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DIETHYLENE GLYCOL DISULFIDE FROM CASTOR BEAN CELL SUSPENSION CULTURES

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Abstract—Diethylene glycol disulfide was isolated from castor bean cell suspension cultures. Incubation of suspension cultures with $Na_2{}^{35}SO_4$ resulted in the incorporation of radioactivity into the isolated diethylene glycol disulfide. Diethylene glycol disulfide was detected in cells (430 nmol/g cells) and in cell-free growth medium (41.5 nmol/ml).

INTRODUCTION

Thioethylene glycol (β-merceptoethanol) (ESH) is a reagent used commonly for reducing disulfides to the thiol form. For this reason it has been widely used in the isolation and purification procedures of enzymes which are susceptible to oxidation and are active only in the reduced form. Like other thiols, ESH can be oxidized to its disulfide form, diethylene glycol disulfide [di-(2-hydroxyethyl)disulfide] (ESSE). Neither ESH nor ESSE has ever been shown to be formed in biological systems. Instead, the tripeptide glutathione is considered to be the most important non-protein thiol in living systems. Changes in the glutathione thiol–disulfide status [1] are considered to be of potential importance in regulating biological activities. It was shown that an increase of the GSH content occurs just prior to nuclear division in *Chlorella* [2]. A few reports indicate that non-protein SH (presumably GSH) content increases during cell growth cycle in the passage from the G_1 to the S stage [3, 4]. In addition, the non-protein and disulfide content of seeds and spores changes during their formation and germination. An increase in GSH concentration was reported in pea seeds during germination together with an increase in the protein thiol/disulfide ratio [5]. Fahey *et al.* showed that GSSG and GSS-protein content increase in aged *Neurospora* spores and decreased immediately after germination. Similar behaviour was observed in wheat and *Artemia* [6, 7]. They also recently reported that large amounts of GSSG inhibited protein synthesis in extracts of wheat embryo [8]. The role of glutathione in biological systems has been discussed thoroughly in a recent review by the Kosowers [9].

Our interest in the thiol–disulfide status in plant systems arose from an observation in our laboratory that kaurene synthetase, a known enzyme in the pathway leading to the formation of gibberellins [10], was inhibited by a low MW, heat-stable entity that we found in castor bean cell suspension culture (CB-I) [11]. This compound was found to be a disulfide. The objective of this work was to isolate and identify this entity.

RESULTS

Incubation of CB-I culture with $Na_2{}^{35}SO_4$

Extraction with ethyl acetate of CB-I cell suspension culture that was previously incubated for 3 days with 1 mCi of $Na_2{}^{35}SO_4$ resulted in large amounts of radiolabelled material in the organic extract. TLC of the concentrated extract on silica gel developed with ethyl acetate showed that more than 95% of the ethyl acetate extractable radiolabelled materials remained at the origin of the plate. In addition, scanning of the TLC plate for radioactivity indicated the presence of numerous radiolabelled overlapping materials that migrated on the plate. Therefore, two additional chromatographic separations were employed using the same chromatography conditions. After each run, the narrow zone of the gel containing a UV-quenching material at R_f 0.6–0.7 was scraped off and extracted with ethyl acetate. Only a single spot was detected on the TLC plate under UV light following the third chromatographic separation. This UV quenching spot was developed into a greenish-yellow spot by spraying the plate with *p*-anisaldehyde reagent [12]. TLC showed that the ^{35}S radioactivity peak on the TLC plate coincided with the material which was observed under UV light (and developed yellow-green colour with *p*-anisaldehyde). TLC also showed a considerable amount of radiolabelled materials which did not co-migrate with the visible spot.

Quantitative measurement of ESSE in CB-I culture

ESSE was measured in extracts of the cells and in ethyl acetate extracts of cell-free growth medium from a 9-day-old culture. Analysis showed that the cell-free growth medium contained 430 nmol ESSE/g cells and the growth medium contained 41.5 nmol/ml. The ethyl acetate extract of the CB-I cells and the cell-free medium did not contain any detectable amount of thiols. Unlike its ethyl acetate extract, the cell-free medium contained 13.6 nmol/ml of thiols. This indicated that the organic extraction discriminates efficiently between the disulfide ESSE and the thiols present in the cell-free growth medium. We could not find any evidence to suggest that disulfides other than ESSE were extracted by ethyl acetate. Several attempts to obtain ESSE by ethyl acetate extraction of soluble enzyme preparations from CB-I cells invariably resulted in a lower recovery of ESSE compared with direct extraction of the cells by ethyl acetate. This indicates that at least some of the ESSE is associated with

cell debris or large particles. Again, free thiols could be measured in the cell protein extract but none were present in the ethyl acetate extract of the cellular protein. We calculated the amount of free thiols to be *ca* 0.07 pmol/g wet wt of CB-I cells. Drying the cells for 2 hr at 80° showed that CB-I cells dry wt is 8.125% of the wet wt of the cells. Since the MW of ESSE is 154 we can calculate that the amount of ESSE in CB-I cells is 0.082% of the dry wt of the cells.

DISCUSSION

The results presented here clearly demonstrate the presence of ESSE in CB-I cell suspension cultures. The evidence is based on the comparison of the properties of the isolated material to those of an authentic standard of ESSE. Briefly, identical UV absorption spectra, identical ^{1}H NMR spectra, similar mass spectra and co-migration on TLC and GC. In addition, element analysis and high-resolution MS indicate that the proportion between the elements that are present in the isolated material agrees precisely with the relative amounts of the elements that are expected to be present in ESSE. Based on that we concluded that the isolated material is indeed ESSE. To the best of our knowledge, this compound, despite its wide use in biological investigations, was never described as a product of a biological system.

