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Biocatalytic properties of horseradish root extract peroxidase (HRP)

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Abstract: The objective of the present work was to examine biocatalytic properties of peroxidase in horseradish acclimatized in our country. We have found that horseradish root extract's peroxidase (HRP) has K_M 2.5 mM and V_{max} 5.36 $mM \cdot s^{-1}$. Maximum activity (pH_{opt}) was estimated at pH 6.0 and enzyme is more stable in alkali, than in acid. The optimum temperature (T_{opt}) for HRP is 40°C and the enzyme is not stable to temperature influence. The horseradish root's extract retains enzymatic activity within 21 days.

Keywords: Horseradish peroxidase (HRP), enzyme activity, biocatalytic properties

INTRODUCTION

Horseradish (*Armoracea rusticana* Gaerth.) is a rootcrop plant of the Brassicaceae family, one of the oldest known condiments, valued for its extremely pungent, fleshy roots. Roots of this plant are rich in peroxidase, heme containing enzyme belonging to oxidoreductases (EC 1.11.1.7). Applications of horseradish peroxidase (HRP) as noted Hollmann and Arends [1] include removal of peroxide, phenols, amines, indols and other heterocyclic compounds from materials such as food stuffs and industrial waste, as oil refineries, plastics, resins, textiles, iron and steel, forestry industries waste water containing phenolic compounds, synthesis of various aromatic chemicals, in biocatalysis and in radical polymerization. Veitch [2] have reported the decolorization and removal of textile dyes from polluted water and dyeing effluents by using soluble and immobilized peroxidases. Azevedo *et al.* [3] note that about 82% of the commercially available HRP is used in kits to test for levels of glucose, uric acid, cholesterol and lactose. Since the 2000s scientists have developed a new method in the treatment of cancer gene-directed enzyme prodrug therapy strategies [3, 4] where HRP plays a key role.

In this work we have studied some properties of peroxidase from horseradish acclimatized to severe Mongolian climate and estimated peroxidase activity as well as its biocatalytic properties including T_{opt}, pH_{opt}, K_M, heat inactivation, effects of acid and bases on the stability of HRP.

EXPERIMENTAL

General: All chemicals used in this study were chemically pure. Reagents for buffer solution, hydrogen peroxide, acids and bases were purchased from Tsetsuuh Trade Co Ltd. (Mongolia). Benzidine was obtained from Sigma-Aldrich (Korea). All experiments were carried out with a 3-5 repetition and average results were taken.

Plant material: Acclimatized horseradish plant samples was collected from Ulaanbaatar area (Mongolia). The roots were stored in a freezer until further studies. For extraction chopped horseradish roots were mixed with a fourfold volume of cold distilled water (1:4 w/v) for 30 min and then filtered. The filtrate was collected and used for the next procedures.

Enzyme assay: Peroxidase activity was measured by A.N. Boyarkin colorimetric assay [5], using benzidine and H_2O_2 as substrate. The assay mixture contains 1 ml of enzyme solution, 2 ml of benzidine (184 mg of benzidine was dissolved in 200 ml of 0.1M acetate buffer with pH=4.7 at 60°C) and 1 ml of 3% H_2O_2. Color development rate is measured at 750 nm, after adding H_2O_2. Protein concentration was measured by the Lowry method [6] using standard BSA solutions for calibration. One unit (U) of enzymatic activity is defined as the amount of 1 mg enzyme protein which changes extinction of assay mixture in 1 second by 0.001.

Study of biocatalytic properties: The optimum pH value for HRP activity was found by assaying enzyme activity at different pH (4.0-9.0, 0.1 M) levels at intervals of 0.5 units. The assay was carried out by taking universal buffer. Optimum temperature value for HRP activity was found by assaying enzyme activity at 20-65°C at intervals of 5 degrees and temperature stability was measured at 55°C, 60°C and 65°C. The samples of crude HRP were prepared

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according to the previous procedure and their activities were measured under the circumstances. We determine K_M value from the Lineweaver-Burk (or double reciprocal) plot 1/V from various substrate concentrations (ranging from 1/100 to 1/500 M, H_2O_2). Effect of acids and bases on the stability of HRP was found by incubating with acid (pH=2.3, 4.0, HCl) and alkali (pH=8.7, 9.7, NaOH). Enzyme stability was measured by storing horseradish extract at 20°C, 5°C and -5°C for 21 days.

