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Effect of high-pressure treatment on the activity of some polyphenoloxidases

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The activity of a commercial polyphenoloxidase extracted from mushrooms was found to decrease steadily with increasing applied pressure (100–800 MPa) and time (1–20 min) in phosphate buffer at pH 6.5. Complete inactivation was only achieved on treatment at 800 MPa for at least 5 min. In contrast, relatively mild heat treatment (60°C for 30 min) at atmospheric pressure led to total inactivation. The loss in activity was irreversible. Sephadex chromatography indicated that pressure treatment caused some aggregation of the enzyme.

Polyphenoloxidases extracted (but not purified) from mushrooms and potatoes responded differently to pressure. The enzyme in potatoes steadily lost activity with increasing applied pressure although after 10 min at 800 MPa about 40% of the activity remained. The mushroom extract exhibited a marked increase in activity after treatment at 400 MPa for 10 min (about 140% of the value of the untreated sample) and even after 10 min at 800 MPa considerable activity remained (about 60% of the original value). Possible reasons for these differences are discusssed.

When mushrooms were pressurized, considerable browning was observed immediately after pressurization, even at 200 MPa. Potatoes and apples browned to a limited degree on pressure treatment up to 600 MPa and browned still further on subsequent storage. Treatment at 800 MPa, of both potatoes and apples, caused litte browning but both foods took on a cooked appearance. It is concluded that high-pressure treatment alone is not a realistic means of inactivating these enzymes in food. Copyright © 1996 Elsevier Science Ltd.

INTRODUCTION

Polyphenoloxidases (EC 1.14.18.1), also known as tyrosinases, are copper-containing enzymes which catalyse two types of reactions, both involving oxygen. The first reaction corresponds to hydroxylation of monophenols to *o*-diphenols and the second to dehydrogenation of *o*-diphenols to *o*-quinones (Whitaker, 1972) and the biochemical mechanisms involved have been characterized (Zawistowski *et al.*, 1991).

These enzymes, which can exist in multiple forms (Jolley *et al.*, 1969*a*,*b*; Montgomery & Sgarbieri, 1975) are found in both plants and animals (Marshall *et al.*, 1984; Mayer, 1987; Mayer & Harel, 1979; Simpson *et al.*, 1987, 1988; Vámos-Vigyázó, 1981) and can give rise to the undesirable enzymic browning in many foods of plant origin.

Several methods have been used to inhibit enzymic browning in plant foods. For example, the exclusion of oxygen (Berk, 1976) or the addition of antioxidants such as ascorbic acid or sulphur dioxide (Golan-Goldhirsh & Whitaker, 1984; Embs & Markakis, 1965) are widely used. In some cases heat is used to inactivate the enzyme (Schwimmer, 1981).

In the last few years numerous studies have been reported involving the use of high pressures to preserve food since many organisms are sensitive to pressure, though spores are relatively resistant (Sale *et al.*, 1970; Butz *et al.*, 1990). Thus bacteria are destroyed by pressure treatment without the loss of flavour and colour often associated with heat treatment (Galazka & Ledward, 1995).

However, as well as destroying bacteria, high pressures may modify protein structure and thus modify enzymic behaviour (Galazka & Ledward, 1995). In recent years there has been much speculation about the use of high pressures (up to 800 MPa) to inactivate polyphenoloxidase enzymes, although the reported results are not clear cut. For example, Asaka & Hayashi (1991) found an increase in activity in Bartlett pears when subjected to pressures of 200–500 MPa, whereas Knorr (1993) found that pressures of 100–400 MPa led

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to a decrease in activity in potatoes. There appear to be no reports in the literature indicating the complete inactivation of these enzymes by pressure alone, although Eshtiaghi *et al.* (1994) have recently reported that at pH 7 in phosphate buffer, potato polyphenoloxidase is inactivated when treated at 900 MPa and 45°C for 30 min. At normal temperatures these pressures only inactivate the enzyme at low pH.

Potential benefits of high-pressure processing have been highlighted by many authors (Hayashi, 1992) and include retention of flavour, colour and nutritional quality, low energy usage, no environmental pollution and elimination of chemical additives. In view of its potential, the present study was undertaken to evaluate the effect of pressure on a commercially available mushroom tyrosinase and the tyrosinases found in three selected foods.

