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# ROLE OF A FLAVONOID IN THE PEROXIDE-DEPENDENT OXIDATION OF GLUTATHIONE CATALYSED BY PEA EXTRACTS

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**Key Word Index**—*Pisum sativum* cv Massey Gem; Leguminosae; peas; glutathione oxidation; peroxidase, kaempferol-3-(*p*-coumaroyltriglucoside); hydrogen peroxide; metabolism.

**Abstract**—Crude pea extracts catalysed $H_2O_2$-dependent oxidation of glutathione but gel filtration through Sephadex G-25 abolished activity. Activity was restored by recombining the protein with a flavonoid [tentatively identified as kaempferol-3-(*p*-coumaroyltriglucoside)], isolated from peas. Protein fractions which supported peroxidase activity with pyrogallol as electron donor also supported $H_2O_2$-dependent oxidation of glutathione in the presence (but not in the absence) of the flavonoid with the concomitant consumption of $O_2$. In the absence of glutathione, active protein fractions also supported $H_2O_2$-dependent alteration of the spectral characteristics of the flavonoid. Some properties of these reactions were examined. It was concluded that these activities cannot be attributed to glutathione peroxidase and that a peroxidase belonging to EC class 1.11.1.7 is involved.

## INTRODUCTION

Glutathione (GSH) peroxidase ($H_2O_2$:GSH oxidoreductase, EC 1.11.1.9) has been described in many heterotrophic organisms [1–3] and purified from several sources [3]. The enzyme, at least from mammalian sources, contains selenium in the selenol groups of four selenocysteinyl residues per mol of native enzyme [3, 4]. In animals it is proposed that GSH peroxidase together with superoxide dismutase functions in catalysing the reduction of toxic intermediates of $O_2$ metabolism [4]. In plants, GSH peroxidase activity *per se* has not been demonstrated; such evidence as does exist is circumstantial. In this regard a mitochondrial contraction factor (C-factor) has been described in rat tissues and maize rootlets [5]. The C-factor from rat was later identified as GSH peroxidase [6] but the nature of the maize C-factor was not determined. Flohe and Menzel [7] reported that crude extracts of spinach leaves catalysed $H_2O_2$-dependent oxidation of GSH, an activity consistent with GSH peroxidase activity. However, this reaction is also catalysed by reaction sequences in plants which do not involve GSH peroxidase [8]. In addition, Smith and Shrift [2] found no evidence for GSH peroxidase activity in plants as determined by a procedure involving ($H_2O_2$ plus GSH)-dependent oxidation of NADPH in the presence of GSSG reductase.

This paper describes a re-evaluation of the reported occurrence of GSH peroxidase activity in plants, a matter pertinent to whether Se is essential for plant nutrition [9] and a postulated mechanism of light/dark modulation of some chloroplast enzymes [10]. We report that crude extracts of pea seedlings catalyse $H_2O_2$-dependent oxidation of GSH but that this activity cannot be attributed to GSH peroxidase since procedures which remove the flavonoid kaempferol-3-(*p*-coumaroyltriglucoside) (KGC) abolish this activity.

## RESULTS

### *Characterisation of* $H_2O_2$*-dependent oxidation of GSH by crude extracts*

In the absence of any additions, crude extracts from pea shoots catalysed $H_2O_2$-dependent oxidation of GSH at a mean rate of 345 units/mg protein. Extracts prepared in medium containing insoluble PVP (125 mg/ml) were *ca* 45% less active; insoluble PVP was also inhibitory if added directly to incubation mixtures (60% inhibition at 100 mg/ml). The specific activity was enhanced by $(NH_4)_2SO_4$ fractionation (50–80%) but, unlike authentic GSH peroxidase from other sources, subsequent dialysis or gel filtration on Sephadex G-25 almost completely abolished the activity catalysing $H_2O_2$-dependent oxidation of GSH in pea shoot extracts. However, activity was restored when the supernatant fraction from the boiled $(NH_4)_2SO_4$ treatment (100° for 5 min) was recombined with the dialysed or Sephadex-treated preparation. Similarly, when an $(NH_4)_2SO_4$ fraction (50–80%) was subjected to gel filtration on a Sephadex G-25 column, some fractions, which eluted after the protein, supported $H_2O_2$-dependent oxidation of GSH when recombined with the Sephadex-treated protein. Since the active fractions exhibited absorption maxima at *ca* 270 nm and 310 nm, the flavonoids associated with pea shoots were extracted, separated by 2D-PC and assayed for $H_2O_2$-dependent oxidation of GSH in the presence of Sephadex-treated protein (Table 1). The most active compound was **5**. It was purple–blue under UV light and turned yellow with ammonia, characteristic of a flavonol glucoside [11]. Some activity was also observed with compounds **4** and **6** but complete separation from **5** was not achieved. Compound **5** was purified from dried pea shoots as described in Experimental. The final preparation was