In recent years increased attention has been focused on the possible role of oxidation–reduction reactions in the control of biological processes. Particular attention was given to the role of the non-protein thiol glutathione and its disulfide form in these processes. The relationship between the various forms of glutathione: the reduced, oxidized and the mixed disulfide form with proteins has been defined as the GSH status of cells [1]. Since the GSH status of cells was reported to vary during physiological processes (for comprehensive review of this subject see ref. [9] and literature therein) it is of importance to determine the relationship between the GSH status and the ESH status in the biological systems that may produce both GSH and ESH. It is possible that a different entity may affect different enzymes that are modulated by the oxidation–reduction process. It is also possible that one of these two thiols may serve as a pool of reducing power for the other. Therefore, a positive effect by one of these thiols or disulfides may not necessarily indicate a direct interaction with the modulated enzyme but may be the result of an indirect effect of a secondary thiol or disulfide.

EXPERIMENTAL

Growth of suspension cultures. Castor bean cell cultures (CB-I) were maintained on solid and grown in liquid Murashige–Skoog media [13]. Liquid cultures were grown in 250-ml flasks containing 100 ml of medium. The flasks were agitated in a rotary shaker at 24°. Transfer of 10% inoculum to a fresh medium was done every 8 days.

Preparation of cell-free protein extract. 7-Day-old cells were harvested by filtration and washed thoroughly on a filter with 0.1 M Tris–HCl buffer, pH 7.4. The cells were transferred to a pre-cooled mortar and ground in liquid N_2 to a fine powder. PVP was added at a ratio of 0.25 g/g of cells. After mixing, the paste was allowed to warm to 4° and 3 ml of Tris–HCl buffer were added to every g of paste. The mixture was squeezed through four layers of cheese-cloth and the effluent was centrifuged for 30 min at 35 000 *g*. The supernatant was collected and the protein concn adjusted to 10 mg/ml by dilution with Tris–HCl buffer.

Extraction of CB-I cells. Cells were harvested by filtration and washed thoroughly on the filter with Tris–HCl buffer as above. EtOAc (1 ml) was added per g of cells and the cells homogenized with a motor-driven tight fitting glass–Teflon Potter–Elvejhem homogenizer. The homogenized mixture was allowed to stand for 30 min and the organic phase separated by centrifugation. The mixture was extracted ×4 with the same vol. of EtOAc. The combined EtOAc extract was concd under vacuum in a rotary evaporator.

Extraction of growth media. Growth media containing CB-I cells or cell-free growth media were treated the same way. 20% (v/v) of EtOAc was added and the mixture stirred for 1 hr at room temp. The organic phase was separated and removed by centrifugation. This procedure was repeated ×4. The combined EtOAc extract was concd in a rotary evaporator as before.

Separation and analysis of materials by TLC. Large-scale sepn of EtOAc-extractable materials was done on a 2 m thick, 20 × 20 cm Si gel plates containing fluorescent indicator (F-254). The plates were developed for 15 cm with EtOAc. Materials on the plate were detected under UV light. The part of the gel at R_f 0.6–0.7 containing the fluorescence-quenching material was scraped off and extrd with EtOAc. Analysis of materials was done using analytical TLC Si gel plates with fluorescent indicator. Materials on the plates were detected by exposing the plates to UV light, to I_2 vapor or by spraying with *p*-anisaldehyde reagent [12]. Radiolabelled materials were detected by scanning for radioactivity. For quantitative analysis, zones of the gel were scraped off and counted for radioactivity.

Synthesis and isolation of ESSE. A soln of 2.5 M of ESH, pH 12, was prepared. To 10 ml of this soln, 20 mg of $CuSO_4$ were added and air was bubbled through for 3 hr. The soln was then extracted ×5 with EtOAc. ESSE was purified by prep. TLC as previously described.

Spectral characteristics. UV (H_2O) λ_{max} 247 nm, λ_{min} 230 nm. ^{1}H NMR ppm ($CDCl_3$): δ 2.79 (2 H, *t*, J = 5.9 Hz, $-CH_2-S$); δ 3.81 (2 H, *t*, J = 5.9 Hz, CH_2 O); δ 3.2 (H, *s*, OH). (D_2O): δ 2.8 (2 H, *t*, J = 5.9 Hz, $-CH_2-S$); δ 3.8 (2 H, *t*, J = 5.9 Hz, $-CH_2-O$); δ 4.7 (H, *s*, H_2O). The signal obtained in $CDCl_3$ at δ 3.2 was not observed in D_2O. Low-resolution MS *m/z* 156 (M^+ 11%), 154 (M^+ 100), 136 (34), 110 (60), 92 (75), 79 (35), 64 (61), 60 (59). High-resolution MS M^+ *m/z* 156.0058 and M^+ *m/z* 154.0133 calcd for $C_4H_{10}O_2S_2$. The higher MW represents the natural abundance of ^{34}S.

Identical spectral characteristics were obtained for both the isolated ESSE and the synthetic ESSE.

Element analysis showed (%) C = 31.03, H = 6.85, S = 40.81, and O = 21.31. The above quantitative analysis predicts a minimum formula of C_2H_5OS.

Materials. $Na_2{}^{35}SO_4$ carrier free was purchased from the Nuclear Research Center, Negev.

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