Phytochemistry, Vol. 27, No. 7, pp. 1993 1998, 1988.
Printed in Great Britain.

0031 9422/88 $3.00+0.00
Pergamon Press plc.

ACID INVERTASE FROM *TROPAEOLUM* LEAVES

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(*Received* 21 *September* 1987)

Key Word Index—*Tropaeolum majus*; Tropaeolaceae; invertase; inhibition by fructose; inhibition by glucose; protein activation.

Abstract—The invertase of *Tropaeolum majus* leaves was partially purified. Its optimum pH was *ca* 5.25 and its K_m values for sucrose, raffinose, and stachyose were 5.3, 17 and 25 mM respectively. The products of the reaction were *in vitro* inhibitors of the enzyme. Fructose was a partial competitive inhibitor (K_1 49 mM) and glucose was a non-competitive classical inhibitor (K_1 57 mM). Proteins were activators of the enzyme. As activation by proteins suppressed the inhibitory effects of the reaction products, product inhibition does not appear to be involved in the physiological regulation of the enzyme.

INTRODUCTION

In any study of the metabolism of sucrose it is important to distinguish between cell wall, vacuolar and cytoplasmic invertases. The cell wall invertases appear to function as a part of the transport mechanism of sucrose [1, 2]. Among the soluble invertases, the neutral or alkaline invertases are thought to be cytoplasmic enzymes. As the activities of these enzymes rise on maturity of the storage tissues, when the activity of the acid invertase disappears, neutral invertases probably take over the role of the acid soluble invertases. Examples of this situation are provided by sugar cane [3] and carrot root invertases [4]. Another argument advanced in favour of the physiological role of neutral invertases is the cytoplasmic localization attributed to these enzymes. However, to our knowledge, the localization of neutral invertases has never been studied and the interpretation of a cytoplasmic localization rests almost exclusively on the optimum pH value of the enzyme [1, 5].

Even less clear is the situation with regard to acid invertases. These enzymes appear to occur more frequently than neutral invertases. Generally, acid invertases occur as vacuolar enzymes. This is true for immature storage tissue and leaf tissue [6–9]. However, acid invertase of sugar cane cell suspension cultures has been reported as an extravacuolar enzyme [10]. The physiological function of acid invertase is not clear because of the presence of sucrose synthetase. The reverse reaction of this enzyme produces UPDPGlc and free fructose with conservation of the energy of the glycosidic bond. Sucrose synthetase has been proposed as the enzyme which is involved in the use of sucrose for biosynthetic reactions, and invertase as the enzyme which hydrolyses sucrose for the energetic needs of the cell. An argument in favour of an important physiological role of the acid invertases is the vacuolar co-existence of invertase and sucrose in *Beta vulgaris* [6] and endosperm [11] and leaves [8] of *Ricinus communis*. Also the existence of tissues showing only invertase activity and lacking sucrose synthetase activity [12] and the occurrence of an acid invertase in sugar cane leaf sheaths whose activity is regulated by fructose [13] supports the importance of these enzymes in the plant metabolism. Another important fact is the well known increase of the acid invertase content in some developing tissues [14], an increase also produced by hormonal action [15]. Finally, 1′-fluorosucrose is a substrate for invertase but not for sucrose synthetase. 1′-Fluorosucrose is not metabolized by plant tissues [16].

Studies performed in our laboratory have shown the occurrence of more than one type of acid soluble invertase. Some of these enzymes appear to be modulated by their reaction products [13] but others, such as the invertase of *R. communis* [17], possess a mechanism to avoid this inhibition. Thus the regulatory mechanisms of the metabolism of sucrose appears to vary from plant-to-plant. However, we do not know the extent nor the types of variation among plants. This paper reports on the molecular and kinetic properties of the invertase from *Tropaeolum majus* leaves. The effect of both reaction products, fructose and glucose, is also reported.

