Characterization of Vacuolar Transport of the Endogenous Alkaloid Berberine in Coptis japonica

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Alkaloids comprise one of the largest groups of plant secondary metabolites. Many of them exhibit strong biological activities, and, in most cases, they are accumulated in the central vacuole of alkaloid-producing plants after synthesis. However, the mechanisms involved in alkaloid transport across the tonoplast are only poorly understood. In this study, we analyzed the vacuolar transport mechanism of an isoquinoline alkaloid, berberine, which is produced and accumulated in the vacuole of cultured cells of Coptis japonica. The characterization of berberine transport using intact vacuoles and a tonoplast vesicle system showed that berberine uptake was stimulated by Mg/ATP, as well as GTP, CTP, UTP, and Mg/pyrophosphate. Berberine uptake was strongly inhibited by NH4+ and bafilomycin A1, while vanadate, which is commonly used to inhibit ATP-binding cassette transporters, had only a slight effect, which suggests the presence of a typical secondary transport mechanism. This is contrary to the situation in the plasma membrane of this plant cell, where the ATP-binding cassette transporter is involved in berberine transport. Model experiments with liposomes demonstrated that an ion-trap mechanism was hardly implicated in berberine transport. Further studies suggested that berberine was transported across the tonoplast via an 

H+/berberine antiporter, which has a $K_m$ value of 43.7 $\mu$M for berberine. Competition experiments using various berberine analogs, as well as other classes of alkaloids, revealed that this transporter is fairly specific, but not exclusive, for berberine.

Alkaloids, which are nitrogen-containing low-$M_r$ substances, are a major class of plant secondary metabolites that show a wide variety of chemical structures and biological activities. Some are used in both modern and traditional medicine. For instance, vincristine and taxol are widely used as anticancer drugs and morphine is an indispensable analgesic in clinical medicine. They also play important roles in plants as endogenous biological barriers to protect against pathogens or herbivores due to their strong antimicrobial, antifungal (Iwasa et al., 1998; Mahady et al., 2003), and anti-insect activities (Steppuhn et al., 2004). The importance of alkaloids to both humans and plants has attracted the attention of many researchers. Many systems for the production of alkaloids have been established (Kutchan, 1995) and biosynthetic pathways have been actively studied (Facchin, 2001). However, the transport and accumulation mechanisms of these alkaloids in plant cells are still largely unknown.

On the other hand, alkaloid-producing plant cells seem to be insensitive to their own metabolites, probably because they have a detoxification mechanism to prevent the cytotoxicity of alkaloids. However, such plant detoxification of secondary metabolites is not well understood. One possible explanation is the compartmentation of alkaloids into the plant vacuole. Many alkaloids are presumed to be synthesized in the cytosol and on the endoplasmic reticulum (ER) and then transported through the tonoplast to be sequestered in the vacuolar matrix. A simple model for alkaloid accumulation in plant vacuoles is the ion-trap mechanism (Matile, 1976). According to this model, alkaloids, which are in a lipophilic state in the neutral pH of cytosol, can freely pass through the tonoplast by simple diffusion. However, under the acidic conditions inside the vacuole, they are protonated to form hydrophilic cations, and therefore become unable to permeate through the membrane and are trapped inside. Contrary to this hypothesis, several studies have demonstrated that the vacuolar transport of alkaloids was managed by specific carriers in an energy-requiring manner, which may involve a proton-antiport carrier system (Deus-Neumann and Zenk, 1984, 1986; Mende and Wink, 1987; Wink and Mende, 1987). A third possible mechanism was recently proposed; i.e. a directly energized transporter,
the ATP-binding cassette (ABC) transporter, is present
on the tonoplast and might be responsible for the
transport of secondary metabolites (Klein et al., 2000).
However, there have been few detailed biochemical
analyses of the vacuolar membrane transport of en-
dogenous alkaloids.

