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Promotive Effect of Supersonic Oscillation on Carthamin Bleaching Induced by Peroxidatic Enzyme Oxidation

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ABSTRACT

The effect of supersonic oscillation on carthamin bleaching by peroxidatic enzyme oxidation was investigated in an experimental model system under varied pH and temperature conditions. The loss of the red colour with enzyme after sonosound irradiation (due to the high sensitivity of carthamin towards peroxidative species) was greater than with no added enzyme preparation. The progress curves from light absorption spectra indicated that the oscillatory promotion is pH- and temperature-dependent. The rates of carthamin bleaching at three different pH values were as follows (enzyme-containing and non-treated control = 1·0): pH 3·0 = 1·32, pH 5·0 = 1·27, pH 7·0 = 2·03. Another experiment at three given temperatures showed the following ratios (enzyme-containing and non-treated control = 1·0): 20°C = 0·93, 30°C = 1·27, 40°C = 1·45. The data are briefly discussed.

INTRODUCTION

Reports have recently been published concerning sonochemical effects on organic synthesis on a laboratory scale with newly established supersound

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techniques (Abdulla, 1988). Supersonic irradiation of solutions of inorganic and organic substances has potentially serious effects on the molecular structures (Abdulla, 1988; Suslick, 1989). McKee *et al.* (1977) reported that cytosine, uracil, guanine, thymine and adenine were all degraded into unknown products in aqueous solution under 1 MHz of supersound. It was suggested that the rate-limiting step was an attack of OH^- on the bases. Staas & Spurlock (1975) demonstrated that 0·8 MHz ultrasound decomposed a variety of amino acids over a 6-h period of irradiation. Cystine was oxidized to cysteine. The other amino acids were deaminated and/or decarboxylated presumably by the action of free radicals on the α-carbon atom. The synthesis of amino acids was proposed by Sokol'skaya (1975, 1978) in which a nitrogen-saturated solution of HCHO was irradiated with ultrasound at 850 kHz for 12 h. Glycine was detected to be the major product, accompanied by a smaller amount of alanine, lysine and glutamic acid. Notwithstanding the considerable number of reports from pure and applied chemistry, this useful sono-technique has not yet been applied to cell-free and/or enzyme systems. It therefore follows that the effects of supersound on biocatalytic reactions are little known. Information on this becomes more essential and important in view of the recent findings that hydrolysis, oxidation and/or reduction are promoted by supersonic radiation.

Accepting that sound waves contribute to chemical events, we have focused attention here on the supersonic oscillation of a biooxidative reaction. Carthamin bleaching, which is induced by a peroxidative enzyme (Kanehira & Saito, 1990), seemed to be a very promising area to study sonochemical effects on the enzyme process. The rapid decrease of its red colour can be easily observed in aqueous media, which is followed by a simultaneous consumption of atmospheric oxygen.

Initial evidence from this experiment will be presented below and discussed briefly with regard to the possible effects of supersonic irradiation on an enzyme reaction.

MATERIALS AND METHODS

Materials

Carthamin, used as an enzyme substrate, was from our laboratory collection. Seeds of dyer's saffron were obtained commercially. Other chemicals and reagents were purchased from several commercial sources as described previously (Kanehira & Saito, 1990).

Preparation of enzyme extract

Seeds were soaked for about 15 h in tap water, rinsed for 1 h in disinfectant solution at room temperature (20–22°C) and then maintained in a growth-box at 28°C on moistened vermiculite in the dark. At intervals water was supplied to maintain appropriate moisture levels.

Derooted hypocotyls were gathered from 7-day-old etiolated seedlings. Weighed hypocotyls (300 g fresh wt) were homogenized with 250 ml of 50 mM citrate-phosphate buffer (pH 7·0) containing 20 mM sodium D-araboascorbate and 0·1 mM 2-mercaptoethanol. The slurry was passed through nylon-cloth and the filtrate centrifuged for 20 min at 12 000 × *g* at 2 ± 1°C. $(NH_4)_2SO_4$ was added to the supernatant to obtain a saturated solution of 40% and then centrifuged at 12 000 × *g* for 5 min at 2 ± 1°C. The resultant pellet was suspended in 3 ml of 50 mM citrate-phosphate buffer (pH 7·0) and desalted with Sephadex G-25 gel. The liquid was again saturated with $(NH_4)_2SO_4$ to obtain an 80% solution. The precipitate obtained was then suspended in 1 ml of 50 mM citrate-phosphate buffer (pH 7·0) and placed in a Sephadex gel column (1·5 × 90 cm, bed vol. 156 ml), previously equilibrated with 50 mM citrate-phosphate buffer (pH 7·0). The column was eluted with the equilibration buffer. Fractions (24–35, 5 ml each) were retained for assaying enzyme activity.

