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The increasing medicinal relevance of plant natural products had lead to a relatively comprehensive understanding of specific secondary biosynthetic pathways (De Luca and Laflamme, 2001; Kutchan, 1995). The use of modern techniques and methods in molecular biology and in vitro culture allows for a rapid and precise discovery of genes and enzymes involved in such processes (Kutchan, 1998). It is now realistic to attempt to discover each enzyme involve in a secondary biosynthetic pathway with the help of continuously improved biotechnological and biochemical tools. The final aim is often the deviation of the normal pathway routes to obtain tailored natural product profiles through the over expression or silencing of genes in selected points of the biosynthesis pathway (Frick et al., 2006; Larkin et al., 2006; Magnotta et al., 2006; Frick et al., 2004).

The present work was the first attempt to discover, isolate and characterize candidate cDNAs encoding enzymes involved in the biosynthetic pathway of the terpenoid isoquinoline (ipecac) alkaloids from tissue cultures of Psychotria ipecacuanha plant species.

From P. ipecacuanha plants, until now, no cDNA library or gene isolations have been reported in the literature. From Alangium lamarckii leaves, the enzyme deacetylipecoside synthase (DIS), which condenses dopamine and secologanin to the (R)-configured deacetylipecoside, was purified and partially characterized (De-Eknamkul et al., 2000) and the presence of deacetylisopecoside synthase (DIIS), which leads ultimately to the synthesis of emetine and cephaeline with the (S)-configuration, was also proposed (De-Eknamkul et al., 1997). However, clones for these key enzymes as well as for any other specific step in the ipecac alkaloid biosynthetic pathways have not yet been isolated.

4.1. Tissue culture and alkaloid content

The tissue culture technique allowed, according to the objectives of this study, a successful maintenance and multiplication of plant material. Costa Rican Psychotria ipecacuanha plants were cultured under the in vitro conditions described in Hidalgo and Palma (1993). Without additional requirements, healthy in vitro plants were grown and could enable the multiplication of more than 4000 plants per year for various uses (fig. 3.1).
The generation and multiplication of root cultures was attempted considering the literature available (Yoshimatsu and Shimomura, 1991a; Yoshimatsu and Shimomura, 1993). Nevertheless, as it is well known, there are not universal culture conditions for all plant species. It could, therefore, be possible that for the Costa Rican plant material under the examined conditions in the section (3.1.2.), the root induction required the supplementation of 0.5 - 1.0 µg/l α-naphthalene acetic acid (NAA) and the root development was optimal without growth regulators in the culture media (fig. 3.4).

Ipecac alkaloids, the principal secondary metabolites present in *P. ipecacuanha* plants, were clearly identified in both leaves and roots, separately cultivated. Several intermediates were detected by their mass spectrometric fragmentation patterns (3.2.2.), but the absolute configuration was not determined. The fact that cephaeline, emetine and ipecoside were found leads to assumption of the presence of the, so far known, biosynthetic pathways for both (R) and (S)-configurations in the cultured tissues. Although ipecoside was not detected in leaves, the presence of O-methylippecoside (and/or its isoform) might suggest the presence also of the pathway with the (R)-configuration in this tissue (fig. 1.1). In *P. ipecacuanha*, nothing is known about the transport of the alkaloids, nevertheless the results indicated that the biosynthetic pathways seem to be active in leaves as well as in roots and therefore the alkaloids are found in both independently cultivated tissues.

A comparison of alkaloid contents among different cultured tissues and parental plants showed differences in amount and composition. In roots, cephaeline was 1.1-2.7 times more concentrated than emetine, whereas in leaves the cephaeline concentration was around 5 times higher than that of emetine. In roots of parental plants, similar levels of cephaeline and emetine were found (Yoshimatsu and Shimomura, 1993). The alkaloid concentrations in the tissues analyzed herein were measured as µg alkaloid per 100 mg fresh weight material. The ratio of cephaeline to emetine indicated that 20 times more cephaeline than emetine accumulated in roots, whereas only 1.3 times more cephaeline was present in leaves. Comparing alkaloid concentrations between the tissues, cephaeline was around 5 times lower in roots than leaves; in the literature almost no differences are reported. Emetine was approximately 50 times lower in roots than in leaves (fig. 3.10). On the contrary, the literature showed 3 times more emetine in roots than in leaves (Yoshimatsu and Shimomura, 1993). It was not possible to obtain an absolute quantification of emetamine, ippecoside, O-methylippecoside, psychotrine and O-methylpsychotrine in this work due to a lack of
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standard compounds (fig. 3.7). The relative concentration determined for each alkaloid did not permit to establish a significant comparison of both tissues (fig. 3.11).