To study the mechanism of alkaloid transport in
plant vacuoles, we have been using a yellow isoquino-
line alkaloid, berberine, which was the first alkaloid to
have its biosynthesis fully described at the enzyme
level (Zenk, 1995). Berberine is stable and widely used
as an antibacterial and antimalaria drug in many
countries (Yamamoto et al., 1993; Iwasa et al., 1998).
While it is one of the most widely occurring alkaloids
in many plant families, we used cell cultures of Coptis
japonica (Ranunculaceae) as a model system. C. japon-
ica is a perennial medicinal plant grown in Asian
countries, and berberine is highly accumulated in its
rhizome as its main alkaloid. Cultured C. japonica
cells produce berberine and accumulate it exclusively in
the vacuole (Sato et al., 1990, 1994). Moreover, exogenous
berberine added to the culture medium is actively
taken up by C. japonica cells (Sato et al., 1990, 1994),
and is also transported into the vacuole and stably
accumulated in the lumen (Sato et al., 1993; Sakai et al.,
2002). Our previous studies to identify the berberine
transporter in C. japonica cells showed that CjMDR1,
a multidrug-resistance protein-type ABC transporter,
was involved in the transport of berberine at the
plasma membrane (Shitan et al., 2003). However, it is
still unclear how berberine is transported across the
tonoplast.

In this study, we analyzed the vacuolar transport
mechanism of berberine by using intact vacuoles and
tonoplast vesicles of C. japonica cells. Berberine trans-
port into tonoplast vesicles was stimulated by Mg/ATP
and was inhibited by NH₄⁺ and bafilomycin A₁,
but not by vanadate, which suggested that uptake
involved an H⁺/berberine-antiporter.

RESULTS
Uptake of [³H]Berberine by Isolated Vacuoles

To clarify the mechanism of the vacuolar uptake
of berberine, we first isolated intact vacuoles from
2-week-old C. japonica suspension cultures. Vacuoles
of C. japonica cells appear to be bright yellow because
of the high concentration of endogenous berberine,
which reaches more than 72 mM. Their diameter was
11 to 28 μm and the average volume was calculated to
be 4.9 pL. The vacuolar pH of C. japonica cells was
approximately 5.5. These isolated vacuoles were incu-
bated with radiolabeled berberine in an assay buffer
of pH 7.8, which mimicked the endogenous state of
vacuoles in terms of the ΔpH across the tonoplast.
Incubated vacuoles were passed through a silicon oil
layer by centrifugal filtration and [³H]berberine in the
vacuoles recovered from the bottom layer was esti-
mated by scintillation counting. Burst vacuoles, which
were prepared by sonication in the presence of Triton,
were used as a negative control. As a result, the clear
uptake of berberine by intact vacuoles was observed in
the absence of Mg/ATP, and berberine uptake was
somewhat stimulated by Mg/ATP, but not by Mg/
ADP and Mg/ATP-γ-S, a nonhydrolyzable analog of
ATP (Fig. 1). This suggests that berberine is apparently
taken up by intact vacuoles, probably due to the preexis-
ting ΔpH in the assay condition (incubation buffer, pH 7.8),
and that ATP enhances berberine transport into the vacuole,
whereas the effect is seemingly indirect. However, further detailed analyses of
berberine uptake using various inhibitors were ham-
pered because of the instability of the vacuoles, i.e. it
was very difficult to perform the assays using solvents
such as dimethyl sulfoxide (DMSO). For this reason,
we decided to further analyze berberine transport
using tonoplast vesicles.

ATP-Dependent Uptake of Berberine by
Tonoplast Vesicles

We purified tonoplast vesicles by fractionating mi-
crosomes of C. japonica cells on a discontinuous Suc
density gradient. Vanadate-sensitive ATPase and

