New Assay Method for Protoporphyrinogen Oxidase Inhibitors Using Chloroplasts Isolated from Spinacia oleracea L.

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A new assay method for protoporphyrinogen oxidase inhibitors, using stromal extracts of chloroplasts and a fluorescence micro-plate reader, was developed. The I_{50} value of S-23142 which had been confirmed to a specific protoporphyrinogen oxidase inhibitor was 10^{-10} M (100 pm) in this assay. These results indicate that this new assay system is very useful and sensitive for screening protoporphyrinogen oxidase inhibitors.

Haem and chlorophyll have in common a cyclic tetrapyrole structure called a porphyrin. The last common step in haem and chlorophyll biosynthesis is oxidation of protoporphyrinogen IX (Protoph) to protoporphyrin IX (Protop IX), this oxidation being catalyzed by an enzyme called protoporphyrinogen oxidase (Protox EC 1.3.3.4). In plants, Protox activity has been observed in both plastids and mitochondria, but its character is still not fully understood. On the other hand, Protox is the target enzyme for photo-bleaching herbicides such as S-23142 (N-[4-chloro-2-fluoro-5-propargyloxy]-phenyl-3,4,5,6-tetrahydrophthalimide) and the diphenyl ether type herbicide such as acifluorfen (AF). The mode of action of these herbicides has been deduced to involve the accumulation of a strong photosensitive photosensitizer, protoporphyrin IX (Protop IX). However, the reason for Protop IX accumulation and the manner of Protox inhibition are ambiguous in detail.

An analysis of the structure–activity relationship for this class of herbicide might give useful information for assessing the interaction between an inhibitor and the target site, although there are very few bioassay methods for this purpose. In this paper, we describe a novel assay method for Protox inhibitors, using the stromal fraction of chloroplasts isolated from Spinacia oleracea L and combined with fluorescence measurement by the micro-plate reader.

Spinach chloroplasts were isolated by the method of Mills et al. with slight modifications. Spinach leaves (50 g) were cut into small pieces, which were directly immersed in 150 ml of an ice-cold extraction medium (330 mM sorbitol, 50 mM Tricin [N-Tris (hydroxymethyl)methylglycine]-KOH at pH 7.9, 2 mM EDTA, 1 mM MgCl_{2}, and 0.1% BSA) and homogenized for 5 s with a Polytron (PT 10/35; Kinematica, Basel, Switzerland). After filtration through four layers of gauze, the chloroplasts were pelleted by centrifugation at 2500 × g for 1 min. A stromal extract was then prepared by the method of Furbank and Lilley, the isolated chloroplasts being osmotically shocked by being resuspended in a 1/13 dilution of the extraction medium and centrifuged for 15 min at 9000 × g. The resulting pellet of envelope-free chloroplasts was retained for the Chi estimation. The supernatant (stromal extracts) was subjected to ultra-filtration, using a Centricon 10 (Amicon Grace Co., MA, U.S.A.). The concentrated stromal extracts (5 mg of protein/ml) were immediately sub-divided into 2 ml aliquots and placed in small serum tubes for storage in liquid N_{2}. The activity loss under these conditions was less than 1% per day for all the measured activities, although bioassays on a rethawed sample of the stromal extracts need to be completed within 3 h. The protein content in each extract was determined by the method of Bradford, using BSA as a standard.

Protogen was prepared from Proto IX (Porphyrin Products, Logan, UT U.S.A.) by reducing with sodium amalgam as previously described. The reduced porphyrin solution was adjusted to pH 8.2 by 40% H_{3}PO_{4} and then filtered from a glass filter and a membrane filter (0.2 micron cellulose acetate filter; Corning Glass Works, N.Y., U.S.A.). A colorless solution was stored in liquid N_{2} and proved to be stable for at least 3 months.

