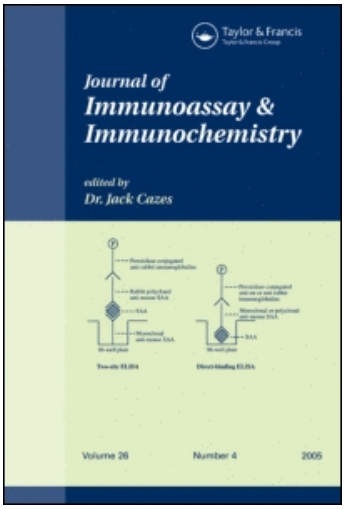

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[a] Siberian Division of Russian Academy of Sciences, Siberian Institute of Plant Physiology and Biochemistry, Russia

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AN INFLUENCE OF STRESS PROTEIN CSP 310 AND ANTISERUM AGAINST THIS PROTEIN ON LIPID PEROXIDATION IN CEREAL MITOCHONDRIA

A. V. Kolesnichenko,* O. I. Grabelnych, V. V. Tourchaninova, V. V. Zykova, N. A. Koroleva, T. P. Pobezhimova, and V. K. Voinikov

Siberian Institute of Plant Physiology and Biochemistry,
Siberian Division of Russian Academy of Sciences,
P.O. Box 1243, Irkutsk-33, 664033, Russia

ABSTRACT

It is determined that an addition of an anti-CSP 310 antiserum to isolated winter wheat and maize mitochondria caused more significant increasing of spontaneous lipid peroxidation than the addition of stress protein CSP 310. It is shown that, at function of different mitochondrial respiratory chain complexes, the lipid peroxidation in winter wheat and maize mitochondria take place with different intensities. Under the functioning of mitochondrial respiratory chain complex IV, the maximum output of lipid peroxidation products, dienic conjugates is detected.

The presence of antiserum against CSP 310 in incubation media induces lipid peroxidation more than the presence of CSP 310 in mitochondria isolated from stressed plants under

* Corresponding author.

these conditions. Based on data obtained, it is possible to conclude that in vivo endogenous CSP 310, during a cold stress, has an antioxidant activity the same as other known uncoupling proteins.

INTRODUCTION

It is known that the development of chilling injury symptoms is coincident with fatty acids peroxidation.[1] Peroxide and malondialdehyde levels are often increased by a freezing and thawing stress, suggesting peroxidation of lipids[2] that resulted in changes of structural and functional membrane with membrane proteins changes.[3] It was found that the level of oxidative stress and defense mechanisms are differently expressed in tolerant and low temperature susceptible populations of maize when seedlings are grown at a temperature near the lower growth limit.[4] Shewfelt and Erickson[3] proposed that lipid peroxidation would alter the physical properties of membrane lipids, and thereby inhibit the function of membrane-bound proteins, contributing to the development of chilling injury.

On the other hand, it is known that about 1-2% of oxygen reduced in mitochondria by iron-sulfur centers in complex I and partially by reduced ubiquinone and cytochromes b in complex III is constitutively converted to superoxide, which is a powerful oxidant radical.[5,6] There is experimental evidence to indicate that mitochondria are a major source of superoxide in chilling-sensitive plant tissues at low temperatures.[7]

Recently, the presence of proteins which caused uncoupling of oxidation and phosphorilation in plant mitochondria, such as PUMP, StUCP, and some others[8] was determined. If, in mammals, the main function of these proteins is to prevent overcooling by means of thermogenesis, Skulachev[9] suggested that uncoupling proteins in plants could have an antioxidant influence on the mitochondrial membrane lipid peroxidation by means of "softly" uncoupling that caused decreasing of oxidant radical formation by mitochondria. This point of view was supported by recent studies.

