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PREPARATION OF DEHYDROBENZYLISOQUINOLINES BY IMMOBILIZED (*S*)-TETRAHYDROPROTOBERBERINE OXIDASE FROM PLANT CELL CULTURES

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Key Word Index—*Berberis wilsoniae* var. *subcaulialata*; Berberidaceae; (*S*)-tetrahydroprotoberberine oxidase (STOX); immobilization; 1,2-dehydrobenzylisoquinolines

Abstract—(*S*)-Tetrahydroprotoberberine oxidase (STOX) has been isolated in enriched (7.4-fold) form from a high yielding cell suspension of *Berberis wilsoniae* var. *subcaulialata* in a three step procedure and was immobilized by several different methods. The properties of immobilized STOX were compared with immobilized *Berberis* cells and with the soluble enzyme. Although pH and temperature optima were shifted by immobilization, K_M-values with (*S*)-norreticuline remained unaffected. The stability of immobilized STOX was 50 times better than the free enzyme. A cyclic process is described using the stereospecific enzymatic oxidation of (*S*)-norreticuline to 1,2-dehydronorreticuline followed by sodium borohydride reduction for the transformation of racemic norreticuline to the (*R*)-enantiomer.

INTRODUCTION

In the biosynthesis of 1-benzylisoquinoline alkaloids, reticuline plays a pivotal role. Biogenetically this compound is formed via norcoclaurine in the (*S*)-enantiomeric form [1]. (*S*)-Reticuline [derived from (*S*)-norcoclaurine] has been established as the biogenetic precursor also for those 1-benzylisoquinoline-derived alkaloids showing the (*R*)-configuration such as the opium alkaloids thebaine, codeine and morphine [2]. From feeding experiments with (*R*)- and (*S*)-[^{3}H, ^{14}C]-reticuline, Battersby and coworkers [3] concluded that dehydroreticuline served as an intermediate during the interconversion of the (*S*)- to the (*R*)-form of reticuline. It was not until 1978 that Borkowski *et al.* [4] unequivocally showed that 1,2-dehydroreticuline is a naturally occurring compound and that it is efficiently incorporated into the morphinan alkaloids.

Regarding the chemical synthesis of the analgesic, (−)-morphine, the correct stereochemistry is the crucial point. The unnatural enantiomer, (+)-morphine, shows a more than 10^3-fold weaker affinity for its physiological opiate receptor. In attempts towards the chemical total synthesis of (−)-morphine, the yield of the desired enantiomer was usually increased by transforming the (*S*)-benzylisoquinoline intermediates to the (*R*)-form by a procedure affording classical separation of the enantiomers but in low yield [5–7].

In a continuation of our work on the biosynthesis of benzylisoquinoline alkaloids, we have found here that (*S*)-tetrahydroprotoberberine oxidase (STOX) in addition to its activity towards the oxidation of (*S*)-tetrahydroprotoberberines [8] can also oxidize (*S*)-benzylisoquinolines to their corresponding 1,2-dehydroforms (Fig. 1). This ability to transform a variety of alkaloids makes STOX interesting not only from a biosynthetic view point, but from a biotechnological one as well. To exploit the biotechnological potential of an enzyme which cannot commercially and easily be obtained, it is advantageous to bring it into a form which can be reused several times for catalytic transformation. This is usually achieved by attachment of the enzyme to an appropriate support.

We report here methods for the immobilization of STOX together with a procedure for the conversion of racemic or (*S*)-norreticuline to 1,2-dehydronorreticuline and subsequently to (*R*)-norreticuline.

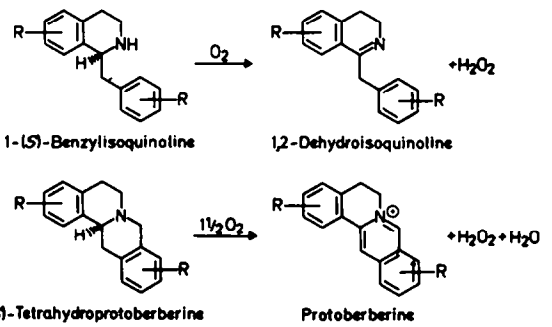

Fig. 1. (*S*)-Tetrahydroprotoberberine oxidase (STOX) catalysed oxidation of different substituted benzylisoquinolines and tetrahydroprotoberberines to the corresponding dehydroforms and protoberberines respectively. The enzyme is specific for substrates with the (*S*)-configuration. *N*-Methyl-benzylisoquinolines are also accepted as substrates.

