A flow injection (FI) spectrophotometric procedure is presented for determining hydrogen peroxide for pharmaceutical use and in swimming pool water samples. Crude extracts of several vegetables such as peach, yam, manioc, artichoke, sweet potato, turnip, horseradish and zucchini were investigated as the source of peroxidase (donor: hydrogen peroxide oxidoreductase, POD; EC 1.11.1.7). Of these, a zucchini crude extract was found to give highest specific activity and was used directly as the carrier solution. This enzyme catalyses the oxidation of guaiacol in the presence of hydrogen peroxide to tetraguaiacol, which shows strong absorbance at 470 nm. For the optimum extraction conditions found, the peroxidase activity in the crude extract did not vary for at least 5 months when stored at 4 °C and decreased by only 2–3% during an 8 h working period at 25 °C. The recovery of hydrogen peroxide from two samples ranged from 97.8 to 103.0% and a rectilinear calibration curve for hydrogen peroxide concentration from 1.6 × 10⁻³ to 6.6 × 10⁻⁴ mol l⁻¹ was obtained. A detection limit of 2.1 × 10⁻⁶ mol l⁻¹ and a sample throughput of 32 h⁻¹ were attained. The relative standard deviations were < 0.2% for hydrogen peroxide solutions containing 2.0 × 10⁻⁴ and 4.0 × 10⁻⁴ mol l⁻¹ (n = 10) and a paired t-test showed that all results obtained for water samples using this FI procedure and permanganate titration agreed at the 95% confidence level.

The determination of hydrogen peroxide is of great interest in a large variety of samples in industrial and environmental fields and in clinical control.1,2 Several methods have been proposed for the determination of hydrogen peroxide, including oxidation–reduction titrometry,3,4 chemiluminescence,5 spectrophotometry,6–8 and electrochemical.9–14 Nevertheless, there are no procedures for determining hydrogen peroxide using crude extracts as a source of peroxidase.

The first biosensor for hydrogen peroxide was constructed using a bovine liver membrane containing catalase with an oxygen electrode.15 Recently, the development of several biosensors with the use of novel biological materials as biocatalysts has received considerable attention with the aim of replacing pure enzymes. Several tissue biosensors for determining hydrogen peroxide, such as asparagus tissue,16 grape tissue,17 pineapple,18 kohlrabi,19 tobacco callus20 and horseradish root,21–23 have been used as peroxidase sources. This class of biocatalytic materials maintains the enzyme to be used in these biosensors in its natural environment, which results in considerable stabilization of the desired enzymatic activity.

Peroxidase (donor: hydrogen peroxide oxidoreductase, POD; EC 1.11.1.7), includes a class of enzyme extensively distributed in higher plants (e.g., horseradish, turnip, fig sap), animals (e.g., tryptophan pyrrolase, iodine peroxidase of thyroid) and microorganisms (e.g., cytochrome c peroxidase of yeast).24 Peroxidase catalysis is associated with four types of activity: peroxidic, oxidative, catalytic and hydroxylation.24–27

We have developed several biosensors and enzymatic batch and flow injection procedures for determining phenolic compounds,28–30 l-dopa and carbipoda,31 methylidopa and dopamine32 and sulfite33 using crude extracts of various vegetables in place of isolated enzymes. The use of such biological materials is very attractive because of their high stability, high enzyme activity concentration, very low cost and fewer cofactor requirements in comparison with the pure enzymes.

In this paper, a simple, sensitive and rapid flow injection (FI) enzymatic procedure is reported for determining hydrogen peroxide for pharmaceutical use and in swimming pool water samples. A crude extract of zucchini (Cucurbita pepo) was used as the enzymatic source of peroxidase (POD; EC 1.11.1.7) directly in the carrier solution. In the peroxidic reaction, this enzyme catalyses the oxidation of various hydrogen donors such as guaiacol in the presence of hydrogen peroxide to tetraguaiacol, which shows a strong absorption at 470 nm (see Fig. 1). The use of an insoluble polyvinylpyrrolidone such as Polyclar SB-100 to remove natural phenolic compounds from the solution in the preparation of the crude extract (homogenate) of zucchini led to a substantial increase in the enzyme activity, storage time and stability of the baseline.

