Plant Cell Reports

Alternative final steps in berberine biosynthesis in *Coptis japonica* cell cultures

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Received October 30, 1987 – Communicated by K. Hahlbrock

ABSTRACT

In **Coptis japonica** cell cultures an alternative pathway has been discovered which leads from (S)-tetrahydrocolumbamine via (S)-canadine to berberine. The two enzymes involved have been partially purified. (S)-Tetrahydrocolumbamine is stereospecifically transformed into (S)-canadine under formation of the methylenedioxy bridge in ring A. This new enzyme was named (S)-canadine synthase. (S)-Canadine in turn is stereospecifically dehydrogenated to berberine by an oxidase, (S)-canadine oxidase (COX), which was partially purified (25-fold). This enzyme has many physical properties in common with the already known (S)-tetrahydroprotoberberine oxidase from **Berberis** but grossly differs from the latter enzyme in its co-factor requirement (Fe) and its substrate specificity. Neither (S)-norreticuline nor (S)-scoulerine serves as substrate for the **Coptis** enzyme, while both substrates are readily oxidized by the **Berberis** enzyme. The four terminal enzymes catalyzing the pathway from (S)-reticuline to berberine are housed in **Berberis** as well as in **Coptis** in smooth vesicles with a density of ρ = 1.14 g/ml. These vesicles have been enriched and characterized by electron microscopy.

INTRODUCTION

Berberine is the first plant alkaloid whose biosynthesis is completely known at the enzyme level (Zenk et al., 1985). Recently the early steps in the pathway leading from tyrosine to reticuline, the universal precursor for isoquinoline alkaloids, had to be revised (Stadler et al., 1987) in that norcoclaurine was recognized as the first alkaloid intermediate in reticuline biosynthesis (Loeffler et al., 1987). The berberine pathway has been worked out mainly using cell cultures of different **Berberis** species as the experimental material. Cell suspension cultures of **Coptis japonica**, on the other hand, have been optimized to such a degree that the commercial production of berberine seems now feasable (see Fujita and Tabata, 1987). In order to understand the alkaloid biosynthesis at the biochemical and cytological level in this tissue we set out to compare the pathway for berberine formation in **Coptis** with the established pathway in our **Berberis** system. This study was justified due to the report of Yamada and Okada (1985) on a crude enzyme from **Coptis** which converted (S)-tetrahydroberberine (= (S)-canadine) to berberine. This unpurified enzyme was strikingly different from the (S)-tetrahydroprotoberberine oxidase (STOX) reported from our laboratory (Amann et al., 1984) in that the **Coptis** enzyme dehydrogenated only (S)-canadine while other tetrahydroprotoberberines were reported to be inactive. In further contrast to the STOX enzyme, their enzyme did not produce hydrogen peroxide but rather H_2O as one of the reaction products. Our analysis of the **Coptis** system reported here led to the surprising result that the terminal two steps in the biosynthesis of berberine in **Berberis** and **Coptis** are biochemically completely different while similar at the cytological level.

MATERIAL and METHODS

CELL CULTURES - **B. stolonifera** was cultivated in Linsmaier and Skoog (1965) medium as previously described (Amann et al., 1986), and **C. japonica** under conditions as given by Fukui et al. (1982).

