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PROTEIN DEGRADATION AS A SOURCE OF PRECURSORS FOR FLAVONOID BIOSYNTHESIS IN BUCKWHEAT COTYLEDONS

Intracellular pools of L-phenylalanine, the principal precursor of flavonoids and most other phenolics, are not homogenous in their metabolic origin. Phenylalanine molecules primarily formed via the shikimic acid pathway may, instead of being used for protein biosynthesis, be channelled into phenolic pathway after their deamination. At the same time phenylalanine units are continuously released from proteins during their catabolism. It has been suggested that released amino acids are not returned to the protein precursor pool (Bidwell et al., 1964; Huffaker, Peterson, 1974; Becker et al., 1981) but are reused in other syntheses. This assumption is supported by the investigations where precursors of some secondary products have been shown to originate, apparently, from protein breakdown. For example, the main source of labelled L-phenylalanine for alkaloid synthesis in *Penicillium cyclopium* in long-term chase experiments was supposed to be protein degradation (Nover, 1979). As for higher plants, differences in the half-life of chlorogenic acid formed from exogenous labelled cinnamic acid and phenylalanine in *Xanthium* leaves suggested that breakdown of proteins provided a source of precursor for chlorogenic acid biosynthesis (Taylor, Zucker, 1966).

The first attempts to differentiate quantitatively phenylalanine of primary (derived directly from the shikimic acid pathway) origin from that of secondary (derived from protein breakdown) origin used in the formation of flavonoids were made in our laboratory (Margna et al., 1989). Buckwheat seedlings were treated with two inhibitors which are able to block selectively the formation of aromatic amino acids and deamination of phenylalanine. A comparison of levels of shikimate, free phenylalanine, and flavonoids in the treated tissues provided data on the pool sizes of both primary and secondary phenylalanine, and allowed us to make conclusions on the production of flavonoids from these metabolically different precursor pools.

So far no data are known on the direct measurements of protein turnover in buckwheat seedlings, except for a study on the breakdown of storage protein in germinating buckwheat seeds (Дунаевский, Белозерский, 1988). The main storage component of these seeds, 13S globulin, has been shown to undergo a complete degradation within the first 4 days of germination. However, the question whether catabolism of newly synthesised proteins may be sufficient to contribute significantly to flavonoid biosynthesis remains at present unanswered.

The aim of the present work was to follow changes in the content and radioactivity of both soluble and insoluble proteins of buckwheat seedlings and to compare these changes with the corresponding data on flavonoids in order to estimate the role of protein degradation in flavonoid biosynthesis.

Material and methods

Plant material

The experiments were carried out with isolated buckwheat (*Fagopyrum esculentum* Moench cv. Victoria) cotyledons excised from 80-hr-old etiolated seedlings grown in water. The detached cotyledons were incubated in a phytotron (continuous light from white fluorescent tubes, fluence rate 28 $W \cdot m^{-2}$, temperature 25 °C) in Petri dishes on filter paper moistened with a solution of [1-^{14}C]-L-phenylalanine (concentration 10 mM, specific activity 10.0—20.6 $GBq \cdot mol^{-1}$). The label was introduced into the acting solution by complementing it with a radioactive preparation of [1-^{14}C]-D,L-phenylalanine. The duration of pulse feeding was 16 hr in the first experiment and 1 hr in all subsequent series. After the pulse the treated plant material was washed with water in order to remove the external label and was then transferred either to distilled water or to a 10 mM solution of nonradioactive L-phenylalanine. Samples were taken before and at the end of the pulse feeding and at various intervals up to 72 hr after the beginning of incubation.

Analytical methods

The content of individual flavonoids (rutin, orientin, isoorientin, vitexin, and isovitexin) in ethanolic extracts was determined spectrophotometrically after their paper chromatographic separation (Margna, Margna, 1969; Margna, Vainjärv, 1983) and expressed in nmols per pair of cotyledons. The radioactivity of flavonoids was assayed in the ethanolic eluates after paper chromatography in a Beckman LS-100 liquid scintillation counter. On the basis of radioactivity data the amount of flavonoids formed from exogenous phenylalanine was calculated.

Soluble proteins were extracted in 50 mM Tris-HCl buffer (ph 8.6) containing 3 mM EDTA, 14 mM β-mercaptoethanol, 30 mM ascorbic acid, 0.5 M sucrose and 1% (v/v) Triton X-100. Extracts were separated by centrifugation. Proteins were precipitated from combined supernatants with ethanol (final concentration 67%). After staying overnight at 5 °C, the precipitates were washed with 70% and 96% ethanol and dissolved in 0.1 N NaOH (each extraction 10 min at 100 °C followed by a further 50 min extraction at room temperature).