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Table 1. Promotion of $H_2O_2$-dependent oxidation of GSH by flavonoids from pea leaf tissue in the presence of treated protein extract

| Compound* | $R_f$ Solvent IV | $R_f$ Solvent V | Activity† (units/mg protein) |
|---|---|---|---|
| 1 | 0 84 | 0.04 | 0 |
| 2 | 0.71 | 0.03 | 0 |
| 3 | 0.80 | 0 23 | 26 |
| 4 | 0.66 | 0.17 | 52 |
| 5 | 0.66 | 0.25 | 894 |
| 6 | 0.69 | 0.36 | 233 |
| 7 | 0.51 | 0.25 | 0 |
| 8 | 0.77 | 0 14 | 0 |
| 9 | 0.82 | 0.26 | 0 |

*The compounds were extracted from dry pea leaf tissue with MeOH for 3 days and resolved by 2D-PC in solvents V and IV respectively. After PC the compounds were eluted from the paper with MeOH, dried and dissolved in 0.1 M K-$P_i$ buffer pH 7.

†Activity was determined as described for (KGC plus $H_2O_2$) dependent oxidation of GSH except that KGC was replaced with compounds 1–9 as shown. Reaction mixtures contained treated extract (0.19 mg protein/ml).

homogeneous as determined under UV light or with $FeCl_3/K_3Fe(CN)_6$ reagent [12] during PC in the following solvents ($R_f$ values in parentheses): I (0.28), II (0.45), IV (0.64), V (0.34). The UV data ($\lambda_{max}$ nm) for compound **5** were as follows; MeOH, 265, 300 sh, 313; + NaOMe, 272, 310 sh, 368; + $AlCl_3$, 274, 300 sh, 304, 394; + $AlCl_3$–HCl, 274, 302, 322, 394; + NaOAc, 273, 302 sh, 311, 374; + NaOAc–$H_3BO_3$, 298, 304 sh, 315. On the basis of these properties and published spectra of pea flavonoids [13], compound **5** was tentatively identified as KGC.

*Properties of the activity catalysing (KGC plus $H_2O_2$)-dependent oxidation of GSH*

Fractionating crude extract by $(NH_4)_2SO_4$ precipitation and gel filtration resulted in large losses of activity and negligible improvement in specific activity (Table 2). However, material prepared in this way, which is referred to as treated extract, was free from NADH peroxidase, NADPH peroxidase and *o*-diphenol oxidase activities. Very low catalase activity (0.06 μmol/min/mg protein) was observed. Treated extract exhibited very active pyrogallol peroxidase activity (105 μmol/min/mg protein). It also supported $O_2$ consumption in the presence of KGC, GSH and $H_2O_2$. Some $O_2$ consumption occurred in the absence of $H_2O_2$ but not in the absence of KGC or treated extract. Omission of $H_2O_2$ decreased the rate of $O_2$ consumption by 90% (Fig. 1). The rate of $O_2$ consumption in the presence of all three substrates was proportional to the amount of treated extract at 25–100 mg protein/ml and activity was abolished by pretreating the extract at 100° for 5 min. The pH optimum in K-$P_i$ buffer was 7.2. The reaction exhibited relatively high affinity for $H_2O_2$ ($V_{max}/2$ *ca* 30 μM) but concentrations > 0.1 mM were inhibitory (Fig. 2). The effect of concentration of GSH and KGC were consistent with Michaelis–Menten kinetics; the concentrations of GSH and KGC which supported $V_{max}/2$ as determined by $O_2$ consumption were 70 μM and 0.22 mg/ml respectively. GSH could be re-

