BIOLOGICAL MARKERS OF RADIATION INJURY: A CHROMOGENIC TEST FOR IRRADIATED ORIENTAL FRUIT FLY, *BACTROCERA PHILIPPINENSIS*, AS QUARANTINE DIAGNOSTIC KIT

ABSTRACT

A biochemical marker for gamma-irradiated Oriental fruit fly, *Bactrocera philippinensis* (earlier designation: *Dacus dorsalis*) was isolated and identified in denaturing polyacrylamide gel electrophoresis (SDS-PAGE). The biomarker, Gs-protein, is the biosynthetic product of a radiosensitive gene locus of *B. philippinensis*. The gene appears to be sensitive to gamma radiation even at a low dose of 25 Gy as demonstrated by the loss of the Gs-protein band from SDS-PAGE gel analysis of pupal homogenates and total soluble protein fractions of irradiated larvae. Isolated Gs-protein has a tyrosinase enzyme activity capable of converting tyrosine into an intermediate product, DOPA. An apparent molecular weight of 109 kDa was calculated from SDS-PAGE preparation of the Gs-protein using high molecular weight proteins as standards.

In practical terms, a convenient chromogenic test for tyrosinase present in homogenates of *B. philippinensis* at different stages of development starting from 3-day old larvae, has been devised to detect whether the fruit fly had been irradiated at a dose of at least 100 Gy. Using homogenate material from fruit fly to be tested in the field, such a simple test, based on the activity of the tyrosinase enzyme with substrate, produces a positive dark brown to black coloration on a test spot mounted on acetate film containing 2-methyl DOPA.

INTRODUCTION

The presence of a suitable biological marker for irreversible radiation injury in an insect pest was identified in pupae of the Oriental fruit fly, *Bactrocera philippinensis*. A radiation sensitive protein marker was identified in SDS-PAGE gel profile of *B. philippinensis* pupae (1). Cobalt-60 irradiation of mature larvae at even as low a dose as 25 Gy was enough to lose the protein marker of pupae as shown in the SDS-PAGE profile. From further analysis, we conclude that the radiation sensitive protein band is a tyrosinase enzyme which we initially termed Gs-protein (4). In another study (2), it has been shown that gamma radiation causes the loss of phenoloxidase (tyrosinase) activity in larvae of Carribbean fruit fly. This supports our findings on the radiosensitivity of Gs-protein isolated from larvae of *B. philippinensis*.

Irradiation of fresh fruits for importation is one of the methods to disinfest the fresh produce. Imported fresh fruits are usually screened for the presence of insect pest(s) and quarantine laws in fruit-importing countries, are very specific and stringent against

entry of these organisms into the importing country. Efforts have been made to find an assay that will identify fruit fly as having been sufficiently irradiated.

In this paper,, we present a diagnostic tool adapted specifically for *B. philippinensis* from the method used by Nation et al.(3) to identify irreversibly irradiated fruit fly. The protocol uses methyl-DOPA to produce a dark brown to black coloration with sample test materials from fruit fly.

METHODS

IRRADIATION:

For DOPA experiment, eggs of *Bactrocera philippinensis* were irradiated at 100 Gy at the PNRI Multipurpose Cobalt-60 Irradiation Facility (dose rate = 301.5075 Gy/h). After irradiation, eggs were allowed to continue their life cycle up to the time that the control eggs treated likewise but without irradiation developed into fully grown insect at room temperature.

For SDS-PAGE isolation purposes, mature larvae of *B. philippinensis* were irradiated at 25 to 100 Gy and allowed to developed through its different life cycle stages at room temperature.

SDS-PAGE ELECTROPHORESIS:

Pupae were homogenized in sodium phosphate buffer (pH 7.6) and centrifuged at 1500 x g to separate the soluble fraction. In an SDS (3.3%)-acrylamide gel (8.5%) and continuous buffer system of 0.1 M Tris-10% glycine (Sigma), Gs protein was isolated from the total soluble proteins. Electrophoresis was at 25 mA for 1.5 h. Gels were stained with Coomassie Brilliant Blue (Sigma) and destained in methanol (10%)-acetic acid (7%) mixture (4)

MOLECULAR WEIGHT DETERMINATION

High molecular weight markers (Sigma) (α-macroglobulins: 230 kDa, β-galactosidase: 113 kDa, bovine serum albumin: 66 kDa, egg albumin: 45 kDa) served as standards in determining molecular weight of Gs-protein from SDS-PAGE gel (4).

