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ACTIVATION OF Ca^{2+}- DEPENDENT ATP-ASE OF PLASMATIC MEMBRANES OF WHEAT SEEDS BY SECONDARY HORMONE OF CYTOKININE (SHC) AND 14-3-3 PROTEINS

It was established the effect of Secondary hormone Cytokinine (SHC) with 14-3-3 proteins to activate Ca^{2+}- dependent ATP-ase of plasmatic membranes from aleuron layer of wheat seeds. This enzyme is inhibited by vanadate. The activation Ca^{2+}- dependent ATP-ase causes the increasing of the level of the cytosolic Ca^{2+} ions.

At first time the cytokinine secondary hormone (CSH) was purified by hydrophobic chromatography on column with octylsepharose 4B and by reverse phase chromatography on column type RP-18. Purified CSH shows it's physiological and biochemical effects at concentrations 100 times less and much quicker than cytokinine. It was shown that CSH is very close by its properties to fusicoccine.

One of the interesting effects of CSH is its ability with 14-3-3 proteins to activate Ca^{2+}- dependent ATP-ase of plasmatic membranes from aleurone layer of wheat seeds. The activation this enzyme let to increasing of the level of the cytosolic Ca^{2+} ions.

INTRODUCTION

Investigation of signal transduction is a one of the hot point of modern biology. One of the important phytohormone is cytokinine. The proliferation of plant cells dependens on cytokinine. However, the signal transduction of cytokinine is stayed unclear. Among problems of cytokinine signal transduction one of the main is the question of existence of hormonal secondary cytokinine. In this reason one of the main tasks of our investigation is the isolation purification and investigation of CSH.

Also, in recent decades it was established that 14-3-3 proteins are a one of the important component of the signal transduction systems. It was shown that they are important regulatory proteins of the cells. Namely 14-3-3 proteins are molecular targets for hormonal and other signals. 14-3-3 proteins play key role in regulation of such important life processes as ion transport, and cells proliferation [1, 2].

It's well known that cytokinine, 14-3-3 proteins and important intracellular messenger - cytosolic Ca ions participate in cell division. We suppose that cytokinine through CSH and 14-3-3 proteins activate the mechanism of the level of the cytosolic Ca.

There is only one way of increasing of the level of the cytosolic Ca^{2+}. It is the activation of Ca^{2+} pump – Ca^{2+} dependent ATP-ase of plasmatic membrane. The study of the mechanism of activation of this pump is the main task of our investigation [3, 4].

MATERIALS AND METHODS

Plant materials

The object of our investigation were dry seeds of wheat (*Triticum aestivum*) "Saratovskaya-29" cultivar.

The dry unembryonated seeds of wheat were milled and then bran was isolated from flour by sifting. Bran was homogenized in chilled porcelain mortar in 0,05M tris-HCl buffer (pH~7.4) in ratio 1:4 (w/v). Then homogenate was centrifuged at 10000g during 10 min.

For basis of isolation of plasmatic membrane we have taken the method /5/. It was taken unembryonated dry seeds. They were grinded in porcelain mortar at with 0.05 M Tris-HCl buffer pH 7.4 with 0.25 M sucrose. The homogenate was centrifuged at 2000 g during 20 minutes for removing the nuclei and large cell aggregates. Then supernatant was centrifuged in gradient of sucrose concentration (0.8-1.4 M) at 15000 g during 2 hours. Then the fractions which contain plasmatic membranes were used for investigation.

The activity of ATP-ase was determined by measuring of formed inorganic phosphate (P_i) by Toribara and Warner /6/. The specific activity of ATP-ase was expressed as mkM of formed P_i/mg protein/ 1hour.

RESULTS AND DISCUSSION

First of all it is necessary to develop the effective method of purification of CSH. For the basis of our method of purification of CSH was taken the method

of Sultanbaev et al /7/. Usually for purification of CSH it was taken 2kg of dry seeds of wheat *Triticum aestivum* "Saratovskaya-29" cultivar. The seeds were germinated during 24 hours in sterile tap water with 0.1 mM 6- benzylaminopurin (6-BAP) for induction of CSH. After that the seeds were homogenized in mortar with cooled ethanol in ratio 1:3 (w/v). Then the homogenate was centrifuged at 10000 x g during 10 min on refrigerated centrifuge. After that the ethanol extract was used for the further purification. The data of purification are presented at figure 1. As shown from the figure 1 CSH was eluted in the second peak by 50% of ethanol. For final purification we additionally introduced the new step – the purification of CSH by reverse phase chromatography on column type RP-18.