MATERIALS AND METHODS

Materials

L-Tyrosine, DL-3-4-dihydroxyphenylanine (DL-DOPA) and tyrosinase were obtained from Sigma Chemical Co. Fresh mushrooms, potatoes and apples were obtained from a local supermaket. All chemicals used were analytical grade.

Preparation of extracts

Chilled mushrooms or peeled potatoes were cut into small pieces (~10 mm^3) and homogenized with double their volume of 0.1 M sodium phosphate buffer, pH 6.8, containing 0.1 M sodium fluoride. The mixture was filtered through double muslin and centrifuged at 2224 *g* for 10 min at 5°C to remove insoluble material.

After centrifugation the mushroom extract was diluted 5 times with the buffer. The potato extract was not diluted since its activity was in the appropriate range for the assay.

Pressurization

The enzyme solution (500–1000 units/ml in 0.5 M phosphate, pH 6.5) or extract was sealed in a plastic bag (polyethylene) and subjected to pressures of 100–800 MPa for 1–20 min at ambient temperature in a lab model Stansted high-pressure rig (Stansted Fluid Power Ltd, Stansted, UK) as described previously (Defaye *et al.*, 1995). Activity was measured within 30 min.

Some samples pressure treated at 800 MPa were left in solution, at 4°C for 4 and 7 days and the activity reassessed.

Heat treatment

To compare pressure and heat treatments the enzyme solutions were heated in water baths at 20, 40 and 60°C for 30 min.

Enzyme assay

Commercial enzyme

The activity of tyrosinase from mushrooms was determined spectrophotometrically according to the Sigma method with modification.

A reaction cocktail containing deionized water (9 ml), 50 mM potassium phosphate, pH 6.5 (10 ml) and 1 mM L-tyrosine (10 ml) was mixed and adjusted to pH 6.5 at 25°C with 1 M HCl or 1 M NaOH if necessary. Air was bubbled through the reaction cocktail for 30 min. The mixture (2.9 ml) was pipetted into quartz cuvettes in a thermostatted spectrophotometer at 25°C until constant absorbance at 280 nm was achieved. To the blank was added 0.1 ml of potassium phosphate buffer, and to the sample 0.1 ml of the enzyme solution (500–1000 units/ml in phosphate). The solutions were immediately mixed by inversion of the cuvettes and the increase in absorbance at 280 nm recorded at 1 min intervals over 30 min.

Extract

Since there is a lag phase in colour development with L-tyrosine (see Results and Discussion), DL-DOPA was used as the substrate in this part of the study.

Activity was measured spectrophotometrically by observing the increase in absorbance at 475 nm due to the formation of DOPA-quinone from DL-DOPA. The reaction mixture containing 0.1 M sodium phosphate buffer, pH 6.8 (1.8 ml), 4 mg/ml DOPA in phosphate buffer (1.0 ml) was pipetted into quartz cuvettes in a thermostatted spectophotometer at 30°C. To the blank was added 0.2 ml of phosphate buffer (pH 6.8) and to other cuvette 0.2 ml of the enzyme solution. The cuvettes were immediately inverted and the increase in absorbance at 475 nm recorded over 10 min at 30 s intervals

Each activity quoted is the mean of at least three separate experiments; individual values were always within 10% of this mean.

Sephadex chromatography

0.5 ml of commercial enzyme solution (2 mg/ml), native and after pressurization at 800 MPa for 1 min and after heating at 60°C for 30 min, was separated on Sephadex G-100 at a flow rate of 30 ml/h. The eluting buffer (50 mM phosphate, pH 6.5) was chilled in ice and the absorbance of the fractions (75 drops) determined at 220 nm.

Whole tissue studies

Potatoes, mushrooms and apples were cut into pieces (~10 mm^3), sealed in polyethylene bags and subjected to pressures of 200, 400, 600 and 800 MPa for 10 min.

After treatment the samples were held at room temperature (~22°C) and the colour assessed visually. Photographs were also taken of the freshly processed and stored products.

RESULTS AND DISCUSSION

Commercial enzyme

There was an initial induction or lag period when the enzyme was assayed with the substrate tyrosine. The time of treatment had little effect on this lag phase but it was affected by the pressure. It is seen that treatment at 100 MPa had no effect on the duration of the lag phase, but it doubled at 200–400 MPa; above 400 MPa it increased rapidly with increasing pressure (Fig. 1). The activity of the enzyme was calculated from the slope of the absorbance–time plots following this lag phase.