Table 2. Effect of fractionation procedures on the activity catalysing (KGC plus $H_2O_2$)-dependent oxidation of GSH

| Treatment* | Protein (mg) | Specific activity (units/mg protein) |
|---|---|---|
| Crude extract | 554 | 328 |
| $(NH_4)_2SO_4$ (50–80%, dialysed) | 75.2 | 439 |
| Sephadex G-200 | 15.2 | 425 |
| | | 992† |

*Procedures were as described in Experimental for preparation of treated extract.

†Activity determined in the absence of $NaN_3$.

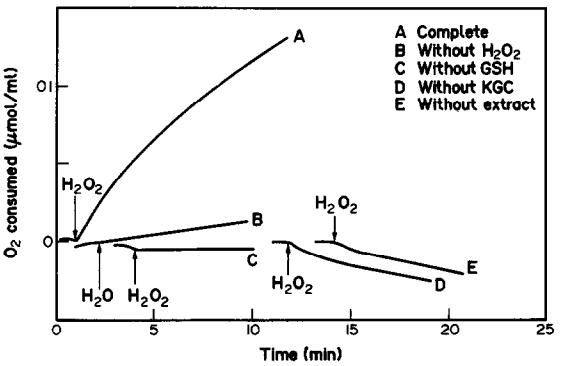


Fig. 1. Time course of $O_2$ consumption by treated extract in the presence of KGC, $H_2O_2$ and GSH. Incubation mixture A initially contained 1.4 mM GSH, KGC (0.1 mg/ml) and treated extract (52 μg protein/ml) and the reaction was initiated after 2 min with 0.5 mM $H_2O_2$ as shown. Incubations B–E were as for A except that B lacked $H_2O_2$, C lacked GSH, D lacked KGC and E lacked treated extract.

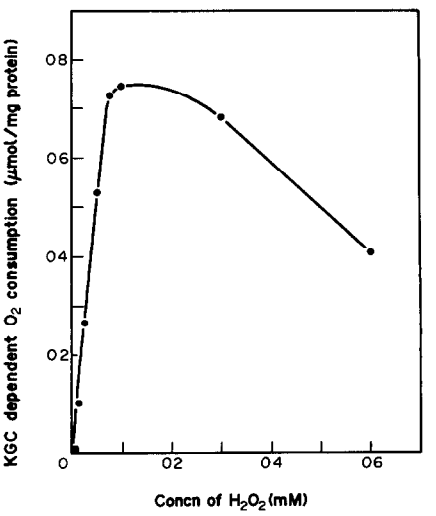


Fig. 2. Effect of concentration of $H_2O_2$ on $O_2$ consumption by treated extract (44 μg protein/ml) in the presence of KGC (0 1 mg/ml) and 1.4 mM GSH.

placed by L-cysteine and thioglycollate (but not by mercaptoethanol) although the rates with these substrates relative to GSH were considerably less (Table 3). The system did not support $O_2$ consumption at significant rates when $H_2O_2$ was replaced with *t*-butylhydroperoxide (Table 3). The reaction was extremely sensitive to potassium cyanide and ascorbate, less sensitive to sodium azide (Table 4) and insensitive to iodoacetate (0.4 mM) and zinc chloride (1 mM). On the other hand, manganese chloride enhanced activity (Table 4).