Figure 1. Effects of Mg/ATP and different compounds on the uptake of
berberine into isolated C. japonica vacuoles. A, Isolated protoplasts of
C. japonica cells. B, Isolated vacuoles of C. japonica cells. Bars = 50
μm. C, Vacuoles were incubated in the presence of 1 μM [³H]berberine
and 5 mM of the compounds listed. The pH of the incubation medium
was adjusted to 7.8 and berberine uptake was measured after 20 min.
Double bars indicate the data of two independent experiments. In the
negative control, burst vacuoles were used for berberine uptake.
KNO₃-sensitive ATPase activities were measured as marker enzymes for plasma membrane and tonoplasts, respectively. Tonoplast-rich membrane vesicles were recovered at 0/20% Suc fraction (Fig. 2A). The enrichment of the tonoplast in this fraction was also confirmed by immunodetection of vacuolar pyrophosphatase (V-PPase), a tonoplast marker, whereas plasma membrane H⁺-ATPase and luminal binding protein (BiP), an ER marker, sedimented at positions different from V-PPase (Fig. 2B). The tonoplast vesicle fraction showed clear [³H]berberine uptake, while other fractions, which contain a large portion of plasma membrane and ER, did not show the uptake activity even in the presence of ATP (Fig. 2C). These results suggested that the tonoplast vesicles had the berberine transport activity, which was not observed in plasma or ER membranes. Using the tonoplast vesicles, we measured the time course of [³H]berberine uptake (Fig. 2D). Rapid uptake was observed in the presence of Mg/ATP, whereas no uptake was seen in the absence of Mg/ATP, which indicates that Mg/ATP is required for berberine uptake in this assay system. This berberine transport activity was reproducibly observed among each preparation of the tonoplast vesicles, i.e. the uptake within the range between 12 and 18 pmol/µg protein was observed for 5 min in the presence of Mg/ATP.

We also investigated the pH dependency of berberine uptake by this assay method. The tonoplast vesicles were incubated in reaction mixtures of different pH values within the range of physiological conditions, from pH 6.5 to 8.0. Figure 3 shows that berberine uptake rose as pH increased from 6.5 and had an optimum pH around 7.5, which is in the common cytosolic pH.

Next, we investigated whether the effect of energization on the vacuolar transport of berberine was exclusive to Mg/ATP, or if it could be substituted by other nucleotides. Figure 4 shows that, although some of these effects were only slight, Mg/GTP, Mg/CTP, and Mg/UTP could each stimulate berberine uptake (23%, 14%, and 9% of Mg/ATP, respectively), while the addition of Mg/AMP had no effect, confirming that the hydrolysis of these nucleotide triphosphates was necessary for berberine uptake. Notably, Mg/pyrophosphate was also able to stimulate berberine uptake.

Figure 2. Time-dependent uptake of berberine into tonoplast vesicles. A, Tonoplast vesicles were prepared by Suc gradient fractionation. Vanadate-sensitive ATPase (●) and KNO₃-sensitive ATPase (○) activities were measured as marker enzymes for plasma membrane and tonoplast content, respectively. B, Plasma membrane H⁺-ATPase, V-PPase, and BiP were immunodetected to confirm the purity of tonoplast vesicles. C, Each membrane fraction was incubated with 50 µM of [³H]berberine in the presence (●) or absence (○) of 5 mM Mg/ATP. D, Tonoplast vesicles (0/20% Suc fraction) were incubated with 50 µM of [³H]berberine in the presence (●) or absence (○) of 5 mM Mg/ATP. Berberine uptake was monitored at 25°C, as described in “Materials and Methods.” Data are an average of three replicates and are presented as pmol/µg protein.
uptake to about 35% the amount of Mg/ATP, suggesting the involvement of a transport mechanism that depends on the H\textsuperscript{+}-gradient across the tonoplast.

Table I shows a comparison of berberine uptake in the presence of various inhibitors. The addition of NH\textsubscript{4}Cl, which abolishes the ΔpH across the membrane, inhibited berberine uptake by 80%, and the V-type ATPase inhibitor bafilomycin A1 reduced uptake by 68%. On the other hand, vanadate, which is a typical inhibitor of ABC transporters, exhibited only 10% inhibition, which excludes the possible involvement of an ABC transporter as the main transport mechanism of berberine into vacuoles of C. japonica.

Basic compounds, such as amines and alkaloids, may be trapped by membrane vesicles via a differential pH shift, i.e. when the inside of a membrane vesicle is acidic, an amine compound that permeates through the lipid layer under the neutral condition of the cytosol is protonated under the acidic conditions inside vesicles, which forms a polar quaternary amine that cannot permeate back across the lipid layer and is thus trapped inside (ion-trap mechanism). Since the quaternary nitrogen atom of berberine is part of an aromatic six-member ring structure, berberine does not take protonation form, the positive charge remaining stable under physiological conditions (structure is shown in Table II), and the permeability of berberine through the lipid bilayer is thought not to be very high. To exclude the possibility of an ion-trap mechanism for the transport and accumulation of berberine by vacuoles, we measured the uptake of berberine into liposomes that contain a stable pH gradient (intracellular acidic) and compared it to the uptake of a model compound, methylamine, which is transported into vacuoles by an ion-trap mechanism (Churchill and Sze, 1983). In the presence of the pH gradient, methylamine was taken up and accumulated in liposomes, i.e. 6-fold accumulation was observed compared to that in the absence of the pH gradient. Berberine accumulation with the pH gradient was 1.6-fold higher than that without the pH gradient (Fig. 5).

