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Evaluation of Combined Effects of Heat Treatment and Antioxidant on Peroxidase Activity of Crude Extract of Green Peas

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ABSTRACT

The combined effects of heat treatment and antioxidant addition on residual peroxidase activity and isoenzyme patterns of green peas were compared with heat treatment alone.

The changes of pea peroxidase isoenzyme patterns during heat treatment of crude extracts with and without antioxidants were observed by analytical thin-layer isoelectric focusing (pH 3·5–9·5). The determination of peroxidase activities in the heat-treated samples was in agreement with an overall decrease in the number of isoenzymes detected by specific staining of the polyacrylamide gels.

The antioxidants during heat treatment decreased the apparent number of peroxidase isoenzymes as well as the residual peroxidase activity. The antioxidants, TBHQ, BHA, BHT, and α-tocopherol were tested at concentrations ranging from 4×10^{-6} M *to* 1×10^{-2} M.

INTRODUCTION

Peroxidase, one of the most heat-resistant enzymes found in plants, is conventionally used as an index of processing adequacy for blanching or other thermal processing of food (Richardson & Hyslop, 1985). Some problems, however, in the use of peroxidase as an indicator of adequate blanching for vegetables have been indicated. Under certain conditions of limited heat treatment, peroxidase can regain activity during storage, which results in quality changes in foods. Although the rate and extent of reactivation vary from one sample to another, it is known that the faster the temperature is raised to a given value, the greater the extent of regeneration of peroxidase (Vetter *et al.*, 1959; Resende *et al.*, 1969; Lu & Whitaker, 1974). Numerous studies indicated that the regeneration of peroxidase activity could be partially attributable to the presence of multiple forms of isoenzymes with different thermal stabilities (Wang & DiMarco, 1972; Delincée & Shaefer, 1975; Burnett, 1977).

Yet, considerable evidences indicate that peroxidase may not be directly responsible for the quality deterioration during frozen storage of vegetables, and thus heating to complete inactivation of peroxidase will lead to overheating (Böttcher, 1975; Williams *et al.*, 1986).

In an attempt to optimize the thermal inactivation and to prevent subsequent regeneration of peroxidase activity, the antioxidant was combined with heat treatment. The objective of this study was to determine whether the combination of heat treatment and antioxidant was an effective method for inactivation of green pea peroxidase. The effectiveness of the peroxidase inactivation was measured by the residual peroxidase activity and changes in peroxidase isoenzyme patterns. Utilizing analytical thin-layer isoelectric focusing on polyacrylamide gel plates, isoenzyme patterns of heat treated samples both with and without antioxidants and the unheated controls, were identified and compared.

EXPERIMENTAL

Materials and reagent

The four antioxidants, tertiary butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *d*-α-tocopherol, acrylamide, *N,N'*-methylen-bisacrylamide (BIS), *N,N,N'*-tetra-methyl-ethylene-diamine (TEMED), Coomassie Blue Brilliant Blue R-250, ammonium persulfate, dimethylformamide, 4-aminoantipyrine, hydrogen

peroxide (30%) and phenol were purchased from Eastman Kodak Co., Rochester, New York.

Bovine serum albumin, hemoglobin (horse) and *o*-dianisidine were purchased from Sigma Chemical Co., St. Louis, Missouri.

The Multiphor (LKB 2117-301) basic unit for electrofocusing was used with a constant power supply (LKB 2103). The carrier ampholyte solutions ('Ampholine'—various pH ranges) and all other equipment for analytical isoelectric focusing in thin-layers of polyacrylamide gel were obtained from LKB-Produkter AB, Bromma, Sweden.

Fresh green peas (*Pisum sativum*, variety Frisky) were grown by the Department of Horticulture, University of Illinois.

Sample preparation

Fresh green peas were ground in a Waring Blender with dry ice for 10 min until pea powder was obtained. The pea powder was stirred with the same weight of 10 mM sodium phosphate buffer (pH 7·0) for 2 h at 4°C. The crude extract was filtered through four layers of cheesecloth and centrifuged at 40 000 *g* for 30 min in a Beckman model JA-17. The supernatant was centrifuged at 105 000 *g* for 30 min at 4°C in an International Preparative Ultracentrifuge, Model B-60. The greenish supernatant was dialyzed against 1 mM phosphate buffer for 16 h with more than three changes of buffer solutions, and centrifuged at 40 000 *g* for 20 min. The final superantant (I) was further diluted to adjust the protein concentration to approximately 1·5–3·0 mg/ml. The diluted supernatant (II) was used in all subsequent experimental steps. The protein concentration of the eluate was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard. All the extraction and dialysis procedures were performed at 4°C.

