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Altered feedback sensitivity of acetohydroxyacid synthase from valine-resistant mutants of tobacco (*Nicotiana tabacum* L.)

J.M. Relton[1], R.M. Wallsgrove[1], J.-P. Bourgin[2], and S.W.J. Bright[1]

[1] Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts. AL5 2JQ, UK, and
[2] Laboratoire de Biologie Cellulaire, INRA, F-7800 Versailles, France

Abstract. Acetohydroxyacid synthase (EC 4.1.3.18) has been extracted from leaves of three valine-resistant (Val^r) tobacco (*Nicotiana tabacum*) mutants, and compared with the enzyme from the wild-type. The enzyme from all three mutants is appreciably less sensitive to inhibition by leucine and valine than the wild-type. Two of the mutants, Val^r-1 and Val^r-6, have very similar enzymes, which under all conditions are inhibited by less than half that found for the wild-type. The other mutant, Val^r-7, has an enzyme that only displays appreciably different characteristics from the wild-type at high pyruvate or inhibitor concentrations. Enzyme from Val^r-7 also has a higher apparent K_m for pyruvate, threefold greater than the value determined for the wild-type and the other mutants. The sulphonylurea herbicides strongly inhibit the enzyme from all the lines, though the concentrations required for half-maximal inhibition of enzyme from Val^r-1 and Val^r-6 are higher than for Val^r-7 or the wild-type. No evidence has been found for multiple isoforms of acetohydroxyacid synthase, and it is suggested that the valine-resistance of these mutant lines is the result of two different mutations affecting a single enzyme, possibly involving different subunits.

Key words: Acetohydroxyacid synthase – Amino acid (branched-chain) – Feedback regulation – Mutant (*Nicotiana*) – *Nicotiana* – Valine resistance.

Introduction

Valine inhibits the growth of higher plants, both as seedlings (Miflin 1969a) and protoplasts (Bourgin 1976). This inhibition is relieved by isoleucine. Such inhibition has been interpreted as being due to the effect of valine on acetohydroxyacid synthase, the first enzyme in the common pathway leading to the biosynthesis of leucine, valine and isoleucine. Studies on the enzyme isolated from barley have demonstrated concerted feedback inhibition by valine and leucine (Miflin 1969b, 1971), and synthesis of branched-chain amino acids in isolated chloroplasts has been reported to be regulated by exogenous valine and isoleucine (Schulze-Siebert et al. 1984). Hence, metabolic control of branched-chain amino-acid biosynthesis involves allosteric regulation of acetohydroxyacid synthase by the end products of the pathway, and there is no evidence to indicate additional repression and-or deactivation controls such as seen in bacteria. The recent discovery that two potent new classes of herbicides act through inhibition of this enzyme, namely the sulphonylureas (Ray 1984) and the imidazolinones (Shaner et al. 1984), has focused renewed attention on acetohydroxyacid synthase.

Inhibition of growth of cultured plant cells by amino acids or their analogues has been successfully exploited for the selection of mutant cell lines in which the feedback sensitivity of regulatory enzymes is reduced. The use of analogues has led to resistant cell lines with altered properties of anthranilate synthetase (Widholm 1972; Scott et al. 1979), chorismate mutase (Palmer and Widholm 1975), dihydrodipicolinate synthase (Negrutiu et al. 1984) and threonine dehydratase (Strauss et al. 1985). Resistance to lysine plus threonine has been used to select mutants with altered regulation of aspartate kinase, in barley (Bright et al. 1982; Rognes et al. 1983; Arruda et al. 1984), and maize (Hibberd et al. 1980). Valine-resistant tobacco lines have been obtained by selection of protoplast-

Table 1. Percent inhibition of acetohydroxyacid synthase from tobacco by leucine (Leu), valine (Val), isoleucine (Ile) and 2-oxobutyrate. All results represent the mean of three (a) or two (b) separate experiments. The specific acitivity in the control assays was 30–50 pkat·mg^{-1} protein

Amino acid (0.1 mM)	Tobacco line			
	XHFD-8	Val^r-1	Val^r-6	Val^r-7
a) 50 mM Pyruvate				
Leu	41	9	7	9
Val	21	10	7	3
Ile	4	3	2	0
Leu + Val	51	19	8	28
Leu + Ile	45	8	0	15
Val + Ile	23	7	2	17
Leu + Val + Ile	53	17	2	26
10 mM 2-Oxobutyrate	54	53	58	56
b) 5 mM Pyruvate				
Leu	23	10	16	22
Val	32	15	21	17
Ile	15	11	6	14
Leu + Val	63	33	30	53
Leu + Ile	49	20	19	45
Val + Ile	28	18	16	35
Leu + Val + Ile	67	33	30	45
10 mM 2-Oxobutyrate	67	57	63	64

derived cell cultures (Bourgin 1978, 1983); some of these proved to have reduced uptake of valine and other amino acids (Bourgin et al. 1985).