The objective of the induction assay with methyl jasmonate was to increase alkaloid production (Gundlach et al., 1992; Memelink et al., 2001) in order to facilitate alkaloid biosynthetic gene isolation based upon differential expression (Eiler et al., 1987; Roewer et al., 1992; Yamazaki et al., 2003). A clear induction effect in either roots or leaves was not observed for any of the alkaloids. In an eventual further investigation, however, the potential increase of cephaeline and emetine concentration in leaves at 48 hours after induction might be analyzed more closely.

Although the concentration of the ipecac alkaloids could not be estimated, and induction in the alkaloid production was not detected, the identification of several ipecac alkaloids in tissue cultures of *P. ipecacuanha* indicate the presence an active biosynthetic pathway. The probability of isolating clones involved in the alkaloid biosynthesis in these tissues was considered probable.

4.2. cDNA library construction

The cDNA library construction was a critical step to obtain clones involved in ipecac alkaloid biosynthesis. In the preparation of the library, the source tissue for RNA isolation should have a maximal expression of the alkaloid biosynthetic genes compared to house keeping genes. This facilitates transcript (cDNA) identification. Different methods can be applied to increase the chance of detecting genes of interest, including the use of a specific developmental stage, selection of plant tissues in which biosynthesis is high, the use of gene induction by addition of compounds such as methyl jasmonate, as well as technical methods like the analysis of gene expression by array technologies in which transcriptome analysis and alkaloid profiling are compared (Ziegler et al., 2006; Shukla et al., 2006; Zulak et al., 2006) or the correlative analysis of extensive metabolic profiling against the gene expression profiling (Fridman and Pichersky, 2005).

A cDNA library was generated from *P. ipecacuanha* root cultures. Advantages considered for this tissue include the avoidance of the highly expressed genes of the photosynthetic apparatus (clearly observed in the work of Shukla et al., 2006), the higher alkaloid production found in roots than in unorganized tissues such as callus or cell cultures (Jha et al., 1988; Teshima
et al., 1988) and the detection of at least five ipecac alkaloids in the Costa Rican *P. ipecacuanha* root cultures. Ultimately, the cDNA library was generated from mRNA isolated from cultured roots and estimated by plaque titration to contain 30,000 primary clones.

### 4.3. cDNA identification and analysis

The expressed sequence tag (EST) library was prepared from a randomly selected sample of the cDNA library in order to collect enough sequence information on cDNA clones. The similarity and identities with known sequences was determined by comparison using the software BLAST against the NCBI data base bank. From the *P. ipecacuanha* cDNA library 1050 single-pass sequences with an average of 324 nucleotides long were analyzed for this study. To judge the quality of the cDNA library preparation, the classification according to the matching homology (fig. 3.14) helped to evaluate the cDNA library contained representative clones. Compared to similar studies, the *P. ipecacuanha* EST library did not show obvious differences in the distribution of the EST classifications to *Papaver somniferum* (Ziegler et al., 2005) or *Catharanthus roseus* (Murata et al., 2006). Around 45 % of the ESTs were classified into no-homology and unknown sequences (fig. 3.14-A) and within general cellular activity classification, 41 % of the sequences showed homology to those genes with metabolism function (fig. 3.14-B). A classification of the homologous metabolic sequences differs from study to study, depending on the objectives of the work. Of the *P. ipecacuanha* ESTs that showed homology to those sequences assigned to metabolism in the public databases, 9 % correspond to secondary metabolism (fig. 3.14-B).

Among the 1050 ESTs, sequences with homology to various types of proteins including ABC-transporters, cytochromes P450 and transcription factors with similarities between 72 and 98 % were identified, including a sequence with specific homology to a flavonoid *O*-methyltransferase from *C. roseus* (77 %). More directly related to alkaloid biosynthesis was a homolog to 10-hydroxygeraniol oxidoreductase from *C. roseus* (77 %) and a cytochrome P450 (secologanin synthase) (68 %) (table 3.1). Both are involved in the biosynthesis of terpenoid isoquinoline and terpenoid indole alkaloids through the synthesis of secologanin. The ESTs that were tentatively most related to terpenoid alkaloid biosynthesis were a strictosidine synthase-like and a raucaffricine β-D-glucosidase-like sequence.
Several ESTs with homology to important proteins involved with general and specific steps of several groups of alkaloid biosynthesis were in this work clearly identified. The correlation between the intensity of the expressed genes and a detailed quantification of the alkaloid intermediates under given developmental stages, plant tissues or gene inductions conditions could allow the targeting of clones that have not been previously identified. Macroarray and microarray gene expression analysis methods could make the detection of genes encoding enzymes that catalyze specific steps of ipecac alkaloid biosynthesis systematic and facile.