ENZYME PREPARATION - Cells of **C. japonica** were harvested after 10 days of cultivation and frozen in liquid nitrogen. Typically 100 g fresh weight were thawed while stirring in 200 ml 10 mM phosphate buffer (pH 7.5, 5 mM ß-mercaptoethanol). After 30 min the mass was pressed through cheese-cloth and centrifuged for 10 min at 13 500 x g. The clear supernatant was applied to an XAD-2 column (XAD-2, 0.31 - 1 mm, Serva Heidelberg, column: 2.5 x 15 cm, flow rate: 100 ml/h), equilibrated with 10 mM phosphate buffer (pH 7.5, 5 mM ß-mercapotethanol). The eluate of the column (230 ml) was applied to a DEAE-Sephacel column (Pharmacia, 2.5 x 18 cm, flow rate 35 ml/h) equilibrated with the buffer described above. The protein was eluted with a linear gradient of KCl (0 - 1 M KCl, 8 h) in fractions of 5 ml. The oxidase was detected in fractions 125 - 142 (92 ml) which were pooled and dialyzed against 10 mM phosphate buffer (pH 7.5, 5 mM ß-mercaptoethanol). The dialyzed enzyme solution was applied to a QAE-column (QAE fast flow, FPLC-system Pharmacia, 1.0 x 10 cm) at a flow rate of 1 ml/min equilibrated with the standard phosphate buffer. The enzyme was eluted with a linear gradient from 0 - 0.3 M KCl (70 min, flow rate: 1 ml/min) in 1 ml fractions. The enzyme was detected in fractions 44 - 64 (20 ml), pooled and dialyzed again against the standard buffer. The resulting protein solution was bound to a red A-column (Procion red (Ciba-Geigy) bound to TSK-gel (Merck)) at a flow rate of 15 ml/h (column: 1.5 x 30 cm, equilibrated with standard buffer). The protein was incubated on the column for 2 h and afterwards the enzyme could be eluted with the equilibration

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buffer (36 ml), about a 25-fold purification resulted with a specific activity of 6.5 pkat/mg. The molecular weights of the enzymes were determined by gel filtration HPLC (G-3000 SW, LKB, 21.5 x 600 mm). The isoelectric point was determined by preparative isoelectric focusing in a granulated flat agarose gel from pH 4.5 - 6.0 (LKB).Protein determinations were performed according to Bradford (1976).

ENZYME ASSAYS - The reaction mixture (250 µl) included 25 µmoles borate buffer (pH 8.9), 1 nmole (R,S)-[8,14-tritium]-canadine (20 000 cpm) and enzyme (up to 300 µg protein). The assay mixture was incubated for 20 min at 30°C and the reaction terminated by addition of 300 µl dextran coated charcoal (1 g/10 ml). The suspension was centrifuged for 5 min in an Eppendorf centrifuge and an aliquot of 250 µl removed for scintillation counting (Rotiszint 22, Roth). The optical assay was performed using the stereochemically pure isoquinoline alkaloids according to Amann et al. (1986). (S)-Canadine synthase was assayed as stated previously (Rueffer and Zenk, 1985), however, using (R,S)-[3-methoxy-tritium]-tetrahydrocolumbamine.

VESICLE ISOLATION AND ELECTRON MICROSCOPY - The vesicles were isolated by isopycnic sucrose density gradient centrifugation and characterized by electron microscopy according to Amann et al. (1986). Atomic absorption spectroscopy (graphite column mode) was performed on a Perkin Elmer 1100B instrument.

RESULTS

ENZYMOLOGY OF BERBERINE FORMATION - **C. japonica** cells grown under conditions of high berberine production were used as an enzyme source. Crude protein extracts were analyzed for their ability to oxidize a variety of tetrahydroprotoberberines. Using the very sensitive tritium removal assay as well as the optical assay it could be shown that (S)-canadine was by far the best substrate (100%) followed by (S)-scoulerine (16%), (R,S)-tetrahydrojatrorrhizine (15%), (R,S)-coreximine (7%), (R,S)-stylopine and (R,S)-tetrahydrocolumbamine (each 6%), and by (R,S)-tetrahydropalmatine (5%). Tetrahydroprotoberberines with (R)-configuration were not utilized as substrates. The presence of various protoberberines in this crude and colored protein extract may have influenced the oxidase activity in a negative way and the results have to be interpreted more in a qualitative manner. Regardless of this potential interference, the experiment demonstrates the presence of oxidase activity similar to the one previously observed in **Berberis**. There was, however, one major difference in the substrate specificity. While the **Berberis** oxidase catalyzes the dehydrogenation of (S)-norreticuline with high activity to 1,2-dehydronorreticuline (Amann et al., 1987), the **Coptis** enzyme clearly did not transform this substrate. This behaviour as well as the observations of Yamada and Okada (1985) made it likely that the **Coptis** oxidase was different from the **Berberis** enzyme. In order to investigate this hypothesis we set out to purify this enzyme. In a three step procedure the canadine dehydrogenating enzyme from **Coptis** could be purified about 25-fold. One major obstacle in purifying the enzyme was its lability. The enzyme has a half life of about 2 days in dilute solution. During the purification procedure it was noticed that the enzyme exists in two forms, a high molecular form (ca. 150 kD) and a low one (ca. 58 kD). Both enzyme activities were isolated and tested for their substrate specificities.
As shown in Table 1, both enzyme forms catalyzed exclusively the oxidation of (S)-canadine and not of (R)-canadine. Stylopine and tetrahydrocolumbamine were dehydrogenated as well, while scoulerine and norreticuline were not. The 58 kD enzyme which was present in the cells predominantly (ca. 60%) was further characterized and compared to the enzyme from **Berberis wilsoniae.**

