J. Biochem. Mol. Biol. Vol. 29, No. 2 pp. 163~168 March 31, 1996

Purification of the Glycosylated Polyphenol Oxidase from Potato Tuber

Do-Yoon Kwon and Woo-Yeon Kim*

Department of Biotechnology, Chung-Ang University, Ansung 456-756, Korea
(Received December 29, 1995)

Abstract: Glycosylated polyphenol oxidase was purified from potato tuber using ammonium sulfate fractionation, Sephadex G-100, and concanavalin A Sepharose column chromatography. Two or three types of polyphenol oxidase were separated on concanavalin A Sepharose. Type I and II polyphenol oxidases did not bind to concanavalin A Sepharose. Type I seemed to be an aggregated form of polyphenol oxidase. Type III polyphenol oxidase, which is presumed to be glycosylated because it was bound to concanavalin A Sepharose and eluted with α-D-methyl glucopyranoside, was further purified by chromatography on Econo-Pac Q and Superose 12. Glycosylated polyphenol oxidase was purified 130-fold from the dissolved ammonium sulfate pellet resulting in about 6 μg of the enzyme from 100 g of potato tuber periderm. The molecular weight of the glycosylated enzyme determined by SDS-polyacrylamide gel electrophoresis was about 64,000. Optimum temperature and pH of both type II and type III potato polyphenol oxidases were 20℃ and pH 7.0, respectively. Glycosylated form of polyphenol oxidase (type III) preferred catechol to catechin as a substrate, whereas type II enzyme showed the reverse substrate preference.
Key words: glycosylation, polyphenol oxidase, potato, purification.

Polyphenol oxidase, which is a copper containing enzyme and is responsible for melanization in animals and browning in plants, is also known as phenol oxidase, phenolase, tyrosinase, *o*-diphenol oxidase, and catechol oxidase depending on its substrate specificity. Polyphenol oxidase catalyzes either one or two reactions involving molecular oxygen (Bartlett *et al.*, 1972; Butt, 1979; Matheis, 1987a). The first type of reaction is hydroxylation of monophenols leading to the formation of *o*-dihydroxyphenols (EC 1.14.18.1., monophenol monooxygenase). The second type of reaction is oxidation of *o*-dihydroxyphenols to *o*-quinone (EC 1.10.3.1., 1,2-benzenediol : oxygen oxidoreductase). Polyphenol oxidases are believed to be ubiquitous in the plant kingdom. They have been detected in most known fruits and vegetables. Although these enzymes have been studied for many years (Arnon, 1949; Mayer and Harel, 1979; Vamos-Vigyazo, 1981), the physiological significance of them in plants is unclear. A couple of roles have been postulated, these could be members of the host defense mechanism, where activation of the latent polyphenol oxidase is triggered by the infection of pathogens (Montalbini *et al.*, 1981; Felton *et al.*, 1989), or they coud be mediators of photosynthetic electron transport, which might be related to the finding of polyphenol oxidase in PSII particles (Tolbert, 1973; Lax and Vaughn, 1991).

In addition to the research on the physiological roles of polyphenol oxidase, this enzyme has been concerned in the food industry because it is one of the major factors involved in the enzymatic browning of fresh and processed fruits and vegetables and the off-flavor generation in canned or frozen horticultural products (Paulson *et al.*, 1980). It is no wonder that ever since its discovery this enzyme has been the subject of extensive research. However, the molecular structures and relationship between the various forms of the enzymes purified by different laboratories from the same or different species are still not clear (Vamos-Vigyazo, 1981).

Because postharvest enzymatic browning is a serious problem in potato processing, potato tuber polyphenol oxidase, which is the major enzyme responsible for browning, has been studied extensively (Matheis, 1987 a, 1987b). However, potato tuber polyphenol oxidase has not been purified to homogeneity as judged by polyacrylamide gel electrophoresis. In this study, we purified the glycosylated potato tuber polyphenol oxidase to homogeneity and compared the glycosylated enzyme to a nonglycosylated form in terms of optimum pH, optimum temperature, and substrate specificity.