PROTEIN CONCENTRATION: BRADFORD METHOD

Protein concentration of Gs-protein was measured following the Bradford method (6). Gs-protein was isolated from SDS-PAGE gel and eluted with water and mixed with Coomassie Blue dye in accordance to the method of Bradford. Bovine serum albumin (Sigma) served as standard protein.

Tyrosinase Enzymatic Assay

Gs-protein was extracted from unstained SDS-PAGE gel, centrifuged and lyophilized prior to enzyme assay.

Tyrosinase enzyme assay was performed, firstly, by oxygenating tyrosine (Sigma) in phosphate buffer (pH 6.5) for 5 minutes and the subsequent addition of 50 μl of Gs-protein solution to 1.45 ml of oxygenated tyrosine solution. Rate of reaction was recorded at 280 nm for 10 min in a spectrophotometer (JASCO). Purified mushroom tyrosinase (Sigma) was used as activity standard. The protocol for tyrosinase enzyme assay that was followed can be found in the Worthington Manual for Enzyme and Related Biochemicals (5).

Absorption Spectra

Optical absorption, in the visible spectrum, of pupal total homogenate and SDS-PAGE isolated-Gs-protein were recorded spectrophotometrically. Starting from 400 nm, changes in absorbance in the visible range were recorded until 300 nm. The samples were in phosphate buffer (pH 7.5) and spectrophotometric readings were taken against phosphate buffer serving as blank.

Total Reflection X-Ray Fluorescence (TXRF) Spectroscopy

Isolation and extraction from SDS-PAGE of Gs-protein was done following the protocol mentioned above with the exception that all reagents were prepared with triple distilled copper-free water. Gs-protein was analyzed semi-quantitatively for the presence of bound copper using TXRF spectrometry by the staff of Applied Physics Research Section of the Atomic Research Division, PNRI, under Ms. Virginia S. Calix.

Chromogenic Diagnostic Kit for Field Use

A spot test for field use was prepared using 2-methyl DOPA (10 ng/ml) in phosphate buffer (pH 6.5) applied as a spot into an acetate film (3). About 10 μl of 2-methyl-DOPA was applied on the film and allowed to air-dry (for best results, an overnight drying period is recommended). A single fruit fly, at any variable developmental stage, was squashed in water with the pestle part of a Dounce homogenizer and a drop of the resulting material was applied onto the dried 2-methyl-DOPA solution. The spot was allowed to dry and within 30 minutes full development of color from dark brown to black was observed for control insect (unirradiated).

Results and Discussion

A biochemical marker for irreversible radiation injury, designated as Gs-protein, was isolated from pupae of *Bactrocera philippinensis* in SDS-PAGE gel. Electrophoresis

of fruit fly pupae in SDS-PAGE gel showed a protein band which disappeared after Cobalt-60 irradiation of mature larvae (Fig. 1). This may suggest that the gene locus for Gs-protein underwent some mutational event which prevented it from synthesizing Gs-protein or that the Gs-protein could have been drastically changed to lose its band-site in the gel. When eggs were irradiated at doses ranging from 25 to 100 Gy and allowed to continue its development, the Gs-protein from pupae of irradiated larvae disappeared from the gel protein profile in SDS-PAGE (Fig. 2). These facts are indicative that Gs-protein is a radiosensitive biomarker whose gene locus underwent radiation-induced mutational change even at a low dose of 25 Gy.

Molecular weight determination of Gs-protein showed an apparent molecular weight of 109 kDa (Fig. 3). The visible absorption spectra of total homogenate showed a λmax of 365 nm indicating the presence of highly absorbing chromophoric group(s) which could be used in a chromogenic test (Fig. 4). Using the Bradford Protein Method, the presence of 5 μg of Gs-protein per mature pupa was determined.

From our initial TXRF study on the presence of copper in Gs-protein molecule, we have shown copper to be present in SDS-PAGE isolated Gs-protein but at a lower concentration compared with copper found in mushroom tyrosinase (4). Efforts are being undertaken to isolate a purified Gs-protein and analyze its copper content in fruit fly.