Substrate	Enzyme activity in % 155 kD	58 kD
(S)-Canadine	100	100
(R)-Canadine	0	0
(R,S)-Stylopine	11	26
(R,S)-Tetrahydrocolumbamine	5	5
(R,S)-Scoulerine	0	0
(R,S)-Norreticuline	0	0

Table 1 Substrate specificity of the high and low molecular weight forms of **Coptis** (S)-canadine oxidase. The assay conditions were given in material and methods.

Both enzymes are very similar; in their physical properties they show the same pH (**Berberis**: pH 8.9, **Coptis**: pH 8.7) and temperature optimum (40°C), they both occur in a higher and lower M_r form, they have similar isoelectric points (pH 5.7/pH 5.3), they catalyze the dehydrogenation of only the (S)-forms of their substrates, they both exist in specific vesicles (see below), and the products of the reaction are the quaternary protoberberine and hydrogen peroxide. One difference is, however, while the oxidase from **Berberis** is a flavin enzyme (Amann et al., 1984), the **Coptis** enzyme clearly contains iron as shown by atomic absorption spectroscopy of the electrophoretically pure enzyme and by its inhibition by orthophenanthroline (50% inhibition at 0.05 mM), a behaviour which is not shown by the **Berberis** enzyme. The major difference, however, is seen in the substrate specificity of both enzymes. While the **Berberis** enzyme is fully active towards (S)-scoulerine, (S)-canadine and (S)-norreticuline, the **Coptis** enzyme catalyzes only the oxidation of (S)-canadine. We therefore propose to call this enzyme (S)-canadine oxidase (COX) or (S)-tetrahydroberberine oxidase (Yamada and Okada, 1985), in contrast to the **Berberis** enzyme which we have termed (S)-tetrahydroprotoberberine oxidase (STOX) because of its much wider substrate range.
Having now established that (S)-canadine is the preferred substrate for the **Coptis** oxidase catalyzing the last step in berberine formation, the question arises at which stage of biosynthetic events is the methylenedioxy group present in berberine formation. It has been established beyond any doubt that in the **Berberis** system the introduction of the methylenedioxy group occurs at the quaternary alkaloid level and columbamine is transformed into berberine by action of berberine synthase as a last step in its biosynthesis (Rueffer and Zenk, 1985). Due to the substrate specificity of the oxidase (COX) the **Coptis** enzyme introducing the methylenedioxy group must act on the corresponding tetrahydroprotoberberine, namely (S)-tetrahydrocolumbamine, thus forming (S)-canadine. It has been previously known that the terminal steps of berberine biosynthesis are located exclusively in specific vesicles contained in the cells of protoberberine containing plants (Amann et al., 1986), none of these enzymes are present in the cytosol. Therefore vesicles were isolated from both **Berberis stolonifera** as well as from **Coptis japonica** employing the method of Amann et al. (1986). Incubation of the