Decreased tyrosinase activity was observed with both increasing pressure and time. Complete inactivation was only seen after 5 min at 800 MPa (Fig. 2). As expected, heat treatment also reduced the activity, a 60% reduction was observed after treatment at 40°C for 30 min. The loss of activity was almost 100% at 60°C after 30 min.

It has been reported that latent or inactive forms of polyphenol oxidase can be activated by treatments such as acid–base shock, anionic detergents (SDS—sodium dodecylsulphate), urea, protease enzymes and fatty acids. It has also been reported (Asaka & Hayashi, 1991) that the enzyme in Bartlett pears can be activated by pressure. The results though are not unequivocal since it has been claimed that mushroom tyrosinase can be activated by SDS (Rodriguez & Flurkey, 1992; Yamaguchi *et al.*, 1970); some reports (Angleton & Flurkey, 1984) claim that it is not activated by SDS.

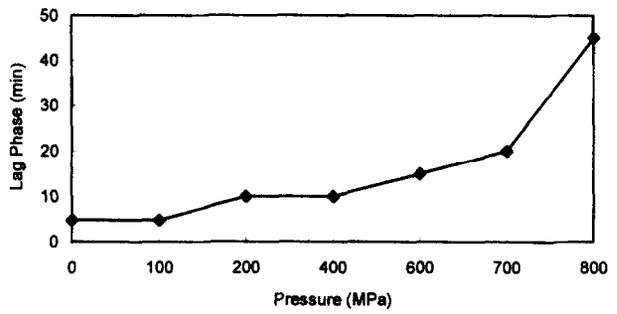

Fig. 1. Effect of pressure on length of the lag phase observed with the mushroom tyrosinase assay following treatment of the enzyme at the pressures indicated.

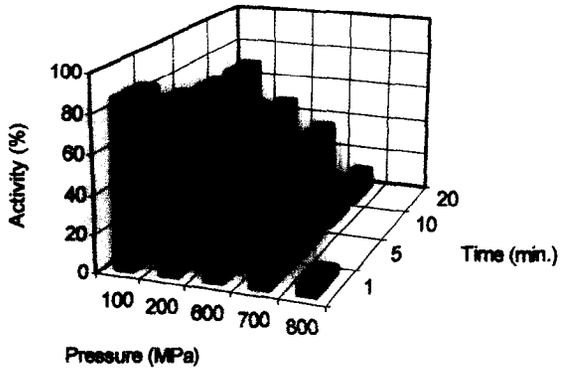

Fig. 2. Inactivation of tyrosinase from mushrooms as a function of pressure and time.

The decrease in activity of the mushroom tyrosinase seen on pressure treatment in this study suggests the absence of latent enzyme activity, or at least activity that can be activated by pressure.

Pressure-induced changes in molecular size of this tyrosinase were seen on Sephadex chromatography. Pressure treatment appears to lead to the formation of some higher molecular weight aggregates (Fig. 3). Numerous studies have shown that the application of pressure will lead to conformational changes in many proteins (Heremans, 1982; Weber & Drickamer, 1983). As a general rule, reversible effects are observed at relatively low pressures (100 and 200 MPa); these usually involve dissociation of oligomeric proteins into subunits. At pressures higher than about 200 MPa, many proteins tend to unfold and reassociation of subunits and dissociated oligomers can occur. Several authors (Defaye *et al.*, 1995; Hawley & Mitchell, 1975; Silva & Weber, 1993) have found that, under certain conditions, pressure-induced dissociation and/or denaturation is reversible, but after pressure release the renaturation process can be slow and both hysteresis behaviour and conformational drift have been observed. It is now well established that a globular protein may give a modified molecular conformation following pressure treatment. The pressure-treated form of ovalbumin has been shown to differ from the native form (Hayakawa *et al.*, 1992) and it has been demonstrated that pressure induces dimerization of metmyoglobin (Defaye & Ledward, 1995). As well as leading to changes in size, the renaturation process often gives rise to stable, soluble forms of the protein that have modified secondary structures, there is usually some loss of α-helix content. No detailed conformational analysis was carried out on the tyrosinase, but the aggregation and probable change in secondary structure has presumably modified the active site thus decreasing its activity. It is perhaps noteworthy that whereas relatively mild treatment at 60°C for 30 min appears to totally inactivate the commercial enzyme, even after high-pressure treatment at 700 MPa there is still some residual activity (Fig. 2). The destruction of the active site is obviously not an all or nothing phenomenon but rather increased severity of treatment leads to increased modification.

