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The effect of a natural inhibitor isolated from the tealeaf on the energy processes in model systems☆

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Abstract

Food spoilage is a significant problem and constitutes a major economic loss. The formation of coloured spoilage in food products is largely due to the activity of two classes of redox enzymes: copper-containing phenol oxidases and heme-containing peroxidases. This effect was studied with an inhibitor of phenol oxidases and peroxidases that was isolated from leaves of the tea plant and applied in two model systems, namely cultures of the unicellular alga, *Chlorella vulgaris* and excised roots of wheat seedlings. The data demonstrated that the tealeaf inhibitor can affect algal and plant cells through changes in their energy status as exemplified by the rates of oxygen consumption, oxygen evolution and heat production. It is hypothesized that the effects can be caused not only by the modulation of the activity of phenol oxidases but also alterations in membrane ATPase systems.

Keywords: Phenol oxidases; Peroxidases; Inhibitor from tealeaf; Heat production rate; Superoxide anion radical

1. Introduction

Food spoilage is a significant problem and constitutes a major economic loss. The quality of food can be lost due to undesirable physiological reactions such as dehydration and the browning of vegetables and fruit. The formation of coloured spoilage in food products is largely due to the activity of two classes of redox enzymes: Copper-containing phenol oxidases and heme-containing peroxidases. For many years, the food industry extensively used sulphur dioxide as a cheap and potent browning inhibitor. However, such use of sulphites may be harmful to the health of the population. Presently, the food industry is looking for alternative means to prevent food spoilage. One possible set of candidates is the natural plant inhibitors of phenol oxidases and peroxidases. A research group from the Republic of Georgia has demonstrated that a phenolic extract isolated from tealeaves is able to inhibit both phenol oxidases and peroxidases activity [1]. The two model systems for investigating the physiological responses were (i) photosynthesising *Chlorella vulgaris* was chosen due to its functionally, namely their structural features and sensitivity to the action of different compounds and (ii) excised roots of wheat seedlings because they constitute a convenient model for the study of dark metabolic processes. The aim of this research is, then, to study the physiological activity of a tealeaf inhibitory preparation on these systems. The investigation focuses on energetic processes in microalgal and plant cells that are exposed to inhibitors of plant origin, which may affect the phenol oxidases and peroxidases con-

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nected with the electron transport chains in their energetic systems.

2. Experimental

2.1. Biological material

The unicellular microalga *C. vulgaris* was used as a photosynthetic model. It was grown in Tamiya medium, pH 6.8–7.2 [2], at 30 °C, and illuminated at 1×10^4 lx with a light and dark photoperiod of 12 h each. Cell suspensions were aerated with air containing 0.3% CO_2. The optical density was maintained at 1×10^8 to 1.5×10^8 cells/ml. Wheat seedlings were grown in distilled water with 0.25 mM $CaCl_2$.

The inhibitor of phenol oxidases and peroxidases isolated from tealeaves is of phenolic nature. The inhibitor was used in final concentrations from 0.1 to 0.5 mg ml^{-1}. The tealeaves were field-collected in west Georgia.

2.2. Isolation of natural inhibitor from tealeaves

The method of separation and purification of the tealeaf inhibitor was first described in [3]. Five hundred grams of green tea leaves were powdered in liquid nitrogen, then 2 L of boiling water containing 10% K_5 was added and the mixture placed in a water bath at 80 °C for 30–40 min. The aqueous extract was concentrated in a rotary evaporator at 40–50 °C to 200 ml and centrifuged for 20 min at $1000 \times g$. Then 100 ml of the concentrated extract was applied to a Sephadex LH-20 column, which was equilibrated and washed with distilled water until the eluate showed negative reaction with vanillin reagent [3]. The elution of the inhibiting ingredients was carried out with 60% acetone. The eluates were collected and combined, and the acetone was evaporated at 30–35 °C. The remaining solution was freeze-dried and the obtained preparation tested as inhibitory agent.

2.3. Quantitative determination of proanthocyanidins

Both flavan 3,4-diols and their polymeric forms were determined according to the method of Popov and Shlipakova [3], based on their conversion to anthocyanidins by heating in acidic solution.

