Acetolactate Synthase of Suspension-Cultured Carrot Cells Resistant to Bensulfuron Methyl*

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Abstract: Activities of acetolactate synthase (ALS) of 10^{-8} M bensulfuron methyl (BSM)-resistant carrot cells were compared with those of susceptible (normal) cells. ALS activity, assayed by measuring acetoin formed by decarboxylation of acetolactate, was inhibited in normal cells by low concentrations of BSM. However, nearly or more than half of the total activity was not inhibited by 10^{-6} M BSM at pH lower than 7.5. Acetoin amounts produced in undecarboxylated samples almost coincided with those inhibited by high concentrations of BSM, 10^{-6} and 10^{-4} M, in normal and resistant cells, respectively. This might indicate the presence of a direct acetoin producing system in ALS assay. Net ALS activity could be obtained by subtracting the undecarboxylated value from the total activity.

Optimum pHs were broad, around 8.5 for ALS and 7.0 for direct acetoin producing activity. ALS was saturated at 40 mM pyruvate and apparent Km value for ALS was 5-6 mM.

ALS from resistant cells was inhibited by BSM less than the normal cells. The BSM concentrations inhibiting ALS activity by 50% (I_{50}) were 1\times10^{-8} M and 6\times10^{-7} M for normal and resistant cells, respectively. As this I_{50} value of the resistant cells was about 10 times higher than that reported previously (5.2\times10^{-8} M), the probability is that insensitivity of ALS to BSM of the resistant cells developed during long-term culture.

Key words: Acetolactate synthase (ALS), bensulfuron methyl, carrot cells, herbicide resistance.

Introduction

The primary action of bensulfuron methyl (methyl 2-[[3-(4,6-dimethoxypyrimidi-2-yl)ureido] sulfonyl]-o-toluate, BSM) in plants, like other sulfonylurea herbicides, is well established as the inhibition of acetolactate synthase (ALS, or aceto-hydroxy acid synthase), a key enzyme for biosynthesis of branched chain amino acids[2,3,6,13]. ALS is inhibited by sulfonylureas noncompetitively with the substrate, pyruvate[4,6]. Sulfonylurea herbicide resistant cultured cells have been selected in some plants[3,8,19], and recently, some weed biotypes resistant to these herbicides were also found in the crop fields[5,9,14]. The mechanism of the resistance was found to be the development of ALS less-sensitive to the herbicides[2,3,5,14,19]. Cross-resistances among sulfonylurea, imidazolinone and triazoropyrimidine sulfonanilide herbicides have been investigated in the resistant plants and cells based on their growth and ALS activities[5,14,15,19]. Further, cells resistant to feedback inhibition by valine and/or leucine have been found[19].

BSM resistant cells were also selected from

* Parts of this study were presented at the 30th annual meeting of the Weed Science Society of Japan in April, 1991 and the First International Weed Control Congress, Melbourne in February, 1992.
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(Received August 13, 1992)
susceptible carrot cells and their resistance mechanism was assumed to involve BSM-insensitive ALS. Then, we found an increase in the ratio of ALS activity which was not inhibited by high concentrations of BSM even in normal cells after long-term culture of the cells. In this paper we investigate further the reason for this and ALS in the resistant cells.

Materials and Methods

BSM (99% pure) was the generous gift of Du Pont Japan, Ltd. Cells induced from hypocotyls of carrot (Daucus carota L. cv, US Harumakigosun) were suspension cultured in Linsmaier and Skoog's medium (LS medium) containing 1 mg/l 2, 4-D at 28°C and 120 rpm by subculturing at 7-day intervals. The 10^-8 M BSM-resistant cells selected previously were maintained in BSM-free medium for about 2 years and were about 100 times more resistant than untreated (normal) cells.

ALS assay was carried out using both normal and resistant cells at their linear growth phase, 4 days after inoculation. ALS was extracted from the cells homogenized with 50mM phosphate buffer (pH 7.0) containing 0.1 mM MgSO_4, 0.5 mM DTT, 0.05 mM FAD, and supernatant after centrifugation (20000×g, 15 min) was used as crude enzyme. ALS activities were assayed with some modifications of Anderson and Hibberd's method. The reaction mixture consisted of 50 mM phosphate buffer (pH 8.0), 40 mM Na-pyruvate, 5 mM MgCl_2, 0.1 mM thiamine pyrophosphate (TPP) and the enzyme solution was incubated at 30°C for 30 min as a standard assay, and the acetolactate formed was measured after decarboxylation to acetoin with H_2SO_4. Undecarboxylated blanks were made by adding color developing reagents just after completion of the enzyme reaction without H_2SO_4 addition.

