International Food Research Journal 24(4): 1422-1428 (August 2017)
Journal homepage: *http://www.ifrj.upm.edu.my*

Study of phenol oxidases in fruits and vegetables:Co-occurrence of thermostable peroxidases and alkaline tyrosinase in a novel source *Coccinia grandis*

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Article history

Received: 19 July 2016
Received in revised form: 25 August 2016
Accepted:27 August 2016

Keywords

Tyrosinase
Catechol oxidase
Peroxidase
Characterization

Abstract

Tyrosinase and peroxidase content in various fruits and vegetables was compared. Avocado pulp was found to be a rich source of tyrosinase yielding around 4.5U/g. Avocado seeds, *Datura metel* and *Coccinia grandis* were found to be the next best sources. The tyrosinase rich sources gave comparatively poor yield of peroxidase with the exception of *C. grandis*. Tyrosinases from Avacado (ATy) functioned optimally at pH 6.5 and was stable over a broad range of pH from 5.5-9. *C. grandis* tyrosinase (CTy) was an alkaline tyrosinase with an optimum pH of 9.5-10. Optimum temperature of C grandis peroxidase (CPox) was 50 °C. ATy, CTy and CPox retained 100% activity at 40 °C, 60 °C and 75 °C respectively for 30min. The K_M for Catechol was 3.2-3.3 mM for both CTy and ATy. K_M for Dopa was 3.3 and 1.27mM for ATy and CTy respectively. CPox exhibited a strong affinity for hydrogen peroxide with a K_M of 87 µM.

Introduction

Critical role of reactive oxygen species (ROS) as components of the signaling pathways and as key regulators of cellular responses of plants to environmental factors is well documented. The plant defense mechanisms involve activities of antioxidant enzymes such as peroxidases, catalases and polyphenoloxidases. These antioxidant enzymes are known to be involved in oxidation of phenolics to quinones and related derivatives which are toxic to pathogens. Peroxidases and phenol oxidases are members of oxidase super family and are widely distributed in plants. Apart from their significance in plant metabolic processes, these enzymes have potential applications in biotechnology. Phenol oxidases are of great interest for many applications in biotechnology, food processing, medicine, textile, pulp and paper industry (Selinheimo *et al.,* 2007; Popa and Bahrim, 2011; Dorantes and Zuniga, 2012). Polyphenol oxidases (PPOs) are copper containing enzymes found in various plants, animals and fungi. Enzyme nomenclature differentiates between monophenol oxidase (tyrosinase, EC 1.14.18.1) and catechol oxidase or o-diphenol:oxygen oxidoreductase (EC 1.10.3.2). Tyrosinases have the ability to hydroxylate monophenols to o-diphenols and catechol oxidase oxidizes o-diphenols to their corresponding o-quinones (Mayer, 2006; Sullivan 2014). PPOs from different species are diverse in terms of their primary structure, glycosylation pattern, tissue distribution, and cellular location. Action of tyrosinase on tyrosine results in formation of melanin which causes browning of fruits and vegetables. The study of tyrosinase has economic significance in the food industry and is of interest to researchers due to its possible role in the plant defense system.

Peroxidases (EC 1.11.1.7; phenolic donor:hydrogen-peroxide oxidoreductase) are heme containing enzymes that catalyze the oxidation of a range of organic compounds by hydrogen peroxide. The attributes of broad substrate specificity, multifunctional properties and availability of peroxidases confer advantage to the enzyme in several biotechnological applications. Peroxidases are useful in chemical synthesis processes, as redox indicators and in detoxification processes (Veitch, 2004; Belcarz *et al.,* 2007; da Silva *et al.,* 2010). HRP (Horseradish Peroxidase) has been used as a reporter in diagnostic assays, biosensors and histo-chemical staining. As its applications are immense, researchers across the globe are exploring various plants for their peroxidase content. Peroxidases are implicated to enhance the degradation of phenols using hydrogen peroxide and quinones generated by PPO catalysis (Yoruk and Marshall, 2003). Search for sources with co-occurrence of both peroxidase and phenol oxidase activity of these enzymes is of interest for researchers. A potential reservoir of these enzymes of biotechnological significance may not only be promising to industries, it could be beneficial for agricultural community as well.

