ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. XLII — nr 2
1973

Changes in the electrophoretic pattern of glucosidases during apple seeds stratification

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(Received: July 16, 1972)

Abstract

The technique of localization of glucosidases on disc electropherograms, based on reduction of 2,3,5-triphenyltetrazolium chloride by enzymatically liberated sugar is described. The changes in electrophoretic patterns of amygdaline, phloridzin and p-nitrophenyl-β-glucoside hydrolysing glucosidases during apple seed stratification were studied. The changes were correlated with earlier described changes in endogenous apple seed amygdaline and phloridzin contents.

INTRODUCTION

The main glucosides of apple seeds are amygdaline and phenol glucoside — phloridzin. As reported by Duczmal (1963), large amounts of hydrogen cyanide produced by apple seeds during stratification are the result of hydrolytic degradation of amygdaline. Its largest production is observed during the first 60 days, later a decrease is noted and the plateau remains until the end of stratification. Phloridzin appears in the apple embryos in the 7-th week of stratification, its amount gradually rising (Kamiński, Pieniążek 1968), and reaching its maximum during the greening of cotyledons of the germinating embryos.

The usually applied methods of preparative separation of plant hydrolases consisting in ion exchange chromatography and molecular sieving resulted in some highly purified fractions of apple seeds glucosidases (Podstolski, Lewak 1970). However, inactivation of some glucosidases during long lasting procedure and the large amount of seeds required have to be considered as the disadvantages of these methods. By using polyacrylamide gel electrophoresis we have been able to avoid the disadvantages. It was also possible to investigate the substrate specificity and the changes in the electrophoretic pattern of glucosidases directly on the electropherograms.

MATERIAL AND METHODS

Material

Apple seeds cv. Antonovka, 1970 collection, from Experimental Station of Institute of Pomology at Sinołęka, were used throughout the experiments. The seeds were stratified and acetone powder was prepared as described earlier (Podstolski, Lewak 1970).

Methods

Extraction of glucosidases

The acetone powder obtained from 100 seeds was extracted in ice bath with 12 ml of 0.08 M acetate buffer pH 6.5, containing 10% of mannitol. The extraction was carried out for 60 min., with magnetic stirring. The preliminary centrifugation 15 min. at 16 000 g at 0—5°, was followed by 60 min. at 120 000 g (VAC-60 ultracentrifuge). The resulting supernatant was used for electrophoretic assay.

Polyacrylamide gel electrophoresis

Electrophoresis was carried out according to Reisfeld et al. (1962) with the following modifications:

I. small pore gel was prepared in pH 3.3 buffer.
II. spacer gel was prepared in pH 5.5 buffer.
III. electrophoresis was run in β-alanine-acetic acid buffer pH 3.5. The current was adjusted to 2 mA per tube for the first 20 min. and 5 mA per tube during the following 110 min.

Visualisation of glucosidase activity bands

The electropherograms were taken out of the tubes and incubated with 5 ml of a solution of the respective glucosides in acetate buffer pH 5.0. The following 5 mM glucoside solutions were used: amygdaline, phloridzin, p-nitrophenol-β-glucoside and salicine. The incubation was carried out at 30° for 30 and 60 min. for each substrate and the gels were then transferred to 5 ml of 0.05% solution of 2,3,5-triphenyltetrazolium chloride in 0.5 N KOH at 45° for 20 minutes. Enzymatic hydrolysis of the substrates resulted in the appearance of red bands due to the reduction of 2,3,5-triphenyltetrazolium chloride by liberated glucose.

RESULTS

The routine methods of detection and determination of glucosidase activity are based on the assay of aglycone liberated during enzymatic hydrolysis (Conchie et al. 1967; Barham et al. 1971). Taking into

account the reductive properties of simultaneously liberated sugar, we elaborated the metod of visualisation of glucosidases separated electrophoretically. This method consists in reduction of 2,3,5-triphenyltetrazolium chloride by glucose liberated durnig incubation from the respective glucosides by glucosidases separated on polyacrylamide gel. The reduction of 2,3,5-triphenyltetrazolium chloride causes the formation of red, insoluble bands at the site of glucosidase activity. The same conditions were thus applied to visualise the products of hydrolysis of various glucosides and therefore the evaluation of the activity of various glucosidases towards different substrates was relatively simple.

The investigation of the changes of the glucosidases electrophoretic pattern throughout stratification of apple seeds was performed at 10 days intervals. The dynamics of changes regarding the intensity of the bands with various substrates were different. Therefore the diagrams of the electrophoretic pattern are presented at intervals which allow to notice the differences in pattern and/or intensity.