**Experimental**

**Apparatus**

A DuPont Instruments (Newtown, CT, USA) Model RC-5B centrifuge, provided with a Model SS-34 rotor, was used in the preparation of the crude extract of the zucchini. A Hewlett-Packard (Boise, ID, USA) Model 8452A UV–visible spectrophotometer with a quartz cell (optical path 1 cm) was used in POD activity and protein determinations. An eight-channel Ismatec (Zurich, Switzerland) Model 7618–40 peristaltic pump supplied with Tygon pump tubing was used for the propulsion of the fluids. The manifold was assembled with polyethylene tubing (0.8 mm id). A Micronal (São Paulo, Brazil) Model B352 automatic proportional com-
mutator was used for inserted reagent and sample solutions. Spectrophotometric measurements were carried out using a Femto (São Paulo, Brazil) Model 435 spectrophotometer with a glass flow cell (optical path 1.0 cm) connected to a Cole Parmer (Niles, IL, USA) Model 12020000 two-channel strip-chart recorder. The effect of temperature on the enzymatic reaction was evaluated using a Tecnal (Piracicaba, Brazil) Model TE184 thermostatically controlled water-bath.

Reagents and solutions

All reagents were of analytical-reagent grade and all solutions were prepared with water from a Millipore (Bedford, MA, USA) Milli-Q system (Model UV Plus Ultra-Low Organics Water). Guaiacol and peroxidase from horseradish (type VI, P8375) were purchased from Sigma (St. Louis, MO, USA). Hydrogen peroxide was purchased from Aldrich (Milwaukee, WI, USA); a 1.00 × 10−1 mol l−1 stock standard solution was prepared daily in 0.1 mol l−1 phosphate buffer (pH 7.0) and standardized by a conventional method. Working standard solutions of 1.6 × 10−3−6.6 × 10−4 mol l−1 were prepared from the stock standard solution by dilution with 0.1 mol l−1 phosphate buffer (pH 7.0).

Polyclar SB-100 used as a protective and/or stabilizing agent in the crude extract preparation was kindly donated by GAF (Wayne, NJ, USA). This polyvinylpyrrolidone was first purified essentially as described elsewhere. Guaiacol phosphate buffer (pH 7.0).

Commercial hydrogen peroxide samples for pharmaceutical use were purchased from a local drug store. Swimming pool water samples without any chlorine species were collected and an iced cooler for storage was used during transport to the laboratory.

Healthy zucchini (Cucurbita pepo), a variety of squash with a long, narrow shape and a greenish rind, purchased from a local producer were selected, washed, hand-peeled, chopped and cooled in a refrigerator at 4 °C.

Zucchini crude extract preparation

A 25 g amount of the frozen peeled zucchini was homogenized in a liquefier with 100 ml of 0.1 mol l−1 phosphate buffer (pH 6.0) containing 2.5 g of Polyclar SB-100 for 2 min at 4–6 °C. The homogenate was rapidly filtered through four layers of cheesecloth and centrifuged at 13 500 rpm for 15 min at 4 °C. The resulting supernatant was stored at this temperature in a refrigerator and utilized as the enzymatic source after the centrifugation process. The resulting supernatant was stored at this temperature in a refrigerator and utilized as the enzymatic source after the centrifugation process.

Peroxidase activity present in the crude extract was determined in triplicate by measurement of the absorbance at 470 nm of tetraguaiacol produced by the reaction between 0.2 ml of 0.05 mol l−1 guaiacol solution and 0.1 ml of 10.0 mmol l−1 hydrogen peroxide solution in 0.1 mol l−1 phosphate buffer (pH 7.0) at 25 °C. The initial rate of guaiacol peroxidation reaction (Fig. 1) was a linear function of time for 1.5–2.0 min. One activity unit is defined as the amount of enzyme that causes an increase of 0.001 absorbance per minute under the conditions described above.

Total protein concentration was determined in triplicate by the method of Lowry et al. using bovine serum albumin as a standard.