2.4. Paper chromatography of the aglycons (anthocyanidins)

This was carried out with the developing solvent mixture: acetic acid–hydrochloric acid–water, 30:3:10 (v/v/v) on a high quality filter paper. The spotting of samples was done with vanillin reagent. The obtained spots were cut out and eluted with methanol for 2 h before the further step of spectral analysis. The UV–vis spectra (200–600 nm) of proanthocyanidins and anthocyanidins were recorded in methanolic solutions on a Specord UV–vis spectrophotometer (Germany).

2.5. Determination of the chemical nature of the inhibitor

The freeze-dried preparations showed characteristic effects for the qualitative reaction of tea proanthocyanidins with vanillin reagent. The quantitative determination of the proanthocyanidins was carried out according to Popov et al. [3]. Then the anthocyanidins were formed by hydrolysis of the isolated proanthocyanidins in hot acidic solution. Paper chromatographic analysis of these anthocyanidins showed two spots on the paper with Rf values of 0.32 and 0.49. After extraction these two substances evidenced absorption maxima in spectral analysis at 546 and 536 nm ($A_{\max \text{methanol}}$). The addition of $AlCl_3$ to the methanolic solution of anthocyanidins caused a bathochromic shift of the above maxima by 27 ($A_{\max}$ = 573 nm) and 28 nm ($A_{\max}$ = 564 nm). These data coincided with both chromatographic and spectral characteristics of delphinidin and cyaniding analyzed as standards. All these experimental data obtained underlined that the inhibitors have a phenolic nature and are proanthocyanidins.

2.6. Analytical methods

Direct calorimetric measurements of the heat flow rates of *Chlorella* cells were performed using an LKB batch heat conduction colorimeter (Biological Activity Monitor BAM; successor of LKB is Thermometric AB, Järfälla, Sweden) operated at 30 °C [4]. Two LKB 2277-201 ampoule cylinders were used in the experiments. The sensitivity of detector is 0.4v/W; the baseline noise is better than ±0.1 μW. The calorimetric system was electrically calibrated daily on the range 100 μW.

1.5 ml cell suspension was placed in a 3 ml glass vial, the control vial contained 1.5 ml distilled water. The thermal equilibration time of the calorimeter was 20 min, so that the onset of measuring the heat production rates corresponds to 20 min after addition of the inhibitor to the *Chlorella* culture. The data were registered by the digital amplifier and calculated by Galex-151002 software (programmed by I. Lukoyanov, Kazan, 2002). The number of repetitions per point is 3–5. In all calorimetric experiments the standard deviation was less than 5%.

Oxygen uptake and evolution rates were measured by a polarographic method using a Clark-type electrode [5]. 3.2 ml samples were placed in a measuring tube located in a tight-fitting water bath and equilibrated to 30 °C for 5 min. Then a black box was fitted over the bath to determine the oxygen uptake rate in the dark for 3 min. The box was removed and the cells were illuminated to record the oxygen evolution rate for a further 3 min period.

The amount of superoxide released by cells in suspension was monitored by incubating cells with 1 mM epinephrine (ICN Biomedical Inc.) for 10 min as described in [6]. Independent samples were taken at definite time intervals.

The main pigments of *C. vulgaris* were isolated with 85% acetone. The quantity and characteristics of the pigments were determined spectrophotometrically at the wavelengths: 665, 649 and 452.5 nm according to [7].

3. Results

As can be seen in Fig. 1, the rate of heat production measured the changes in the metabolic activity of the algal cells caused by the tealeaf inhibitor added at the 20 min point on the *x*-axis. The inhibitor caused a decrease in metabolic rate.

Fig. 2 shows data on the alteration of the heat production rates of the *Chlorella* cells after incubation for 24 h with the tealeaf inhibitor. At a concentration of 0.5 mg ml^{-1}, the inhibitor caused an initial steep drop in the heat production rate which then remained at approximately 70% during the first hour before then slowly returning to the norm values of 100%.