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The present work focuses on screening of various locally available fruits and vegetables for determining the yield of peroxidases and tyrosinases in these plant sources. Characterization of tyrosinase from Avocado and C grandis was also carried out. Peroxidase was also characterized in the extract of C grandis.

Materials and Methods

Materials

Radish, potato, Kohlrabi (*Brassica oleracea gongylodes*), button mushroom (*Agaricus bisporus*), Solenostemon rotundifolius (*Coleus*), Delhi apple and Misri apple were procured from central market Udupi, Karnataka, India. Other fruits and vegetables namely *Morinda citrifoliae* (Noni), *Datura metel, Curcuma longa, Curcuma amada, Persea americana* (Avocado), *Carica papaya, Cucumis sativus* (cucumber), *C grandis, Averrhoa bilimbi* (Bilimbi), *Averrhoa carambola* (carambola), *Ficus racemosa* (fig) were collected from the local farms. All the reagents used were of analytical grade. Thermoscientific Genesys 10 UV-Visible spectrophotometer was used for measuring the absorbance.

Extraction

Vegetables/fruits were washed, peeled and the tissues were macerated in chilled D/W to pasty consistency. The extracts were centrifuged at 6000 rpm for 10min. The supernatants were analyzed for enzyme yield and stored at -20°C.

Peroxidase assay

Appropriately diluted enzyme was added to the reaction mixture containing H_2O_2 (2 mM) and Phenol/Aminoanitpyrine mixture (85 mM/1.25 mM respectively) in 40 mM buffer (acetate buffer, pH 4.5; phosphate pH 6.4; carbonate buffer pH 8.5) and incubated at 37±0.2°C. The A_{510nm} was read at 10 min. One unit of activity is the amount of enzyme which produces one micromole of the quinoneimine dye (Ɛ=13600; Khuchareontaworn *et al.*, 2010) in one minute under the defined conditions.

Tyrosinase (Dopa oxidase) assay

Appropriately diluted enzyme was added to the reaction mixture containing Dopa (8 mM) in 40 mM buffer (acetate buffer, pH 4.5; phosphate pH 6.4; carbonate buffer pH 8.5) and incubated at 37±0.2°C. The A_{475nm} was read at 10 min.

Catechol oxidase assay

Enzyme was assayed using 18 ɛmM Catechol as substrate (phosphate buffer pH 8.5 was used for alkaline buffer). One unit of activity is the amount of enzyme which produces one micromole of dopachrome in one minute under the defined conditions (□=3700 as reported by Behbahani *et al.,* 1993).

Alpha hydroxy acid oxidation

Carambola extract was incubated with 4 mg/ml of Citric/ Malic/ Lactic/ Succinic acid in acetate buffer pH 3.5, 40 mM at ambient temperature (30±1°C). Change in absorbance was monitored at 240 nm.

Estimation of phenolics

Phenolics were estimated using folin Cio-calteau reagent and sodium carbonate (Agbor *et al.,* 2014). Catechol was used for calibration.

Localization of peroxidase activity on polyacrylamide gel

Disc electrophoresis was carried out under non-denaturing conditions (Stacking gel – 5% T, 3.33% C; Resolving gel – 8% T, 3.33% C). Specified plant extracts were loaded and electrophoresis was performed at 100V. The gel was removed, rinsed with D/W and equilibrated in 50 mM PO_4 buffer, pH 6.4 for 10 min. The gel was then immersed in Phenol-AAP mixture/Ortho phenylene diamine with 50 mM H_2O_2 till pink/brown bands were observed.

Effect of pH and temperature

Enzyme activity and stability studies were performed at various pHs ranging from 3.6-12. For characterization of tyrosinase, pH of the phosphate buffers were adjusted in the alkaline ranges of 6-11, as sodium carbonate buffer system promoted auto oxidation of the substrates. Effect of temperature on the activity and stability were studied from 25-70°C at pH 7 for ATy and CPox and at pH 8 for CTy.

Effect of substrate concentration

The effect of varying concentrations of the phenol and H_2O_2 were studied for peroxidase. K_M was calculated using Michaelis-Menten and Lineweaver-Burke (LB) plots. K_M values of phenol oxidase for both Dopa and Catechol were also determined. Molar absorptivity of the respective products was considered for calculating the Vmax.

