Pergamon

PII: S0031-9422(96)00620-6

Phytochemistry, Vol. 44, No. 4, pp. 563–566, 1997

0031-9422/97 $17.00+0.00

CHARACTERIZATION OF A DIOXYGENASE FROM *TRIGONELLA FOENUM-GRAECUM* INVOLVED IN 4-HYDROXYISOLEUCINE BIOSYNTHESIS

CATHERINE HAEFELÉ, CLAUDE BONFILS* and YVES SAUVAIRE†

Laboratoire de Recherche sur les Substances Naturelles Végétales, UPR ES 1677, CP 024, Université Montpellier II, 34095 Montpellier Cedex 05, France; *INSERM U 128, CNRS, BP 5051, 34033 Montpellier, France

(*Received in revised form* 15 *July* 1996)

Key Word Index—*Trigonella foenum-graecum*; Fabaceae; fenugreek; biosynthesis; 4-hydroxyisoleucine; 2-oxoglutarate dependent dioxygenase.

Abstract—A study was made of the enzyme in fenugreek implicated in the biosynthesis of 4-hydroxyisoleucine, which is an unusual amino acid known for its insulin stimulating effect. 4-Hydroxyisoleucine was detected by HPLC following isoleucine incubation with a cell-free extract from etiolated 6-day-old fenugreek seedlings in the presence of various cofactors. The reaction showed that 4-hydroxyisoleucine formation is dependent on the presence of Fe^{2+}, 2-oxoglutarate, ascorbate and oxygen. This suggests that a 2-oxoacid dependent dioxygenase plays a key role in this biosynthetic pathway. Copyright © 1997 Published by Elsevier Science Ltd

INTRODUCTION

4-Hydroxyisoleucine is an unusual amino acid that was isolated and identified for the first time by Fowden *et al.* in 1973 [1] and its conformation was established by Alcock *et al.* in 1989 [2]. Studies have shown that hydroxyisoleucine represents up to 80% of free amino acids in fenugreek seeds, but is absent from the seed reserve proteins [3]. We have shown that this amino acid possesses insulin-stimulating properties both *in vitro* and *in vivo* in rats and dogs [4]. It is present in most of the *Trigonella* genus, except for *T. cretica* [5]. Fowden *et al.* [1] obtained evidence of a relationship between isoleucine and hydroxyisoleucine, but the nature of the enzymatic system involved was not established.

In the present study we identified the enzyme responsible for hydroxylation of isoleucine into hydroxyisoleucine. Of the various oxygenases present in plant tissues, cytochrome *P*450 has been suggested as a potential candidate for catalysing such a reaction [6]. This hypothesis was not confirmed in our previous investigations [7] in which we noted that isoleucine hydroxylation by fenugreek microsomes was not dependent on the presence of NADPH and was not inhibited by carbon monoxide. This led us to consider the possible involvement of a dioxygenase. In hydroxylation reactions catalysed by dioxygenases, one atom of molecular oxygen is incorporated in the substrate, while the other oxygen atom is incorporated in 2-oxoglutarate, resulting in the formation of succinate and release of carbon dioxide. Dioxygenases are capable of hydroxylating an aliphatic carbon chain in a stereospecific manner as, for example, lysyl and prolyl hydroxylases [8]. In the present study we observed that 4-hydroxylation of isoleucine by a fenugreek cell extract required 2-oxoglutarate, Fe^{2+}, ascorbate and oxygen as cofactors and cosubstrates for full activity.

RESULTS AND DISCUSSION

Choice of material

We first studied variations in 4-hydroxyisoleucine in young fenugreek plantlets as a function of age. Figure 1 shows the increased hydroxyisoleucine content in tissues during growth. We noted a significant increase in the hydroxyisoleucine content between days 6 and 7, and we used this material in subsequent trials.

Enzyme extraction procedure

To measure isoleucine hydroxylase activity accurately, 4-hydroxyisoleucine, which is present in high quantities on fenugreek tissues, must be removed from plant extracts. We chose to remove this by dialysis of the crude extract using a Spectra Por MWCO: 1000 membrane. A preliminary time-course dialysis study showed that a minimum of 36 hr was required for

† Author to whom correspondence should be addressed.

