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# PURIFICATION AND CHARACTERIZATION OF THE BERBERINE BRIDGE ENZYME FROM *BERBERIS BEANIANA* CELL CULTURES

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**Key Word Index**—*Berberis beaniana*; Berberidaceae; berberine bridge enzyme; (*S*)-reticuline; (*S*)-scoulerine.

**Abstract**—The berberine bridge-forming enzyme (BBE) has been found in 66 samples taken from differentiated plants and from cell suspension cultures. It was purified 450-fold from *Berberis beaniana* cell cultures by gel-filtration, DEAE and phenyl-Sepharose chromatography, electrophoresis and isoelectric focusing. The enzyme was shown to be homogeneous by gel electrophoresis ($M_r$ = 52 kD ± 4). The enzyme, which requires the presence of oxygen, catalyses the conversion of the (*S*)-enantiomers of reticuline, protosinomenine and laudanosoline to the corresponding (*S*)-tetrahydroprotoberberines and released stoichiometric amounts of $H_2O_2$. Within the cells the enzyme is located in a particle with the density $\rho$ = 1.14 g/ml.

## INTRODUCTION

The keystone in the biosynthesis of a great number of isoquinoline alkaloids in the plant kingdom is reticuline. This compound is also a biogenetic precursor of the protoberberine skeleton. Reticuline is converted *via* scoulerine to various protoberberines as has been previously determined by precursor feeding experiments using differentiated plants [1–3]. During this conversion of the benzylisoquinoline the so-called berberine bridge is formed, i.e. the N–Me group of reticuline is converted to carbon 8 of the protoberberine molecule. Rink and Böhm [4] were the first to describe a cell-free system prepared from *Macleaya microcarpa* cell suspension cultures which could convert reticuline to scoulerine. The enzyme which was named the berberine bridge enzyme (BBE), was purified 7-fold for the preliminary study of its properties. In our continuing work on the enzymology of isoquinoline alkaloid biosynthesis, we have developed a new and convenient assay procedure for the BBE, and attempted to purify and characterize this important enzyme.

## RESULTS

### *Assay principle*

The formation of the berberine bridge involves the oxidation of the N-methyl group of reticuline and subsequent ring closure, most probably proceeding *via* an imminium species (Fig. 1). The loss of a hydrogen atom during this process was the basis for the development of a very sensitive assay of this enzyme using [–N–$CT_3$]-labelled (*S*)-reticuline as a substrate. Enzyme-containing samples were analysed by first incubating them with the labelled substrate, and then by assaying the aqueous part of the incubation mixture for radioactivity. Water could be separated from the rest of the test sample for this purpose either by sublimation [5], or, more conveniently, by the charcoal method [6]. Any (*S*)-tetrahydroprotoberberine oxidase (STOX) present in crude extracts could, however, subsequently act on the reaction product (*S*)-scoulerine to form dehydroscoulerine with the additional removal of one tritium atom

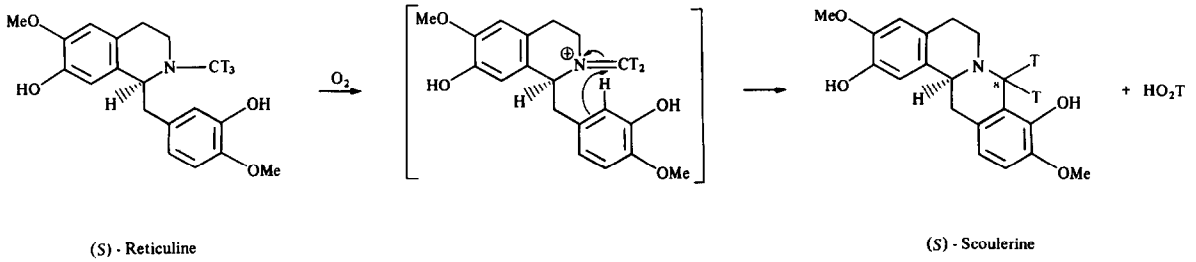



Fig. 1. Reaction sequence catalysed by the berberine bridge enzyme showing the assay principle and also the postulated iminium intermediate.