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RESULTS

Occurrence of STOX

A screening of differentiated plants as well as cell cultures of different plant families was done not only to learn about the taxonomic distribution of the enzyme but also to find the most suitable plant material for the isolation of STOX. The plant species tested were all known to contain reticuline-derived alkaloids. Enzymatic activity of STOX was determined by ^{3}H-release assay or by means of an optical assay previously described [9]. As shown in Table 1, the highest activities of cell-free crude extracts from whole plants were detected in the roots of a *Berberis* species. Likewise, the roots of *Papaver somniferum* also seemed to be a richer source of STOX as compared with the leaves. Plant species which are known to contain no protoberberine alkaloids (*Nicotiana tabacum* or *Catharanthus roseus*) served as controls and were found to lack in the target enzyme. Of 13 cell suspension cultures tested, *B. wilsoniae* var. *subcaulialata* proved to be the most suitable regarding both the specific enzymatic activity and the overall yield of STOX.

Preparation of STOX

Highest enzyme activities could be obtained from cell cultures of *B. wilsoniae* after a cultivation period of 16 days in Fernbach flasks. Since the STOX was intended for preparative purposes, a partially purified enzyme extract free of interfering activities such as that of the berberine bridge enzyme [10] was readily achieved. Detailed investigations have shown that the quaternary protoberberines, abundant in this *Berberis* cell culture, [11] exert an inhibitory effect on STOX. To remove most of these interfering alkaloids, the deep yellow crude enzyme extract was first passed through a column filled with Amberlite XAD-2. The eluant was subsequently applied to an anion exchange gel, eluted with a two-step gradient, dialysed and used for immobilization studies. A typical preparation procedure is summarized in Table 2.

Immobilization of STOX activity

Different methods were applied to obtain immobilized STOX. Carrier binding by covalent attachment was done with STOX purified as described above according to the procedures given by the manufacturers. Coupling to the rigid glass support (controlled pore glass; CPG-10) could be achieved by introducing amino groups onto the glass surface followed by classical glutaraldehyde activation [12]. The enzymatic activity of the immobilized enzyme was determined by means of an optical test as 13.7% of the free STOX. Reduction of the Schiff base originating between an amino function of the STOX enzyme and the aldehyde by sodium borohydride did not negatively influence the enzymatic activity of the CPG-bound STOX. Good immobilization yields of 17.8% were also obtained by covalently coupling STOX to Sepharose via the activated tresyl residue. Entrapment of STOX in a lattice of an alginate matrix by the $CaCl_2$-method yielded the well-known beadlike enzyme preparation showing still about 10% of enzymatic activity as compared to the soluble enzyme.

In a quite different approach to immobilize the STOX activity, the cell-free preparation of the enzyme was avoided. As described for several plant cells, it is in principle possible to immobilize the whole cell containing

Table 1. Occurrence of (*S*)-tetrahydroprotoberberine oxidase in plant cell cultures and whole plants of six plant families

Differentiated plants (pkat/g dry wt)		Plant cell cultures (nkat/l medium)	
Annonaceae:	*Annona reticulata* (305)	Berberidaceae:	*Berberis wilsoniae* var. *subcaulialata* (36); *B. stolonifera* (32); *B. beaniana* (24); *B. aristata* (18); *B. aggregata* (21)
Berberidaceae:	*Berberis wilsoniae* var. *subcaulialata* leaves (120), root (350)	Menispermaceae:	*Cissampelos mucronata* (25)
Papaveraceae:	*Chelidonium majus* (85); *Papaver somniferum* leaves (63), root (192); *Corydalis cava* (60); *Eschscholzia lobbii* (31)	Papaveraceae:	*Eschscholzia californica* (4); *Argemone platyceras* (2); *Papaver somniferum* (2)
Fumariaceae:	*Fumaria parviflora* (102)	Ranunculaceae:	*Thalictrum glaucum* (2); *T. foetidum* (2); *T. flavum* (2); *T. dipterocarpum* (1)

Table 2. Three-step preparation of (*S*)-tetrahydroprotoberberine oxidase (STOX) from 1280 g (fresh weight) *Berberis wilsoniae* var. *subcaulialata* cell suspension culture