The reason for the marginal difference in berberine accumulation in liposomes between in the presence and absence of the pH gradient is not clear. One possible explanation is that membrane potential or berberine solubility might be affected by NH\textsubscript{4}Cl, which altered hydrophobic interaction between berberine and liposome, resulting in lower accumulation in the absence of the pH gradient. Using the tonoplast vesicles, on the other hand, berberine accumulation with ATP was approximately 4-fold higher than that without ATP, as shown in Figure 2D. Taken together, berberine was unlikely to be accumulated by an ion-trap mechanism, but rather a specific carrier was necessary for berberine transport in the C. japonica tonoplast.

Kinetic Determinants and Competitive Inhibition of Vacuolar Transport of Berberine

The uptake of berberine by tonoplast vesicles exhibited $K_m$-type saturation kinetics, as shown in Figure 6. The $K_m$ and $V_{max}$ values were calculated to be 43.7 μM and 13.5 pmol/μg protein per min, respectively.

Next, we carried out a competitive inhibition experiment in the berberine uptake assay system. Table II shows the effects of berberine analogs, as well as nicotine and vinblastine, on [3H]berberine uptake by tonoplast vesicles. A protoberberine-type alkaloid, reticuline, and another berberine analog, palmatine, inhibited berberine uptake, whereas norlaudanosoline, tetrahydropalmatine, and a pyrrolidine-type alkaloid, nicotine, did not show competitive inhibition. Interestingly, vinblastine, which is an indole alkaloid and shows no structural resemblance to berberine, also inhibited berberine uptake. This result suggests that the antiporter shows a preference for protoberberine-type alkaloids, but is not exclusive to berberine.

### DISCUSSION

Biosynthetic reactions of alkaloids occur in various organelles in plant cells, which may vary according
to the type of the alkaloid involved, e.g. quinolizidine alkaloids of legumes appear to be biosynthesized in mesophyll chloroplasts of green leaves (De Luca and St. Pierre, 2000). However, the final intracellular destination of most biosynthesized alkaloid molecules is vacuoles. In plants, four major uptake mechanisms have been proposed for secondary metabolite transport across the tonoplast, i.e. H⁺/berberine antiporter, ion trap, conformational trap (Rataboul et al., 1985), and directly energized transport by ABC transporters (Martinoa et al., 2000). The first three use the electrochemical gradient of H⁺ across the tonoplast, whereas ABC transporters directly use the energy of Mg/ATP hydrolysis to pump their substrates into the vacuolar lumen, i.e. independent of electrochemical force. This study demonstrated that an H⁺ electrochemical gradient was necessary for berberine transport across C. japonica vacuoles. The liposome experiment showed that the ion-trap mechanism was not applicable to berberine accumulation in this plant, but rather a certain carrier-mediated transport mechanism is involved. Based on these results, we conclude that berberine was transported across the tonoplast via an H⁺/berberine antiporter, which uses the proton gradient formed by the two vacuolar proton pumps, vacuolar H⁺-ATPase and PPase, localized at the tonoplast (Fig. 7).