RESULTS

Sugars and invertase in developing leaves

The sugar (fructose, glucose, sucrose and total sugars) and invertase contents during the development of the leaves of *T. majus* is shown in Fig. 1. The leaves were numbered starting from the tips of the stalk. In this way the first easily visible leaf was leaf number 1. For the determinations, groups of 3 consecutively numbered leaves were processed. Thus, group 1 was comprised of

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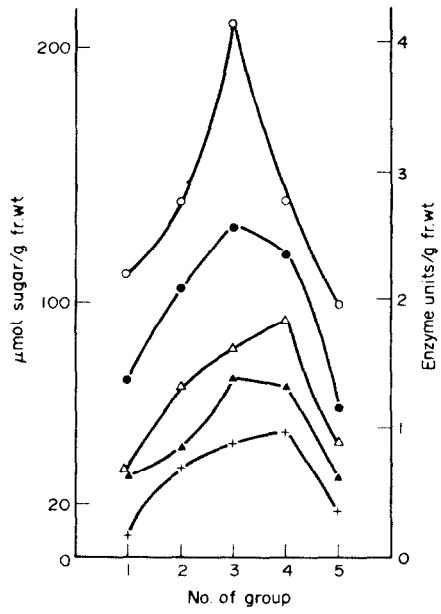

Fig. 1. Changes in invertase (●——●), glucose (▲——▲), fructose (△——△), sucrose (+——+) and total sugars (○——○) during leaf development.

leaves number 1, 2 and 3. Leaves number 13, 14 and 15 were senescent. As leaves 7, 8 and 9 (group 3) contain the maximum level of invertase, they were used throughout this paper.

Partial purification

The purification of the invertase is depicted in Table 1. The enzyme was purified about 75-fold. Attempts at further purification using ion exchange columns (DEAE-Sephadex), adsorption chromatography (hydroxylapatite column), and precipitation with organic solvents and acids were unsuccessful because of the loss of enzyme activity. As the enzyme showed two activity bands on PAGE, the extracts were preincubated at 37° for 30 minutes. This treatment produced a 10% loss of the enzyme activity and just one activity band on PAGE. Consequently the preincubated enzyme was used throughout this work.

Effect of pH on enzyme activity

The activity of the enzyme was measured at a pH range of 3 to 8. The optimum pH was 5.25. Subsequent experiments were run at pH 5.25 unless otherwise stated.

Effect of enzyme concentration

Increasing the enzyme concentration produced a proportional increase in the initial rate of the reaction, at least from 5 to 40 μl (1–8 mg of protein) of the enzyme preparation. This result indicates the absence of reversible inhibitors in the enzyme preparation [18].

Progress curves

Progress curves were straight lines at least up to 1 hr even at the most dilute concentration of sucrose used (5 mM).

Determination of the activation energy

The activity of the enzyme was determined at various temperatures between 10 and 50°. The activation energy was calculated to be 8400 cal/mol. Arrhenius plots were straightline graphs (not shown). This result is different from that of the sugar cane leaf sheaths [13] whose plots showed a break at 30°.

Effect of substrate concentration

This was measured for three substrates (Fig. 2). The kinetics correspond to simple cases of the Michaelis–Menten equation. The K_m of sucrose was calculated to be 5.3 mM. Raffinose (K_m 17 mM) and stachyose (K_m 25 mM) were substrates of the enzyme, but the preferred substrate was sucrose.

Substrate specificity

The enzyme preparation was unable to hydrolyse α-methylglucoside, trehalose, melezitose, maltose, melibiose, cellobiose, turanose, inulin or levan. According to these results the enzyme attacks only the β-fructofuranosides which have terminal fructose residues. Consequently the invertase is a β-fructofuranosidase.