A sustained reduction of oxygen uptake by *Chlorella* cells was observed at an inhibitor concentration of 0.5 mg ml^{-1} (Fig. 3). The maximum reduction of ∼70% corresponds to that seen in Fig. 2 for the heat production rate. The effect started 10 h after the addition of the inhibitor to the culture and continued for about 30 h. A lower inhibitor concentration of 0.1 mg ml^{-1} decreased the oxygen uptake rate by only 10–15% compared with the control. Excised roots of wheat seedlings also responded by a decreased oxygen uptake to the presence of the inhibitor (Fig. 4). The effect became immediately visible and lasted for 4–5 h. The maximum reduction (down to 75%) equalled that in *Chlorella* cells (Fig. 3). The data depicted in Fig. 5 showed that the tealeaf inhibitor considerably decreased the rate of generation of the superoxide anion by *Chlorella* cells. The effect was observed for a longer time period that the metabolic effects and was practically without any change at values around 33% of the control in the first 2 h and even 22% in the third.

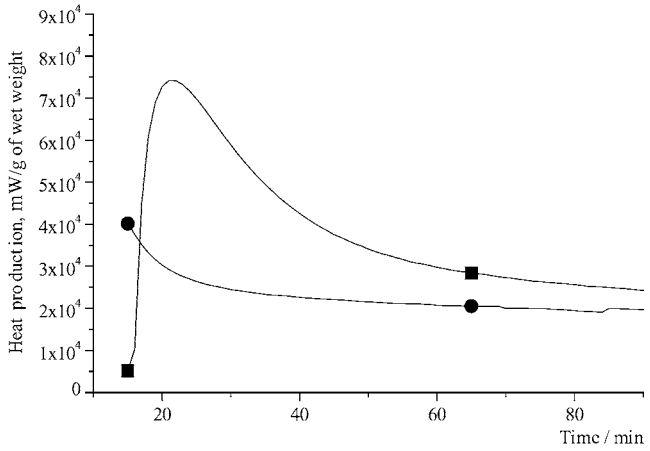

Fig. 1. Rate of heat production of *Chlorella* suspension's under the action of INH. (—) Control; (●) INH 0.5 mg ml^{-1}.

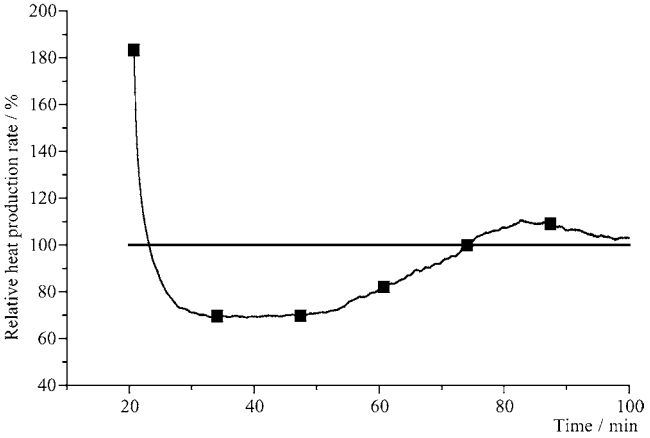

Fig. 2. Rate of heat production of *Chlorella* cells after 24 h incubation with INH. (—) Control; (■) 0.5 mg ml^{-1}.

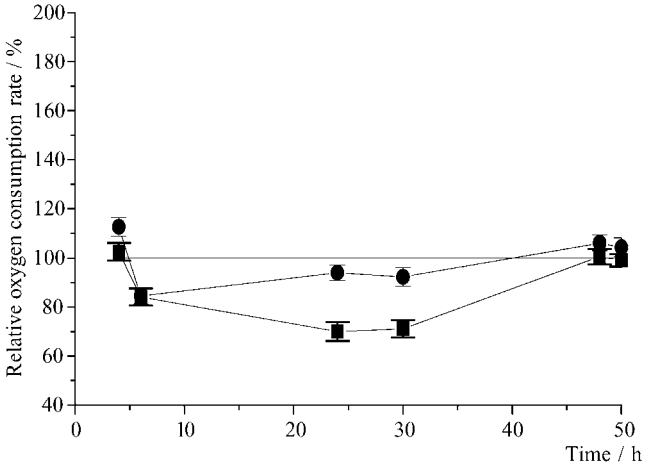

Fig. 3. Effect of INH on the rate of oxygen uptake by *Chlorella* cells. (—) Control; (■) 0.5 mg ml^{-1}; (●) 0.1 mg ml^{-1}.

