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LOCALIZATION OF BENZYL GLUCOSINOLATE AND THIOGLUCOSIDASE IN *CARICA PAPAYA* FRUIT*

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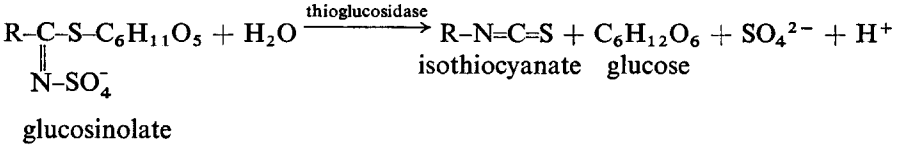
Key Word Index—*Carica papaya*; Caricaceae; papaya; benzyl glucosinolate; thioglucosidase; localization within the plant.

Abstract—Macerated papaya seeds and pulp contained benzyl isothiocyanate, produced by the enzymatic hydrolysis of benzyl glucosinolate by thioglucosidase. The substrate and enzyme were localized in different areas. In mature papaya seeds, thioglucosidase was found in sarcotestae but not in endosperms, while the reverse was true for benzyl glucosinolate, which constituted more than 6% (w/w) of the endosperms. Both the enzyme and substrate were present in embryos and the amount of the latter was 3·9% (w/w). In immature papaya pulp, benzyl glucosinolate was localized principally, if not exclusively, in the latex, ranging from 7·3 to 11·6% of the dry wt of latex fluid. No thioglucosidase activity was found in papaya latex. The possible significance of the localization of this enzyme-substrate system and aspects concerning functions of papaya latex are discussed.

INTRODUCTION

NATURALLY occurring isothiocyanates are usually distinctive in flavor and detected only after the disintegration of the tissues of certain higher plants.[1,2] The substrate, glucosinolates, are hydrolyzed by the enzyme thioglucosidase (myrosinase), and isothiocyanates are formed according to the following reaction:



Localization of the substrate and the enzyme in Cruciferae, Capparidaceae, Resedaceae, Tropaeolaceae and Limnanthaceae was studied by Guignard as early as the 1890s.[3,4] The thioglucosidase-containing cells (idioblasts) were histochemically distinguished with Millon's reagent or other staining techniques. These early studies concerning the anatomical distribution of the enzyme and substrate have been reviewed by Kjaer.[5]

Benzyl isothiocyanate (BITC) was identified in macerated papaya (*Carica papaya* L.) seeds by Ettlinger and Hodgkins.[6] Benzyl glucosinolate, the parent compound of BITC, appears to be the only thioglucoside of Caricaceae.[7] Localization of benzyl glucosinolate

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[1] ETTLINGER, M. G. and KJAER, A. (1968) *Recent Advances in Phytochemistry* (edited by MABRY, T. J., ALSTON, R. E. and RUNECKLES, V. C.), Vol. I, p. 59, Appleton-Century-Crofts, New York.
[2] TANG, C. S. (1971) *Phytochem.* **10**, 117.
[3] GUIGNARD, L. (1890) *J. Botanique* **4**, 385.
[4] GUIGNARD, L. (1893) *J. Botanique* 7, 345.
[5] KJAER, A. (1960) *Progr. Chem. Org. Nat. Prod.* **18**, 122.
[6] ETTLINGER, M. G. and HODGKINS, J. E. (1956) *J. Org. Chem.* **21**, 204.
[7] GMELIN, R. and KJAER, A. (1970) *Phytochem.* **9**, 591.

and thioglucosidase in papaya seeds[8] was suggested by the observation that the characteristic BITC flavor could be detected by chewing the whole seed but not by testing sarcotesta alone, or by chewing a seed from which the sarcotesta was washed away.[9] In macerated papaya pulp, the concentration of BITC was reported to decrease from 291 to 4 ppm during fruit maturation.[2] Since it is generally observed that only green, unripe papayas, but not the yellow, ripe ones produce latex, it seems possible that latex actually serves as a source of either benzyl glucosinolate or thioglucosidase. In the present report, the above observations are substantiated by quantitative studies of BITC in different parts of payapa seeds and latex from immature papaya fruit. The possible significance of these localizations and certain aspects on the latex function are also discussed.