Heat treatment and antioxidant addition

Antioxidants used for this experiment were BHA, BHT, TBHQ, and α-tocopherol. An aliquot (20 μl) of antioxidant dissolved in absolute ethyl alcohol was added to the enzyme solution (supernatant II) immediately prior to heat treatment. The maximum amount of ethyl alcohol added to the sample was 1% of the total sample volume used. An identical amount of ethyl alcohol was added to controls. The concentrations of antioxidants used were 4×10^{-6} M to 1×10^{-2} M.

For heat treatment, samples (3 to 6 mg protein/2 ml), with and without antioxidant, were placed in culture tubes (15 × 100 mm) and heated in a temperature controlled water-bath for 1, 2, 5, 10 or 20 min at 90°C. The

reaction was terminated by cooling in ice and the samples were centrifuged at 40 000 *g* for 20 min. The supernatant (III) of each heated sample was assayed to determine residual peroxidase activity. The heat-treated samples (supernatant III), both with and without antioxidant, were applied to analytical thin-layer isoelectric focusing on polyacrylamide gel (PAG) plates. All treatments were repeated three times.

Analytical isoelectric focusing (IEF)

Analytical IEF in thin-layers of polyacrylamide gel (PAG) was performed following the modified method of Righetti & Drysdale (1976) and Davies (1975).

To obtain better resolution and minimize the streaking effect, which was often observed with samples of crude extracts, concentrations of acrylamide and Ampholine were increased. The final gel plates were pH 3·5–9·5 with Ampholine 3% (w/v) and T = 7%, C = 3%, 110 mm × 245 mm × 2 mm.

Previous reports indicated that atmospheric CO_2 could substantially reduce the pH of basic pH ranges and cause cathodic drift or nonlinearity of the gradient (Chrambach *et al.*, 1973; Delincée & Radola, 1976). In this experiment, to minimize the absorption of atmospheric CO_2 into the gel, 100 ml of 0·01M NaOH was added to the buffer tank of the Multiphor system.

The detailed methods for the preparation of PAG plates, sample applications, running time and electrofocusing parameters, and staining and destaining procedures were exactly followed as described by Lee & Klein (1988).

Peroxidase activity

here as a typical example. The results were based on four replications (two The peroxidase activity was measured spectrophotometrically following the procedures described in Worthington Enzymes (1978), using 0·002 5M 4-aminoantipyrine with 0·17M phenol solution and 0·001 7M hydrogen peroxide in 0·2M phosphate buffer, pH 7·0, as substrate. The reaction mixture consisted of 1·5 ml of 0·001 7M hydrogen peroxidase in 0·2M potassium phosphate buffer and 1·4 ml of phenol/4-aminoantipyrine. The mixture was equilibrated at 25°C, and the reaction was initiated after addition of 0·1 ml of enzyme solution. The change in absorbance at 510 nm was recorded using a Beckman Model 25 Spectrophotometer at 25°C. One unit of peroxidase activity was defined as a change of 0·001 absorbance unit (510 nm) per minute under the experimental conditions used.

RESULTS AND DISCUSSION

Combined effects of antioxidant and heat treatment on residual peroxidase activity

To examine if the antioxidants alone had any inhibitory or denaturing effect on peroxidase, the peroxidase activity assay was performed with unheated, crude extracts (supernatant II), and four antioxidants at various concentrations. The antioxidants tested in this experiment were TBHQ, BHA, BHT and α-tocopherol, with concentrations ranging from 4×10^{-6}M to 1×10^{-2}M. As shown in Table 1, peroxidase activity of crude extracts of green peas was not inhibited by antioxidant alone at the concentrations of the antioxidants tested. Mean values of the residual activity ranged from 100·0% to 92·3%.

