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Influence of the concentration of enzyme, phosphate ions and calcium chloride on the heat-resistance of horseradish peroxidase

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1. SYNOPSIS

The objective of this work was to study the effect of the concentration of the enzyme, of calcium chloride and of phosphate ions on the heat resistance of horseradish peroxidase in aqueous solution at pH 11.5. The influence of the concentration of phosphate ions and calcium chloride was also studied at pH 7.0. It was found that decreasing the phosphate ion concentration increased the heat resistance at both pH, that the enzyme concentration did not affect its resistance, and that low concentrations of calcium chloride increased the resistance at pH 11.5, but not at pH 7.0.

2. NOTATION

A = Residual enzymic activity (ΔOD^{-1})
A_o = Initial enzymic activity (ΔOD^{-1})
α = Fraction of the heat-resistant enzyme
D = Decimal reduction time (min^{-1})
D_r = Decimal reduction time at the reference temperature T_r (min^{-1})
T_r = Reference temperature (65 °C)
z = Temperature increase required for a ten-fold decrease of the decimal reduction time (°C)

Subscripts:
s = heat-resistant isoenzyme
l = heat-labile isoenzyme

3. INTRODUCTION

The problem of enzyme stabilisation has received considerable attention in recent years.
A special application of enzyme thermal inactivation kinetics in food processing stems from their potential as Time-Temperature Integrators (TTI). It has been shown (1) that if the thermal inactivation kinetics follows a mathematical model with a temperature-sensitivity constant equal to that of the target micro-organism, then the F value of a thermal treatment can be calculated from the TTI response. The z value for micro-organisms varies between 5 and 12, depending on species, strain and the environment (2).
In a separate unpublished work the authors have verified that the z values for the thermal inactivation of horseradish peroxidase decreased significantly from around 30 °C (3) to around 10 °C in the pH range 11 - 12 and the decimal reduction times in the temperature range 50 - 80 °C decreased greatly (around 20 to 50 times).
The purpose of this work was to study simple ways of stabilising the enzyme in solution at this high pH. The following factors were studied: (i) concentration of sodium phosphate in the range 0 - 0.1 M in solution (without calcium chloride) at pH 7.0 and 11.5, (ii) concentration of peroxidase in the range 0.02 - 1.0 mg/ml in solution (without calcium chloride and with sodium phosphate at 0.1 M) at pH 11.5, (iii) concentration of calcium chloride in the range 0 - 0.6 M in solution (without sodium phosphate) at pH 11.5, (iv) joint effect of calcium chloride and sodium phosphate at pH 7.0. Sodium phosphate is normally used to buffer solutions at pH 7.0 and a previous study (4) has shown that they affect the inactivation kinetics at this pH.
The results could be useful both in the development of TTI systems and in biocatalysis. There is a great potential in the use of an enzyme catalyst in this pH range, but its applicability

requires an adequate heat resistance. Other methods of stabilisation can also be developed such as immobilisation, freeze-drying and use of organic solvents (5). This work is also necessary for those methods because the stability of the immobilised or freeze-dried forms depends on the characteristics of the original solution.

4. MATERIALS AND METHODS

The standard solution used was prepared by dissolving horseradish peroxidase (Ec 1.11.1.7, Sigma, St. Louis, Missouri, U.S.A.) in a sodium phosphate solution prepared with distilled water, with final concentrations of 0.08 mg enzyme per ml of solution and 0.1 M of sodium phosphate. The pH at room temperature was measured with a pH meter (Crison, 2001) and adjusted to 7.0 or 11.5 by adding a 0.1 M solution of sodium hydroxide. Solutions with other concentrations of enzyme (0.02, 0.04, 0.16, 0.32 and 1 mg/ml) were prepared in the same way.

The sodium phosphate solution was prepared as described in (6), to a concentration of 0.1 M. Solutions with different ionic strength were prepared by diluting this standard solution with distilled water to obtain the concentrations of 0.0005, 0.001 and 0.002 M prior to the pH adjustment with sodium hydroxide.