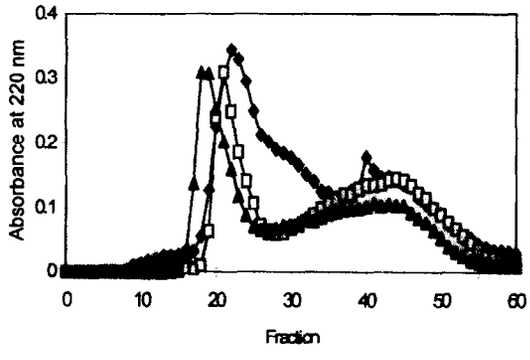

Fig. 3. Elution profile of tyrosinase from mushrooms on Sephadex G-100. (◆), native; (▲), pressure-treated (800 MPa for 1 min); (□), heat-treated (60°C for 30 min).

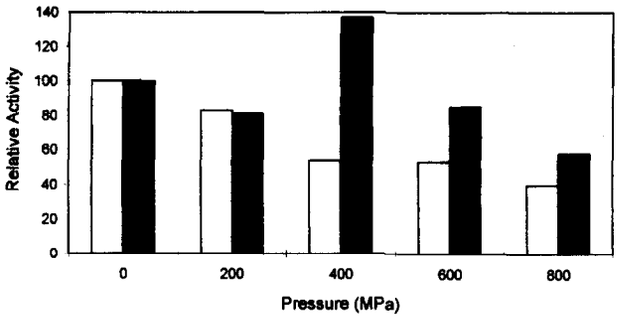

Fig. 4. Effect of high-pressure treatment for 10 min on the tyrosinase activity in extracts of (□) potato and (■) mushroom. All determinations were within 10% of the mean value shown.

On storage at 4°C for 7 days there was no measurable increase in activity of the sample pressure treated at 800 MPa, indicating that the loss of activity was irreversible.

Extract

It is well stablished that polyphenoloxidases from different sources may have different molecular sizes and conformations. For this reason it was considered worthwhile to compare an extract prepared from mushrooms with one from another food (potatoes). This would also enable a comparison to be made of the effects of high pressure on both a purified enzyme and a crude extract. Since the induction phase seen with the conversion of the mono to the diphenols complicated the assay and would be very extended with the impure extract, it was decided to use DOPA as the substrate in this study (Friedman & Daron, 1977).

As expected in these studies the absorbance increased with time for about 4 min after which it decreased as a result of further autoxidation and polymerization of DOPA (Friedman & Daron, 1977).

A reduction in the activity of the crude extract of potato was observed with increasing pressure (Fig. 4). This agrees with the results of Knorr (1993) who found a reduction in tyrosinase activity when potato cubes were subjected to pressure.

In contrast, when the crude extract of mushroom was treated at 400 MPa an enhancement in activity was observed (Fig. 4) although treatment at 200 MPa, led to a significant decrease. This was not observed in the purified, commercial preparation (Fig. 2). Pressures above 400 MPa led to the expected decrease.

Owing to the unexpected nature of the result observed at 400 MPa, the value quoted is the mean of 10 experiments. All others treatments were the mean of three experiments.

The enhancement in activity observed in the crude extract of mushroom at 400 MPa for 10 min may arise from changes due to interactions with other constituents in the extract or from the release of membrane-bound enzymes, and may relate to the pressure-activated latent activity found in Bartlett pears (Asaka & Hayashi, 1991).

Treatment of whole foods

The above results were found either after processing a commercially available mushroom enzyme or the crude extracts obtained from the foods. However, many subsidiary experiments showed that, when some whole fruits or vegetables were subjected to pressure, the pressure *per se* caused browning. The results are summarized in Table 1, and it can be seen that pressure treatment of mushrooms at 200 MPa for 10 min, caused the samples to brown quite markedly. Increasing pressure caused the intensity of colour to increase and was accompanied by the exudation of a large amount of black–brown fluid. The tissue became very soft. There was little browning in cut mushrooms left for 1 h, unpressurized at room temperature (Table 1).