Isolated Gs-protein exhibited a tyrosinase activity (Fig. 5) which supports our initial theory that Gs-protein is a copper-containing enzyme having a tyrosinase activity. This was eventually proven to be true when, with 2 methyl-DOPA as substrate, a total homogenate of unirradiated pupae exhibited a dark brown to black color reaction (Fig. 6). Development of color for unirradiated fruit fly starts from as early as 3-day old larva. 100 Gy irradiated fruit fly eggs did not develop beyond 6-day old larva. Initial studies with isolated Gs-protein also showed a dark color reaction with DOPA. This color reaction between fruit fly homogenate and 2 methyl-DOPA is the basis of a chromogenic test that we have developed specifically for *B. philippinensis* based on the initial color test of Nation, et al.(3). This chromogenic test is a handy diagnostic kit for field work in determining the efficacy of radiation dose for *Bactrocera philippinensis* found in fruits.

The loss of tyrosinase activity in irradiated eggs indicates that mutation at the gene locus of Gs-protein due to radiation dose of 100 Gy has resulted in the post-

translational damage of the enzyme which renders it to be non-functional or that the gene locus has been somatically mutated so that Gs-protein is not synthesized at all. Our findings indicate that irradiation of egg even before Gs-protein is synthesized has resulted in the loss of the Gs-protein from SDS-PAGE gel profile (I). This study demonstrates that Gs-protein is a radiosensitive protein of *Bactrocera philippinensis*.

Irradiation of fruit fly eggs at 100 Gy drastically reduced the subsequent survival rate of the fly (1). Exported fruits undergo screening by the quarantine services of importing countries for the possible presence of any residual viable infestation, beyond the egg stage. This chromogenic test would be a handy diagnostic kit for use by quarantine officers to determine whether flies in larval and pupal stages have been sufficiently irradiated or not, which if insufficient, may give rise to subsequent viable residual infestation in the fruit-importing country.

REFERENCES

1. Yulo-Nazarea, M. T. and Manoto, E.C. A biochemical marker for detection of irradiated pupae of the Oriental fruit fly (*Dacus dorsalis*) In: Proceedings of an International Symposium on Management of Insect Pest: Nuclear and Related Molecular and Genetic Techniques. IAEA Press, Vienna. pp.641-648. (1993).
2. Mansour, M. and Franz, G. Effect of gamma radiation on phenoloxidase activity on larvae of the Mediterranean fruit fly (Diptera: Tephritidae). *J. Econ. Entom.* J94-381 (1994).
3. Nation, J. L., Smittle, B. J. and Milne, K. Radiation-induced changes in melanization and phenoloxidase in Carribean fruit fly larvae (Diptera: Tephritidae) as the basis for a simple test of irradiation. *Physiol., Biochem. and Toxicol.* 88 (2): 201-205 (1995).
4. Yulo-Nazarea M.T. and Nato, Jr., A. Q. Detection method for irradiated fruit fly (*B. philippinensis*) for quarantine purposes Technical Paper. INIS#PH9500600, IAEA.
5. Charles C. Worthington, (ed.). Polyphenol oxidase (Tyrosinase 1.14.18.1) *In: Worthington Manual for Enzyme and Related Biochemicals.* Worthington Biochemical Corporation, Freehold, New Jersey. pp. 288-291 (1988).
6. Ausubel, F. et al. (Eds.) Quantitation of Proteins In: Short Protocols in Molecular Biology. John Wiley and Sons, New York. p. 283 (1989).

PROJECT PERSONNEL

Ma. Teresa Nazarea
Alejandro Nato, Jr.

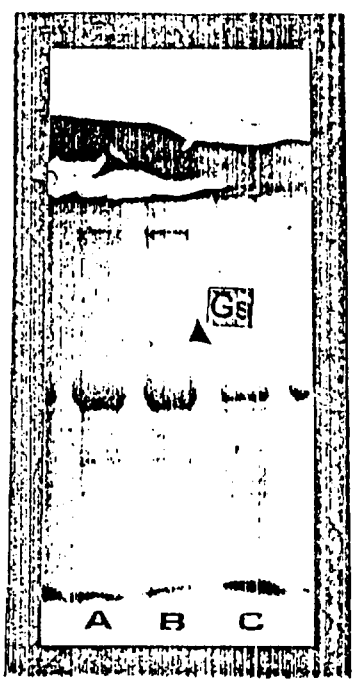

Figure 1. SDS-PAGE protein profile of pupae (4 day old) *B. philippinensis* at 100 Gy (A) and 0 Gy (B) irradiation dose. Arrow indicates Gs-protein.

