments, separate extracts were prepared from four L^+ genotypes; the ratios were 0·25, 0·20, 0·16 and 0·18 respectively.

[7] L. CORKILL, *N.Z. J. Sci. Tech.* **23B**, 178 (1942).
[8] S. S. ATWOOD and J. T. SULLIVAN, *J. Heredity* **34**, 311 (1943).

TABLE 1. β-GLUCOSIDASE ACTIVITY OF ENZYME EXTRACTS

Source of enzyme extract	Substrate: Linamarin/ Lotaustralin	Substrate: Salicin, arbutin, amygdalin, cellobiose
Higher plants		
Trifolium repens L. N.Z. certified white clover leaves	+	+
Linum usitatissimum L. Linseed flax shoots	+	+
Almond emulsin	+	+
Photinea serrulata Lindl. Leaves	−	+
Sambucus nigra L. Elder leaves	−	+
Trifolium pratense L. Red clover leaves	−	+
Triticum vulgare L. Sprouted wheat	−	+
Vicia faba L. Sprouted broad beans	−	+
Conium maculatum L. Hemlock leaves	−	+
Populus alba L. Poplar shoots	−	+
Micro-organisms		
Rumen protozoa (mixed bovine)	+	+
Pithomyces chartarum (Berk and Curt) M. B. Ellis. Syn. *Sporidesmium bakeri* (Syd.)	+	+

Conditions are given in experimental section.
+ Strong hydrolysis, intense glucose spot on chromatogram.
− Little or no hydrolysis, glucose not detectable on chromatogram.

The presence of at least two β-glucosidases in New Zealand certified white clover leaves was also shown by mixed substrate tests,[9] using linamarin and salicin (Table 2). If two enzymes are involved, the rate of hydrolysis of the mixed glucosides should exceed that of either glucoside alone. It will be seen that this was the case.

Purification of linseed linamarase

The procedure of Coop[5] was employed for extraction and preliminary purification of linamarase from linseed meal. This extract is termed "crude linamarase" hereafter. Several techniques were employed for obtaining purification:

(1) Using DEAE-cellulose columns, crude linamarase extracts were applied in 0·005 M citrate buffer, pH 6·0, and the buffer concentration was raised stepwise to 0·1 M and 0·2 M. Assays were made on the eluted fractions for ability to hydrolyse linamarin, salicin and

[9] M. DIXON and E. C. WEBB, *Enzymes*, 1st Ed. p. 93. Academic Press, New York. p. 782 (1958).

Table 2. Mixed substrate test on unpurified extract of white clover herbage

Substrate	Rate of hydrolysis* (mμmoles glucose released per hr)
Salicin (1·75 μmole)	35·5
Linamarin (2·00 μmole)	38·3
Salicin (1·75 μmole)+Linamarin (2·00 μmole)	54·4

* Incubation mixture included 15 μmoles potassium phosphate buffer, pH 6·0, and 50 μl enzyme extract (0·66 mg protein) in a total volume of 1·20 ml. Samples were incubated at 27° and liberated glucose was measured by the Nelson method at 0, 4, 9 and 20 hr.

amygdalin. Figure 1 shows the results of one experiment, where it will be seen that a slight activity against amygdalin was separated from activities against linamarin and salicin, with the latter two not being resolved. When an aliquot of the fraction active against amygdalin was tested for cellobiase activity, using the technique employed for compilation of Table 1,

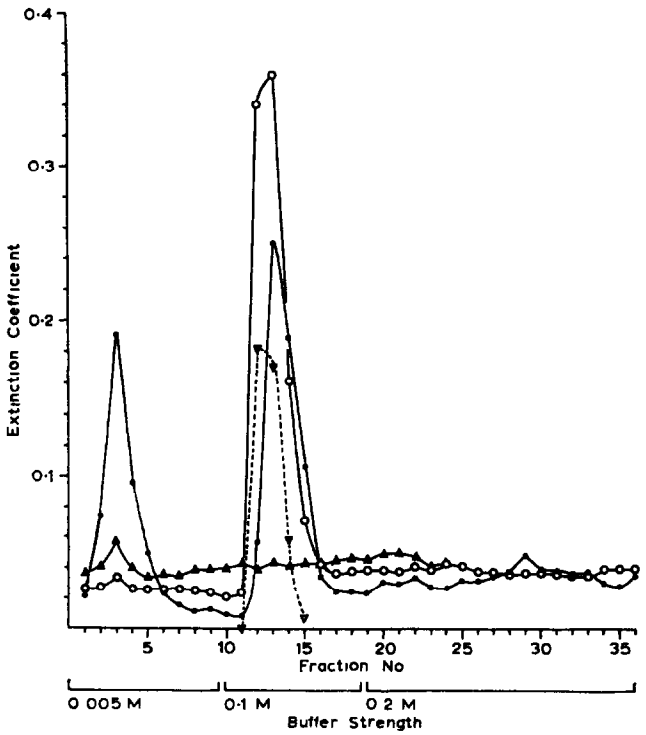

Fig. 1. DEAE-cellulose chromatography of crude linseed linamarase.