Inhibitory effect of sugars

Table 2 shows the effect of some sugars on the enzyme reaction. Fructans were not inhibitors and this appears

Table 1. Purification of the soluble acid invertase from *T. majus* leaves

Fraction	Total enzyme units	Specific activity (units/mg protein)	Purification	Yield (%)
Crude homogenate	552	0.067	1	100
Centrifugation at 15000 *g*	471	0.078	1.2	85
$(NH_4)_2SO_4$ precipitation	216	0.143	2.1	39
Centrifugation at 15600 *g*	211	1.7	2.6	38
Sephadex G-150 filtration	157	5.1	75.0	28

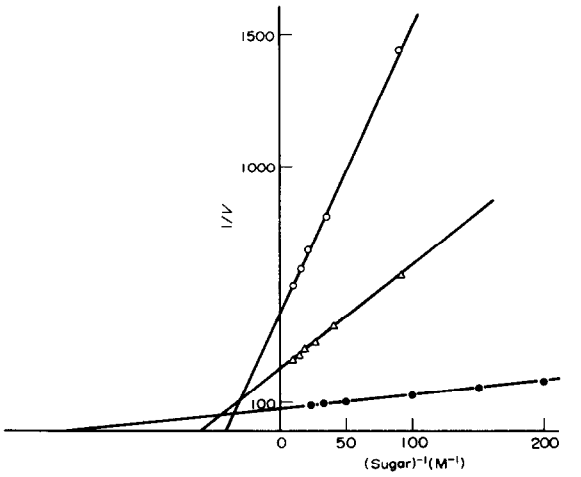

Fig. 2. Lineweaver–Burk plots obtained with sucrose (●--●), raffinose (△——△) and stachyose (○——○) as substrate. Reducing power was measured by the method of Nelson [25].

to be true of all plant invertases [13, 17]. Turanose at a relatively low concentration was a strong inhibitor of the enzyme and β-methyl fructoside had a slightly inhibitory action even at high concentration. All the other sugars tested were inhibitors of the enzyme.

Effect of the reaction products

Both reaction products, fructose and glucose, were inhibitors of the enzyme. Fructose produced a competitive inhibition of the invertase (Fig. 3). The sugar is a partial competitive inhibitor according to replots of slope or apparent K_s against fructose concentration. The K_i

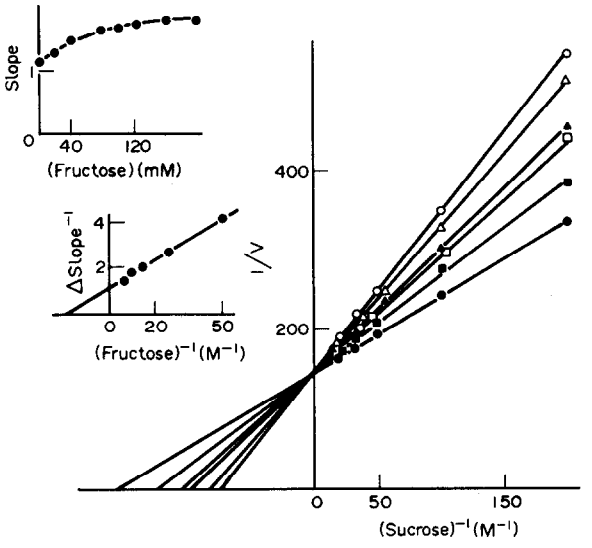

Fig. 3. Partial competitive inhibition of soluble acid invertase by fructose, and replots of slope and $1/\Delta_{slope}$ versus fructose concentration. Glucose was measured by glucose oxidase method [34]. Reactions were run at 37° and pH 5.25. ●——●, without inhibitor; ○——○, 200 mM, △——△, 160 mM, ▲——▲, 80 mM, □——□, 40 mM, and ■——■, 20 mM fructose.

Table 2. Inhibitory effect of some sugars on *T. majus* invertase

Sugar	Concentration (mM)	Inhibition (%)
Turanose	20	100
Maltose	80	30
β-Methyl-fructoside	336	27
Trehalose	50	23
Cellobiose	40	22
α-Methyl-glucoside	50	20
Lactose	40	17
Melibiose	40	13
Melezitose	40	10
Galactose	50	10
Levan	1%	0
Inulin	3%	0

was calculated to be 49 mM on the basis of a plot of $1/\Delta$ slope vs $1/[I]$. The factor a which expresses the change of K_s when I occupies the enzyme was found to be 1.005.

