

Synthesis of Sinapine during Seed Development of *Sinapis alba*

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Z. Naturforsch. **35 c**, 539–543 (1980); received March 26, 1980

Sinapis alba, Sinapine, Phenylalanine, Seed Development, Substance Accumulation

Sinapine (sinapoyl-choline) is accumulated in the cotyledones of *Sinapis alba* during the main growth phase of the developing embryos. It is degraded during germination of older embryos which have not reached full maturation and of mature seeds, but remains stable in young embryos. The main product of degradation is sinapoyl-glucose. [^{14}C]phenylalanine is not only incorporated into sinapine during the accumulation phase in situ but just so into isolated embryos which do not accumulate the substance further. Synthesis takes place in the cotyledones. Their synthesizing capacity (incorporation of [^{14}C]phenylalanine) is correlated with the growth rate of the cotyledones.

Introduction

Sinapine (= sinapoyl-choline) is the predominant secondary plant substance in the seeds of many Brassicaceae especially *Sinapis alba* [1]. The substance is accumulated in the cotyledones during seed formation and is very rapidly degraded in the progress of seed germination [2–5]. The possible pathway of biosynthesis was recently analyzed by taking into account the enzymes considered to be related to sinapine biosynthesis [5]. Yet questions remain on the course of synthesis and degradation – yielding the net accumulation rate – just as on the localisation of the process in developing pods or seeds. These questions, however, are of importance to understand the different regulation processes involved in the developing embryo [7] and the germinating seed. The following experiments reveal aspects of the location and time of synthesis by incorporation of labelled precursors into sinapoyl-choline and sinapoyl-glucose [5]. For this purpose

[^{14}C]phenylalanine was employed as it is the first substance of the sinapine-pathway and a potent precursor [6].

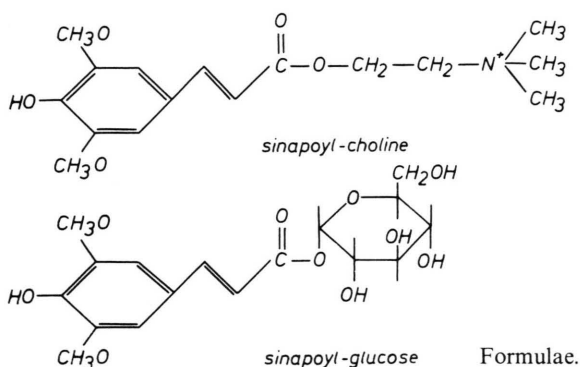
Materials and Methods

All experiments were carried out with *Sinapis alba* (seeds from Landwirtschaftliche Ein- und Verkaufsgenossenschaft, Raiffeisen, Heidelberg-Kirchheim). Plants were regularly cultivated in a climatic chamber under continuous light (light intensity 12 000 lux, temp. $26 \pm 0.5^\circ\text{C}$, rel. humidity 60%). Under these conditions flowers appear very early, about 19 to 20 days after sowing. Pollination of the flowers was carried out with a small brush as soon as 10 flowers of one individual plant had opened. After pollination the developing seeds were observed over a period of 26 days. After an additional period of 10 days the seeds are desiccated, yellow and fully ripe.

Labelling of sinapic acid esters was performed using two methods:

a) Detached shoots with young siliques were brought into a small test tube containing radioactive precursor in 5 ml Knop-solution.

b) Seeds were separated from the pods. The seed coat was removed and the green embryos isolated. These embryos were transferred onto filterpaper soaked with the radioactive solution in small petri-dishes. With this method only half of the radioactivity was necessary to obtain high labelling. After variously long periods the seeds (or embryos) were frozen at 20°C . For extraction 20 seeds were ground in an icecold mortar with 2 ml aqua dest. for 5 min, then centrifuged at 17 000 rpm for 15 min. The supernatant was filled up to 6 ml and the proteins in the supernatant removed by boiling and filtering. The remaining clear solution was kept at -20°C .



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0341-0382/80/0700-0539 \$ 01.00/0



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For separation of the different substances thin layer electrophoresis (TLE) on polyamid DC 6.6 was employed with two different buffer systems, either 0.1 M sodiumacetat pH 6.9 or glycine buffer pH 12 having the following composition: glycine 3.42 g, NaCl 2.67 g, 1 N NaOH 54.5 ml in 1 000 cm³ H₂O. Electrophoresis is driven at 400 V and 10 °C for 45–60 min. This method produced better results than paper- or thinlayer chromatography and additionally could be shown to be only technique allowing a clear separation of the administered phenylalanine and sinapoyl derivatives.