Stepwise elution was performed with 0·005 M, 0·1 M, 0·2 M phosphate buffer (pH 6·0) was indicated. Symbols: ●, protein, E_{280}; ○, linamarase activity; ▲, activity against amygdalin; ▽, activity against salicin. β-Glucosidase activities were measured by the glucose oxidase method on 200 μl aliquots.

a positive result was obtained. Thus β-disaccharase activity was separated from β-glucosidase activity by this procedure.

(2) Purification on Sephadex columns by the gel filtration technique[10] was also investigated. Results of a typical experiment are shown in Fig. 2, from which it will be seen that β-disaccharase activity against amygdalin and cellobiose was again separated from β-glucosidase activity against linamarin and salicin.

Crude linamarase contained appreciable concentrations of polysaccharides, and the linamarase fractions from both the DEAE-cellulose and Sephadex columns were still heavily contaminated with these substances.

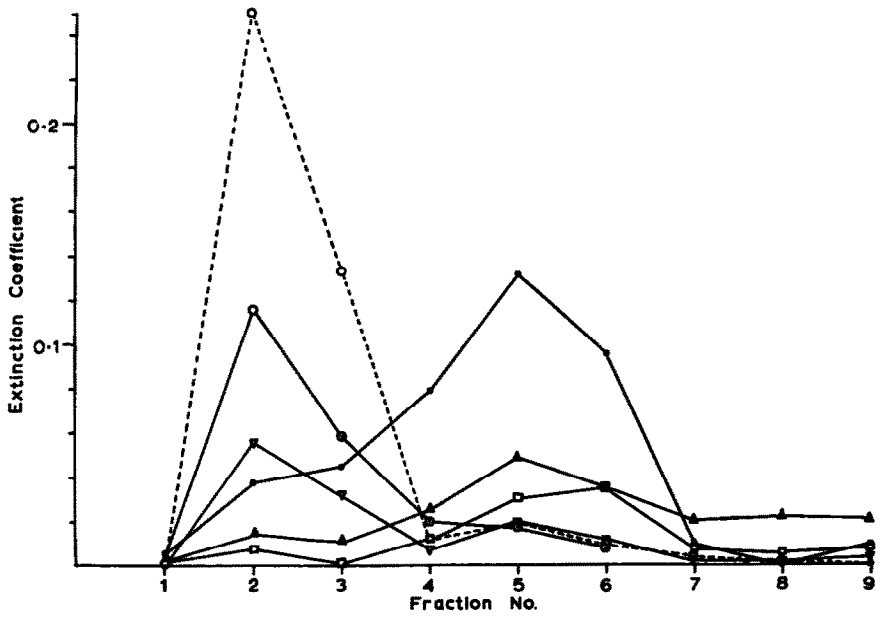

FIG. 2. FRACTIONATION OF CRUDE LINSEED LINAMARASE BY GEL DIFFUSION ON SEPHADEX G-200.
Symbols: ●, protein, E_{280}; ○, linamarase activity; ▽, activity against salicin; ▲, activity against amygdalin; □, cellobiase activity; ⊙, polysaccharide (anthrone method). β-Glucosidase activities were measured by the glucose oxidase method on 200 μl aliquots.

(3) The most successful technique for linamarase purification was column electrophoresis on Sephadex G25 as described by Loontiens and de Bruyne.[11] Electrophoresis of crude linamarase was carried out for 40 hr in 0·1 M sodium acetate buffer, pH 5·0, after which the column was eluted with acetate buffer. It was found that linamarase migrated faster towards the anode than the main protein band. A 105-fold purification from protein was achieved, with recovery of 60 per cent of the activity applied. Also the linamarase band contained negligible polysaccharide. No advantage accrued from preliminary purification by the DEAE-cellulose and Sephadex column techniques described above.