*To whom correspondence should be addressed.
Tel : 82-334-675-0405, Fax : 82-334-675-0405.

Materials and Methods

Materials

Potato (*Solanum tuberosum* L. cv Daeji) was obtained from Rural Development Adminstration of Korea and stored at 4℃ before use. Miracloth was purchased from Calbiochem (CA, USA). Tris, polyvinylpolypyrrolidone, glycine, benzamidine-HCl, phenylmethylsulfonyl fluoride, EDTA, α-D-methyl glucopyranoside, CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), tyramine, vanillic acid, chlorogenic acid, caffeic acid, 3,4-dihydroxy benzoic acid, catechol, L-DOPA (L-3,4-dihydroxyphenylalanine), resorcinol, catechin, hydroquinone, gallic acid, and pyrogallol were purchased from Sigma. Sephadex G-100, concanavalin A Sepharose, and Superose 12 were obtained from Pharmacia (Sweden). Econo-Pac Q was from Bio-rad (CA, USA). All other reagents were of analytical grade.

Polyphenol oxidase assay and protein determination

The polyphenol oxidase activity was determined using L-DOPA as substrate at 20℃ by measuring the initial rate of quinone formation. The standard assay mixture contained 46.6 mM HEPES-NaOH, pH 7.0, 9.3 mM L-DOPA, and 0.1 ml of enzyme in 1.5 ml. Reaction mixtures were incubated at 20℃ for 3 min. Absorbances at 475 nm were measured spectrophotometrically. One unit of polyphenol oxidase activity is defined as that amount of enzyme that catalyzes an increase in absorbance of 0.001 per min at 20℃. Proteins were measured by the microprotein assay of Bradford (1976). Bovine serum albumin was used as a standard.

Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970). The resolving gel was 12% acrylamide. Gels were stained with Coomassie Blue.

Purification of polyphenol oxidase

All purification steps with the exception of FPLC were performed at 4℃. One hundred grams of potato tuber periderm was frozen in liquid nitrogen and ground to a fine powder with a Waring Blendor. This powder was suspended in 200 ml of buffer A (50 mM sodium phosphate, pH 6.0, 20 mM ascorbic acid, 1 mM benzamidine-HCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 M glycine, 25 mM EDTA, 5% glycerol, 1% polyvinylpolypyrrolidone). The slurry was homogenized at 12,000 rpm for 1 min with a Polytron PT 3000 homogenizer (Kinematica AG, Swiss) and filtered through one layer of Miracloth. The filtrate was centrifuged at 14,500×g for 30 min in a Beckman JA-14 rotor. Polyphenol oxidase was precipitated from the supernatant by the slow addition of 0.26 g of solid ammonium sulfate per ml of solution maintaining pH 6.0 by addition of 5 N NaOH solution. After 30 min, the precipitate was recovered by centrifugation at 12,000 ×g for 30 min. The pellet was resuspended in 6 ml of 50 mM sodium phosphate, pH 7.0 containing 20 mM ascorbic acid using a Dounce homogenizer and centrifuged at 18,000×g for 30 min to remove insoluble materials. Four-ml aliquots of the dissolved ammonium sulfate pellet were applied twice to 100 ml of Sephadex G-100 column previously equilibrated with 20 mM Tris-HCl, pH 7.0 containing 5% glycerol. The flow rate was 0.5 ml/min. Polyphenol oxidase-containing fractions (6 ml) were pooled and loaded onto 40 ml of concanavalin A Sepharose previously equilibrated with 20 mM Tris-HCl, pH 7.0 containing 0.5 M NaCl. The column was washed with two bed volumes of the above buffer and polyphenol oxidase was step-eluted with the above buffer containing 0.2 M α-D-methyl glucopyranoside at a flow rate of 1 ml/min. The fractions enriched in polyphenol oxidase were pooled, dialyzed against 20 mM Tris-HCl, pH 7.0 containing 2 mM CHAPS, and loaded onto Econo-Pac Q. The resin was washed with 3 column volumes of the above buffer, and polyphenol oxidase was eluted with a 7.5-ml linear gradient of 0~1 M NaCl in the above buffer. The fractions enriched in polyphenol oxidase were pooled and, 0.4-ml aliquots were consecutively applied to Superose 12 previously equilibrated with 20 mM Tris-HCl, pH 7.0 containing 0.15 M NaCl. The flow rate was 0.3 ml/min.