RESULTS

The quantities of BITC in macerated sarcotesta, endosperm and embryo are summarized in Table 1. BITC was not detected in sarcotesta extract if incubated alone. However, upon the addition of potassium benzyl glucosinolate, the extract exhibited a strong thioglucosidase activity, indicating the presence of the enzyme, and the near or total absence of the substrate in sarcotesta. The thioglucosidase of the crude sarcotesta extract was later partially purified by ammonium sulfate fractionation, the protein fraction which precipitated between 50 and 70% saturation exhibited a specific activity of 478 μmol BITC/min/mg protein, approximately a 2-fold increase over the original extract.

TABLE 1. THE CONTENTS OF POTASSIUM BENZYL GLUCOSINOLATE AND THE PRESENCE OF THIOGLUCOSIDASE IN DIFFERENT PARTS OF PAPAYA SEEDS

	mg benzyl isothiocyanate/g tissue*		mg potassium benzyl glucosinolate/g tissue†	presence of thioglucosidase
	without adding thioglucosidase	with thioglucosidase		
Sarcotesta	—	0	0	+
Endosperm	0	21	63	−
Embryo	13	13	39	+

* Average of two experiments.
† Calculation based on the ratio of MWs of potassium benzyl glucosinolate and benzyl isothiocyanate.

In contrast to the sarcotesta, the endosperm appears to be devoid of thioglucosidase but rich in benzyl glucosinolate, as the macerated endosperms alone did not produce any detectable amount of BITC, whereas 21 mg was found in 1 g of endosperm after the addition of thioglucosidase. This amount of BITC is equivalent to 63 mg of potassium benzyl glucosinolate prior to enzymatic hydrolysis. A different situation existed in embryo; it contained both the substrate and the enzyme. As a result, BITC was rapidly produced in the macerated embryo without any addition of thioglucosidase. The quantity of BITC was 13 mg/g of wet tissue, equivalent to 39 mg of the potassium benzyl glucosinolate in 1 g of intact embryo.

[8] FOSTER, L. T. (1943) *Bot. Gaz. Ital.* **105**, 116.
[9] TANG, C. S. (1970) *J. Chem. Educ.* **47**, 692.

Table 2 indicated that benzyl glucosinolate was highly concentrated in the latex samples, ranging from 7·3 to 11·6% of the dry wt of latex fluid. The concentration of thioglucoside in latex decreased gradually during the maturation process, which coincided with the reduction of BITC in the macerated papaya pulp during fruit ripening.[2]

TABLE 2. CONTENTS OF POTASSIUM BENZYL GLUCOSINOLATE IN PAPAYA LATEX

	Wt. of papaya (g)*	Potassium benzyl glucosinolate (mg)/latex (g dry wt)		Wt. of papaya (g)*	Potassium benzyl glucosinolate (mg)/latex (g dry wt)
Set 1	255	101	Set 2	112	116
	398	89		217	108
	433	85		434	73

* Set 1 and Set 2 papayas were collected from two different trees. The heavier fruits, presumably were more mature than the lighter ones.

The ratio of BITC in macerated outer and inner rinds of immature papaya was rather inconsistent from experiment to experiment, probably because of the variations in sampling technique. However, repeated experiments indicated that the outer rind contained more than 10 times of BITC in comparison with that of the inner rind.

DISCUSSION

According to Guignard's classical study on the localization of 'active principles' of crucifers,[3] thioglucosides are generally accumulated in all parenchymatous tissues and the enzyme, myrosinase, is confined in 'special cells' (idioblasts). It appears that, these special cells are present in all tissues of isothiocyanate-producing plants.[5] It was also reported by Guignard that in the seeds of certain crucifers, such as *Lunaria biennis* L. and *Matthiola incana* R. Br., myrosinase is localized principally, if not exclusively, in the integument which has little or no thioglucoside. In papaya seeds, benzyl glucosinolate is concentrated in endosperm, the essential energy reserving unit for seed germination. The enzyme thioglucosidase is predominantly located in the gelatinous portion of the outer integument, which is free from benzyl glucosinolate. The distribution of this enzyme in the integument, however, is not exclusive due to its presence in the embryo.

