BIOLOGIA PLANTARUM (PRAHA)

33 (4) : 277–286, 1991

Enzymes of Auxin Biosynthesis and Their Regulation I. Tryptophan and Phenylalanine Aminotransferase in Pea Plants

SULTANA D. TERZIIVANOVA-DIMOVA* and M. KUTÁČEK

Institute of Experimental Botany, Czechoslovak Academy of Sciences,
Ke dvoru 15, 166 30 Praha 6, Czechoslovakia

Abstract. The transaminations of L-tryptophan (L-trp) and of L-phenylalanine (L-phe) are catalysed *in vitro* by the same non-specific aminotransferase. The transaminations procceed at the same pH (pH 8.5) and temperature (45 °C) optima, have parallel increases in activity with addition of the coenzyme pyridoxal phosphate (PRP) and have identical elution characteristics in gel chromatography. The enzyme from pea seedlings has a relatively weak affinity for both amino acids (K_M L-trp = 4.16×10^{-1} mmol l^{-1}; K_M L-phe = 2.10×10^{-1} mmol l^{-1}). Differences in affinity for a series of keto acids in the pea enzyme were observed, with pyruvate having the strongest and glyoxylate the weakest affinity. Transamination of L-trp and L-phe was demonstrated by enzyme extracts from pea, maize and tomato, but was not detected in kohlrabi. The amino acids L-asparagine (L-asn), L-phe, L-lysine (L-lys), L-methionine (L-met) have distinct inhibitory effects on the transamination of L-trp. Indolylacetylaspartate and tryptophol were shown to be competitive inhibitors. The regulation at the molecular level of L-trp transaminase activity is discussed.

The deamination of L-trp or L-phe to indolylpyruvate (IPyA) and phenylpyruvate (PPyA) is usually regarded as the first step of auxin biosynthesis in higher plants. It may be catalysed by several systems among which L-tryptophan aminotransferase (TAT) has been most frequently investigated (Gamborg and Wetter 1963, Truelsen 1972, 1973, Matheron and Moore 1973, Wightman and Rauthan 1974, Liu *et al.* 1978, Noguchi and Hayashi 1980).

However, even a purified TAT preparation displays only low affinity for L-trp and catalyses as well the transamination of other amino acids, aromatic and aliphatic, with simultaneous utilisation of various keto acids as amino group acceptors. Thus, L-trp is one of the substrates of aminotransferase, but not the main one.

Very little is known about the regulation of TAT activity. Forest and Wightman (1972) found, that L-asp inhibits L-trp transamination.

Received June 10, 1990; accepted October 10, 1990

* Present address: Institute of Plant Physiology "M. Popov", Bulgarian Academy of Sciences, 1113 Sofia

In this study we have compared the transamination of L-trp (TAT) and of L-phe (PAT). We have further investigated the effect of several natural plant components on the *in vitro* activity of TAT and PAT: a) the effect of a group of amino acids, b) the effect of some natural indole compounds, including indolylacetylaspartic acid (IAAsp).

MATERIAL AND METHODS

Plant material

Sterilized pea seeds (*Pisum sativum* L., cv. Jupiter) were germinated under aseptic conditions and then cultivated at 25 °C in darkness. Plants of maize (*Zea mays* L., cv. Ta 37/71 o_2), kohlrabi (*Brassica oleracea* var. *gongylodes* L., cv. Moravia) and tomato (*Lycopersicon esculentum* L., cv. Stupické) were grown under similar conditions.

Preparation and purification of the enzyme extract

The preparation was in principle the same as that developed by Truelsen (1972). The enzyme extract was obtained from the shoots of 7–9 d old etiolated plants, either from fresh material, or – more often – from acetone powder (Chen and Boll 1968) in which form the enzyme is stable for one month of storage at –20 °C. The enzyme was extracted with a cooled (4 °C) TRIS-HCl buffer (50 mM, pH 9.5, containing 10 mmol l^{-1} of EDTA and 1 mmol l^{-1} of 2-mercaptoethanol) either from crushed fresh plant material using a 1:2 ratio (m/v) or from the acetone powder. After 30 min of extraction at 4 °C the mixture was centrifuged at 17 000 *g* for 30 min at –4 °C.