Attempts to purify clover linamarase by the techniques described above for linseed linamarase have not been successful. Difficulties appeared to arise from aggregation of leaf

[10] P. FLODIN, *Dissertation, Uppsala*, 85 pp. (1962).
[11] F. G. LOONTIENS and C. K. DE BRUYNE, *Naturwissenschaften* **19**, 614 (1963).

proteins after extraction;[12] also linamarase activities of clover leaf extracts were much lower than those of linseed extracts and the enzyme was less stable.

Substrate specificity of purified linseed linamarase

The substrate specificity of linseed linamarase purified by column electrophoresis was examined. The relative activities against salicin and linamarin of successive fractions eluted from the column are shown in Fig. 3; the results for salicin have been multiplied by a factor of 2·33 to emphasize the close match of the curves.

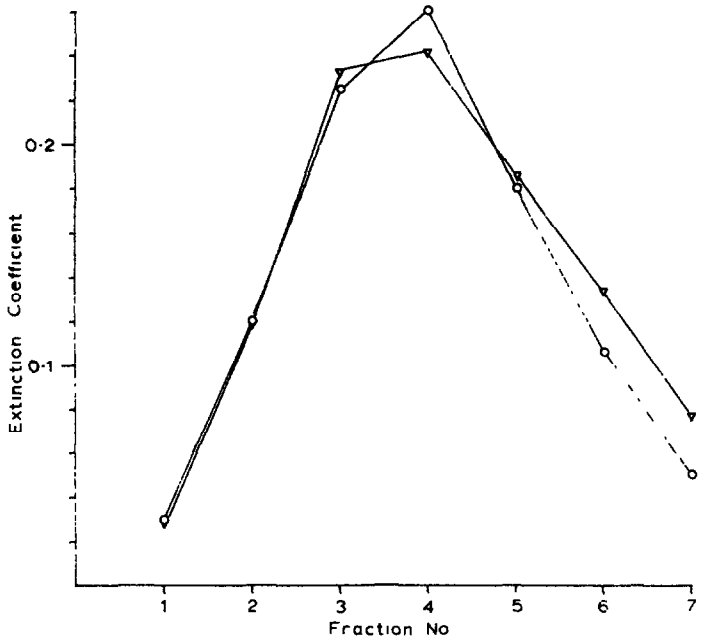

FIG. 3. ACTIVITIES AGAINST SALICIN AND LINAMARIN/LOTAUSTRALIN OF FRACTIONS FROM COLUMN ELECTROPHORESIS.

Symbols: ○, linamarase activity; ▽, activity against salicin, adjusted by a factor of 2·33. The subsequent 28 fractions contained 99 per cent of the protein. β-Glucosidase activities were measured by the glucose oxidase method on 200 μl aliquots.

These results are consistent with the presence in the purified extract of only one β-glucosidase capable of hydrolysing both salicin and linamarin. Another result which supports this conclusion was the finding that the same ratio of linamarin/salicin activities of 2·33 was observed with different purified linamarase preparations from the same batch of linseed meal. On the other hand, variation in the ratios of linamarin/salicin activities was observed in preparations of crude linamarase and in partially purified fractions obtained by the use of DEAE-cellulose or Sephadex (Figs. 1 and 2), indicating the presence of more than one β-glucosidase at these stages of purification.

The activity of purified linseed linamarase against five other glucosides is shown in Table 3. Of particular interest is the contrast between the activity against prunasin (D-mandelo-nitrile-β-D-glucoside) and amygdalin (D-mandelonitrile-β-D-gentiobioside).

[12] M. A. STAHMANN, *Ann. Rev. Plant Physiol.* **14**, 145 (1963).

TABLE 3. HYDROLYSIS OF GLUCOSIDES BY PURIFIED LINSEED LINAMARASE

Glucoside	% hydrolysis*
Linamarin	76·2
Prunasin	51·6
Amygdalin	0·0
Phloridzin	30·0
Arbutin	34·2
Salicin	32·7
β-Methylglucoside	1·1

* Incubation mixture contained 0·36 μg protein, 75 μmoles potassium phosphate buffer at pH 5·7 and 1 μmole glucoside in a total volume of 0·75 ml. Mixture was incubated for 4 hr at 30° and liberated glucose was measured by the Nelson method.

The activity of the purified enzyme was also tested against two isomeric cyanoglucosides, dhurrin (L-*p*-hydroxymandelonitrile-β-D-glucoside) and taxiphyllin (D-*p*-hydroxymandelonitrile-β-D-glucoside).[13] Liberation of *p*-hydroxybenzaldehyde was followed spectrophotometrically[14] and Fig. 4 shows results of a typical experiment. Dhurrin was hydrolysed at one-tenth of the rate of taxiphyllin.