Papain is one of the most important proteolytic enzymes used in food and medicinal industry. It is a product of dried papaya latex collected from the immature papaya fruit. Table 2 shows that the papaya latex also contains large percentage of potassium benzyl glucosinolate. While high concentration of this compound in latex fluid does not exclude its possible existence in tissues other than laticifers, it seems possible that this thioglucoside, as to the case of papain, is essentially localized in latex. The following observations may be cited as supporting evidence: (a) ripened papaya which was usually devoid of latex also contained little BITC in its pulp macerate;[2] (b) when the cross section of an immature papaya fruit was cut with a sharp knife, latex exuded essentially from the outer rind of the pulp. Subsequent determinations indicated that the latex-rich outer rind contained at least 10 times more BITC in comparison with the inner rind.

Laticifers would appear to fit best in the class of excretory structures where non-functional by-products are gathered, although quantitative and qualitative variations of latex

composition in different plants suggest the possibility that laticifers may have more than one function.[10] The germicidal,[11] insecticidal[12] activities of isothiocyanates have been well established. Benzyl isothiocyanates was found to be lethal at 15 ppm toward *Phytophthora palmivora*, one of the most important papaya fungal pathogens in Hawaii (Tang and Aragaki, unpublished data). Although there is good reason to believe that isothiocyanates function to protect plants against parasites, direct evidence is lacking that the glucosinolate–thioglucosidase system is involved.[13] Consequently, it is premature to describe papaya laticifers as a defense system which store, and transport the precursor of a germicidal compound. It is also premature to generalize this type of localization to all latex containing plants, which number 12 500 species belonging to 900 genera.[10]

The isothiocyanates are chemically active toward nucleophilic groups such as $-NH_2$, –OH and –SH, their various inhibitory effects on biological systems have also been reported.[14] It is likely, therefore, that these compounds would act as inhibitors, even in plants which produce them. Localization of the enzyme and substrate in different organs or tissues appears to be a plausible mechanism to control production of these toxicants.

The biochemistry of papain has been intensively studied, but the *in vivo* function of this proteolytic enzyme is not known. Since both benzyl glucosinolate and papain are present in lacticifers, one possibility is that papain could deter uncontrolled enzymatic production of BITC by inactivating any small quantities of thioglucosidase in latex through proteolytic hydrolysis. BITC would be produced only if damage of tissues causes sufficient amount of the thioglucosidase to interact with benzyl glucosinolate in the latex. Further studies on these possible interactions would contribute to the better understanding of the physiological significance of papaya latex.

EXPERIMENTAL

Preparation of thioglucosidase. A partially purified thioglucosidase (Myrosinase) solution was prepared as follows: Ripe papaya (*Carica papaya* L.) fruit was purchased from a local supplier. 100 g fresh seeds were rinsed with distilled H_2O, the sarcotestae were ruptured by squeezing the seeds between 2 layers of cheesecloth in 200 ml of ice-cold citric acid–Na_2HPO_4 buffer, pH 6·4 (6·15 mmol citric acid and 27·7 mmol Na_2HPO_4), containing 0·1 mol NaCl and 20 g Polyclar AT. The aqueous extract was centrifuged at 7710 *g* for 15 min. The supernatant was fractionated by $(NH_4)_2SO_4$ and the protein which precipitated between 50 and 70% saturation was collected and dialyzed against the same buffer. The enzyme assay was carried out in an 8 ml capped culture tube containing 3·2 μmol potassium benzyl glucosinolate, 2 μmol L-ascorbic acid, 0·6 ml citric acid–Na_2HPO_4 buffer, appropriate amount of enzyme solution, and H_2O to make a final vol. of 2 ml. After incubation at 37° for 5 min, the mixture was extracted with 4 ml of $CHCl_3$ and the amount of BITC in the $CHCl_3$ layer was determined by GLC analysis.[2] Protein concentration was determined by the method of Lowry *et al.*[15] The potassium benzyl glucosinolate solution used in this enzyme assay was prepared from dried papaya seeds according to Gmelin and Kjaer,[7] except that the glucosinolate was eluted from the anionotropic aluminum oxide (Woelm) column with 0·1 N KOH instead of Me_4NOH.