Conditions which supported (KGC plus $H_2O_2$)-dependent oxidation of GSH and $O_2$ consumption by treated extracts also supported the spectral modification of KGC. Addition of $H_2O_2$ to reactions containing treated extract, GSH and KGC resulted in a decrease in the absorbance of KGC at 313 nm and an increase at 265 nm. Neither absorbance change occurred in the absence of treated extract and omitting $H_2O_2$ inhibited the decrease in absorbance at 313 nm by 70%. However, in the absence of GSH, addition of $H_2O_2$ caused a decrease in the absorbance of KGC at both 265 nm and 313 nm. Since this response did not occur in the absence of treated extract this implies that the oxidation of GSH and the associated $O_2$ consumption are dependent on the product(s) formed from KGC and $H_2O_2$ in a reaction involving a peroxidase. Storage of treated extract at 2° for 1 month resulted in the abolition of $O_2$ consumption in the presence of KGC, GSH and $H_2O_2$ but $H_2O_2$-dependent change in absorbance at 313 nm of KGC in the absence of GSH was not affected. When treated extract was subjected to ion exchange chromatography on CM-cellulose, fractions containing pyrogallol peroxidase activity (134 μmol/min/mg protein) also supported the $H_2O_2$-dependent spectral changes described above for the treated extract. Moreover, this activity was inhibited by 1 mM potassium cyanide and 1.4 mM GSH (100% and 83% inhibition respectively). However, CM-cellulose treated fractions containing pyrogallol peroxidase activity did not support $O_2$ consumption in the presence of KGC, GSH and $H_2O_2$ when recombined with any other fraction eluted from CM-cellulose before and after the application of 800 mM potassium chloride.

KGC is reportedly associated with unwashed pea chloroplasts [14] although contamination of the preparation of KGC from other subcellular compartments was not excluded. Nevertheless, since chloroplasts synthesize $H_2O_2$ in the light through the action of light-dependent reduction of $O_2$ and superoxide dismutase [15, 16], this raises the question whether the activity supporting $O_2$ evolution and GSH oxidation in the presence of KGC, and $H_2O_2$ is associated with chloroplasts. In this event, light-generated reducing equivalents could be directed from $H_2O$ into $H_2O_2$ via NADPH, GSH and KGC. However, washed sonicated chloroplasts (92% intact prior to sonication) did not catalyse $H_2O_2$-dependent oxidation of GSH either in the presence or absence of KGC (0.1 mg/ml) with or without 1 mM sodium azide. Further, sonicated chloroplasts did not support $H_2O_2$-dependent spectral changes of KGC at 265 nm or 313 nm in the presence or absence of 1 mM sodium azide.

Table 3. Effect of various thiols and peroxides on $O_2$ consumption by treated extract in the presence of KGC

| Additions* | | $O_2$ consumed |
|---|---|---|
| Thiol | Peroxide | (units/mg protein) |
| GSH | $H_2O_2$ | 0.49 |
| L-Cysteine | $H_2O_2$ | 0.22 |
| Thioglycollate | $H_2O_2$ | 0.05 |
| 2-Mercaptoethanol | $H_2O_2$ | 0 |
| GSH | *t*-Butylhydroperoxide | 0.03 |

*All thiols were supplied at a concentration of 1.4 mM and the peroxides at 0.5 mM. All incubation mixtures contained 0.1 mg/ml KGC and 50 μg/ml treated protein.

Table 4. Effect of various compounds on $O_2$ consumption by treated extract in the presence of $H_2O_2$, KGC and GSH

| Compound | $O_2$ consumed (units/mg protein) |
|---|---|
| No additions | 0.61* |
| $NaN_3$ (0.1 mM) | 0.50 |
| (1 mM) | 0 32 |
| KCN (0.1 mM) | 0.03 |
| (1 mM) | 0 |
| L-Ascorbate (0.1 mM) | 0 |
| $MnCl_2$ (0.05 mM) | 0 73 |
| (0.1 mM) | 0.96 |
| (1 mM) | 0.75 |

*Incubations were conducted under standard conditions for $O_2$ consumption in the presence of 52 μg protein/ml of treated extract.

## DISCUSSION

The $H_2O_2$-dependent oxidation of GSH catalysed by crude pea extracts cannot be attributed to GSH peroxidase since the reaction was dependent on the presence of a flavonoid substrate, the properties of which are consistent with those of KGC [14]. This explains the decreased activity of extracts prepared or incubated in the presence of PVP since PVP binds phenolic compounds [19]. Many other properties including the specific requirement for $H_2O_2$ (inactive with *t*-butylhydroperoxide), the lack of specificity for GSH (active with cysteine and thioglycollate), confirm that GSH peroxidase activity is not involved [1]. The presence of a very active pyrogallol peroxidase in treated extracts which support both GSH oxidation and $O_2$ consumption in the presence of $H_2O_2$, GSH and KGC and the sensitivity of these reactions to potassium cyanide and sodium azide suggest the involvement of a peroxidase belonging to EC class 1.11.1.7.