### 4.4. Strictosidine synthase-like cDNA (ipstr-like)

#### 4.4.1. Sequence analysis

The partially sequenced cDNA had 78.2% identity to a strictosidine synthase-like cDNA from *Arabidopsis thaliana*. The complete sequence of the *P. ipecacuanha str*-like cDNA clone (*ipstr*-like) was 1767 bp long with an open reading frame of 1056 bp. The identity of the clone was reduced to around 40% when compared to the characterized strictosidine synthase (STR1) from *O. pumila, R. serpentina* and *C. roseus*. The identification of a clone with similarity to STR1 could indicate the isolation of the gene involved in the terpenoid indole alkaloid biosynthesis, which is not expected for *P. ipecacuanha* plants, or a gene with a similar function from a common diverged origin, which could condense dopamine (instead of tryptamine) with secologanin, to synthesize the key intermediate deacetylipecoside (by DIS) or deacetylisoipecoside (by DIIS) along the ipecac alkaloid pathway. Both STR1 and DIS were similar with respect to temperature optimum, pH optimum, exhibiting high substrate specificity, sharing one common substrate secologanin, using the Pictet-Spengler reaction type and similar molecular size, 30 kDa for DIS and 38 kDa for STR1 (39.6 kDa for ipSTR-like). (Hemscheidt and Zenk, 1980; Kutchan, 1989; De-Eknamkul *et al.*, 2000).

The recombinant ipSTR-like protein was purified to near homogeneity (fig. 3.21), but did not show any activity with the substrate secologanin together with dopamine or tryptamine. Tryptophan, *L*-dopa, tyramine and tyrosine, also tested together with secologanin, did not serve as substrates for the protein.

Recently, the crystallization and structure elucidation of STR1 from *Rauvolfia serpentina* have been achieved. Several residues were identified in the structure that may be responsible for the binding or possibly function in the enzyme activity (Ma *et al.*, 2006). Two small helices held in proximity by a disulfide bridge between Cys-89 and Cys-101 appear to be conserved in the STR1 family and seem to be a distinct feature of the family. The covalently
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bound Cys residues play an important role in the integrity of the substrate binding pocket and to the overall structure (Ma et al., 2006). Based on the algorithms of the program MegAlign used for the sequence comparison, section (2.1.7), both Cys residues were also conserved in the ipSTR-like protein. In the same comparison, the residues that form the hydrophobic pocket and the two polar residues within the active site in the ipSTR-like protein did not match to any single residue in the STR1 sequence. This includes Glu-309, proposed to be the essential residue for the catalysis by its amine deprotonation in STR1. However, with exception of the residues Val-167 and Phe-226 for STR1, the nature of the amino acids was conserved in ipstr-like cDNA (table 4.1). It is not aim of this work to elucidate the function of residues or the structure of ipSTR-like, but the presence of a glutamate residue next to the position, at the C-terminus, where the essential residue (Glu-309) occurs in STR1 was present in the ipSTR-like amino acid sequence (fig. 3.16).

<table>
<thead>
<tr>
<th>Position</th>
<th>89</th>
<th>101</th>
<th>149</th>
<th>151</th>
<th>167</th>
<th>176</th>
<th>179</th>
<th>180</th>
<th>208</th>
<th>226</th>
<th>276</th>
<th>277</th>
<th>307</th>
<th>309</th>
<th>324</th>
</tr>
</thead>
<tbody>
<tr>
<td>STR1</td>
<td>Cys</td>
<td>Cys</td>
<td>Trp</td>
<td>Tyr</td>
<td>Val</td>
<td>Val</td>
<td>Ile</td>
<td>Met</td>
<td>Val</td>
<td>Phe</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>ipSTR-like</td>
<td>Cys</td>
<td>Cys</td>
<td>Phe</td>
<td>Asn</td>
<td>Ser</td>
<td>Phe</td>
<td>Leu</td>
<td>Val</td>
<td>Phe</td>
<td>Gly</td>
<td>Ile</td>
<td>His</td>
<td>Ala</td>
<td>Ser</td>
<td>Leu</td>
</tr>
<tr>
<td>STR1</td>
<td>di</td>
<td>di</td>
<td>hy</td>
<td>po</td>
<td>hy</td>
<td>hy</td>
<td>hy</td>
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<td>ch</td>
<td>po</td>
<td>ca</td>
<td>hy</td>
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<tr>
<td>ipSTR-like</td>
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<td>✓</td>
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</tr>
</tbody>
</table>