We report here on the properties of acetohydroxyacid synthase from the valine-resistant tobacco lines Val^r-1, Val^r-6 and Val^r-7, that do not have altered valine uptake. A preliminary report of some of this work has been presented (Wallsgrove et al. 1984).

Materials and methods

Plant material. Valine-resistant tobacco (*Nicotiana tabacum* L.) lines Val^r-1, Val^r-6 and Val^r-7 (Bourgin et al. 1985), and a wild-type line (XHFD-8) were grown in compost in a controlled-environment cabinet.

Chemicals. Amino acids and biochemicals were obtained from Sigma (Poole, Dorset, UK). Chlorsulphuron, sulphometuron methyl and the imidazolinone AC 252, 214 were kindly donated by Dr. D. Pearson of ICI (Jealotts Hill Research Station, Bracknell, Berks., UK). Stock solutions of these compounds were prepared in 5 mM K-phosphate pH 7.5.

Enzyme extraction. Young leaves were harvested, washed in distilled water and homogenised in 5 vol. of buffer A (50 mM K-phosphate pH 7.5, 1 mM $MgSO_4$, 1 mM pyruvate, 1 mM L-leucine, 1 mM L-valine, 0.5 mM thiamine pyrophosphate, 0.1 mM flavin adenine dinucleotide [FAD], 10% [v/v] ethanediol) with 0.05% (v/v) Triton X-100, 0.2 g·g^{-1} fresh weight polyvinylpolypyrolidone, and acid-washed sand. The homogenate was centrifuged at 20000·*g* for 30 min, and the supernatant fractionated with $(NH_4)_2SO_4$. Protein precipitating between 25 and 50% saturation was collected by centrifugation, redissolved in a small volume of buffer A and stored as 1-ml aliquots at −70° C until needed. Prior to assay, the thawed extract was desalted on a small column of Sephadex G-25 equilibrated with 50 mM K-phosphate pH 7.5, 1 mM $MgSO_4$ and 10% (v/v) ethanediol.

Fast protein liquid chromatography (FPLC). The enzyme preparation (500 µl containing about 3 mg of protein) was applied to a Mono Q anion-exchange column (FPLC system; Pharmacia, Milton Keynes, UK) equilibrated with 20 mM 2-amino-2(hydroxymethyl)-1,3-propanediol (Tris)-HCl pH 7.5, 1 mM $MgSO_4$, 150 mM KCl and 10% (v/v) ethanediol. The column was eluted with 5 ml of this buffer followed by 20 ml of a linear KCl gradient (0.15–0.60 M) at a flow rate of 1 ml·min^{-1}. Fractions, 1.0 ml, were collected and assayed for activity. All solutions were degassed and filtered through a 0.22-µm-mesh filter prior to use.

Acetohydroxyacid-synthase assay. The standard assay mixture comprised 30 mM K-phosphate pH 7.5, 50 mM pyruvate, 10 mM $MgSO_4$, 0.25 mM thiamine pyrophosphate, 0.23 µM FAD, plus enzyme, in a volume of 0.5 ml. After incubation at 30° C for 1 h, the reaction was stopped by the addition of 100 µl 3 M H_2SO_4, the tubes heated at 60° C for 15 min, and acetoin determined by the method of Westerfeld (1945). A standard curve of A530 versus acetoin concentration was prepared; under the conditions of the assay, an absorbance of 1.0 was equivalent to 65 nmol acetoin.

Protein was determined by the method of Bradford (1976).