(KGC plus $H_2O_2$)-dependent oxidation of GSH catalysed by treated extract consists of two component reactions involving (i) oxidation of KGC by $H_2O_2$ and (ii) GSH-dependent modification of the phenolic oxidation product accompanied by $O_2$ consumption and GSH oxidation. The catalysis of flavonoid oxidation with $H_2O_2$ by peroxidases has been reported previously [17]; the $H_2O_2$-dependent changes in the $\lambda_{max}$ of KGC in the presence of treated extract (in the absence of GSH) could involve a reaction of this type. In addition, peroxidases also catalyse the aerobic oxidation of GSH in the presence of various phenolics and $Mn^{2+}$ in a peroxidase–oxidase

reaction [18, 19]. The stimulation by $Mn^{2+}$ of $O_2$ consumption by treated extract in the presence of $H_2O_2$, KGC and GSH and the inhibition of this reaction by ascorbate (Table 4) are consistent with the properties of this type of reaction [19], although, unlike peroxidase–oxidase reactions, both GSH oxidation and $O_2$ consumption in the presence of KGC required the addition of $H_2O_2$. Catalase also catalyses the oxidation of GSH in the presence of $Mn^{2+}$ and certain phenolics [18]. However, the involvement of catalase in the reaction we describe involving KGC is unlikely since 1 mM sodium azide, which completely inhibited catalase, caused only 47% inhibition of $O_2$ consumption in the presence of $H_2O_2$, KGC and GSH. It is not known whether more than one enzyme is involved in the $O_2$ consumption reaction but several observations are consistent with the possibility that more than one protein is required. They include the preparation of a fraction by ion-exchange chromatography on CM-cellulose which supports $H_2O_2$-dependent modification of KGC but not $O_2$ consumption. In addition, the latter reaction (but not the former) is more labile. The nature of the GSH inhibition of $H_2O_2$-dependent modification of KGC, catalysed by CM-cellulose treated protein, is not known.

Our failure to detect GSH peroxidase in plants confirms the earlier study of Smith and Shrift [2] and implies that if Se is essential for plant growth it must be required for some other function. Moreover, since sonicated chloroplasts did not support $H_2O_2$-dependent oxidation of GSH in the presence of KGC this suggests that the system we describe for the reduction of $H_2O_2$ by GSH via KGC is an unlikely mechanism for light-dependent detoxification of $H_2O_2$ in illuminated chloroplasts.

## EXPERIMENTAL

*Plant material.* Pea seeds (*Pisum sativum* cv Massey Gem) were grown as before [20] and harvested 15–20 days after imbibition. Chloroplasts were prepared and washed × 4 as in ref. [21] and sonicated as in ref. [20].

*Extraction and purification of KGC.* Dried pea shoots (40 g) were extracted in MeOH (500 ml). Pea flavonoids were extracted as per ref. [22] and the flavonoid fraction evaporated to dryness. The residue was resuspended in MeOH and applied to a Sephadex LH-20 column. The column was equilibrated and eluted with MeOH. The fractions containing KGC were subjected successively to PC in solvents I–IV KGC (*ca* 0.1 mg/g dry wt) was eluted with MeOH and stored in 10 mM K-$P_i$ buffer pH 7 In initial experiments, prior to establishing the identity of KGC, flavonoid fractions were monitored for their activity to support $H_2O_2$-dependent oxidation of GSH in the presence of protein fractionated from crude extracts with $(NH_4)_2SO_4$ (50–80% saturated) and desalted by passage through Sephadex G-25 KGC was characterized spectrophotometrically as per [11].