The stromal extracts (0.5–1 mg of protein) were suspended in 250 μl of a reaction medium containing 100 mM Tris–HCl at pH 7.5, 1 mM EDTA, and 5 mM DTT in a 96-well microplate (Corning, N.Y., U.S.A.) and the mixture was preincubated for 2 min at 25°C. The reaction was

![Fig. 1. Time–Course of Proto IX Production by Stromal Fractions.](image)

Proto IX synthesis was assayed as described in the Materials and Methods section. The circles in this figure indicates a heated (O) and unheated (●) enzyme (0.5 mg/tube), respectively. The heat-inactive enzyme was obtained by heat treatment of stromal extracts at 85°C for 30 min. The values represent the average of three independent measurements and the range of the experimental error was within ±2%.

Abbreviations: Protox, protoporphyrinogen oxidase; Protogen, protoporphyrinogen IX; Proto IX, protoporphyrin IX.

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Fig. 2. Effect of Enzyme Concentration on the Rate of Enzymatic oxidation in This Assay System.
Proto IX synthesis was assayed as described in the Materials and Methods section. The values represent the average of three independent measurements, the range of experimental error being within ±5%.

Fig. 3. Dose–Response Curve for Protox Activity in the Stromal Fraction Incubated with S-23142 and AF in Darkness for 30 min.
Protox activity is expressed as a percentage of the control value. The control activity of Protox was 37–55 nmol Proto IX·mg protein⁻¹·h⁻¹. The circles in this figure indicate S-23142 (●) and AF (○), respectively. The values represent the average of ten independent measurement, the range of experimental error being within ±2%.

started by adding 2.5 μmol Protogen, and the medium was incubated for 30 min in darkness. The Proto IX production was fluorometrically monitored with the micro plate reader (CytoFluor 2300, Millipore Co., MA, U.S.A.) at λₜₐₚ = 410 nm and λₙₐₕ = 640 nm.

The time-course for Proto IX biosynthesis by the stromal fractions is shown in Fig. 1. Proto IX was rapidly synthesized from Protogen, and this production of Proto IX was increased with the passage of time. Very little Proto IX formation from Protogen was observed when the assay was used with heated stromal fractions in this bioassay conditions (Fig. 1). This pattern of Proto IX formation from the heated stromal fraction is quite similar to those observed with same assay system without a stromal fraction (data not shown). Figure 2 shows the Proto IX production at various concentrations of the enzyme and of Protogen, and indicates that the Proto IX production was proportional to the enzyme concentration on 0.2–1 mg protein/250 μl. The specific activity of Protox in the stromal fractions was estimated to be 37–55 nmol Proto IX·mg protein⁻¹·h⁻¹. By the above results, we confirmed that Proto IX was enzymatically synthesized from Protogen.

We also confirmed the usefulness of this bioassay method as a screening system for Protox inhibitors by using S-23142 and AF. Figure 3 shows the dose-dependent inhibition for Proto IX synthesis by S-23142 and AF. The Proto IX synthesis was completely inhibited by 10⁻⁹ (1 nM) S-23142 and 10⁻⁷ (100 nM) AF, respectively. The S-23142 and AF concentrations for 50% inhibition (I₅₀) of Proto IX were 10⁻¹⁰ M (100 pm) and 5 × 10⁻⁷ M (5 nM), this value being similar to that given the previous report.⁹ The I₅₀ values for S-23142 and AF in this assay system were 50 to 100 times lower than the I₅₀ values in chloroplasts and etio-plasts that were the previously reported.¹⁰ These results suggest that this assay system would be very useful and sensitive for screening a Protox inhibitor.

It has been reported that Proto IX is synthesized from the Protogen in chloroplast envelopes and thylakoid membranes isolated from spinach.¹⁰ In fact, we confirmed the high activity of Proto IX biosynthesis in chloroplast membrane fractions. The activity in chloroplast membranes is 30 times higher than that in the stromal fractions, but the Protox assay system, using the membrane fractions is not suited for screening purposes because Proto IX production can not be detected by a plate reader without a separation procedure by HPLC and/or centrifugation due to strong fluorescence of endogenous porphyrin such as chlorophylls.

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