For example, it was found that the inhibition of PUMP activity in potato tuber mitochondria significantly increased mitochondrial H_2O_2 generation.[10] It was also found that substrates of uncoupling protein, such as linoleic acid and other fatty acids, reduce mitochondrial H_2O_2 generation[10, 11] and superoxide anion generation.[12]

Previously, we isolated and purified, from winter rye, a cold stress protein, CSP 310;[13] furthermore, its presence in mitochondria has been demonstrated. This protein caused uncoupling of oxidation and phosphorylation in mitochondria during cold stress in low temperature tolerant

winter cereals.[14] Though the mechanism of uncoupling of oxidation and phosphorylation caused by this stress protein in mitochondria is not known, it was suggested that this protein can have an influence on lipid peroxidation in plant mitochondria. Really, in previous work, it was shown that an addition of exogenous CSP 310 induced lipid peroxidation in ascorbate-dependent and NADH-dependent systems, but did not significantly induce spontaneous lipid peroxidation.[15]

On the other hand, it was shown that CSP 310 could strongly associate with mitochondria and eliminate from mitochondrial proteins spectrum after pronase E treatment.[16] Therefore, we can suppose that this protein associates with mitochondrial outer membrane and that the influence of endogenous CSP 310 on lipid peroxidation in isolated mitochondria can be eliminated by addition of anti-CSP 310 antiserum to the mitochondrial incubation medium.

Therefore, in our current study, we compared the influence of CSP 310 and anti-CSP 310 antiserum on lipid peroxidation in winter wheat and maize mitochondria from non-stressed and cold-stressed plants, and attempted to determine how this influence depends on the functioning of different mitochondrial respiratory chain complexes.

EXPERIMENTAL

Three-day-old etiolated shoots of winter wheat (Triticum aestivum L., cv. Zalarinka) and maize (Zea mays L.), grown on moist paper at 26^0C, were used in this work. Winter wheat and maize seedlings were stressed for 1 h at -1^0C and 4^0C, respectively.

Mitochondria were extracted from winter wheat and maize shoots by differential centrifugation;[17] they were used for organelle purification. For organelle purification, the mitochondrial sediment obtained by means of differential centrifugation was resuspended in a medium of the following composition: 250 mM sucrose, 18 mM Na_2SO_4, pH 7.4. Purification was performed on a sucrose gradient.

Stress protein CSP 310 was isolated and purified from stressed (-1^0C, 1 h) winter rye shoots as described previously.[13] The purification of prepared substances was controlled by SDS-PAGE electrophoresis.

For induction of lipid peroxidation, 1 mg of mitochondrial protein was resuspended in 1 mL of reaction mixture contained 125 mM KCl, 18 mM KH_2PO_4, 1 mM $MgCl_2$, and 5 mM EDTA, pH 7.4. The following oxidizing substrates were used: 10 mM malate in the presence of 10 mM glutamate for complex I of mitochondrial respiratory chain, 10 mM succinate in the presence of 10 mM glutamate for complex II, 1 mM NADH for

complex III, and 2 mM ascorbate plus 0.2 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) for complex IV of the mitochondrial respiratory chain. In this case, electron transport in complex I was blocked by 0.003 mM rotenone.

The rate of lipid peroxidation was determined by measuring the primary products of lipid peroxidation – conjugated diene formation. Mitochondria were incubated in the incubation medium mentioned above. Each sample contained 0.9 mL of the incubation medium and 0.1 mL mitochondrial suspension. To measure the dienic conjugate contents, mitochondria lipids were extracted by hexane-isopropanol (1:1 v/v) mixture (9 mL per 1 mL of the sample) by shaking. After shaking, 1 mL H_2O was added to the mixture to stratify the hexane and isopropanol phases.

Measurements of dienic conjugate contents were made in hexane phase at 233 nm with a spectrophotometer "SF-46" ("LOMO", USSR).[15] The dienic conjugate contents in sample were calculated according to 233 nm molar extinction coefficient to polyunsaturated fatty acids conjugated dienes 2.2 x 10^5 x M^{-1} sm^{-1}.[18] The data obtained were analyzed statistically, i.e., arithmetic means and SE were determined.

The mitochondrial proteins were analyzed by the Lowry method.[19]

RESULTS AND DISCUSSION

Because the analysis of CSP 310 influence on energetic activity of winter wheat mitochondria shows that its uncoupling influence on energetic parameters of mitochondria was detected during mitochondria incubation within 1.5 hours;[14] the analysis of CSP 310 and antiserum against this stress protein (anti-CSP 310 antiserum) influence on a lipid peroxidation in isolated winter wheat mitochondria was performed during their incubation within 2 hours.