*Preparation of extracts.* Pea shoots were extracted in 0.1 M K-$P_i$ buffer pH 7 (0.5 g fr. wt/ml) and the supernatant soln (referred to as crude extract) recovered by centrifugation (15000 *g* for 30 min). $(NH_4)_2SO_4$ (0.291 g/ml) was added and pptd material discarded. Additional $(NH_4)_2SO_4$ (0.194 g/ml) was added and pptd protein was recovered, dissolved in 20 mM K-$P_i$ buffer pH 7 and dialysed against 100 vols of the same buffer. The soln was passed through Sephadex G-200 in 20 mM K-$P_i$ buffer pH 7, eluted material, which supported $O_2$ consumption or GSH oxidation in the presence of $H_2O_2$, KGC and GSH, is referred to as treated extract. In some experiments treated extract was dialysed against 20 mM K-$P_i$ buffer pH 6 and applied to a CM-cellulose column which, after flushing, was eluted with a linear gradient of KCl (0–400 mM) in the same buffer.

*Enzyme assays.* $H_2O_2$-dependent oxidation of GSH (without added KGC) was determined by a modification of the method of [23] for the estimation of GSH peroxidase activity. Reaction mixtures (5 ml) containing 500 μmol K-$P_i$ buffer pH 7, 7 μmol GSH, 5 μmol $NaN_3$ and enzyme extract were preincubated at 30° for 5 min. Reactions were initiated by addition of 4 μmol $H_2O_2$. Samples were withdrawn at intervals and treated with 4 ml $H_3PO_4$ (1.67%) Particulate material was removed by centrifugation and GSH determined with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) [23]. Activity was determined from slopes of time plots of the decrease in $\log_{10}$ μmol of GSH. Activity, corrected for controls lacking enzyme and $H_2O_2$, is expressed in units which are defined as 0.001 ($\log_{10}$ μmol GSH oxidized/min).

(KGC plus $H_2O_2$)-dependent oxidation of GSH was as described above for $H_2O_2$-dependent oxidation of GSH except that incubation mixtures also contained exogenous KGC (0.1 mg/ml). The activity supporting this reaction was also determined polarographically by $O_2$ consumption in the presence of KGC, $H_2O_2$ and GSH. Incubations (1 ml) were conducted at 25° in $O_2$ electrodes [20] initially containing 1.4 μmol GSH, 0.1 mg KGC, 100 μmol K-$P_i$ buffer pH 7 and enzyme extract. After 2 min preincubation, reactions were initiated with 0.5 μmol $H_2O_2$. In both assays, exogenous KGC was supplied at non-saturating concns: this was dictated by the low affinity of the system(s) for KGC. Enzyme activity was corrected for controls lacking $H_2O_2$, GSH, KGC and enzyme extract (Fig. 1) and is expressed as the initial rate of $O_2$ consumption in μmol/min (units)

NADP peroxidase (EC 1.11.1.2) and NAD peroxidase (EC 1.11.1.1) activities were determined by oxidation of NAD(P)H at 340 nm in reaction mixtures containing 0 1 mM NAD(P)H, 0.5 mM $H_2O_2$ and enzyme extract in 0.1 M K-$P_i$ buffer pH 7. Pyrogallol peroxidase activity was determined as in ref [24] except that incubations were conducted in 0.1 M K-$P_i$ buffer pH 7 and initiated with 0.8 mM $H_2O_2$. Catalase activity was determined polarographically [21], and *o*-diphenol oxidase was determined as described by Pierpoint [25].

*Other methods.* Protein in crude extracts and $(NH_4)_2SO_4$ fractions was pptd with TCA, washed twice with $Me_2CO$, solubilized in 2 M NaOH at 60° for 1 hr and measured as in ref [26]. More purified protein from Sephadex G-200 and CM-cellulose columns was determined as in ref. [27]. The concns of solns of $H_2O_2$ and *t*-butylhydroperoxide were determined immediately prior to use by titration with $KMnO_4$. PC was conducted on 3 MM paper using the following solvents: I, $H_2O$; II, *n*-BuOH–HOAc–$H_2O$ (4:1:5); III, as for II; IV, HOAc (15%); V, *t*-BuOH–HOAc–$H_2O$ (3·1·1).

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