When the subcellular localization of biosynthetic enzymes of berberine is considered, another transport mechanism may be possible for endogenously biosynthesized berberine. The terminal steps of berberine biosynthesis are reportedly located exclusively in specific vesicles that are observed in Berberis and other berberine-producing plant cell cultures (Amann et al., 1986; Galneder et al., 1988). These vesicles are described as highly specific and unique compartments, which are probably derived from the ER, and later fuse with the central vacuole (Bock et al., 2002). A similar mechanism was also proposed for another isoquinoline alkaloid, sanguinarine, by a recent study using cultured opium poppy (Papaver somniferum) cells (Alcantara et al., 2005). According to this scheme, endogenous berberine is thought to be synthesized in these specific vesicles and transported to the vacuole by a vesicle transport mechanism. This mechanism seems to be very similar to that of storage protein accumulation in vacuoles via precursor-accumulating vesicles (Mitsuhashi et al., 2001). The antiporter mechanism for berberine transport demonstrated in this article may not conflict with vesicle-mediated transport. Precursors of berberine are more hydrophobic than berberine and the H⁺/berberine antiporter contributes to retain these intermediates inside such vesicles, since the antiporter showed relatively broad substrate specificity (Table II). When these vesicles fuse to the large central vacuole, the membrane of vesicles is integrated into the tonoplast. Molecular cloning of an H⁺/berberine antiporter and its characterization should provide further information to prove this hypothesis about the mechanism of berberine accumulation in C. japonica cells.

It is not yet clear why C. japonica cells have two different types of transporters for berberine; i.e. ABC transporter (CjMDR1) at the plasma membrane (Shitan et al., 2003) and H⁺/berberine antiporter at the tonoplast, but the difference in their Vₘₐₓ might offer one possible explanation. Compared to the antiporter at the tonoplast, CjMDR1 appears to pump berberine relatively slowly (the Vₘₐₓ of CjMDR1 for berberine is 0.08 nmol/mg protein) to the cytosol from the apoplast (Shitan et al., 2003), whereas the transport across the tonoplast by the H⁺/berberine antiporter seems to be more rapid (the Vₘₐₓ of the antiporter is 13.5 nmol/mg protein). If this difference in transport efficiency is valid for C. japonica cells in vivo, the rapid

### Table I. Effects of various inhibitors on ATP-dependent berberine uptake

<table>
<thead>
<tr>
<th>Condition</th>
<th>Berberine Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>+5 mM MgATP</td>
<td>100%</td>
</tr>
<tr>
<td>+5 mM MgATP + NH₄⁺ (10 mM)</td>
<td>20.0 ± 6.0**</td>
</tr>
<tr>
<td>+5 mM MgATP + bafilomycin A1 (1 μM)</td>
<td>32.0 ± 26.0*</td>
</tr>
<tr>
<td>+5 mM MgATP + vanadate (1 mM)</td>
<td>90.0 ± 8.0</td>
</tr>
</tbody>
</table>

### Table II. Effects of various compounds, including protoberryne alkaloids and other alkaloids, on berberine uptake

<table>
<thead>
<tr>
<th>Compound</th>
<th>Berberine Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100%</td>
</tr>
<tr>
<td>Reticuline</td>
<td>27.9 ± 17.4**</td>
</tr>
<tr>
<td>Norlaudanosoline</td>
<td>107.1 ± 17.4</td>
</tr>
<tr>
<td>Palmatine</td>
<td>62.8 ± 5.8**</td>
</tr>
<tr>
<td>Tetrahydropalmatine</td>
<td>100 ± 27.9</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>48.8 ± 23.3*</td>
</tr>
<tr>
<td>Nicotine</td>
<td>76.7 ± 57.1</td>
</tr>
</tbody>
</table>

* Asterisk indicates statistically significant difference compared to control (*, P < 0.05; **, P < 0.01).
transport by the antiporter would largely contribute to the detoxification of this cytotoxic alkaloid; i.e. the cytosolic concentration of berberine in \textit{C. japonica} cells can be kept at a low level by the scheme shown in Figure 7. Therefore \textit{C. japonica} cells are capable of safely accumulating berberine in vacuoles even if they are cultured in the presence of 750 $\mu$M berberine in the medium, which is highly toxic to tobacco (\textit{Nicotiana tabacum}) and other plant cells, but not to \textit{C. japonica} cells (Sakai et al., 2002).

In bacteria, several antiporters that accept berberine as their substrate have been reported, although Na$^+$ is the counterpart instead of H$^+$, and their roles as drug efflux pumps for the purpose of detoxification have been demonstrated. For example, NorM from \textit{Vibrio para-haemolyticus} and its homolog in \textit{Escherichia coli}, YdhE, which are both multidrug and toxic compound extrusion (MATE)-type transporters, efflux several toxic compounds, including berberine, and confer multidrug resistance to these microorganisms (Xu et al., 2003). In plants, a plasma membrane-localized MATE-type transporter of Arabidopsis (\textit{Arabidopsis thaliana}), AIDTX1, which also recognizes berberine as an exogenous substrate (Li et al., 2002), has been reported so far as an H$^+$ / berberine antiporter of \textit{C. japonica}, although the MATE family in Coptis may be different from these known MATE members since the former may only be responsible for the endogenous alkaloid, not other exogenous compounds.

