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A comparative study of effects of nitrogen and argon on tyrosinase and malic dehydrogenase activities

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Abstract

Tyrosinase and malic dehydrogenase were treated with argon and nitrogen. Their activities were measured with time. The activities of tyrosinase and malic dehydrogenase when treated directly with argon, and when treated as a mixture of the enzymes and their substrates, were lower than with nitrogen treatments. The results suggest that argon reduces the activities of tyrosinase and malic dehydrogenase more than nitrogen. Tyrosinase activity with argon treatment was reduced by up to 14.2% more than nitrogen treatment when treated directly, and by up to 22.6% in the mixture of the enzyme and substrate. Malic dehydrogenase activity with argon treatment was reduced by up to 9.5% more than nitrogen treatment when treating the enzyme directly, and by up to 13.9% in the mixture of the enzyme and substrate.

Keywords: Argon; Nitrogen; Tyrosinase; Malic dehydrogenase

1. Introduction

Recently, use of noble gases such as argon (Ar), helium (He), neon (Ne), xenon (Xe), and radon (Ra) has been introduced into the preservation of foods. Since the 1960s up to 100 patents and a few publications (Spencer, 1995) have appeared describing the use of argon and other noble gases for packaging, but most of this literature generally describes the gases to be completely inert and equivalent to nitrogen in their non-reactivity. By contrast, a few literature citations (Behnke, 1969; Colten, 1990; Doebbler, 1967; Schreiner, 1964, 1965, 1966, 1968; Spencer, 1995) are known in which it has been suggested that argon and other noble gases may have properties unique for application to biological systems or food, but each of these citations shows different results and fails to substantiate or even suggest the conclusion upon which the invention is premised. Some experiments (Spencer, 1995) have indicated that noble gases are biochemically active, probably due to their enhanced solubility in water compared with nitrogen and possible interference with enzymatic oxygen receptor sites. Thus, they may offer preservative qualities in the gas packaging and processing of foodstuffs. So far there has been little research ongoing into the biochemical and physiological effects of noble gases on specific enzymes related to browning of fresh fruits and vegetables and respiratory metabolism.

Tyrosinase (*monophenol, dihydroxyphenol oxidoreductase* EC 1.14.18.1) is a copper-containing enzyme that catalyses two reactions in which molecular oxygen is the hydrogen receptor, phenol is the hydrogen donor, and AH_2 represents the hydrogen donor:

$$\text{monophenol} + O_2 + AH_2 = o\text{-dihydroxyphenol} + H_2O + A$$

$$o\text{-dihydroxyphenol} + 1/2\,O_2 = o\text{-quinone} + H_2O.$$

It is one of the phenolic oxidative enzymes which cause browning in many fruits, vegetables, and other foods in the presence of oxygen (Mayer, 1987; Mayer & Harel, 1991; Robinson, 1991; Vamos-Vigyazo, 1981). It has been reported to be present in mushrooms (Kumar & Flurkey, 1992). Selective substrates have been used to differentiate the phenolic oxidative enzymes with moderate

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success. Tyrosine and Dopa are relatively specific substrates for tyrosinase (Marr, 1984; Mayer & Harel, 1979).

Malic dehydrogenase (EC 1.1.1.37) is one of the key enzymes in the Krebs cycle of respiration metabolism. The tricarboxylic cycle (Krebs cycle) is completed when the oxidation of L-malic acid to oxaloacetic acid is accomplished by the enzyme malic dehydrogenase (Mathews & van Holde, 1990). The reaction is the fourth oxidation-reduction reaction to be encountered in the cycle and involved in the exchange of energy; the oxidising agent for the enzyme is NAD^+.

$$C_4H_6O_5 \text{ (malic acid)} + NAD^+ = C_4H_4O_5 \text{ (oxaloacetic acid)} + NADH + H^+$$

At pH 7.0 the equilibrium constant is 1.3×10^{-5}; thus the equilibrium is very much to the left. The further reaction of oxaloacetate with acetyl-CoA in the condensation reaction is strongly exergonic in the direction of citrate synthesis. This tends to drive the conversion of malate to oxaloacetate by displacing the equilibrium through the continuous removal of oxaloacetate.