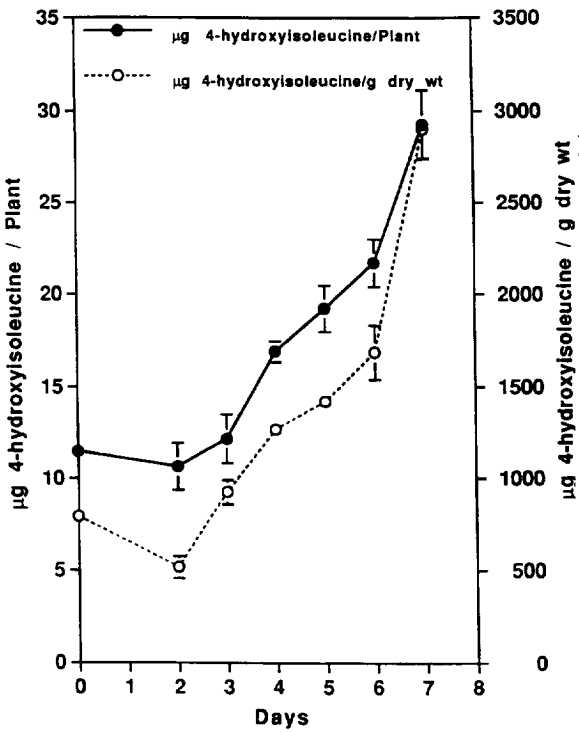

Fig. 1. 4-Hydroxyisoleucine content during plantlet growth. The experiment was conducted as described in the Experimental section. Results are shown as the mean ± S.D. of four replicates.

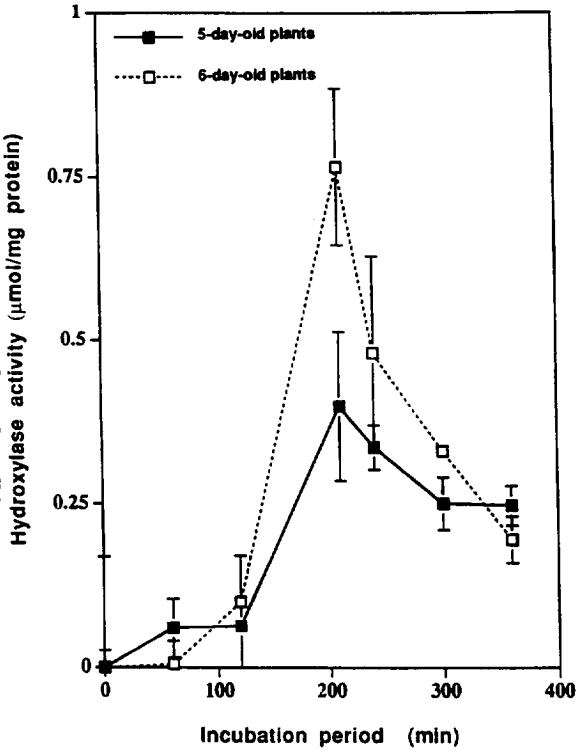

Fig. 2. Isoleucine hydroxylase activity in 5- and 6-day-old plants. Two dialysed enzymic extracts were incubated under standard conditions at pH 7.5. Hydroxylase activity was tested at different times and compared with a control. Hydroxyisoleucine synthesis was quantified by HPLC. Data are shown as the mean ± S.D. of three replicates.

separation of endogenous amino acids and proteins without affecting hydroxylase activity. Amino acids were not totally eliminated from the extracts, but their concentration was low enough to enable detection of hydroxyisoleucine synthesis after HPLC of the incubation medium.

Isoleucine hydroxylase activity

The dialysed plant extracts were incubated with isoleucine in the presence of various cofactors. Enzymic trials were carried out as described in the Experimental section (measurement of enzymic activity) and hydroxyisoleucine synthesis was quantified by HPLC. In the light of the results of previous experiments concerning the extraction, isolation and characterization of various 2-oxoglutarate dependent dioxygenases [9–12], we decided to test hydroxylase activity under the following conditions: 5 mM isoleucine, 0.5 mM Fe^{2+}, 5 mM 2-oxoglutarate and 5 mM ascorbate. As indicated in Fig. 2, we observed *in vitro* biosynthesis of hydroxyisoleucine by the plant extract in the presence of cofactors required by 2-oxoglutarate dependent dioxygenase. This result strongly suggests that a dioxygenase is involved in hydroxyisoleucine biosynthesis. Hydroxylase activity was not linear as a function of the incubation period and there was a long latent phase. This pattern may be explained by the fact that the activity was measured using a crude extract. To assess the effects of different cofactors, the enzymic extract (2.13 mg ml^{-1} of proteins) was incubated with and without the cofactors required for enzymatic activity (5 mM Fe^{2+}, 5 mM 2-oxoglutarate and 5 mM ascorbate) with oxygen or under anoxic conditions at 30° for 210 min.