Figure 1 shows the typical example of effects of heat treatment and antioxidant on residual peroxidase activities. Among all the concentrations tested, 4×10^{-4}M and 4×10^{-3}M reacted similarly. Also, it was relatively easy to obtain uniform straight lines of peroxidase activities with these concentrations. Therefore, the antioxidant, TBHQ, 4×10^{-4}M was shown here as a typical example. The results were based on four replications (two treatments × two replications). When the crude extract of peas was heated at

TABLE 1
Effect of Antioxidants on Crude Extracts (Unheated) of Green Peas at Different Concentrations

Antioxidants	*Concentration (mole/litre)*	*Peroxidase activity (units/ml)*[a]	*Residual activity (%)*[a]
Control	0	4 100 ± 283	100·0
BHA	1×10^{-2}	3 945 ± 64	96·5 ± 8·2
	4×10^{-3}	3 905 ± 64	95·0 ± 5·1
	4×10^{-4}	4 195 ± 148	98·5 ± 2·2
BHT	1×10^{-2}	4 040 ± 268	100·0 ± 2·8
	4×10^{-3}	4 083 ± 208	99·5 ± 0·7
	4×10^{-4}	3 900 ± 15	95·4 ± 6·6
TBHQ	4×10^{-3}	3 990 ± 197	97·3 ± 0·6
	4×10^{-4}	4 042 ± 266	98·5 ± 2·1
	4×10^{-5}	3 954 ± 186	96·5 ± 2·1
	4×10^{-6}	4 036 ± 192	98·5 ± 2·1
α-Tocopherol	1×10^{-2}	3 807 ± 151	93·0 ± 2·8
	4×10^{-3}	3 780 ± 178	92·3 ± 2·2
	4×10^{-4}	3 900 ± 282	95·1 ± 2·8

[a] Values are means and standard deviations, $n = 4$.

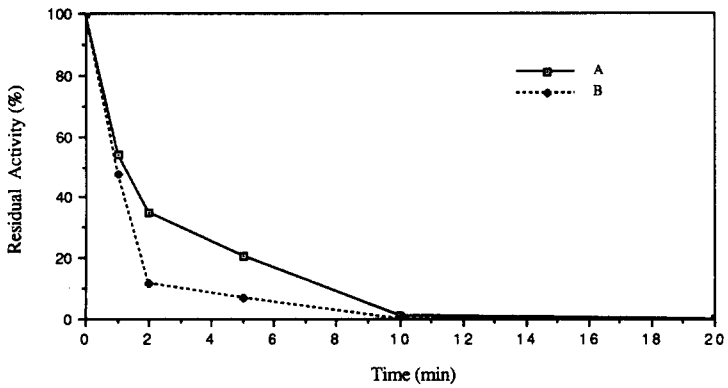

Fig. 1. Combined effects of heat treatment and antioxidant on residual peroxidase activity. A—heat treatment only, B—heat treatment with antioxidant.

90°C, 50% of the peroxidase activity was inactivated after 1·25 min of heat treatment. After 2 min of heat treatment, 34·8% of the peroxidase activity remained. Even after 5 min of heating, 20·4% of the peroxidase was left. When TBHQ was added, 50% of peroxidase activity was inactivated in less than 1 min. Further, residual peroxidase activities were reduced to 11·7% and 6·9% after 2 min and 5 min of heat treatment, respectively. Thus, the residual enzyme activity was noticeably reduced by the presence of antioxidant.

TABLE 2

Effects of Antioxidant Concentrations on Residual Peroxidase Activity of Crude Pea Extract (Heat-treated at 90°C for 2 min)

Antioxidant	*Concentration (mole/litre)*	*Peroxidase activity (units/ml)*[a]	*Residual activity (%)*[a]
Control	0	1 800 ± 250	100·0
BHA	1×10^{-2}	932 ± 259	51·6 ± 1·5
	4×10^{-3}	829 ± 265	42·8 ± 1·7
	4×10^{-4}	884 ± 253	51·7 ± 2·9
BHT	1×10^{-2}	987 ± 205	54·3 ± 3·4
	4×10^{-3}	792 ± 248	43·5 ± 3·1
	4×10^{-4}	945 ± 228	52·6 ± 0·5
TBHQ	4×10^{-3}	1 280 ± 191	72·4 ± 5·5
	4×10^{-4}	797 ± 225	44·0 ± 1·5
	4×10^{-5}	733 ± 140	41·3 ± 2·8
	4×10^{-6}	717 ± 200	39·6 ± 1·6
α-Tocopherol	1×10^{-2}	548 ± 234	24·0 ± 4·5
	4×10^{-3}	640 ± 231	35·0 ± 4·5
	4×10^{-4}	623 ± 172	34·5 ± 1·6

[a] Values are means and standard deviations, $n = 6$.

