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Inhibition of Glutamine Synthetase from Pea by Tabtoxinine-β-lactam

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ABSTRACT

Tabtoxinine-β-lactam, a hydrolytic product of tabtoxin produced by *Pseudomonas syringae* pv. *tabaci*, apparently inactivates pea seed glutamine synthetase. Inhibition of the enzyme's initial velocity is linear over a range of 0.5 to 5 millimolar tabtoxinine-β-lactam in the presence of 10 millimolar glutamate. A method for the purification of glutamine synthetase from dried peas is presented which gives a 30% yield with a 2,000-fold increase in specific activity. A method for obtaining highly purified tabtoxinine-β-lactam and tabtoxin in good yields is also presented. The authenticity and purity of tabtoxinine-β-lactam and tabtoxin were verified by chromatography, biological activity, and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy.

The phytopathogenic bacterium *Pseudomonas syringae* pv. *tabaci* and certain other closely related pathovars produce a toxin which induces chlorosis in plants (5, 11, 12, 16). This toxin, called tabtoxin, is a dipeptide composed of either threonine or serine and the novel β-lactam-containing amino acid, tabtoxinine-β-lactam (2-amino-4-[3-hydroxy-2-oxo-azacyclobutan-3-yl]-butanoic acid). Originally, it was hypothesized that chlorosis was caused by a rapid buildup of ammonium, resulting from the inhibition of GS[1] (EC 6.3.1.2) by tabtoxin. Semipurified tabtoxin did, in fact, inhibit the activity of crude GS from pea (14). This hypothesis was also supported by the following observations: (a) MSO, which was already known to inhibit GS, also caused chlorosis and ammonium accumulation (7, 10); (b) tabtoxin- and MSO-induced chlorosis and ammonium accumulation are light-dependent reactions (3) which can be inhibited by dichlorodimethyl phenylurea (Durbin, unpublished); and (c) glutamine negated the inhibitory effects of both MSO and tabtoxin in several biological systems (2). Preliminary studies using purified materials failed to show inhibition of GS by tabtoxin (19). This has been clarified by the present study and the recent observation that unidentified peptidases in tobacco will hydrolyze tabtoxin to yield tabtoxinine-β-lactam (19). Inasmuch as this compound can also cause chlorosis and, more important, appeared to inhibit GS, we postulated that chlorosis development via an inhibition of this enzyme depended upon the generation of tabtoxinine-β-lactam.

This report establishes that purified tabtoxinine-β-lactam, but not tabtoxin, inhibits purified GS from pea by apparently irreversibly inactivating the enzyme. A simple, rapid procedure for the purification of tabtoxin and tabtoxinine-β-lactam is also reported.

[1] Abbreviations: GS, glutamine synthetase; MSO, L-methionine-*S*-sulfoximine; NMR, nuclear magnetic resonance.

MATERIALS AND METHODS

Enzyme Purification. Commercially obtained, dried split peas (1 kg) were milled to a fine powder and soaked 24 h at 4°C in 2 L 20 mM imidazole (pH 7.5), 10 mM $MgCl_2$, and 1 mM β-mercaptoethanol. The crude enzyme preparation was clarified by centrifugation (14,000*g* for 30 min), and a fraction containing the enzyme activity was precipitated by adjusting the pH to 5.2 with 5 M sodium acetate buffer (pH 4). The precipitate was collected immediately by centrifugation (14,000*g* for 10 min), resuspended in 50 ml 20 mM imidazole and 10 mM $MgCl_2$, and the pH adjusted to 6.5 with 4 M imidazole (pH 9.5). Two percent aqueous protamine sulfate was added dropwise until no additional precipitate formed; this precipitate was discarded after centrifugation (14,000*g* for 30 min). The enzyme was concentrated by precipitation with 50% saturated $(NH_4)_2SO_4$, followed by centrifugation (40,000*g* for 10 min). The precipitate was resuspended in 50 ml of 20 mM imidazole (pH 8.2) containing 10 mM $MgCl_2$ (column buffer) and dialyzed against this buffer (4 L) for 24 h. The enzyme preparation was applied to a column of DEAE-Cellulose (1 × 20 cm) previously equilibrated with column buffer. The column was washed with 0.5 to 1 L of column buffer and the enzyme then eluted with a linear gradient of 0 to 0.5 M KCl in column buffer (400 ml). GS eluted between 20 and 100 mM KCl. Fractions containing GS activity were pooled, and the enzyme concentrated by precipitation with 50% saturated $(NH_4)_2SO_4$. The precipitate was resuspended in 1 ml of 20 mM imidazole buffer (pH 7.5) containing 1 mM $MgCl_2$ and dialyzed overnight against 200 volumes of this buffer. The preparation was passed through a column of Sepharose 6B (1.8 × 118 cm) equilibrated with the above buffer plus 0.02% NaN_3. Fractions containing enzyme activity were pooled and concentrated as described above. The gel filtration chromatography step was repeated once.