The objective of this research was to investigate effects of nitrogen and argon on tyrosinase and malic dehydrogenase to understand whether these gases have any effects on physiological and respiratory metabolism.

2. Materials and methods

2.1. Materials

Tyrosinase and malic dehydrogenase were obtained from Sigma Co. The tyrosinase solution was prepared regularly at 0.2 mg tyrosinase/ml with 0.1 M sodium phosphate (pH 6.0). Malic dehydrogenase was a suspension in 3 M $(NH_4)_2SO_4$–0.01 M KH_2PO_4 solution, 1.4 mg protein/ml (pH 7.3). The gases, nitrogen and argon, were obtained from Air Products PLC. Nitrogen was oxygen-free in a cylinder filled to 230 bar maximum at 15°C and argon was pureshield argon in a cylinder filled to 230 bar maximum at 15°C.

2.2. Tyrosinase assays and treatments with nitrogen or argon

Tyrosinase activities were assayed in 0.1 M sodium phosphate (pH 6.0) using 1 mM tyrosine as substrate. The increase in absorbance at 280 nm was monitored for tyrosine. One unit of activity was defined as an increase in 0.1 absorbance unit. Enzyme activity was defined as units of enzyme/mg protein/min. For treatments with nitrogen or argon, two different experiments were set up. The first one was to use nitrogen or argon directly to treat tyrosinase. An 8 ml vial, kept in an ice bath, was flushed continuously with nitrogen or argon at 10 ml/min. After flushing for 5 min, tyrosinase was placed in the vial using a syringe equipped with a long needle through the outlet for the gas; then the duration of treating with the gases was timed. Tyrosinase activity was then assayed at 25°C in a reaction mixture of 3 ml that included 1 mM tyrosine and 0.1 M sodium phosphate (pH 6.0) with a spectrophotometer at 30, 120, 300, 600, and 1200 s, respectively, after treatment with the gases. The second experiment used nitrogen or argon to treat the mixture of tyrosinase and its substrate. An 8 ml vial was flushed continuously with nitrogen or argon at 10 ml/min. After flushing for 5 min, the substrate was placed in the vial before the enzyme was added to the substrate in the same vial. Tyrosinase activity in the reaction mixture was assayed at 25°C at 30, 60, 120, 300, 600, 1200, and 1800 s, respectively, after the reaction mixture was treated with the gases. Fig. 5 (see below) shows the system used for treatment and assay.

2.3. Malic dehydrogenase assays and treatments with nitrogen or argon

Malic dehydrogenase activities were assayed at 25°C by the oxidation of reduced nicotinamide adenine dinucleotide (NADH). Solutions prepared were 0.1 M sodium phosphate buffer at pH 7.4, 10 ml cold, distilled water containing 5 mg NADH, and 0.06 M oxaloacetic acid. The oxaloacetic acid was dissolved in a small amount of water, adjusted to pH 7.4 and made up to required volume with buffer. The reaction mixtures were prepared as follows: 0.2 ml NADH, 2.8 ml 0.06 M oxaloacetic acid in 0.1 M sodium phosphate buffer at pH 7.4, and 50 μl malic dehydrogenase. Malic dehydrogenase activities were measured by the decrease in absorbance at 340 nm for 120 s, which was associated with NADH oxidation. One unit of activity was defined as an decrease in 0.001 absorbance unit. Enzyme activity was defined as units of enzyme/mg protein/min. For treatments with nitrogen or argon, two different experiments were set up. The first one used nitrogen or argon to directly treat malic dehydrogenase. An 8 ml vial kept in an ice bath was flushed continuously with nitrogen or argon at 10 ml/min. After flushing for 5 min, malic dehydrogenase was placed in the vial through the outlet for the gas, then the duration of treating with the gases was timed. Malic dehydrogenase activity was assayed (25°C) at 340 nm at 60, 180, 300, and 600 s, respectively, after being treated with the gases. The second experiment used nitrogen or argon to treat a mixture of malic dehydrogenase, its substrate and co-enzyme (NADH). An 8 ml vial was flushed continuously with nitrogen or argon at 10 ml/min. After flushing for 5 min, the mixture of the substrate and co-enzyme (NADH) was placed in the vial before addition of the enzyme. Malic dehydrogenase

