Phytochemistry, 1967, Vol. 6, pp. 1501 to 1505. Pergamon Press Ltd. Printed in England

THE ELECTROPHORETIC SEPARATION OF THE β-GLUCOSIDASES OF ALMOND "EMULSIN"

D. R. HAISMAN, D. J. KNIGHT and M. J. ELLIS

The Fruit and Vegetable Preservation Research Association, Chipping Campden, Glos., and Meat Research Institute, Low Temperature Research Station, Cambridge

(*Received* 1 *February* 1967, *in revised form* 26 *April* 1967)

Abstract—Three specific enzymes, amygdalin hydrolase, prunasin hydrolase and hydroxynitrile lyase have, been isolated by continuous electrophoresis in a free buffer film from a purified, lyophilized preparation of almond β-glucosidase. Amygdalin hydrolase and prunasin hydrolase, although quite specific to their natural substrates, also displayed some activity towards salicin and *p*-nitrophenyl-β-D-glucopyranoside.

INTRODUCTION

THE specificity of β-glucosidase preparations towards particular substrates has been a controversial topic for many years. Opinions have differed according to the standpoint adopted. Those workers who have examined the natural substrates for the enzyme, such as the cyanogenic glycosides in the kernels of stone fruit, have concluded that the hydrolysis of each glycoside required a special enzyme, e.g. amygdalase, salicinase etc.[1,2] Others, using synthetic or alien substrates, have postulated that all β-glucosides are hydrolysed by one enzyme, a β-glucosidase.[3,4]

Partial separation of β-glucosidase activities has been achieved by column chromatography on alumina,[5] filter paper electrophoresis,[6] cellulose column chromatography,[7] ion-exchange chromatography[8] and precipitation and adsorption procedures.[9] Some of these experiments[6,7] yielded β-glucosidases which appeared to be specific to aryl β-glucosides. Mao and Anderson[9] found that *Sorghum* contained two β-glucosidases, only one of which was active towards the endogenous substrate dhurrin (*p*-hydroxy-(−)-mandelonitrile β-D-glucopyranoside). Helferich and Kleinschmidt[8] found that almond emulsin contained several glucosidases, two of which displayed different activities towards salicin (*o*-hydroxymethylphenyl β-D-glucopyranoside) and phenyl β-D-glucopyranoside.

A study of the kinetics of the hydrolysis of amygdalin (D(−)-mandelonitrile β-D-gentiobioside) by almond β-glucosidase showed that the reaction proceeded by three steps, via the monoglucoside, prunasin and mandelonitrile to the final products, glucose, benzaldehyde and hydrocyanic acid.[10] Each step was catalysed by a particular enzyme, respectively amygdalin hydrolase, prunasin hydrolase and hydroxynitrile lyase.

[1] H. E. ARMSTRONG, E. F. ARMSTRONG and E. HORTON, *Proc. Roy. Soc.* (*London*) **B80**, 321 (1908).
[2] H. E. ARMSTRONG, E. F. ARMSTRONG and E. HORTON, *Proc. Roy. Soc. London* **B85**, 359 (1912).
[3] R. WILLSTATTER, R. KUHN and H. SOBOTKA, *Z. Physiol. Chem.* **129**, 33 (1923).
[4] S. VEIBEL, *The Enzymes, Chemistry and Mechanism of Action*, Vol. 1, p. 583. Academic Press, New York (1950).
[5] L. ZECHMEISTER, G. TOTH, P. FURTH and J. BARSONY, *Enzymologia* **9**, 155 (1941).
[6] M. A. JERMYN, *Australian J. Sci. Res. B.* **5**, 433 (1952).
[7] J. H. HASH and K. W. KING, *J. Biol. Chem.* **232**, 395 (1958).
[8] B. HELFERICH and T. KLEINSCHMIDT, *Z. Physiol. Chem.* **340**, 31 (1965).
[9] C.-H. MAO and L. ANDERSON, *Phytochem.* **6**, 473 (1967).
[10] D. R. HAISMAN and D. J. KNIGHT, *Biochem. J.* **103**, 528 (1967).

The implication that almond β-glucosidase contained at least three highly specific enzymes has now been substantiated by the complete separation of the enzymes using continuous flow electrophoresis. With this technique, electrophoresis takes place in an enclosed buffer film; the absence of supporting material eliminates losses by absorption and allows relatively large samples to be separated under closely controlled conditions.