During this experiment, it was found that, in a spontaneous system of lipid peroxidation in winter wheat mitochondria, the induction of lipid peroxidation was marked after 1 hour of mitochondria incubation at 27^0C (about 140 %). An addition of 1 mg CSP 310 per 1 mL of mitochondria incubation medium contained 1 mg of mitochondrial protein during the first 30 min of incubation caused only slight (10 %) induction of lipid peroxidation, as compared to the control variant (Fig. 1). Then, after 30 min of incubation, and to 1 h 30 min, increasing rate of a product of lipid peroxidation-dienic conjugates (up to 60 % to control) generation was detected. Two hours of mitochondria incubation with CSP 310 caused decrease of dienic conjugate formation in mitochondria (to 30 % to control) (Fig. 1).

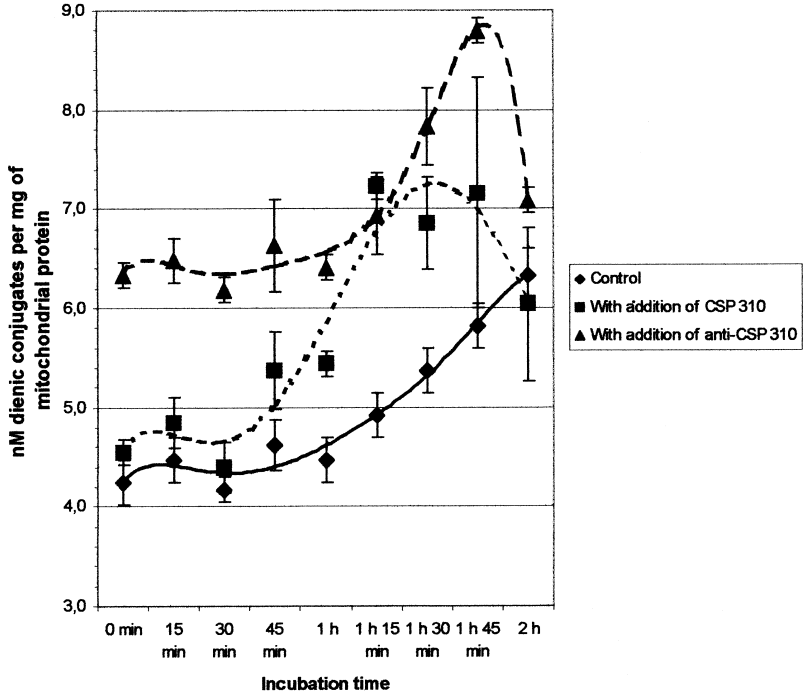

Figure 1. An influence of CSP 310 and antiserum against this stress protein on spontaneous lipid peroxidation in isolated winter wheat mitochondria during their incubation for 2 h at 27 ^{0}C.

If an addition of CSP 310 to incubated mitochondria has only little influence on lipid peroxidation during the first 30 min of incubation, an addition of 2 mg anti-CSP 310 antiserum per 1 mL of mitochondrial incubation medium immediately caused increasing of dienic conjugates formation from 4.2 nM in control variant to 6.4 nM per mg of mitochondrial protein, i.e., approximately 50 % (Fig. 1). The content of dienic conjugates under this treatment during the first hour of mitochondria incubation was approximately at one level. The further incubation of mitochondria with anti-CSP 310 antiserum caused increasing of dienic conjugate concentration up to 8.8 nM per mg of mitochondrial protein (Fig. 1). After two hours of mitochondria incubation with this antiserum, as well as at incubation with stress protein CSP 310, the decreasing of dienic conjugate content in mitochondria to 7.0 nM per mg of mitochondrial protein (Fig. 1) was detected.

Thus, if spontaneous lipid peroxidation in mitochondria was characterized by monotonic increasing of dienic conjugate content after 1 hour of

incubation, in presence of CSP 310 and antiserum against this stress protein, the maximum of dienic conjugate content was detected at 1 hour 30 min-1 hour 45 min of mitochondria incubation. It is necessary to note that the induction of lipid peroxidation in the presence of antiserum against CSP 310 in incubation media, was higher than in the presence of CSP 310.