	Volume (ml)	Activity (nkat)	Protein (mg)	Specific activity (nkat/mg)	Yield (%)	Purification (-fold)
Cell-free extract	2720	1469	4379	0.34	100	–
XAD-2	2700	2247	3678	0.61	153	1.8
Ion exchange chromatography	340	1948	785	2.48	132	7.4

The increase in activity is due to the adsorption of inhibitory low molecular substances by the XAD resin.

the enzymes of interest without losing the biosynthetic potential of the cell [13]. Of the various procedures attempted the acrylamide polymerisation with intact cells of *B. wilsoniae* was successful. This method, which had predominantly been used with microorganisms [14] resulted in a partial disintegration of the *Berberis* cells as seen by the release of the vacuolar protoberberines into the supernatant after polymerization. The polymerized cells still showed more than 5% of the soluble STOX activity as compared to extracts from control cells.

An overview of the results obtained with the various methods is given in Table 3. The relative good yields and the stability of STOX immobilized to controlled pore glass made this method appear superior to the others. Additional advantages were the unlimited availability of the carrier, the inexpensive activation and coupling procedure as well as the excellent physical stability of this inorganic carrier all of which are of central importance for large scale operation.

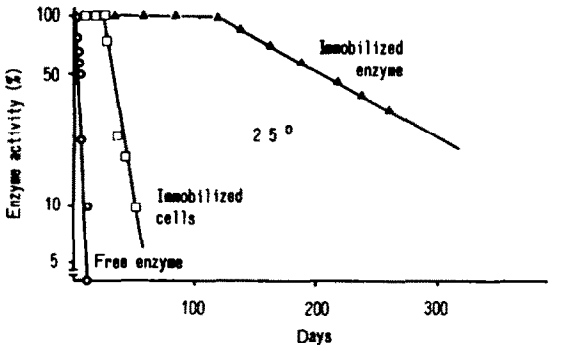

Fig. 2. Stability of free and immobilized (*S*)-tetrahydroprotoberberine oxidase at 25° storage temperature. With sterile filtered, free enzyme (o-o) 50% of the starting activity was present after four days, with *Berberis* cells entrapped in polyacrylamide (□-□) after 30 days, with enzyme immobilized on activated glass (▲-▲) after 200 days.

Properties of immobilized STOX

The immobilization of an enzyme to a support generally influences the physico-chemical properties in a more or less favourable way. The general aim of immobilization is not only to get the catalyst in a reusable and easy to handle form but also to improve some possibly critical and undesirable characteristics of the enzyme. A central aspect of immobilization is the possibility to increase the lifespan of the enzyme of interest. To study the influence of the immobilized status on the stability of STOX, we compared the enzymatic activities of the free enzyme, enzyme covalently bound to controlled pore glass and enzyme entrapped in its natural surrounding in the form of whole *Berberis* cells in a polyacrylamide gel. The covalent attachment of STOX to the glass matrix drastically influences the enzyme's stability (Fig. 2). While free enzyme loses 50% of its original activity within 4 days, the covalently bound form shows its initial activity after more than 100 days and the 50% level is not reached before 200 days at room temperature. Polyacrylamide entrapped *Berberis* cells also seem to be a suitable form to increase the half-life of the enzyme.

The improved stability of STOX can also be seen from measuring the temperature dependence of the enzymatic activity. A comparison of the temperature optima of CPG-bound STOX with free enzyme shows a significant shift towards elevated temperatures. Immobilized STOX showed a maximal turnover rate at 50°, that is 10° above the optimum for free enzyme. Acrylamide entrapped *Berberis* cells showed an intermediate value of 47°.

The differing microenvironments of freely diffusible enzyme in solution and an enzyme entrapped in a gel or covalently bound to a carrier matrix often markedly influences kinetic aspects of the enzymatic catalysis and those properties due to the interaction of essential charged groups. To study some of these effects we first compared the influence of the pH on the three different forms of STOX. The pH-optima of STOX catalysis are shifted towards more alkaline conditions (Fig. 3). This interesting observation is not favourable concerning the preparative biotechnological aspect. The reason is that the solubility of almost all substrates that can undergo oxidation by STOX is much better under acidic conditions, so that the pH-shift by immobilization moves in an unwanted direction.

The altered spatial flexibility, the changed microenvironment and the interactions with the immobilization matrix can change the kinetic behaviour of an enzyme. Comparing the concentration dependence of the initial turnover rate using (*S*)-norreticuline as substrate, we found that both the soluble enzyme as well as the CPG-

Table 3. Immobilization of intact cells and a cell-free preparation of (S)-tetrahydroprotoberberine oxidase (STOX) by entrapment and covalent attachment. Activities of immobilized STOX are given as percentage of activity of the enzyme in solution.

