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Peroxidase-Active Cell Free Extract from Onion Solid Wastes: Biocatalytic Properties and Putative Pathway of Ferulic Acid Oxidation

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The exploitation of food residuals can be a major contribution in reducing the polluting load of food industry waste and in developing novel added-value products. Plant food residues including trimmings and peels might contain a range of enzymes capable of transforming bioorganic molecules, and thus they may have potential uses in several biocatalytic processes, including green organic synthesis, modification of food physicochemical properties, bioremediation, etc. Although the use of bacterial and fungal enzymes has gained attention in studies pertaining to biocatalytic applications, plant enzymes have been given less consideration or even disregarded. Therefore, we investigated the use of a crude peroxidase preparation from solid onion by-products for oxidizing ferulic acid, a widespread phenolic acid, various derivatives of which may occur in food wastes. The highest enzyme activity was observed at a pH value of 4, but considerable activity was retained up to a pH value of 6. Favorable temperatures for increased activity varied between 20–40°C, 30°C being the optimal. Liquid chromatography-mass spectrometry analysis of a homogenate/H_2O_2-treated ferulic acid solution showed the formation of a dimer as a major oxidation product.

[**Key words:** biocatalysis, bioremediation, ferulic acid, onion, peroxidase]

Modern biocatalysis aims at the development of novel, precise tools to improve a wide range of processes that reduce energy and raw material consumption, and generate less waste and toxic by-products. Enzymes can be putatively considered as useful biocatalysts, but the main drawbacks that hinder broad applicability are high production costs and low yields (1).

In recent years, the use of enzymes has gained wider acceptance, mainly because of the fact that enzymes from various plant and microbial sources have several advantages over conventional physical and chemical treatment processes. These advantages include; selective removal of particular pollutants, application to xenobiotic recalcitrant compounds, high reaction rates, activity over a wide range of pH and salinity, reduction in sludge volume, and the overall simplicity of controlling the processes (2, 3).

Few plant tissues have been tested as peroxidase sources for use in bioremediation processes. Horseradish peroxidase (HRP) is the most studied enzyme in this regard and has been used mainly for the treatment of aqueous phenols and chlorophenols (4–9), and other pollutants such as polychlorinated biphenyls (10) and bisphenol A (11). Moreover, crude and partly purified soybean peroxidase has been used in the treatment of phenols and chlorophenols (12–15) and bitter gourd (*Momordica charantia*) peroxidase for the treatment of textile dyes (16).

To the best of our knowledge, the use of onion peroxidase has never been examined in bioremediation studies. Our investigation aimed at providing some basic information on the biocatalytic properties of a crude homogenate obtained from solid onion waste and to clarify the oxidation pathways of ferulic acid, a model compound chosen on the basis of its structural features. A wide spectrum of similar useful substances may occur in various food industry wastes, such as olive mill wastewater (17), which includes caffeic acid (CA), p-coumaric acid (COuA), and derivatives thereof.

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Abbreviations: 4-AAP, 4-aminoantipyrine; BGP, bitter gourd peroxidase; CA, caffeic acid; CouA, *p*-coumaric acid; DAD, diode array detector; DMF, dimethyl formamide; ESI, electrospray ionisation; FA, ferulic acid; FA-OP, ferulic acid oxidation product; HRP, horseradish peroxidase; LC-MS, liquid chromatography-mass spectrometry; OSWH, onion solid waste homogenate; POD, peroxidase; SA, sinapic acid; SBP, soybean peroxidase; S.D., standard deviation; TCA, trichloroacetic acid; TMP, tomato peroxidase; TNP, turnip peroxidase.

MATERIALS AND METHODS

Chemicals All solvents used for chromatography were HPLC grade. Ferulic acid (FA) and 4-aminoantipyrine (4-AAP) were from Sigma Chemical (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2, 30%) and trichloroacetic acid (TCA) were from Merck (Germany).

Preparation of the solid onion waste homogenate (OSWH) The onion solid waste used in this study was obtained from a local catering facility (Chania, Crete) after processing of brown-skin onion

bulbs. The waste consisted of the apical trimmings of the bulbs, as well as the outer dry and semi-dry layers. The material was transferred to the laboratory immediately after processing and ground in a domestic blender. An aliquot of 2 g of the ground tissue was suspended in 15 ml phosphate/citrate buffer solution (pH 7) under continuous stirring, and the suspension was centrifuged at 3000×*g* for 20 min and then filtered through filter paper to remove cell debris. The clear supernatant obtained was treated with activated charcoal for decolorization and filtered through celite under vacuum. The clear filtrate was used as the crude enzyme source.

Peroxidase activity The assay mixture contained 0.25 ml 4-AAP (10 mM in water), 0.1 ml substrate (FA) in dimethyl formamide (DMF), 0.1 ml H_2O_2, 0.5 ml of the buffer (50 mM), and 0.1 ml enzyme extract. Absorbance was monitored at 515 nm for over 2 min against a suitable blank. One enzyme unit was defined as $A_{515}\ s^{-1}$, using the slope (initial velocity) of the line produced after plotting A_{515} against the reaction time. Control reactions omitting H_2O_2 or using heat-inactivated homogenate were also carried out. In assays performed at different temperatures, all constituents of the reaction mixture were pre-incubated either in a freezer (5°C) or in a temperature controlled water bath (30–60°C). At pH 2 and 8, a potassium chloride/HCl and a boric acid/NaOH buffer were used, respectively. In the pH range of 3–7, a phosphate/citrate buffer was used. For all determinations, a computer-controlled HP 8452A diode-array spectrophotometer was employed.