Earlier, it was established that the mechanism of uncoupling action of stress-related protein CSP 310 is substrate-dependent and differs from the mechanism of other known uncoupling proteins, and that the key point of CSP 310 uncoupling action is the complex I of the mitochondrial respiratory chain.[20] According to this fact, it was interesting to find out how the lipid peroxidation in winter wheat mitochondria depends on the functioning of different mitochondria respiratory chain complexes and an influence of CSP 310 and antiserum against CSP 310 presence in incubation media on this process.

For estimation of CSP 310 influence on lipid peroxidation at functioning of separate respiratory chain complexes, substrates at which oxidation separate electron transport complexes of a respiratory chain participant were used. These substrates were malate for complex I, succinate for complex II, NADH for complex III, and ascorbate plus TMPD for complex IV. The electron transport in complex I was blocked by 0.003 mM rotenone.

The analysis of CSP 310 and antiserum against CSP 310 influence on lipid peroxidation in mitochondria, isolated from non-stressed winter wheat shoots, shows that, if electrons were transferred through complexes I, II, or III, the intensity of lipid peroxidation in mitochondria is rather low. In all these cases, the content of dienic conjugates in mitochondria were approximately identical — about 4 nM per mg of mitochondrial protein (Fig. 2). At the same time, the content of dienic conjugates, when using of a substrate for complex IV of respiratory chain, was higher — about 7 nM per mg of mitochondrial protein (Fig. 2).

It is possible to suppose that this phenomenon deals with the fact that, during the function of the first three respiratory chain complexes, electrons are transferred through ubiquinone complex,[21] which is an effective antioxidant system.[22] An addition of 1 mg CSP 310 per mL of mitochondria incubation medium caused only slight induction in all cases (Fig. 2). The significant difference from the control (about 25 %) was marked only with use of the I complex substrates. At the same time, an addition of antiserum against CSP 310 to incubation media caused more considerable induction of lipid peroxidation than addition of CSP 310. In this case, an increase of the lipid peroxidation rate was about 29, 24, 36, and 27 % when using substrates of respiratory chain complexes I, II, III, and IV, respectively (Fig. 2).

Thus, on the basis of data obtained, it is possible to draw a conclusion that, at functioning of different complexes of mitochondrial respiratory chain, the lipid peroxidation in winter wheat mitochondria takes place with different

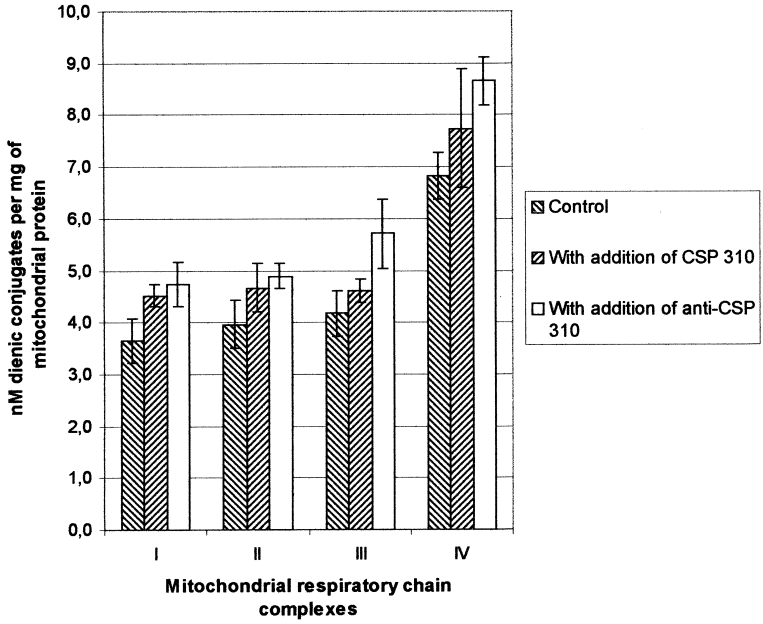

Figure 2. An influence of CSP 310 and antiserum against this stress protein on lipid peroxidation at functioning of different mitochondrial respiratory chain complexes in mitochondria, isolated from non-stressed winter wheat shoots.

intensity. Under the functioning of complex IV of mitochondrial respiratory chain, the maximum output of lipid peroxidation products – dienic conjugates – is detected. The presence of antiserum against CSP 310 in incubation medium induces lipid peroxidation more than the presence of CSP 310.