At 0 – 4 °C and pH above 6.8 the active enzyme fraction precipitated at between 60 to 80 % of saturation with ammonium sulphate. It was separated by centrifugation at 17 000 g for 15 min at –4 °C. The pellet of proteins was dissolved in a minimum amount of 50 mM TRIS-HCl buffer and applied onto a Sephadex G 200 column (86 cm × 1.7 cm). For elution (8 ml h^{-1} at 4 °C) the last mentioned buffer was used. Absorbance of the effluent was measured at $\lambda = 280$ nm. After detection of the enzyme activity in individual fractions the fractions were combined, dialysed against distilled water (3 × 21 ml) and freeze-dried. The purification increased the original specific activity 18.4 fold (Truelsen 1972).

Abbreviations: L-trp – L-tryptophan, L-phe – L-phenylalanine, L-asp – L-aspartic acid, L-lys – L-lysine, L-met – L-methionine, PRP – pyridoxal phosphate, IAA – indolyl-3-acetic acid, IPyA – indolyl-3-pyruvic acid, IAAsp – indolylacetylaspartic acid, ILA – indolyl-3-lactic acid, IAN – indolyl-3-acetonitrile, T-NH_2 – tryptamine, PPyA – phenylpyruvic acid, PyA – pyruvic acid, KG – α-ketoglutarate, T-OH – tryptophol, TAT – L-tryptophan aminotransferase, PAT – L-phenylalanine aminotransferase.

Determination of L-trp transamination activity

The reaction product, IPyA, was stabilized as a borate complex (Truelsen 1972). The standard reaction mixture of 3 ml final volume contained L-trp (6 mmol l^{-1}), 2-ketoglutaric acid (KG) (6 mmol l^{-1}), EDTA (10 mmol l^{-1}) in 100 mM borate buffer of pH 8.5 (according to Sörensen) and the enzyme extract (usually 0.5 ml). If pyridoxal-5-phosphate (PRP) was added as coenzyme, it was applied in 0.22 mmol l^{-1} concentration. Incubation took place at 45 °C, usually for 3 h. The reaction was stopped by addition of 100 µl of 50 % TCA. The precipitated proteins were centrifuged at 5 000 *g* at room temperature. The borate complex of IPyA formed was determined spectrophotometrically at $\lambda = 328$ nm. In control experiments the reaction mixture was incubated without addition of the enzyme extract. The product formed, IPyA, was determined using the calibration curve.

Determination of L-phe transamination activity

The procedure for the determination of the enzymatically formed PPyA from L-phe (6 mmol l^{-1}) was the same: the keto acid was stabilized in the form of the borate complex, the absorption maximum of which in the UV region corresponds to $\lambda = 300$ nm.

Determination of the L-glu: oxaloacetate aminotransferase

The activity of L-glu-oxaloacetate aminotransferase (GOT) was determined using the method according to Langer *et al.* (1975). The concentration of the substrates (L-asp and KG) was 6 mmol l^{-1}.

Specificity of the enzyme towards keto acids

The specificity of the enzyme towards keto acids as substrates was investigated in the case of KG, pyruvate (PyA), PPyA, oxaloacetate and glyoxylate. The affinity of the enzyme to keto acids was expressed in K_M values obtained by the Lineweaver-Burk graphical method (Lineweaver and Burk, 1934).

Determination of the effects of amino acids and natural indoles

The enzyme was preincubated with the substance investigated (usually 9 mmol l^{-1}) for 10 min, before addition of the substrate. The change in the activity was determined in % of the activity in comparison with a control (= 100 %). When the type of inhibition of L-asp, IAAsp and tryptophol (TOH) was determined graphically, the experimental values were unified using the method of least squares, i.e. by linear regression analysis.

In the case of IAAsp the following parameters were controlled by TLC: 1) stability of IAAsp during the incubation with the enzyme, 2) possible formation of IAAsp from IAA and L-asp in the course of the incubation with the enzyme.

Determination of proteins

The proteins were determined with Coomassie Brilliant Blue G 250 (Bradford 1976).

RESULTS

Chromatographic analysis of L-trp and L-phe aminotransferse

In the effluent from the Sephadex G 200 column TAT and PAT coincided (Fig. 1).