RESULTS AND DISCUSSION

It was found that almond β-glucosidase preparations could be separated by cellulose acetate electrophoresis into several active components, but the separation was insufficient to reveal any differences in specificity. The stability of the enzyme was markedly dependent on the pH of the buffer used.

In acetate buffer at pH 5·0 no measurable change in the activity occurred over a period of seven days, but in sodium diethylbarbiturate/acetate buffer, pH 8·6, the enzyme was rapidly inactivated, making post-electrophoretic measurement of the activity very difficult.

From the data obtained in the cellulose acetate electrophoresis experiments, approximate values were obtained for the electrophoretic mobilities of the different components of the enzyme preparation. On the basis of these mobilities an attempt was made to separate the enzymic components by continuous flow electrophoresis in 0·025 M acetate buffer at pH 5·0. Thirteen active fractions were obtained. When the activities of the fractions towards the four substrates amygdalin, prunasin, mandelonitrile and salicin were examined, it was found that a partial separation of activities had been achieved, although there was still considerable overlap between fractions. A band of fractions which were only active towards mandelonitrile had migrated towards the anode. A band active towards amygdalin but not towards prunasin had moved towards the cathode. A third band, which appeared almost electrically neutral, moving only slightly towards the cathode, hydrolysed prunasin but not amygdalin. Both the amygdalin hydrolase and the prunasin hydrolase bands displayed some activity towards salicin.

A second experiment, using modified conditions, achieved a much better separation. Thirty active fractions were obtained, and their activities towards five different substrates are shown in Fig. 1. This time, three fractions were obtained which were quite specific with respect to the indigenous substrates amygdalin (22–26), prunasin (38) and mandelonitrile (46). In addition, a few intermediate fractions (30–36) displayed some activity towards both amygdalin and prunasin.

The two other substrates which were tested, salicin and *p*-nitrophenyl β-D-glucopyranoside, do not occur naturally in the kernels of stone fruit. It was found that most of the active fractions hydrolysed these substrates, the peak activities coinciding with the amygdalin hydrolase and the prunasin hydrolase fractions.

The distribution of the activity towards salicin is in agreement with the variation in the salicin activity of different β-glucosidase components noted by Helferich and Kleinschmidt.[8] Subsequent experiments have shown that the β-glucosidase preparation we have used displays a slight activity towards phenyl β-D-glucopyranoside.

None of the fractions displayed activity towards cyanidin-3 β-D-glucopyranoside. Other workers have found that almond β-glucosidase does not act on anthocyanins.[11]

The function of the cyanogenic glucosides which occur in the higher plants is speculative, but there is evidence that they are metabolic intermediates in the biosynthesis of certain

[11] J. B. HARBORNE and H. S. A. SHERRATT, *Biochem. J.* **65**, 24p (1957).

amino-acids. It has been shown that the nitrile moieties of linamarin and lotaustralin in trefoils are derived from the α-carbon and nitrogen of valine and isoleucine, and after hydrolysis to hydrocyanic acid are utilized in the synthesis of asparagine.[12] Similarly, phenylalanine is an effective precursor of prunasin in cherry-laurel leaves[13] and it is probable that prunasin is also an intermediate in amino-acid synthesis.