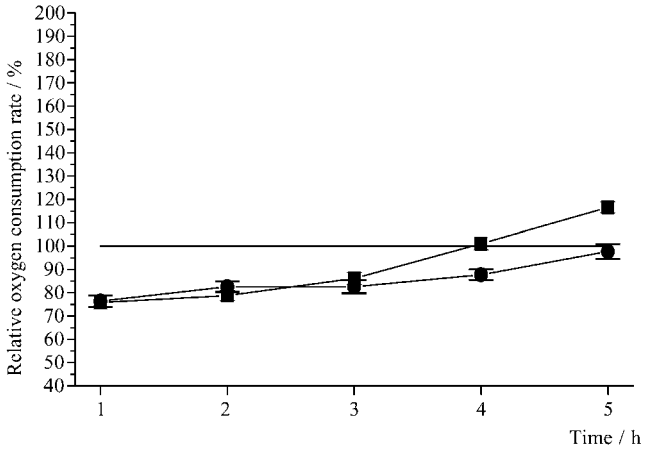

Fig. 4. Effect of INH on the oxygen uptake rate of wheat roots. (—) Control; (■) 0.5 mg ml^{-1}; (●) 0.1 mg ml^{-1}.

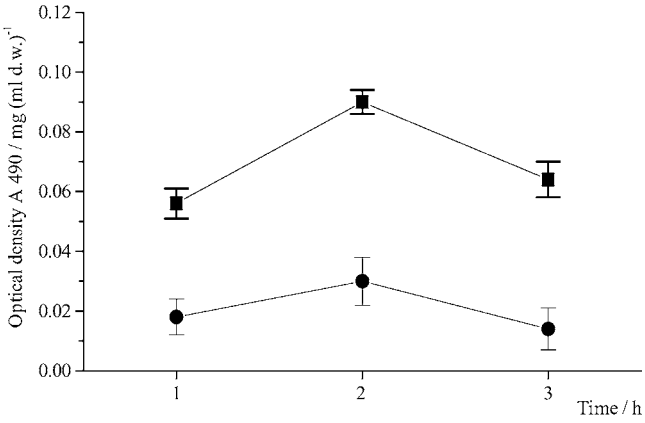

Fig. 5. Rate of superoxide anion generation by *Chlorella* cells. (■) Chlorella; (●) chlorella + INH.

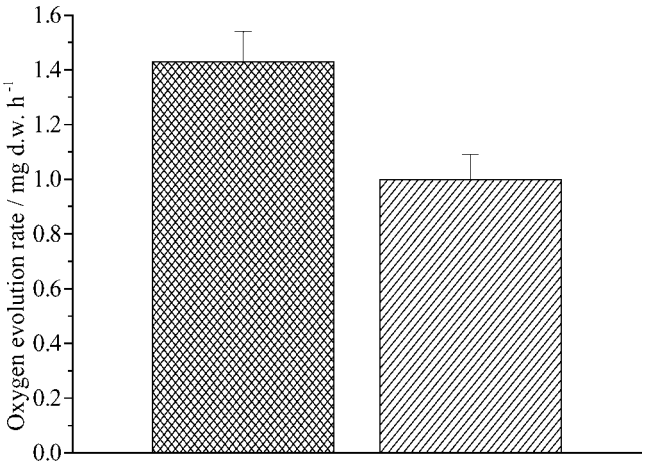

Fig. 6. The rate of oxygen evolution by *Chlorella* cells in light after addition of INH. (▨) Control; (▨) INH.

In terms of the apparent photosynthesis process, the data in Fig. 6 show that the rate of oxygen evolution decreased by 30% in *Chlorella* cells treated with the inhibitor. This value altered between 25 and 30% in different experiments.

The content of chlorophyll *a* in cells treated with the inhibitor was lower than that in the control with 79 and 73% of the control at 0.1 and 0.5 mg ml^{-1} inhibitor, respectively (Fig. 7). Chlorophyll *b* and the carotenoid content in treated material showed no significant difference from the control (Fig. 7).