The activity of crude linamarase against dhurrin and taxiphyllin is also shown in Fig. 4. The reason for the lag phase observed here was not elucidated. In contrast to the results with purified linamarase, dhurrin and taxiphyllin were hydrolysed at the same rate. Hence two β-glucosidases, separable by column electrophoresis, are present in crude linamarase each of which acted preferentially against one of the isomeric cyanoglucosides.

Action of emulsin on lotaustralin and linamarin

In Fig. 5 results are given for the hydrolysis of a mixture of linamarin and lotaustralin by emulsin. It will be seen that appreciable hydrolysis of the cyanoglucosides occurred, but markedly lower amounts of hydrogen cyanide than glucose were found. Checks established that this disparity was not due to methodological error. Furthermore the disparity between glucose and hydrogen cyanide levels was not observed when the hydrolysis of linamarin/lotaustralin by crude linamarase was measured under the same experimental conditions.

DISCUSSION

β-Linkage of the glucose in linamarin, and presumably lotaustralin, is based largely on the original synthesis[15] and low α_D value, −29°. Infra-red studies confirm the presence of β-links for the following reasons:

(1) All compounds containing α-linked glucose so far listed give infra-red spectra with a characteristic peak at 845 ± 9 cm^{-1}; the absence of this peak is considered good evidence for

[13] G. H. N. TOWERS, A. G. MCINNES and A. C. NEISH, *Tetrahedron* **20**, 71 (1964).
[14] C. BOVÉ and E. E. CONN, *J. Biol. Chem.* **236**, 207 (1961).
[15] E. FISCHER and G. ANGER, *Ber.* **52**, 854 (1919).

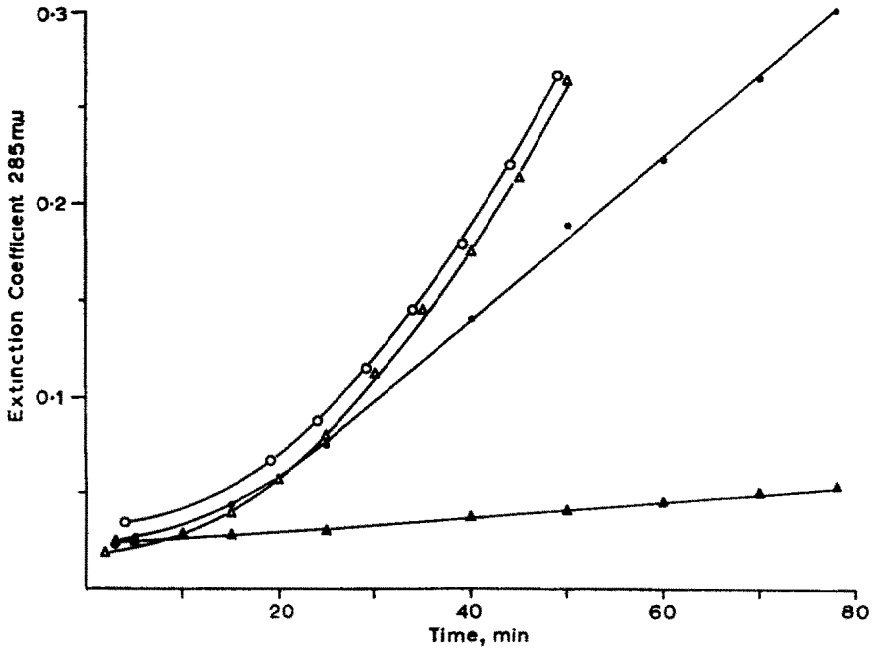

FIG. 4. HYDROLYSIS OF TAXIPHYLLIN AND DHURRIN BY CRUDE AND PURIFIED LINSEED LINAMARASE.
Symbols: ○, taxiphyllin, crude enzyme; ▽, dhurrin, crude enzyme; ●, taxiphyllin, purified enzyme; ▲, dhurrin, purified enzyme.
Digest contained in a total volume of 2·25 ml, 90 μmoles potassium phosphate buffer, pH 6·0, and 3 μmoles substrate, together with 1·08 μg protein (for purified linamarase) or 114 μg protein (for crude linamarase). Incubation temperature, 30°.