activity was assayed (25°C) at 60, 180, 300, and 600 s, respectively, after the reaction mixture was treated with the gases. Malic dehydrogenase activity was also measured at 25°C in an air-treated control.

2.4. Data handling

All data were obtained from means of three individual experiments where there were three replicates for each treatment. Data were subjected to the *t*-test for significant difference between argon and nitrogen treatments at the 0.05 level.

3. Results

3.1. Effects of nitrogen or argon on tyrosinase

Fig. 1 shows the effect of nitrogen or argon on tyrosinase. It indicates that both nitrogen and argon reduced the tyrosinase activities, compared to tyrosinase activity without gas treatment. The activity of the argon-treated tyrosinase was lower than that of the nitrogen-treated tyrosinase. The activity of nitrogen-treated tyrosinase stayed relatively constant, while the argon treatment showed a gradual fall. Tyrosinase activity with argon treatment was 98.2% of nitrogen treatment at the 30 s, 97.6% at the 120 s, 89.4% at the 300 s, 86.6% at the 600 s, and 85.8% at the 1200 s separately. However, a further reduction in the activity of argon-treated tyrosinase could not be enhanced with increase in the period of argon treatment. There was a significant difference between argon and nitrogen treatments ($P < 0.05$).

3.2. Effects of nitrogen or argon on the reaction of tyrosinase and its substrate

Fig. 2 shows the effect of nitrogen or argon on the reaction of tyrosinase and its substrate. It indicates that both nitrogen and argon slowed down the reaction of tyrosinase with its substrate. The activity of nitrogen-treated tyrosinase stayed relatively constant. After 30 s, the activity of argon-treated tyrosinase was lower than the nitrogen treatment. The activity of argon treatment was 97.4% of nitrogen treatment at the 60 s, 91.4% at the 120, 80.1% at the 300 s, 86.2% at the 600 s, 77.4% at the 1200 s and 82.4% at the 1800 s separately. There was a significant difference between argon and nitrogen treatments ($P < 0.05$).

3.3. Effects of nitrogen or argon on malic dehydrogenase

Fig. 3 shows the effect of nitrogen or argon on malic dehydrogenase. It indicates that both nitrogen and argon slightly reduced the malic dehydrogenase activities, compared to malic dehydrogenase activity in air. The activity of the argon-treated malic dehydrogenase was lower than that of the nitrogen-treated malic dehydrogenase. The activity of argon treatment was 91.9% of nitrogen treatment at the 60 s, 90.5% at the 180 s, 90.6% at the 300 s, and 91.6% at the 600 s separately. However, increasing the duration of gas treatment up to

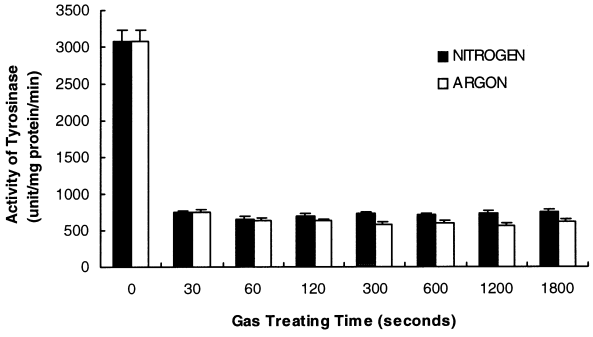

Fig. 2. Effects of nitrogen and argon on the reaction of tyrosinase and its substrate. Vertical bars represent S.E. One unit of activity was defined as an increase in 0.1 absorbance unit. Enzyme activity was defined as units of enzyme/mg protein/min.