Results and Discussion

Roots, tubers and fruits of many plants are known to be rich sources of antioxidant enzymes. Sweet potato, Horse radish and vegetables belonging to

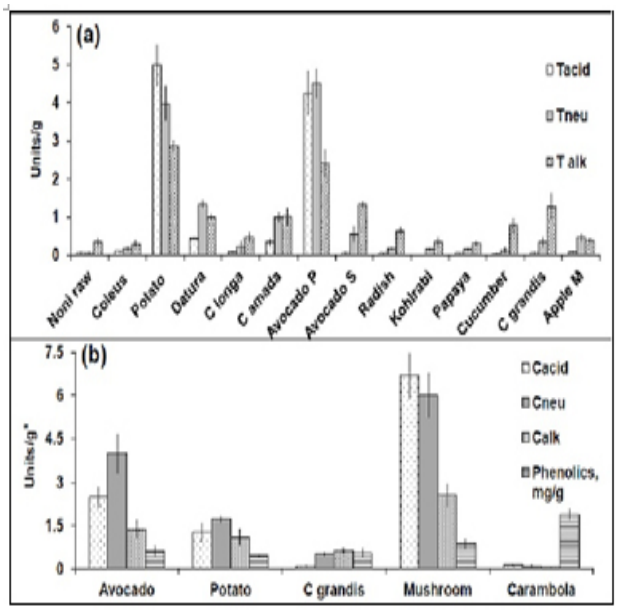

Figure 1. Oxidation of Dopa and Catechol by phenol oxidases;
(a) Tyrosinase activity: Tacid at acidic pH; Tneu at neutral pH; Talk at basic pH.
(b) Catechol oxidase: Cacid at acidic pH; Cneu at neutral pH; Calk at basic pH.

Brassicaceae family are well documented as reservoirs of peroxidases. Studies on phenol oxidases from many fruits and vegetables have been extensively reported in the literature. Activity of these enzymes is reported to be cultivar dependent (Vamos-Vigyazo and Haard, 1981). In the present investigation, many locally available plant sources were assayed for tyrosinase and peroxidase content. Mushroom and potato are well known sources of phenol oxidase and Horse radish and Kohlrabi are reservoirs of peroxidases (Shetty *et al.,* 2012). Mushroom, Potato and Kohlrabi were included in the study to compare the yield of the antioxidant enzymes from other sources. Neutral, acidic and basic isoforms of these enzymes are known to exist in various plant sources (Vamos-Vigyazo and Haard, 1981; Triplett and Mellon, 1992; Yoruk and Marshall, 2003; Thongsook and Barrett, 2005). The isoforms may differ with respect to their pH optima and often one isoform may be more pronounced in a particular source. The assays were carried out at pHs 4.5, 6.8 and 8.6. Avocado seed comprises around 18-22% weight of the fruit and hence the seeds and the fruit pulp were separately assayed. The results for tyrosinase assay are shown in Figure 1a. Avocado pulp was found to be an excellent source of tyrosinase comparable to the yield in potatoes, which is one of the well known sources. The enzyme yield was around 4.3-4.5U/g in the pulp of Avacodo at acidic and neutral pH values and in the seeds it was around 2.4 U/g at alkaline pH. The yield was lower at higher pH values in the pulp, while the

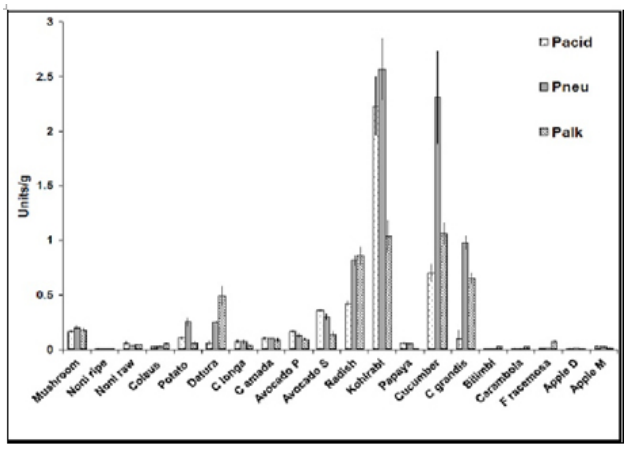

Figure 2. Yield of Peroxidase in various fruits and vegetables; Pacid - at acidic pH; Pneu - at neutral pH; Palk - at basic pH.