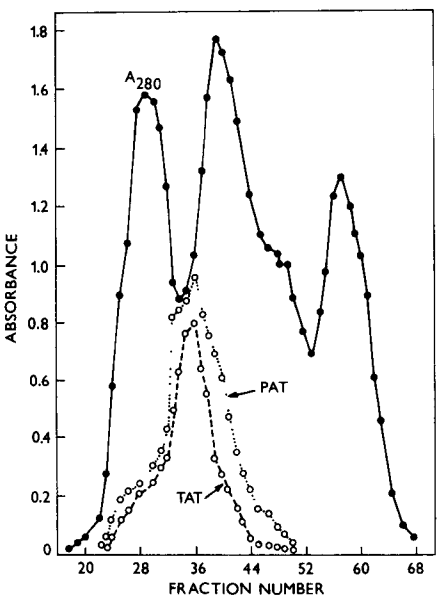

Fig. 1. Gel chromatography and L-tryptophan and L-phenylalanine aminotransferase (TAT and PAT) from pea plants on a Sephadex G 200 fine column (138 × 5 cm). Fractions 3 ml, 8 ml h^{-1} flow, TAT activity (328 nm), PAT activity (300 nm).

The activities of TAT and PAT were compared with the most common aminotransferase GOT. In pea the activity of TAT is 13 times lower than the activity of GOT.

Basic conditions of transamination of L-trp and L-phe

Kinetic data for TAT and PAT from pea are shown in Table 1. For both substrates the pH optimum of the reaction was 8.5, optimum temperature 45 °C.

Table 1
Basic kinetic data of L-tryptophan and L-phenylalanine aminotransferase (TAT and PAT) from pea plants.

Data	TAT	PAT
Optimal pH	8.5	8.5
Optimal temperature	45 °C	45 °C
Linear increase of enzyme activity	3 h	3 h
Activation by PRP 0.22 mmol l^{-1}	+62.1%	+59.3%
K_M (KG 3 mM)	0.41 mmol l^{-1}	0.21 mmol l^{-1}
K_M (KG, PRP)	0.10 mmol l^{-1}	0.07 mmol l^{-1}
Inhibition by L-asp 9 mmol l^{-1}	–83.3%	–79.1%

Although the transamination of L-trp and L-phe takes place even without addition of the coenzyme PRP, its addition increases the transamination of both amino acids. L-phe is more actively transaminated.

Specificity of the enzyme to keto acids

The specificity of the enzyme to keto acids was determined with L-trp and L-phe as substrates (Table 2). The results, in K_M values, show differences in the affinity of the apoenzyme to keto acids in the same order with both substrates. In the case of L-phe lower K_M values of keto acids were observed. For the enzyme from pea the most active acceptor of the amino group was PyA.

Effect of amino acids on the L-trp and L-phe transamination

The activity of transamination of L-trp and L-phe was inhibited by those amino acids which were active substrates in transamination. L-asp is the

Table 2
Substrate specificity of pea L-tryptophan aminotransferase (TAT) and L-phenylalanine aminotransferase (PAT): affinity to keto acids (NH_2-acceptors).

Keto acid	TAT K_M 0.1 mM	PAT K_M 0.1 mM
Pyruvate	2.14	1.74
Phenylpyruvate	3.86	–
2-Ketoglutarate	4.16	2.10
Oxaloacetate	6.88	5.32
Glyoxylate	7.13	6.57

Table 3
Effect of amino acids on the activity of L-tryptophan and L-phenylalanine aminotransferase (TAT and PAT) from pea plants. Comparison with the activity of amino acids as substrates.

Amino acid	Inhibition (in % of control = 100 %)		Substrate activity of amino acid (L-trp = 100 %)
	TAT	PAT	
L-aspartic acid	− 83.3	− 79.1	+ 95
L-lysine	− 79.2	− 70.4	+ 288
L-methionine	− 65.2	− 81.0	+ 216
L-alanine	− 54.1	− 52.0	+ 202
L-asparagine	− 39.2	− 42.7	+ 213
L-histidine	− 28.2	− 16.1	+ 82
L-valine	− 15.0	− 15.1	+ 62
L-cysteine	− 14.5	− 17.6	+ 4
L-glutamic acid	− 9.2	− 8.4	−
L-tyrosine	− 6.0	− 8.3	+ 131
D-phenylalanine	− 5.2	− 11.8	−
D-tryptophan	− 4.8	− 5.9	−

strongest inhibitor, while L-glu is only weakly active. D-amino acids affected the transamination reaction negligibly (Table 3). The mutual effect of L-trp and L-phe on the transamination of the other amino acid was observed. L-phe (9 mmol l^{-1}) inhibited TAT by 87 %, L-trp (9 mmol l^{-1}) PAT by 15 %.

