Effect of Chlorsulfuron, a Potent Inhibitor of Acetohydroxyacid Synthase, on Metabolism of *Claviceps purpurea*

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Chlorsulfuron (CS) is a potent sulfonylurea herbicide inhibiting specifically acetohydroxyacid synthase which catalyzes the first step in the biosynthesis of branched-chain amino acids in plant cells, bacteria and yeast. The peptide portion of some ergot alkaloids contain inter alia branched-chain amino acids. The influence of CS on growth and alkaloid formation in *Claviceps* was studied. In an ergotoxine strain growth is inhibited by chlorsulfuron in the range of 10–100 μM. Under CS influence ergosine is accumulated besides ergotoxines. Growth inhibition caused by CS could be reversed by addition of L-valine (~ 35 mM), but neither by leucine nor isoleucine. Ergosine did not occur under these conditions. Acetohydroxyacid synthase (AHAS) from *Claviceps purpurea* was partially purified. AHAS preparations are not inhibited even by high concentrations of chlorsulfuron.

Ergot alkaloids comprise an important group of useful therapeutic agents. A common feature of the peptide ergot alkaloids (ergopeptines) is the unique cyclol portion. Obviously essential intermediates in ergopeptine biosynthesis are d-lysergyl tripeptides. In the case of the ergotoxine group branched-chain amino acids are constituents of the peptide portion *e.g.* L-valine is the amino acid adjacent to lysergic acid in all ergotoxines. Besides proline the ergotoxine alkaloids differ in the third amino acid *e.g.* valine (ergocornine) leucine (α-ergokryptine) and isoleucine (β-ergokryptine) (Table I).

The biosynthesis of ergot peptide alkaloids is at least partially controlled by the relative amino acid concentration in the internal pool of the cells. Addition of appropriate amino acids or their analogues to the culture broth significantly determines the proportion of the alkaloid mixture in a given strain [1–5].

A number of sulfonylurea herbicides *e.g.* chlorsulfuron are potent and specific inhibitors of acetohydroxyacid synthase (AHAS) (EC 4.1.3.18) in bacteria [6], yeasts [7] and higher plants [8]. AHAS catalyzes the first step in the biosynthesis of branched-chain amino acids, valine, leucine and isoleucine. Chlorsulfuron causes a 50% inhibition at nM levels of AHAS from many different plant species [9]. The growth inhibition due to sulfonylurea herbicides may be reversed by the addition of a single branched-chain amino acid or combinations of them. The reversal pattern differs for plants and bacteria [10].

In the present communication we describe the effect of chlorsulfuron on ergot fungi, especially that strain, which produces ergotoxine alkaloids. It is feasible that a depletion of leucine and valine may influence the yield of ergocornine and ergokryptine. On the other hand, an addition of an alkaloid-specific amino acid may reverse growth inhibition caused by the herbicide and alter the quality of the alkaloid mixture. Apparently, there is no report on the influence of this specific AHAS inhibitor on growth and metabolism of filamentous fungi.

Abbreviations: AHAS, acetohydroxyacid synthase; CS, chlorsulfuron; MS, mass spectrometry.

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Materials and Methods

Organisms and culture technique

Strains of *Claviceps purpurea* (Fr.) Tul. were used for these investigations: Strain Pepty 695/S accumulates a mixture of ergocornine and ergokryptine besides clavines and ergonovine. Strain MUT 170 accumulates preferentially ergosine besides clavine alkaloids; Strain Pepty 695/ch-I produces a mixture of agroclavine and elymoclavine and is derived *via* the secoclavine producer Pepty 695/ch from Pepty 695/S by regeneration of protoplasts [11].

The stock cultures were maintained on asparagine/sucrose agar and the submerged cultivations were performed as described [11, 12].