reverse was true in seeds. C grandis, avocado seeds, Datura metel and Curcuma amada were the next best sources. The activity of tyrosinase was lower in other fruit and vegetable extracts. In radish, kohlrabi, papaya, cucumber, C grandis and apples, tyrosinase activity was significantly more at higher pH values in comparison to lower pH values. It is interesting to note that all these fruits and vegetables belong to the clade Rosid. Tyrosinase activity in mushroom was higher at acidic and neutral pH yielding around 13.4 and 12.7-U/g respectively. Catechol oxidizing ability and phenolic content in the extracts from these reservoirs were also determined (Figure 1b). Mushroom phenol oxidase yielded around 6-6.7 U/g of catechol oxidase activity at neutral and acidic pH. Although dopa oxidation abilities were relatively same in avocado pulp and potato, phenol oxidase of avocado pulp was more effective in oxidizing catechol in comparison to potato. Of the species tested, kohlrabi and cucumber were found to be the best sources of peroxidase yielding 2.6 and 2.3U/g respectively at neutral pH followed by C grandis which gave around 0.93U/g (Figure 2). Most of the tyrosinase reservoirs were relatively poor in peroxidase content with the exception of C grandis which yielded around 1.3 U/g of tyrosinase. Neutral forms of peroxidases were found to predominate in cucumber and C grandis. Peroxidases and tyrosinases were found to be absent/ negligible in Noni, F racemosa and Delhi apple at the specified pH values. Bilimbi and carambola which belong to family oxalidacea showed negligible content of the three enzymes at the pHs employed. While pH of all the extracts (0.7-1 w/v) was in the range of 4.2-6.3, carambola and bilimbi extracts exhibited pH values of 1.9-2.05. Enzyme assays were carried at a pH of 3 in carambola extract. Peroxidation and catechol oxidation were found to be negligible. However,

dopa oxidase activity of 0.22U/g was observed. Phenolic content of the selected fresh extracts is given in Figure 1b. Keeping quality of juices/extracts is affected by generation and accumulation insoluble products. The extracts of potato and mushroom which exhibited high content of dopa oxidases were found to accumulate significant amount of dark colored insoluble residue. The supernatant gave poor yield of phenolics. However, the homogenous suspension gave same yield as that of the fresh extract indicating precipitation of polymerized phenolics/ tannins. Content of phenolics in mushroom, and potato was 0.77 and 0.46 mg/g respectively. Despite having phenolics content of 0.66mg/g, avocado extract which is a rich and balanced source of both dopa and catechol oxidase did not accumalate significant amount of insoluble residue. This could be due to the presence of significant amounts of proanthocyanidins in avocado which are known to inhibit tyrosinase activity (Chai et al, 2015). Phenolics content in the fresh extracts of bilimbi and carambola was found to be high amounting 0.87 and 1.84 mg/g in fresh extracts and 0.66 and 1.1 respectively after 10 days of storage. Both the extracts accumulated yellow colored precipitates on storage. Lignins are relatively yellow in color at acidic pH values (Soest and Wine, 1968; Jingjing, 2011). The insoluble residues accumulated in carambola, avocado and potato extracts were washed twice with D/W, centrifuged and re-suspended in 0.01N sulfuric acid (pH 2). The precipitates of avocado and potato extracts remained brown, while residue from carambola remained yellow. Carambola becomes hard on storage unlike many other vegetables/fruits such as avocado which become mushy or soft. Possibility of phenolics being used up for lignifications cannot be ruled out as phenolics are known to be the precursors in lignifications process. Peroxide generation is reported to be one of the factors involved in lignifications (Kuźniak and Urbanek, 2000; Sharma *et al.,* 2012). The acidic pH of the carambola indicates presence of organic acids in the extract. The glycolic acid oxidase and ά- hydroxy acid oxidase enzymes are known to be present in significant amounts in several plant species which oxidize ά- hydroxy acids with generation of peroxide (Blanchard et al., 1946; Zelitch and Ochoa, 1953; Maurinoa and Engqvista, 2015). Ability of carambola extract to oxidize ά- hydroxy acids namely, malic, citric, lactic and succinic acid was tested and generation of H_2O_2 was monitored at 240 nm. There was no change in absorbance in presence of citric and lactic acid. The extract on incubation with malic / succinic acid, resulted in an increase of extinction at 240 nm indicating generation of H_2O_2. Both succinic acid and malic acids absorb at 240nm. The control