Effect of indoles on the L-trp and L-phe transamination

TOH inhibited TAT and PAT distinctly, more than indolyl-3-lactic acid (ILA) (Table 4). Indolylacetonitrile (IAN) and tryptamine (T-NH_2) inhibited the transamination less distinctly. When 9 mM IAA was used, no feedback effect on the activity of the enzyme could be observed. The inhibition of TAT

Table 4
Inhibition of L-tryptophan and L-phenylalanine aminotransferase (TAT and PAT) from pea plants with indole derivatives in % of control (= 100 %)

Indole 9 mM	Inhibition	
	TAT	PAT
Tryptophol	− 83.1	− 72.4
Indolyl-3-lactic acid	− 50.3	− 42.3
Indolyl-3-acetonitrile	− 32.4	− 28.0
Tryptamine	− 19.6	− 24.8
Indolyl-3-acetic acid	0	0

under the effect of TOH had a competitive character (Fig. 2), while TOH inhibited PAT non-competitively.

Effect of L-asparate and indolylacetylaspąrate on the L-trp and L-phe transamination

The effect of L-asp and IAAsp on TAT and PAT activities is shown in Table 5. IAAsp is a competitive inhibitor of TAT (Fig. 3) while L-asp is a non-competitive one. L-asp and IAAsp inhibit PAT non-competitively (a mixed

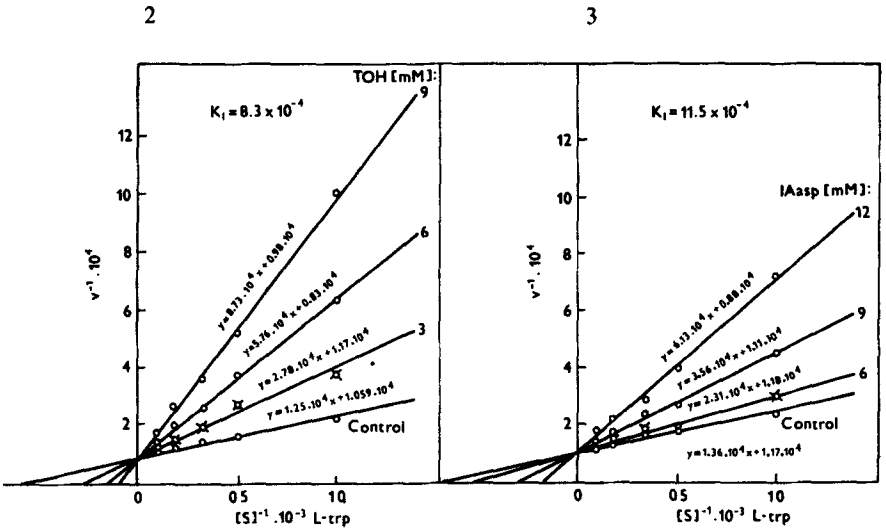

Fig. 2. Lineweaver-Burk plot of L-tryptophan aminotransferase (TAT) inhibition by tryptophol (TOH).

Fig. 3. Lineweaver-Burk plot of L-tryptophan aminotransferase (TAT) inhibition by indolylacetylaspartate (IAasp).

Table 5

Inhibition of L-tryptophan and L-phenylalanine aminotransferase (TAT and PAT) from pea plants with aspartic acid (L-asp) and indolylacetylaspartic acid (IAAsp) in % of control (= 100 %)

Concentration [mM]	Inhibitor	Inhibition [%]	
		TAT	PAT
3	L-asp	- 21.3	- 28.9
	IAAsp	- 29.1	- 30.1
6	L-asp	- 54.2	- 56.1
	IAAsp	- 61.5	- 60.1
9	L-asp	- 84.4	- 73.1
	IAAsp	- 88.0	- 84.3
12	L-asp	- 90.0	- 86.7
	IAAsp	- 95.2	- 98.9

Table 6
Comparison of the activity of L-tryptophan and L-phenylalanine aminotransferase (TAT and PAT) in individual plants. Without and in addition of PRP (0.22 mmol l^{-1})