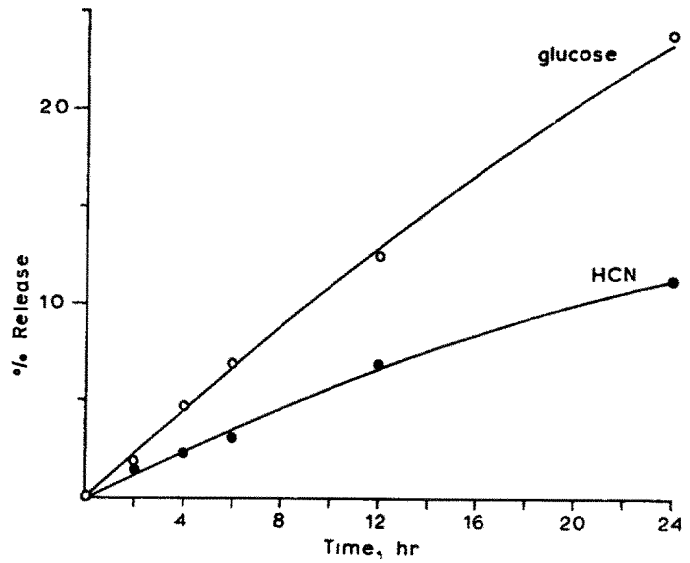

FIG. 5. HYDROLYSIS OF LINAMARIN/LOTAUSTRALIN BY EMULSIN.
For conditions see experimental section.

the presence of only β-links. This peak was absent from the spectra of linamarin and lotaustralin and β-methyl glucoside used as a reference.

(2) β-Linked glucose also gives a peak at 891 ± 7 cm^{-1}. Since a few α-linked compounds (e.g. cyclomaltoheptaose) give a broad peak in this region,[16] this peak does not provide conclusive evidence for β-linkage. Nevertheless the presence of a strong peak in this region of the spectra of linamarin and lotaustralin also supports the presence of a β-link in the cyanoglucosides.

The survey of β-glucosidase activity in unpurified extracts from different plants (Table 1) shows that, although the extracts were all rich in β-glucosidase activity so far as other glucosides were concerned, the ability to hydrolyse linamarin and lotaustralin is restricted to certain species. Strong hydrolysis of linamarin and lotaustralin was observed in extracts from linseed flax and white clover, two species which contain these glycosides. Weaker hydrolysis of linamarin/lotaustralin was observed with almond emulsin (prepared from another cyanoglucoside containing tissue), but no hydrolysis was detected with unpurified extracts from *Sambucus nigra* (which contains sambunigrin, L(+)-mandelonitrile-β-D-glucoside[2]) or *Photinea serrulata* (which contains prunasin, D(−)-mandelonitrile-β-D-glucoside). A random selection of 5 species known not to contain cyanoglucosides yielded extracts which did not hydrolyse linamarin/lotaustralin. These differences between plant extracts did not apply to non-plant β-glucosidases. Thus extracts from protozoa and a fungus hydrolysed all of the glucosides; an extract from a bacterial species, *Leuconostoc mesenteroides* has also been found to hydrolyse these glucosides.[17]

The presence of a specific β-glucosidase for the hydrolysis of linamarin and lotaustralin in white clover and linseed flax was thus indicated. Examination of unpurified extracts from white clover genotypes segregating for linamarase lent support to the hypothesis that more than one β-glucosidase was present in these tissues.

In the case of linseed linamarase, it was possible to make a more detailed assessment of the β-glucosidases present and to examine the substrate specificity of purified linamarase. Thus the separations of crude linamarase carried out on DEAE-cellulose (Fig. 1) and Sephadex (Fig. 2) columns both yielded two fractions, one of which hydrolysed linamarin and salicin and the other amygdalin and cellobiose. This represented a separation of β-glucosidase from β-disaccharase activity. Evidence from partial fractionation of extracts of barley[18] and *Aspergillus niger*[19] suggested the presence in these extracts of separate enzymes hydrolysing β-diglucosides and phenolic glycosides respectively. Results from Hutson's study[20] of β-glucosidase activity in alfalfa (*Medicago sativa*) seed are also indicative of the hydrolysis of these two classes of β-glucosides by separate enzymes. The present work, however, appears to be the first clear-cut separation of β-disaccharase activity from the enzymes hydrolysing β-monoglucosyl-aglycone glucosides. The question of whether activity towards gentiobiose in the case of amygdalin was distinct from cellobiase was not further investigated.