For further study of the combined effect of antioxidant and heat treatment on inactivation of peroxidase activity, all four antioxidants at various concentrations were examined using 2 min of heat treatment at 90°C (Table 2). This treatment was selected because the fastest decrease in peroxidase activity occurred during the first 2 min of heating. The reductions in peroxidase activity with increasing concentrations of the antioxidants BHA and BHT were similar. At the levels used in this experiment, a 50% further reduction in peroxidase activity as compared to the heat-treated control was observed. TBHQ was apparently more effective at low concentrations (4×10^{-4}M, 4×10^{-5}M, 4×10^{-6}M) than at a higher concentration (4×10^{-3}M). At equal concentrations (4×10^{-4}M), α-tocopherol was most inhibitory, followed by TBHQ, BHA and BHT. At relatively high levels (1×10^{-2}M), α-tocopherol was more effective than BHA and BHT. α-Tocopherol has been considered by other researchers to be a weak antioxidant (Hamilton & Tappel, 1963). In this experiment, α-tocopherol, however, proved to be an effective antioxidant when combined with heat treatment.

Combined effects of antioxidants and heat treatment on peroxidase isoenzyme patterns

The change of peroxidase isoenzyme patterns during heat treatment of crude extracts with and without antioxidant was clearly shown on analytical thin-layer IEF on PAG plate (pH 3·5–9·5). In Fig. 2, typical isoenzyme patterns of unheated (0 time) and heat-treated crude extracts, with and without the antioxidant, TBHQ (4×10^{-4}M), are shown. Analytical IEF profiles of unheated (control) samples of crude extracts of fresh peas indicated the presence of 14 peroxidase isoenzymes with pI values from 3·8 to 9·3. After 1 min heating without antioxidant, only minor isoenzymes (pI values 9·3 and 7·7) were inactivated, even though the residual peroxidase activity was reduced to 54·2%. Even after 10 min, six isoenzymes were still present. However, in the presence of the antioxidant (TBHQ), the inactivation of peroxidase isoenzymes was more pronounced. After 5 min of heat inactivation at 90°C, five isoenzymes remained in the antioxidant (TBHQ) treated samples, as compared to nine isoenzymes in the samples without antioxidant. It should be noted that after 20 min of heat treatment with antioxidant (TBHQ), almost no trace of peroxidase was observed. Furthermore, the intensity of many isoenzyme bands was greatly decreased, indicating the effectiveness of antioxidant on inhibition of peroxidase activity during heat treatment. In contrast, without antioxidant, even after 20 min of heat treatment, activity of the most heat-resistant isoenzymes (pI values 6·9, 4·1, and 3·8) was persistent. The patterns of isoenzymes with four different antioxidants after 2-min heat treatment were similar.