Only sinapic acid, sinapine and sinapoyl-1-glucose [5, 8] (*i. e.* substance X in our earlier paper [2]) were identified. Beside these 4 to 5 other light or deep blue fluorescing substances could be seen on the electropherogram.

Quantitative measurements were carried out using electrophoresis with glycine buffer as the substances in question show a bright yellow fluorescence at pH12 and therefore could be scanned with a Joice Loible chromoscan. Within the range of measurement the area under the scanning curve is linearly correlated to the amount of substance.

The radioactivity was measured subsequent to electrophoresis: the polyamid-layer was scraped off and suspended in 7.5 ml scintillation liquid and counted in a Tricarb Scintillationcounter. The radioactive substances were purchased from Radiochemical Centre Amersham: 5 µCi/ml L-[U-¹⁴C]phenylalanine and 5 µCi/ml L[U-¹⁴C]tyrosine. Tyrosine was only a very weak precursor. Therefore experiments with this amino acid are not mentioned in the results.

Further details of methods are presented in an earlier paper [2].

Results

Fig. 1 shows the variation of fresh weight of intact seeds and isolated embryos of *Sinapis alba* from 10 to 25 days after pollination. It is obvious that at the end of seed-development most of the fresh material is contributed by the embryo or more exactly by the cotyledones, which start to grow by the 12th day. Seed coat and endosperm lose water from the 15th day leading to a drastic decrease of the seed's total fresh weight.

The content of sinapine per embryo increases parallel to embryo growth and is not correlated to the fresh weight of the whole seed. Under our con-

ditions it reaches highest values on the 22nd day after pollination (Fig. 2 [6]). Subsequently the amount of sinapine remains constant until germination. Only in intact plants will the sinapine level increase steadily, in detached shoots, however, the total amount of sinapine per embryo will remain constant at the further stages of development. This may be caused by a starvation effect in detached shoots. But even under such conditions where accumulation stops, the synthesis of sinapine continues, which is shown by the incorporation of considerable amounts of [¹⁴C]phenylalanine (Fig. 3). After 24 and 48 h labelling-periods the greatest amount of activity of an aqueous (protein-free) extract is found to be incorporated in sinapine. Only one or two other labelled substances with a blue fluorescence are found in the extract. The content of free phenylalanine is very low, sinapic acid and sinapoyl-glucose are not labelled after 24 and 48 h. The total distribution of labelled substances is identical in both cases. But after 48 h the activity is three times that as after 24 h.

The total activity incorporated per seed is rather low, therefore we performed experiments with isolated embryos. To understand this phenomenon it was necessary to follow the metabolism of sinapine in such embryos.

The behaviour of the isolated embryos depends on their age. In general all embryos "germinate" immediately after isolation and removal of the seed

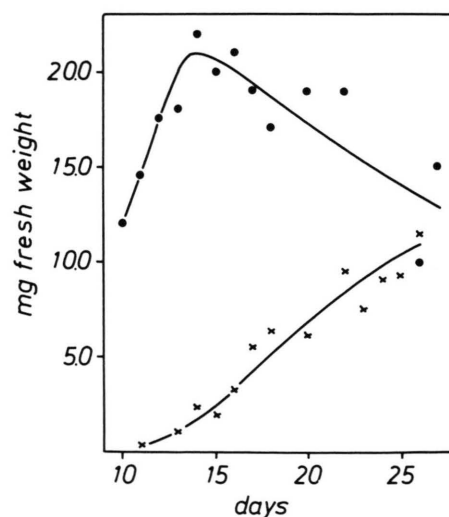


Fig. 1. Variation of fresh weight of whole seeds (●-●) and embryos (x-x) of *Sinapis alba* between 10 and 25 days after pollination. Each point representing the value of 10 seeds. Ordinate: fresh weight of one seed or embryo.