Plant material	TAT (K_M 0.1 mmol l^{-1})		PAT (K_M 0.1 mmol l^{-1})	
	− PRP	+ PRP	− PRP	+ PRP
Pisum sativum *(Fabaceae)*	4.16	1.04	2.10	0.72
Zea mays *(Poaceae)*	6.82	3.04	4.96	1.97
Lycopersicon esculentum *(Solanaceae)*	9.07	7.45	7.11	4.25
Brassica oleracea var. *gongylodes* *(Brassicaceae)*	0	0	0	0

type of inhibition). It was found that IAAsp was not cleaved after 4 h incubation at 45 °C and pH 8.5, and further, that it was not formed from IAA and L-asp in the presence of the enzyme extract.

Transamination of L-trp and L-phe in individual plant species

The activity of TAT and PAT was demonstrated in a series of plants of different families (Table 6). The most active aminotransferase was found in pea plants, followed by maize and finally by tomato. This order for L-trp coincides with that found for L-phe, which is transaminated more actively in all the plants. The plants of the *Brassicaceae* family did not display aminotransferase activity towards L-trp and L-phe even in the presence of external PRP.

DISCUSSION

A characteristic quality of the plant aminotransferase catalysing the transamination of L-trp is its low specificity. The much used term "L-trp transaminase" evokes an erroneous idea of enzyme specificity.

The investigated enzyme transaminates a whole group of amino acids and L-trp is not the most suitable substrate; several other amino acids surpass L-trp in their affinity to the enzyme. In our study we present evidence about the identity of the enzyme catalysing the transamination of L-trp and L-phe, the transamination of L-phe is being more active. The low affinity of L-trp to the enzyme is apparent also from the relatively high Michaelis constant (K_M for L-trp = 4.16 10^{-1} mmol l^{-1}). An additional consequence of the low affinity of L-trp to the enzyme is the competition of individual amino acids for the active

centre. An inhibition of L-trp transamination by amino acids with higher affinity to the enzyme results from this competition.

It is assumed that TAT is the primary enzyme of the IPyA pathway of IAA synthesis (Wightman and Cohen 1968). This pathway is considered to be most widespread system of IAA synthesis in higher plants. In our opinion *in vivo* an effective "two-step" regulation could be assumed between unequal pools of L-trp and IAA (Kutáček 1985):

1) Reduction of L-trp flow into the pathway of IAA synthesis in order to maintain the concentration gradient between the L-trp pool and the IAA hormonal level.

2) Regulation of the actual level of IAA in plants according to the physiological requirements, by metabolic turnover of the intermediary product indolylacetaldehyde and by the terminal indolylacetaldehyde oxidase activity (Brown and Purves 1980, Kutáček 1985, Kutáček and Terziivanova-Dimova 1991).

A direct correlation between the L-trp and IAA levels seems in tissue cultures dubious (Moloney and Elliott 1982, El Bahr *et al.* 1984).

TAT may have an important role in the first step of the regulation of IAA synthesis, contributing on the molecular level to the reduction of the L-trp flow into the biosynthetic pathway. The kinetic properties of the enzyme, especially its low affinity to L-trp, the interaction of L-trp with other amino acids, eventually with indoles (indolylacetylaspartic acid) are tools in the regulation of the non-specific enzyme activity.

TAT activity is widely spread in higher plants (*e.g.* Truelsen 1973). It was surprising that in seven-day-old plants of the *Brassica* genus it was not possible to detect any L-trp and L-phe transaminase activity. This lack of enzyme activity suggests that these plants acquire the necessary IAA by another pathway than *via* IPyA. In these plants IAA synthesis by the indolylacetaldoxime pathway was confirmed (Kutáček and Procházka 1964, Kutáček and Kefeli 1968, Mahadevan and Stowe 1972).

To get a more precise picture of the regulation of TAT activity and of its exact role in IAA synthesis, further detailed studies, especially of isoenzymes and their compartmentation, are needed.

REFERENCES

Bradford, M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. – Anal. Biochem. **72** : 248–254, 1976.

Brown, H. M., Purves, W. K.: Indoleacetaldehyde reductase of *Cucumis sativum* L. Kinetic properties and role in auxin biosynthesis. – Plant Physiol. **65** : 107–113, 1980.