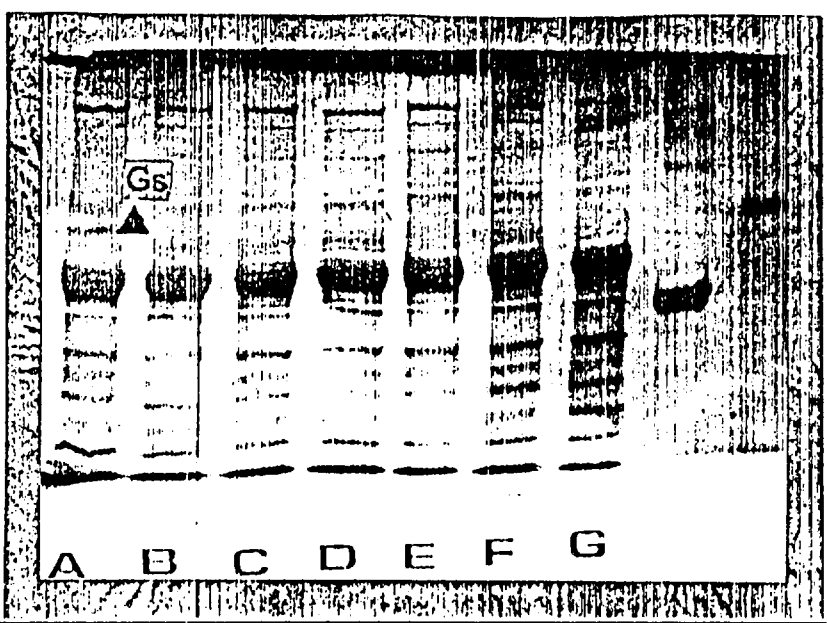

Figure 2. Irradiation of matured larvae of *B. philippinensis* at doses ranging from 25-50 Gy. SDS-PAGE protein profile of matured pupae of irradiated larvae at 0 Gy (A), 25 Gy (B), 30 Gy (C), 35 Gy (D), 40 Gy (E), 45 Gy (F), and 50 Gy (G). Arrow indicates Gs-protein.