Analytical Procedures. The γ-glutamyl transferase and phosphate liberation assays of Shapiro and Stadtman (13) were used. The transferase assay was used to monitor enzyme activity during purification and the phosphate liberation assay was used in characterizing the inhibition of GS by tabtoxinine-β-lactam. Authentic γ-glutamyl hydroxamate was used as a standard for the transferase assay and for which 1 unit of enzyme activity was defined as the synthesis of 1 μmol γ-glutamyl-hydroxamate/min. The Pi liberation assay was modified to include stopping the reaction (0.2 ml) with 1 ml of 52 mM $FeSO_4$ in 0.03 N H_2SO_4, and developing the color by adding 1 ml of 8 mM $(NH_4)_6Mo_7O_{24}$ in 1.1 N H_2SO_4. The *A* at 660 nm was determined after 5 min. Inorganic K-phosphate was used as a standard. Enzyme activity was expressed as μmol Pi released per min ($A_{660\ nm}$ = 0.6/μmol Pi).

Protein concentration was determined by the method of Bradford (1) using BSA as a standard. Enzymes and chemicals were

obtained from Sigma Chemical Co.[2]

Purification of Tabtoxin and Tabtoxinine-β-lactam. *P. syringae* pv. *tabaci* (ATCC 11528) was grown with agitation in Woolley's medium (21) at 24°C. For tabtoxin production, the incubation period was approximately 3 d, at which time the pH had risen to 7.6 to 7.8 (3.45 OD at 560 nm). For tabtoxinine-β-lactam production, the bacteria were grown for only about 36 to 40 h (1.5 OD at 560 nm). The cells were then removed by centrifugation (8,000*g* for 10 min) and resuspended in the same medium modified to contain 0.5 g sucrose/l and 0.05 g yeast extract/l. The culture was then incubated as above for an additional 24 h. In either case, the cells were next removed by centrifugation (8,000*g* for 10 min) and the supernatant passed through a column of Amberlite CG-120, H^+-form (3 × 33 cm). The column was washed with Milli Q water (Millipore), and the biologically active fraction was eluted with 4% NH_4OH. A 30-ml fraction was collected at the pH change and immediately concentrated *in vacuo* (concentrated HCl was added to the receiver to aid in rapidly removing the ammonia). Ethanol (90% final concentration) was added, the preparation held at −20°C overnight, and the resulting precipitate removed by centrifugation at 10,000*g* for 20 min. The supernatant was concentrated *in vacuo* to 1 ml and then passed through a column of LH-20 (2 × 90 cm) using 70% aqueous methanol as the eluant (0.5 ml/min flow rate). Fractions (7 ml) were collected and tested for conductivity and amino acid content. Ninhydrin-positive fractions with less than 10 μMHo conductance were pooled and evaporated to dryness *in vacuo*. Final purification was done by preparative HPLC using a column of Ultrasphere 5 μ ODS (10 mm × 25 cm) with degassed Milli Q water (pH adjusted to a 3.25 with acetic acid) as the solvent (2 ml/min flow rate). The effluent was monitored at 225 nm. The tabtoxin or tabtoxinine-β-lactam peak (0.5 mg/run) was collected and evaporated to dryness *in vacuo* and stored at −20°C. Their authenticity was confirmed by biological activity (chlorosis induction on tobacco leaves), amino acid analysis of tabtoxin, tabtoxinine-β-lactam, and their corresponding δ-lactam forms, isotabtoxin and tabtoxinine-δ-lactam, using a sodium W2 ion-exchange resin (Beckman) at 60°C with 0.2 M sodium citrate buffer (pH 3.28) (16), and ^1H and ^{13}C NMR spectroscopy.