The residue which remained after the extraction of Tris-soluble material was washed repeatedly with 96% ethanol and cold trichloroacetic acid (TCA), then extracted with 0.3 N NaOH as described above. Proteins in combined extracts were precipitated by adding crystalline TCA (concentration after neutralization 10%). After staying overnight at 5 °C, samples were washed with 96% ethanol and redissolved in 0.1 N NaOH as in the case of soluble proteins. This base-extractable fraction was referred to as insoluble proteins.

Samples were assayed for protein content (Lowry et al., 1951) using bovine serum albumin dissolved in sodium hydroxide as the standard. The radioactivity of the samples was measured in a Beckman LS-100 liquid scintillation counter.

All experiments were run in 3 replicate series. In each series, three sets of 25—30 pairs of cotyledons were assayed per time of incubation.

Results

16-hr pulse

In the first experiment, buckwheat cotyledons were treated with radioactive phenylalanine for 16 hr followed by an incubation in the presence of the cold (nonradioactive) amino acid.

At the beginning of the experiment 80-hr-old etiolated cotyledons contained an approximately equal amount of soluble and insoluble proteins (Fig. 1). During incubation in the light opposite changes in the content of these protein fractions were observed: the amount of insoluble proteins showed a clear-cut increase while the content of soluble proteins continued to decrease till the end of the experimental period. The total amount of proteins remained practically unchanged. Continuous synthesis of insoluble proteins was confirmed by a rise in their radioactivity. At the same time changes in the radioactivity of soluble proteins paralleled the decrease of their content. During 3 days of incubation this fraction lost more than 40% of its initial label. Similar changes were revealed in an analogous experiment with labelled L-lysine (results not shown).

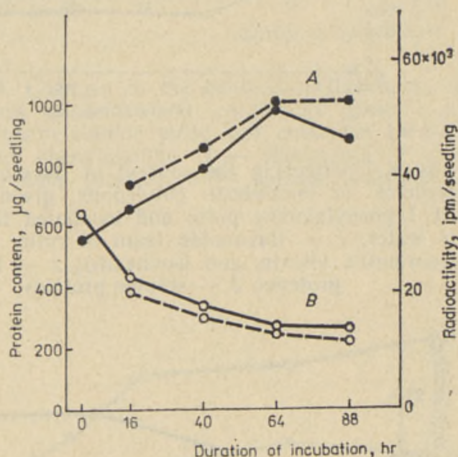

Fig. 1. Changes in the content and radioactivity of proteins in buckwheat cotyledons fed with ^{14}C-L-phenylalanine during the first 16 hr of the experiment and incubated thereafter in cold phenylalanine. *A* — insoluble proteins, *B* — soluble proteins; solid lines — content, broken lines — radioactivity.

It must be taken into consideration, however, that synthesis of soluble proteins most likely did not stop in the cotyledons but continued simultaneously with catabolic processes. The actual degradation level of soluble proteins could thus be even over 40%. Previous analyses at our laboratory have shown that the feeding of exogenous phenylalanine to buckwheat cotyledons results in an about 27-fold temporary rise in the level of the free amino acid (during 16 hr). After the subsequent transfer of the cotyledons into water, most of the excess free phenylalanine disappeared during the following 24 hr (Margna, Vainjärv, 1981). In pulse chase experiments, however, the treated material should maintain a high level of free phenylalanine up to the end of the experiment, and the initial label, although diluted, therefore remains in the tissues for a considerable time. This excess labelled phenylalanine was evidently the source of radioactivity appearing in insoluble proteins after the 16-hr pulse. Consequently, it is logical to assume that at the same time at least some label had entered soluble proteins as well, partly masking the catabolic release of labelled phenylalanine from these compounds.

In order to minimize these interpretational complications, the pulse of feeding was reduced to 1 hr in the subsequent experiments.

1-hr pulse followed by an incubation in water

In comparison with the initial level of flavonoids at the beginning of the radioactive pulse, an about twofold increase in the content of these compounds was observed in the cotyledons during this experiment. At the same time the fraction of insoluble proteins showed an almost 40%

increase while the content of soluble proteins decreased more than two-fold (Fig. 2). The sum of soluble and insoluble proteins remained relatively stable throughout the experiment.