enzyme with columbamine after breaking the vesicle membranes by brief freezing and thawing showed in the case of **Berberis** that this was the substrate for the methylenedioxy bridge-forming berberine synthase (20.1 pkat/mg protein). Columbamine, however, was absolutely not transformed by the **Coptis** vesicular enzyme. **Coptis**, however, transformed with equal efficacy tetrahydrocolumbamine to canadine (24.6 pkat/mg protein). This new enzyme which is contained in the **Coptis** vesicles catalyzes a new reaction in that the methylenedioxy bridge is introduced at the tertiary, tetrahydro stage. We name this enzyme (S)-canadine synthase. Since in the case of **Berberis** the four terminal enzymes involved in berberine biosynthesis, the berberine bridge enzyme, scoulerine-9-O-methyltransferase, as well as STOX and berberine synthase are located in specific vesicles (Zenk et al., 1985), we investigated whether this is also the case with **Coptis**. Vesicles were isolated from **B. stolonifera** and from **C. japonica** and all four enzymes analyzed in these preparations. As demonstrated in Fig. 1, both in **Berberis** and **Coptis** vesicles are present which have a density of ρ = 1.14 g/ml, as determined by sucrose density gradient centrifugation. These vesicles isolated from both species contain in each case the four terminal enzymes transforming (S)-reticuline to berberine.

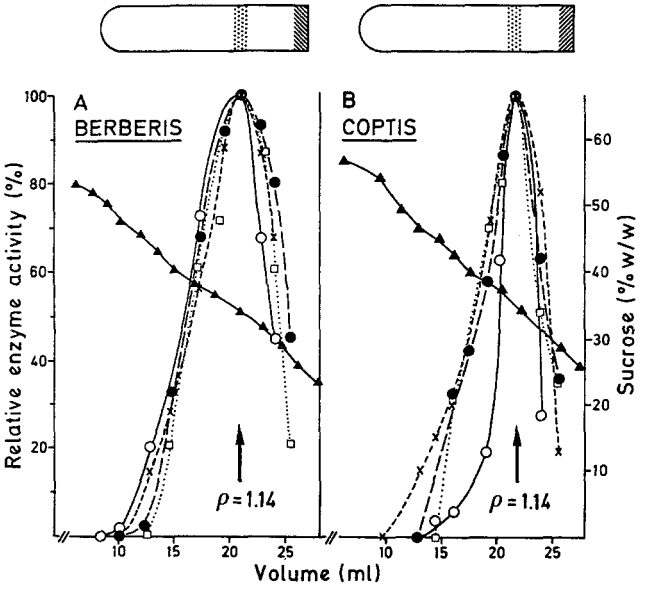

Fig. 1 Typical fractionation profile of a sucrose density gradient separation of vesicles isolated from **A. Berberis:** Berberine bridge enzyme (●--●); (S)-scoulerine-9-O-methyltransferase (□··□); (S)-tetrahydroprotoberberine oxidase (x--x); berberine synthase (o-o); sucrose concentration (▲-▲). **B. Coptis:** Berberine bridge enzyme (●--●); (S)-scoulerine-9-O-methyltransferase (□··□); (S)-canadine synthase (o-o); (S)-canadine oxidase (x--x); sucrose concentration (▲-▲).

Vesicles from **Berberis** as well as from **Coptis** contain the berberine bridge enzyme, thus catalyzing the formation of (S)-scoulerine from (S)-reticuline. The (R)-enantiomer of reticuline is not used as substrate in either system. Also present in both vesicular bands is the SAM: (S)-scoulerine-9-O-methyltransferase, yielding (S)-tetrahydrocolumbamine from (S)-scoulerine. Therefore, in both plant species these two steps leading from (S)-reticuline to (S)-tetrahydrocolumbamine are the same. After this intermediate, the pathways diverge. While in **Berberis** tetrahydrocolumbamine is first oxidized and then the methylenedioxy bridge introduced, in **Coptis** the methylenedioxy forming enzyme acts on the tetrahydroprotoberberine first to yield (S)-canadine which is subsequently oxidized to the final product of both pathways, berberine.