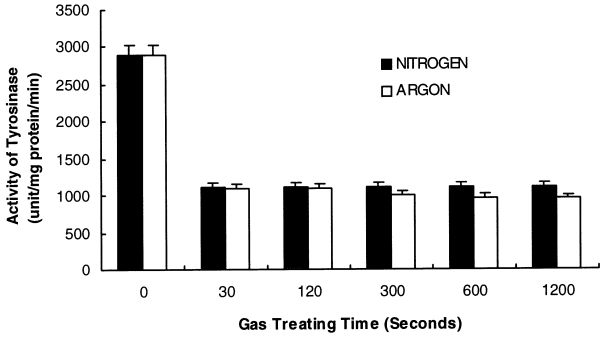

Fig. 1. Effects of nitrogen and argon on tyrosinase. Vertical bars represent S.E. One unit of activity was defined as an increase in 0.1 absorbance unit. Enzyme activity was defined as units of enzyme/mg protein/min.

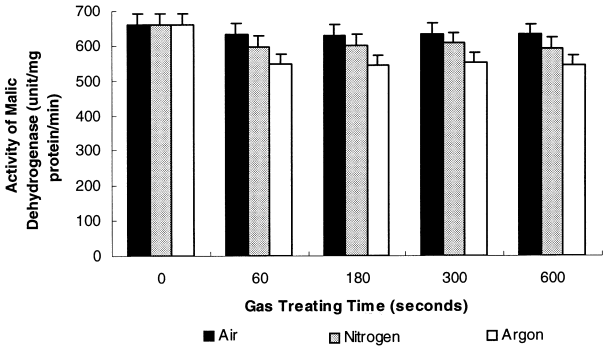

Fig. 3. Effects of nitrogen and argon on malic dehydrogenase activity. Vertical bars represent S.E. One unit of activity was defined as an increase in 0.001 absorbance unit. Enzyme activity was defined as units enzyme/mg protein/min.

10 min did not enhance the effect. There was significant difference between argon and nitrogen treatments ($P < 0.05$).

3.4. Effects of nitrogen or argon on the reaction of malic dehydrogenase and its substrates

Fig. 4 shows the effect of nitrogen or argon on the reaction of malic dehydrogenase and its substrates. It indicates that both nitrogen and argon slowed down the reaction of malic dehydrogenase and its substrate slightly. Since, during gas treatment, the reaction of malic dehydrogenase and its substrate progressed, all the activities of control, nitrogen treatment, and argon treatment decreased. However, there were some differences between the controls and treatments. The activities of argon treatments were lower than nitrogen-treatments. The activity of argon treatment was 95.1% of nitrogen treatment at the 60 s, 93.0% at the 180 s, 86.1% at the 300 s, and 86.6% at the 600 s separately. There was significant difference between argon and nitrogen treatments ($P < 0.05$) (Fig. 4).

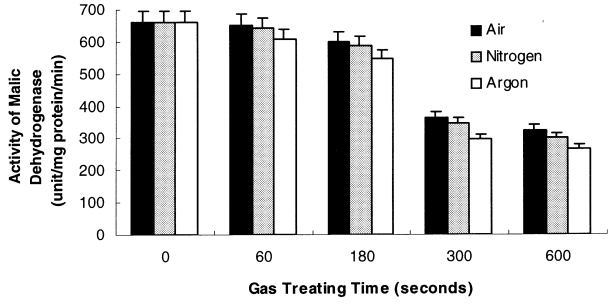

Fig. 4. Effects of nitrogen and argon on the reaction of malic dehydrogenase and its substrate. Vertical bars represent S.E. One unit of activity was an decrease in 0.001 absorbance unit. Enzyme activity was defined as units of enzyme/mg protein/min.