^1H (300 MHz) and ^{13}C (75 MHz) pulsed, Fourier transform NMR spectra were collected on tabtoxinine-β-lactam using a Bruker WM-300 NMR spectrometer. The proton-decoupled ^{13}C NMR spectrum was collected on a 2 ml sample (2.5 mg ml^{-1}, 10 mm tube) using a 45° pulse and a 2.5-s recycle time. Data from 18,000 scans were averaged, and then were multiplied by an exponential to increase signal to noise (1 Hz line broadening). The resulting spectrum had seven resonances (176.95, 176.34, 86.78, 57.41, 53.73, 32.1, and 27.7 ppm from sodium 3-[trimethyl-silyl] propinate). The ^1H NMR spectrum was collected on a 10 mg ml^{-1} sample in D_2O using the following acquisition parameters; 0.5 ml, 5 mm tube, 45° pulse, 2.5-s recycle time, and 1,000 scans. The spectrum contained the following resonances, 4.14, 2.8, 2.75, 2.69, 2.65, 2.35, 2.17, 2.04, and 1.97 ppm, from trimethylsilane. These NMR spectra will be reported in detail and with assignments later, but they are completely consistent with the proposed structure of tabtoxinine-β-lactam (15, 17).

RESULTS

GS Purification. The enzyme was purified to a specific activity of 58 units/mg protein. This purification procedure typically yields 30% recovery instead of the 11 to 13% yield reported by Elliott (6), and results in a greater than 2,000-fold increase in specific activity of the enzyme (Table I). The addition of RNA to the crude enzyme, as used by Elliott (6), did not improve the yield or purification of our preparation during the pH 4 precipitation step. The 20 to 50% ethanol fractionation step used by Varner and Webster (20) significantly increased the specific activity of the enzyme preparation from DEAE-Cellulose ion exchange chromatography; however, yields of only 20% from this procedure precluded its use. A similar ethanol precipitation treatment at the purified GS stage did not substantially increase specific activity, and it also reduced the total activity. Affinity chromatography of partially purified GS using ADP-agarose, Reactive Blue 2-agarose, and Affi-Gel Blue ligands failed due to either failure to bind or elute GS in acceptable yields. The pH, ionic strength, and concentration of the competing nucleotide were independently varied over a wide range of conditions. Chromatofocusing was also abandoned due to enzyme yields of 10%.

Tabtoxin and Tabtoxinine-β-lactam Preparation. The culture supernatant from pv. *tabaci* grown for 3 d in Woolley's medium contained tabtoxinine-β-lactam, tabtoxin, and several unidentified, ninhydrin-positive substances (Fig. 1A). In contrast, the

Table 1. *Purification of GS from Pea Seed*

	Total Protein	Total Units	Specific Activity[a]	Yield
	mg			%
1. Crude extract	49,600	1,344	0.03	100
2. Acid precipitation	568	1,021	1.80	76
3. Protamine sulfate	100	1,008	10.10	75
4. DEAE-Cellulose	30	712	23.70	53
5. Sepharose 6B	12	564	47.00	42
6. Sepharose 6B	7.2	417	58.06	31

[a] μmol γ-glutamyl hydroxamate formed mg^{-1} protein min^{-1} at 24°C.

