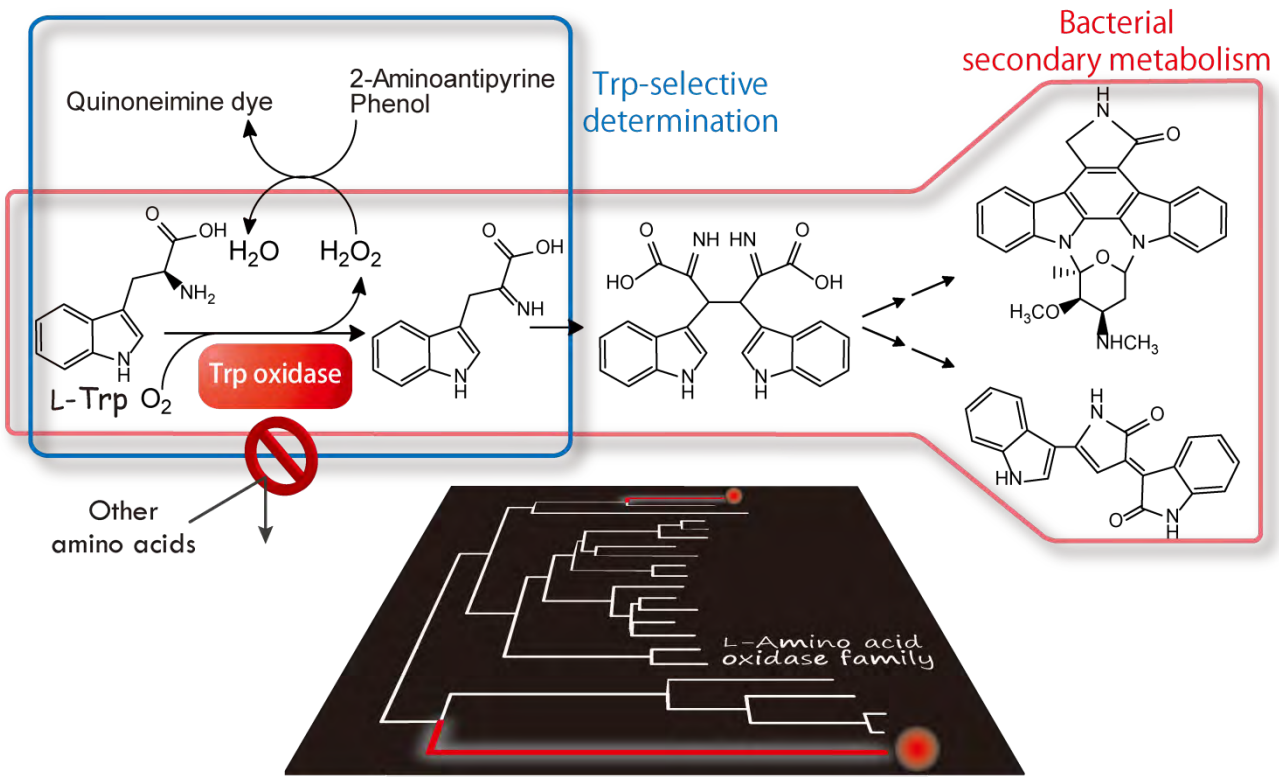

Selective tryptophan determination using tryptophan oxidases involved in bis-indole antibiotic biosynthesis

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Abstract

A novel tryptophan assay was developed using tryptophan oxidases. While many L-amino acid oxidases (LAAOs) have been reported to catalyze tryptophan oxidation, most of them have broad substrate specificity and oxidize multiple amino acids. To obtain a tryptophan-specific LAAO, we focused on bis-indole antibiotic biosynthesis, a bacterial secondary metabolic pathway. A putative LAAO from *Streptomyces* sp. TP-A0274, StaO involved in staurosporine biosynthesis, was heterologously expressed, biochemically characterized, and shown to serve as a selective tryptophan oxidase for the first time. In addition, another LAAO, VioA involved in violacein biosynthesis in *Chromobacterium violaceum*, was characterized for comparison with StaO. Interestingly, these enzymes share similar properties, namely narrow substrate specificity and high affinity for L-tryptophan, despite the phylogenetic distance between them. Owing to these features, uncommon among known LAAOs, StaO and VioA assays can be used for selective and accurate quantification of L-tryptophan. Indeed, the assays provided tryptophan concentrations in human plasma as accurately as those obtained by HPLC. Therefore, these enzymes were clearly shown to offer an effective method for determining tryptophan in biological samples rapidly, inexpensively, and accurately. The results shown here also suggest the possibility of metabolism-oriented screening as a strategy to obtain enzymes highly selective for individual biomolecules.

Key words

tryptophan oxidase; L-amino acid oxidase; staurosporine; violacein

Introduction

Amino acids are key metabolites in our body, and their quantification has attracted significant attention in recent years in part because of their role as biomarkers for the diagnosis of a variety of diseases. For example, the quantitation of phenylalanine and methionine in blood are established methods for the diagnosis of phenylketonuria and homocystinuria, respectively [1; 2]. It has also recently been reported that amino acids in blood are useful biomarkers for certain cancers and inflammatory bowel disease [3; 4]. Thus, accurate measurement of amino acids in biological samples is clearly important from a medical viewpoint.