Plant alkaloids are often translocated from the source organ to a sink organ (Hashimoto and Yamada, 1994; Hartmann, 1999). Berberine in \textit{C. japonica} is also translocated from root tissues to rhizome, which involves transport across several membranes, i.e. at the plasma membrane in root and rhizome and at the tonoplast in cortex cells of rhizome. Together with our previous work on CjMDR1 at the plasma membrane, this study on the tonoplast should help to clarify the entire molecular mechanism of the translocation of alkaloids in plants.

### MATERIALS AND METHODS

#### Chemicals

Chemicals used in this study were purchased from Wako Pure Chemicals (Osaka) or Nakalai Tesque (Kyoto).

![Figure 5](image)

**Figure 5.** Berberine uptake into liposomes in the presence and absence of a pH gradient. A, $[^{14}C]$Methylamine uptake into liposomes. B, $[^{3}H]$Berberine uptake into liposomes. A pH gradient was generated as described in “Materials and Methods.” Liposomes were incubated at 25°C for 10 min with 20 $\mu$M $[^{14}C]$methylamine (approximately 1 $\mu$Ci/mL) or 50 $\mu$M $[^{3}H]$berberine, and uptake was determined as described for the measurement of berberine transport. To destroy the pH gradient, NH$_4$Cl (10 mM) was added to the incubation mixture. Results are mean ± SD of three replicates. Asterisk indicates statistically significant difference (*, $P < 0.05$; **, $P < 0.01$).

![Figure 6](image)

**Figure 6.** Uptake of $[^{3}H]$berberine into tonoplast vesicles shows $K_m$-type saturation kinetics. A, Tonoplast vesicles were incubated in the presence of $[^{3}H]$berberine. B, Representative saturation experiments are illustrated as a Hanes-Woolf plot. $K_m$ value measured in three independent experiments ranged between 10 and 50 $\mu$M.
Vacuolar Transport of Alkaloid

Figure 7. Proposed model for berberine transport and accumulation. Exogenous berberine is taken up relatively slowly by CjMDR1, an ABC transporter, while the H+/berberine antipporter effluxes berberine very rapidly from the cytosol to the vacuole via a proton gradient formed by V-ATPase and V-PPase, resulting in a low berberine concentration in the cytosol.

Cultured Cells

High berberine-producing cultures of Coptis japonica, which were originally induced from the rootlets of C. japonica Makino var. dissecta (Yamabe), were maintained as described (Sato and Yamada, 1984). Two-week-old cells were used for the experiments.

Isolation of Vacuoles

Protoplasts were isolated from cultured cells by the procedure of Sato et al. (1990). Isolation of vacuoles from protoplasts was carried out by the procedure of Sato et al. (1992). Isolated purified vacuoles were used immediately for the uptake experiment.

Preparation of Tonoplast Vesicles

The tonoplast vesicles used in this study were prepared from C. japonica cells by the procedure of Rocha Facanha and de Meis (1998) with some modifications. Cells were collected and homogenized in 2 mL/g of ice-cold 0.1 M EDTA, 0.1 M Tris-HCl adjusted to pH 7.6 and autoclaved. Isolated membrane vesicles were stored at -80°C in resuspension buffer containing 10 mM MgCl₂, 2 mM ATP, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Vanadate-sensitive ATPase and KNO₃-sensitive ATPase activities were measured as marker enzymes for plasma membrane and tonoplast contents, respectively. For the berberine uptake, the membrane vesicles obtained from 0/20% Suc fraction were used as the tonoplast vesicles. The purity of tonoplast vesicles was checked by immunodetection. The antibodies used for immunodetection were against plasma membrane H⁺-ATPase, vacuolar H⁺-pyrophosphatase from Arabidopsis (Arabidopsis thaliana), and ER luminal BiP (see “Acknowledgments” for the sources of antibodies).