Results

Extraction of tobacco leaves using the procedure of Miflin (1971) proved unsuccessful, little or no acetohydroxyacid synthase being recovered from either wild-type or mutant plants. Inclusion of polyvinylpolypyrolidone, 0.2 g·g^{-1} fresh weight, was absolutely required for retention of enzyme activity, and it was found that inclusion of pyruvate, thiamine pyrophosphate and FAD improved both yield and stability. Ethanediol increased the stability of the enzyme, and leucine and valine were necessary in the extraction medium to retain feedback sensitivity. The finally optimised extraction medium was very similar to that described by Chaleff and Mauvais (1984). The yield of enzyme activity declined sharply with older leaves, and for all the work described here only young, expanding leaves (less than 5 cm long) were used. The extracted enzyme, when stored at −70° C, retained activity and feedback sensitivity for 4–5 d – after this, the feedback sensitivity rapidly decreased and less than 50% of the initial activity remained after one month at −70° C.

The enzyme from wild-type leaves was inhibited by leucine, and to a lesser extent, valine (Table 1). At high (50 mM) pyruvate concentrations

Table 2. Affect of sulphonylurea herbicides on the kinetic properties of acetohydroxyacid synthase from mutant and wild-type tobacco leaves. ND = not determined. K_m values represent the mean of four (wild-type) or two (mutants) determinations

	K_m^{app} (pyruvate) (mm)	IC_{50}[a] ($\mu g \cdot l^{-1}$)	
		Chlorsulphuron	Sulphometuron methyl
Wild-type	1.68	6	3
Val^r-1	1.68	ND	ND
Val^r-6	1.63	12	6
Val^r-7	4.65	6	3

[a] IC_{50} – concentration giving 50% of maximum inhibition

the inhibition by these two amino acids was additive whilst at low (5 mM) pyruvate there was evidence of synergistic (greater than additive) inhibition when both were present. All three mutant lines contained enzyme that was markedly less inhibited by leucine and valine, alone or together, at a pyruvate concentration of 50 mM. With 5 mM pyruvate, enzyme from Val^r-1 and Val^r-6 was less sensitive than the wild-type enzyme to leucine or valine alone, and was inhibited only half as much as the wild-type by (leucine plus valine). Enzyme from Val^r-7, however, was almost indistinguishable from the wild-type at 5 mM pyruvate, being slightly less inhibited by valine alone and by leucine plus valine. For the enzyme from all sources, isoleucine was only inhibitory when assayed with 5 mM pyruvate, and did not potentiate inhibition by either of the other amino acids. 2-Oxobutyrate, the substrate for the second reaction catalysed by acetohydroxyacid synthase, did not give rise to a coloured product but was an inhibitor of acetolactate synthesis (Table 1). No differences were observed between wild-type and mutant enzymes.

The effect of increasing concentrations of valine on the enzyme are shown in Fig. 1a. At the highest concentration tested (10 mM), the wild-type was inhibited by 34%, and the enzymes from Val^r-6 and Val^r-7 by 10–12%, there being no appreciable difference between the mutants. However, the effect of leucine plus valine, at equimolar concentrations (Fig. 1b) was quite different. At all concentrations the enzyme from Val^r-6 was markedly less inhibited than the wild-type, with a maximum inhibition of 30% compared with 70%. Enzyme from Val^r-7 was only distinguishable from the wild-type at the higher concentrations of amino acids, and was always more inhibited than Val^r-6. Maximum inhibition was 50%. Enzyme from Val^r-1 gave essentially similar results to Val^r-6 (data not shown).

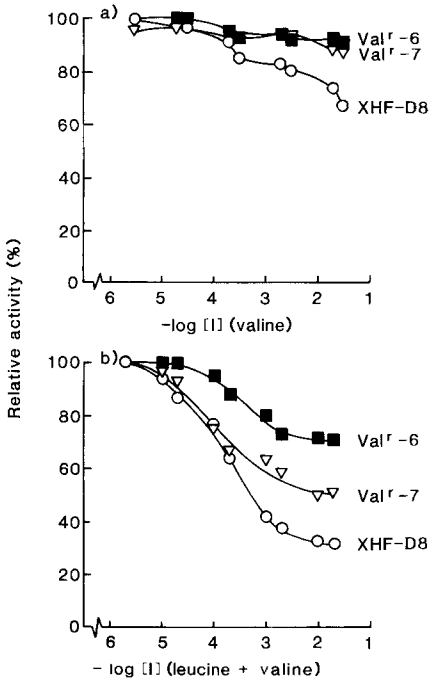

Fig. 1a, b. Inhibition of acetohydroxyacid synthase from mutant and wild-type tobacco by **a** valine, **b** leucine plus valine (equimolar concentrations)