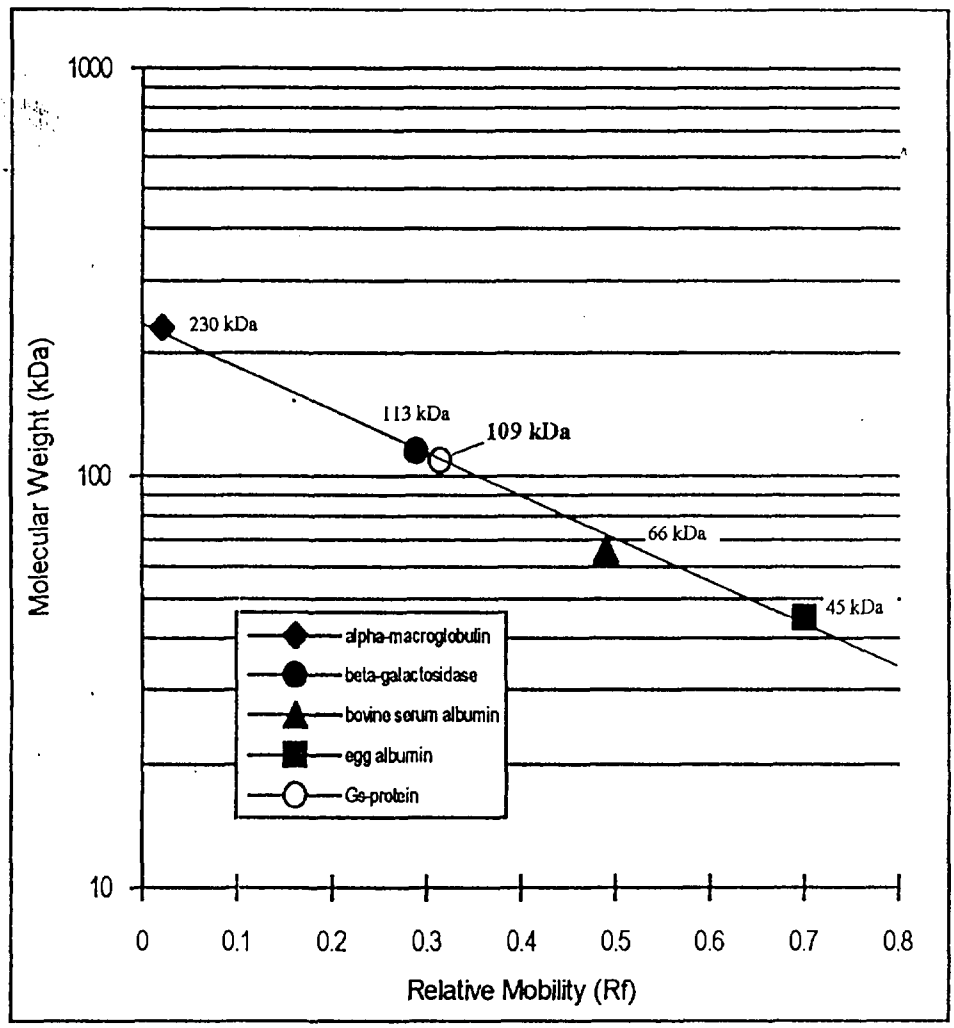

Figure 3. Molecular weight determination of Gs-protein by SDS-PAGE using high molecular weight markers: α-macroglobulin (230 kDa), β-galactosidase (113 kDa), bovine serum albumin (66 kDa), and egg albumin (45 kDa). SDS-PAGE gel was prepared at 7.5% concentration with tris-glycine (pH 8.3) buffer. Bands were stained with Coomassie Brilliant Blue dye.

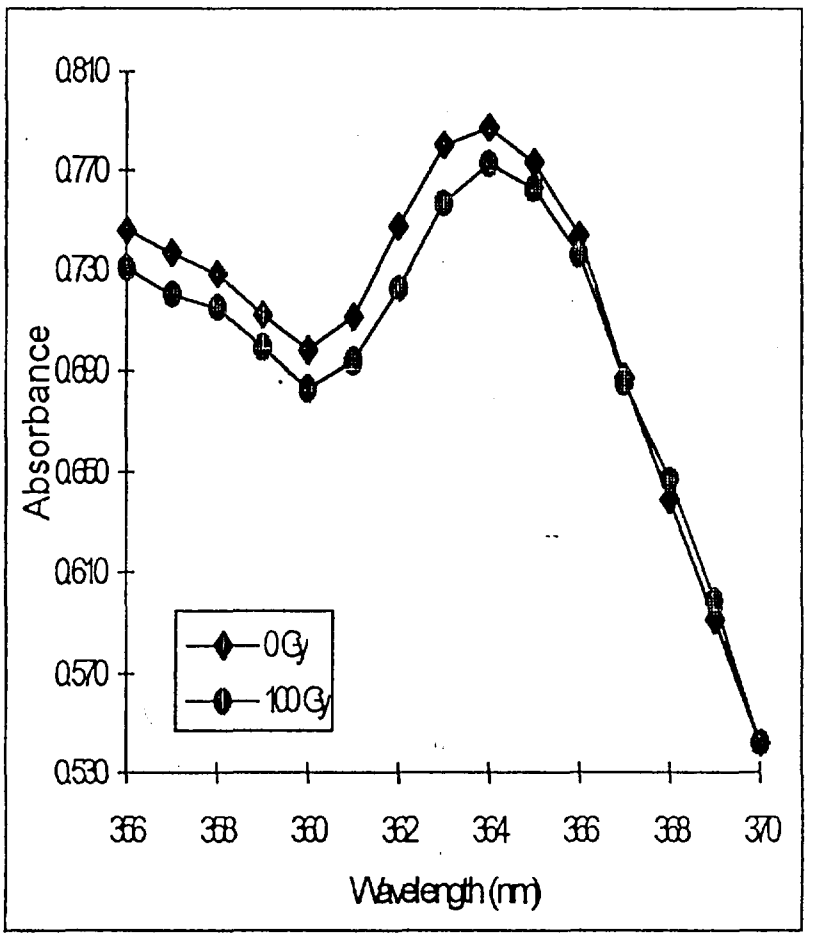

Figure 4. Absorption spectra of homogenate soluble fraction ranging from 355-370 nm visible range. Maximum peak for both 0 Gy and 100 Gy samples is observed at 364 nm.