RESULTS AND DISCUSSION

We have determined HRP activity dependence from the horseradish root diameter (Table 1).

Table 1. Dependence of peroxidase activity from horseradish root diameter

Root diameter, mm	Peroxidase activity, U
4.4±0.1	10.52±0.48
10.6±0.4	44.36±1.01
19.15±2.85	14.20±0.37

These results show that the enzymatic activity is the greatest in the roots with average dimensions, while in thin and thick roots enzymatic activity decreases. Studies of MacAdam *et al.* [7], Djakovic and Jovanovic [8] have confirmed that peroxidase activity is directly dependent on the intensity of plant growth, leaf growth parameters as leaf elongation rate and segmental elongation rate. So we believe that the increase in enzyme activity in the roots with average dimensions associated with this process and may be associated with period of plant and root intensive growth.

We have determined K_m value from the Lineweaver-Burk plot (Fig. 1).

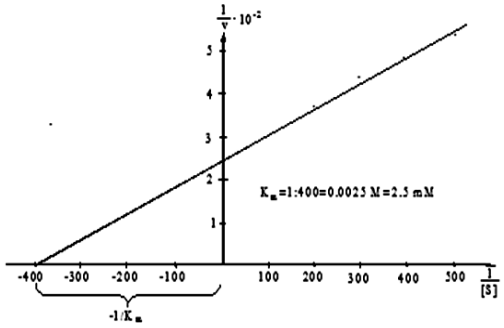

Fig. 1. Lineweaver-Burk plots for HRP activity at various concentrations of H_2O_2

From this plot we calculated K_M value as 2.5 mM and V_{max} as 5.36 mM·s^{-1}. Qiu *et al.* [9] defined kinetic parameters of HRP and K_m value was estimated as 0.43 mM and V_{max} as 0.35 µmol·min^{-1}·mg^{-1}. This shows that in our case there was an increase of K_m and V_{max} values of peroxidase of acclimatized horseradish.

HRP experienced peak activity (pH_{opt}) at pH 6.0, with >80% of the maximum rate observed between pH 5.2 and 7.0 (Fig. 2).

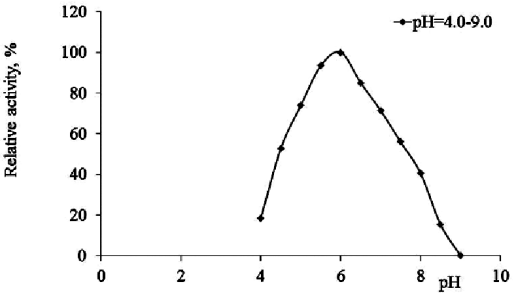

Fig. 2. Optimum pH of HRP

In order to evaluate the stability of HRP in acids and bases HRP incubated over a wide range of pH (pH=2.3, 4.0 for acid stability, pH=8.7, 9.7 for alkaline stability) and then the activity was measured (Fig. 3 and 4).

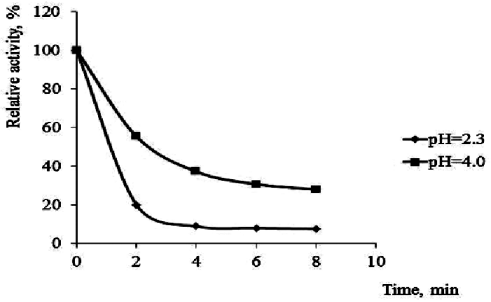

Fig. 3. Acid stability of HRP

From these results we can see that HRP is more stable in alkali, than in acid. In acid peroxidase begins to lose activity after 2 minutes of incubation whereas in alkali peroxidase is stable and retains greater than 50% (in pH=8.7 relative activity 57.1% and in pH=9.7 relative

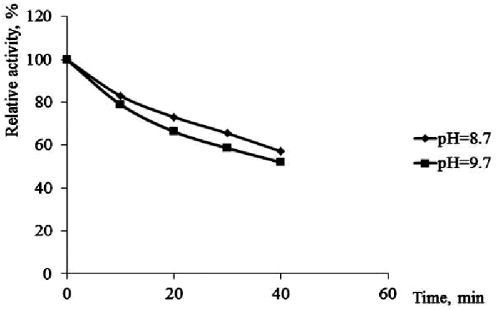

Fig. 4. Alkali stability of HRP

activity 52%) activity in 40 minutes.