The effect of the sulphonylureas chlorsulphuron and sulphometuron methyl on the enzyme from wild-type and mutants is shown in Fig. 2. Enzyme from all the lines was inhibited by at least 95% by either compound at 1 $mg \cdot l^{-1}$. At lower concentrations the enzyme from Val^r-6 was somewhat less inhibited than the wild-type or Val^r-7 enzymes, the concentration giving 50% inhibition being doubled (Table 2). The imidazolinone AC 252, 214 (Shaner et al. 1984) inhibited enzyme from both wild-type and mutants equally, with 50% of maximum inhibition produced by 1.5–2 $mg \cdot l^{-1}$ (data not shown).

The kinetics of acetohydroxyacid synthase with respect to pyruvate were examined, and the enzymes from both wild-type and mutants followed Michaelis-Menten kinetics even at low pyruvate concentrations, with no evidence of sigmoidal or biphasic responses (Fig. 3 shows the wild-type). Apparent K_m values are given in Table 2, and whereas enzyme from Val^r-1 and Val^r-6 gave values similar or identical to that for the wild-type, the K_m^{app} for the enzyme from Val^r-7 was reproducibly three-fold higher.

Chromatography of the extracts on a Mono Q anion-exchange column resulted in a broad peak of activity eluting between 0.3 and 0.5 M KCl (Fig. 4). No differences in the elution profile were found for any of the mutants, and in all cases frac-

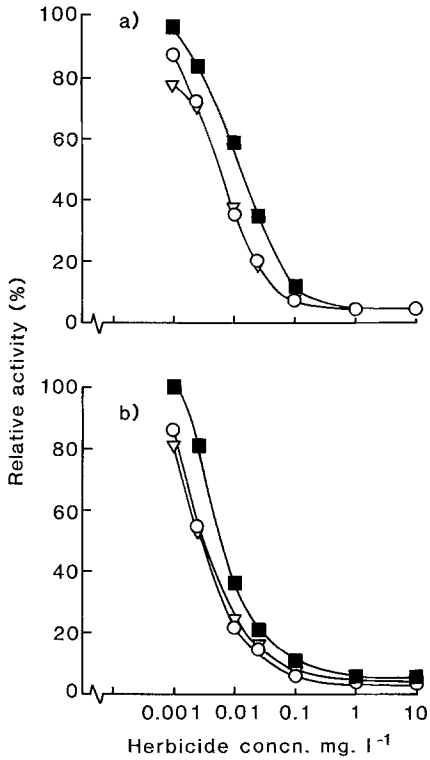

Fig. 2a, b. Inhibition of acetohydroxyacid synthase from mutant and wild-type tobacco by **a** chlorsulphuron, **b** sulphometuron methyl. ■—■ = Val^r-6; ▽—▽ = Val^r-7; ○—○ = wild-type

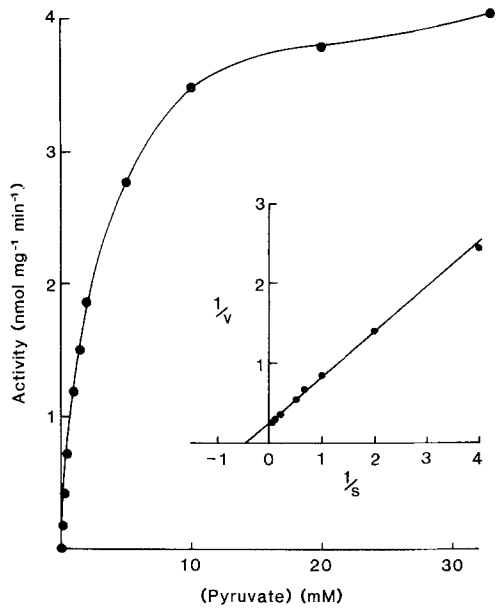

Fig. 3. Relationship between pyruvate concentration and enzyme activity for acetohydroxyacid synthase from wild-type tobacco. *Inset:* double-reciprocal plot of the same data