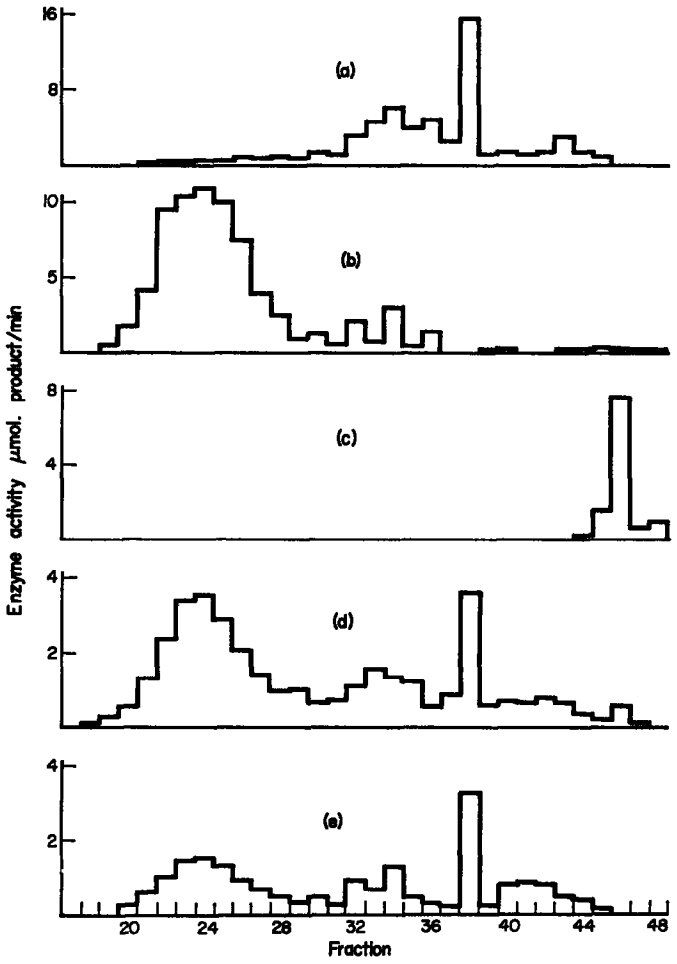

FIG. 1. THE DISTRIBUTION OF β-GLUCOSIDASE ACTIVITY TOWARDS DIFFERENT SUBSTRATES.
(a) Prunasin; (b) Amygdalin; (c) Mandelonitrile; (d) Salicin; (e) *p*-nitrophenyl glucoside.

Our experiments on almond β-glucosidase show that the endogenous enzymes active in the degradation of the cyanogens in the plant tissues are highly specific to their particular substrates along the metabolic pathways. No doubt this specificity is essential to ensure control of the biosynthesis in the plant.

Although the reasons for the absolute specificity of the β-glucosidases to their natural substrates are easily understood, the mechanisms making such selectivity possible present

[12] Y. P. ABROL and E. E. CONN, *Phytochem.* **5**, 237 (1966).
[13] C. MENTZER, J. FAURE-BONVIN and M. MASSIAS, *Bull. Soc. Chem. Biol.* **45**, 749 (1963).

unusual features. Each enzyme, while specific to a unique endogenous substrate, also displays more general catalytic activity towards extraneous substrates. Thus amygdalin hydrolase is quite specific to the gentiobioside amygdalin, has no effect on the closely related glucoside prunasin, and yet will catalyse the hydrolysis of quite different aryl glucosides such as salicin and *p*-nitrophenylglucoside. It is interesting to compare our results with those of Mao and Anderson,[9] who found that *Sorghum* contained two β-glucosidases, one active towards aryl glucosides, and the other towards benzyl glucosides (including dhurrin, the natural substrate). This would appear to be an example of yet another selective mechanism.

The significance of the intermediate fractions 30–36 which display activity towards all four phenyl glucosides is uncertain. The original β-glucosidase sample was applied opposite fraction 34, and it is possible that the activity in these fractions results from an overlap of the purified components concentrated in fractions 36 and 24. On the other hand, they may contain a further unidentified enzymic species which displays general activity in addition to its

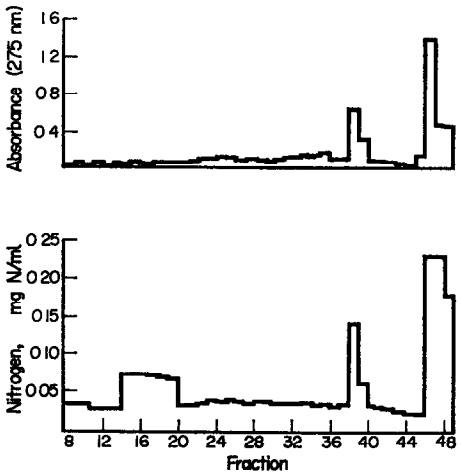

FIG. 2. THE ABSORBANCE (275 NM) AND KJELDAHL NITROGEN CONTENT OF DIFFERENT ELECTROPHORETIC FRACTIONS.

specific function. Alternatively the fractions may represent a truly non-specific β-glucosidase.

Apart from differences in the enzymic activity of the fractions collected, differences were also observed in the composition of the protein components. Figure 2 shows the absorbance at 275 nm and the Kjeldahl nitrogen contents of the fractions. Prunasin hydrolase and hydroxynitrile lyase both showed marked absorbance at 275 nm indicating a high proportion of aromatic amino-acids, and have a high nitrogen content. Amygdalin hydrolase, by contrast, is low in both nitrogen and aromatic residues, and would appear either to have a different chemical composition, or else, based on enzymic activity, a very much greater turnover number. Another protein component was found in the lower fractions but this was not active towards any of the substrates used in these experiments and may be an inactive impurity.