4. Discussion

The application of natural inhibitors isolated from plants, may prevent food spoilage and help to maintain the levels of beneficial biologically active components that naturally exist in food products. The formation of spoilage in them is largely due to the activity of two classes of redox enzymes:

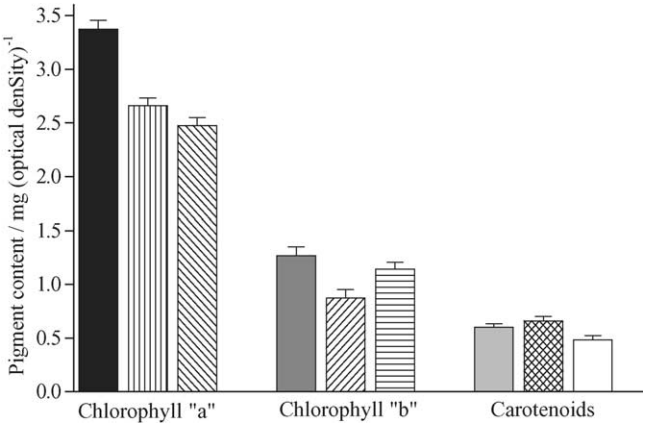

Fig. 7. Change of content of main pigments by *Chlorella* cells after 48 h exposition with INH. Chlorophyll "*a*": (▨) control; (▨) 0.1 mg/ml^{-1}; (▨) 0.5 mg/ml. Chlorophyll "*b*": (▨) control; (▨) 0.1 mg/ml; (▨) 0.5 mg/ml. Carotenoids: (▨) control; (▨) 0.1 mg/ml; (▨) 0.5 mg/ml.

phenol oxidases and peroxidases. Substances isolated from tealeaves are of phenolic nature and may inhibit both enzymatic activities [1]. Here, the effect on energetic processes in model algal and plant cells by such inhibitors of phenol oxidases and peroxidases were studied by means of the rate of heat production as an integral indicator that reflects changes in catabolic as well as anabolic processes [8,9]. It was shown that the heat production rate of *Chlorella* cells was reduced after the addition of the inhibitor, which is a sign of depressed energetic activity of the cells.

Suppression of oxygen uptake rate by the action of the inhibitor was observed for both the *Chlorella* cells and the wheat roots. Excised roots of seedlings responded more quickly than *Chlorella* to the presence of the inhibitor shown as an earlier decrease of the oxygen uptake rate (see Figs. 3 and 4). This time difference between the two model systems may be explained by the different permeability of their cell walls. *Chlorella* cells are covered by close cellulose walls containing chitinase that may result in less permeability than the cell walls of higher plants. The decrease in oxygen uptake in both systems caused by this specific inhibitor proves that peroxidases and phenol oxidases systems are associated with the respiration of plants. Moreover, the content of phenol oxidases in mitochondria is a further evidence of the involvement of these systems in plant respiration. But one should keep in mind that the decrease in oxygen uptake by plant cells induced by this inhibitor can be also caused by a depression of H^+ATP of the plasma membrane [5].

The data obtained on the action of the inhibitor on the generation rate of superoxide are interesting. As shown in Fig. 5 the generation of superoxide in *Chlorella* cells is sharply decreased by the inhibitor. It is necessary to remark that the effect of the inhibitor practically did not change with time after incubation of *Chlorella* cells for at least 24 h. Probably, NAD(P)H oxidase is the source of superoxide and the inhibitor reduces the activity of this ferment, an effect that may

be essential to protect food from spoiling during long storage, when the generation of active oxygen forms is inhibited [10,11].

Moreover, the data show that the inhibitor decreased the rate of photosynthesis by about 30%, determined as oxygen evolution. Probably, the reduction of the oxygen evolution rate is related with the effect of inhibitor on phenol oxidase activity, as this enzyme system is found in chloroplasts [12,13]. This may be confirmed by the fact that some correlation was observed between the rate of photosynthesis and the reduction of the amount of chlorophyll under the influence of the inhibitor [14].

Thus, the data obtained demonstrate that the tealeaf inhibitor can affect the plant cells through changes in their energy status in terms of the rates of oxygen consumption, oxygen evolution, and heat production. These changes may be caused by the modulation of the activity of phenol oxidases and ATPase systems.

5. Conclusions

Our data support the opinion that oxidative processes play the main role in food losses. The inhibitor isolated from tealeaves appears to be a natural powerful antioxidant which can depressed the oxidative processes in terms of the generation of active forms of oxygen.

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