Enzymatic Synthesis of Two Isoxazolylalanine Isomers by Cysteine Synthases in *Lathyrus* Species

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Two isoxazolylalanine isomers, β-(isoxazol-5-5-on-2-yl)-L-alanine (BIA, 1) and β-(isoxazol-5-5-on-4-yl)-L-alanine (TAN-950A, 2) were confirmed to be derived from O-acetyl-L-serine (OAS) and isoxazol-5-one by cysteine synthases (CSases) with a different ratio in different plant parts. Some properties of this enzyme in the biosynthesis of both isomers are described.

Key words *Lathyrus sativus;* β-(isoxazol-5-5-on-2-yl)-L-alanine; β-(isoxazol-5-5-on-4-yl)-L-alanine; O-acetyl-L-serine; cysteine synthase; biosynthesis.

β-(Isoxazol-5-5-on-2-yl)-L-alanine (BIA, 1) has been isolated as a prominent metabolite during the seedling stage of the grass pea (*Lathyrus sativus* L.) and of some other closely related legumes. I has shown antimycotic activity, and recently it has been demonstrated that 1 is the biosynthetic precursor of the neurotoxin β-N-oxaethyl-l-α,β-diaminopropanoic acid (β-ODAP) found in *L. sativus* (Fig. 1). It has been also reported that β-ODAP and 1 affect x-aminoo-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors. On the other hand, its isomer, β-(isoxazol-5-5-on-4-yl)-L-alanine (TAN-950A, 2), was isolated as a novel antifungal antibiotic from the culture filtrate of *Streptomyces platensis* strain A-136, and was demonstrated to show antimycotic activity against the pathogenic yeast *Candida albicans*.

In our previous studies on the biosynthesis of heterocyclic β-substituted alanines in higher plants, we have demonstrated the formation of BIA (1) by cysteine synthases (CSases) purified from *L. sativus*.

This paper reports the enzymatic synthesis of the two isomers, 1 and 2, from O-acetyl-L-serine (OAS) and isoxazol-5-one by CSases purified from *Lathyrus* plants. Some properties of this enzyme in the biosynthesis of both isomers are also described.

MATERIALS AND METHODS

**Materials** Grass pea (*Lathyrus sativus* L.) and sweet pea (*L. odoratus* L.) seeds were sown in moistened vermiculite and grown in the dark or light for 5–6 d at 26–28°C. Seedlings were harvested, washed, and the cotyledons removed; they were then cooled for 1 h at 0–4°C before enzyme extraction. Green shoots were harvested from 12–14-d-old plants growing in the greenhouse of the medicinal plant gardens of our faculty. *Streptomyces platensis* strain A-136 was kindly provided by Takeda Chemical Ind., Ltd. Sephadex G-25, Sephacryl S-200, and DEAE-Sephadex Fast Flow were purchased from Pharmacia-LKB. All other chemicals used were purchased from local suppliers and were of the highest commercial grade available.

**Activity Assays** The formation of 1 and 2 was determined using an automatic amino acid analyzer coupled to a UV detector (265 nm) under standard operating conditions as described previously. I and 2 were eluted from the column after about 22 and 37.5 min, respectively, at a flow rate of 0.275 ml per min. The presence of 1 and 2 in the final reaction mixtures and in the seedlings or in the culture filtrate was also established by HPLC (Wakosil 5C18 column) as previously described. The unit of enzyme activity (kat) used in this paper is equivalent to 1 mol of 1 or 2 produced per s. Protein was determined by a dye-binding method.

**Purification of CSases from *Lathyrus* Plants** All operations were carried out at 0–4°C. CSases were purified from fresh etiolated seedlings (300–350 g,

![Fig. 1. Proposed Biosynthetic Pathways for 1 and 2](image-url)
cotyledons removed) or from green shoots (360 g) by almost the same procedures described previously.7) The highly purified CSase isoenzymes A and B in 50 mM K-Pi buffer (pH 8.0) were used directly as the source of enzyme activity for the formation of 1 and 2 in all further experiments. Specific activities of both enzymes were almost the same as those of our previous study on the formation of t-cysteine.7) Purification of the enzyme from S. platensis strain A-136 was also performed as described above.