With potatoes, the application of pressures from 200 to 600 MPa for 10 min induced some slight browning and when left at room temperature for 1 h they continued to brown (Table 1). There was no major difference in the appearance of any of the samples. However, when treated at 800 MPa for 10 min the potatoes took on a cooked yellowish appearance, but there was no further change in colour during 1 h at room temperature and normal pressure. This would suggest that the result found with the crude extract can not be applied directly to the potatoes since, in the extract, there is a steady decrease in activity with increasing pressures, with some residual activity even after treatment at 800 MPa. It must be remembered that the pH of the phosphate-buffered systems will decrease under pressure

Table 1. The degree of browning* observed in three foods immediately after pressure treatment (fresh) and following 1 h storage at 22°C (stored)

	Colour intensity					
	Fresh			Stored		
Pressure (MPa)	Apple	Mushroom	Potato	Apple	Mushroom	Potato
0	1	1	1	2	2	2
200	3	5	2	4	5	3
400	3	5	2	4	5	3
600	3	5	2	4	5	3
800	1	5	1	1	5	1

*1, No browning; 2, slightly brown; 3, brown; 4, dark brown; 5, very dark brown.

(Heremans, 1982), and although the pH of the whole food may also decrease, the extent may differ. However, it is very unlikely such an effect could explain the difference between the behaviour of the extracts and the whole foods.

Apples browned quite markedly following treatment at pressures in the range of 200–600 MPa for 10 min and, on leaving for a further 1 h at room temperature and normal pressure, they became even browner (Table 1). After treatment at 800 MPa for 10 min the apples took on a cooked yellowish appearance, but no further colour change was observed during subsequent storage at room temperature for 1 h and normal pressure. Thus their behaviour was similar to that observed with the potatoes.

This paper has demonstrated that polyphenoloxidase activity in several foods is modified by pressure. However, even with the limited number of foods studied it is readily apparent that high pressure alone is not a realistic means of inhibiting the enzymes. They only become inactivated at pressures which cause irreversible damage to the tissue itself.

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REFERENCES

Angleton, E. L. & Flurkey, W. H. (1984). Activation and alteration of plant and fungal polyphenoloxidase isoenzymes in sodium dodecylsulphate electrophoresis. *Phytochemistry*, **23**, 2723–5.

Asaka, M. & Hayashi, R. (1991). Activation of polyphenoloxidase in pear fruits by high pressure treatment. *Agric. Biol. Chem.*, **55**, 2439–40.

Berk, Z. (1976). Control of enzymic browning. In *Introduction to the Biochemistry of Foods*. Elsevier Science, Amsterdam, pp. 252–6.

Butz, P., Traugott, U., Ludwig, H., Ries, J. & Weber, H. (1990). The high pressure inactivation of bacteria and bacterial spores. *Pharm. Ind.*, **52**, 487–91.

Defaye, A. B. & Ledward, D. A. (1995). Pressure induced dimerization of metmyoglobin. *J. Food. Sci.*, **60**, 262–5.

Defaye, A. B., Ledward, D. A., MacDougall, D. B. & Tester, R. F. (1995). Renaturation of metmyoglobin subjected to high isostatic pressure. *Food. Chem.*, **52**, 19–22.

Embs, R. J. & Markakis, P. (1965). The mechanism of sulfite inhibition of browning caused by polyphenol oxidase. *J. Food Sci.*, **30**, 753–8.

Eshtiaghi, M. N., Stute, R. & Knorr, D. (1994). High-pressure and freezing pretreatment effects on drying, rehydration, texture and color of green beans, carrots and potatoes. *J. Food Sci.*, **59**, 1168–70.

Friedman, M. E. & Daron, H. H. (1977). Tyrosinase—An introductory experiment with enzymes. *J. Chem. Educ.*, **54**, 256–7.

Galazka, V. B. & Ledward, D. A. (1995). High pressure treatment of food. *Food Technol. Int. Eur.*, 123–5.

Golan-Goldhirsh, A. & Whitaker, J. R. (1984). Effect of ascorbic acid, sodium bisulfite, and thiol compounds on mushroom polyphenol oxidase. *J Agric. Food Chem.*, **32**, 1003–9.

Hawley, S. A. & Mitchell, R. M. (1975). An electrophoretic study of reversible protein denaturation: chymotrypsinogen at high pressures. *Biochemistry*, **14**, 3257–64.