[7]. Thus, measurements of the BBE activity could be twice as much as the real values. In order to avoid this problem, morin, a very potent inhibitor of STOX [7] has to be included in the assay in a concentration of 5 $\times 10^{-5}$ M or more. The BBE is not affected by morin concentrations of up to 1 mM, while the interfering STOX enzyme is totally inhibited. Using [N–$CT_3$]-labelled (*S*)-reticuline of high specific activity (7 Ci/mmol), the enzyme activity of BBE could be quantified down to a value of 0.05 fkat; this corresponds to a value of about 6000 cpm released; this method is therefore one of the most sensitive assays ever developed for measuring an enzyme of a secondary metabolic pathway. Figure 2 shows the time course of a tritium removal catalysed with the BBE from [N–$CT_3$]-(*S*)-reticuline, which corresponds approximately to the expected release of 1/3 of the radioactivity. The subsequent addition of purified STOX [7], causes the release of an additional 1/3 of the originally present radioactivity into the aqueous phase.

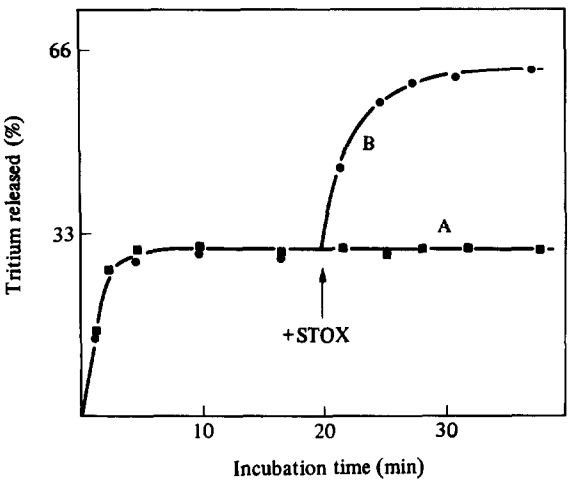



Fig. 2. Kinetics of tritium release into the aqueous incubation medium (A) by purified berberine bridge enzyme from [N–$CT_3$] (*S*)-reticuline (7.0 pkat). The arrow indicates the addition of (*S*)-tetrahydroprotoberberine oxidase (0.3 pkat) in a separate experiment, and the subsequent tritium release (B).

*Occurrence of BBE*

Böhm and Rink [8] have previously demonstrated the occurrence of BBE by TLC in cell-free extracts of seven differentiated plants and one cell culture [4]. In order to extend our knowledge of the taxonomic distribution of the enzyme and to find the most suitable plant material for the attempted purification and characterization of the enzyme we screened 21 differentiated plants and 67 cell suspension cultures for the occurrence of the BBE. As shown in Table 1, the highest activities of crude enzyme extracts from whole plants were detected in the families Papaveraceae and Fumariaceae. The enzyme was also found in a considerable number of the cell cultures screened. Plant species which are known not to contain protoberberine alkaloids were used as controls to test the assay procedure and, as expected, did not show any tritium release from the substrate. The control species used were: *Catharanthus roseus*; *Nicotiana tabacum*; *Nelumbo nucifera*; *Ruta chalepensis*; *Aristolochia clematitis*. Neither the differentiated whole plants nor the cell cultures derived from them yielded enzyme activity. Of all the cell suspension cultures tested, *Berberis beaniana* proved to be the most suitable with regard to its high yield of enzyme per volume of medium, its ease of cultivation, its reproducibility, and its morphological characteristics. This culture was therefore used to investigate the growth parameters and time course of enzyme formation. As shown in Fig. 3, the enzyme is present in the inoculum (day 0) in only very small amounts. The activity increases at the late log phase and peaks at day 11 of cultivation, exactly at the point when the culture enters the stationary phase. A 15-fold increase in enzyme activity can be seen as compared with only a 6-fold increase in dry cell matter.