Amygdaline glucosidases (Fig. 1)

Electropherograms of non-stratified apple seeds revealed 13 intense bands, visible when amygdaline was used as substrate. The intensity of these bands remains unchanged until the 50-th day of stratification. A decrease of the bands of electrophoretic mobility (e.m.) between 0—20, an increase of that at e.m. 25 and appearance of a new band — e.m. 45 were observed after 60 days of stratification. After 70 and 80 days of stratification further progressive decrease of the bands at e.m. 0—20 was noted. The band at e.m. 20 did not appear in the fully stratified seeds. The intensity of the band at e.m. 25 in fully stratified seeds was slightly weaker as compared with that after 60 days of stratification, however, it still remained very high. There were no marked changes of the bands at e.m. 45 between 60, 70 and 80 days of stratification.

Phloridzin glucosidases (Fig. 2)

Thirteen bands of phloridzin hydrolysing glucosidases were observed on electropherograms of nonstratified seeds, however, there were only traces of activity of the bands localised between e.m. 0 and 20. The bands at e.m. 7, 9 and 11 disappeared completely after 30 days of stratification, whereas the intensity of that at e.m. 17 rose markedly. After 50 days of stratification the band at e.m. 11 reappeared and a new one with e.m. 45 was visible. During longer stratification the intensity of the glucosidase band at e.m. 25 increased stepwise, reaching maximum after 70 days.

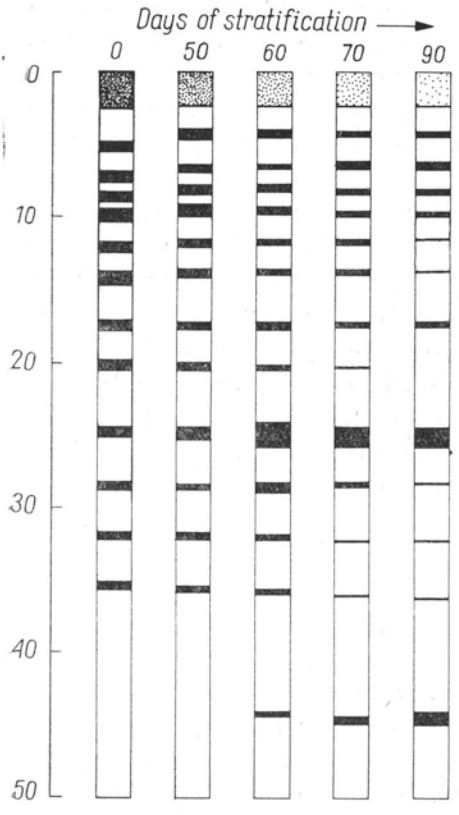

Fig. 1. Changes in electrophoretic pattern of amygdaline hydrolysing glucosidases during apple seed stratification

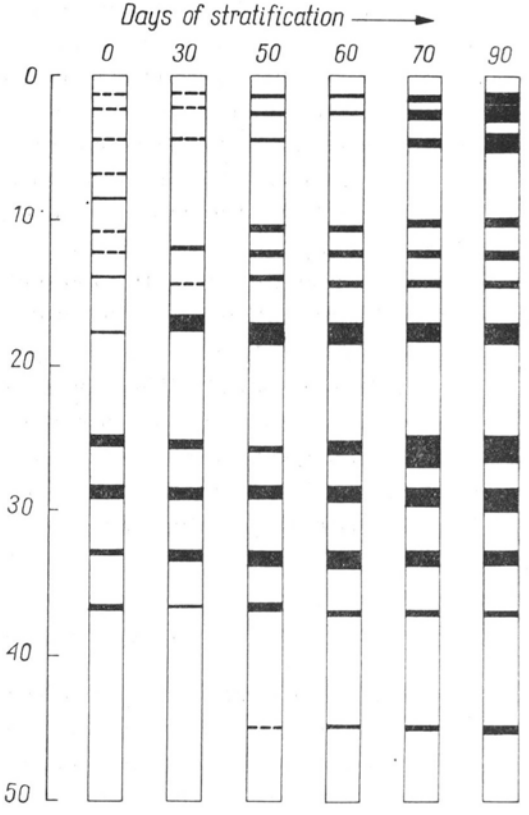

Fig. 2. Changes in electrophoretic pattern of phloridzin hydrolysing glucosidases during apple seed stratification

The intensity of the bands at e.m. 28, 32 and 45 rose but slightly and that of bands which show electrophoretic mobility — 1, 2 and 4 increased markedly.

Salicine and p-nitrophenol-β-glucoside (PNPG) glucosidases

The activity of glucosidase bands towards non-native substrates such as salicine and PNPG was assayed in order to check their specifity.