Various methods have been developed to quantify amino acids, including instrumental analyses by high-performance liquid chromatography (HPLC), gas chromatography, and capillary electrophoresis [5]. However, these instrumental methods are time-consuming when analyzing multiple samples, and require bulky and expensive apparatuses. Because these drawbacks often limit the application of amino acid measurements at present to large clinics and laboratories that possess the required resources, a novel method is desired in order to expand their use to smaller clinics with more limited resources. Enzymatic assays can provide promising solutions for measuring amino acids easily and rapidly [3], because multiple samples can be simultaneously and rapidly analyzed without the need for expensive analytic instruments such as HPLC. Enzymes can be incorporated into diagnostic reagent kits, allowing the target molecule to be optically measured by clinic biochemical analyzers that are widespread in hospitals. In this regard, an enzymatic assay for phenylalanine has been widely adopted for neonatal mass screening of phenylketonuria in Japan and in several other countries [2; 6]. Furthermore, colorimetric enzyme assays can be incorporated into test strips that are available in small clinics and patients' homes, permitting the analytical results to be easily visualized.

L-Tryptophan is an essential amino acid that plays various roles in the body. One of its roles is as a precursor of serotonin, a neurotransmitter found in the central nervous system, and a significant correlation has been reported between plasma tryptophan concentrations and depressive illness [7]. Tryptophan concentrations have also been reported to be related to fibromyalgia [8]. In addition, tryptophan has recently been identified as a useful biomarker for diagnosing inflammatory bowel disease [3]. Because of this clinical background, an assay by which tryptophan can be easily quantified is expected to bring many medical

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Abbreviations: CV, coefficient of variation; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; HPLC, high-performance liquid chromatography; IPA, indole-3-pyruvic acid; LAAO, L-amino acid oxidase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UPLC, ultra-performance liquid chromatography

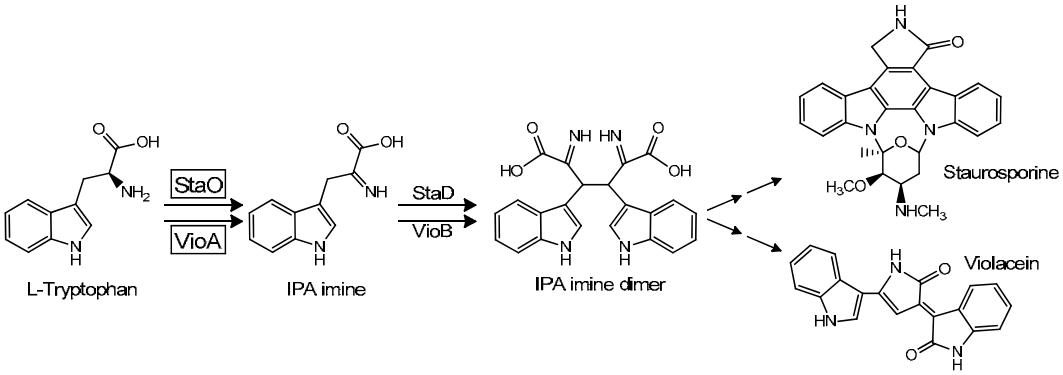

Fig. 1. Biosynthetic pathways of staurosporine in *Streptomyces* sp. TP-A0274 and for violacein in *C. violaceum.* IPA, indole-3-pyruvic acid.

benefits. Colorimetric assays are often used to detect tryptophan in a sample containing few impurities, but this method cannot be used for biological samples containing other indole derivatives that interfere with the assay [9]. Whereas a number of instrumental procedures that measure amino acids can be applied to tryptophan-specific determination [10; 11; 12; 13], enzymatic assays have been less well developed for measurement of tryptophan. Tryptophan 2-monooxygenase (EC 1.13.12.3) has been employed to construct a tryptophan sensor in some studies [14]. However, the use of this sensor is limited because of the interference by L-phenylalanine and the complicated flow-injection manifold required that includes multiple pumps and an O_2 electrode.

L-Amino acid oxidases (LAAO; EC 1.4.3.2) catalyze the oxidative deamination of L-amino acids, resulting in the formation of hydrogen peroxide, 2-oxo acids, and ammonia. This class of enzymes is potentially useful for amino acid quantification because a product of the enzyme-catalyzed-reaction is hydrogen peroxide, which can be easily detected using colorimetric assays. In spite of this advantage, LAAO has not been widely applied to selective quantification due to its broad substrate specificity: LAAO from snake venom, one of the best-studied LAAOs, exhibits nonspecific reactivity toward a wide range of amino acids [15; 16; 17]. Most of the LAAOs purified from other animals, fungi, and bacteria have been shown to react with multiple amino acids [18; 19; 20; 21; 22; 23; 24]. An exception is LAAO from *Osteichthyes*, which exhibits high specificity toward L-lysine [25; 26]. An enzyme from a basidiomycete, *Coprinus* sp. SF-1, has been reported to be relatively specific for tryptophan [27]. However, its substrate specificity is insufficient for selective quantification because of its reactivity with phenylalanine and tyrosine. Moreover, no structural information concerning the primary sequence is available for this enzyme, making the enzyme unavailable without laborious cultivation and purification from the native basidomycete.

In a few bacteria, tryptophan is used as a precursor of bis-indole antibiotics, indolocarbazoles such as staurosporine in *Streptomyces* sp. TP-A0274 [28] and violacein in *Chromobacterium violaceum* [29] (Fig.1). The previous studies presumed that tryptophan is oxidized by an LAAO as the first step of these biosynthetic pathways.