Results and Discussion

Purification of the glycosylated polyphenol oxidase

One of the problems for the purification of polyphenol oxidase from potato tuber was the relative abundance of patatin, which is the major potato storage protein. The reason that only potato tuber periderm was used in this experiment is that the ratio of polyphenol oxidase to patatin in tuber periderm is higher than that in the rest of tuber. Another thing to consider in the purification of polyphenol oxidase in plants is to remove phenolics because these compounds are changed to quinones by polyphenol oxidase, which could react covalently with the enzyme, resulting in discoloration and aggregation of the enzyme (Butt, 1980). Sephadex G-100 was used to remove some small molecular weight proteins including patatin and phenolics from the dissolved ammonium sulfate pellet. There were two peaks, which were monitored at 280 nm, in Sephadex G-100 filtration. The first peak contained most proteins, which

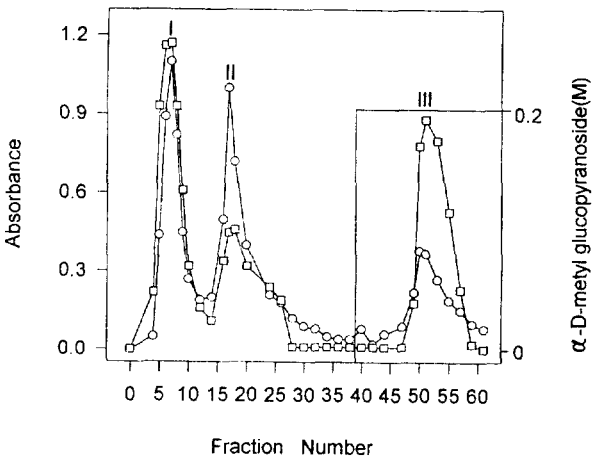

Fig. 1. Elution profile of concanavalin A Sepharose column chromatography. Active fractions from Sephadex G-100 column chromatography were applied to concanavalin A Sepharose column equilibrated with 20 mM Tris-HCl, pH 7.0 containing 0.5 M NaCl and glycosylated proteins were step-eluted with 0.2 M α-D-methyl glucopyranoside. Fractions were collected and polyphenol oxidase activity was determined. ○–○, absorbance at 280 nm (protein); □–□, absorbance at 475 nm (polyphenol oxidase activity); ——, concentration of α-D-methyl glucoside.

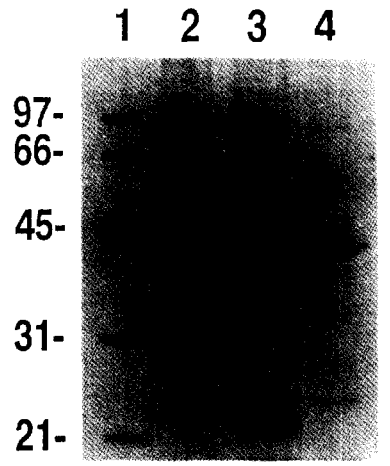

Fig. 2. SDS-polyacrylamide gel electrophoresis of polyphenol oxidase resolved by chromatography on concanavalin A Sepharose. Aliquots (20 μg) were analyzed by 12% Laemmli gel. The gel was stained for protein with Coomassie Blue. Lane 1 : molecular weight markers; lanes 2~4, concanavalin A Sepharose peaks I, II, and III, respectively. Molecular weight markers used were phosphorylase b (97,400), serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and trypsin inhibitor (21,500), respectively.

were visualized in SDS-polyacrylamide gel, whereas the second peak did not contain any protein (data not shown). Most phenolic compounds were presumably eluted in the second peak.