Enzyme source	Immobilization method	Immobilization matrix	Enzyme activity [rel. %]
Berberis cells	Entrapment	Polyacrylamide	5.2
		Ca^{++} alginate	9.7
Cell-free enzyme preparation	Covalent attachment	Sepharose -tresyl activated	17.8
		-aminohexyl	7.8
		Activated glass	13.7

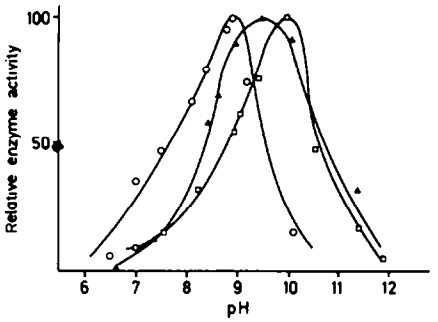

Fig. 3. pH-profile of the catalytic activity of soluble (*S*)-tetrahydroprotoberberine oxidase (o-o), CPG-bound enzyme (▲-▲) and of whole *Berberis* cells immobilized by polymerisation in acrylamide (□-□).

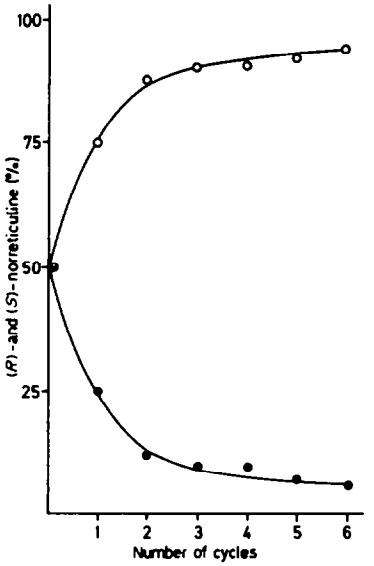

Fig. 4. Formation of (*R*)-norreticuline (o-o) starting with a racemic mixture of norreticuline. Cycles of stereospecific enzymatic oxidation followed by non-specific reduction with sodium borohydride were repeated six times. Individual determination of the (*R*)- and (*S*)-enantiomer (●-●) could be achieved by radioimmunoassay.

bound form showed the typical hyperbolic Michaelis–Menten Kinetics. The K_M-values were almost identical: 0.15 mM for free enzyme, 0.12 mM for the immobilized STOX. Differences were found, though, for V_{max}. The immobilization increased this constant by a factor of 18–2.1 nmol/sec. A comparison of some characteristic parameters of free and immobilized STOX-enzyme is given in Table 4.

Enzymatic transformation of racemic norreticuline into the (R)*-enantiomer*

Chemical transformation of (−)-(*S*)-norreticuline into the (*R*)-form is a time consuming, multistep, low yield procedure including aromatization, reduction and separation of the enantiomers [7]. A process using the stereospecific enzymatic oxidation of (*S*)-norreticuline combined with a simple borohydride reduction was developed to solve this longstanding problem. STOX immobilized to CPG was used as the catalyst. An alkaline aqueous solution of racemic norreticuline was pumped over a column with immobilized STOX. The eluant containing the dehydro derivative was reduced by addition of sodium borohydride and the ratio of the (*R*)- and (*S*)-enantiomers was determined using the sterospecific radioimmunoassays (RIA) developed by our group [15]. Repeating this procedure by again passing the norreticuline solution over the column, we could stepwise increase the percentage of (*R*)-norreticuline (Fig. 4). In the first cycle exactly the theoretically expected ratio of the norreticuline enantiomers (R:S) of 75:25 was obtained. After six cycles a ratio of 97.5:2.5 was calculated from the immunoassay data. The theoretical value is 99.2:0.8. Problems were only encountered by the adsorption of the enzymatically formed dehydronorreticuline to the matrix necessitating thorough washing steps.