**POD solution in phosphate buffer**

A 270 units ml−1 POD solution in 0.1 mol l−1 phosphate buffer (pH 7.0) was prepared daily by dilution of 10 ml of a 6750 units ml−1 POD solution with 0.1 mol l−1 phosphate buffer (pH 7.0) in a 250 ml calibrated flask using the same buffer solution.

**Sample preparation and FI enzymatic procedure**

Appropriate dilution of hydrogen peroxide for pharmaceutical use and swimming pool water samples with 0.1 mol l−1 phosphate buffer solution (pH 7.0) containing guaiacol at a convenient concentration was performed in order to obtain a concentration of the hydrogen peroxide in the range 1.6 × 10−5–6.6 × 10−4 mol l−1.

The single channel spectrophotometric flow system used was similar to that reported previously. In this work, a 270 units ml−1 POD solution in 0.1 mol l−1 phosphate buffer (pH 7.0) was used as the carrier solution at a flow rate of 1.0 ml min−1. A 0.05 mol l−1 guaiacol–hydrogen peroxide solution contained in the sample loop (50 cm, 250 μl) was injected and transported by the enzymatic carrier stream. A 25 cm tubular coiled reactor maintained in a 25 °C water-bath was placed in the analytical path in order to provide better reaction conditions and the tetraguaiacol formed (Fig. 1) was measured in the flow-through spectrophotometric cell at 470 nm.

**Results and discussion**

**Selection and preparation of the crude extract**

Peroxidase is widely distributed in the plant kingdom, in certain animal tissues and also in microorganisms. This enzyme has been isolated from several sources such as horseradish, turnip and soybean. The main source of peroxidase, commercialized by various companies, is horseradish.

In this work, vegetable crude extracts such as peach (Prunus persica), yam (Alocasia macrorhiza), manioc (Manihot utilissima), artichoke (Cynara scolymus L.), sweet potato (Ipomoea batatas L.), turnip (Brassica campestris ssp. rapifera), horseradish (Armoracia rusticana) and zucchini (Cucurbita pepo) were obtained and characterized. The activity and total protein of the crude extracts of these vegetable materials varied according to the extraction procedure and medium used. The buffer-to-tissue ratio was an important factor in the preparation of POD from all these enzymatic sources. In this study, the enzyme was extracted using ratios varying from 2:1 to 6:1 ml g−1 and the highest specific activity for each was obtained at a ratio 4:1 ml g−1.

The effect of buffer pH on the extraction of POD was also investigated in the pH range 5.0–7.5. The highest enzymatic activity for each was obtained at pH 6.0. To minimize the effect of the natural phenolic compounds responsible for the decrease in the POD activity in these crude extracts, Polyclar SB-100 at a mass ratio of 1:10 g g−1 was used. The enzyme activity of the crude extract of zucchini obtained using this PVP did not vary for at least 5 months when stored in a refrigerator at 4 °C, whereas that of a sweet potato crude extract decreased by 5–7% and those of turnip, horseradish and artichoke crude extracts decreased by 15–20% under the same experimental conditions.

Table 1 shows the activity (units ml−1), total protein (mg ml−1) and specific activity (units mg−1 of protein) obtained in triplicate using different vegetable crude extracts. As can be seen, the peach crude extract showed the lowest specific activity.
whereas with the zucchini crude extract the highest enzymatic activity was obtained. To the best of our knowledge, no work has been published on obtaining peroxidase from zucchini. This is surprising, since the specific activity of this crude extract was about 35% higher than that obtained for horseradish crude extract, a common source of commercial peroxidase. Therefore, the crude extract of zucchini was used in subsequent experiments.

Storage time and crude extract stability

For the optimum extraction conditions described above, the peroxidase activity in the crude extract did not vary for at least 5 months when the extract was stored in a refrigerator at 4 °C and decreased by only 2–3% after an 8 h working period at 25 °C. Similar long storage times and low background absorbance of the crude extract obtained with Polyclar SB-100 in the present work were also obtained in previous studies,29–33 showing the advantage of the medium, preparation method and biological material used in this work in comparison with other substances normally used such as sodium azide and L-cysteine.35

