BIOCHEMICAL CHARACTERIZATION OF DIHYDROFLAVONOL 4-REDUCTASE FROM PLANT AND MICROBIAL SPECIES

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Most of the enzymes involved in the flavonoid biosynthesis pathway have been functionally expressed in microorganisms such as bacteria and yeast (3, 5). This has allowed the elucidation of the biochemical properties of several enzymes as well as the metabolic pathways that lead to the biosynthesis, among others, of anthocyanins (5) and proanthocyanins (6).

Dihydroflavonol 4-reductase (DFR) plays a dominant role in the formation of colored pigments (anthocyanins) and plant-defense molecules (tannins). The first suitable *in vitro* expression system for this enzyme was recently developed using yeast (3). Here we demonstrate the functional expression of various plant DFRs in *E. coli*, something that has consistently failed in the past (3). Since *E. coli* is the bacterial species of preference for many applications in the biotechnology industry, our results open the way for the construction of the metabolic circuits that lead to anthocyanins and proanthocyanins.

The *in vitro* biochemical studies presented here show that Dihydroquercetin (DHQ) and Dihydromyricetin (DHM) are the universal substrates for the recombinant DFR, as it has also been demonstrated for many native DFR enzymes isolated directly from plants. We also demonstrated that Dihydrokaempferol (DHK) was accepted by recombinant DFR isolated both from plants accumulating pelargonidin derivatives, such as strawberry (4), as well as from some that do not. For example, recombinant DFR from *A. thaliana* was able to utilize dihydrokaemferol, albeit with low activity, even though pelargonidin has never been detected in *A. thaliana* plants. This data is in agreement with a recent investigation of *A. thaliana* DFR which concluded that the enzyme is capable of utilizing dihydrokaemferol in Arabidopsis plants only when flavonoid 3-hydroxylase- the enzyme that hydroxylates flavanones and dihydroflavonols- is inactive.

One of the most interesting findings of the present work is that cyanidin rather than pelargonidin is the dominant anthocyanin pigment in *I. purpurea* and *I. nil* (7). However, unlike a recent report by Zufall & Rausher (7) to the opposite, in our hands the recombinant DFR from *I. purpurea* gave no leucopelargonidin when DHK was used as a substrate. It is very likely that this inconsistency between our results and the ones reported by Zufall & Rausher is due to the use of a different *I. purpurea* cell line for the isolation of the DFR cDNA. Zufall & Rausher demonstrated that even though the *I. purpurea* and *Ipomoea quamoclit* DFRs have a 95% similarity on the DNA and 93% similarity on the DNA level, the later can only utilize DHK (and not DHQ) as a substrate. In our current work, we demonstrate that DFR isolated from *I. nil* can utilize both DHQ and DHK as substrates, even though DHK is reduced at a much lower rate than DHQ. This difference in substrate specificity occurs despite the fact that the *I. nil* and *I. purpurea* DFR used in this study show 95% similarity at the DNA level and 96% similarity at the amino acid level. It is possible that evolutionary changes in the enzymes from *I. purpurea* and *I. nil* account for the substrate specificities demonstrated. A similar case, attributed to evolutionary differences has been demonstrated for the DFR enzymes from *I. purpurea* and *I. quamoclit* (7). The *I. purpurea* and *I. nil* enzymes used in our studies differ in 13 amino acids located throughout the proteins as well as a 17 amino acid insertion in the *I. nil* protein at its C-terminus. Interestingly enough, this insertion is very similar to the insertion that Zufall and Rausher report for the *I. quamoclit* DFR enzyme- an enzyme that can efficiently reduce DHK but not DHQ (7). What is even more surprising is that no differences were found between the *I. nil* and *I. purpurea* enzymes in the 13 amino acid region that has been implicated in determining the substrate specificities DFRs demonstrate (2).

Finally, all recombinant proteins that we tested also exhibited FNR activities. In general, flavanones were not as efficiently reduced as dihydroflavonols. The results showed that eriodictyol served as a universal substrate for all recombinant proteins. Unlike the earlier report on the flavanone reductase (FNR) activity of the DFR enzymes from *M. domestica* and *P. communis* (1) all the DFR proteins that could accept DHK were also able to reduce NAR. However, apiferol was not detected in assays using *Arabidopsis* DFR, which was not surprising considering the low activity towards DHK.

In conclusion we have demonstrated that DFR proteins functionally express in *E. coli,* something that excludes the need for post-translational modifications that could not take place in prokaryotes. This opens up the possibility of more thorough biochemical studies of these important enzymes as well as the construction of the metabolic network that lead to anthocyanins and proanthocyanins in prokaryotic species. In all cases, the FNR activity is a ubiquitous property of plant DFR enzymes. Furthermore, we demonstrate the possibility that a 17 amino acid insertion at the C-terminus of the protein could be involved in the determination of substrate specificities that some of the DFR enzymes demonstrate. Our immediate goal is to further investigate this possibility by generating chimeric and mutant enzymes as well as the elucidation of the crystal structure of this protein.

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