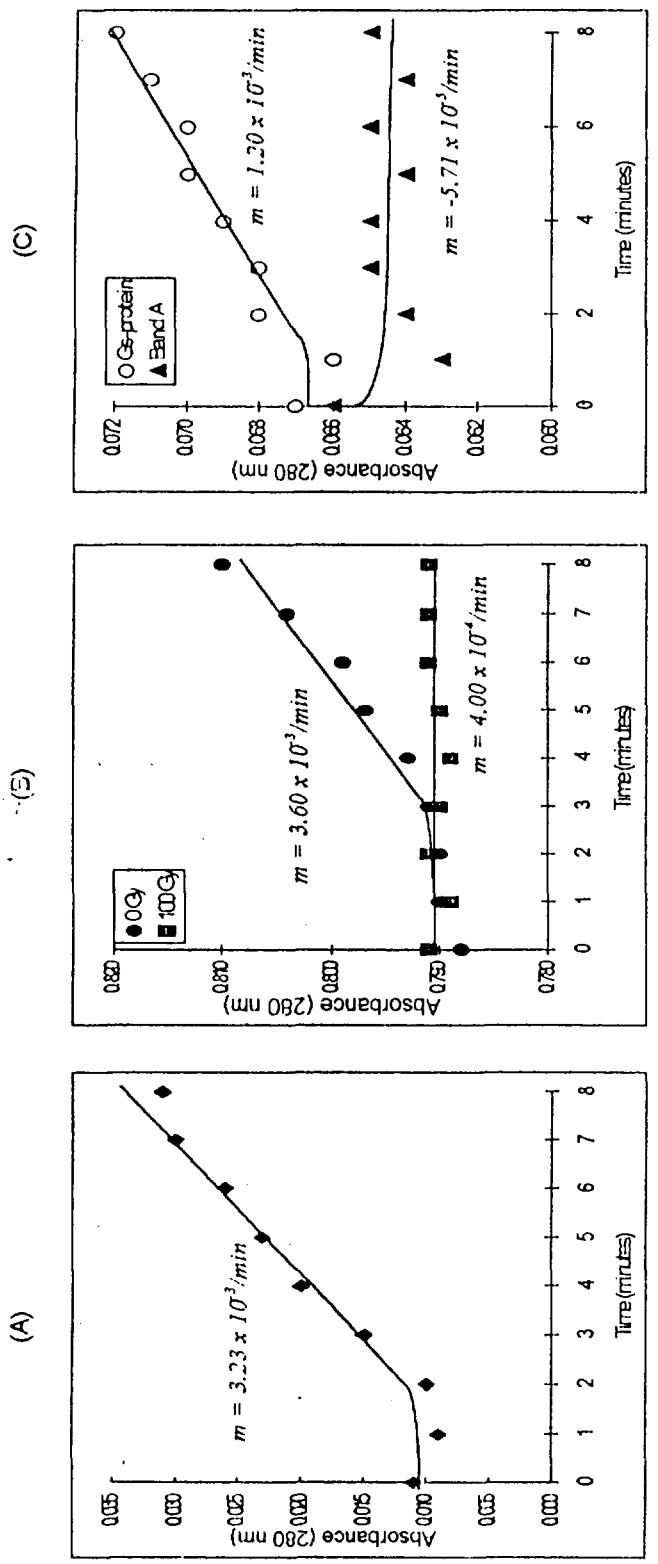

Figure 5. Tyrosinase activity of (A) mushroom tyrosinase (◆), (B) total soluble fractions of *B. philippinensis* pupae from unirradiated [0 Gy (●)] and irradiated [100 Gy (■)] larvae, and (C) Gs-protein (O) and Band A (▲) isolated from SDS-PAGE gel by elution with distilled water