Method for assaying enzyme activity

Standard assay was performed by adding 0·1 ml of appropriately-diluted enzyme solution (531 μg protein/ml) to a reaction mixture containing 10 ml of 66 μM carthamin in 50 mM citrate-phosphate buffer (pH 6·7), 0·1 ml of 0·03 mM H_2O_2 and 0·1 ml of 0·03 mM $FeCl_3$. Final volume was 10·3 ml. The mixtures were incubated, if not otherwise stated, at 30°C for 0–120 min with continuous vibration in a supersound oscillator (Cho-Onpa Kogyo, type UE-600Z 26S-2A) at 26 kHz. The colour decrease was measured at 521 nm with a Shimadzu double-beam spectrophotometer, type UV 150–02. Oxygen consumption was monitored with an Orion oxygen electrode, model 97-08. Protein contents were determined using the Folin-Ciocalteu method modified by Lowry *et al.* (1951).

RESULTS AND DISCUSSION

To test whether or not enzymic bleaching of carthamin is affected by supersound treatment, a mixture consisting of a given amount of carthamin and dyer's saffron enzyme in citrate-phosphate buffer was exposed to sono-

oscillation in the presence of Fe^{3+} and H_2O_2 under varied conditions. Figure 1 shows the data at three different pH values, 3·0, 5·0 and 7·0, at 30°C. It is evident from the diagram that the sono-vibration strongly affects both enzymic and non-enzymic bleaching of carthamin. Sonic treatments always resulted in higher average values of carthamin loss than those of no irradiation. The sono-effect is more pronounced at lower pH, especially in enzyme reactions. The promotive effect ranges of enzyme containing extracts were as follows (non-treated control = 1·0): pH 3·0 = 1·32, pH 5·0 = 1·27, pH 7·0 = 2·03.

In the supersonic acceleration of carthamin bleaching, temperature also acted as a decisive factor (Fig. 2). At the three temperature levels examined, the highest rate is seen at 40°C. The value falls off gradually in proportion to the temperature decrease. The promotive effect is emphasized markedly in the enzyme bleaching. The ratio calculated from each enzyme-containing system is as follows (non-sono-sound treated test in control = 1·0): 20°C = 0·93, 30°C = 1·27, 40°C = 1·45. Based on our previous work (Kanehira & Saito 1990), participation of oxygen in the carthamin bleaching should also be clarified using this procedure. However, under the conditions of the present study, oxygen consumption could not be measured by the electrode method. This paper is the first report dealing with supersound treatment of the carthamin bleaching system under varied pH and/or temperature conditions. Current evidence clearly indicates that an enzyme reaction is positively stimulated by the sonication.

Sound waves are thought to affect chemical reactions when they induce 'cavitation', the rapid growth and sudden collapse of bubbles within a liquid (Abdulla, 1988; Suslick, 1989), though this can be conceptually divided into two processes (rarefaction phase and compression half-phase), In the former, a cavitation nucleus undergoes isothermal expansion, during which time it passes into the cavity from the liquid. In the latter, the bubble is swiftly and adiabatically compressed, leading to intense, localized pressure and temperature differentials, as well as electric discharge, owing to the development of non-uniformly distributed and uncompensated charge on the surface of the bubbles. Margulis (1981) suggested that, in such a process, remarkably high differentials of pressure, temperature and electrostatic potential are possible. Richards and Loomis (1927) listed the following sonochemical effects: (1) hydrolysis of dimethyl sulphate in basic solution is accelerated, (2) HgI (yellow) is converted to HgI_2 (red) at relatively low temperature, (3) reduction of KI by aqueous H_2SO_3 is promoted. Spurlock and his co-workers (Spurlock & Reifsneider, 1970; Reifsneider & Spurlock, 1973; Fayter & Spurlock, 1974) proposed that most sonochemical reactions carried out in water are induced by the thermolytic or electronic breakdown of H_2O to give H_2O_2.