Table 1. Occurrence of berberine bridge enzyme in different plant cell cultures and whole plants representing five plant families

| Plant cell cultures (pkat/l. medium) |
|---|
| Berberidaceae: *Berberis beaniana* (320); *B. vulgaris* (250); *B. stolonifera* (230); *B. wilsoniae* var. *subcaulialata* (210); *Berberis henryana* (210); *B. turcomanica* (110); *B. heteropoda* (84); *B. regeliana* (63); *B. julianae* (54); *B. valdisepala* (52); *B. canadensis* (47); *B. gagnepainii* (38); *B. notabilis* (24); *B. vernae* (22); *B. thibetica* (18); *B. aristata* (12); *B. papillifera* (10); *B. amurensis* (5, 8); *B. para-virescens* (5, 5); *B. circumserrata* (5, 1); *B. dictyophylla* (3, 0); *Podophyllum versipelle* (2, 1); *P. emodi* (0). |
| Ranunculaceae: *Thalictrum squarrosum* (240); *T. glaucum* (210); *T. sparsiflorum* (180); *T. dipterocarpum* (90); *T. dasycarpum* (67); *T. calabricum* (31); *T. minus* (23); *T. coerulum* (16); *T. tuberosum* (12); *T. simplex* ssp. *galioides* (5, 7); *T. alpinum* (3, 0); *Aquilegia alpina* (1, 9); *Ranunculus acer* (0); *R. carpaticus* (0); *R. flammula* (0). |
| Menispermaceae: *Cissampelos mucronata* (370). |
| Papaveraceae: *Glaucium flavum* (240); *G. rubrum* (190); *Bocconia cordata* (86); *Eschscholzia tenuifolia* (68); *E. lobbii* (46); *Argemone platyceras* (35); *Papaver somniferum* (32); *Eschscholzia californica* (29); *Meconopsis robusta* (26); *Argemone intermedia* (13); *Chelidonium majus* (13); *Glaucium corniculatum* (2, 7); *G. vitellinum* (2, 4); *Hypecoum leptocarpum* (1, 3); *Roemeria rhoeadiflora* (0). |
| Fumariaceae: *Fumaria capreolata* (240); *Adlumia fungosa* (180); *Corydalis vaginans* (91); *Fumaria officinalis* (71); *Corydalis aurea* (37); *Dicentra spectabilis* (21); *Corydalis ophiocarpa* (4, 1); *Fumaria muralis* (2, 4). |
| **Differentiated plants (pkat/g dry wt)*** |
| Papaveraceae: *Chelidonium majus* (19); *Eschscholzia californica* (2, 4); *E. lobbii* (14); *Papaver fugax* (1, 5); *P. somniferum* leaves (2, 9); roots (103). |
| Fumariaceae: *Dicentra spectabilis* (2, 5); *Fumaria parviflora* (8, 7). |

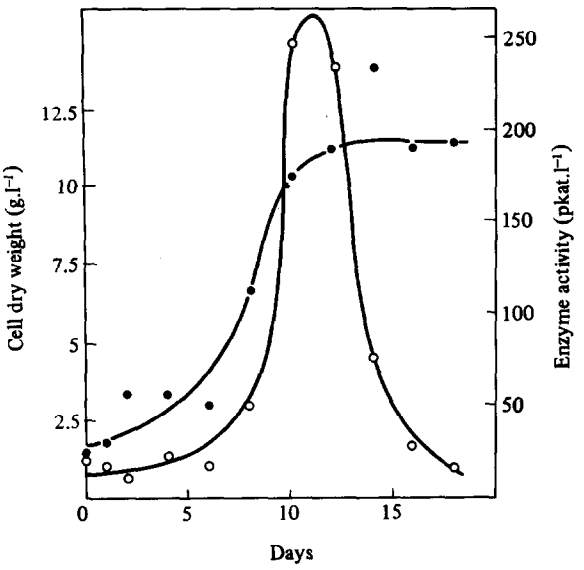



Fig. 3. Time course of berberine bridge enzyme activity (○) and cell dry weight (●) in a suspension culture of *B. beaniana* (1 l. flask containing 250 ml medium).

Total activity of the enzyme then decreases drastically during the stationary phase. For the purification of the enzyme, cells harvested on days 10–12 were used.

*Purification of BBE*

Cell cultures of *B. beaniana* contain high amounts of protoberberines mainly jatrorrhizine [9]. In the early phases of this work, we recognized that these quaternary alkaloids exert a strong inhibitory effect on the BBE. In order to remove most of these interfering cationic substances the deep yellow crude enzyme solution was treated first with carboxymethyl-Sepharose and subsequently with dextran-coated charcoal. The resulting protein solution was then fractionated using standard procedures and yielded, after isoelectric focusing, a single protein band in SDS gel electrophoresis. The enzyme so obtained, had been purified 450 times and contained 0.7% of the originally activity present in the crude extract. The data for a typical purification procedure are summarized in Table 2.