Fractions containing more polyphenol oxidase and less patatin were used for the separation on concanavalin A Sepharose column. As shown in Fig. 1, polyphenol oxidase activities were detected in peak I and II of the flow-through. Patatin, which is a glycoprotein (Park *et al.*, 1983), and an additional polyphenol oxidase (peak III) were eluted with α-D-methyl glucopyranoside from concanavalin A Sepharose. Type I polyphenol oxidase (peak I) was eluted earlier than type II enzyme (peak II) on Sepharose 4B, which is the matrix of concanavalin A Sepharose. This indicates that type I polyphenol oxidase is much larger than type II enzyme. Type I polyphenol oxidase might be an aggregated form of the enzyme. In fact, peak I fractions appeared to be turbid and the pellet obtained after centrifugation of the turbid sample showed polyphenol oxidase activity. Therefore, there are at least two distinct types of polyphenol oxidase based on the glycosylation of the enzyme because only type III polyphenol oxidase (peak III) was bound to concanavalin A Sepharose and eluted with α-D-methyl glucopyranoside. Concanavalin A is a lectin and binds molecules which contain α-D-mannose, α-D-glucose, and sterically related residues. The glycosylation of polyphenol oxidase is supported by the fact that broad bean, spinach, peach, and potato polyphenol oxidases are associated with carbohydrates (Robb *et al.*, 1965; Balasingham, 1970; Flurkey and Jen, 1980; Flurkey, 1986). A Coomassie-stained gel of the three polyphenol oxidase peaks is shown in Fig. 2. Peak I fraction (lane 2) from concanavalin A Sepharose column seems to contain relatively less contaminated proteins compared to peak II (lane 3) and peak III (lane 4). However, it was very hard to purify polyphenol oxidase from peak I and II due to precipitation of polyphenol oxidase during the ion exchange column chromatography. Glycosylated form of the polyphenol oxidase appeared to be more soluble and less aggregated than the enzymes found in the flow through from concanavalin A Sepharose column. Therefore, peak III fractions were used for further purification even though this peak contained much more patatin.

In order to keep the glycosylated form of the enzyme more soluble, 20 mM Tris-HCl buffer, pH 7.0 containing 2 mM CHAPS was used for the purification of polyphenol oxidase on Econo-Pac Q. Polyphenol oxidase was eluted with a linear gradient of 0~1 M NaCl. The fractions containing more polyphenol oxidase and less patatin were pooled and separated on Superose 12. Purified polyphenol oxidase was clearly visible following chromatography on Superose 12 (Fig. 3, lane 1). Major contaminant protein, patatin (M_r=43,000) was separated from polyphenol oxidase (see lanes 3 and 4). A Coomassie-stained gel of polyphenol oxidase at various stages of purification is shown in Fig. 4. Fig. 4 clearly shows again that patatin is a major problem for the purification of potato tuber polyphenol oxidase.

Table 1. Purification of potato polyphenol oxidase. The purification was based on 100 g of potato tuber periderm

Fraction	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Dissolved $(NH_4)_2SO_4$ pellet	6	26.4	49,999	1,893.9	100.0
Sephadex G-100	8	23.4	45,784	1,956.6	91.6
Concanavalin A Sepharose	16.5	15.2	51,060	3,359.2	102.1
Peak I	5.5	4.3	21,624	5,028.8	43.2
Peak II	5	4.5	9,837	2,186.0	19.7
Peak III[a]	6	6.4	19,599	3,062.3	39.2
Econo-Pac Q	3	2.1	8,599	4,094.8	17.2
Superose 12	3	0.006	1,500	250,000.0	3.0

[a]Only Peak III was chromatographed on Econo-Pac Q and Superose 12.