Experiments with mitochondria isolated from non-stressed maize shoots shows that an addition of 1 mg CSP 310 per mL of incubation medium caused strong induction of dienic conjugate formation for I (60 %) and II (120 %) complex functioning. For complex IV, minor induction (7 %) was detected. Only with complex III, an addition of CSP 310 experimental concentration reduced dienic conjugates formation as compared to the control (Fig. 3). An addition to maize of mitochondria antiserum against CSP 310 showed, as a whole, the same picture as with winter wheat mitochondria. With functioning of complexes I and II, the induction of lipid peroxidation was higher than in control variant on 33 and 67 %, respectively. When using substrates of complexes III and IV of respiratory chain, there was not detected any induction of lipid peroxidation, as compared with the control variant (Fig. 3).

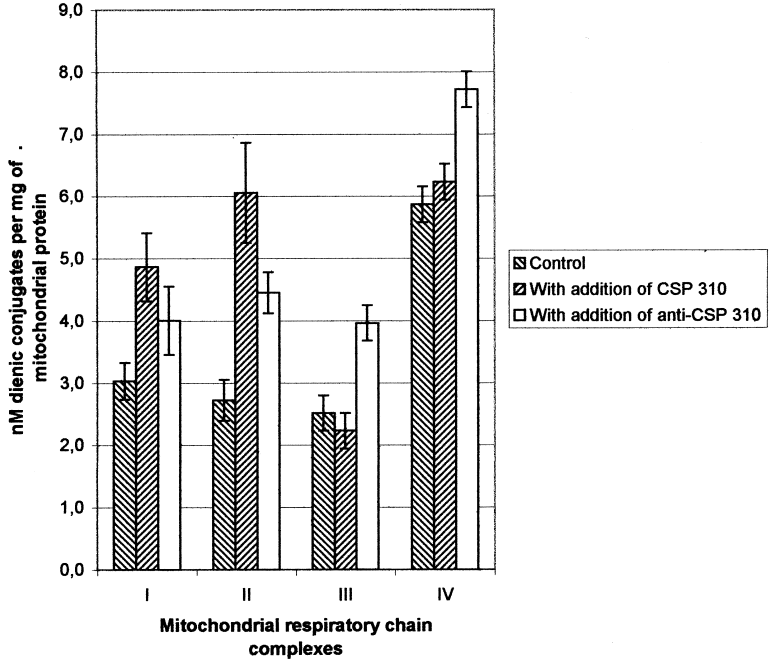

Figure 3. An influence of CSP 310 and antiserum against this stress protein on lipid peroxidation at functioning of different mitochondrial respiratory chain complexes in mitochondria, isolated from non-stressed maize shoots.

It is necessary to note that, if addition of CSP 310 to incubated winter wheat mitochondria caused only slight induction of lipid peroxidation (Fig. 2), an addition of CSP 310 to maize mitochondria with functioning complexes I and II of respiratory chain caused strong induction of lipid peroxidation (Fig. 3). Probably, this fact can be explained by the lower content of endogenous CSP 310 in maize cytoplasm, as compared with winter wheat;[23] therefore, contact with cytoplasmatic CSP 310 during the procedure of mitochondria isolation caused lower association of CSP 310 with mitochondria in maize than in wheat.

Based on data obtained, it is possible to conclude that an addition of CSP 310 and antiserum against this protein to incubation media of isolated product from non-stressed maize shoots mitochondria also induces lipid peroxidation. It is necessary to note that, with use of substrate of complex III, an addition of CSP 310 decreased dienic conjugate formation. It is possible that this protein has no influence on this complex of maize mitochondrial respiratory chain.