**Physicochemical Properties and Kinetics** Some properties of the purified CSase isoenzymes in the biosynthesis of both isomers were studied by methods described previously.9)

**RESULTS AND DISCUSSION**

In this work, we studied the formation of two isoxazolylalanine isomers, 1 and 2, in vivo, using etiolated seedlings and the green shoots of Lathyrus plants or the culture filtrate of S. platensis A-136, as well as in vitro by isolated enzymes. We found that only 1 is present in Lathyrus plants and that only 2 is present in the culture filtrate of S. platensis A-136. Therefore, we have studied the formation of 1 and 2 by enzymes obtained from Lathyrus plants or S. platensis A-136. We demonstrated the enzymatic synthesis of both isomers 1 and 2 by two CSase isoenzymes highly purified from Lathyrus plants. Lathyrus plant enzymes can catalyze the formation of both isomers in vitro; however, in vivo Lathyrus plants do not synthesize 2.

CSase isoenzyme A purified from different sources exhibited different relative rates of synthesis for 1 and 2 (Table 1). When the enzyme was obtained from green shoots of L. sativus, the rate of synthesis of 1 (0.4 nkat/ml) was about 4 times greater than that of 2. When the enzyme was obtained from etiolated seedlings of L. sativus, the rate of synthesis of 1 (0.15 nkat/ml) was about 200 times greater than that of 2. CSase isoenzyme A purified from the green shoots of L. odorus synthesized 2 at almost the same rate as 1, but the enzyme from etiolated seedlings formed 2 at a rate of about 2.5% that of 1. The enzyme activities catalyzing the formation of 1 and 2 could not be separated from the CSase activities purified from the plant material.

The optimum pH for the formation of 1 and 2 by CSase isoenzyme A from the green shoots of L. sativus and L. odorus was 7.8—8.0 using 50 mM K-Pi buffer, similar to the formation of t-cysteine. The synthase activity for both 1 and 2 was dependent upon the concentrations of OAS and isoxazolin-5-one used, in line with our previous findings.6,7) CSase isoenzyme A from the green shoots of L. sativus has K_m values of 3.8 and 2.1 mM for OAS under the formation of 1 and 2, respectively. The K_m values for OAS are within the range of 1.5—7.1 mM determined for plant CSases.8) A relatively low final concentration (ca. 2.5 mM) of isoxazolin-5-one was sufficient to give a maximum rate of 2 formation by CSase isoenzyme A in the presence of a fixed concentration (12.5 mM) of OAS, and ca. 5 mM of isoxazolin-5-one was sufficient for CSase isoenzyme B. Isoenzyme A purified from L. sativus could synthesize more 2 than isoenzyme B from the same source, while the relative synthetic rates of 2 were less than those of 1, compared with that of t-cysteine by each enzyme, respectively.

On the other hand, enzyme preparations obtained from S. platensis A-136 could not synthesize either 1 nor 2, and CSase activity was difficult to obtain from this source. Since both isomers were highly synthesized by watermelon CSase isoenzyme A over-expressed in E. coli10) at almost the same ratio, we cannot exclude the possibility that an enzyme system, different from CSase, may be operative in S. platensis A-136.

From the above results and from our earlier work it can be suggested that plant CSases can catalyze the formation of both isomers 1 and 2 from OAS and isoxazolin-5-one, and that a control system, other than enzyme specificity and favouring the formation of the natural occurring isomer, may be operative in vivo but not in vitro. Also, when plant CSase is expressed in E. coli, this control mechanism may not be active, resulting in the formation of both isomers at almost the same rate.10) Perhaps a similar mechanism, favouring the formation of one of the potential isomers, might explain the formation of the isomeric uracilalanines willardine and iso-willardine by CSases in different plants, as previously described.11)

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**REFERENCES**


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Table 1. Relative Synthesis Rates of 1 and 2 by CSase Isoenzyme A from L. sativus and L. odorus

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. sativus</td>
<td>151</td>
<td>0.78</td>
</tr>
<tr>
<td>Etiolated seedlings</td>
<td>403</td>
<td>103</td>
</tr>
<tr>
<td>Green shoots</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. odorus</td>
<td>44.1</td>
<td>1.10</td>
</tr>
<tr>
<td>Etiolated seedlings</td>
<td>77.5</td>
<td>80.7</td>
</tr>
<tr>
<td>Green shoots</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The relative rates of synthesis were presented as pkat/ml by each enzyme.