It has previously been found by electron microscopic observation (Yamamoto et al., 1986) that berberine producing strains of **C. japonica** contain an abundance of cytoplasmic vesicles 0.5 - 1 µm in diameter. These vesicles are obviously similar or identical with those vesicles isolated from **Berberis** and three other alkaloid containing members of different plant families which have been shown to contain exclusively all the enzymes necessary for protoberberine formation (Amann et al., 1986). We therefore attempted here a direct comparison of the functionally characterized vesicles (see above) of **C. japonica** with those isolated from **B. stolonifera** both of which have a density of ρ = 1.14 g/ml. Electron micrographs of the vesicle fraction are shown in Fig. 2.

As can be seen, there is an abundance of vesicles 0.1 - 1 µm in diameter which make up this vesicular band. They undoubtedly contain the enzymes for protoberberine synthesis. As has been observed previously for **Berberis** (Amann et al., 1986), the **Coptis** vesicles are also yellow in color and chromatographic analysis shows that they contain berberine, coptisine, palmatine, and jatrorrhizine in the same ratio as found in vacuoles (data not shown). This indicates that the vesicles are the origin of synthesis of protoberberines and are also involved in the transport of these quaternary alkaloids.

DISCUSSION

The pathway to berberine had been previously completely worked out at the enzyme level using mainly **Berberis** cell cultures (Zenk et al., 1985). The main feature of the **Berberis** pathway is the 5-step formation of (S)-reticuline from dopamine and 4-hydroxyphenylacetaldehyde and the subsequent entrance of this central intermediate into a smooth vesicle. (S)-Reticuline, having entered this compartment, is immediately transformed to (S)-scoulerine which is subsequently methylated at the 9-O-position to yield (S)-tetrahydrocolumbamine. This intermediate in **Berberis** is first oxidized by STOX to columbamine and subsequently in a final step the methylenedioxy group is formed at the level of the quaternary protoberberine to result in berberine. This route, as can be judged from the catalytic properties of the STOX enzyme, is realized in the plant families **Berberidaceae**, **Papaveraceae**, and **Menispermaceae**. It seems to be therefore the main pathway in the plant kingdom. In this paper, however, we present an alternative route to berberine which corresponds to a previously postulated but experimentally, at the enzyme level, not proven pathway (Barton et al., 1965). Yamada and Okada (1985) have made the important observation that in crude extracts of **C. japonica** an oxidase is found which is in its properties different from the previously described STOX enzyme (Amann et al., 1984): the **Coptis** enzyme dehydrogenates exclusively (S)-canadine and in contrast to the **Berberis** oxidase does not yield hydrogen peroxide but water. Indeed, we confirm in this paper that the **Coptis** oxidase is different from the **Berberis** oxidase. However, the original observations on the **Coptis** oxidase have to be modified. First, in the crude **Coptis** extract there are several oxidases acting on tetrahydroprotoberberines. The main oxidase described in this paper was shown to dehydrogenate mainly (S)-canadine, but in contrast to previous assumption, also acted on other (S)-tetrahydroprotoberberines such as stylopine and tetrahydrocolumbamine. The main difference between the **Coptis** and the **Berberis** oxidase is, however, that