Glucose was a non-competitive inhibitor of the enzyme (Fig. 4). Replots of $1/v_{App}$ or slope against glucose concentration showed that the inhibition was of the non-competitive classical type. The K_i for glucose was 57 mM.

M_r determination

The M_r of the invertase from *T. majus* was 74 000 as determined by gel filtration (not shown) according to the method of ref. [19].

Effect of chemicals

Various chemicals were assayed as possible enzyme inhibitors. Table 3 shows that most of these chemicals possess an inhibitory action. At 10 mM, Cu^{2+}, Ca^{2+} and

Table 3. Effect of some chemicals on the activity of the β-fructofuranosidase of *T. majus* leaves

Chemical	Concentration (mM)	Inhibition (%)
NaH_2PO_4	100	0
KNO_3	10	0
Inositol	10–100	8–20
Urea	10–100	4–15
NH_4^+	10–100	8–38
Tris	10–100	5–51
EDTA	10–100	9–23
Na_2BO_3	5–50	4–25
Mg^{2+}	5–20	7–12
Ca^{2+}	5–50	30–39
Ba^{2+}	10–50	22–96
Hg^{2+}	5–50	72–97
Cu^{2+}	10–100	85–96
Co^{2+}	10–100	80–92
Zn^{2+}	10–100	23–59
Mn^{2+}	5–20	7.6–14

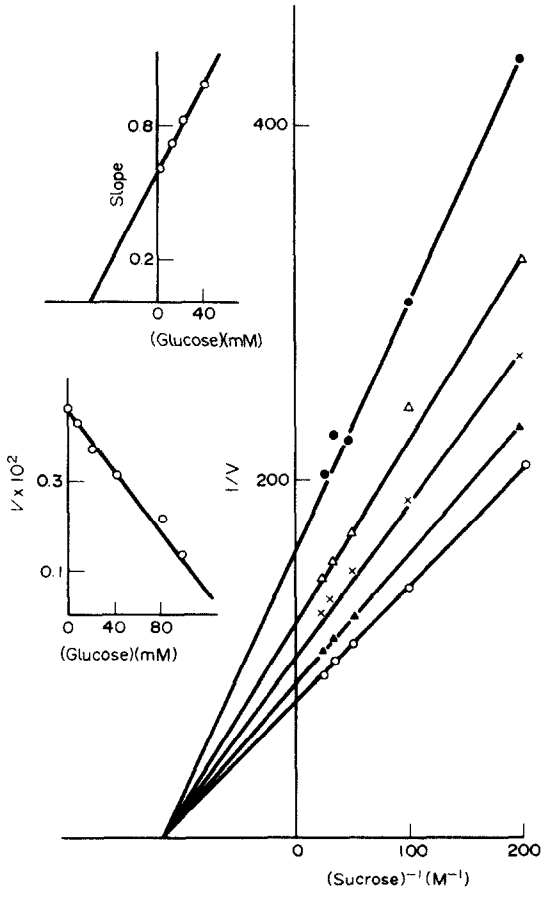

Fig. 4. Non-competitive inhibition of soluble acid invertase by glucose, and replot of slopes versus concentration of glucose. The reactions were run at 37° and pH 5.25. Fructose was measured by the fructose dehydrogenase method. ○——○, Control; ▲——▲, 10 mM, ×——×, 20 mM, △——△, 40 mM, and ●——●, 80 mM glucose.