Preparation of cell-free extracts and purification of AHAS

20 g lyophilized 3–4 days old mycelium was ruptured by grinding with dry ice in a mortar and suspended in 0.1 M potassium phosphate buffer pH 7.0 containing 20% glycerol, 5 mM dithioerythritol, 1 mM EDTA and 5 mM $MgCl_2$ (buffer A). The suspension was centrifuged at 15000 × *g* for 30 min. The enzyme solution was fractionated by slow addition of a saturated solution of $(NH_4)_2SO_4$ yielding a 45–55% saturation. Following centrifugation the pellet was dissolved in 50 mM potassium phosphate buffer containing the above mentioned additions (buffer B) and immediately applied to a Sepharose 6B column. After elution with buffer B the active fractions were pooled and chromatographed on a DEAE-Sepharose Cl–6 B column using buffer B as eluent. A gradient was not necessary [13]. The active fractions were pooled and used for the enzyme assay.

Assay for acetohydroxyacid synthase

The reaction mixture contained in 1 ml final volume: 50 µmol sodium pyruvate, 5 µmol $MgCl_2$, 0.5 µmol thiamine pyrophosphate, 0.5 µmol FAD, 10–40 µg protein and 0.5 ml 0.1 M citrate sodium phosphate buffer, pH 6.6.

The mixture was incubated for 1 h at 40 °C. The reaction was stopped by addition of 50 µl of 6 N H_2SO_4 and subsequently incubated at 60 °C for 15 min. After cooling 0.5 ml of 5% α-naphthol dissolved in 2.5 N NaOH, and 0.5 ml of 0.5% creatine were added. Samples were incubated for 20 min at 60 °C and after cooling and centrifugation the absorbance was measured at 530 nm. Enzyme activity is expressed as µmol acetoin mg protein^{-1} h^{-1}.

Analytical procedures

Total alkaloid determinations [12] were carried out using van Urk's reagent. The quantity of alkaloids were calculated according to standard curves using elymoclavine or the appropriate peptide alkaloids as references.

The quantitative determinations of various compounds of the alkaloid mixtures were performed after TLC (silica gel "Merck" PF_{254}) separation [15]. Solvent systems I: chloroform : methanol (8 : 2); II: ethylacetate : N,N-dimethylformamide : ethanol (13 : 1.9 : 0.1 v/v). The latter solvent system is especially suited for separation of ergotamine and ergosine.

Protein was determined according to Bradford [16]. For dry weight determination washed mycelium was dried in an oven at 60 °C for 24 h.

Mass spectrum of ergosine was recorded on an electron attachment mass spectrograph of the Research Institute "Manfred von Ardenne", Dresden. Ergosine *m/z:* no M^+-peak, 280 (cyclol fragment), 267, 266, 224, 223, 221, 210 and 209 (leu-prolactam), 196, 195, 181, 167.

Chemicals

Solutions of chlorsulfuron and Glean® were filter-sterilized and added prior to inoculation to the fermentation medium.

Results

For these experiments we used at first an ergocornine- and ergokryptine-accumulating ergot strain, designated Pepty 695/S (Table I).

Cultivation was performed in presence of either chlorsulfuron or Glean®. Growth and alkaloid formation were measured after 12–14 d of cultivation (Table II). Essentially the same results were obtained using either CS or its formulated product Glean®. The sulfonylurea herbicide did not inhibit at nM levels the development of the given ergot strain. Growth inhibition was observed in the range of 20–100 µM CS. Interestingly ergotoxine accumulation was strongly reduced in the presence of 10–20 µM chlorsulfuron but total alkaloid yields and mycelial growth was not affected to the same degree. Mixtures of branched-chain amino acids (5 mg/100 ml) did not alleviate CS-induced growth inhibition and ergotoxine depression. Surprisingly after addition of CS in the range of 20–40 µM to the culture medium another peptide ergot alkaloid was

Table I. Potential intermediates in the course of biosynthesis of some ergot peptide alkaloids.

Alkaloid	Intermediate
Ergotamine	d-Lysergyl–Ala–Phe–Pro
Ergosine	d-Lysergyl–Ala–Leu–Pro
Ergocornine	d-Lysergyl–Val–Val–Pro
α-Ergokryptine	d-Lysergyl–Val–Leu–Pro
β-Ergokryptine	d-Lysergyl–Val–Ile–Pro
Ergocristine	d-Lysergyl–Val–Phe–Pro

Table II. Effect of chlorsulfuron on growth and alkaloid formation of ergot strain Pepty 695/S.