*Properties of BBE*

The pure enzyme showed a pH optimum at pH 8.9, as depicted in Fig. 4. The enzyme had a broad temperature optimum between 40 and 50°. The activation energy for tritium release over the temperature range from 20 to 30° was determined to be 48.6 kJ/mol. The isoelectric point of the homogeneous enzyme is at pH 4.9. Identical values were obtained both with isoelectric focusing and with chromatofocusing. The molecular weight of the protein was determined by SDS gel electrophoresis to be 54 kD. By gel filtration on AcA 54 the enzyme shows a distribution coefficient which corresponds to a molecular weight of 49 kD, assuming a globular shape. We have to

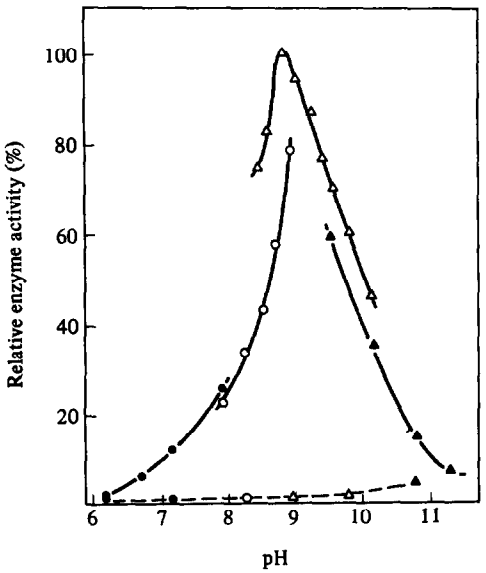



Fig. 4. PH profile of the catalytic activity of purified berberine bridge enzyme. Buffers used (60 mM); ●—●, Na–$KPO_4$; ○—○, Tris–HCl; △—△, glycine–NaOH; ▲—▲ $NaHCO_3$–$Na_2CO_3$. [control (– – –): heat-denatured enzyme].

Table 2. Summary of purification procedure for the berberine bridge enzyme from *Berberis beaniana* cell suspension cultures

| Step | Total protein (mg) | Total activity (pkat) | Specific activity (pkat/mg) | Purification (-fold) |
|---|---|---|---|---|
| 1. Crude extract | 726 | 10530 | 14.5 | 1 |
| 2. CM-Sepharose filtrate and charcoal supernatant | 387 | 7720 | 19.9 | 1.4 |
| 3. DEAE-Sephacel | 145 | 10270 | 70.8 | 4.9 |
| 4. AcA-54 | 32.7 | 1810 | 55.4 | 3.8 |
| 5. DEAE-Sephacel | 11.5 | 1780 | 155 | 10.7 |
| 6. Ultrafiltration and Phenylsepharose eluate | 3.9 | 868 | 222 | 15.3 |
| 7. Disc electrophoresis | 0.14 | 294 | 2100 | 145 |
| 8. Isoelectric focusing | 0.011 | 73.5 | 6680 | 460 |