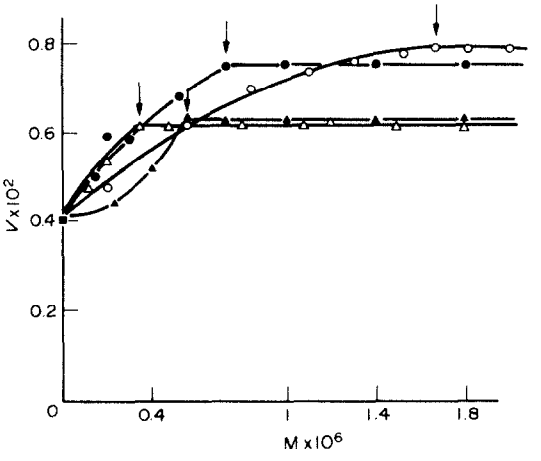

Fig. 5. Activation of *T. majus* invertase by proteins. ▲——▲, Alkaline phosphatase; ●——●, BSA; ○——○ β-lactoglobulin; △——△, human γ-globulin. The concentrations at maximal activation as shown by arrows were: Alkaline phosphatase 5.5×10^{-7} M (75% activation); BSA 7.3×10^{-7} M (58% activation); human γ-globulin 3.4×10^{-7} M (45% activation); β-lactoglobulin 17×10^{-7} M (87% activation).

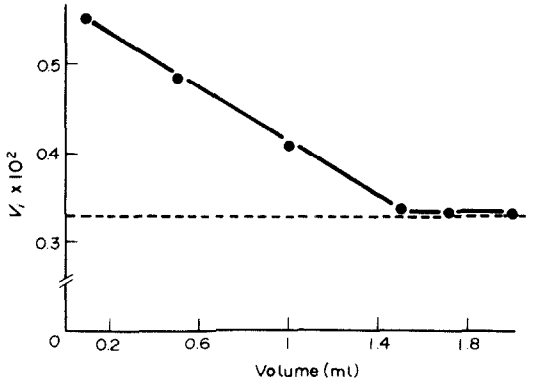

Fig. 6. Effect of dilution on the activity of the enzyme-BSA complex. (Each reaction mixture contained 20 μl (0.12 enzyme units) of invertase, 40 μl of 0.2 M sodium acetate buffer, pH 5.2 and BSA to reach a concentration of 1.05×10^{-6} M (10 μl of a 0.5 mg/ml BSA solution). The enzyme-activator mixture was diluted after 15 min. Sucrose was kept at a constant concentration of 60 mM with variable final volumes of the incubation mixtures as shown in the figure. Incubations were run at 37° for 20 min. Reducing power was determined as stated in the enzyme assay.

Hg^{2+} were strong inhibitors. Other bivalent cations such as Zn^{2+}, Ba^{2+}, and Mg^{2+} were also inhibitory. However, some anions such as NO_3^- reported as a physiological effector of the invertases from *Hevea brasiliensis* latex [20] and from sweet potato [21], were not effectors of the invertase from *T. majus*. The same lack of effect was found in the case of sodium dihydrogen phosphate, reported as activator of the invertase of *Streptococcus mitis* [22] and *H. brasiliensis* [20].

Effect of proteins

The activity of the invertase from *T. majus* was affected by proteins. The activation produced by BSA, β-lactoglobulin, alkaline phosphatase and human γ-globulin is shown in Fig. 5. The activation level and the molar concentration of protein required to attain the maximal activation of the enzyme depends on the nature of the protein tested. Reports on protein activation have been published in the case of the invertases from *Raphanus sativus* [23] and *Ricinus communis* [17]. In the case of *R. communis* invertase, it has been shown that proteins produce the activation by forming a dissociable complex with the enzyme. The dissociation by dilution of the BSA–*T. majus* invertase complex is shown in Fig. 6. The dissociation curve is of the same type of that produced on dilution by the BSA–*R. communis* invertase complex. When the proteins were added to incubation mixtures, the inhibitory effect of the reaction products, fructose and glucose, was suppressed.

DISCUSSION

The invertase from *T. majus* leaves is a β-fructosidase as are most, if not all, of the higher plant acid invertases.