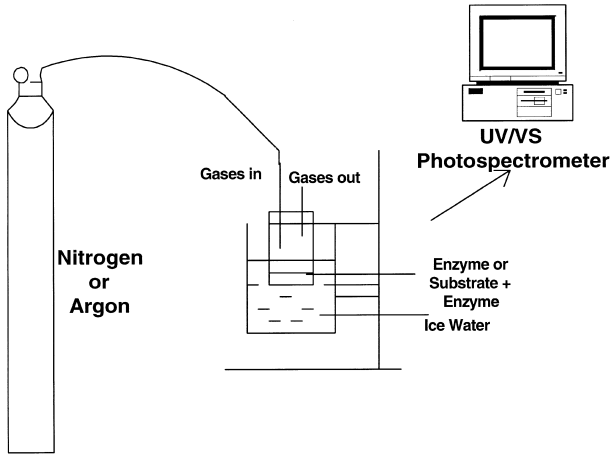

Fig. 5. The system for using nitrogen and argon to treat tyrosinase and malic dehydrogenase and assaying tyrosinase and malic dehydrogenase activity.

4. Discussion

Although, recently, some experiments (Spencer, 1995) have indicated that noble gases are biochemically active and use of noble gases such as argon (Ar), helium (He), neon (Ne), xenon (Xe), and radon (Ra) has been introduced into the preservation of foods, there are no detailed data on their physiology and biochemistry, especially on effects of noble gases on specific key enzymes such as tyrosinase and malic dehydrogenase related to browning and respiration metabolism.

Argon is two and half times more soluble in water than nitrogen, having slightly better solubility than oxygen (Weast, Astle & Beyer, 1983). Argon is reported to be biochemically active, probably due to its enhanced solubility in water compared with nitrogen and possible interference with enzymatic oxygen receptor sites (Spencer, 1995). Behnke (1969) suggested that high pressure inert gases inhibit tyrosinase in non-fluid (i.e. gelatin) systems by decreasing oxygen availability, rather than by physically altering the enzyme. In our studies, treating the mixture of tyrosinase and its substrate reduced tyrosinase activity more than the control in air. This may be because argon has a better ability than nitrogen to reduce the level of dissolved oxygen whose presence is necessary for tyrosinase to catalyse the reaction. Our results also indicate that treating tyrosinase directly with argon has a better effect than nitrogen. Therefore, we suggest that argon could inactivate some chemically-active sites on the enzyme more effectively than nitrogen.

In non-competitive inhibition, the molecules of inhibitors could bind to a second site (inhibiting site) on the enzyme or might distort the enzyme so that the substrate could not bind to the active site properly and the catalytic process is not as efficient (Mathews & van Holde, 1990). Our study, treating malic dehydrogenase with argon and nitrogen, indicates that argon treatment has a slightly greater effect on malic dehydrogenase than nitrogen treatment. This may be because the greater solubility of argon compared to nitrogen could generate a greater affinity with the inhibiting site on the enzyme than nitrogen.

Although argon treatments have a greater effect on tyrosinase and malic dehydrogenase than nitrogen, the effect may be limited. However, a gaseous inhibitor for the enzymes related to browning and respiration could have an important role in maintaining the quality of fresh fruits and vegetables to replace some chemical treatments causing potential health risks. Our research on sliced apple treated with argon and nitrogen in modified atmosphere packaging (data unpublished) indicates that argon treatment affects the respiratory level inside packaging more than nitrogen treatment.

As the gases used may contain trace amounts of impurities such as CO and oxides of nitrogen, these might inhibit tyrosinase and interfere with the oxidising

power of NAD for the malate enzyme, the purity of the gases used would be an important factor for consideration.

Before commercial application, there is a need for research and technology development to reach a better understanding of the scientific and technological issues affecting fresh produce using noble gases and to support the growth of the industry through improved quality and shelf-life. In addition, noble gases would be permitted for food use in the UK as miscellaneous additives, and so users will be able to exploit their use in food grade gas mixtures. Recently, with the development of MAP technology, there has been great interest in the potential benefits of using argon and other noble gases for MAP since the application of noble gases may be a new way to improve the quality of MAP of fresh foods.

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