END-PRODUCT INHIBITION OF *PISUM* PHENYLALANINE AMMONIA-LYASE BY THE *PISUM* FLAVONOIDS

T.H. ATTRIDGE, G.R. STEWART* and Harry SMITH
Department of Physiology and Environmental Studies
University of Nottingham School of Agriculture
Sutton Bonington, Loughborough
Leics., England

Received 16 June 1971
Revised version received 9 July 1971

1. Introduction

The biosynthesis of flavonoids is in many plants regulated by light [1]. Over recent years, evidence has accumulated from precursor incorporation experiments [2, 3] and enzyme studies [4–8] that this regulation is mediated largely through changes in the total activity of phenylalanine ammonia-lyase (PAL). In most cases, light brings about an increase in PAL synthesis [4, 6], but in peas there is evidence for a light mediated activation of PAL occurring before any changes in PAL synthesis. The major flavonoids synthesized in pea seedlings are kaempferol-3-triglucoside (KG), kaempferol-3-*p*-coumaroyltriglucoside (KGC), quercetin-3-triglucoside (QG) and quercetin-3-*p*-coumaroyltriglucoside (QGC), and the levels of KGC and QGC, the likely end products, are closely correlated with the changes in PAL activity [8, 10]. In this communication we report evidence for end-product inhibition of PAL from peas by the flavonoids.

2. Materials and methods

Pea seedlings were grown in a dark room at 25° for 7 days and then placed under a fluorescent white light source (700 lux) for 6 hr. The apices of 600 seedlings were homogenized in 15 ml of 0.5 M borate buffer pH 8.8. The homogenate was centrifuged at 30,000 *g* for 15 min and the supernatant desalted through a Sephadex G-25 column equilibrated with the extraction buffer. PAL activity was estimated by one of two methods; spectrophotometrically by measuring the increase in absorbance at 270 nm due to the production of cinnamic acid; or by using a radioactive assay similar to that of Koukol and Conn [11], counting samples in a Beckman LS 100 scintillation counter with quench correction by the external standard method. Crystalline QGC was prepared from light-grown pea seedlings by Dr. D.B. Harper using thin-layer chromatography [12].

3. Results and discussion

Kaempferol, quercetin and QGC are all inhibitory to PAL activity at low concentrations (fig. 1). Experiments on the relationship between reaction velocity and substrate concentration in the presence of quercetin have shown kinetics similar to those described as "mixed-type inhibition" by Dixon and Webb [13]. However, in view of the unusual relationship between substrate concentration and reaction velocity described below, it would seem inadvisable to make any conclusions regarding the site

* Present address: Department of Botany, The University, Manchester, England.

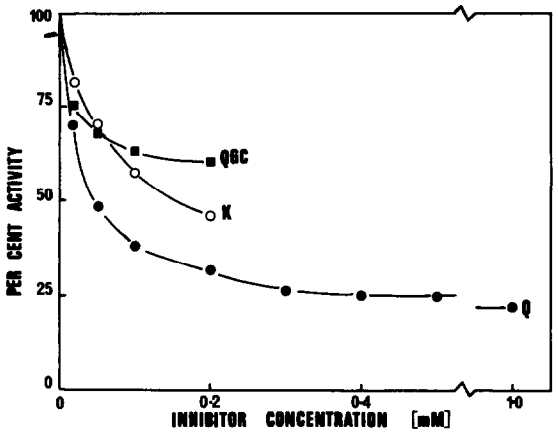

Fig. 1. Inhibition of PAL by the *Pisum* flavonoids. K = kaempferol; Q = quercetin. Radioactive assay.

Table 1
Partial desensitization of PAL.

Treatment	mM phenylalanine		mM phenylalanine + 0.03 mM quercetin	
	dpm/hr/mg	%	dpm/hr/mg	%
None	9891	100	6064	60.6
Heat	7103	100	6183	87.0
$HgCl_2$	4359	100	3806	89.3
Freeze–thawing	7259	100	6166	84.9

Enzyme preparations were incubated at 45° for 30 min (heat), or at room temperature for 60 min in 10^{-6} $HgCl_2$ followed by desalting through Sephadex G-25 ($HgCl_2$), or frozen and thawed three times (freeze–thawing), or left untreated (none). Enzyme activity is expressed as dpm/hr/mg protein and as percent of control.

of action of the inhibitor. Since it is well-known that polyphenolic compounds are often capable of denaturing proteins, the enzyme was preincubated with quercetin for varying periods up to 90 min before addition of substrate, but no significant losses in activity were observed. Although quercetin is not completely stable at pH 8.8, very little breakdown occurred within this time period.

The enzyme can be partially desensitized to inhibition by quercetin by preincubation with mercuric chloride, by freeze–thawing treatment, and by high temperature incubation (table 1). Such evidence has previously been used to argue that the binding site for the inhibitor is both distinct from, and more labile than, the catalytic site [14] and it could thus be suggested that PAL shows such second-site regulation.