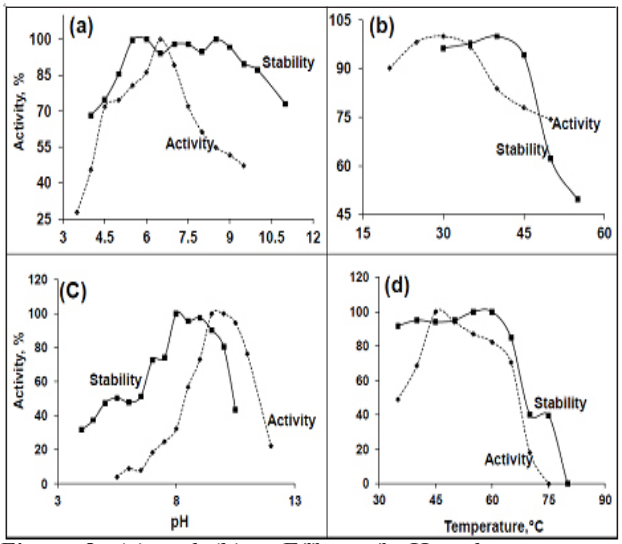

Figure 3. (a) and (b) – Effect of pH and temperature on Avocado tyrosinase; (c) and (d)- Effect of pH and temperature on tyrosinase of C grandis.

tubes containing respective acids with heat denatured enzyme did not show any change in absorbance. The result therefore indicates presence of succinic / malic acid oxidases in carambola extract.

Restraint in the study of cucumber peroxidase was its instability. It was found that the activity in the extract decreased by 65- 70% when stored for 7 days at -20°C and assayed. Both C grandis and avocado are known to have medicinal valuable properties (Manal *et al.,* 2013; Patel and Ishnava, 2015). Although avocado is a well known source of tyrosinase and C grandis a widely consumed vegetable in India, report on the characterization of its antioxidant enzymes are scarce. Tyrosinase from both Avocado (ATy) and C grandis (CTy) were characterized. Characterization of Peroxidase from C grandis (CPox) was also undertaken.

The pH activity and pH stability profiles of ATy are shown in Figure 3a. The enzyme was maximally active at pH 6.5 and was relatively stable retaining more than 95% activity in the pH range of 5.5-9. Temperature optimum of the enzyme was at around 30°C and was stable up to 40°C (Figure 3b). The pH activity profile of ATy did not show the typical bell shaped curve. At values of pH 4.5 to 5.5 the activity remained steady indicating possible presence of one or more acidic isoforms of tyrosinase. The KM values for Dopamine and Catechol were calculated to be 3.3 and 3.25 mM respectively. CTy exhibited an optimum pH of 9.5-10 and stability in the range of pH 8-9 (Figure 3c). The enzyme was highly active in alkaline side of the pH yielding an activity of 2.1U/g at its pH optimum. The enzyme was maximally active at 45°C and was found to be thermostable as it could retain its activity even at 60°C for 30min (Figure 3d).

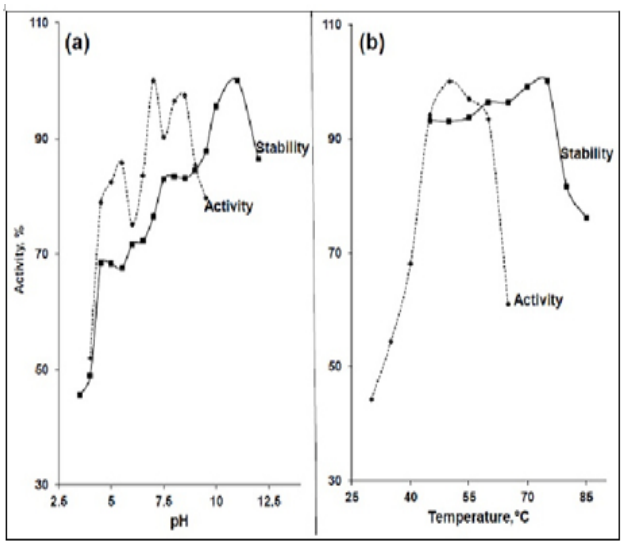

Figure 4. (a) and (b) Effect of pH and temperature on C grandis peroxidase.