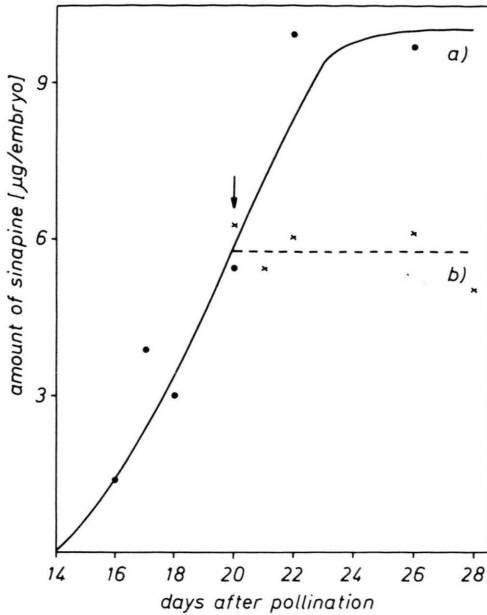


Fig. 2. Accumulation of sinapine in the embryos (cotyledones) of *Sinapis alba* from intact plants (a) and from shoots detached 20 days after pollination (b). In this case sinapine accumulation ceases. Such plants are used for labelling with [^{14}C]phenylalanine. Sinapine was isolated by thin layer electrophoresis (TLE) in glycine buffer pH 12 and measured with a chromoscan.

coat. Whole seeds containing immature embryos do not germinate. Embryos, 14 to 16 days of age grow at about the same rate as embryos in situ, while older embryos grow much faster. 20 day-old embryos double their fresh weight in about 60 h, whereas the growth of embryos in the intact seed is terminated soon after (Fig. 4).

Sinapine accumulation is equally dependent on the age of the embryo (Fig. 5). In embryos being 14 or 16 days old accumulation continues but to a lesser degree as in intact plants. Embryos being 18 days old are found to show a clear decrease while after 20 days the decline of the sinapine content is as dramatic as during the germination of mature seeds [2]. One may assume that in the young embryos sinapine esterase [9–11] or related enzymes are the limiting factors in the degradation process.

Further labelling experiments were carried out with 16 and 20 day-old embryos by applying [^{14}C]phenylalanine for various lengths of time. In addition to the labelled substances found for whole-plant-labelling two further blue fluorescing bands with radioactivity are found in isolated embryos. The remaining labelling pattern is analogous to that

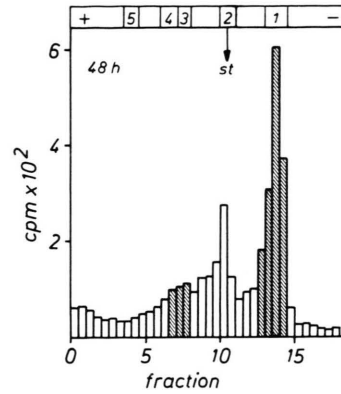


Fig. 3. Distribution of radioactivity in TLE. The plants are extracted at 48 h following administration of [^{14}C]phenylalanine ($5 \mu\text{Ci/ml}$). Seeds were used on the 20th day after pollination. Radioactivity was taken up by the detached shoots. Only the seeds were extracted. The numbers give the distribution of fluorescing bands and other substances in TLE. 1) sinapine, 2) two blue fluorescing bands, 3) phenylalanine, 4) dark blue fluorescing band, 5) sinapic acid. st, start point of electrophoresis.

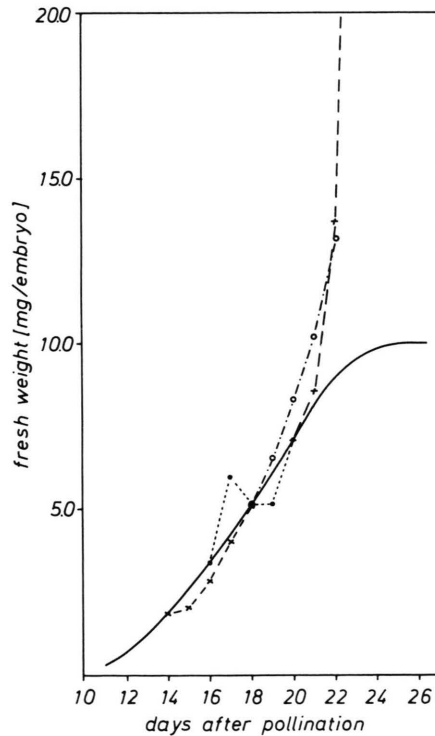


Fig. 4. Variations of fresh weight of embryos isolated at different stages of development — same values as Fig. 1. Embryos isolated after 14 \times — \times , 16 \circ — \circ , 18 \circ — \circ and 20 $+$ — $+$ days. Growth (germination) is faster the older the embryos are at the time of isolation.