Linamarase preparations purified a further 105-fold by column electrophoresis retained activity against salicin; the close match of relative activities against the two substrates of successive fractions eluted from the column left little doubt that only one β-glucosidase was

[16] S. A. BARKER, E. J. BOURNE, M. STACEY and D. H. WHIFFEN, *J. Chem. Soc.* **171** (1954).
[17] R. W. BAILEY and E. J. BOURNE, *Nature, Lond.* **191**, 277 (1961).
[18] F. B. ANDERSON, W. L. CUNNINGHAM and D. J. MANNERS, *Biochem. J.* **90**, 30 (1964).
[19] C. R. KRISHNA-MURTI and B. A. STONE, *Biochem. J.* **78**, 715 (1961).
[20] D. H. HUTSON, *Biochem. J.* **92**, 142 (1964).

involved (Fig. 3). Purified linseed linamarase exhibited appreciable activity against two other cyanoglucosides, prunasin and taxiphyllin, which contain D-mandelonitrile and D-*p*-hydroxymandelonitrile aglycones respectively (Table 2, Fig. 4). Dhurrin, (L-*p*-hydroxymandelonitrile aglycone) was hydrolysed at one-tenth the rate of taxiphyllin; an additional β-glucosidase capable of hydrolysing dhurrin more rapidly was present in crude extracts. Activity against arbutin was of the same order as that against salicin, which is consistent with structural similarities. The lack of activity against phloridzin is readily appreciated on steric grounds, because of the bulky substitution of the aglycone in the ortho-position, and the low activity against β-methylglucoside may well be due to unsuitable conformation because of the small aglycone. Lack of activity against amygdalin confirms that the enzyme has no gentiobiase activity.

Linseed linamarase as purified by these methods is thus a β-glucosidase with a moderate degree of aglycone specificity, which can best be envisaged as arising from steric relationships in the vicinity of the active centre. β-Glucosidase activity of linseed linamarase is clearly not dependent on the presence of a nitrile group in the aglycone. Factors controlling the specificity of β-glucosidases have been fully discussed elsewhere.[21, 22, 23]

The overall purification of linseed linamarase achieved in this study was approximately 500-fold: this compares favourably with that obtained for other plant glucosidases.[24, 25, 26] The column electrophoretic technique employed did not permit the preparation of sufficient purified enzyme for a study of its composition and physico-chemical properties. Increasing the scale of the purification procedure is under investigation.

Earlier reports[5, 6] of the weak hydrolytic action of emulsin against linamarin/lotaustralin were confirmed in this study (Table 1, Fig. 5). It should be emphasized that in order to demonstrate this it was necessary to use a relatively large amount of a purified emulsin preparation with very high activity against salicin (β-glucosidase value,[22] 10–12). The rate of hydrolysis of salicin under these experimental conditions would have been approximately 1300 times greater than observed here for linamarin/lotaustralin. Also, whereas in Table 3 76·2% linamarin/lotaustralin was hydrolysed by linamarase containing 0·36 μg protein in 4 hr, it will be seen from Fig. 5 that only 4·8% of these cyanoglucosides were hydrolysed by 4 mg purified emulsin over the same period (although substrate concentrations were 1·33 mM and 26·6 mM respectively). The lower recovery of hydrogen cyanide than of glucose may be due to enzymatic decomposition of the hydrogen cyanide, e.g. by the action of a nitrilase.[27] This is being further investigated.

EXPERIMENTAL

Glucosides

Linamarin and lotaustralin were used as a mixture of approximately equivalent proportions, isolated from white clover by the second method of Melville and Fraser.[28] Coop[5] showed that both white clover and linseed flax linamarases readily hydrolysed both of these

[21] A. GOTTSCHALK, *Adv. Carbohydrate Chem.* **5**, 49 (1950).
[22] S. WEIBEL, *The Enzymes I*, Part 1, p. 583 (Edited by J. B. SUMNER, K. MYRBACK). Academic Press (1950).
[23] M. A. JERMYN, *Rev. Pure & Appl. Chem. (Australia)* **11**, 92 (1961).
[24] L. ZECHMEISTER and M. ROHDEWALD, *Fortschr. Chem. Org. Naturst.* **8**, 341 (1951).
[25] B. HELFERICH and T. KLEINSCHMIDT, *Z. Physiol. Chem.* **334**, 60 (1963).
[26] F. J. JOUBERT, *Arch. Biochem. Biophys.* **91**, 11 (1960).
[27] K. V. THIMANN and S. MAHADEVAN, *Arch. Biochem. Biophys.* **105**, 133 (1964).
[28] J. MELVILLE and J. G. FRASER, *N.Z. J. Sci. Tech.* **33A**, 56 (1951).

glucosides. Synthetic linamarin used in the infra-red studies was a gift from the Division of Plant Industry, C.S.I.R.O., Canberra. Prunasin, isolated from peach leaves, was a gift of Dr E. E. Conn, Department of Biochemistry and Biophysics, University of California, Davis; taxiphyllin, isolated from *Taxus canadensis* Marsh, was a gift of Dr G. H. Towers, Atlantic Regional Laboratory, Halifax; and dhurrin, isolated from *Sorghum vulgare*, was a gift of Dr L. Anderson, Biochemistry Department, University of Wisconsin. β-Methyl glucoside was purchased from Bios Laboratories, Inc.; other glucosides used were obtained from commercial sources.