The solutions containing calcium chloride were prepared by diluting the enzyme in a calcium chloride solution instead of the sodium phosphate solution. The enzyme concentration was 0.08 mg/ml in all cases, the calcium chloride concentrations being 0.001, 0.0025, 0.005, 0.01, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 M.

The enzymic activity analysis followed the procedure suggested by Worthington (7), which is based on the measurement of the increase in optical density of a substrate solution. The substrate had a final concentration of oxygen peroxide (27% w/w) of 0.9 mM, a concentration of phenol of 83.09 mM and a concentration of aminoantipyrine of 1.19 mM. The substrate was buffered to a pH of 7.0 with a 0.1 M sodium phosphate solution. When analysing samples with calcium chloride, the buffer used was an imidazole (glyoxaline) - HCl solution (0.1 M), prepared as described in (6). The optimum temperature for this analysis given in (3) is 25 °C. An aliquot of 100 µl of the enzyme solution to be analysed was mixed with 2.9 ml of substrate. The increase in optical density at 510 nm was measured in a Unicam 8625 UV/VIS kinetic spectrometer every second for one minute.

The thermal inactivation was performed by placing glass tubes of 9 mm diameter and 10 cm height containing a maximum of 3 ml of the enzyme solution in a thermostatic bath previously equilibrated at the specified inactivation temperature. At pre-determined time intervals 250 µl were pipetted into a glass tube of the same size which was immediately immersed in an ice bath. After cooling, 0.375 ml of the buffer (sodium phosphate or imidazole) were added to each sample. Duplicates were always used.

The thermal inactivation kinetics was represented by a two fraction model, as described in (3), (4) and (5). This model considers that the enzyme consists of two iso-enzyme populations, one being more heat resistant than the other and both being inactivated according to a first order decay. At a constant temperature the mathematical model is therefore:

$$\frac{A}{A_o} = \alpha \cdot 10^{-\frac{t}{D_S}} + (1-\alpha) \cdot 10^{-\frac{t}{D_l}} \qquad 1$$

The kinetic constants in equation 1 were determined by non-linear regression of the experimental results, using commercial software (STATA 3.0). The correlation coefficient was always greater than 0.975, being higher than 0.99 in most cases. When the temperature-sensitivity was analysed, the following relationships were used for the heat resistant and heat labile isoenzymes, respectively:

$$D_S = D_{r_S} \cdot 10^{-\frac{T-T_r}{z_S}} \qquad D_l = D_{r_l} \cdot 10^{-\frac{T-T_r}{z_l}} \qquad 2$$

5. RESULTS AND DISCUSSION

Table 1: Kinetic constants (85 °C, pH 7.0)

[phosphate] (M)	α (%)	D_s (min)	D_l (min)
0.0	34±4	106±8	8±2
0.0005	41±3	95±4	2±1
0.001	37±4	84±4	0.8±0.4
0.002	25±7	67±9	2.4±1

[phosphate] indicates the concentration of phosphate ions

Table 2: Kinetic constants (55 °C, pH 11.5)

[phosphate] (M)	α (%)	D_s (min)	D_l (min)
0.0001	5±1	389±9	28±11
0.1	3±2	34±7	0.5±0.5

Table 3: Kinetic constants (65 °C, pH 11.5)

[peroxidase] (mg/ml)	α (%)	D_s (min)	D_l (min)
0.02	2.4±0.7	84±23	9±1
0.04	3.6±1	106±31	6.9±0.7
0.08	2.9±1.5	64±21	7.9±0.8
0.16	6.5±2.9	63±15	12±2
0.32	1.4±1	170±27	12±1
1.0	1.9±0.6	169±41	15±11

[peroxidase] indicates the concentration of enzyme

Table 4: z values (55 - 65 °C, pH 11.5)

[peroxidase] (mg/ml)	z_s (°C)	z_l (°C)
0.04	8.5±1	11±2
0.08	6.7±0.8	22±4
0.16	14.7±8	11.8±1.7