DISCUSSION

There is a growing interest in new synthetic applications of enzymes in preparative bioorganic chemistry due to the substrate- and stereospecificity they can achieve. The discovery [8] of STOX in *Berberis* species as an enzyme oxidizing exclusively (*S*)-benzylisoquinolines has opened up a new possibility in the attempts toward an economical total synthesis of morphine. The crucial step, the trans-

Table 4. Characteristic parameters of (*S*)-tetrahydroprotoberberine oxidase (STOX) as free enzyme, of STOX immobilized onto controlled pore glass and of the STOX activity of whole *Berberis* cells entrapped in a polyacrylamide gel

	Immobilized plant cells	Immobilized enzyme	Free enzyme
pH-optimum	9.8	9.4	8.9
Temperature optimum [°c]	47	50	40
K_M [mmol/l]	n.d.	0.12	0.15
V_{max} [nmol/sec]	n.d.	2.1	0.12
Stability at 25° [d]*	30	200	4

*Time (in days) required for 50% of the enzyme activity to be lost.

formation of (*S*)-benzylisoquinolines into the corresponding dehydro forms can now easily be accomplished by enzymatic means.

The occurrence of STOX is not restricted to the Berberidaceae but it is also formed in species of six other plant families [9 and unpublished results]. A screening of representative species of protoberberine-containing plant families by an immunological dot blot procedure using an anti-STOX antiserum firmly corroborates the occurrence data found with the enzymatic STOX assay (Table 1). These results were confirmed by work done with a cell culture of Ranunculaceae *Coptis japonica*, a species yielding high amounts of berberine. In cell-free extracts of this plant, STOX was also detected [16], which, however, differed from the *Berberis* oxidase by its lower M_r (58 000) and the lack of detectable H_2O_2 generation during catalysis. Since these authors did not study the substrate specificity of their crude STOX preparation, one can only speculate about using this enzyme for the synthesis of 1,2-dehydrobenzylisoquinolines.

Our preparation of STOX was achieved with relative ease. This was surprising because STOX occurs strictly compartimentalized in a special class of vesicles harbouring a sequence of enzymes involved in alkaloid biosynthesis [9]. After removal of inhibitory protoberberines from the crude extract by the XAD-adsorption step, the enzyme lost its activity in a few days. The problem of enzyme instability was overcome by the immobilization of STOX.

Only a minimum of effort is necessary if one can circumvent the preparation of a cell-free extract and the subsequent enrichment procedure simply by using the whole cells as a catalyst. Though some cells were broken by the polymerisation procedure of the *Berberis* cells into the acrylamide matrix, we could not detect a significant leaching of protein. Low overall yield of STOX activity and the unfavourable physical form, however, led us to look for a better entrapment method. The entrapment of cell-free STOX in calcium alginate was a workable method, but limitation by diffusion processes across the gel beads made it an inappropriate alternative in comparison to methods that covalently bind enzymes to an appropriate water-insoluble support. An enzyme activity yield of 13.7% for an immobilization process cannot be regarded as a good value *per se*, but bringing the longterm stability into calculation one easily sees that a 13.7% activity yield together with a standing half-life of 200 days at 25° is by far more advantageous than the use of the soluble enzyme preparation which loses 50% of its activity during the first four days. A still better stability was found when the immobilized STOX was stored at 4°. After 355 days the preparation still showed 50% of its original activity.

The enzymatic oxidation to 1,2-dehydrobenzylisoquinolines is not restricted to (*S*)-norreticuline as a substrate. Many differently substituted 1-benzylisoquinolines are accepted as substrates including those compounds used in the chemical total synthesis of morphine (unpublished results). The analytical scale procedure described here for the enzymatic chemical synthesis of (R)-norreticuline from a racemic mixture provides an example of the types of chemical problems which can be elegantly solved with a biotechnological process utilizing an enzyme such as STOX. Optimization of this process could be achieved by stereospecific reduction to the (*R*)-enantiomer by either chemical [17, 18] or enzymatic approaches.

EXPERIMENTAL

Materials. Plants were obtained from the Munich Botanical Garden or grown in our institute. The plant organs were frozen in liquid N_2, powdered and immediately used for crude extract preparation. Cell cultures were provided by our cell culture laboratory. *Berberis wilsoniae* var. *subcaulialata* was grown as rdviously described [9] and frozen in liquid N_2. Norreticuline was synthesized according to [6]. Activated Sepharoses and DEAE-Sephacel were obtained from Pharmacia, Amberlite XAD-2 and controlled pore glass (CPG-10) were provided by Serva.