Reaction between guaiacol, hydrogen peroxide and peroxidase

The flow injection procedure for determining hydrogen peroxide is based on the catalytic oxidation of guaiacol by peroxidase in the presence of hydrogen peroxide to tetraguaiacol, which shows strong absorption at 470 nm (Fig. 1). Hence, when hydrogen peroxide–guaiacol solution is inserted in the flow injection system the formation of tetraguaiacol is based on the catalytic oxidation of guaiacol by peroxidase in the presence of hydrogen peroxide to tetraguaiacol, which shows strong absorption at 470 nm (Fig. 1). Therefore, in this study all guaiacol standard and sample preliminary batch study showed that 0.05 mol l⁻¹ increased with increase in hydrogen peroxide concentration. A decrease in absorbance signal was observed when hydrogen peroxide–guaiacol solution was inserted in the flow injection system of tetraguaiacol increased with increase in hydrogen peroxide concentration. A preliminary batch study showed that 0.05 mol l⁻¹ guaiacol and 5.0 × 10⁻³ mol l⁻¹ peroxidase do not react in a time range of 0–3 h. Therefore, in this study all guaiacol standard and sample solutions were injected together (same solution) in the flow injection system containing zucchini crude extract as the carrier solution.

Effect of enzyme concentration, pH and temperature

The effect of the POD concentration from 12 to 380 units ml⁻¹ on the analytical signal (absorbance) for 5.0 × 10⁻² mol l⁻¹ guaiacol and 2.0 × 10⁻³ mol l⁻¹ hydrogen peroxide was investigated. The absorbance signal increased linearly with increase in enzyme solution concentration up to 300 units ml⁻¹ POD. Therefore, a concentration of 270 units ml⁻¹ was adopted in this work.

The effect of pH in the range 5.0–7.5 on the absorbance of 5.0 × 10⁻² mol l⁻¹ guaiacol and 2.0 × 10⁻³ mol l⁻¹ hydrogen peroxide solution and 270 units ml⁻¹ POD enzyme was also studied. The optimum pH value for POD activity was 7.0.

The effect of temperature was studied between 15 and 65 °C. The enzyme exhibited the highest activity in the range 25–45 °C, after which a gradual decline in its activity owing to heat inactivation was observed between 45 and 65 °C. Therefore, a temperature of 25 °C was selected for further experiments. A commercial POD from horseradish under the above experimental conditions showed an optimum pH of 6.5 and maximum activity in the temperature range 20–40 °C.

Flow injection parameters and reaction conditions

The effect of varying the sample loop length from 25 to 100 cm (125–500 μl) on the analytical response was initially evaluated. The best sample loop length was found to be 50 cm (250 μl).

Table 1 Activity, total protein and specific activity obtained from various vegetable crude extracts

<table>
<thead>
<tr>
<th>Vegetable crude extract</th>
<th>Activity/ units ml⁻¹</th>
<th>Total protein/ mg ml⁻¹</th>
<th>Specific activity/ units mg⁻¹ protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peach</td>
<td>262</td>
<td>2.45</td>
<td>107</td>
</tr>
<tr>
<td>Yam</td>
<td>1929</td>
<td>5.84</td>
<td>303</td>
</tr>
<tr>
<td>Manioc</td>
<td>1738</td>
<td>2.67</td>
<td>651</td>
</tr>
<tr>
<td>Artichoke</td>
<td>8905</td>
<td>4.61</td>
<td>1932</td>
</tr>
<tr>
<td>Sweet potato</td>
<td>8688</td>
<td>3.21</td>
<td>2707</td>
</tr>
<tr>
<td>Turnip</td>
<td>4226</td>
<td>0.49</td>
<td>8 624</td>
</tr>
<tr>
<td>Horseradish</td>
<td>3381</td>
<td>0.33</td>
<td>10 245</td>
</tr>
<tr>
<td>Zucchini</td>
<td>6750</td>
<td>0.49</td>
<td>13 776</td>
</tr>
</tbody>
</table>

Table 2 Calibration equations obtained for hydrogen peroxide and related parameters as a function of guaiacol concentration in mol l⁻¹