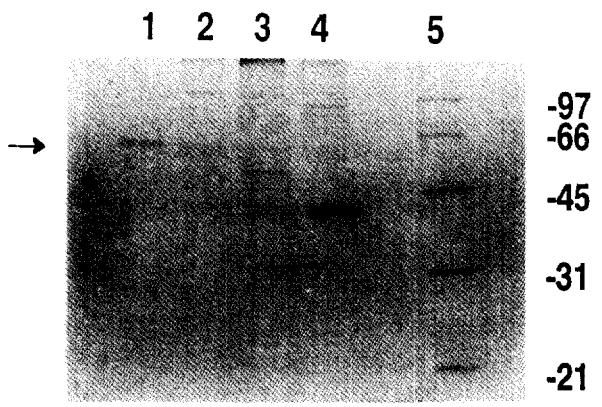

Fig. 3. SDS-polyacrylamide gel electrophoresis of potato tuber polyphenol oxidase resolved by chromatography on Superose 12. The gel was stained for protein with Coomassie Blue. Sample from each fraction was TCA-precipitated for gel electrophoresis. Lanes 1~4, fractions from Superose 12 column; lane 5, molecular weight markers.

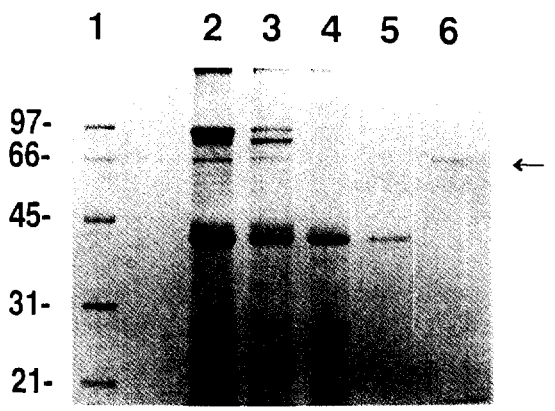

Fig. 4. SDS-polyacrylamide gel electrophoresis of potato tuber polyphenol oxidase at various stages of purification procedure. Lane 1 : molecular weight markers; lane 2 : dissolved ammonium sulfate pellet; lane 3 : Sephadex G-100 peak; lane 4 : concanavalin A Sepharose peak III; lane 5 : Econo-Pac Q peak; lane 6 : Superose 12 peak concentrated by TCA precipitation. The arrow in the right margin indicates the purified polyphenol oxidase.

The purified polyphenol oxidase indicated by the arrow in the right margin of the figure could be visualized only after concentration of the sample by TCA (trichloroacetic acid)precipitation (compare lanes 5 and 6). Typical results obtained during the purification steps are shown in Table 1. Glycosylated polyphenol oxidase was purified 130-fold from the dissolved ammonium sulfate pellet resulting in about 6 μg of the enzyme from 100 g of potato tuber periderm. The overall yield for the glycosylated polyphenol oxidase was 3% from the dissolved ammonium sulfate pellet. The reason for the low purification yield is that the loss of polyphenol oxidase was severe due to aggregation during purification and only glycosylated form was used for further purification.

Reports for the purification of plant polyphenol oxidase to homogeneity are rare probably due to aggregation of the enzyme caused by the quinones produced by the enzyme in the presence of endogenous phenolic compounds during purification (Butt, 1980). Broad bean polyphenol oxidase has been purified to homogeneity and the relationship between 59 kDa and 43 kDa polypeptide was elucidated clearly (Robinson and Dry, 1992). In the case of potato, polyphenol oxidase from the glandular trichomes of wild potato, *Solanum berthaultii* was purified to homogeneity using preparative isoelectrofocussing (Kowalski *et al.*, 1992), whereas potato tuber polyphenol oxidase has not been purified clearly (Matheis, 1987a). Compared to M_r of 59,000 in the case of polyphenol oxidase from wild potato glandular trichomes, molecular weight of the potato tuber enzyme has been reported to be 36,000 to 135,000. In the case of the glycosylated polyphenol oxidase purified here, the molecular weight based on the mobility on SDS-polyacrylamide gel is about 64,000 as shown in Fig. 3 and 4. Based on the cDNA size, Hunt *et al.* (1993) estimated molecular mass of potato leaf polyphenol oxidase as about 67 kDa polypeptide, which is processed to a mature form with M_r of 59,000~60,000. The apparent molecular weight difference might be partly caused by glycosylation.