Table 5: Kinetic constants (65 °C, pH 11.5)

[calcium] (M)	α (%)	D_s (min)	D_l (min)
0	3±1	64±20	7.9±0.8
0.001	3±1	880±110	52±1
0.0025	7±2	383±113	51±6
0.005	11±3	377±157	44±4
0.01	7±2	528±156	36±3
0.1	5±2	226±99	17±3
0.2	9±2	174±47	11±2
0.3	3±1	118±40	7.9±0.8
0.4	1.0±0.7	86±52	7±1
0.5	1.1±0.7	62±11	6.0±0.3
0.6	7±3	62±19	6±1

[calcium] indicates the concentration of calcium chloride

Table 6: Kinetic constants (85 °C, pH 7.0)

[calcium]/ [phosphate] (M)	α (%)	D_s (min)	D_l (min)
0 / 0	34±4	106±8	8±2
0 / 0.005	14±7	64±22	6±2
0.001 / 0	37±13	77±12	14±7
0.001 / 0.005	7±2	72±16	3.0±0.7

Tables 1 and 2 show the effect of the concentration of phosphate ions at pH 7.0 and 85 °C and at pH 11.5 and 55 °C, respectively. It is clear in both cases that the enzyme thermal resistance is increased by decreasing the concentration of sodium phosphate.

Table 3 shows that the enzyme concentration does not have a significant effect at 65 °C and pH 11.5 in the range studied. Several experiments were then performed at 55, 60 and 70 °C with three concentrations of the enzyme to find the temperature-sensitivity factor z, the results showing a negligible effect of the enzyme concentration at a 95% confidence level (table 4).

The effect of the concentration of calcium chloride at pH 11.5 and 65 °C is shown in terms of its influence on the D values of both isoenzymes in table 5. It is clear that a great increase in the resistance results from adding a small amount of calcium chloride. As the concentration of calcium chloride increases, the resistance decreases, with the D values reaching again those without calcium chloride. Several experiments were then performed with calcium chloride concentrations of 0.01 M and 0.3 M at other temperatures (55, 60, 70 and 80 °C) to study the effect on the temperature-sensitivity. It was found that the z values were not affected by the calcium chloride concentration. The z-values for the heat-resistant isoenzyme were 9.5 °C and for the heat-labile isoenzyme 11.9 °C at both concentrations.

The effect of calcium chloride was further studied by performing inactivation experiments at pH 7.0 with different combinations of concentrations of calcium chloride and sodium phosphate, which allowed possible interactive effects to be identified. The results are shown in table 6. It can be seen that adding phosphate buffer decreased the stability regardless of the content of calcium chloride, but the stabilising effect of the small amount of calcium chloride at the concentration of 0.001 M was not observed at pH 7.0. While at pH 11.5 this concentration of sodium phosphate increased the D values around 10 times, at pH 7.0 the difference between adding this solution or not is minimal. The factorial analysis indicated that interactive effects were negligible.

This result clearly shows the importance of the structural conformation of the enzymic matrix on the mechanisms of thermal inactivation and on the heat resistance. The very different z values at pH 7.0 and 11.5, which were not affected by any of the factors studied, indicated that the mechanisms of thermal inactivation are different, possibly changing from conformational at neutral pH to covalent at pH 11.5. Although small amounts of calcium chloride eventually lead to a more structured matrix at pH 11.5, at neutral pH there is no effect. Tomazic and Klibanov (8) and Combes *et al* (9) have shown that the effect of a given additive on different enzymes can be very different, even opposite. This present work shows that the specific effect of additives is also related to the actual conformation of the enzyme and therefore to the characteristics of its environment.

6. CONCLUSIONS

The effect of specific agents on the heat-resistance of horseradish peroxidase was found to be specific to the enzyme system, different results being obtained at pH 7.0 and 11.5 for the effect of calcium chloride. The optimal thermal resistance at pH 11.5 was found with a low concentration of calcium chloride (0.001 M) and of sodium phosphate (0.0001 M). The z value was not affected by any of the factors studied.

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