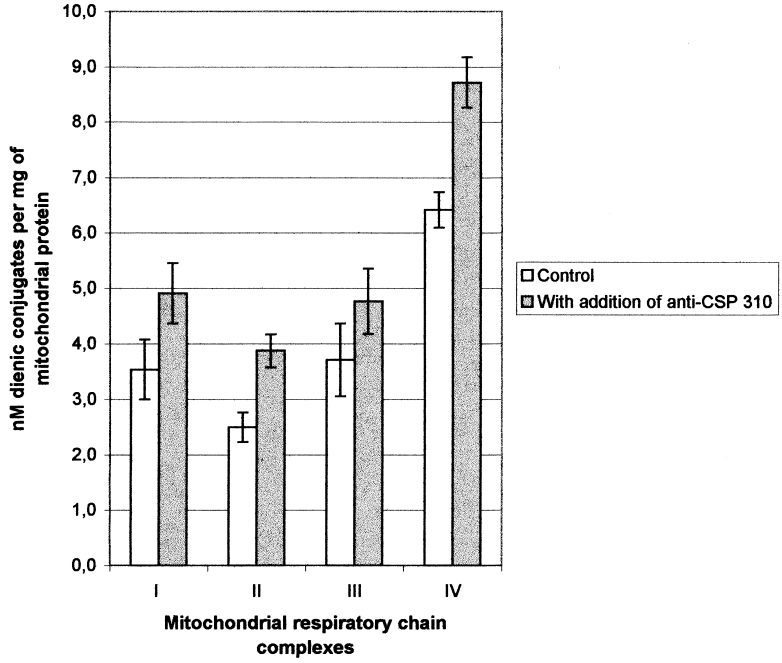

Figure 4. An influence of antiserum against CSP 310 on lipid peroxidation at functioning of different mitochondrial respiratory chain complexes in mitochondria, isolated from cold-stressed (-1^{0}C, 1 h) winter wheat shoots.

The analysis of an influence of cold stress on the rate of lipid peroxidation in winter wheat mitochondria with functioning of different respiratory chain complexes, shows that, if electrons are transferred through complexes I, II, or III, the intensity of lipid peroxidation was rather low and approximately identical (Fig. 4). At the same time, when electrons were transferred through a complex IV, the intensity of lipid peroxidation was about 80 % higher (Fig. 4).

It is necessary to note that only with complex II of respiratory chain, the absolute value of lipid peroxidation in mitochondria, isolated from stressed shoots, was lower than in mitochondria isolated from non-stressed shoots. The rate of dienic conjugate formation with other respiratory chain complexes, functioning of mitochondria isolated from stressed shoots did not differ from mitochondria isolated from non-stressed shoots (Fig. 2, Fig. 4).

An addition of antiserum against CSP 310 to the incubation medium of mitochondria isolated from stressed winter wheat shoots caused more

significant induction of lipid peroxidation than with mitochondria isolated from non-stressed shoots. Increasing lipid peroxidation products formation under this treatment were 40, 58, 26, and 37 % with use of substrates of respiratory chain complexes I, II, III, and IV, respectively (Fig. 4). It is necessary to note that antiserum against CSP 310 caused an increase of lipid peroxidation rate, both in the control and stressed mitochondria, except for complex II, to the similar absolute values – about 5 nM dienic conjugates per mg of mitochondrial protein with complexes I and III, and 9 nM dienic conjugates per mg of mitochondrial protein with complex IV of mitochondrial respiratory chain (Fig. 2, Fig. 4).

Thus, based on data obtained, it is possible to conclude that *in vivo* endogenous CSP 310 during a cold stress has an antioxidant activity, the same as other known uncoupling proteins.

An analysis of cold stress influence on intensity of lipid peroxidation in mitochondria isolated from stressed (1 hour at 4 ^{0}C) maize shoots shows that cold stress increased dienic conjugate formation in mitochondria at functioning of all respiratory chain complexes. The greatest increase of dienic conjugates formation was detected with functioning of respiratory chain complex IV in maize mitochondria (Fig. 5). An addition of antiserum against CSP 310 to mitochondrial incubation media caused more considerable induction of lipid peroxidation than in control mitochondria – up to 6 nM dienic conjugates per mg of mitochondrial protein at functioning of respiratory chain complexes I, II, and III and up to 14 nM dienic conjugates per mg of mitochondrial protein at functioning of complex IV.