In this study, we focused on these biosynthetic pathways to obtain tryptophan-specific LAAOs. Through enzymatic characterization, the obtained LAAOs showed unusually high specificity for L-tryptophan, enabling selective quantification of L-tryptophan. By using these enzymes, we developed an enzymatic assay that can determine tryptophan in biological samples rapidly, accurately, and selectively.

Materials and Methods

Bacterial strains and plasmids

Escherichia coli JM109 and *E. coli* BL21 (DE3) were used as hosts for cloning and heterologous expression of tryptophan oxidases, respectively. *Streptomyces* sp. TP-A0274 and *C. violaceum* NBRC 12614 were used as the sources of *staO* and *vioA*, respectively. Chaperone plasmids pG-kJE8, pGro7, pKJE7, pG-Tf2, and pTf16 were purchased from Takara (Shiga, Japan).

Cloning of tryptophan oxidase genes

pTYMCsta [28], which carries *staO*, was digested by *Stu*I, and the resulting fragment was cloned into the *Hinc*II site of pTYM19 [30]. N- and C-termini of *staO* were modified using the QuikChange site-directed mutagenesis kit (Agilent, CA, USA) and the following primers: StaO-N-sense (5’-tactggaggaaacatatgacggcaccc-3’), StaO-N-anti (5’-caagggtgccgtcatatgtttcctcca-3’), StaO-C-sense (5’-gaccggtcggcgaagctttcttcgacctg-3’), and StaO-C-anti (5’-gcaggtcgaagaaagcttcgccgaccggt-3’). *staO* was cut from the resultant plasmid by *Nde*I and *Hin*dIII digestion and was inserted into pET-26b to construct an expression plasmid, pET26b-StaO.

vioA was amplified by PCR using *C. violaceum* genome as the template and the following two primers: 5’-attctagacatatgaagcattcttccgatatctg-3’ and 5’-aataagcttcgcggcgatgcgctg-3’. The amplified fragment was inserted into pET-28a after *Nde*I and *Hin*dIII digestion to construct an expression plasmid, pET28a-VioA.

Heterologous expression and purification of tryptophan oxidases

E. coli BL21 (DE3) harboring pET26b-StaO or pET28a-VioA was cultured aerobically in LB medium containing 50 μg/ml kanamycin at 37˚C. Gene expression was induced by the addition of IPTG of 0.1 mM when the optical density at 600 nm reached 0.6-0.8. The culture temperature during the induction was shifted from 37°C to 16°C, if necessary, to minimize inclusion body formation. About 4 hours (37°C) or 16 hours (16°C) after the addition of IPTG, cells were harvested by centrifugation. When a chaperone plasmid was coexpressed, 20 μg/ml chloramphenicol, 0.5 mg/ml L-arabinose, and/or 5 ng/ml tetracycline were added to the medium. For purification, StaO was induced at 16˚C with the coexpression of pG-kJE8, while VioA was induced at 16˚C without coexpression of any chaperones.

Enzyme solutions were maintained at 4 ˚C throughout purification. Cells were disrupted by sonication and centrifuged to remove cell debris. The supernatant was applied to an open column packed with 5 ml of Ni-Sepharose Fast Flow (GE Healthcare, Buckinghamshire, UK). After the elution of unbound proteins with 20 mM Tris-HCl and 50 mM imidazole-HCl (pH 8.0) containing 300 mM NaCl, the His-tagged protein was eluted with 20 mM Tris-HCl and 500 mM imidazole-HCl (pH 8.0) containing 300 mM NaCl. For StaO purification, 10% (v/v) glycerol was added to the solutions. The Ni-Sepharose fraction of StaO was applied to a MonoQ HR 5/5 column (GE Healthcare; bed volume, 5 ml) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 10% (v/v) glycerol. Proteins were eluted with a gradient of NaCl from 0 M to 1 M using an ÄKTA purifier system (GE Healthcare). The purified fractions of StaO and VioA were dialyzed against 20 mM Tris-HCl (pH 8.0) to remove salt if necessary. Glycerol was added to the purified fractions at a final concentration of 20% (v/v), and they were stored at 4˚C or -80˚C until use.

Detection of tryptophan oxidase activity

Tryptophan oxidase activity was measured by a colorimetric assay. Reaction mixtures contained 20 mM Tris-HCl (pH 9.0), 5 mM L-tryptophan, 1 mM 4-aminoantipyrine, 1 mM phenol, 15 U/ml horseradish peroxidase (Wako, Osaka, Japan), and tryptophan oxidase solution in a final volume of 200 μl. Absorbance at 505 nm was monitored at 30˚C by a microplate reader, Infinite M200 (TECAN, Seestrasse, Switzerland), to detect the production of hydrogen peroxide. To investigate substrate specificity, 0.5 mM L-alanine, L-cysteine, L-aspartic acid, L-glutamic acid, L-phenylalanine, glycine, L-histidine, L-isoleucine, L-lysine, L-leucine, L-methionine, L-asparagine, L-proline, L-glutamine, L-arginine, L-serine, L-threonine, L-valine, L-tryptophan, L-tyrosine, D-tryptophan, or 5-hydroxy-L-tryptophan was used as the substrate in the reaction mixture.

Gel filtration

To estimate the molecular mass, gel filtration was performed using a Superdex 6 HR 10/300 column (GE Healthcare) equilibrated with 20 mM Tris-HCl (pH 8.0) buffer containing 150 mM NaCl at a flow rate of 0.5 ml/min. A gel filtration standard (Oriental Yeast, Tokyo, Japan) was used as molecular markers for the calibration. Each measurement of standards or samples was performed in triplicate.