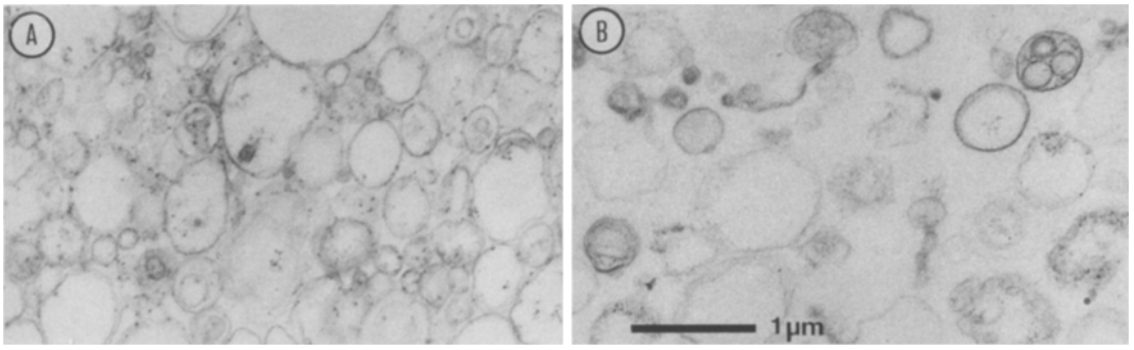

Fig. 2 Electron micrographs of ultra thin sections of the particulate fraction after sucrose density gradient centrifugation. This fraction is predominantly composed of vesicles with a diameter ranging from 0.1 to 1.0 µm. **A. Coptis; B. Berberis.**

the latter oxidizes with high efficiency norreticuline and scoulerine. Both enzymes produce as one of the reaction products hydrogen peroxide. This fact was missed by Yamada and Okada (1985), since these authors used crude enzyme extracts from **Coptis** probably containing catalase, which degraded the reaction product. Both enzyme systems are housed in smooth vesicles which are the site of synthesis of protoberberines and also seem to be the carriers to transport these quaternary alkaloids to the vacuoles, the final storage compartment. In spite of this and the physical similarities of both enzymes as shown in Fig. 2, the final steps of berberine biosynthesis in the **Berberis** and the **Coptis** system are fundamentally different as shown in Fig. 3.

The fact that there are two alternative pathways for one and the same secondary plant product should serve as a warning to us not to generalize pathways even if this metabolic route is completely established at the enzyme level for one species. This fact will increase the number of biotechnologically interesting enzymes involved in secondary metabolism in plants, over the enormous number already expected by assuming that each product is formed only by one and the same pathway.

ACKNOWLEDGEMENT - This work was supported by Sonderforschungsbereich 145 of Deutsche Forschungsgemeinschaft, Bonn, and Fonds der Chemischen Industrie. We thank Dr. T. M. Kutchan for her linguistic help in the preparation of this manuscript and Miss S. Kunz for excellent technical assistance.

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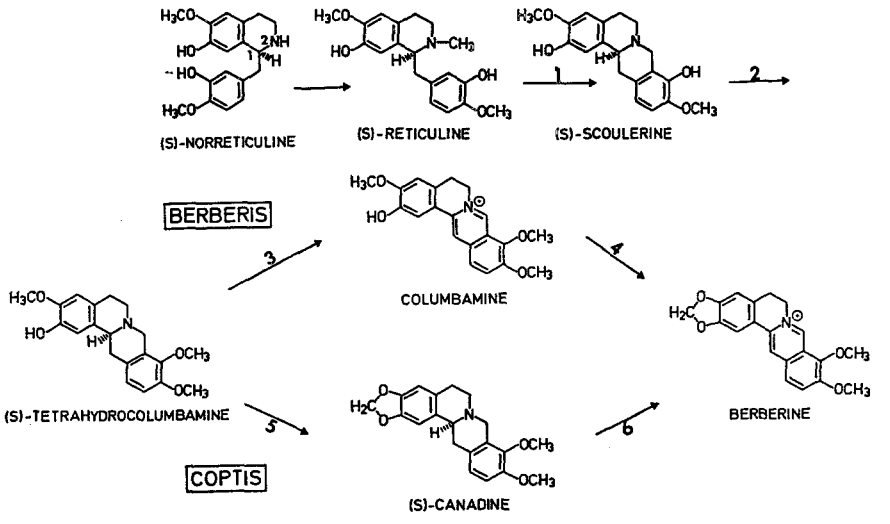

Fig. 3 Biosynthetic sequence leading from (S)-reticuline to berberine catalysed by two common enzymes: **1.** berberine bridge enzyme, **2.** (S)-scoulerine-9-0-methyltransferase, and two enzymes specific for the **Berberis** pathway: **3.** (S)-tetrahydroprotoberberine oxidase and **4.** berberine synthase. The alternative **Coptis** pathway is characterized by **5.** (S)-canadine synthase and **6.** (S)-canadine oxidase.