Table 2. Substrate specificity of the two types of potato polyphenol oxidase. Relative absorbance values were indicated showing the absorbances at 475 nm with L-DOPA as 100%

Substrate (10 mM)	Relative activity	
	Type II	Type III
Monohydroxyphenol		
L-tyrosine	3	1
tyramine	2	1
vanillic acid	0.7	0
Dihydroxyphenol		
chlorogenic acid	11.5	10
caffeic acid	18	16
3,4-dihydroxy benzoic acid	1	1
catechol	53	67
	126[a]	158[a]
L-DOPA	100	100
(±)catechin	70	44
	130[b]	86[b]
(+)catechin	71	42
	124[b]	80[b]
resorcinol	0.7	1
hydroquinone	0.7	0
Trihydroxyphenol		
gallic acid	1.7	2
pyrogallol	20	18

[a]Absorbance was measured at 406 nm.
[b]Absorbance was measured at 433 nm.

Characterization of the glycosylated polyphenol oxidase

The optimum temperature of the three types of polyphenol oxidase was determined by measuring the initial rate of absorbance increase at 475 nm after addition of 0.1 ml of enzyme to 1.4 ml of 10 mM L-DOPA previously incubated at various temperature for 10 min prior to the addition of the enzyme. The optimum temperature was found to be 20℃. There is no significant difference between the three types of polyphenol oxidase even though slight thermal stability is shown in the case of type III polyphenol oxidase between 40 and 55℃ (data not shown). The optimum pH of the enzyme was determined after addition of 0.1 ml of enzyme to 1.4 ml of L-DOPA prepared in various buffer solutions. The optimum pH of the three types of polyphenol oxidase was found to be 7.0.

Substrate specificities of both type II and type III polyphenol oxidases were compared. Both types of the polyphenol oxidase did not use monohydroxy phenolics like tyrosine, tyramine, and vanillic acid as shown in Table 2. This might indicate that both types of polyphenol oxidase did not show monophenol monooxygenase (EC 1.14.18.1) under the assay conditions described in "Materials and Methods". Matheis (1987a) reported that potato tuber polyphenol oxidase has both monophenol monooxygenase (hydroxylase) activity and catecholase type activity, and adjustment of potato juice from pH 6 to pH 7.8 resulted in considerable loss of hydroxylase activity. It has been postulated that apparent absence of hydroxylase activity in many enzyme preparations is probably at least the result of methods of isolation and extraction, and hydroxylase activity may be detected by more sensitive means such as by tritium release (Mayer, 1987). The polyphenol oxidase purified here might need a lag time for hydroxylase activity as shown in animal tyrosinase (Hearing and Ekel, 1976). Therefore, it may not be possible to detect hydroxylase activity within 3 min, which is the enzyme reaction time in this experiment. L-DOPA, catechol, and catechin were more markedly oxidized by both types of polyphenol oxidase than chlorogenic acid, caffeic acid, and a trihydroxy phenol like pyrogallol. Because quinones produced from catechol and catechin have maximum absorbances at different wavelength, absorbances were measured at 406 nm for catechol and 433 nm for catechin in addition to 475 nm as shown in Table 2. It is noteworthy that type III polyphenol oxidase oxidized catechol more preferentially, whereas type II enzyme oxidized catechin more. This might indicate a difference in primary structure in addition to glycosylation between type II and type III polyphenol oxidases. Probably it is necessary to have the purified nonglycosylated form to explain the difference clearly.