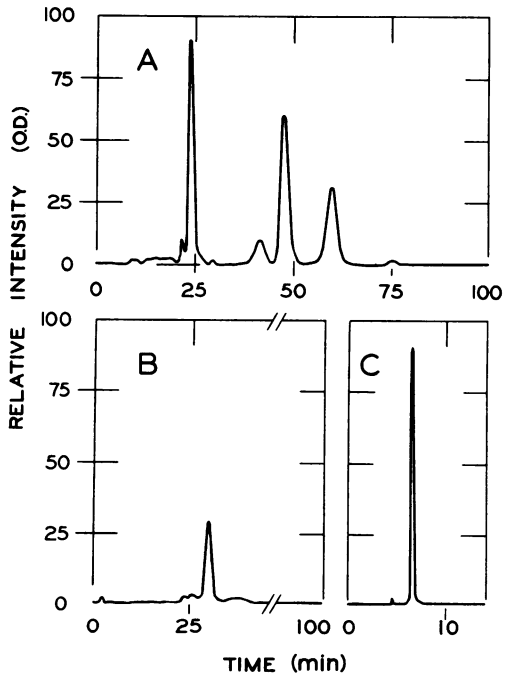

FIG. 1. Elution profiles of: A, Supernatant from Woolley's medium after 3 d incubation. The tabtoxinine-β-lactam peak elutes at 29 min and tabtoxin at 47 min. B, Supernatant from Woolley's medium containing 0.5 g sucrose/l and yeast extract after 24 h incubation. Note that the peak at 24 min (threonine) in A is insignificant in B. C, HPLC of purified tabtoxinine-β-lactam (6.5 min); tabtoxin elutes at 10.5 min in this system. See text for details.

[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

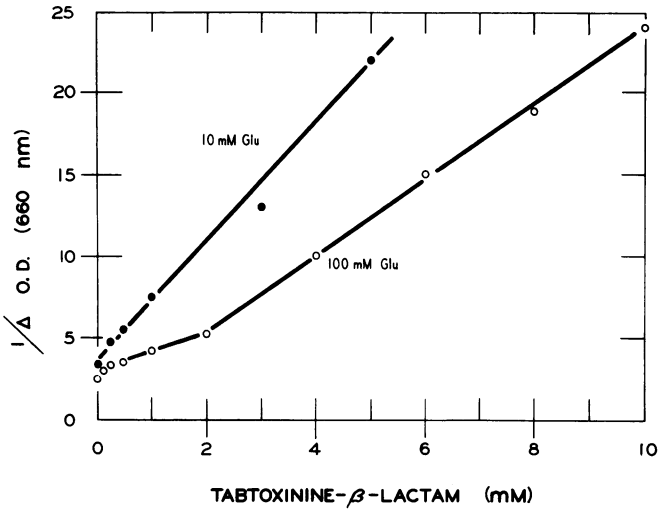

FIG. 2. Response of pea GS to various concentrations of tabtoxinine-β-lactam.

supernatant from Woolley's medium with yeast extract and 0.5 g/l sucrose contained exclusively tabtoxinine-β-lactam after the 24-h incubation period (Fig. 1B). The final yields of tabtoxin and tabtoxinine-β-lactam ranged between 200 and 250 μmol (58–72 mg) and 150 and 200 μmol (28–37 mg) l^{-1} medium, respectively. Purified tabtoxin (not shown) and tabtoxinine-β-lactam (Fig. 1C) exhibited single symmetrical peaks when monitored at 225 nm during HPLC and, based on amino acid analysis and ^{1}H and ^{13}C NMR spectroscopy, were of very high purity; the tabtoxinine-β-lactam preparation contained <5% carbon and <1% nonexchangeable proton contamination.

Inhibition of Pea GS by Tabtoxinine-β-lactam. Using the phosphate liberation assay initiated with 10 or 100 mM glutamate, the initial velocity of GS was determined at various concentrations of tabtoxinine-β-lactam (Fig. 2). Within the range of 0.5 to 5 mM, tabtoxinine-β-lactam at 10 mM glutamate inhibited the reaction in an apparently linear fashion when analyzed in the presence of 10 mM glutamate. The inhibition is curvilinear in the presence of 100 mM glutamate. The initial enzyme velocity was reduced to 45% of the control activity when 2 mM tabtoxinine-β-lactam was added to the reaction.