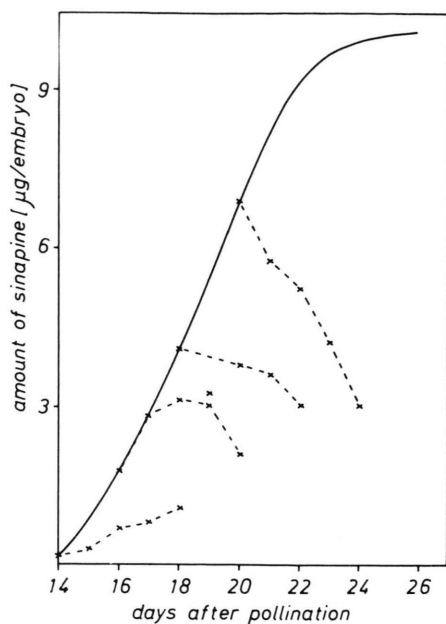


Fig. 5. Variations of sinapine accumulation in isolated embryos after isolation and removal of the seed coat. — accumulation of sinapine *in situ* (same as Fig. 2). The embryos are isolated at 14, 16, 18 and 20 days after pollination. Sinapine content/embryo decreases in older, further developed embryos. Determination as in Fig. 2. 16 and 20 day-old isolated embryos are used for labelling experiments.

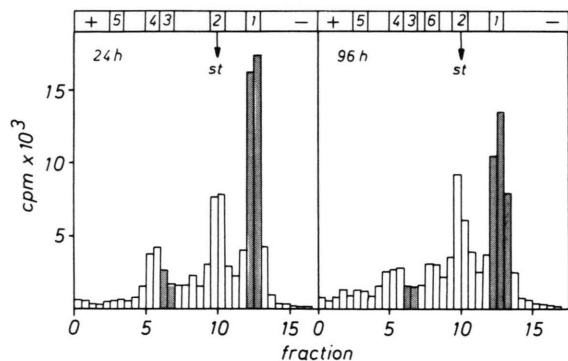


Fig. 6. Distribution of radioactivity in TLE. Radioactivity [^{14}C]phenylalanine, 2.5 Ci/ml) was offered to 16 day-old isolated embryos for 24 and 96 h. For each experiment 20 embryos were used. The distribution of radioactivity does not change significantly. After 96 h a small amount of activity was found in sinapoyl-glucose. This substance was formed from 24 to 96 h after isolation. Numbers and methods were the same as in Fig. 3. 6, sinapoyl-glucose.

of seeds *in situ* and remains nearly unchanged for 96 h after supply of [^{14}C]phenylalanine (Fig. 6). During this time the content of labelled and unlabelled sinapoyl-glucose increases, but the activity is much lower than for sinapine.

The specific activity of sinapine (Fig. 7) increases throughout this time period even when further accumulation terminates in the isolated embryos or, as in 20 day-old embryos, when the sinapine-content decreases rapidly. Therefore sinapine synthesis permanently occurs in embryos which are not fully developed, independent whether they accumulate sinapine or not.

For the following experiment we used seeds from radioactively labelled pods after full maturation, *i. e.* 35 days after pollination. The distribution of radioactivity in seeds at 24 h after germination is completely identical to that found in labelled embryos (Fig. 8). The accumulated substances of 20 day-old

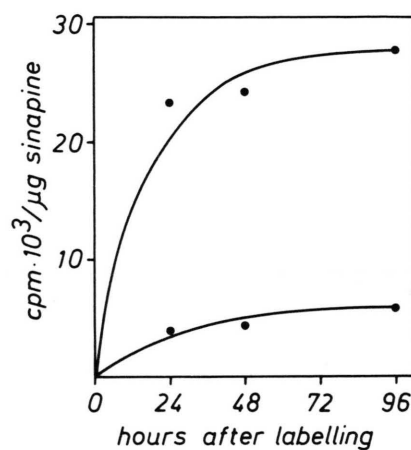


Fig. 7. Increase of specific activity of sinapine in 16 and 20 day-old embryos during the experimental phase.