The physiological role(s) of polyphenol oxidase in plants has not been established clearly and at present it is not known why polyphenol oxidase is latent in some species but not in others (Mayer, 1987). In the case of potato, polyphenol oxidase from glandular trichomes of wild type potato, *Solanum berthaultii* might be involved in defense against insects. Polyphenol oxidase in this glandular trichomes is supposed to be responsible for the O_2-requiring polymerization of trichome exudate, which results in entrapment of insect pests (Tingey, 1991). However, the physiological role(s) of potato tuber polyphenol oxidase is still unknown even though high polyphenol oxidase activity is found in potato tubers. We are presently trying to develop purification procedures for the nonglycosylated form and the latent form of polyphenol oxidase, if present. The development of such methods may provide a means to understanding of the signals for browning reactions and the physiological role(s) of potato tuber polyphenol oxidases in the future.

Acknowledgement

We greatly acknowledge Dr. Jongkee Kim (Department of Horticultural Crops Breeding, Chung-Ang University) for many helpful discussions. We also thank Dr. Hyun-Mook Cho (Rural Development Adminstration of Korea) for providing potatoes. This work was supported by the grant from Genetic Engineering Program (1993), Ministry of Education, Republic of Korea.

References

Arnon, D. I. (1949) *Plant Physiol.* **24**, 1.

Balasingham, K. and Ferdinand, W. (1970) *Biochem. J.* **118**, 15.

Bartlett, D. J., Poulton, J. E. and Butt, V. S. (1972) *FEBS Lett.* **23**, 265.

Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248.

Butt, V. S. (1979) *Recent Adv. Phytochem.* **12**, 433.

Butt, V. S. (1980) in The Biochemistry of Plant, Vol. 2 (Davies, D. D. ed.) pp. 81-123, Academic Press, San Diego, California.

Felton, G. W., Donato, K., Del Vecchio, R. J. and Duffey, S. (1989) *J. Chem. Ecol.* **15**, 2667.

Flurkey, W. H. (1986) *Plant Physiol.* **81**, 614.

Flurkey, W. H. and Jen, J. J. (1980) *J. Food Biochem.* **4**, 29.

Hearing V. J. and Ekel, T. M. (1976) *Biochem. J.* **157**, 549.

Hunt, M. D. Eannetta, N. T., Yu, H., Newman, S. M. and Steffens, J. C. (1993) *Plant Mol. Biol.* **21**, 59.

Kowalski, S. P., Eannetta, N. T., Hirzel, A. T. and Steffens, J. C. (1992) *Plant Physiol.* **100**, 677.

Laemmli, U. K. (1970) *Nature* **227**, 680.

Lax, A. R. and Vaughn, K. C. (1991) *Plant Physiol.* **96**, 26.

Matheis, G. (1987a) *Chem. Mikrobiol. Technol. Lebensm.* **11**, 5.

Matheis, G. (1987b) *Chem. Mikrobiol. Technol. Lebensm.* **11**, 33.

Mayer, A. M. (1987) *Phytochemistry* **26**, 11.

Mayer, A. M. and Harel, E. (1979) *Phytochemistry* **18**, 193.

Montalbini, P., Buchanan, B. B. and Hatcheson, S. W. (1981) *Physiol. Plant Pathol.* **18**, 51.

Park, W. D., Blackwood, C., Mignery, G. A., Hermodson, M. A. and Lister, R. M. (1983) *Plant Physiol.* **71**, 156.

Paulson, A. T., Vanderstoep J. and Porrit, S. W. (1980) *J. Food Sci.* **45**, 341.

Robb, D. A., Mapson, L. W. and Swain, T. (1965) *Phytochemistry* **4**, 731.

Robinson, S. P. and Dry, I. B. (1992) *Plant Physiol.* **99**, 317.

Tingey, W. M. (1991) in Naturally Occurring Pest Bioregulators (Hedin, P. A. ed.) pp. 126-135, American Chemical Society, Washington, D.C.

Tolbert, N. E. (1973) *Plant Physiol.* **51**, 234.

Vamos-Vigyazo, L. (1981) *CRC Crit. Rev. Food Sci. Nutr.* **15**, 49.