Results and Discussion

In ALS assay, undecarboxylated samples contained acetoin to varying degrees. Its ratio to decarboxylated total activity was more than half at low pH and rather less at higher pH (Fig. 1) in both normal and resistant cells. The undecarboxylated values were very close to the values inhibited by high concentration of BSM, 10^-6 M in the normal cells. When pyruvate concentration was varied, acetoin amounts in undecarboxylated samples increased as pyruvate concentration increased (Fig. 2). And the curves of the undecarboxylated values almost coincided with those inhibited by BSM of high concentrations (10^-6 M and 10^-4 M in ALS of normal and resistant cells,

![Graph](image.png)

Fig. 1. Optimum pH of ALS and acetoin producing activity in normal (A) and resistant (B) cells. ● Total, ○ Total in the presence of 10^-6 M BSM, ▲ Direct acetoin production, ▽ Net ALS.
respectively). This might indicate presence of a direct acetoin producing system in ALS assay. Imidazolinone insensitive pyruvate decarboxylase activity is shown to contribute apparent ALS activity in crude enzyme preparation\(^\text{17}\). Therefore, net ALS activity could be obtained by subtracting the undecarboxylated direct acetoin producing activity from the total activity.

Optimum pHs were broad, around 8.5 for ALS and 7.0 for direct acetoin producing activity in both normal and resistant cells (Fig. 1). Pyruvate saturation curves were hyperbolic\(^\text{17}\) and the enzyme activity was saturated at 40 mM pyruvate in both types of cells (Fig. 2). This result also showed that maximal velocities (V\(_{\text{max}}\)) were 300 and 310 nmol/mg protein/hr, and apparent Km values (Michaelis constant) were about 5 mM and 6 mM, for ALS of normal and resistant cells, respectively. When BSM concentration was varied in the standard assay, ALS of the resistant cells was inhibited by the herbicide less than normal (Fig. 3). The BSM concentrations inhibiting ALS activity by 50% were \(6 \times 10^{-7}\) M and \(1 \times 10^{-8}\) M for the resistant and normal cells, respectively. This value of the resistant cells was about 10 times higher than that of the previous study (\(5.2 \times 10^{-8}\) M)\(^\text{20}\). These results suggest that the ALS of the resistant cells became even less sensitive to BSM during long-term culture. This probability might also apply to the increase in ALS activity which was not inhibited by BSM, i.e. the direct acetoin producing activity, in both types of cells.

This decrease in sensitivity might be the result of reduction of binding affinity of BSM to ALS enzyme. The binding of sulfonylurea herbicides to ALS have been shown to be unrelated to the substrate and cofactors\(^\text{19}\). Mutation of one or two amino acids in ALS has also shown to cause reduction of binding affinity and to result in resistance to sulfonylureas\(^\text{19}\). It is unlikely that

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**Fig. 2.** Saturation curves for pyruvate in ALS and acetoin producing activity from normal (A) and resistant (B) cells. Symbols are same as in Fig. 1 except (○) total in the presence of \(10^{-4}\) M BSM in (B).

**Fig. 3.** Inhibition of ALS by BSM from normal (○) and resistant (●) cells.
BSM-resistance was a result of ALS overexpression in the resistant cells, because the specific activities (310 and 300 nmol/mg protein/hr) in the resistant and normal cells, respectively, were very similar. Also, the Km values for pyruvate, 5 mM and 6 mM, were very close and did not seem to be affected by BSM. From these results it is suggested that the BSM-insensitive ALS was the result of lower affinity of BSM at a binding site other than a pyruvate binding or catalytic site*4,14,16).

It is confirmed that the BSM-resistance mechanism primarily involves the development of ALS being insensitive to BSM in the resistant carrot cells20).

References

ベンスルフロンメチル抵抗性ニンジン細胞のアセトラクテート合成酵素

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要 旨

10^{-8} M ベンスルフロンメチル（BSM）抵抗性ニンジン細胞のアセトラクテート合成酵素（ALS）の活性を通常細胞と比較した。細胞は、2,4-D を1 mg/l 含む LS 培地で懸濁培養し、約 2 年間 1 週間ごとに植え替えた。対数増殖期（植え替後 4 日目）の細胞より ALS を抽出し、pH、ビリビン酸濃度および BSM 濃度を変えて活性を測定した。活性は、生じたアセトラクテートを硫酸で脱炭酸しアセトインに変
え比色定量したが、脱炭酸しないプランクにも相当量のアセトインが検出された（第 1, 2 図）。このプランクの値は、pH7.5 以下では脱炭酸した値（全活性）の約 1/2 以上を占めたが、高い pH では低い値を示した。また、この値は、ビリビン酸濃度に依存して増加し高濃度の BSM で阻害されなかったので、ビリビン酸によりアセトラクテートを経ないアセトイン生成によるものと考えられた。そこで、脱炭酸した値から脱炭酸しないプランクの値を差し引いたもののがより正確な ALS 活性を示すと考えられた。

その結果、通常、抵抗性細胞とも、ALS の至適 pH は 8.5 付近、飽和ビリビン酸濃度は約 40 mM、見掛けの Km は 5〜6 mM であった（第1, 2図）。BSM による ALS の阻害は、抵抗性細胞（I_{50} 6×10^{-7} M）が通常細胞（I_{50} 1×10^{-8} M）より小さく、約 60 倍の差があった（第 3 図）。この抵抗性細胞の値は既に発表されたもの（5.2×10^{-8} M）より約 10 倍大きく、長期間培養中に ALS の不感受性化が進んだ可能性もあると考えられる。

キーワード: アセトラクテート合成酵素(ALS), ベンスルフロンメチル, ニンジン細胞, 除草剤抵抗性