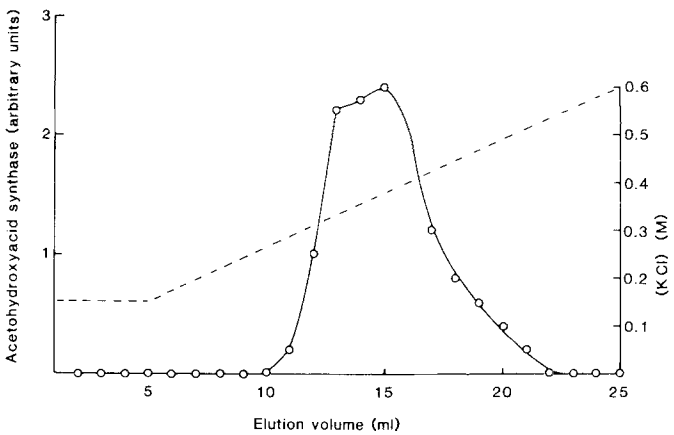

Fig. 4. Elution of tobacco acetohydroxyacid synthase from a Mono Q anion-exchange column (wild-type enzyme)

tions from the beginning and end of the peak displayed identical patterns of inhibition as the starting material. There was no evidence for any separation of isoforms with differing kinetic properties. Recovery of enzyme activity from the column was in excess of 60%, the specific activity of the peak fractions being 11-fold greater than the applied enzyme.

Discussion

The valine resistance of each of the tobacco lines Val^r-1, Val^r-6 and Val^r-7 is transmitted as a dominant Mendelian character, which has been denoted Vrl (Bourgin et al. 1985). From the results reported here, we conclude that the mutation in each case results in an acetohydroxyacid synthase that is less sensitive to feedback inhibition by valine. Genetic analysis has indicated that resistance in Val^r-1 and Val^r-6 is the result of mutation at a common allele, whereas the resistance of Val^r-7 segregates independently. This is consistent with our results, in that the enzymes from Val^r-1 and Val^r-6 are very similar (with respect to feedback inhibition and Km), and enzyme from Val^r-7 is distinctly different. However, there is no evidence of two or more isoforms of acetohydroxyacid synthase in tobacco, so we do not seem to be observing mutation of two independently-coded forms of the enzyme. One possibility would be that acetohydroxyacid synthase is made up of two (or more) subunits, and the Val^r-1/6 and Val^r-7 mutations involve different subunits. To date, the enzyme has not been sufficiently purified from any plant to allow analysis of subunit composition. Acetohydroxyacid synthase I from *Escherichia coli* contains two subunits of similar molecular weight (Grimminger and Umberger 1979), and exists as the dimer or the tetramer, the latter being catalytically active. Isoenzyme II from *Salmonella typhimurium* is composed

of two large and two small subunits (Schloss et al. 1985), products of the overlapping *ilvG* and *ilvM* genes.

Acetohydroxyacid synthase from Val^r-7 has only a slightly reduced sensitivity to inhibition by leucine and valine compared with the wild-type, and the difference is most apparent at high pyruvate or high inhibitor concentrations. As for the enzyme, previous studies on the effect of valine on protoplasts from the mutants indicated that Val^r-7 was intermediate in sensitivity between the wild-type and Val^r-1 (Bourgin et al. 1985). Enzyme from Val^r-7, alone of the mutants, has an altered K_m^{app} for pyruvate, yet is as sensitive as the wild-type to sulphonylureas. Val^r-6 enzyme is marginally less sensitive to these compounds, which appear to be competitive inhibitors with respect to pyruvate (Schloss 1984).

Previous studies on the barley enzyme demonstrated a sigmoidal response to increasing pyruvate concentrations (at low pyruvate concentrations) (Miflin 1971). This was not apparent with the tobacco enzyme, even down to 0.1 mM pyruvate. The K_m^{app} for the barley enzyme, (14 mM), is very much higher than that for the tobacco enzyme.

Tobacco mutants have now been isolated with two different, and independent, alterations in the properties of acetohydroxyacid synthase. The mutants described here demonstrate reduced sensitivity to allosteric effectors but are as sensitive as the wild-type to sulphonylureas and imidazolinones. Herbicide-resistant lines (Chaleff and Mauvais 1984) have enzyme that is almost insensitive to sulphonylureas, but their response to leucine and valine has not been reported.

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