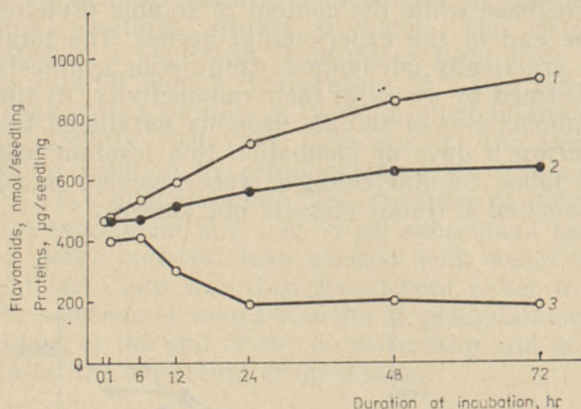

Fig. 2. Changes in the content of flavonoids and proteins of buckwheat cotyledons given a 1-hr ^{14}C-L-phenylalanine pulse and incubated thereafter in water. *1* — flavonoids (sum of rutin, orientin, isoorientin, vitexin, and isovitexin); *2* — insoluble proteins; *3* — soluble proteins.

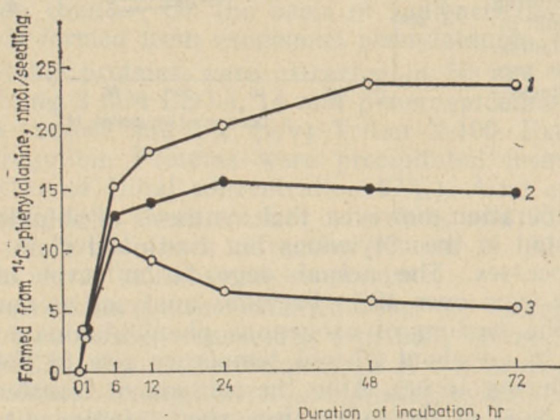

Fig. 3. Incorporation of labelled phenylalanine into flavonoids and proteins of buckwheat cotyledons given a 1-hr ^{14}C-L-phenylalanine pulse and incubated thereafter in water. *1* — flavonoids, *2* — insoluble proteins, *3* — soluble proteins.

Estimations of radioactivity in flavonoids and proteins after the 1-hr pulse served as a basis for calculating the share of corresponding fractions synthesized from exogenous material (Fig. 3). The resulting curves clearly show that within the first 5 hours after pulse feeding incorporation of labelled material into all fractions studied dominated over its possible degradational losses. After this point (6 hr after the beginning of the experiment) incorporation of exogenous phenylalanine into insoluble proteins became insignificant while soluble proteins showed a rapid loss of labelled phenylalanine. This was accompanied by a continuous incorporation of radioactive material into flavonoids. After the 6th hour of incubation up to the end of the experiment flavonoids incorporated approximately 8 nmols of exogenous phenylalanine per pair of cotyledons while at the same time soluble proteins lost about 7 nmols or 60% of the radioactive phenylalanine.

1-hr pulse followed by an incubation in cold phenylalanine

An analogous experiment with the use of a 1-hr pulse of feeding phenylalanine was performed with a different incubation medium after the pulse — in a solution of nonradioactive phenylalanine. As it could be supposed theoretically, the estimated absolute changes in the amount of individual compounds during the incubation period did not coincide exactly with the results of the previous experiment although all tendencies found previously were manifest in the present case, too. Both protein and flavonoid content reached a somewhat higher level (Fig. 4) whereas the share of compounds formed from labelled phenylalanine was less under

these experimental conditions (Fig. 5). It must be emphasized that the total amount of proteins and flavonoids produced from exogenous phenylalanine in the present experiment includes both labelled and nonlabelled material. The labelled fraction originates from the radioactive phenylalanine taken up from the initial incubation medium during the 1-hr pulse feeding period. After the beginning of the chase period the excess free labelled precursor gets gradually diluted in the second, nonlabelled source of exogenous phenylalanine. The share of nonradioactive exogenous precursor in the overall production of respective compounds cannot be estimated under present experimental conditions.

The relative decrease in the radioactivity of soluble proteins between 6 to 72 hr comprised 60% similar to the previous experiment but was extended for a longer period of time.

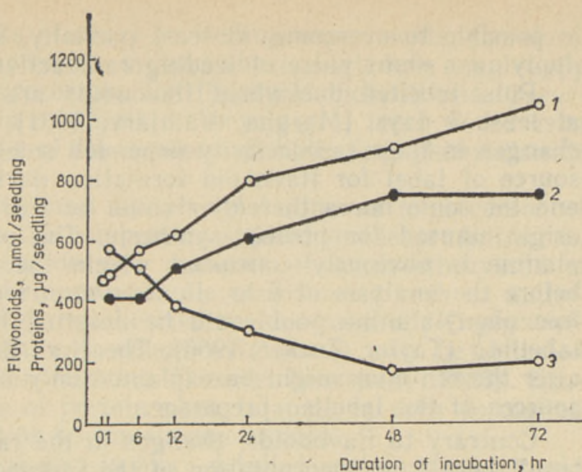

Fig. 4. Changes in the content of flavonoids and proteins of buckwheat cotyledons given a 1-hr ^{14}C-L-phenylalanine pulse and incubated thereafter in cold phenylalanine. *1* — flavonoids, *2* — insoluble proteins, *3* — soluble proteins.