Chen, J., Boll, W. G.: Tryptophan synthetase in shoot and root tissue of pea seedlings. – Can. J. Bot. **46** : 1031–1041, 1968.

El Bahr, M. K., Kutáček, M., Opatrný, Z., Li Sok Nam, Prasad, R.: Changes in L-tryptophan and auxin content during the life cycle of normal and tumorous tobacco cell culture. – Biochem. Physiol. Pflanz. **179** : 739–748, 1984.

Forest, J. C., Wightman, F.: Amino acid metabolism in plants II. Transamination reaction of free amino acids in cell free extract of cotyledons and growing tissues of bushbean seedlings (*Phaseolus vulgaris* L.). – Can. J. Biochem. **50** : 538–542, 1972.

Gamborg, O. L., Wetter, L. R.: An aromatic amino acid transaminase from mung bean. – Can. J. Biochem. Physiol. **41** : 1733–1740, 1963.

Kutáček, M.: Auxin biosynthesis and its regulation on the molecular level. – Biol. Plant. **27** : 145–153, 1985.

Kutáček, M., Terziivanova-Dimova, S. D.: Proposed enzymes of auxin synthesis and their regulation III. Some properties of pea indolylacetyldehyde oxidase. – Biol. Plant., in press.

Kutáček, M., Kefeli, V. I.: The present knowledge of indole compounds in plants of the *Brassicaceae* family. – In: Wightman, F., Setterfield, G. (ed.): Biochemistry and Physiology of Plant Growth Substances, Pp. 127–152. The Runge Press, Ottawa 1968.

Kutáček, M., Procházka, Ž.: Methodes de détermination et d'isolement des composés indoliques chez les Cruciferes. – In: Régulateurs Naturels de la Croissance Végétale. (Colloques de CNRS. Vol. 123.) Pp. 445–456. CNRS, Paris 1964.

Langer, I., Stránský, P., Kutáček, M.: Differences of cytoplasmic transaminase activity in normal and opaque maize (*Zea mays* L.) seedlings. – Theor. appl. Genet. **46** : 19–23, 1975.

Lineweaver, H., Burk, D.: The determination of enzyme dissociation constant. – J. amer. chem. Soc. **56** : 658–666, 1934.

Liu, S. T., Katz, C. D., Knight, C. A.: Indole-3-acetic acid synthesis in tumorous and nontumorous species of *Nicotiana*. – Plant Physiol. **61** : 743–747, 1978.

Mahadevan, S., Stowe, B.: Conversion of 3-indole-acetaldoxime to glucobrassicin and sulphoglucobrassicin by woad (*Isatis tinctoria* L.). – In: Carr, D. (ed.): Plant Growth Substances 1970. Pp. 117–126. Springer Verlag, Heidelberg 1972.

Matheron, M. E., Moore, T. O.: Properties of an aminotransferase of pea (*Pisum sativum* L.) – Plant Physiol. **52** : 63–67, 1973.

Moloney, M. M., Elliott, M. C.: Tryptophan and indole-3-acetic acid accumulation in *Acer* cell cultures and its relationship with cell autolysis. – Planta **156** : 326–331, 1982.

Noguchi, T., Hayashi, S.: Peroxisomal localization and properties of tryptophan aminotransferase in plant leaves. – J. biol. Chem. **255** : 2267–2269, 1980.

Truelsen, T. A.: Indole-3.pyruvic acid as an intermediate in the conversion of tryptophan to indole-3-acetic acid. Some characterizations of tryptophan transaminase from mung bean seedlings. – Physiol. Plant. **26** : 289–295, 1972.

Truelsen, T. A.: Indole-3-pyruvic acid as an intermediate in the conversion of tryptophan to indole-3-acetic acid II. Distribution of tryptophan transaminase activity in plants – Physiol. Plant. **28** : 67–70, 1973.

Wightman, F., Cohen, D.: Intermediate steps in the enzymatic conversion of tryptophan to IAA in cell. – In: Wightman, F., Setterfield, G. (ed.): Biochemistry and Physiology of Plant Growth Substances. Pp. 273–288. The Runge Press, Ottawa 1968.

Wightman, F., Rauthan, B. S.: Evidence for the biosynthesis and natural occurence of the auxin phenylacetic acid in higher plants. – In: Plant Growth Substances 1973. Pp. 15–27, Hirokawa, Tokyo 1974.