These results are similar to commercial peroxidase which pH_{opt} was found at 6.0-6.5 and stable in the pH range of 5.0-9.0 [10].

Next, we studied the optimum temperature value and thermal stability of peroxidase. HRP show the optimum reaction temperature (T_{opt}) clearly at 40°C, with >60% of the maximum rate observed between 20°C and 60°C (Fig. 5).

Temperature stability was determined by heating at 55°C, 60°C and 65°C. The results show that after 5 minutes of heating at 65°C begins denaturation and inactivation of the enzyme, and at 60°C this process

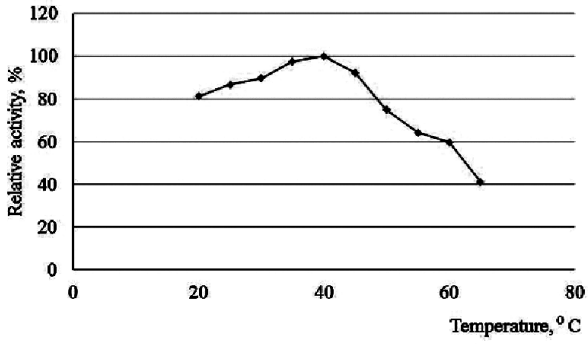

Fig. 5. Optimum temperature of HRP

occurs after 10 minutes and at 55°C after 15 minutes of heating, respectively (Fig. 6).

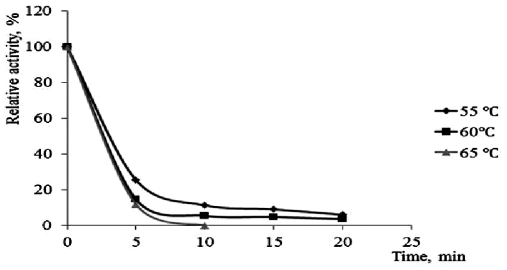

Fig. 6. Temperature stability of HRP

Azevedo *et al.* [11] determined thermal stability for the soluble enzyme at pH 7 (phosphate buffer) in comparison with immobilized peroxidase in aqueous-organic solvent mixture. The results of this work show that free peroxidase deactivates at 50°C with a half-life of 124 minutes. On the other studies Hendrickx *et al.* [12] investigated the influence of water activity on thermal stability of horseradish peroxidase. The results of this work show that dehydrated enzyme reveals much more thermostability than in solution. Our results confirm these findings and show that horseradish extract peroxidase markedly unstable to temperature influence.

Enzyme stability was measured by storing horse radish extract at 20°C, 5°C and -5°C for 21 days and enzyme activity was expressed by relative activity (Fig. 7).

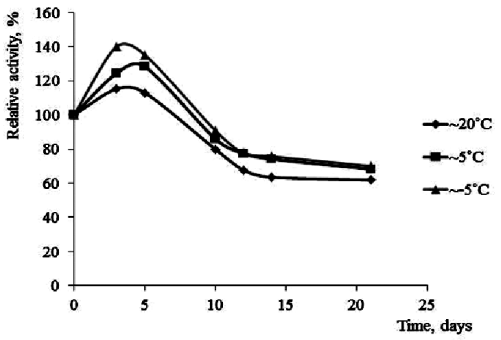

Fig. 7. Enzyme storing stability

During the first three days enzymatic activity is increased by 15.2% (for 20°C), 28.3% (for 5°C) and 40.0% (for -5°C) respectively. We believe that this may be due to the induction of this enzyme.

CONCLUSIONS

In conclusion, peroxidase from acclimatized horseradish has kinetic parameters: K_M 2.5 mM and V_{max} 5.36 mM·s^{-1}. pH_{opt} of HRP was at 6.0 and enzyme is more stable in alkali, than in acid. T_{opt} at 40°C and the enzyme is not stable to heat. The horseradish root's extract retains enzymatic activity within 21 days of not depending on the storage temperature.

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