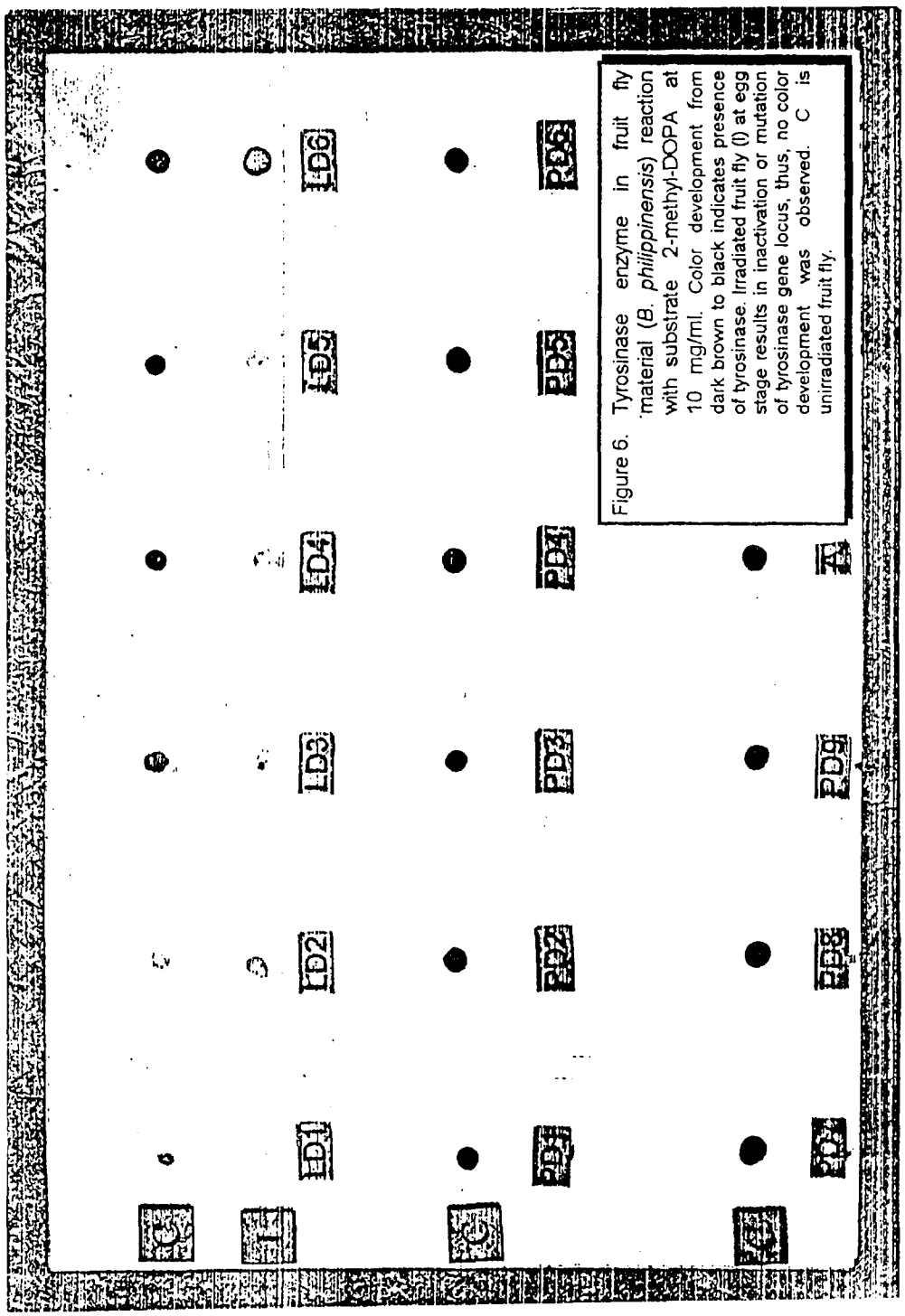

Figure 6. Tyrosinase enzyme in fruit fly material (*B. philippinensis*) reaction with substrate 2-methyl-DOPA at 10 mg/ml. Color development from dark brown to black indicates presence of tyrosinase. Irradiated fruit fly (I) at egg stage results in inactivation or mutation of tyrosinase gene locus, thus, no color development was observed. C is unirradiated fruit fly.