Incubation of GS with 2 mM tabtoxinine-β-lactam and all assay components except glutamate (for a period of 150 s) reduced the enzyme activity to 10% of the initial uninhibited level. Incubation for 30 min under these conditions completely eliminated GS activity. In contrast, after incubation with the complete assay mixture containing tabtoxinine-β-lactam and 100 mM glutamate, approximately 30% of the initial GS activity remained after 150 s. Complete loss of GS activity was observed after 60 min under these conditions. GS incubated without tabtoxinine-β-lactam under the conditions of the two experiments described above retained 100% of the initial activity. No GS activity was recovered after dialysis of these samples against 500 volumes of assay mixture for 24 h, nor was any activity restored after precipitation with 50% ethanol followed by resuspension in assay mixture.

GS activity was inhibited by tabtoxinine-β-lactam but not by tabtoxin. Aliquots of GS were incubated for 60 min in the assay mixture containing 0.5 mM of either presumed inhibitor but minus glutamate. The GS activity in the samples containing tabtoxin retained 100% of the initial enzyme activity (10 munits); however, the sample containing tabtoxinine-β-lactam retained only 35% (3.5 munits) of the initial enzyme activity.

DISCUSSION

The procedure reported here for purification of GS produces a highly purified preparation in good yield and is simpler and faster than that of Elliott (6). We used common, dried split peas because seed peas are now almost always treated with fungicides that can inhibit GS. The production and purification procedures for tabtoxin and tabtoxinine-β-lactam result in products of greater than 95% purity and in good yield. The proof of purity for tabtoxin has improved over previous preparations and the yield is increased 5- to 6-fold (15). Procedures for the production and purification of tabtoxinine-β-lactam have not been reported before.

We conclude that the inactivation by tabtoxinine-β-lactam is time dependent and that the rate of inactivation is slowed by the addition of glutamate. Glutamate and tabtoxinine-β-lactam may compete for the same site on the enzyme. The failure to recover any activity after extensive dialysis (or ethanol precipitation) of the inactivated enzyme strongly supports the hypothesis that the enzyme is irreversibly inactivated. The time dependence of the activation is also consistent with irreversible inactivation. These basic tests indicate that a steady-state does not exist between free and bound inhibitor.

The inhibition of GS by tabtoxinine-β-lactam appears to be similar to that caused by MSO (9). Glutamine synthetase is inactivated by both compounds and, in both cases, enzyme activity is not restored by dialysis or ethanol precipitation of the inactivated complexes. Recently, Maurizi and Ginsburg (8) reported that both adenylated and unadenylated forms of GS from *Escherichia coli* can be reactivated by a 25°C treatment at pH 4.0 and high ionic strength. Reactivation is accompanied by the stoichiometric release of 0.9 to 1 eq of MSO phosphate and ADP/subunit of the enzyme from the inactive complex. Such reactivation experiments are in progress using pea GS inactivated by tabtoxinine-β-lactam.

This is the first detailed report on the inhibition of GS by tabtoxinine-β-lactam. Previous work had shown that GS activity preferentially decreases both in tabtoxin-treated and infected plant tissues (7, 18). Further, a preliminary study stated that tabtoxinine-β-lactam but not tabtoxin inhibit GS, but no data were presented (19). These reports, along with the results presented here that purified tabtoxin does not inhibit GS whereas tabtoxinine-β-lactam irreversibly inactivates it, strongly support the concept that *in planta* tabtoxinine-β-lactam is the moiety that inhibits GS. This compound could arise by either hydrolysis of tabtoxin (19) or as a biosynthetic end product (4) or both. These findings support the hypothesis that GS is critically involved in the toxin's mechanism of action.

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