Infra-red studies

Samples were examined as paraffin mulls using a Perkin Elmer Model 21 spectrophotometer.

Enzyme extracts

For studies of β-glucosidase activity in plant tissues other than linseed meal, the tissue (25 g wet wt.) was ground, in an end-runner mill, at 2° in citrate buffer (50 ml, 0·1 M, pH 6·0) and, after filtering and centrifuging, the extract was dialysed, for 16 hr at 2°, against two changes of 400 ml citrate buffer and finally distilled water. Rumen protozoa were isolated from fresh rumen contents by differential centrifuging, ground in citrate buffer with Ballotini beads and the extract centrifuged and dialysed as before. *Pithomyces chartarum* mycelia, from glucose agar or ryecorn cultures,[29] were disrupted in a Hughes press, extracted with citrate buffer and centrifuged and dialysed as above. Almond emulsin (purified by the methods of Helferich and co-workers, as quoted by Weibel,[22] to a β-glucosidase value of 10–12) was obtained from Seravac Ltd. (Colnbrook, Bucks., England).

β-Glucosidase assays

For the general survey (Table 1), extracts were tested in digests containing glucoside (2 mg), extract (0·2 ml except almond emulsin, 1 mg) and citrate buffer (0·2 ml, 0·1 M, pH 6·0). The digests were incubated, under toluene, for 48 hr at 25° and analysed by paper chromatography both before and after incubation for liberated glucose; appropriate controls were included. Chromatograms were developed with ethyl acetate:pyridine:water (2:1:2) and glucose located with acetone silver nitrate–alcoholic sodium hydroxide.[30] From the known sensitivity of this spray reagent and the volumes of digest applied to the papers, it was estimated that the hydrolysis of 0·3–0·5% of the substrate during 48 hr would have yielded sufficient glucose to be detected on the papers. For the purpose of the survey it was found sufficient to compare the intensities of the glucose spots visually.

Modifications of the glucose oxidase method of Hugget and Nixon[31] were used for estimation of β-glucosidase activity of enzyme extracts. Reagents were as follows:

A. *o*-Dianisidine (1%) in absolute ethanol.
B. Glucose oxidase (12·5 mg, Soluble, Type II, Sigma Chemical Company) and 0·5 mg horse-radish peroxidase (Worthington Biochemical Corporation) in 100 ml 0·5 M phosphate buffer, pH 7·0.
C. Reagent B (100 ml) plus 1 ml reagent A.

[29] A. B. Lloyd and R. T. J. Clarke, *N.Z. J. Agr. Res.* **2**, 1084 (1959).
[30] W. E. Trevelyan, D. P. Procter and J. S. H. Harrison, *Nature, Lond.* **166**, 444 (1950).
[31] A. St. G. Hugget and D. A. Nixon, *Lancet* ii, 368 (1957).

For the emulsin studies, 0·1 ml of enzyme digest was diluted to 0·5 ml with water, heated for 2–3 min on a boiling water-bath, cooled, 2·5 ml reagent C added and the mixture incubated for 1 hr at 37° and read at 420 mμ. Reagent blanks and glucose standards (50 μg) were also run.

For assay of clover linamarase, 0·2 ml enzyme extract was incubated at 30° with 2 μmoles substrate and 50 μmoles phosphate buffer, pH 6·0, in a total volume of 0·7 ml, with toluene, for a suitable time (1–16 hr). Reagent C (1·5 ml) was added and the previously described procedure followed. Because of the high colour of some of the clover preparations, colour blanks (without substrate) were also run.

In the case of linseed linamarase, it was found possible to combine the initial incubation with the glucose oxidase step. To 0·2 ml enzyme extract was added 1·5 ml reagent C and 0·5 ml (2 μmoles) substrate. The above procedure was then followed.

The glucose oxidase method was unsuitable in several instances because of interference from liberated aglycones. In these cases, the Nelson method[32] for glucose estimation was usually employed, after incubation of the digests containing phosphate buffer, pH 6·0, for a suitable time and deproteinization.