In case of enzymes it is often seen that presence of substrates often offers protection to enzymes and hence the optimum temperature of such enzymes is higher than the temperature of its stability. But in the present study, both tyrosinases were stable at temperatures higher than the respective pH optimum. CTy exhibited KM values of 1.27 and 3mM for Dopa and Catechol respectively. Effect of pH and temperature on characteristics of CPox is presented in Figure 4. It was maximally active at pH 7 and the nature of the curve indicated presence of multiple forms of the enzymes. Major activity however, existed in neutral to alkaline region. The enzyme was stable in the alkaline region and retained 95-100% of activity at pH 10-11. Optimum temperature of CPox was 50°C and the enzyme exhibited 93-100% activity at temperatures in the range of 45-60°C. A relatively broad peak in the temperature activity curve and presence of overlapping peaks in the pH-activity profile indicate the possibility of major isoforms of peroxidases being present in the extract of C grandis. CPox was stable at higher temperatures up to 75°C. The enzyme therefore is a thermostable enzyme withstanding temperature as high as 75°C for 30 min.CPox showed high affinity for hydrogen peroxide with a KM value of 90 µM. However, K_M for the substrate phenol was 19.5 mM. The affinity of CPox for organic substrates is therefore low.

Electrophoresis was carried out to test this hypothesis. The extracts of kohlrabi, cucumber and C grandis were loaded for native PAGE and peroxidase activity was localized on the electrophoregram. While two distinct pink bands of peroxidase activity was seen in C grandis, in cucumber and kohlrabi one major band of activity each was seen. Traces of activity were localized above the major bands in all

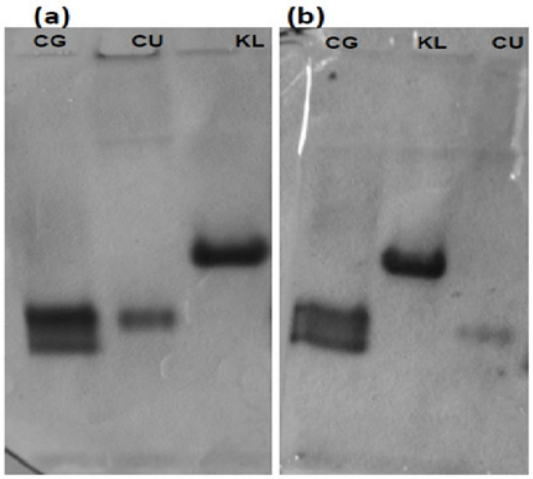

Figure 5. Localization of peroxidase activity using (a) Phenol-AAP and (b) Ortho phenylenediamine as substrates; CG- C grandis; CU-Cucumber; KL-Kohlrabi.

the three extracts as indicated in Figure 5. Thus the electrophoregram supports the interpretation derived from our observations of pH –activity curve about the presence of two or more forms of the enzyme in C grandis.

Conclusion

Cucumber gave high yield of peroxidase. However, the enzyme was found to be highly unstable. Tyrosinase in the Avocado extract showed good pH stability retaining 94-100% of its activity over a broad range of pH 5.5-9. However, the enzyme was found to be thermolabile. *C. grandis* tyrosinase was an alkaline tyrosinase and functioned optimally at pH 9.5-10. The enzyme was also thermostable in comparison to ATy. Peroxidase from *C. grandis* was also found to be thermostable. One common characteristic of all the three enzymes was that pH optimum of each enzyme was less than its respective thermostable temperature. ATy, CPox and CTy were found to be stable on storage at -20 °C and retained 94 -98% of activity after 5 freeze thaw cycles in 30 days. ATy exhibited same affinity for both Dopamine and Catechol. However, the affinity of CTy for Dopamine was 2.35 fold higher than Catechol. Affinity of CPox for its inorganic substrate H_2O_2 was found to be 220 times higher than its organic substrate phenol. In waste treatment processes where high concentration of organic phenolic toxic chemicals are present in the effluents, use of CPox may be beneficial in the initial stages as small quantities of H_2O_2 can be used to initiate the detoxification process.

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