Table. 4.1. Positions of the amino acid residues with possible binding and catalytic relevance in the STR1 and amino acid residues and the nature in the corresponding position of ipSTR-like are compared. The nature or function of the STR1 residues are abbreviated as follow: di, form a disulfide bridge; hy, hydrophobic; po, polar; ch, positively charged; ca, essential for catalysis.

In spite of the similarities with the STR1 sequence, the recombinant ipSTR-like protein did not show STR1, DIS or DIIS enzyme activity. This could be related to the nature of the substrates, the assay conditions or folding problems with the recombinant protein. On the other hand, even if terpenoid indole alkaloids (TIAs) are not synthesized in *P. ipecacuanha*, it should be considered that strictosidine synthase-like cDNAs have been found in plants that do not produce complex alkaloids such as *A. thaliana* (De Luca and Laflamme, 2001, Facchini et al., 2004); these are cDNAs to which the ipstr-like cDNA presented a high homology. The production of MIAs in other *Psychotria* species should also mean the activity of a STR1 in these species (Pasquali et al., 2006; Both et al., 2005; Henriques et al., 2004; Desantos et al., 2001). Therefore the ipstr-like cDNA could represent a very ancestral STR1 that through evolution lost its functionality, a protein of no function in *P. ipecacuanha* from still useful machinery present in closely related species or even just an enzyme with a yet undetected novel activity in *P. ipecacuanha*. 

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4.4.2. cDNA isolation attempt by the degenerate primer method

A potential method for the isolation of a putative gene is the use of the degenerate PCR primers the sequences of which are based upon microsequences of internal peptides of the purified enzyme as well as the amino acid sequence of previously characterized homologous enzymes. Successful results have been obtained with enzyme alkaloid pathways in the isolation of cDNAs encoding a 2-oxoglutarate dependent-dioxygenase involved in the biosynthesis of vindoline (Vazquez-Flota et al., 1997), methyltransferases in the biosynthesis of berberine and morphine alkaloids (Frick and Kutchan, 1999; Ounaroon et al., 2003) and strictosidine glucosidase of the terpenoid indole alkaloid pathway (Warzecha et al., 2000; Geerlings et al., 2000; Gerasimenko et al., 2002). With the aim to isolate cDNAs encoding the deacetylisopicoside (DIIS) and deacetylipecoside (DIS) synthases, the degenerate primer method was attempted based on the STR1 sequence considering the similarities in size, activity conditions and reaction type found for DIS and STR1 (Hemscheidt and Zenk, 1980; Kutchan, 1989; De-Eknamkul et al., 2000). Before the structure elucidation of STR1 was known from Ma et al. (2006), the analysis of the STR1 sequences from O. pumila, R. serpentina and C. roseus established four conserved regions (fig. 3.16) from which degenerate primers were designed (section 2.1.2.2). All combinations of forward and reverse primers were tested by RT-PCR and additional nested PCRs and re-amplifications were applied also with the same primers; however, the isolation of a clone with sequence similarity to STR1 could not be achieved. Once an ipstr-like cDNA was obtained from the P. ipecacuanha EST library, it became obvious from the amino acid sequence comparison that the conserved regions in functional STR1s showed a low homology. Only the conserved region LIKYDP had a 50% homology including the last amino acid, proline, at the carboxyl terminus, which is a critical point in the success for the reverse transcription (Abd-Elsalam, 2003). Finally, from 15 residues with relevance to the binding and catalysis of STR1 indicated by Ma et al. (2006) (table 4.1) only Trp-149, Tyr-151 and Glu-309 were found in the selected conserved regions. Given these homology results, a successful isolation of an ipstr-like cDNA with degenerate primers was, in retrospect, unlikely.
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4.5. Ipecoside glucosidase (Ipe-Gluc)
4.5.1. Isolated clones