Protein determination Protein content was determined according to Bradford (18), using bovine serum albumin as a standard.

Ferulic acid oxidation A solution of FA (0.625 mM) was oxidized with OSWH (total protein content 30 µg/ml) and H_2O_2 (1.6 mM) for 10 min at room temperature (24±2°C, pH 4). Following this, 0.1 ml of a 10% TCA solution in EtOH was added to terminate the reaction and facilitate enzyme precipitation, and the mixture was centrifuged at 5000×*g* for 10 min. The clear supernatant was filtered through 0.45 µm syringe filters (Whatman, Dassel, Germany), and the filtrate was used for chromatographic analyses.

HPLC-DAD analysis The equipment utilized was an HP 1090, series II liquid chromatograph, coupled with an HP 1090 diode array detector and controlled by Agilent ChemStation software. The column was a LiChrosphere RP18, 5 µm, 250×4 mm (Merck), protected by a guard volume packed with the same material. Both columns were kept at 40°C. Eluents A and B were 1% formic acid and acetonitrile, respectively. The flow rate was 1 ml/min, and the elution program was as follows: from 0 to 5 min, 95% A and 5% B; from 5 to 45 min, 0%A and 100% B; from 45 to 55 min, 100% B. The eluate was monitored at 320 nm.

Liquid chromatography-mass spectrometry (LC-MS) The equipment used was a Finnigan MAT Spectra System P4000 pump coupled to a UV6000LP diode array detector and a Finnigan AQA mass spectrometer. Analyses were carried out on a Superspher RP-18, 125×2 mm, 4 µm, column (Macherey-Nagel, Düren, Germany), protected by a guard column packed with the same material, and maintained at 40°C. Moreover, analyses were carried out employing electrospray ionization in negative ion mode, with acquisition set at 12 and 50 eV, capillary voltage 4 kV, source voltage 4.9 kV, detector voltage 650 V, and probe temperature 400°C. Eluents A and B were 2.5% acetic acid and methanol, respectively. The flow rate was 0.33 ml/min, and the elution program used was as follows: from 0 to 5 min, 100% A and 0% B; from 5 to 30 min, 100% B; and from 30 to 35 min, 100% B.

Statistical analyses All experiments were performed at least thrice and the values were averaged and given along with the standard deviation (±S.D.). For all statistics, Microsoft Excel 2000 was used.

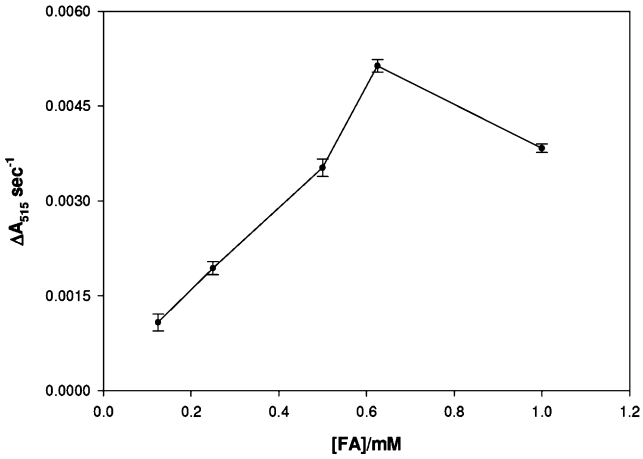

FIG. 1. Activity variation in response to ferulic acid concentration. Reaction conditions: H_2O_2, 0.4 mM; total protein, 36.3 µg/ml; pH=4; T=24±2°C.

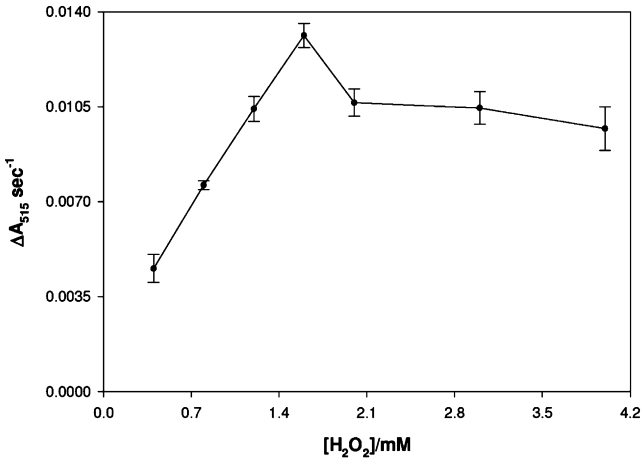

FIG. 2. Activity variation in response to H_2O_2 concentration. Reaction conditions: FA=0.625 mM; total protein=42.3 µg/ml; pH=4; T=24±2°C.

RESULTS

Biocatalytic properties Preliminary experiments using quercetin, which is a physiological substrate for onion POD (19), showed that maximum activity was attained at pH 4 (unpublished). For this reason, initial investigations pertaining to the effect of FA and H_2O_2 concentration on enzyme activity were performed at this pH. Increases in FA concentration induced proportional effects on the enzyme activity up to a concentration of 0.625 mM (Fig. 1). Thereafter, a gradual limited decline in activity was observed. Likewise, increases in H_2O_2 concentration were shown to promote enzyme activity up to 1.6 mM. Further concentration increases were shown to be inhibitory (Fig. 2) as manifested by a decline in enzyme activity.

Examination of the effect of pH changes from 2 to 8 revealed high enzyme activity between pH 3 and 5, whereas a significant decline was seen when the enzymatic reactions were carried out at pH 7 and 8 (Fig. 3). Maximum activity