Table 1 shows that, like other 2-oxoglutarate dependent dioxygenases, the enzyme required 2-oxoglutarate, Fe^{2+} and ascorbate [8]. In the presence of

Table 1. Influence of various cofactors and cosubstrates on isoleucine hydroxylase activity*

Incubation mixture†	Product formed (µmol)	Hydroxylase activity (pkat mg^{-1} protein)
Control 1: no cofactors $+O_2$	0.06	0.37
Control 2: cofactors, no O_2	0.05	0.35
OG + Fe^{2+} + Asc + O_2	0.41	3.43
OG	0.24	1.32
Fe^{2+}	0.33	2.05
Asc	0.40	3.43
OG + Asc	0.36	2.62
OG + Fe^{2+}	0.36	2.62
Fe^{2+} + Asc	0.38	2.93

*The data shown are means of duplicate determinations.
†OG, 2-oxoglutarate; Asc, ascorbate.

isoleucine alone, there was almost no activity. There were no significant differences in the other data shown in Table 1. However, the presence of ascorbate and/or Fe^{2+} is more essential than that of 2-oxoglutarate for proper functioning of the enzyme [13, 14]. Isoleucine hydroxylase is not a true 2-oxoglutarate dependent dioxygenase because it does not strictly require 2-oxoglutarate (under the experimental conditions used in the present study). Ascorbate, which has a cyclic C-2 oxo group, seems to play an important role as both a reducing agent and a substrate [13].

Kinetic parameters and pH

Isoleucine hydroxylase activity, as a function of isoleucine concentrations, showed maximum activity at 5 mM substrate (3.3 pkat mg^{-1} protein). In contrast to other 2-oxoglutarate dependent dioxygenases, hydroxylase activity was a maximum at relatively high substrate concentrations. A dioxygenase implicated in vindoline biosynthesis also required 10 mM substrate for maximum activity [9], but other enzymes belonging to this family, such as aspartyl β-hydroxylase [15] and gibberellin C-20 hydroxylase [16], required only 0.1 mM and 0.2 μM of substrate, respectively, to attain maximum activity. When the reaction occurs in an extract, it is possible that the enzyme's access to the substrate is restricted. Moreover, activity is rapidly inhibited when substrate concentrations are increased; the enzyme is almost totally inhibited by 20 mM isoleucine.

The optimum pH for the enzyme present in our plant tissue was 7.5 (the corresponding enzymic activity was 1.5 pkat mg^{-1} protein). In this family of enzymes, the optimum pH can vary from 6.5 for thymidine 2′-hydroxylase [17] to 8.5 for (2*S*)-flavanone 3β-hydroxylase [18]. The pH of lysyl- and prolyl-hydroxylase is quite close to that of the enzyme studied here (pH 7.8).

EXPERIMENTAL

Plant material. Fenugreek seeds (*Trigonella foenum-graecum* L.), cv. Gouka, were harvested from experimental plots at the University of Montpellier II and graded. For germination, seeds were placed in glass boxes (8 × 10 cm) containing vermiculite moistened with H_2O and stored in the dark at 22°.

4-Hydroxyisoleucine extraction and analysis. Whole seeds and plantlets were ground in a mortar for 1 min. The plant material (fr. wt) to solvent volume (EtOH 30%) ratio was maintained for all batches (2 g/5 ml). The homogenate was centrifuged at 20000 *g* for 20 min.

TLC analysis. Qualitative analysis of amino acid was performed by HPTLC using Merck 60 Silica gel (0.25 mm thick). The migration solvent was composed of *n*-BuOH/HOAc/H_2O (3:2:1). Amino acid visualization was performed by spraying the plates with 0.1% ninhydrin in 30% EtOH (sensitivity in the μg range), followed by incubation at 110° for 10 min.