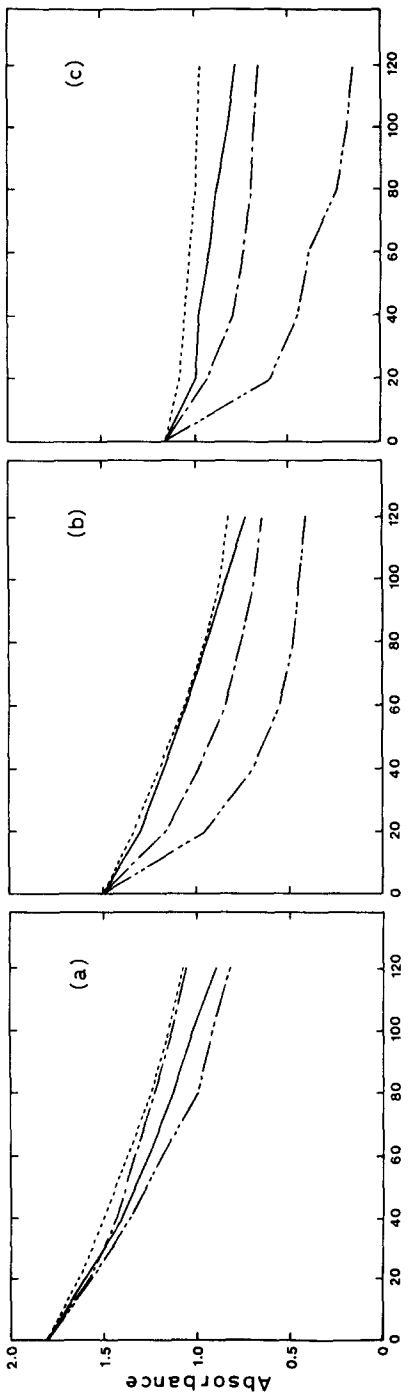

Fig. 1. Effect of supersonic oscillation on carthamin bleaching. Incubation was carried out at 30°C under varied pH levels indicated as follows: (a) pH 3·0; (b) pH 5·0; (c) pH 7·0. Non-treatment with supersound: - - - - - carthamin; —·—·— carthamin + enzyme. Treatment with supersound: —— carthamin; —··—··— carthamin + enzyme. Enzyme activity was determined by the light absorption change at ΔA_{521}. For details of the enzyme assay see section on Materials and Methods.

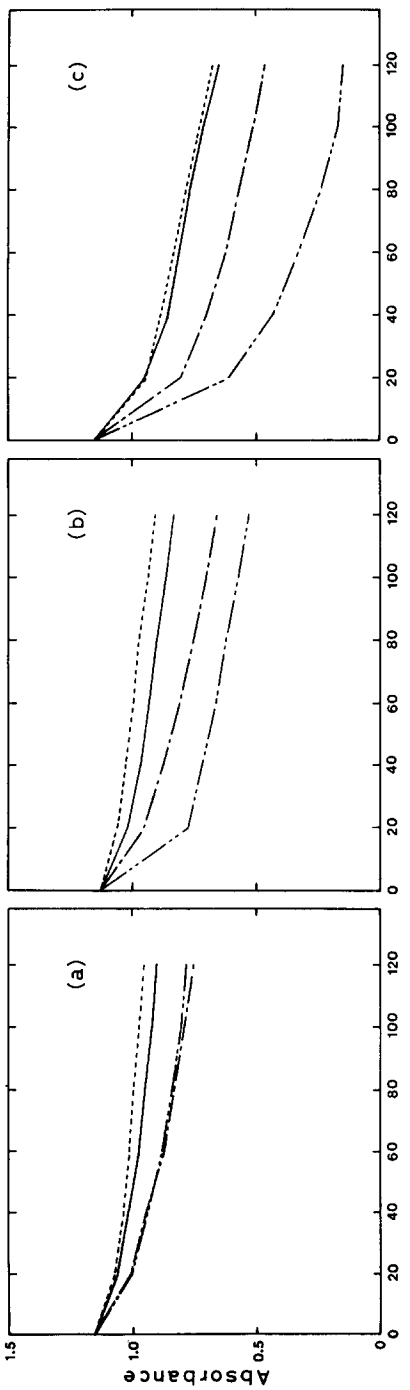

Fig. 2. Effect of supersonic oscillation on carthamin bleaching. Incubation was performed at pH 7·0 under varied temperature levels indicated as follows: (a) 20°C; (b) 30°C; (c) 40°C. Non-treatment with supersound: ----- carthamin; —·—·— carthamin + enzyme. Treatment with supersound; ——— carthamin; —··—··— carthamin + enzyme. Enzyme activity was determined by the light absorption change at ΔA_{521}. For details of the enzyme assay see section on Materials and Methods.

Although the above evidence deals with a wide range of chemical events, it can also cover biological reactions. This is strongly supported by the data indicating that enzymic bleaching activity is promoted by supersonic oscillation. In addition, bleaching activity is always at a markedly high level in enzymic mixtures when compared with that of enzyme-free incubation systems. At higher temperature and lower pH, this characteristic feature is accentuated clearly. A number of mechanisms would presumably be involved in the promotive effect of the supersonic vibration on a peroxidative enzyme reaction system. Among them, two or three can be supposed as follows: (1) carthamin is supplied swiftly to the active site of enzyme by an increase in the molecular movement through the bubble compression, (2) catalytic potentials of the enzyme are elicited by exposure to the intense, localized pressure and temperature differentials, (3) electronic discharge hastens the transfer of its electron, through which the red colour is bleached out. H_2O_2 generated through sonochemical reactions may also contribute to the carthamin bleaching process.

In this work we demonstrated that supersonication is very effective in accelerating enzyme-mediated catabolism *in vitro*. Additional studies should furnish us with further and more conclusive evidence of sonochemical effects on biological systems.

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