Preparation of [³H]Berberine

[³H]Berberine (specific activity, 7.4 mCi/mol) was prepared by labeling berberine with Na[B³H]I. First, 25 mg of cold NaBH₄ were added to 10 mL of Na[B³H]I (Amersham Biosciences, Buckinghamshire, UK) for dilution, which was dissolved in 100 µL of 0.2% NaOH. To this solution was slowly added berberine hydrochloride (50 mg; dissolved in 200 µL of ethanol; stored at 4°C for 8 h. Next, 100 mg I₂ were added to oxidize the tetrahydro-berberine and the mixture was stirred for 1 h. Excess I₂ was decomposed with 120 mg NaHSO₃ by stirring for 30 min. The berberine iodide that precipitated in the solution was recovered by filtration, and berberine chloride was formed by treatment with 200 mg AgCl. The yielded berberine chloride was further purified by preparative thin-layer chromatography, using the solvent system by the procedure of Ikuta and Inoue (1988). Berberine was recovered by elution with methanol, which was evaporated to dryness and dissolved in water for transport assay.

Measurement of Berberine Transport

Uptake of [³H]berberine by intact vacuoles was measured by the centrifuged filtration technique through silicone oil as described in the literature (Heidt and Sauer, 1971) with some modifications. Isolated vacuoles were incubated with 1 µM [³H]berberine at 25°C in vacuole resuspension buffer (0.2 M Glycinebetaine, 1 mM NaH₂PO₄, 2 mM HEPES-NaOH, 1 mg/mL bovine serum albumin, pH 7.8) for 20 min. As the negative control, burst vacuoles prepared by sonication in the presence of 2% Triton-X were used. Filtering centrifugation was carried out at room temperature using 400-µL plastic tubes. The tube contained (from the bottom) 50 µL bottom buffer (25% Percoll, 0.7% sorbitol, 2% Triton-X), 150 µL silicone oil (SH550:SH556 77:23; Toray Dow Corning Co. Ltd, Tokyo), and 70 µL vacuoles in incubation buffer, and was centrifuged at 6,000g for 1 min. The contents of the plastic tube were then frozen in liquid N₂, the bottom layer was recovered by cutting off the tube with a knife, and radioactivity was estimated by a scintillation counter (Beckman Instruments, Fullerton, CA).

Uptake of [³H]berberine by tonoplast vesicles was measured at 25°C for 5 min in 200 µL of reaction mixture containing 50 mM Tris-MES buffer (pH 7.5), 0.1 mM KCl, 5 mM Mg/ATP, 50 µM [³H]berberine, and tonoplast vesicles for 40 min, unless otherwise stated. Inhibitors and competitors were added to the above mixture to give a final volume of 200 µL. After incubation, 130 µL of the reaction mixture were loaded on a Sephadex G-50 fine spin column and centrifuged at 2,000 rpm for 2 min. The radioactivity of 100 µL of filtrate was determined by a liquid scintillation counter. The inhibitors used in this study (ammonium chloride and vanadate) were dissolved in water, whereas bafilomycin A1 was dissolved in DMSO. In the control, DMSO was added to the reaction mixture at a final concentration of 0.1%. DMSO did not affect berberine uptake at this concentration. Statistical analysis was performed using Student’s unpaired t test.

Transport Assay with Liposomes

Liposomes having transmembrane pH gradients were prepared by the procedure of Mayer et al. (1990) with some modifications. Briefly, asolectin (Wako Pure Chemicals) was dissolved in 300 mM citrate buffer (pH 5.5) at a concentration of 5 mg/mL. Multilamellar vesicles were subjected to five freeze-thaw cycles (freezing in liquid N₂ and thawing at 40°C). Transmembrane pH gradients were established by passing the vesicles through a Sephadex G-25 column (PD-10) equilibrated in 20 mM HEPES containing 150 mM NaCl (pH 7.5). Liposomes (1.25 mg/mL) were incubated at 25°C for 10 min with 20 mM [³C]methyamine (approximately 1 µCi/mL) or 50 mM [³H]berberine. The uptake of methyamine or berberine by liposomes was measured by the spin-column assay, as described above, for the measurement of berberine transport.
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LITERATURE CITED


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