Additive	Total alkaloid yield [μg ml^{-1}]	Ergotoxine [μg ml^{-1}]	Dry weight [mg ml^{-1}]
Control	1500	1000	37.5
Chlorsulfuron 5 μM	1600	880	35.0
Chlorsulfuron 10 μM	1600	450	33.0
Chlorsulfuron 20 μM	1300	270	25.0
Chlorsulfuron 50 μM	500	37	15.0
Chlorsulfuron 100 μM	80	10	6.0
Chlorsulfuron 20 μM + 5 mg of each Val, Leu, Ile*	1500	350	27.0
Chlorsulfuron 100 μM + 5 mg of each Val, Leu, Ile	100	8	8.0

* Addition of L-amino acids: mg 100 ml^{-1} culture broth.

found. Many experiments were performed and essentially the same results were obtained as shown in Table III. Ergosine is accumulated, which was identified by chromatographical methods and MS, in the presence of chlorsulfuron besides ergotoxins. In ergosine the amino acid adjacent to lysergic acid is alanine instead of valine in α-ergokryptine (Table I). Apparently a reduction of valine takes place which favours the incorporation of alanine. This particular amino acid is the most prominent component in the free amino acid pool in an ergotamine producer [17] and in our strain Pepty 695/S (data not shown).

An addition of L-valine in rather high concentration as it is used for directed biosynthesis of ergot peptide alkaloids [18] could reverse the detrimental effect of chlorsulfuron (20–40 μM) to strain Pepty 695/S (Table III). Under this condition no ergosine was accumulated.

Leucine and isoleucine in the same dosage had no effect. These results prompted us to investigate the influence of other peptide alkaloid specific amino acids on mycelium cultivated in the presence of chlorsulfuron (Table IV). There was no influence on ergonovine accumulation. Addition of L-alanine and L-phenylalanine alone and in mixture did not change the amount and quality of the peptide alkaloid fraction. It is feasible that the alkaloid spectrum could be altered by administration of high amounts of alanine and phenylalanine triggering the formation of ergotamine. In ergotamine is compared to ergosine leucine replaced by phenylalanine (Table I). We have carefully checked the cultures containing this particular amino acids but no trace of ergotamine was found.

We tested also the influence of CS on metabolic activities of other ergot strains. The clavines ac-

Table III. Reversal of chlorsulfuron induced growth inhibition and alkaloid formation in submerged cultures of ergot strain Pepty 695/S.

Additive	Total alkaloid yield [μg ml^{-1}]	Ergotoxine [μg ml^{-1}]	Ergosine [μg ml^{-1}]	Dry weight [mg ml^{-1}]
None	1300	780	–	31.0
Chlorsulfuron 20 μM	1150	130	400	22.0
CS 20 μM + 300 mg Ile	1300	140	425	29.0
CS 20 μM + 400 mg Leu	1100	125	190	26.7
CS 20 μM + 200 mg Val	1000	680	–	25.5
CS 20 μM + 400 mg Val	1270	715	–	26.0

Addition of L-amino acids: mg 100 ml^{-1} culture medium.

Table IV. Effect of various amino acids on growth and alkaloid formation of *Claviceps* strain Pepty 695/S cultivated in the presence of chlorsulfuron.

Additive	Total alkaloid yield [μg ml^{-1}]	Ergotoxine [μg ml^{-1}]	Ergosine [μg ml^{-1}]	Ergonovine [μg ml^{-1}]	Dry weight [mg ml^{-1}]
Control	1100	800	traces	205	34.0
CS, 20 μM	1100	200	180	215	27.0
CS, 20 μM + 400 mg Ala	1110	165	200	210	27.0
CS, 20 μM + 400 mg Phe	1200	160	190	250	28.0
CS, 20 μM + 400 mg Ala 400 mg Phe	1200	200	170	210	28.0
CS, 20 μM + 400 mg Val	1150	780	traces	200	33.0

Addition of L-amino acids: mg 100 ml^{-1} culture medium.

cumulating strain Pepty 695/ch-I is related to Pepty 695/S and was obtained from regenerated protoplasts [19]. In control experiments an alkaloid yield of 50 μg ml^{-1} chanoclavine-I and 950 μg ml^{-1} elymoclavine was obtained. After addition of varying amounts of chlorsulfuron in the range of 5, 10, 20 and 100 μM an elymoclavine yield of 400, 200, 100 μg m^{-1} and zero, respectively could be found. The inhibition of alkaloid formation caused by CS up to a concentration of 20 μM could be reversed only by addition of 400 mg 100 ml^{-1} L-valine. No change of the alkaloid pattern was observed.