Hayakawa, I., Kajihara, J., Morikawa, K., Oda, M. & Fujio, Y. (1992). Denaturation of bovine serum albumin (BSA) and ovalbumin by high pressure, heat and chemicals. *J. Food Sci.*, **57**, 288–92.

Hayashi, R. (1992). Utilization of pressure in addition to temperature in food science and technology. In *High Pressure and Biotechnology*. Vol. 224, eds C. Balny, R. Hayashi, K. Heremans & P. Manson. Colloque INSERM, John Libbey Eurotext Ltd, pp. 185–93.

Heremans, K. (1982). High pressure effects on proteins and others biomolecules. *Ann. Rev. Biophys. Bioengng*, **11**, 1–21.

Jolley, R. L. Jr, Robb, D. A. & Manson, H. S. (1969*a*). Multiple forms of mushroom tyrosinase. Association–disssociation phenomena. *J. Biol. Chem.*, **244**, 1593–9.

Jolley, R. L. Jr, Nelson, R. M. & Robb, D. A. (1969*b*). The multiple forms of mushroom tyrosinase. Structural studies on the isoenzymes. *J. Biol. Chem.*, **244**, 3251–7.

Knorr, D. (1993). Effects of high-hydrostatic-pressure processes on food safety and quality. *Food Technol.*, **47**, 156–61.

Marshall, M. R., Otwell, W. S. & Walker, B. (1984). Preliminary study on the isolation and activation of polyphenoloxidase from deep sea crab (*Geryon sp.*). *Proc. Ninth Ann. Trop. Subtrop. Conf. Am.*, **9**, 118–20.

Mayer, A. M. (1987). Polyphenoloxidases in plants—recent progress. *Phytochemistry*, **26**, 11–20.

Mayer, A. M. & Harel, E. (1979). Review: Polyphenoloxidases in plants. *Phytochemistry*, **18**, 193–215.

Montgomery, M. W. & Sgarbieri, V. C. (1975). Isoenzymes of banana polyphenol oxidase. *Phytochemistry*, **14**, 1245–9.

Rodriguez, M. O. & Flurkey, W. H. (1992). A biochemistry project to study mushroom tyrosinase—enzyme localization, isoenzymes, and detergent activation. *J. Chem. Educ.*, **69**, 767–9.

Sale, A. J. H., Gould, G. W. & Hamilton, W. A. (1970). Inactivation of bacteria spores by hydrostatic pressure. *J. Gen. Microbiol.*, **60**, 323–34.

Schwimmer, S. (1981). Food color, the phenolases and undesirable enzymatic browning. In *Source Book of Food Enzymology*. AVI Publishing Co., Westport, Connecticut, pp. 267–83.

Silva, J. L. & Weber, G. (1993). Pressure stability of proteins. *Ann. Rev. Phys. Chem.*, **44**, 89–113.

Simpson, B. K., Marshall, M. R. & Otwell, W. S. (1987). Phenoloxidases from shrimp (*Penaeus setiferus*): purification and some properties. *J. Agric. Food Chem.*, **35**, 918–21.

Simpson, B. K., Marshall, M. R. & Otwell, W. S. (1988). Phenoloxidases from pink and white shrimp: Kinetic and other properties. *J. Food Biochem.*, **12**, 205–17.

Vámos-Vigyázó, L. (1981). Polyphenoloxidase and peroxidase in fruits and vegetables. *CRC Crit. Rev. Food Sci. Nutr.*, **15**, 49–127.

Weber, G. & Drickamer, H. G. (1983). The effect of high pressure upon proteins and other biomolecules. *Q. Rev. Biophys.*, **16**, 89–112.

Whitaker, J. R. (1972). Polyphenol oxidase. In *Principles of Enzymology for the Food Sciences*, ed. O. R. Fennema. Marcel Dekker, New York, pp. 571–82.

Yamaguchi, M., Hwang, P. M. & Campbell, J. D. (1970). Latent *o*-diphenol oxidase in mushrooms (*Agaricus bisporus*). *Can. J. Biochem.*, **48**, 198–202.

Zawistowski, J., Biliaderis, C. G & Eskin, N. A. M. (1991). In *Oxidative Enzymes in Foods*, eds D. S. Robinson & N. A. M. Eskin. Elsevier Applied Science, London, pp. 234–9.