conclude that the true molecular weight is in the range of $52 \pm 4$ kD. As judged from SDS electrophoresis, the enzyme consists of only one polypeptide chain. The enzyme is quite stable. Its catalytic activity under sterile conditions at pH 7.4 was determined to have half lives of 6 days when stored at 37°; 18 days at 25°; 150 days at 4°; 320 days at −20°. The product of the enzyme-catalysed reaction using reticuline as a substrate was identified as follows: 0.1 mM of (*R,S*)-reticuline was incubated at pH 8.9 with 520 pkat of enzyme at 25° (total volume 1 l.). The reaction was followed by TLC of aliquots. After 4 days, exactly 50% of the substrate had been converted; on further incubation no subsequent metabolism was observed. The mixture was extracted with chloroform and the extract gave, on TLC purification, 5.6 mg of a product which was identical to scoulerine on several TLC and HPLC systems. Its MS showed a molecular ion at *m/z* 327 (38%) and fragments at *m/z* 178, 176, 150. The $^1$H NMR spectrum showed resonances for H-1 ($\delta$6.82); H-4 ($\delta$6.60); H-11 and H-12 ($\delta$6.71) identical to the authentic substance [10]. The characteristic signals for coreximine (H-12, $\delta$6.56; $H_2$-8, $\delta$4.05) [10] were not present. Acetylation (pyridine–$Ac_2O$) and mass spectrometry of the diacetate gave identical signals when compared to authentic diacetyl scoulerine (*m/z* 411 (18%) 150 (100%)). $[\alpha]_D^{23} = -279°$ (*c* 0.56; MeOH). The reaction product was quantitatively converted by the STOX enzyme, which is completely stereospecific [7], to dehydroscoulerine. By these criteria the product of the reaction catalysed by BBE is unequivocally (*S*)-scoulerine. The isomeric coreximine was never found to be formed by the enzyme under investigation. The stoichiometry of the reaction was investigated by following the lead given by Rink and Böhm [4] that the enzymatic reaction shows absolute oxygen requirement. Following the consumption of oxygen it was observed that 0.94 mole were consumed for each mole of reticuline transformed. Assuming the removal of two hydrogen atoms during the formation of scoulerine, this stoichiometry could be most simply completed by the formation of hydrogen peroxide. Analysis for $H_2O_2$ did indeed show that for each mole of scoulerine formed, one mole of $H_2O_2$ was produced. Figure 5 demonstrates the kinetic relationship between the reactants. Under the conditions chosen (*S*)-reticuline consumption (as measured by a specific radioimmunoassay) paralleled the rate of consumption of oxygen. On the product side, scoulerine and $H_2O_2$ were formed stoichiometrically and simultaneously, while the release of tritium was clearly delayed. A net kinetic isotope effect of $k_H/k_T = 1.64$ was calculated using the initial velocities measured for tritium release and for scoulerine/$H_2O_2$ production. The origin of this effect is not clear; this will be investigated further. Finally, the substrate specificity of the enzyme was determined. (*S*)-Reticuline was up to now the established substrate for BBE. [N–$CT_3$]-(*R*)-Reticuline with high specific activity (3.2 Ci/mmol) was not transformed at all; neither were the N-oxides of (*S*)- or (*R*)-reticuline. Of the three possible isomers of reticuline, only (*S*)-protosinomenine was transformed at the same rate as reticuline to (*S*)-3,9-dihydroxy-2,10-dimethoxytetrahydroprotoberberine, the (*R*)-stereoisomer again being completely inactive. No reaction was observed with orientaline or iso-orientaline. Also, (*R,S*)-laudanidine and laudanosine were completely inactive. Of all the other substrates tested, only laudanosoline was converted to a corresponding tetrahydroprotoberberine, albeit only at 20% of the rate of conversion of reticuline and protosinomenine. In each of these cases analysis of the reaction was performed by measuring the release of $H_2O_2$, and in positive cases as well as in the negative case of laudanidine, the reaction was checked by HPLC. The structural formulae and relative rates of conversion of potential benzylisoquinoline substrates are given in Table 3. Lineweaver–Burk plots gave a $k_m$ value of 0.1 $\mu$M for (*S*)-reticuline and $V_{max}$ of 17 fmol/sec for 7.1 ng of the purified enzyme. One mole of enzyme catalyses the transformation of at least 0.35 mol of substrate per second. As judged from the failure to inhibit the enzyme by established SH-binding inhibitors like *p*-OH-mercuribenzoate, N-ethylmaleimide, iodosobenzoate and acetamide (each at 0.6 mM), obviously no –SH group is essential for the catalytic activity of BBE. However, chelating agents showed strong inhibitory effects on the enzyme. The enzyme was inhibited 50% by: 6 $\mu$M *o*-phenanthroline; 0.4 mM diethyldithiocarbamate; 6 mM $Na_2$ EDTA. $NaN_3$ and NaCN only affected enzymatic activity at