The second clone potentially related to terpenoid alkaloid biosynthesis that was isolated from the cDNA library showed 67% identity to raucaffricine-O-β-D-glucosidase (RG) from *R. serpentina* and 64% strictosidine β-D-glucosidase (SG) from *C. roseus* and *R. serpentina*. The complete sequence of the *P. ipecacuanha* glucosidase cDNA (ipe-gluc) was 1775 bp long with an open reading frame of 1632 bp. The identity of Ipe-Gluc was 54% to RG, and 47% to SGs. The full-length clone was obtained by the rapid amplification cDNA 5'-End (5'-RACE) (section 3.3.4.) using the partial cDNA sequence as a reference and RNA isolated from leaf as template. A total of three glucosidase clones were isolated: two partial cDNAs from cultured root RNA and leaf RNA and a full-length cDNA from leaf RNA. The comparison of the three cDNA sequences showed 12 mismatching residues (table 3.2). In grey are shown matching residues between the first (ph0212_60) and the second (Glucosidase_2) clone and in yellow and red between the second and third (Ipe-Gluc) clone. The differences among the sequences may suggest the presence of isoforms. The ipecac alkaloids can be grouped according to the R- or S-conformation, for which it has been demonstrated that isomerization does not occur (Nagakura *et al.*, 1978; De-Eknamkul *et al.*, 1997; De-Eknamkul *et al.*, 2000). It could be expected that individual glucosidases act separately on each diastereomer.

4.5.2. Sequence analysis

Multiple sequence alignments of β-glucosidases in the family 1 show several conserved regions and motifs with possible binding or catalytic function (Henriott, 1991). In figure 3.19, the principal conserved motifs of β-glucosidases in Ipe-Gluc, RG and two SG sequences are shown. In Ipe-Gluc, the essential residues in each motif are strictly conserved. The motifs found in Ipe-Gluc that are considered signatures for the family 1 glycoside hydrolases are FIFGAGTSSYQIEGA (position 26-40) (Henriott and Davies, 1997) and the motif IYITENGV (position 418-425), which contains glutamate as catalytic nucleophile (Withers *et al.*, 1990; Trimbur *et al.*, 1992; Keresztessy *et al.*, 2001). The highly conserved motif NEP, with the glutamate identified as the acid/base catalysis residue in the cyanogenic β-glucosidase (linamarase) (Keresztessy *et al.*, 1994), was found in position 185-187 in the Ipe-Gluc sequence. The carboxyl terminus of Ipe-Gluc contained the motif DxxRxxY at positions 441-447; aspartate is suggested as necessary for glucoside cleavage (Trimbur *et al.*, 1992).
The comparison of the Ipe-Gluc with closely related glucosidase sequences identified fifty positions at which the residues were conserved in the SGs, but differed in the RG and in the Ipe-Gluc sequence (fig. 3.19). Three of these positions were next to the NEP motif, two flanked the motif and one was two-residues upstream from the motif. Keresztessy et al. (2001) found that the mutation Ala-210Val two residues from the C-terminus of the NEP motif in linamarase (*M. esculenta*) changed the hydrophobic environment of the glutamate in the motif and, therefore, the enzyme activity. A residue that is only conserved in SGs, but not in RG or in Ipec-Gluc, was identified adjacent to the nucleophile motif IYTENGV in the C-terminal direction and at the sixth residue in the DxxRxxY motif. In figure 3.19, some of the mentioned positions are identified with green arrowheads. An overview of the complete sequences shows an evidently higher concentration of this pattern in the C-terminal region, including four consecutive residues at the 3’-end of the sequences. This finding could be the result of higher conserved sequence between SGs genes than RG and Ipe-Gluc, but also the positions near to identified active motifs may take part in the substrate recognition or in the catalytic activity of the enzyme.