For non-stressed maize shoots, addition of antiserum against CSP 310 caused induction of lipid peroxidation to about 4 nM dienic conjugates per mg of mitochondrial protein at functioning of respiratory chain complexes I, II, and III and up to 8 nM dienic conjugates per mg of mitochondrial protein at functioning of complex IV (Fig. 5). It is necessary to note that, in experiments with mitochondria isolated from stressed maize shoots, a higher rate of lipid peroxidation, than in experiments with stressed winter wheat shoots, was detected. If, at functioning of respiratory chain complexes I, II, and III of stressed winter wheat shoots, the dienic conjugates formation was about 4-5 nM dienic conjugates per mg of mitochondrial protein (Fig. 4), for mitochondria isolated from stressed maize shoots, this value was about 6 nM dienic conjugates per mg of mitochondrial protein (Fig. 5).

At functioning of complex IV, the dienic conjugates formation were about 9 and 14 nM dienic conjugates per mg of mitochondrial protein for stressed winter wheat and maize shoots, respectively. Analyzing the influence of antiserum against stress protein CSP 310 on lipid peroxidation intensity in mitochondria isolated from control and stressed maize shoots,

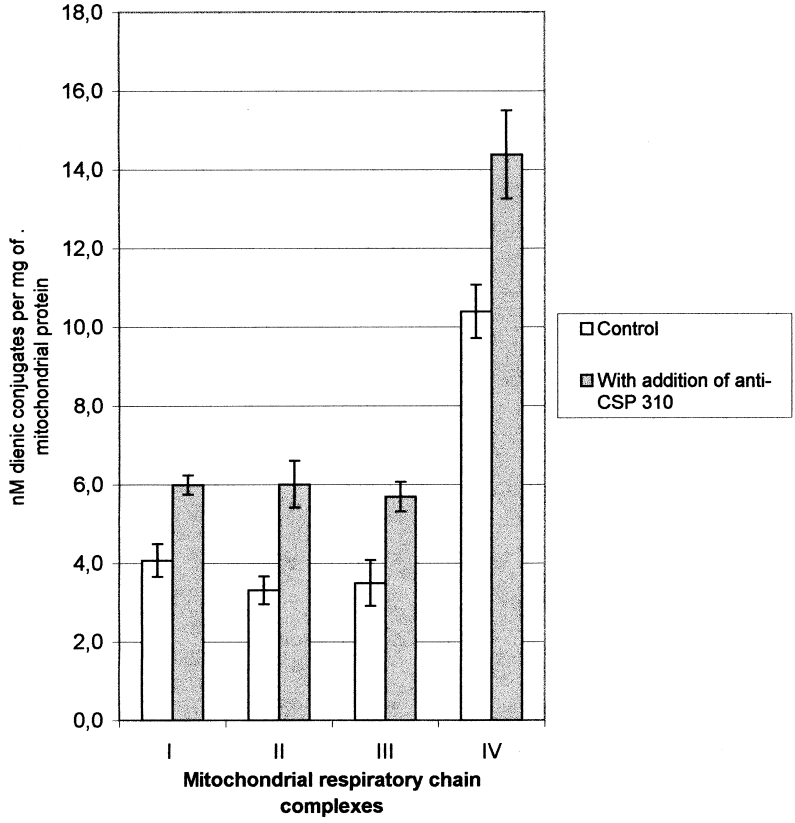

Figure 5. An influence of antiserum against CSP 310 on lipid peroxidation at functioning of different mitochondrial respiratory chain complexes in mitochondria, isolated from chilled (4 ^{0}C, 1 h) maize shoots.

it is possible to conclude that, for stressed shoots, the lipid peroxidation intensity in the presence of an antiserum is higher than in control shoots.

Therefore, the results obtained show that addition of antiserum against CSP 310 to isolated cereal mitochondria caused a significant increase of their lipid peroxidation. The influence of antiserum on lipid peroxidation depends on the functional activity of different mitochondrial respiratory chain complexes.

Anti-CSP 310 antiserum exerts its strongest influence on the I and II complexes. At the same time, the highest values of lipid peroxidation at functioning of winter wheat and maize mitochondrial complex IV were detected.

Thus, based on data obtained, it is possible to conclude that, during cold stress, *in vivo* endogenous CSP 310, as well as other known uncoupling proteins, serve an antioxidant function.

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