EXPERIMENTAL

Electrophoresis

The Elphor Vap I electrophoresis apparatus (Bender and Hobein, GmbH, Munich) was used. On this apparatus the separation takes place in a horizontal chamber, 50 cm square, 0·5 mm high. Buffer is pumped

through the chamber continuously, and the sample solution is metered continuously into the moving buffer curtain at a point depending on the expected range of electrophoretic mobilities of the components. The electric field is applied, at right angles to the direction of buffer flow, by a pair of strip electrodes the length of the chamber, but immersed in a separate circulating buffer system, which prevents interference by electrolysis products. The heat due to the current is removed by a thermostatted refrigeration system which circulates cold air round the chamber. The applied voltage during the experiment was 2150 V, equivalent to 42·0 V/cm (current 129 mA) and the buffer flow was adjusted to 76·4 ml/hr.

Separated fractions of the sample were removed at intervals during the run from 48 points across the chamber.

From the position of the fractions with peak activities the electrophoretic mobilities of the individual enzymes were calculated to be; amygdalin hydrolase $-5{\cdot}05\times10^{-5}$, prunasin hydrolase $+2{\cdot}02\times10^{-5}$ and hydroxynitrile lyase $+6{\cdot}06\times10^{-5}$ cm²/sec/V.

Enzyme

A salt-free lyophilized β-glucosidase preparation from almonds with an activity of 1000 units/mg[14] was obtained from Koch-Light Laboratories Ltd.

Substrates

Commercial samples of amygdalin (D(−)-mandelonitrile-β-gentiobioside), salicin, *p*-nitrophenyl-β-D-glucopyranoside and mandelonitrile were used, but the mandelonitrile was further purified by redistillation under reduced pressure. Prunasin (D(−)-mandelonitrile-β-glucopyranoside) was prepared by the acid hydrolysis of amygdalin, using the method of Caldwell and Courtauld.[15] It was recrystallized from ethyl acetate until found pure by thin-layer chromatography. The cyanidin-3-β-glucopyranoside used was extracted from elderberries, and purified by chromatography on polyvinyl-pyrollidone.

Activity Estimations

Activity estimations were carried out by incubating aliquots of the fractions with fixed concentrations of substrate (final concentrations 13·55 mM prunasin, 5·47 mM amygdalin, 0·38 mM mandelonitrile, 34·93 mM salicin, 1·33 mM *p*-nitrophenylglucoside and 0·36 mM cyanidin-3-glucoside). After a fixed time interval, aliquots were pipetted into acid or other appropriate reagent to stop the enzyme reaction, and the hydrolysis products estimated by reference to calibration curves prepared with standard solutions given the same treatment.

Amygdalin hydrolase activity was determined from the glucose produced, using a simultaneous cyanide estimation to correct for any concurrent prunasin hydrolase activity.[10]

Prunasin hydrolase activity was determined from the mandelonitrile produced[10] and hydroxynitrile lyase activity from the amount of free cyanide produced after correction for acid hydrolysis at pH 5·0. Glucose was estimated by the spectrophotometric measurement of the glucosazone in acetic acid[16] and cyanide by Epstein's method.[17]

The activity towards salicin was determined by estimating saligenin,[14] and towards *p*-nitrophenyl glucoside by estimating *p*-nitrophenol.[18]

The activity towards cyanidin-3-glucoside was determined by incubation at 30° for up to 16 hr. The hydrolysates were extracted with three successive 3 ml portions of *n*-amyl alcohol, the extract made up to a total volume of 10 ml and the absorbance at 550 nm measured.

Blanks were carried out with each estimation.

Acknowledgement—This work formed part of a research programme supported by the Agricultural Research Council.

[14] P. BARUAH and T. SWAIN, *Biochem. J.* **66**, 321 (1957).
[15] R. J. CALDWELL and S. L. COURTAULD, *J. Chem. Soc.* **91**, 666 (1907).
[16] N. WAHBA, S. HANNA and M. M. EL-SADR, *Analyst* **81**, 430 (1956).
[17] J. EPSTEIN, *Anal. Chem.* **19**, 272 (1947).
[18] M. A. JERMYN, *Australian J. Sci. Res. B.* **5**, 409 (1952).