Flavin determination

The flavin content in the enzyme was determined by reversed-phase chromatography after trichloroacetate treatment [31]. The enzyme solution and 20% trichloroacetate were mixed in a 1:1 ratio and kept for 10 min on ice. The mixture was centrifuged, and trichloroacetate was removed from the supernatant by extraction with diethyl ether, while keeping the mixture on ice. Standard solutions of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were also subjected to this extraction before analysis by ultra-performance liquid chromatography (UPLC). The extract was analyzed by a Waters Acquity UPLC System equipped with a BEH C18 column (1.7 μm, 100 × 2.1 mm i.d; Waters, Milford, MA, USA) using 25 mM potassium phosphate (pH 6.5) and 50% acetonitrile as two eluents. Flavin cofactor was detected by a fluorometer at excitation and emission wavelengths of 450 nm and 530 nm, respectively.

Construction of tryptophan standard curves by enzymatic assays

Reaction mixtures contained 20 mM Tris-HCl (pH 9.0), 0-100 μM L-tryptophan, 1 mM 4-aminoantipyrine, 1 mM phenol, 15 U/ml horseradish peroxidase (Wako), and 10 mU/ml tryptophan oxidase solution in a final volume of 200 μl. Absorbance was measured at 505 nm by the microplate reader at the end of the reaction, using an extinction coefficient of 6.4 mM^{-1} cm^{-1} for the quinoneimine dye product [32]. Ni-Sepharose fractions of StaO and VioA were used as the tryptophan oxidase solution. The detection limit of the assay was defined as three times the standard deviation of tryptophan-free blank samples (n = 20).

Instrumental analysis of tryptophan

Analytes were derivatized by the AccQ-Tag Ultra Derivatization Kit (Waters) according to the manufacturer's manual. Subsequently, analytes were subjected to liquid chromatography by the UPLC system equipped with an AccQ-Tag Ultra column (1.7 μm, 100 × 2.1 mm i.d; Waters). AccQ-Tag Ultra Eluent A and B were used as the eluents at a flow rate of 0.7 ml/min under the gradient described in the manufacturer's manual. The elution of derivatized L-tryptophan was detected by monitoring the absorbance at 260 nm.

Human plasma analysis

Six samples of human plasma were purchased from Kohjin Bio (Saitama, Japan) and used in the present work (Table 1). Frozen plasma was thawed and centrifuged to remove precipitates immediately before use. The pH of the thawed plasma was adjusted to 7 by adding KH_2PO_4 solution, if necessary. Microcon YM-10 (Millipore, Bedford, MA, USA) was used to remove proteins in the plasma prior to some enzymatic assays and all instrumental analyses. The assay mixtures containing 20-100 µl of plasma samples were analyzed in the same manner as the standard curve construction. The intraassay coefficient of variation (CV) was determined by measuring tryptophan in three plasma samples with StaO or VioA in a single run (n = 6). The interassay (between-run) CV was calculated from six independent runs.

Phylogenetic analysis

Amino acid sequences were aligned using the MUSCLE program [33] to construct phylogenetic trees by MEGA 5 [34] using the neighbor-joining or maximum likelihood method.

Table 1. Plasma samples used in this study.

Plasma	Donor	Lot number
A	Single donor	MT129165
B	Multiple donors	K08082114P
C	Single donor	MT129163
D	Single donor	R202258M
E	Single donor	N113881F
F	Multiple donors	CB0005A

Results

Expression and characterization of StaO

The biosynthetic pathway of violacein has been biochemically characterized in *C. violaceum* [29]. In this pathway, the precursor tryptophan is oxidized by VioA as the first step, and the indole-3-pyruvic acid (IPA) imine produced is dimerized to IPA imine dimer by VioB (Fig. 1). Subsequently, this unstable dimer is rapidly converted into protoviolacenic acid by VioE [29; 35; 36]. Although the overall pathway for staurosporine biosynthesis has been subjected to extensive study [37; 38; 39; 40], the first reaction step has not been well characterized. The staurosporine biosynthetic gene cluster contains no gene significantly similar to VioA while this cluster harbors *staD*, a gene