4.5.3. Recombinant enzyme

The heterologously expressed Ipe-Gluc enzyme was found partly soluble and partly insoluble in the *E. coli* protein extraction buffer. The formation of inclusion bodies with incorrectly folded protein might be the reason for the insolubility, since no membrane transport signal peptides could be detected. Incubation of soluble recombinant enzyme with the substrates ipecoside, strictosidine, raucaffricine, vincoside lactam, glucosylquercetin, apigenin, naringerin, galloyl, kaempferol, salicin and arbutin (fig. 3.29 and 3.30) with subsequent spectrophotometric detection clearly showed enzymatic deglycosylation of the substrates ipecoside and its methylated form to the respective aglycones (fig. 3.33-3.38). The methylated form of ipecoside was detected as an impurity in both substrate and aglycone product. The absolute configuration was not determined. The concentration of the substrate strictosidine was reduced in assays in the presence of active (fig. 3.30) and inactive enzyme (data not shown), however, only trace amounts of catenamine (fig. 3.40), the reduced aglycone product of strictosidine, were detected in the assay containing active enzyme. Strictosidine glucosidase (SG) activity reported in Gerasimenko et al. (2002) showed activity trends similar to Ipe-Gluc. SG showed activity with strictosidine and no activity with vincoside lactam, raucaffricine and other related compounds. Nevertheless, 0.8 % relative activity was detected.
with ipecoside. Raucaffricine glucosidase (RG) reported in Warzecha et al. (2000) showed maximal activity with raucaffricine and a reduced relative activity with strictosidine (6.5 %), while ipecoside was not accepted as substrate. Taking together the results from Gerasimenko et al. (2002) and Warzecha et al. (2000), the higher structural similarity between the substrates ipecoside and strictosidine compared to raucaffricine (fig. 3.29) and the amino acid sequence similarities between SG, RG and Ipe-Gluc, most evident in the motif regions (fig. 3.19), it is feasible that Ipe-Gluc shows reduced activity with the substrate strictosidine and no activity with raucaffricine. In the biosynthesis of ipecac alkaloids, Ipe-Gluc catalyzes a reaction that until now has not been detected. Nagakura et al. (1978) suggested that the \( N \)-acylated alkaloidal glycosides, ipecoside and alangiside, are metabolic dead-end products from intermediates of the wrong stereochemistry. Later on, however, benzopyridoquinolizine bases, biogenetically derived from alangiside, were isolated from Alangium lamarckii (Fujii and Ohba, 1983). Ipecoside aglycone was not detected in alkaloid extracts of leaves and roots in this work and no reports on related compounds were found in previous studies. The hydrolysis of ipecoside would produce a unique stable aglycone which could be an intermediate for up until now unknown alkaloids. On the other hand, in the biosynthetic pathway of ajmaline, a terpenoid indole alkaloid of \( R. \) serpentina, raucaffricine glucosidase hydrolyzes raucaffricine to vomilenine (Warzecha et al., 1999; Warzecha et al., 2000). Vomilenine is in turn converted to raucaffricine by action of vomilenine UDP-glucose transferase (Ruyter and Stockigt, 1991) (fig 1.3). A putative glucosyltransferase (\( A. \) thaliana) found in the \( P. \) ipecacuanha EST library might suggest the presence of a similar glucose transferase. Since in alkaloid extractions, ipecoside has been isolated, but neither ipecoside aglycone nor any biogenetically derived intermediate have not been reported, a similar cycle to raucaffricine-vomilenine may also be occurring. In Stöckigt (1995), raucaffricine was suggested to take the role of storage compound, but its biosynthetic function is not yet known. Ipecoside glucosidase could be then similar to raucaffricine glucosidase, and this deglycosidation is a step not completely understood in the metabolic pathway of ipecac alkaloids.

4.5.4. Preliminary enzyme characterization

Once ipecoside was established as a substrate for Ipe-Gluc, the pH and temperature optimum for enzyme activity were determined. The pH optima for most \( \beta \)-glucosidases are in the range of pH 4.0 - 6.2 (Esen, 1993). The pH optimum for Ipe-Gluc was determined to be between
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3.5 - 4.0. Figure 3.28-A shows, under the conditions of this work, a minimum ipecoside concentration between pH 3.5 and 5.5; the ipecoside aglycone concentration was increasing from pH 5.5 until its maximum at pH 3.5. Warzecha et al. (2000) assayed RG at pH 5.0 and for assays with SG from C. roseus at pH 6.3. An SG was isolated from cell cultures of Tabernamontana divaricata that showed a relatively constant activity in the pH interval 4.5 - 8.0 with two optima at 4.5 - 5.0 and 7.0 (Luijendijk et al., 1996). Gerasimenko et al. (2002) showed that an SG from R. serpentina had a pH optima between 5.0 - 5.2 and 50 % maximal activity at pH 4.2 that slowly decreased until pH 8.0. For Ipe-Gluc no specific temperature between 20 and 50 °C could be determined as optimal with the substrate ipecoside at pH 3.5 (fig. 3.28-B). The temperature used for assays with RG and SG was around 30 °C (Warzecha et al., 2000; Geerlings et al., 2000). However, Gerasimenko et al. (2002) determined 50 °C to be the optimum temperature for SG from R. serpentina. Finally, test assays with enzyme extracts stored under various conditions showed a stable Ipe-Gluc enzyme; the enzyme maintained around 90 % activity after three weeks (fig. 3.31). Thorough functional characterization and a set of structural data of ipecoside glucosidase would clarify the catalytic mechanism when this enzyme is compared with similar glucosidases.