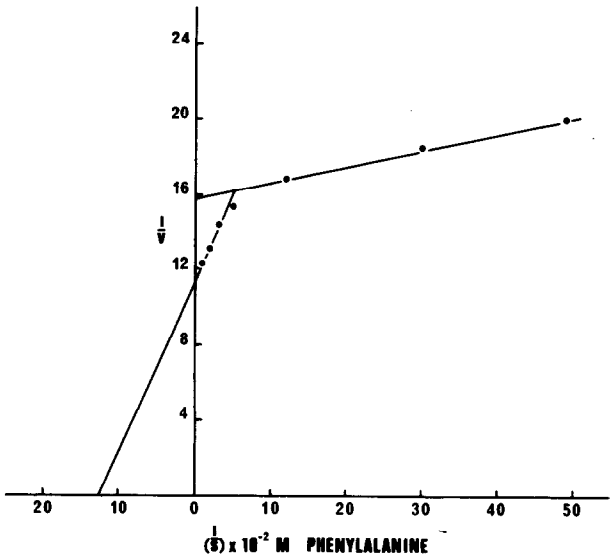

Fig. 2. Relationship between reaction velocity and phenylalanine concentration for PAL from peas. Spectrophotometric assay.

Further evidence for this view comes from the relationship between reaction velocity and phenylalanine concentration. When the data are cast in a Lineweaver-Burke plot (fig. 2), two K_m values can be derived. Kinetics of this unusual pattern have been observed for homoserine dehydrogenase (HDH) from *Rhodopseudomonas rubrum* [15] and from *Zea mays* [16], and also for PAL extracted from potato tubers discs [17]. Using the nomenclature of Datta and Gest [15], K_m^H for PAL averages at 8.0×10^{-4} M, whilst K_m^L averages at 5.2×10^{-5} M. Both Datta and Gest [15] and Bryan [16] have used this behaviour to argue the regulatory role of HDH. Havir and Hanson [17] have reported similar kinetics for PAL from potato tuber discs and have also demonstrated two molecular weight forms of PAL. No evidence has been obtained for two forms of PAL in pea preparations [9]. PAL has further

similarities with HDH in that the relationships between activity and inhibitor concentrations for PAL and the flavonoids (fig. 1) are very similar to those for HDH and amino acids in both of the cited investigations.

The deamination of phenylalanine is the first unique reaction of flavonoid B-ring biosynthesis removing phenylalanine from the predominant pathway to protein biosynthesis and is thus a likely regulatory step. The evidence presented here demonstrates that the activity of PAL from peas can be regulated *in vitro* by the end-products of flavonoid biosynthesis in peas, and that PAL has other properties associated with regulatory enzymes. Whether or not such feedback inhibition is operative *in vivo* is a more difficult question, but the maintenance of relatively constant cellular concentrations of the flavonoids in the leaves of light-grown plants [18] indicates that a mechanism for the regulation of flavonoid biosynthesis must exist.

Acknowledgements

This work was supported by a Research Grant from the Science Research Council to H.S. T.H.A. was a holder of an S.R.C. Research Studentship during this period. The graphical assistance of Mr. A.W. Collings is appreciated by the authors.

References

[1] H. Grisebach, in: Chemistry and Biochemistry of Plant Pigments, ed. T.W. Goodwin (Academic Press, London, 1965) p. 279.
[2] D.B. Harper and H. Smith, Biochim. Biophys. Acta 184 (1969) 230.
[3] D.B. Harper, D.J. Austin and H. Smith, Phytochemistry 9 (1970) 497.
[4] M. Zucker, Plant Physiol. 40 (1965) 779.
[5] F. Durst and H. Mohr, Naturwissenschaften 54 (1967) 319.
[6] G. Engelsma, Planta 75 (1967) 207.
[7] T.H. Attridge and H. Smith, Biochim. Biophys. Acta 148 (1967) 805.
[8] H. Smith and T.H. Attridge, Phytochemistry 9 (1970) 487.
[9] T.H. Attridge, Ph.D. Thesis, Univ. London (1969).
[10] H. Smith and D.B. Harper, Phytochemistry 9 (1970) 477.
[11] J. Koukol and E.E. Conn, J. Biol. Chem. 236 (1961) 2392.
[12] D.B. Harper and H. Smith, J. Chromatog. 41 (1969) 138.
[13] M.M. Dixon and E.C. Webb, Enzymes (Longmans, London, 1958) p. 324.
[14] J. Monod, J.P. Changeux and F. Jacob, J. Mol. Biol. 6 (1963) 306.
[15] P. Datta and H. Gest, J. Biol. Chem. 240 (1965) 3023.
[16] J.K. Bryan, Biochim. Biophys. Acta 171 (1969) 205.
[17] E.A. Havir and K.R. Hanson, Biochemistry 7 (1968) 1904.
[18] W. Bottomley, H. Smith and A.W. Galston, Phytochemistry 5 (1966) 117.