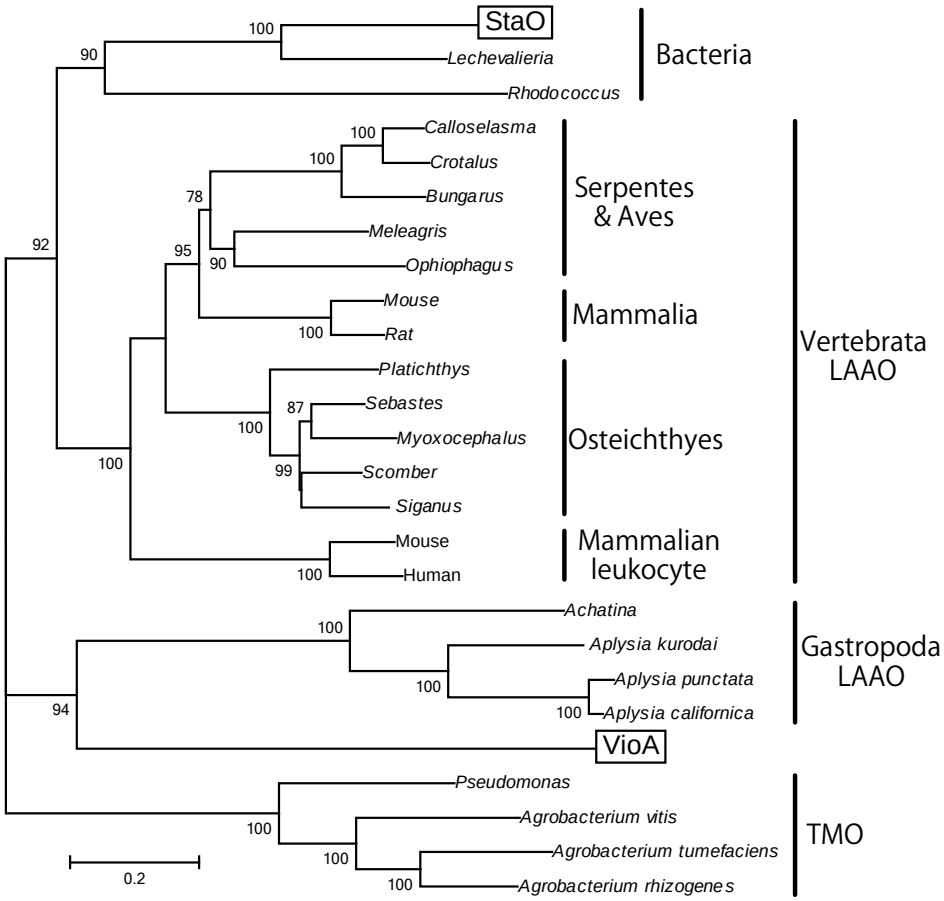

Fig. 2. Phylogenetic tree of the LAAO family constructed by the neighbor-joining method [49] based on amino acid sequences. Tryptophan monooxygenases (TMOs) were used as the outgroup. Bootstrap confidence values (>70) are shown as percentages of 500 bootstrap replicates at nodes reproducibly generated also by the maximum likelihood method. Enzymes from the following organisms were used in the analysis: *L. aerocolonigenes* (BAC15750), *Rhodococcus opacus* (AAL14831), *Calloselasma rhodostoma* (CAB71136), *Crotalus atrox* (AAD45200), *Bungarus fasciatus* (ABN72540), *Meleagris gallopavo* (ACA64754), *Ophiophagus hannah* (ABN72538), *Mus musculus* (NP_598653), *Rattus norvegicus* (NP_001100152), *Platichthys stellatus* (BAI66017), *Sebastes schlegeli* (BAF43314), *Myoxocephalus polyacanthocephalus* (BAG72078), *Scomber japonicus* (CAC00499), *Siganus canaliculatus* (ADW77183), *M. musculus* (NP_034345), *Homo sapiens* (NP_690863), *Achatina fulica* (CAA45871), *A. kurodai* (BAA11867), *A. punctata* (AAR14185), *A. californica* (AAT12273), *Pseudomonas savastanoi* (ZP_07004116), *A. vitis* (YP_002540148), *A. tumefanciens* (CAB44640), and *A. rhizogenes* (AAA22080).

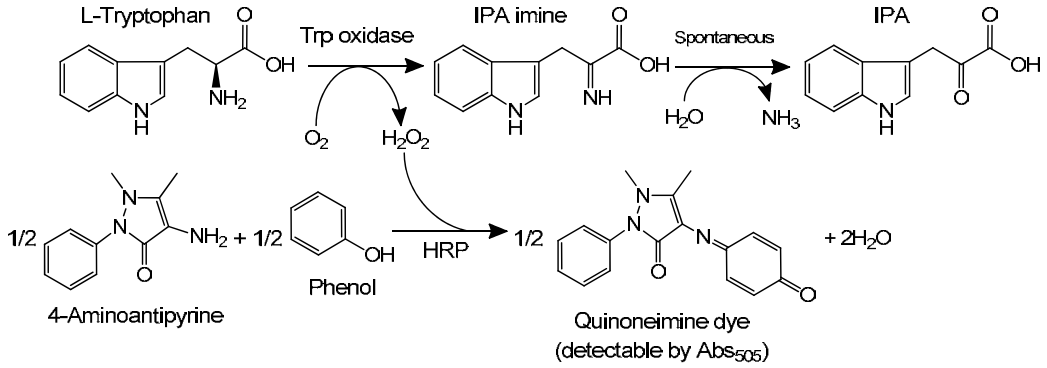

Fig. 3. Scheme for the spectrophotometric assay for measuring L-tryptophan. HRP, horse radish peroxidase.

similar to *vioB* (36% amino acid identity). Although one of the genes in this cluster, *staO*, was found to belong to the LAAO family, StaO and VioA show low homology (16% amino acid identity) and are phylogenetically distant from each other (Fig. 2). RebO from *Lechevalieria aerocolonigenes* shares higher homology to StaO (53% amino acid identity). However, the natural substrate of RebO is 7-chlorotryptophan, and an *in vitro* assay showed that RebO has only weak reactivity and low affinity toward tryptophan [41]. Thus, it remained unclear whether StaO possesses high reactivity toward L-tryptophan.

staO with a C-terminal His-tag sequence was cloned and heterologously expressed in *E. coli*. StaO was poorly expressed in the soluble fraction when induced under 37˚C cultivation because most of the protein was incorporated into inclusion bodies. Neither His-tag attachment at the N-terminus nor removal of the tag significantly affected the solubility (not shown). Soluble expression was improved, however, by lowering the induction temperature to 16˚C. Also, the effects of chaperone coexpression were tested by introducing a Chaperone Plasmid Kit (Takara), revealing that soluble enzyme increases when coexpressed with pG-kJE8 or pGro7. Tryptophan oxidase activity was spectrophotometrically measured by coupling with peroxidase to detect the produced hydrogen peroxide (Fig. 3). Crude extracts of the recombinants produced tryptophan-dependent hydrogen peroxide, while such activity was not detected in those extracts harboring an empty pET-26b vector instead of pET26b-StaO. This result clearly demonstrated that StaO serves as a tryptophan oxidase catalyzing the first step of the staurosporine biosynthesis pathway.