It is affected by its reaction products. Fructose is a classical competitive inhibitor and glucose is a classical non-competitive inhibitor, However, these inhibitions appear to be meaningless on a physiological base becase of their suppression by proteins. The same result was found in the case of *R. communis* invertase. The activation by proteins differs from that reported for *R. communis* invertase because of differences in the required amount of protein needed to attain maximal activation. The invertase of *R. communis* reaches maximal activity at the same molar concentration of proteins independently of the protein used. *T. majus* invertase needs different molar concentrations of the proteins to attain maximal activation. In spite of this difference the *T. majus* invertase has many features in common with the *R. communis* invertase. These are; the simple inhibitory action of the products of the reaction, the similarities in the M_r (the M_r of *R. communis* invertase was reported to be 78 000), the activating effect of proteins, the suppression of the inhibitory effect of the reaction products by the proteins. Further, Arrhenius plots are straight lines for both invertases, without the breaks shown by the invertase of sugar cane leaf-sheaths [13]. According to these results, the soluble acid invertases belong at least to two main groups, the β-fructofuranosidases whose activity appears to be modulated by fructose, and those whose activity is competitively modified by fructose only in absence of proteins. Few studies are available for a better definition of this question and new studies on this point are necessary.

EXPERIMENTAL

Plant material. Fully expanded leaves from *Tropaeolum majus* (leaves nos 7–9 from the tip of the stalk) were used unless otherwise stated.

Enzyme preparation. *Ca* 350 g of *T. majus* leaves were cut into small pieces and homogenized in 250 ml 50 mM NaPi buffer, pH 7.5, containing 1 mM 2-mercaptoethanol, 5 μM $MnSO_4$, 50 mM NaCl, and 87.5 mg of Na_2SO_3. The homogenate was filtered through 2 layers of cheesecloth and centrifuged at 12 100 *g* for 15 min. Solid $(NH_4)_2SO_4$ was added to the supernatant and a fraction which precipitated between 30 and 90% of saturation was collected by centrifugation. The pellet was resuspended in 2 ml of 10 mM NaOAc buffer, pH 5.25, containing 1 mM 2-mercaptoethanol and 50 mM NaCl (Buffer A), and dialysed against the same buffer. The preparation was left to stand over 84 hr. The abundant ppt. produced during this time was removed by centrifugation at 12 100 *g* for 15 min. The supernatant was fractionated on a Pharmacia column K 25/45 filled with Sephadex G-150, preequilibrated and eluted with buffer A. The invertase, which elutes between 84 and 116 ml was pooled and kept at 0–4°. All procedures were carried out at 0–4°. The preparations contained about 4.9 enzyme units/ml and 0.96 mg of protein/ml.

Enzyme units. One enzyme unit was defined as the mg of protein which yields 1 μmol of product/min at 37° at pH 5.25.

Enzyme assay. The reaction mixture consisted of 10 μl 0.6 M sucrose, 40 μl 0.2 M NaOAc buffer, pH 5.25, 20 μl enzyme and water in a final vol of 100 μl. Incubations were performed at 37° and the reactions were stopped by a Cu alkaline reagent [24]. Reducing power release was measured by the method of ref. [25].

Determination of proteins. Proteins were determined by the method of ref. [26] using BSA as standard.

M_r *determinations.* The M_r of the invertase was determined by the method of Andrews [19] using Sephadex G-150. The column (2.5 × 40 cm) was prepared with buffer A. Trypsin (M_r 23 000), β-lactoglobulin (35 800), BSA (68 000) and aldolase (158 000) were used as standards.

Acrylamide gel electrophoresis. Vertical gel electrophoresis was performed according to the procedure of refs [27, 28]. Proteins were stained with Coomassie Blue R-250. Invertase activity stains were performed according to the procedure of ref. [29].

Tissue sugars. Sugars were measured using 3 g of fresh tissues. The leaf tissue was killed in boiling H_2O. Three min later the mixture was homogenized. Cheesecloth was used for separating the fibrous material which was extracted with distilled H_2O. The cheesecloth was carefully washed and squeezed and the extracts were pooled. Sucrose was determined by the method of ref. [30]. Fructose and glucose were measured by the fructose dehydrogenase [31, 32] and glucose oxidase [33] methods respectively.

Effect of the reaction products. The incubation mixtures consisted of 20 μl enzyme, 10 μl 0.05–0.6 M sucrose, 10 μl 0.2–2 M fructose or 0.1–1 M glucose, 40 μl 0.2 M NaOAc buffer, pH 5.25, and distilled water in a final vol of 100 μl. Incubations were made at 37°. Reducing power determinations in presence of fructose and glucose were performed by the method of ref. [33] and by the method of the fructose dehydrogenase [32] respectively.