HPLC analysis. 4-Hydroxyisoleucine was analysed quantitatively on a Shimadzu HPLC apparatus (SCL-6B) equipped with an Alltech Adsorbsphere HS OPA column (100 × 4.66 mm, 5 μm). 40 μl *o*-phthaldialdehyde (OPA) was added to 40 μl of the extract and, after 1 min, 20 μl of this mixture was injected into the column. The elution gradient was composed of a NaOAc soln (65 mM) containing 5% THF, and MeOH containing 5% THF. The fluorescence excitation wavelength was 355 nm and emission was recorded at 410 nm. External calibration was used for 4-hydroxyisoleucine quantification. The 4-hydroxyisoleucine (major isomer) used as a control was isolated from fenugreek seeds in the laboratory and characterized by MS (FAB), ^{1}H NMR and ^{13}C NMR.

Enzymic extract preparation. Whole plantlets (8 g) were ground in a mortar at 4° for 2 min with a K/Pi buffer (pH 7.5) containing 5 mM DTT. The homogenate was centrifuged for 20 min at 20000 *g*. The supernatant was dialysed 3 times with Spectra Por MWCO:1000 membrane in 200 ml K/Pi buffer (pH 7.5) for 12 hr.

Measurement of enzymic activity. Measurements were performed at 30° with 20 ml of dialysed extract (2 mg ml^{-1} protein) containing 5 mM 2-oxoglutarate, 0.5 mM Fe^{2+} and 5 mM ascorbate (final concns) under constant stirring. The substrate, 5 mM isoleucine, was added at the beginning of the reaction. The reaction was stopped by the addition of 100 μl 95% EtOH to 100 μl of extract collected at different times of incubation. Under anoxic conditions, incubation was conducted with isoleucine and the various cofactors under a constant steam of N_2 (flow 0.5 ml min^{-1}) for 3.5 hr, at 30°. Enzymic activity was expressed in pkat mg^{-1} protein. Total protein concn was estimated by the method of ref. [19].

Acknowledgements—We are grateful to Y. Baissac and O. Leconte for their excellent technical assistance.

REFERENCES

1. Fowden, L., Pratt, H. M. and Smith, A., *Phytochemistry*, 1973, **12**, 1707.
2. Alcock, N. W., Crout, D., Gregorio, M., Lee, E., Pike, G. and Samuel, C. J., *Phytochemistry*, 1989, **28**, 1835.
3. Sauvaire, Y., Girardon, P., Baccou, J. C. and Risterucci, A. M., *Phytochemistry*, 1984, **23**, 479.
4. Sauvaire, Y. and Ribes, G., French Patent No. 9210644, European Patent No. 934021353, U.S. Patent No. 8/113951, 1993.
5. Sauvaire, Y., Brenac, P., Guichard, E. and Fournier, N., in *Basic and Applied Aspects of Seed Biology*, Vol. 1, eds D. Côme and F. Corbineau. ASFIS, Paris, 1993, p. 201.
6. West, C. A., in *The Biochemistry of Plants*, Vol.

2, eds P. K. Stumpf and E. E. Conn. Academic Press, New York, 1980, p. 317.
7. Gleize, D., DEA, University Montpellier II, France, 1994.
8. De Carolis, E. and De Luca, V., *Phytochemistry*, 1994, **36**, 1093.
9. De Carolis, E. and De Luca, V., *Journal of Biological Chemistry*, 1993, **268**, 5504.
10. Gilmour, S. J., Bleeker, A. B. and Zeevaart, J. A. D., *Plant Physiology*, 1987, **85**, 87.
11. Chrispeels, M. J., *Methods in Enzymology*, 1984, **107**, 361.
12. Turpeenniemi-Hujanen, T. M., Puistola, U. and Kivirikko, K. I., *Biochemical Journal*, 1980, **189**, 247.
13. Prescott, A. G., *Journal of Experimental Botany*, 1993, **44**, 849.
14. Gronke, R. S., Welch, D. J., VanDusen, W. J., Garsky, V. M., Sardana, M. K., Stern, A. M. and Friedman, P. A., *Journal of Biological Chemistry*, 1990, **265**, 8558.
15. Lange, T. and Graebe, J. E., *Planta*, 1989, **179**, 211.
16. Bankel, L., Lindstedt, G. and Lindstedt, S., *Journal of Biological Chemistry*, 1972, **247**, 6128.
17. Britch, L. and Grisebach, H., *European Journal of Biochemistry*, 1986, **156**, 11.
18. Bradford, M. M., *Analytical Biochemistry*, 1976, **72**, 248.