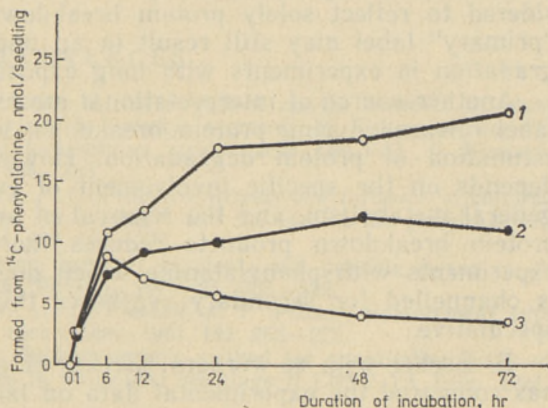

Fig. 5. Incorporation of labelled phenylalanine into flavonoids and proteins of buckwheat cotyledons given a 1-hr ^{14}C-L-phenylalanine pulse and incubated thereafter in cold phenylalanine. *1* — flavonoids, *2* — insoluble proteins, *3* — soluble proteins.

Discussion

In order to prove unequivocally that a fraction of flavonoids has been synthesized from protein-derived material one must be able to follow the route of phenylalanine from proteins to phenolics. Because of experimental and conceptual difficulties, no such attempts have been made so far.

In a standard procedure of feeding experiments the introduction of labelled phenylalanine into proteins is accompanied by its incorporation into phenolics as well. This "primary" label in phenolics interferes with the calculation of the "secondary" label of phenolic compounds that may be derived from protein degradation. On the other hand, disappearance of label from protein fractions due to protein degradation may be masked by its possible recycling or by continuous incorporation of excess exogenous phenylalanine not fully used up during the feeding period. It

is possible to overcome, at least partially, some of these difficulties by applying a short pulse of feeding radioactive precursors.

Pulse-labelled buckwheat flavonoids are metabolically stable within at least 7 days (Margna, Vainjärv, 1981), and therefore the observed changes in their radioactivity depended solely on biosynthesis. The main source of label for flavonoid formation during the short feeding period and for some hours thereafter could be free phenylalanine of exogenous origin unused for protein synthesis. The pool of labelled free phenylalanine is obviously consumed within the first hours after the pulse, before the analysis at 6 hr. In *Xanthium* leaf disks, radioactivity in the free phenylalanine pool could be detected for about 3 hours after 1-hr labelling (Taylor, Zucker, 1966). Therefore the increase in flavonoid label after the 6th hour might be explained only by incorporation of secondary sources of the labelled precursor.

Contrary to flavonoids, changes in the radioactivity of proteins could result both from incorporation of the radioactive amino acid during protein synthesis and from loss of radioactivity due to degradation of labelled proteins. For reasons discussed above, in short-term feeding experiments the availability of radioactive free phenylalanine seems to be restricted to the first hours after the pulse. The further protein synthesis could proceed only from nonlabelled precursors of endogenous or exogenous origin, and the declining radioactivity curve of soluble proteins may be considered to reflect solely protein breakdown. Protein synthesis from the "primary" label may still result in an understimation of the rate of degradation in experiments with long exposures to labelled phenylalanine.

Another source of interpretational errors might be the recycling of the label released during protein breakdown which may result in an underestimation of protein degradation. However, this phenomenon greatly depends on the specific involvement of every individual amino acid in general metabolism, and the removal of an amino acid from the pool of protein breakdown products reduces the chances of its recycling. In experiments with phenylalanine which does not accumulate in cells but is channelled for secondary syntheses this possibility seems to remain speculative.

R. Becker with co-workers (Becker et al., 1981; Winkler et al., 1981) has compared the experimental data on labelling soluble proteins in pea epicotyls with theoretical curves obtained from two different models of protein turnover: a 2-pool one assuming that protein decay products re-enter the protein precursor pool, and another one in which the amino acids released from soluble proteins remain in a degradation pool not equilibrating with the direct precursor pool. Only the latter version was in good agreement with the experimental data. The character of radioactivity changes of soluble proteins of buckwheat is of the type obtained by these authors and might correspond to the model with a special catabolic phenylalanine pool.