<table>
<thead>
<tr>
<th>[Guaiacol]/ mol l⁻¹</th>
<th>Equation</th>
<th>Linearity range/ 10⁻³ mol l⁻¹</th>
<th>Correlation coefficient* (r)</th>
<th>Detection limit/ mol l⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 × 10⁻³</td>
<td>A = 0.014 + 985.44[H₂O₂]</td>
<td>2.4–58.2</td>
<td>0.9986</td>
<td>4.3 × 10⁻⁶</td>
</tr>
<tr>
<td>5.0 × 10⁻³</td>
<td>A = 0.012 +1260.15[H₂O₂]</td>
<td>1.6–65.6</td>
<td>0.9993</td>
<td>2.1 × 10⁻⁵</td>
</tr>
<tr>
<td>5.0 × 10⁻²</td>
<td>A = 0.011 +1571.10[H₂O₂]</td>
<td>1.6–65.6</td>
<td>0.9993</td>
<td>2.1 × 10⁻⁵</td>
</tr>
</tbody>
</table>

* n = 6.

![Graph of absorbance vs. time](image)
With respect to sensitivity and analytical frequency, the optimum compromise was attained using a coiled reactor 250 cm long and a flow rate of 1.0 ml min\(^{-1}\). The dispersion coefficient of the flow injection system was 1.12.

**Effect of guaiacol concentration on the calibration curves**

The effect of guaiacol concentration at 1.0 \(\times\) 10\(^{-3}\), 5.0 \(\times\) 10\(^{-3}\), and 5.0 \(\times\) 10\(^{-2}\) mol l\(^{-1}\) on the linearity of the hydrogen peroxide calibration curves and detection limit (three times the signal blank-to-slope ratio) is shown in Table 2. The best linearity of the calibration curve (absorbance versus concentration of hydrogen peroxide) was attained at a guaiacol concentration of 5.0 \(\times\) 10\(^{-2}\) mol l\(^{-1}\). At this guaiacol concentration, the greatest linearity range and the lowest detection limit of 2.1 \(\times\) 10\(^{-6}\) mol l\(^{-1}\) were obtained. Therefore, this concentration was adopted in all further work.

**Analytical characteristics, recovery and application**

The optimum FI conditions established as described above, i.e., sample loop length of 50 cm (250 \(\mu\)l), coiled reactor length of 250 cm, carrier flow rate of 1.0 ml min\(^{-1}\), enzyme concentration of 270 units ml\(^{-1}\) in phosphate buffer (pH 7.0) and temperature of 25 °C, were adopted in the proposed method. Recoveries of 97.8–103.0% of hydrogen peroxide, from two samples (\(n = 6\)), were obtained using the FI spectrophotometric procedure (Table 3). This is good evidence of the absence of matrix effects in the proposed method. In addition, the RSDs were \(< 0.2%\) for solutions containing 2.0 \(\times\) 10\(^{-4}\) and 4.0 \(\times\) 10\(^{-4}\) mol l\(^{-1}\) of hydrogen peroxide (\(n = 10\)).

**Table 4** Analysis of samples of hydrogen peroxide for pharmaceutical use and swimming pool water using redox titration with potassium permanganate and the proposed FI enzymatic procedure (\(n = 4\), 95% confidence level)

<table>
<thead>
<tr>
<th>Sample</th>
<th>[Hydrogen peroxide]/mg ml(^{-1})</th>
<th>Relative error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen peroxide 1</td>
<td>32.6 ± 0.2</td>
<td>31.1 ± 0.1</td>
</tr>
<tr>
<td>Hydrogen peroxide 2</td>
<td>31.0 ± 0.2</td>
<td>29.6 ± 0.2</td>
</tr>
<tr>
<td>Hydrogen peroxide 3</td>
<td>30.3 ± 0.3</td>
<td>31.0 ± 0.1</td>
</tr>
<tr>
<td>Swimming pool water 1</td>
<td>(4.5 ± 0.3) (\times) 10(^{-3})</td>
<td>(4.6 ± 0.2) (\times) 10(^{-3})</td>
</tr>
<tr>
<td>Swimming pool water 2</td>
<td>(4.5 ± 0.2) (\times) 10(^{-3})</td>
<td>(4.4 ± 0.1) (\times) 10(^{-3})</td>
</tr>
</tbody>
</table>

References


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