Quantitative determination of BITC. Procedures for BITC quantitation were similar to those used previously,[2] except for the sweep co-distillation step which was found unnecessary for seed and latex extracts. GLC was carried out with a Varian 1800 Gas Chromatograph equipped with dual flame ionization detection systems containing 2·5 mm × 3 m stainless steel column packed with 3% OV17 on 80–100 mesh Chromosorb G, AW-DMCS solid support. The oven temperature was 190° and the flow rate of N_2 was 25 ml/min; H_2 25 ml/min and air 300 ml/min.

BITC in sarcotesta extract. 1 g of fresh, mature papaya seeds was cleaned with distilled H_2O and the sarcotestae were ruptured manually in 10 ml citric acid–Na_2HPO_4 buffer. After removing the debris by

[10] ESAU, K. (1965) *Plant Anatomy*, 2nd Edn, p. 318, Wiley, New York.
[11] VIRTANEN, A. I. (1962) *Angew. Chem. Internat. Edn.* **1**, 299.
[12] LICHTENSTEIN, E. P., MORGAN, D. G. and MUELLER, C. H. (1964) *J. Agric. Food Chem.* **12**, 158.
[13] STOESSL, A. (1970) *Recent Advances in Phytochemistry* (edited by STEELINK, C. and RUNECKLES, V. C.), Vol. III, p. 158, Appleton-Century-Crofts, New York.
[14] REID, E. E. (1966) *Organic Chemistry of Bivalent Sulfur*, Vol. 5, p. 79, Chemical Publishing. New York.
[15] LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193**, 265.

low speed centrifugation, the supernatant was incubated at room temp. for 1 hr before being extracted with 20 ml $CHCl_3$. The $CHCl_3$ phase was concentrated under N_2 to 1 ml for GLC analysis of BITC contents.

BITC in endosperm and Embryo. Endosperms and embryos were obtained from 10 air-dried, mature papaya seeds. After manual removal of the outer and inner integuments, the naked seeds were carefully separated into the endosperms and embryos by using a razor blade. Endosperms or embryos were immediately weighed, homogenized with 1 ml citric acid–Na_2HPO_4 buffer in an all-glass tissue homogenizer before being transferred to a capped culture tube. The homogenizer was rinsed 2× with 1 ml of the same buffer and the washings were combined. After incubating at room temp. for 1 hr, the mixture was extracted by vigorous shaking with 6 ml $CHCl_3$ and the $CHCl_3$ layer was used for GLC analysis. In those cases where a thioglucosidase preparation was added, the procedure was the same except 1 ml of thioglucosidase preparation was used for the first washing of the homogenizer instead of the buffer solution.

BITC in latex. Papaya fruit of different sizes were collected from the Waimanalo Experimental Farm. University of Hawaii. The fruit was rinsed with distilled H_2O, dried, and a superficial wound was cut with a razor blade. 2–3 drops of the exuding latex were collected and weighed in a capped culture tube containing 3 ml of the citric–phosphate buffer. After being shaken vigorously the mixture was incubated at room temperature for 1 hr with or without the addition of 1 ml of a thioglucosidase preparation. The BITC formed was extracted with 10 ml of $CHCl_3$ and quantitatively determined by GLC. To determine the dry wt of papaya latex, *ca.* 1 g of latex was collected and dried in a vacuum oven at 100° for 5 hr. The average dry wt was 18·2% of the original latex fluid.

BITC in the inner and outer rinds of papaya pulp. Half size immature papaya pulp was sliced into 3 layers of equal thickness along the culvature of the fruit. After discarding the center layer, BITC contents of the inner and outer layers were determined.