Insoluble proteins of buckwheat cotyledons did not reveal any obvious loss of radioactivity during 72 hr. The experiments do not enable to decide whether the label was stable, or the measured level of radioactivity represented an equilibrium between label uptake and release. In pea epicotyls no appreciable amount of radioactivity from insoluble proteins got into the degradation pool of free amino acids (Winkler et al., 1981). Considering that the major part of insoluble proteins synthesized in etiolated seedlings after the onset of irradiation consists of structural proteins of the newly formed plastids, this protein fraction should be relatively stable in buckwheat cotyledons as well. However, the possibility that some protein degradation might still occur here, too, cannot be fully excluded.

It follows from the results of our 1-hr feeding experiments, therefore, that degradation of soluble proteins in buckwheat cotyledons may really be a significant source of precursors for flavonoid biosynthesis. Occurrence of simultaneous protein synthesis or recycling of label would even increase the numerical values of the amount of liberating phenylalanine units.

It must be noted that the need for phenylalanine as a precursor of phenolics is not restricted to the compounds studied in the present work. In buckwheat cotyledons, considerable amounts of leucoanthocyanidins and lignin, and a number of minor phenolic constituents such as anthocyanins and chlorogenic acid are synthesized besides rutin and C-glycosylflavones. Some new compounds (p-coumaroylglucose) can be detected after feeding exogenous L-phenylalanine (Тохвер, Ыннепалу, 1983). Examination of the possible role of protein phenylalanine in their formation would add valuable information to the flavonoid data.

On the other hand, the next step in more detailed protein studies could be investigation of metabolic activity of different soluble proteins.

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VALKUDE LAGUNEMINE — FLAVONOIDIDE BIOSÜNTEESI EELLASTE ALLIKAS TATRAIDANDITE IDULEHTEDES

Tatraidandite idulehti töödeldi lühiajaliselt [1-^{14}C]-L-fenüülalaniiniga ja jälgiti seejärel muutusi flavonoidide ning valkude sisalduses ja radioaktiivsuses. Selgus, et pärast idulehtede ühetunnist viibimist radioaktiivse fenüülalaniini lahuses jätkus märke lülitumine kõikidesse uuritud ühenditesse veel mõne tunni jooksul. Alates kuuendast tunnist pärast katse algust täheldati lahustuvate valkude sisalduse langust ja nende radioaktiivsuse vähenemist; kolme ööpäeva jooksul kaotasid lahustuvad valgud 60% sisenenud märkest. Ümberarvestatuna katses kasutatud radioaktiivsele fenüülalaniinile vastab see kadu seitsmele nanomoolile idandi kohta. Lahustumatud valgud osutusid metaboolselt stabiilseteks. Samal ajal jätkus flavonoidide süntees. Ajavahemikul kuuendast tunnist katse lõpuni sisestus flavonoididesse veel kaheksa nanomooli märgitud fenüülalaniini idandi kohta. Kuna esialgselt idanditesse sisenenud märgitud fenüülalaniini varud olid kuuendaks tunniks juba kulutatud, võib arvata, et pärast seda flavonoididesse lülitunud radioaktiivne fenüülalaniin oli vabanenud lahustuvate valkude lagunemisel.

Лембе ЛААНЕСТ

РАЗЛОЖЕНИЕ БЕЛКОВ КАК ИСТОЧНИК ПРЕДШЕСТВЕННИКОВ ДЛЯ БИОСИНТЕЗА ФЛАВОНОИДОВ В СЕМЯДОЛЬНЫХ ЛИСТЬЯХ ГРЕЧИХИ

В опытах пульсовой метки с введением радиоактивного L-фенилаланина в семядольные листья гречихи установили, что поглощенный в течение 1-часовой экспозиции свободный меченый фенилаланин включался во флавоноиды и белки не только во время обработки, но и на протяжении нескольких часов после нее. Спустя 6 ч от начала эксперимента содержание и радиоактивность растворимых белков уменьшались, и в течение 3 суток эта фракция потеряла 60% первоначальной метки, что соответствует 7 нмолям экзогенного фенилаланина на проросток. В то же время продолжалось образование флавоноидов, и в них включалось еще 8 нмолей меченого фенилаланина на проросток. Так как запасы первоначально поглощенного меченого фениланина через 6 ч были израсходованы, можно предполагать, что на этом этапе эксперимента источником включенного во флавоноиды меченого фенилаланина было разложение растворимых белков. Нерастворимые белки оказались метаболически стабильными.