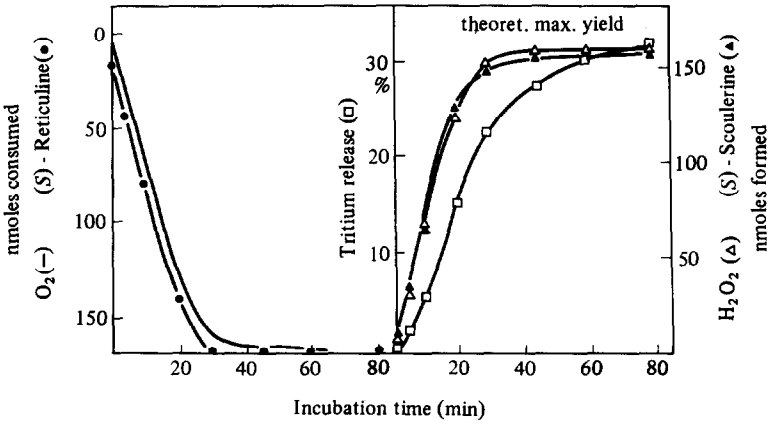
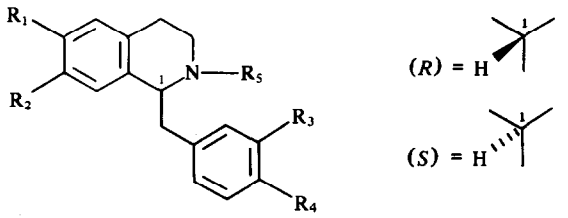



Fig. 5. Kinetics of substrate consumption [A, $O_2$; B, (*S*)-reticuline] and product formation [C, (*S*)-scoulerine; D, $H_2O_2$; E, $^3$H] during the conversion of [N–$CT_3$]-(*S*)-reticuline to (*S*)-scoulerine catalysed by berberine bridge enzyme (170 nmol (*S*)-reticuline; 10 pkat enzyme = 58 $\mu$g; 80 $\mu$mol glycine–NaOH pH 8.9; final volume 1.6 ml).

Table 3. Relative rates of conversion of benzylisoquinolines to tetrahydroprotoberberines catalysed by the berberine bridge enzyme




| Substrate | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | Rate of transformation (%) |
|---|---|---|---|---|---|---|
| (*S*)-Reticuline | OMe | OH | OH | OMe | Me | 100 |
| (*R*)-Reticuline | OMe | OH | OH | OMe | Me | 0 |
| (*S*)-Reticuline-N-oxide | OMe | OH | OH | OMe | Me, O | 0 |
| (*R*)-Reticuline-N-oxide | OMe | OH | OH | OMe | Me, O | 0 |
| (*S*)-Protosinomenine | OH | OMe | OH | OMe | Me | 100 |
| (*R*)-Protosinomenine | OH | OMe | OH | OMe | Me | 0 |
| (*R*, *S*)-Laudanidine | OMe | OMe | OH | OMe | Me | 0 |
| (*R*, *S*)-Laudanosoline | OH | OH | OH | OH | Me | 20 |
| (*R*, *S*)-Laudanosine | OMe | OMe | OMe | OMe | Me | 0 |
| (*R*, *S*)-Orientaline | OMe | OH | OMe | OH | Me | 0 |
| (*R*, *S*)-Isoorientaline | OH | OMe | OMe | OH | Me | 0 |

50 mM concentration. The enzyme was highly resistant to inactivation by $H_2O_2$ and only at 0.7 M was a 50% reduction in catalytic activity observed. As indicated above, protoberberine alkaloids exert a strong inhibiting effect on the BBE. 50% inhibition was observed with 4 $\mu$M berberine and 30 $\mu$M jatrorrhizine, but also with 1 $\mu$M (*S*)-norreticuline, 20 $\mu$M (*R*)-norreticuline, 10 $\mu$M (*S*)-scoulerine and 200 $\mu$M (*S*)-coreximine.

*Intracellular distribution of BBE*

A routine check using isopycnic ultracentrifugation with a linear sucrose gradient showed that more than 90% of the total activity of the BBE in crude preparations is confined to a nearly symmetrical band with a density of 1.14 g/ml (Fig. 6). The maximal activity found does not correspond with the following marker enzymes: cytochrome *c*-oxidase ($\rho$ = 1.185) for mitochondria, or catalase ($\rho$ = 1.215) for glyoxisomes. Freezing of the tissue or grinding of the tissue in buffer without an osmoticum, releases the enzyme quantitatively in a freely soluble form. Thus the enzyme is normally confined to a particle ($\rho$ = 1.14) within the cell.