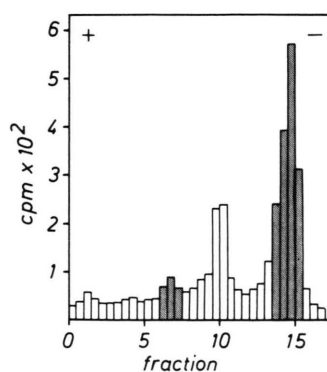


Fig. 8. Distribution of radioactivity in fully-ripe seeds which were labelled with [^{14}C]phenylalanine at the age of 20 days. Subsequently the seeds grew an additional 15 days on the detached shoots. At that time the colour of the seeds turned yellow and the seeds were used for "normal" germination. The distribution of radioactivity is exactly the same as in immature seeds at 24 h after labelling (Fig. 3).

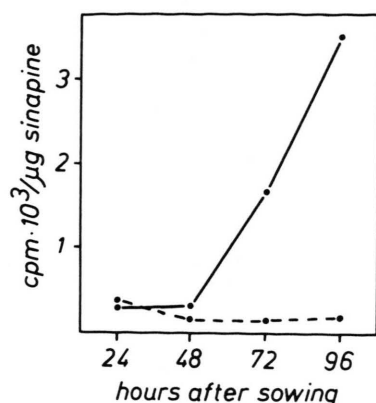


Fig. 9. Variations in specific activity of sinapoyl-choline (sinapine) and sinapoyl-glucose in mature seeds of *Sinapis* during germination. The increase of specific activity means that during the degradation of sinapine synthesis occurs to a minor degree. The specific activity of sinapoyl-glucose nearly constant remains, whereas the total amount of this substance increases considerably.

immature seeds and 35 day-old mature seeds therefore remain unchanged. The total metabolism of sinapine after germination was identical to that described earlier, sinapine decreases whereas sinapoyl-glucose increases [2, 4]. However, the specific radioactivity of sinapine increases while it remains nearly constant for sinapoyl-glucose. During the first phase of germination the specific activity is equal for both substances. Sinapine is therefore newly synthesized from labelled precursors whereas sinapoyl-glucose is very probably interconverted from another labelled substance and this substance can only be sinapine, which total activity decreases rapidly.

Discussion

[¹⁴C]phenylalanine is a suitable precursor for the synthesis of sinapoyl-choline (sinapine). Therefore PAL (phenylalanineammonialyase) which is present in seeds and shows low activity [6, 12] must be involved in the sinapoyl-pathway. Sinapine is not only accumulated but also synthesized in the cotyledones of developing seeds of *Sinapis alba*. Syn-

thesis also continues when accumulation is terminated artificially. Synthesis is reduced during the maturation process of the seeds. As soon as germination of mature seeds or older immature embryos starts sinapine is degraded very quickly. But at the same time when the sinapine content decreases new synthesis of sinapine takes place – indicating that under such conditions a turnover of sinapine is found. In younger embryos sinapine is not degraded and perhaps the specific esterases [9] – responsible for the first steps of interconversion of sinapine to sinapoyl-glucose – are not active or not present at the time of embryo development. As soon as “germination” is possible sinapine degradation takes place. Therefore it is possible that the sinapine degradation enzyme system or the mRNA for these enzymes are formed during the main growth phase of the embryos [12].

The interconversion of sinapoyl-choline to sinapoyl-glucose which was deduced from our earlier experiments [2] and also from experiments with various other Brassicaceae [4] can not be definitively proved with the obtained labelling results. The constant specific activity of sinapoyl-glucose at the time of a strong increase in the total amount indicates the existence of one single labelled precursor. During the initiation of sinapine-turnover the specific activity of sinapoyl-choline and of sinapoyl-glucose occurs at the same rate, so we can assume that sinapine is interconverted to sinapine-glucose [4]. This pathway should include sinapic acid as an intermediate product of a specific sinapine esterase activity [9]. But a labelling of sinapic acid during the catabolic process was not seen, meaning that this substance is not accumulated during the interconversion.

On the other hand sinapic acid was also not labelled during the synthesis in the cotyledones. Therefore it is probably not involved in the pathway of sinapine synthesis, what can be expected according to the scheme of Kühnl and Wellmann [6] who have not found any enzyme which uses sinapoyl-CoA as a substrate for ester formation with choline.

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