For testing β-glucosidase activity towards taxiphyllin and dhurrin, the liberation of *p*-hydroxybenzaldehyde was determined by measuring the increase in light absorption at 285 mμ.[14] The reaction was carried out in cuvettes maintained at 30°. Non-enzymatic decomposition of *p*-hydroxybenzaldehyde cyanhydrin is sufficiently rapid for this step not to have been rate-limiting in the assay.[14]

Purification of linseed linamarase

Coop's procedure[5] for preparing an extract having 20 times the activity of oil-free linseed meal was followed. Linseed was coarsely ground, extracted with ether for 24 hr and the oil-free meal was finely ground. One hundred grammes of this meal was shaken vigorously in lots of 20 g with 2 l. of water and immediately centrifuged, yielding 1500 ml extract. To this was added with stirring 200 ml 0·2 M sodium acetate–acetic acid buffer, pH 4·6. After standing for 2 hr, a copious white precipitate was centrifuged off and discarded. The supernatant was cooled to 4° and one-half its volume of cold ethanol added slowly. The precipitate was centrifuged off and discarded and further cold ethanol was added to the supernatant until the final concentration of ethanol was 60% (v/v). The precipitate was centrifuged off, taken up in distilled water at 4° to give a solution having about 5 mg/ml, and dialysed against water. This extract (designated "crude linamarase") was clarified by centrifugation at 20,000 *g* for 30 min at 0° and stored under toluene at 2° for use in further fractionations. Purification was 20-fold with respect to the oil-free linseed meal and 5-fold with respect to the initial extract. The preparation contained 50% of the enzyme from the linseed meal.

DEAE-chromatography was carried out at 8° on columns 7 × 1 cm using Cellex D (Bio-Rad), equilibrated with 0·005 M citrate buffer, pH 6·0. Crude linamarase (2·1 mg protein) was applied in 1 ml 0·005 M citrate buffer. Stepwise elution was carried out with 0·005M, 0·1 M and 0·2 M phosphate buffer (Fig. 1), with collection of 3·6 ml fractions.

Gel diffusion fractionation was carried out at 8° on 7 × 1 cm columns using various grades of Sephadex (AB Pharmacia). Crude linamarase (2·1 mg protein) was applied in 1 ml 0·005 M phosphate buffer and eluted in the same buffer, with collection of 1·8 ml fractions. Sephadex G 200 and G 100 gave closely comparable fractionations (Fig. 2).

[32] N. NELSON, *J. Biol. Chem.* **153**, 375 (1944).

Column electrophoresis was carried out using the technique of Loontiens and de Bruyne[11] without major modification. Crude linamarase (5·3 mg protein) was applied in 5 ml. 0·1 M acetate buffer (pH 5) to the top of the column (cathode) and displaced with 10 ml buffer solution. After electrophoresis for 40 hr at 300 V and 40 mA, the column was eluted with collection of 3·6 ml fractions. During electrophoresis and elution the column was cooled by passing refrigerant maintained at 4° through the surrounding jacket; buffer solution in the electrode vessels was renewed twice during the run.

Action of emulsin on linamarin/lotaustralin

Two parallel experiments were carried out simultaneously to permit determination of both glucose and hydrogen cyanide. In one experiment, 8 mg linamarin/lotaustralin, 4 mg emulsin and 50 μmoles potassium phosphate buffer (pH 6·0) in a total volume of 1·2 ml were incubated at 30°. Aliquots (0·1 ml) were withdrawn at 0, 2, 4, 6, 12 and 24 hr for the estimation of glucose by the glucose oxidase method. In the second experiment, 6 Widmark flasks were incubated at 30°, each containing one-sixth of the foregoing quantities of linamarin/lotaustralin, emulsin and phosphate buffer, together with 1 ml 0·1 N NaOH in the centre-well. Flasks were withdrawn at the same time intervals and the reaction stopped by addition of 1 ml 0·1 N NaOH. Contents of the centre-well and reaction mixture were combined and hydrogen cyanide determined on suitable aliquots by the method of Aldridge.[33]

Protein and polysaccharide analyses

Protein content of enzyme extracts was determined by the method of Lowry *et al.*[34] In the case of linseed linamarase, absorption at 280 mμ was also used as a measure of protein in monitoring column effluents; checks with Lowry analyses showed close correspondence.

Polysaccharide content of enzyme extracts was determined by an anthrone method,[35] suitable for hexose sugars (anthrone 20 mg/100 ml 70% v/v H_2SO_4).

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