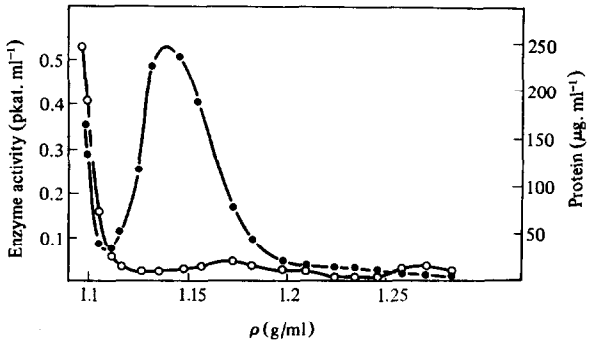



Fig. 6. Distribution of berberine bridge enzyme in linear sucrose density gradient (18–60% w/w; 48 ml).

## DISCUSSION

The berberine bridge enzyme, originally discovered and named by Rink and Böhm [4], has been purified to homogeneity and characterized. The enzyme reaction products and substrate specificity have been determined. In the presence of oxygen the enzyme catalyses the transformation of (*S*)-reticuline and (*S*)-protosinomenine with the concomitant formation of $H_2O_2$ and (*S*)-scoulerine as well as (*S*)-3,9-dihydroxy-2,10-dimethoxy-tetrahydroprotoberberine, respectively. The latter leads by a minor route to the protoberberine alkaloid jatrorrhizine [11]. The only other substrate which was acted upon by the enzyme is laudanosoline, which has not been found to occur in nature. The enzyme is widely distributed, but it only occurs in benzylisoquinoline alkaloid-containing plant species and cell cultures derived therefrom. It fills an important role, channeling the central precursor (*S*)-reticuline into the protoberberine, protopine, benzophenanthridine, phthalideisoquinoline and rhoeadine pathway [3]. From a regulatory point of view it is of interest that the enzyme is localized in a particle with the density of $\rho$ = 1.14 g/ml a value identical with that of the STOX enzyme [7]. No decision can be made yet as to the mechanism by which the enzyme converts the substrate to the tetrahydroprotoberberines; either an ionic or a radical mechanism can be postulated [3]. Although the existence of catalytically active prosthetic group(s) of the

enzyme could not be determined, the inhibition by *o*-phenanthroline and other chelating agents strongly suggests metal ion involvement. These questions have to await the isolation of substantial amounts of BBE.

## EXPERIMENTAL

*Materials.* Whole plant parts were obtained from the Munich botanical garden. The organs were frozen in liquid $N_2$, powdered, and used immediately for the enzyme assay. Cell cultures were provided by the cell culture laboratory of this department. *B. beaniana* was grown as previously described and frozen in liquid $N_2$ [9]. [-N-$^{14}CH_3$] and [N-$CT_3$]-(*R*)- or (*S*)-reticuline were synthetized from the corresponding norreticuline by transfer of the radioactive methyl group of S-adenosyl-L-methionine (Amersham) catalysed by (*R,S*)-norreticuline N-methyltransferase [12]. Alkaloids were synthetized according to standard procedures [e.g. 13]. The identity and optical purity were checked in each case. (*R*)- and (*S*)-reticuline N-oxides were synthesized according to literature procedures [14]. Commercially available were: (*R,S*)-laudanosine (Aldrich), (*R,S*)-laudanosoline (EGA) and (*R,S*)-laudanidine (Merck).