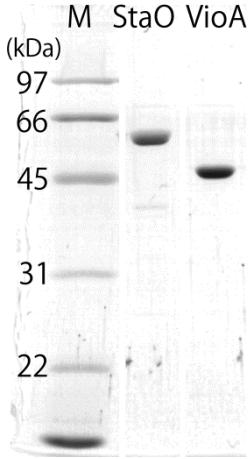

Fig. 4. SDS-PAGE (12%) of StaO and VioA purified by Ni-Sepharose chromatography. Each lane contained 2 μg of the purified enzyme. Proteins were visualized by Coomassie brilliant blue staining. M, molecular mass marker.

Heterologously expressed StaO was purified by Ni-Sepharose chromatography. Although the purified fraction yielded a single band on SDS-PAGE analysis in accordance with the deduced molecular mass of 57 kDa (Fig. 4), this protein unexpectedly yielded two peaks when analyzed by MonoQ chromatography. One of the peaks showed tryptophan oxidase activity while the other did not. Gel-filtration of the active fraction estimated the molecular mass to be 111 kDa, suggesting a dimeric structure for StaO, similar to that of many LAAOs [16; 17; 18; 24; 25; 26]. The molecular mass of the inactive peak was estimated to be greater than 300 kDa, suggesting that this fraction contained an abnormally aggregated form of StaO. The highest activity was observed at pH 8.0-9.0. The specific activity of the active MonoQ fraction was estimated to be 80 mU/mg, a value significantly lower than that of VioA (4.3 U/mg) [29]. The lower specific activity may have been caused by the coexistence of an inactive form of StaO in the purified fraction (see below). Acid extraction of the purified StaO and the subsequent HPLC analysis demonstrated that this enzyme contains 0.3 mol of FAD per mol of protein. This finding indicated that StaO harbors FAD as a cofactor while a substantial amount of StaO in the purified fraction was present as an apoenzyme that lacked flavin. This phenomenon has been observed with other LAAOs [29]. No significant change was observed when FAD or FMN was added to the reaction mixture, suggesting that exogenous flavin does not readily enter the binding site to activate the apoenzyme.

Substrate specificity of StaO and VioA

VioA was heterologously expressed and purified in a similar manner to StaO, and the two enzymes were used in the following tryptophan bioassays.

Strict substrate specificity is one of the most important features required for a selective bioassay. When L-tryptophan in the standard assay mixtures was substituted by another proteinogenic L-amino acid (or

Table 2. Reported relative substrate specificity (%) and the Km values of LAAOs from various organisms.

		StaO[*1]	VioA[*1]	*Coprinus*[*2]	*Rhodococcus*[*3]	*Bothrops*[*4]
Relative activity (%)	L-Tryptophan	[100]	[100]	[100]	[100]	[100]
	L-Alanine	ND	ND	ND	830	19
	L-Cysteine	ND	ND	ND	870	-
	L-Aspartate	ND	ND	-	92	-
	L-Glutamate	ND	ND	-	430	45
	L-Phenylalanine	≈1	ND	7	440	176
	Glycine	ND	ND	-	ND	-
	L-Histidine	ND	ND	ND	300	108
	L-Isoleucine	ND	ND	ND	380	155
	L-Lysine	ND	ND	-	460	-
	L-Leucine	ND	ND	ND	570	185
	L-Methionine	ND	ND	ND	600	187
	L-Asparagine	ND	ND	-	810	-
	L-Proline	ND	ND	ND	ND	ND
	L-Glutamine	ND	ND	-	430	-
	L-Arginine	ND	ND	-	580	110
	L-Serine	ND	ND	-	600	ND
	L-Threonine	ND	ND	-	ND	ND
	L-Valine	ND	ND	ND	270	35
	L-Tyrosine	ND	ND	3	450	45
	D-Tryptophan	ND	ND	ND	ND	-
Km for L-tryptophan (μM)		19	30[*5]	650	-	-

Activity with L-tryptophan was taken as 100%. ND, not detected; minus signs, not reported. [*1] Present study, except for the Km value of VioA; [*2] [27]; [*3] [24]; [*4] [50]; [*5][29].