Crude enzyme extracts. Per gram frozen plant material 2 ml of 50 mM K-Pi pH 7.4 was added. The extract was stirred to a homogenous suspension and centrifuged for 10 min at 10000 *g*. $(NH_4)SO_4$ was added to the supernatant to 75% satn. The ppt. was collected by centrifugation (10 min, 10000 g). The pellet was taken up in extraction buffer, desalted by passage over a Sephadex G-25 column (1 × 12 cm) equilibrated in extraction buffer and STOX activity was assayed.

STOX preparation. All operations were carried out at room temp. 1280 g frozen *Berberis* cells were suspended in 1800 ml 50 mM K-Pi pH 7.4 containing 5 mM mercaptoethanol. The brei was filtered through 4 layers of cheese-cloth and the filtrate cleared from debris by centrifugation at 10000 *g* for 15 min. The yellow protein extract was twice passed over an Amberlite XAD-2 column (5 × 15 cm) to remove the interfering alkaloids. The eluant was applied to an anion exchange column (DEAE-Sephacel; 5.6 × 5 cm). After washing with 0.7 l 50 mM K-Pi pH 7.4 containing 0.1 M KCl, the bound protein containing the STOX enzyme was eluted with 50 mM KPi pH 7.4 containing 0.6 M KCl. Salt was removed by dialysis against 50 mM K-Pi pH 7.4.

Immobilization of cells. 25 g (fr. wt) cells from *Berberis* suspension culture were washed × 4 with H_2O to remove media components interfering with the polymerization, suspended in 10 ml water and added to a solution containing 10 ml acrylamide (30% w/v), 4 ml bisacrylamide (1% w/v), 5.9 ml Tris buffer (1.5 M pH 8.7) 0.1 ml ammoniumperoxodisulphate (10% w/v) and 10 μl TEMED. The gel was cut into square pieces (0.5 × 0.5 cm) and extensively washed with 0.1 M sodium borate buffer pH 9 to remove the protoberberines set free by the polymerization procedure.

Immobilization of STOX. For entrapment in calcium alginate, 1 ml STOX was mixed with 5 ml of 5% sodium alginate and the suspension was dropped via a syringe into a 100 mM $CaCl_2$ solution. After 4 hr of stabilization the beads were separated, washed and tested for STOX activity.

Immobilization on activated Sepharoses were all performed according to the protocol provided by Pharmacia. For immobilization to glutaraldehyde activated CPG-10 according to the method of [12] the glass was silanized with γ-amino propyltriethoxysilane and activated by incubation in a 2.5% solution of glutaraldehyde in 50 mM K-Pi pH 7.4. 3 ml STOX containing 6.3 nkat were added per ml of the thoroughly washed tan glass. After 3 hr of gentle shaking, the glass was washed and the coupling yield was determined.

For transformation of racemic norreticuline into the (*R*)-enantiomer, 40 ml 0.7 mM (*R, S*)-norreticuline in alkaline aqueous solution (pH 9 with NaOH) was pumped at 0.2 ml/min over a column (2.5 × 16 cm) with CPG-bound STOX (0.8 nkat/ml). After washing the column with 40 ml H_2O (pH 9) 10 mg $NaBH_4$ was added to the combined eluant. Remaining $NaBH_4$ was destroyed by acidification (HCl), the pH was readjusted to 9 and the solution was again pumped over the column. The procedure was repeated × 5. Samples for monitoring the en-

antiomeric ratio by radioimmunoassay were taken after each cycle.

Calculations. Coupling yields were determined from the protein bound to the matrix. Activity yields were calculated from the measured activity of the immobilized STOX in relation to the amount of protein bound by the carrier.

Assays. STOX in solution was assayed as described [9]. Activity of immobilized STOX was measured by means of the optical test using (*S*)-norreticuline as a substrate. In polyacrylamide polymerized cells, the calcium alginate entrapped STOX and the CPG-bound enzyme activities were assayed in a batch procedure, in which the particulate matrix was gently shaken in a 0.1 M sodium borate pH 9 solution containing 3 mM (*S*)-norreticuline. STOX activity was calculated from the increase in absorbance at 340 nm of the supernatant. For gel immobilized forms of STOX (Sepharoses), the alkaline (*S*)-norreticuline solution was pumped through the gel and the product was measured in the eluant photometrically.

Berberine bridge enzyme (BBE) was assayed according to [10]. Protein was determined according to the method of ref. [19]. The radioimmunological quantitation of the (*R*)- and (*S*)-norreticuline enantiomers was performed as previously described [15].

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