No bands with e.m. 20 and 45 were noted while using those substrates. The activity of enzymes localised in the bands at e.m. 0—5 towards salicine appeared first after 60 days of stratification and remained low until the end of the process. The activity of these bands towards PNPG is slight, without any rising tendency towards the end of stratification.

The changes of the electrophoretic pattern during seed stratification are quite different for the amygdaline and phloridzin glucosidases. The activity of amygdalinases decreases markedly or even disappears (band at e.m. 20) during the progress of stratification. Only the intensity of the band at e.m. 25 increases during stratification, however, the same band develops when phloridzin used as a substrate. The intensity of the hydrolysing phloridzin glucosidase bands, reveals a marked rising tendency during stratification, what may be seen in the increase of the bands at e.m. 28 and 32 and the appearance of bands at e.m. 1, 2 and 4 towards the end of stratification. The slow-moving bands revealed mainly while using phloridzin as a substrate, represent the enzymes specific towards phloridzin.

DISCUSSION

The changes observed in the activities of phloridzin and amygdaline glucosidases are related with the amounts of the respective glucosides in the course of stratification. The appearance of specific phloridzin glucosidases in the last period of seed stratification (as proved by preparative methods by Podstolski and Lewak, 1970) indicates intense phloridzin turnover during the period immediately preceeding germination.

During the first 60 days of stratification of seeds, the intensity of bands of amygdaline hydrolysing glucosidases is very high. This high activity is parallel to the hydrogen cyanide secretion by seeds as reported by Duczmal (1963). The hypothesis that hydrogen cyanide secretion is due to enzymatic hydrolysis of seed amygdaline being thus confirmed.

The changes in the activity of the phloridzin hydrolysing glucosidases during apple seed stratification can be correlated with the changes in phloridzin content. The rise of intensity of the phloridzin-hydrolysing glucosidases bands appears at the final stage of seed stratification, immediately before germination, when intensive phloridzin biosynthesis appears in the apple seeds (Pieniążek, Grochowska, 1967). However, phloridzin biosynthesis appears already after 50 days of stratification, whereas phloridzin glucosidase activity rises after as long as 70 days.

The breaking of dormancy of apple seeds is due at least in some degree to the change in equilibrium between the inhibitors such as ABA and stimulators, mainly gibberellins and cytokinins. The changes in the content of ABA (Rudnicki, 1969), GA_7 and GA_4 (Sińska, Lewak, 1970) in the course of apple seed stratification were described, whereas the changes of the level of cytokinins remains to be investigated. On the other hand, the synergistic action of phloridzin with $GA_4 + GA_7$ and cytokinins in breaking seed dormancy was reported by Kamiński and

Pieniążek (1968). This action of phloridzin may be presumed to contribute to the physiological effect of endogenous stimulators. The level of phloridzin, influencing the effect of gibberellins and cytokinins, would be, therefore, controlled by the rate of biosynthesis of glucoside and its hydrolysis, due to the enzymes described in this paper.

Towards the end of stratification, when intense biosynthesis of phloridzin appears together with the marked increase of its specific glucosidase activities, the regulative role of phloridzin transformations products seems probable. According to Raa and Overeem (1968), enzymatic hydrolysis or oxydation at position 3 is the first step in phloridzin transformation. The final products of phloridzin transformation are the polymeric compounds, formed by oxydation of 3-hydroxyphloretin (Raa and Overeem, 1968). Such polymeric compounds may be postulated to act as allosteric effectors, affecting the enzymatic systems towards the metabolic process causing the germination of apple seeds.

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This work was partly supported by Grant F.G.-Po-236 from U.S.Department of Agriculture, Agricultural Research Service, according to Public Law 480 and the project No. 09.1.7 co-ordinated by the Institute of Ecology, Polish Academy of Sciences.

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Zmiany w obrazie elektroforetycznym glukozydaz w trakcie stratyfikacji nasion jabłoni

Streszczenie

Opisano metodę lokalizacji glukozydaz na elektroferogramach z żelu poliakrylamidowego, polegającą na redukcji chlorku 2,3,5-trójfenylotetrazoliowego przez uwalnianą enzymatycznie glukozę. Przebadano zmiany w obrazie elektroforetycznym glukozydaz hydrolizujących amygdalinę, florydzynę, salicynę i β-glukozyd-p-nitrofenolu, w trakcie stratyfikacji nasion jabłoni. Zmiany te skorelowano z opisanymi wcześniej zmianami zawartości endogennej amygdaliny i florydzyny w nasionach jabłoni.