Similar experiments were performed with strain MUT 170 which accumulates ergosine (Table I). Surprisingly this strain is not as sensitive as the Pepty 695 series of ergot strains. Growth and alkaloid formation is not affected up to a concentration of 40 μM chlorsulfuron. Administration of CS (100 μM) caused an 75% inhibition of ergosine accumulation which could be completely reversed by 400 mg 100 ml^{-1} valine.

Acetohydroxyacid synthases from a variety of bacteria and plants are extremely sensitive to inhibition by sulfonylurea herbicides [6–8]. Therefore we have isolated and partially purified AHAS from various *Claviceps purpurea* strains, which appeared in the soluble fraction. We examined a number of chromatographic procedures and adopted finally the above described method. Enzyme activity of all fractions were tested in a pH range of 6.0 to 8.5. AHAS eluted always from different columns as single peak showing an pH optimum at 6.6. These findings seem to indicate that no isoenzymes of AHAS exist in *Claviceps purpurea*. End product inhibition of acetohydroxyacid synthase in *Claviceps purpurea* by branched-chain amino acids could not be observed (Table V). Similar results have been reported for AHAS from *Neurospora* [20]. AHAS II activity of

Table V. End product inhibition of acetohydroxyacid synthase by branched-chain amino acids.

Amino acid [mM]	L-Valine AHAS activity*	L-Leucine AHAS activity	L-Isoleucine AHAS activity
None	1.59	1.59	1.59
0.01	1.63	1.61	1.55
0.1	1.60	1.55	1.60
1.0	1.58	1.62	1.64
5.0	1.50	1.51	1.53

* AHAS activity = μmol acetoin mg protein^{-1} h^{-1}.

E. coli was also not inhibited by addition of 10 mM valine [21].

Furthermore AHAS activities in *Claviceps purpurea* strain Pepty 695/S were tested in the presence of various chlorsulfuron concentrations ranging of 10 μM to 1 mM. In these experiments the incubation time was prolonged to 4 h. In no case an inhibition of the enzyme reaction was observed indicating that AHAS from *Claviceps* is apparently insensitive towards chlorsulfuron.

Discussion

We could demonstrate an inhibitory effect of chlorsulfuron in the range of 10–100 μM on growth and alkaloid formation in ergot strains. This is a CS concentration about 1000-fold higher than that required to prevent growth of excised pea roots [9] or *Arabidopsis thaliana* [22]. Under CS influence in ergot strain Pepty 695/S another peptide alkaloid *viz.* ergosine besides ergotoxines is accumulated. In an clavine alkaloids and an ergosine synthesizing strain the composition of the alkaloid fraction was not changed due to chlorsulfuron. Interestingly the sulfonylurea-induced growth inhibition and alteration of the peptide alkaloid spectrum in the ergotoxine producing strain could be reversed only by addition of a rather high dosage of L-valine (~ 35 mM) but neither leucine nor isoleucine. It may be assumed that a depletion of valine in the mycelium caused by CS triggers ergosine formation. The main target site of CS in various organisms is a strong inhibition of acetohydroxyacid synthase, the first common enzyme of pathways leading to branched-chain amino acids. This possibility can be excluded for ergot fungi. AHAS activity in *Claviceps purpurea* is nonsensitive to chlorsulfuron even in the presence of extremely high concentrations (0.5–1 mM). It is feasible that CS interferes with a later step in the pathway leading to L-valine, but other modes of action are also possible. A number of actions of unknown origin induced by μM levels of CS have been described *e.g.* inhibition of RNA synthesis, protein and lipid formation [10, 23] as well as increase in phenylalanine ammonia-lyase and anthocyanin content [24].

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