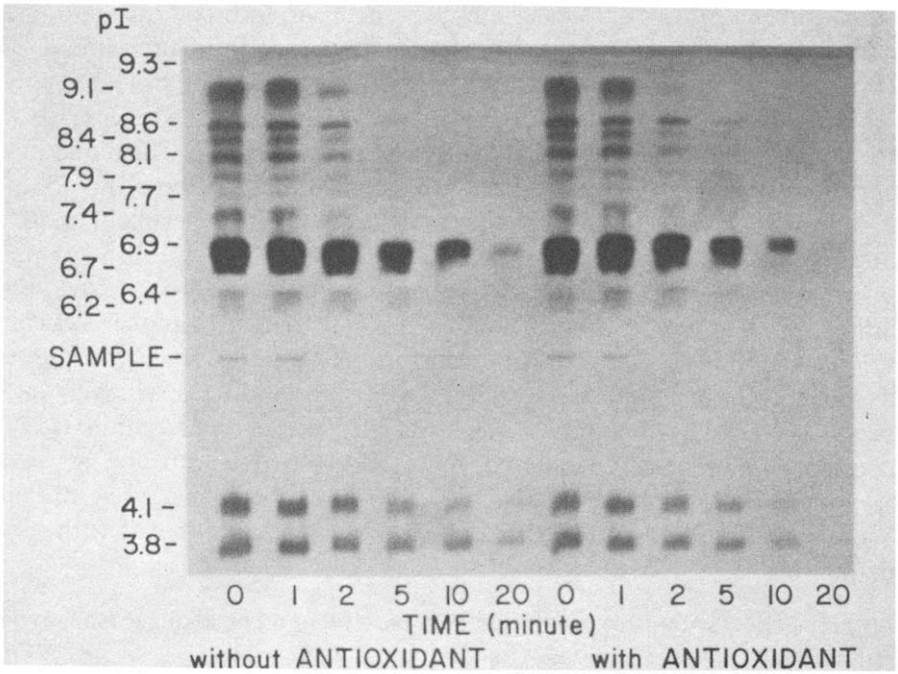

Fig. 2. A typical isoenzyme profile of heat-treated samples with and without antioxidant, TBHQ (4×10^{-4}M).

The residual peroxidase activity of the crude extracts treated with antioxidant and heat for 10 min was not measurable spectrophotometrically. However, the most heat-resistant isoenzymes (pI values 6·9, 4·1, and 3·8) were detected with the enzyme specific staining on the PAG plate. This may be due to the fact that hydrogen donors used in this experiment, *o*-dianisidine and 3-amino-9-ethyl carbazole, are 10 to 20 times more sensitive than other hydrogen donors used as substrates (Delincée & Radola, 1972).

The residual peroxidase activity and the number of isoenzymes after heat and antioxidant treatment are shown in Table 3. The relative activity of the crude extracts decreased drastically in the first minute of heating. However, the number of isoenzymes present did not change substantially until the extract had been heated for 5 min. The relative intensity of each band decreased slightly with each increment in heating time (Fig. 2), suggesting that the loss of activity in the first 5 min was due to partial loss of activity of the isoenzymes, rather than complete inactivation of several isoenzymes. Between 2 min and 5 min, a substantial decrease in the number of isoenzymes occurred. Those isoenzymes with pI values of 6·0, 4·12 and 3·8 appear to be most heat stable, with and without antioxidant.

This study indicated that the addition of antioxidant to crude extracts

TABLE 3
Residual Peroxidase Activity and Number of Isoenzymes of Heat-treated Samples with and without Antioxidant (TBHQ, 4×10^{-4}M)

Treatment	*Time (min)*	*Peroxidase activity (units/ml)*[a]	*Residual activity (%)*[a]	*Number of isoenzymes*
Without antioxidant	0	3825 ± 106	100·0	14
	1	2075 ± 106	54·2 ± 1·3	13
	2	1330 ± 42	34·8 ± 0·1	12
	5	780 ± 14	20·4 ± 1·0	9
	10	30 ± 5	0·8 ± 0·0	6
	20	0	0·0	3
With antioxidant	0	3550 ± 254	100·0	14
	1	1800 ± 28	47·7 ± 4·4	13
	2	415 ± 21	11·7 ± 1·8	12
	5	245 ± 64	6·9 ± 2·1	5
	10	0	0·0	3
	20	0	0·0	0

[a] Values are means and standard deviations, $n = 4$.

during heat treatment more effectively reduced the residual peroxidase activities and the number of isoenzymes than the heat treatment alone. This might be due to the fact that antioxidants were possibly effective in inhibition of both non-enzymatic and enzymatic oxidation of peroxidases. Eriksson & Vallentin (1973) showed that non-enzymatic action of peroxidase increased significantly when peroxidases were heat treated. They postulated that the increase in non-enzymatic activity was due to the exposure of heme groups. The heat treatment caused unmasking of catalytically active heme groups in thermally formed aggregates. These aggregates increased active oxidation sites. Antioxidants might have inhibited this non-enzymatic oxidation in this study. Also, antioxidant might have inhibited the enzymatic oxidation of heme groups from Fe^{III} to Fe^{V}, preventing the formation of the peroxidase-Fe^{V} free radical complex. This complex is an intermediate of the oxidative peroxidase reaction and essential for its catalytic activity (Walsh, 1979). Therefore, when the formation of this intermediate complex was inhibited by antioxidant, the residual peroxidase activity was significantly reduced.

These observations indicated that the use of antioxidant combined with heat treatment will more effectively inactivate the peroxidase activity in green peas than heat treatment alone without prolonged exposure to

thermal processing. This method, therefore, may be useful for thermal inactivation of peroxidase, thus enabling the better retention of heat sensitive nutrients and sensory attributes of foods by reducing the thermal processing time needed.

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