Acknowledgements—This work was supported in part by grants from the Consejo Nacional de Investigaciones Cientificas y Técnicas. Buenos Aires, Argentina, and from the Subsecretaria de Ciencia y Tecnologia.

REFERENCES

1. Hawker, J. S. and Hatch, M. D. (1965) *Physiol. Plant.* **18**, 444.
2. Eschrich, W. (1980) *Ber. Deutsch. Bot. Ges.* **93**, 363.
3. Hatch, M. D. and Glasziou, K. T. (1963) *Plant Physiol.* **38**, 344.
4. Ricardo, C. P. P. and Sovia, D. (1974) *Planta* **118**, 43.
5. Lyne, R. L. and ap Rees, T. (1971) *Phytochemistry* **10**, 2593.
6. Leigh, R. A., ap Rees, T., Fuller, W. A. and Banfield, J. (1970) *Biochem. J.* **178**, 539.
7. Boller, T. and Kende, H. (1979) *Plant Physiol.* **63**, 1123.
8. Vattuone, M. A., Fleischmacher, O. L., Prado, F. E., Lopez Viñals, A. and Sampietro, A. R. (1983) *Phytochemistry* **22**, 1361.
9. Boudet, A. M., Canut, H. and Alibert, G. (1981) *Plant Physiol.* **68**, 1354.
10. Thom, M., Maretzki, A. and Komor, E. (1982) *Plant Physiol.* **69**, 1315.
11. Nishimura, M. and Beevers, H. (1978) *Plant Physiol.* **62**, 44.
12. Hatch, M. D., Sacher, J. A. and Glasziou, K. T. (1963) *Plant Physiol.* **38**, 338.
13. Sampietro, A. R., Vattuone, M. A. and Prado, F. E. (1980) *Phytochemistry* **19**, 1637.
14. Morris, D. A. and Arthur, E. D. (1984) *J. Exp. Botany* **36**, 623.
15. Morris, D. A. and Arthur, E. D. (1985) *Phytochemistry* **23**, 2126.
16. Hitz, W. D., Schmitt, M. R., Card, P. J. and Giaquinta, R. T. (1985) *Plant Physiol.* **77**, 291.
17. Prado, F. E., Vattuone, M. A., Fleischmacher, O. L. and

Sampietro, A. R. (1985) *J. Biol. Chem.* **260**, 4952.
18. Dixon, M. and Webb, E. C. (1966) *The Enzymes*, p 59. Longsman, Essex.
19. Andrews, P. (1965) *Biochem. J.* **96**, 595.
20. Jacob, J.-L., Prevot, J.-C.and D'Auzac, J. (1982) *Phytochemistry* **21**, 851.
21. Matsushita, K. and Uritani, I. (1974) *Plant Physiol.* **54**, 60.
22. Sund, M. L. and Linder, L. (1979) *Arch. Oral. Biol.* **24**, 439.
23. Faye, L. and Lambert, C. (1980) *Biochim. Biophys. Acta* **615**, 392.
24. Somogyi, M. (1945) *J. Biol. Chem.* **160**, 61.
25. Nelson, N. (1944) *J. Biol. Chem.* **153**, 375.
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
27. Ornstein, L. (1964) *Ann. N. Y. Acad. Sci.* **121**, 321.
28. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404.
29. Gabriel, O. and Wang, S-F. (1969) *Anal. Biochem.* **27**, 545.
30. Cardini, C. E., Leloir, L. F. and Chiriboga, J. (1955) *J. Biol. Chem.* **214**, 149.
31. Ameyama, M., Shinagawa, E., Matsushita, K. and Adachi, O. (1981) *J. Bacteriol.* **154**, 814.
32. Avigad, G. and Englard, S. (1965) *J. Biol. Chem.* **240**, 2290.
33. Jorgensen, O. S. and Andersen, B. (1973) *Anal. Biochem.* **53**, 141.