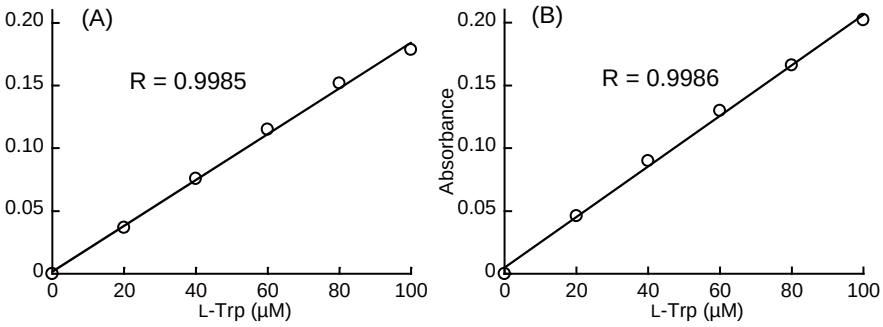

Fig. 5. Standard curves of L-tryptophan (L-Trp) using StaO (A) and VioA (B). The Y axes represent the absorbance change at 505 nm during the reaction. Note that the optical path length was not 1 cm but approximately 0.5 cm because samples were measured by the microplate reader.

glycine) or D-tryptophan at a concentration of 0.5 mM, StaO showed no significant activity except for trace activity toward L-phenylalanine (ca. 1% of that exhibited toward L-tryptophan). In a similar series of experiments, VioA showed no significant reactivity toward amino acids except L-tryptophan. These results indicated that both StaO and VioA have unusually higher substrate specificity than most known LAAOs (Table 2). StaO also oxidized 5-hydroxy-L-tryptophan as a substrate with a relative activity of 8% when compared to L-tryptophan. However, VioA has been reported to show a relatively high activity toward 5-hydroxy-L-tryptophan; activity toward 5-hydroxy-L-tryptophan was shown to be 86% of that toward L-tryptophan [29]. These findings suggested that these two enzymes differ from each other in their preference for L-tryptophan analogs. Nevertheless these reactivities toward 5-hydroxy-L-tryptophan are unlikely to affect the accuracy of the L-tryptophan measurement in blood because the 5-hydroxy-L-tryptophan concentration is less than 1% that of the L-tryptophan concentration in blood [42].

The Km value of StaO for L-tryptophan was estimated to be 19 μM (Table 2). This value is significantly lower than that exhibited by RebO (1.53 mM) [41], clearly indicating the high affinity of StaO toward tryptophan unlike RebO. The comparable Km value of VioA (30 μM [29]) suggested that StaO and VioA share enzymatically similar properties in spite of phylogenetic differences (Fig. 2).

Tryptophan standard curves constructed by using StaO and VioA

To verify whether tryptophan can be quantified by enzymatic assays with StaO or VioA, various amounts of standard L-tryptophan were added to reaction mixtures in the presence of each enzyme, and absorbance changes were monitored by a microplate reader. Absorbance increased after the reaction started until reaching an end point. No residual L-tryptophan was detected by the instrumental analysis in the reaction mixtures, and hence excess incubation time did not significantly influence the final absorbance. Linear standard curves were obtained between final

concentrations of 0-100 μM L-tryptophan and endpoints of absorbance change (Fig. 5). The correlation coefficients were estimated to be 0.9985 and 0.9986 for StaO and VioA, respectively. These results demonstrated that L-tryptophan can be precisely quantified by using StaO and VioA. Detection limits from StaO and VioA assays were calculated to be 1.4 μM and 0.9 μM, respectively, from the standard deviations of each blank sample to which no tryptophan was added. Detection of 1 μM tryptophan was also confirmed by observing reaction mixtures with or without added 1 μM L-tryptophan, which showed significantly different absorbance from each other ($P < 0.01$, $n = 12$). The observed sensitivities are sufficient to analyze human plasma samples (see below). The detection limits of tryptophan previously reported for instrumental analyses in the literature vary widely, from 0.03 μM to 50 μM [10; 11; 12; 13].

Tryptophan quantification in human plasma

Three plasma samples (A-C) were deproteinized by ultrafiltration, and then tryptophan concentrations in the filtrate were measured by the following three methods: StaO assay, VioA assay, and instrumental analysis. Reversed-phase liquid chromatography was employed as the instrumental analysis procedure following derivatization of amino acids by AccQ-Tag [43]. Comparable values were obtained by the three methods for each plasma sample (Fig. 6). This finding clearly shows that enzymatic assays with StaO or VioA can estimate tryptophan concentrations accurately in biological samples.

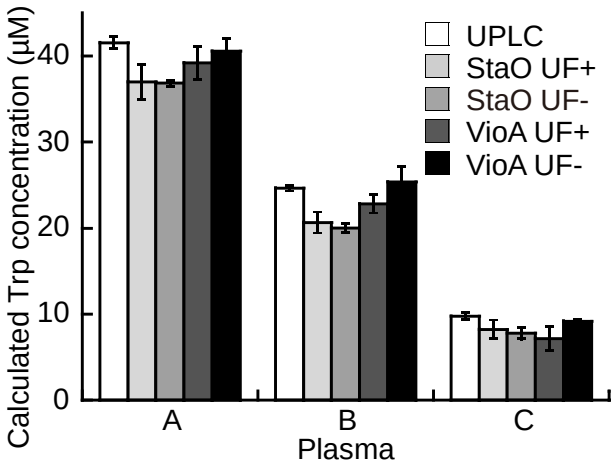

Fig. 6. Measurement of human plasma samples by instrumental analysis, StaO assay, and VioA assay. Three plasma samples were analyzed by the indicated methods after being subjected or not to the ultrafiltration (UF) pretreatment. Error bars indicate standard deviations from at least three independent experiments.

Table 3. CV values (%) of plasma assays by using StaO or VioA.