*Enzyme activities.* The routine assay mixture contained, in a total volume of 0.25 ml: 100 mM glycine–NaOH buffer (pH 8.9); 255 pmol [-N-$CT_3$]-reticuline (180 mCi/mmol or higher), and protein up to 0.1 mg. The mixture was incubated for 60 min at 30°C. The reaction was stopped by the addition of 0.3 ml of a neutralized suspension of activated charcoal (4%) in distilled water. The suspension was agitated for 1 min and the charcoal was removed by centrifugation (all Eppendorf systems). The radioactivity of a 350 $\mu$l aliquot was measured by scintillation counting (in 5 ml Rotiszint, Roth Karlsruhe). Protein preparations from plants or cell cultures were prepared as follows: 2–8 g (fr. wt) of deep-frozen plant material were stirred at room temp in phosphate buffer (100 mM; pH 7.4; 2 ml/g fr. wt) until a homogeneous suspension was obtained. The mixture was pressed through cheesecloth, and the filtrate centrifuged for 10 min at 2 $\times 10^4$ rpm. $(NH_4)_2SO_4$ was added to the supernatant to 70% satn and the suspension was centrifuged. This ppt was taken up in the assay buffer, and this mixture was freed from low molecular weight material by filtration through Sephadex G 25 (1 × 13 cm). The protein-containing eluate was used as the enzyme source for subsequent experiments. For the subcellular localization of the enzyme, *B. beaniana* suspension cultures were freshly harvested, suspended in osmotic buffer (2 ml/g fr. wt), and ground in a mortar. The procedure followed was adapted from ref. [15] using osmotic buffer consisting of 500 mM saccharose; 150 mM tricine–NaOH, pH 7.5; 10 mM KCl; 5 mM thioglycolate; 1 mM $MgCl_2$; 1 mM $Na_2$ EDTA. After centrifugation for 4 hr at $9 \times 10^4$ *g*, fractions of 2.5 ml were collected.

*Enzyme purification.* All operations were carried out at 0–4°. 250 g deep-frozen cells were suspended in 300 ml 20 mM KPi pH 7.4 and extracted as above. Carboxymethyl-Sepharose (Pharmacia, 160 g) preequilibrated in the same buffer was added to the centrifugate. The suspension was shaken for 5 min, filtered, and then a final concn of 5% (v/v) dextran-coated charcoal was added to the filtrate. The suspension was agitated for 1 min and filtered. The liquid was applied to a DEAE-Sephacel column (4.7 × 40 cm) which was pre-equilibrated with the same buffer. The enzyme was retained during elution with this buffer. It was subsequently eluted with buffered 0.3 M KCl at a rate of 1 ml/min. The eluate was applied to an Ultrogel AcA 54 column (5.0 × 93 cm) which was run with the pH 7.4 buffer. The enzyme appeared between 800 and 900 ml of eluant. The fractions were pooled and adsorbed into DEAE-Sephacel (1.4 × 6 cm column). This was eluted using a linear gradient of 0.0–0.3 M KCl and the enzyme came off around 0.18 M. Fractions containing the enzyme (180–220 ml) were pooled and applied to a phenyl-Sepharose column (1 × 6.5 cm), which had previously been equilibrated with standard buffer containing 0.1 M KCl. The enzyme was eluted with EtOH–$H_2O$ (25:75, v/v). The enzyme appeared as a sharp peak in fractions 57–61. Each 650 $\mu$g portion of protein from the previous step was applied to disc gel electrophoresis (9% polyacrylamide) [16]. Bands were cut and eluted and those eluates containing the enzyme were pooled. Samples of this material containing 140 $\mu$g protein in 750 $\mu$l 5 mM phosphate buffer (pH 7.5) with 8.5% glycerol were subjected to isoelectric focusing using cylindrical gels [polyacrylamide 7.3% w/v containing 2.3% (w/v) ampholine pH 4–6.5 from LKB]. Electrode solns used were 0.075 M $NH_2SO_4$ (anode) and 0.075 M triethanolamine (cathode). The separation time was 4 hr at 4° and 200 V. The eluate of the enzyme-containing segments (pH 5.1–4.8) contained the homogeneous protein as judged by discontinuous SDS gel electrophoresis [17].

*Analytical procedures.* Benzylisoquinolinas and tetrahydroprotoberberines were separated by TLC (silica gel 60, Merck) developing with xylene–2-butanone–MeOH–$Et_2NH$ (50:50:6:2). HPLC was conducted with a Spectra Physics instrument on Nucleosil 7 C 18 (4 mm × 25 cm) using MeOH–0.3% $(NH_4)_2CO_3$ (75:25) as solvent system. Flow rate 0.8 ml/min, detection at 283 nm. (*S*)-Reticuline was quantified by a radioimmunoassay specifically directed towards the (*S*)-enantiomer in the 0.05–5 ng range [U. Wieczorek, unpublished]. $H_2O_2$ was determined according to ref. [18]. Oxygen was measured with Clark's oxygen electrode and protein as described in ref. [19]. Relative protein values were determined according to ref. [20]. In both cases bovine serum albumin served as standard.

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