Plasma	StaO assay		VioA assay	
	Intraassay	Interassay	Intraassay	Interassay
D	9.3	6.4	3.6	7.2
E	6.9	5.2	6.3	9.4
F	9.4	8.3	7.7	4.6

Furthermore, plasma samples without deproteinization were also analyzed by enzymatic assays. Interestingly, the values obtained were comparable to those obtained in ultrafiltered plasma (Fig. 6). This result demonstrates that StaO and VioA assays provide simple and rapid tryptophan measurements without any deproteinization pretreatments such as ultrafiltration. CVs of StaO and VioA assays were determined by using another three plasma samples (D-F) without ultrafiltration (Table 3) in order to demonstrate the precision of these assays. When plasma samples were spiked with L-tryptophan, the recovery of L-tryptophan was shown to be 86% and 91% for the StaO and VioA assays, respectively. Considering that L-tryptophan binds to serum albumin [44], it is conceivable that the decrease in the recovery was caused by partial absorption of L-tryptophan by the plasma.

Stability of StaO and VioA

StaO is unstable in the absence of glycerol as >90% activity was lost after one day of storage at 4˚C. Addition of glycerol to StaO solutions significantly improved the stability; no significant decrease in activity was observed after one week of storage at 4˚C in 30% glycerol solution. VioA was relatively stable even in the absence of glycerol, losing <30% activity after one week of storage at 4 °C. Although 80% activity was lost through freezing and thawing of VioA solution without glycerol, no significant inactivation was observed when 10% glycerol was added to the solution prior to freezing. Neither activation nor inhibition was observed by adding glycerol to the enzyme solution or reaction mixtures. Addition of FAD or FMN had no significant effect on the stability of StaO and VioA.

Discussion

In this study, two tryptophan oxidases were employed to develop a selective and accurate assay of tryptophan: one is the previously characterized enzyme, VioA, and the other is a protein whose function had not been clarified, namely StaO. Both enzymes exhibited high substrate specificity toward L-tryptophan, allowing selective detection of L-tryptophan. Such a high specificity toward L-tryptophan is unusual among known LAAOs. While that from *Coprinus* sp. SF-1 [27] is relatively selective for tryptophan, this LAAO also oxidizes phenylalanine and tyrosine (Table 2). Moreover, this enzyme is not suitable for analyzing samples containing a low concentration of L-tryptophan because of its high Km value of 650 μM. Both StaO and VioA are superior to the *Coprinus* enzyme for selectivity and affinity, and they can be easily heterologously overexpressed and purified without any laborious processing. The result also suggests that, as a general rule, bacterial secondary metabolic pathways may provide valuable targets for screening for useful enzymes for quantification of metabolites other than tryptophan.

Some constituents in biological samples such as proteins are known to interfere with some enzymatic analyses, and thus deproteinization is necessary prior to conducting the assays [45]. Likewise, most HPLC analyses require similar pretreatments prior to analysis. In contrast, it was shown that StaO and VioA assays can be used to analyze for L-tryptophan in human plasma samples directly without any deproteinization pretreatments. Thus, StaO and VioA assays allow simpler and easier measurements of L-tryptophan in biological samples that contain a large amount of proteins than are obtained by the more usual enzymatic assays and instrumental analyses. The advantage that makes the assays convenient may be ascribed to the high substrate specificity of StaO and VioA toward L-tryptophan. Color development in the assays can be detected precisely by a spectrophotometer or semi-quantitatively by visual observation, requiring no special analytic instrument. Furthermore, these assays surpass instrumental analyses in the amount of time required to analyze each sample because multiple samples can be simultaneously assayed. Both enzymes are stable when stored at either 4˚C and -80˚C in the presence of glycerol, ensuring the suitability of the enzymes for routine, practical use.

This is the first study experimentally demonstrating that in addition to VioA, StaO functions as a tryptophan oxidase and that both enzymes exhibit high selectivity and affinity toward tryptophan. These characteristics differ from those of *L. aerocolonigenes* RebO, the enzyme most similar to StaO among those biochemically characterized thus far [41]. Therefore, it is instructive to compare the structural and substrate specificity profiles of StaO and RebO in order to understand what differentiates the substrate preferences of these two LAAOs. StaO showed lower reactivity toward 5-hydroxy-L-tryptophan (8%) than that exhibited by VioA (86%). While this compound had previously been assumed to be a precursor of bis-indole antibiotics [46], another study reconstituted the biosynthetic pathway and identified the genuine substrate of violacein not as 5-hydroxy-L-tryptophan but as L-tryptophan [29]. Although the biosynthetic pathway of staurosporine has not been reconstituted, the StaO reactivity revealed here supports the assumption that L-tryptophan is the precursor of staurosporine as well as of violacein.

Interestingly, the high substrate specificity of StaO and VioA is an uncommon feature among known LAAOs. Because StaO and VioA are phylogenetically distant from each other (Fig. 2), it is not likely that both enzymes possess such high specificity toward L-tryptophan by chance. The most probable reason is that these two enzymes were selected during evolution from among many LAAO homologs for a precise physiological role, namely, to catalyze a key step in the biosynthesis of the bis-indole antibiotics. Most known LAAOs contribute to various physiological functions such as providing a toxic defense (snake venom and sea hare ink) [15; 16; 20; 21], antibiosis [18; 22; 47], or degradation of amino acids as a nutrient source [23; 48], for which purposes a broad substrate specificity is an advantage. In contrast to these LAAOs, StaO and VioA serve in a biosynthetic pathway, and high substrate specificity is required for them to participate in the synthesis of the appropriate products, namely staurosporine and violacein, respectively. Therefore, the difference in the physiological role is hypothesized to be the reason for the unusually high substrate specificity of StaO and VioA. The successful results in the present study suggest that metabolism-oriented screening is a promising strategy for obtaining enzymes with high substrate specificity, which are often difficult to obtain through random screening.

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