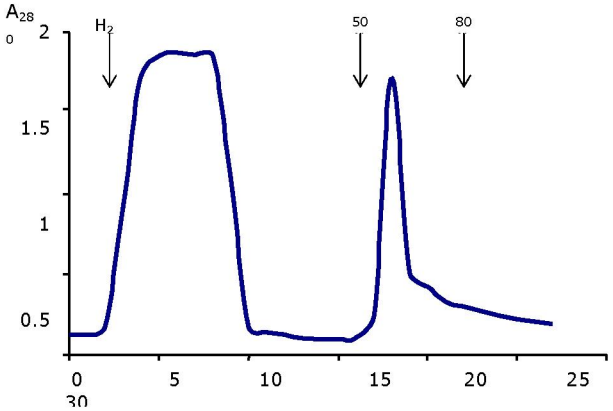

Figure 1 - The hydrophobic chromatography of ethanol extracts from wheat germinated seeds on column with octylsepharose 4B-CL

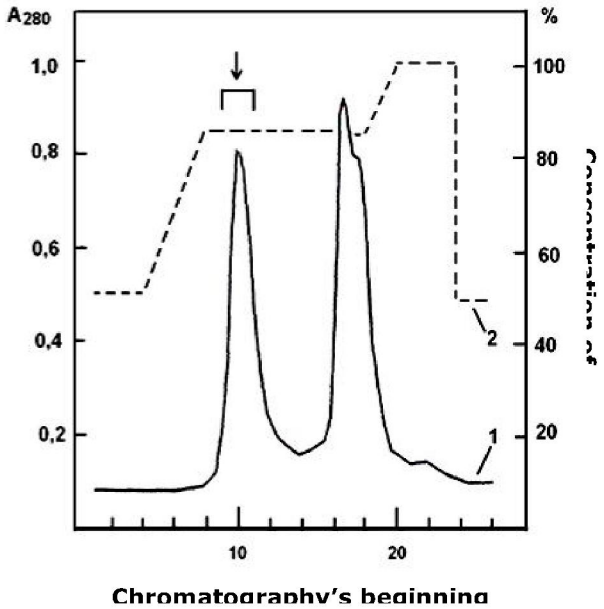

Figure 2 - Purification of CSH by reverse phase chromatography on column type RP-18

The results of purification are presented at the figure 2. As shown on the figure 2 CSH was eluted in the first peak.

While we have enough quantity of high purified CSH at first necessary to carry out its analysis of elements composition. For this CSH was dried up to constant weight in thermostat at 102°C within 12 hours for full removing of traces of water and ethanol. For the analysis was used modern analyzer of the quantitative contents of elements INCA-X-RAY analytical system-Oxford (UK). From the element analysis it is possible to make the next conclusion that the preparation did not contain any traces of nitrogen at all. This speaks about absence of traces of cytokinine and auxine in our preparation.

While it is known that fusicoccine activates H^+ATP-ase of plasmatic membrane /8, 9/, it is necessary to test the effect of related to fusicoccine CSH on the activity of H^+ATP-ase from aleurone layer of wheat seeds.

Unfortunately CSH was not able to activate H^+ATP-ase of plasmatic membrane which was isolated from unembryonated wheat seeds. Whereas when CSH acts on whole wheat seeds the effect of activation of H^+ATP-ase from plasmatic membrane of aleurone layer was achieved. The obtained results are presented on table 1.

As shown on this table it is very surprisingly that CSH causes the activation of ATP-ase of plasmatic membrane of aleuron layer only with Ca^{2+} ions but not with Mg^{2+} ions.

From presented data it is possible to make two important conclusions. The first – for activation of ATP-ase together with CSH it is necessary the second regulator which forms in embryo after action of CSH. The second – the activated ATP-ase related to Ca^{2+}- dependent type of ATP-ase.

It is well known that fusicoccine works together only with 14-3-3 proteins. In this reason we assumed that CSH causes the formation of 14-3-3 proteins in the wheat embryos [10]. And then synthesized 14-3-3 proteins are translocated to the aleuron layer of wheat seeds.

To check this hypothesis we carried out the next experiment. It was taken the embryo parts of wheat seeds and they were soaked by CSH solution 0.23 mkg per ml.

Table 1

The effect of CSH on activity of ATP-ase of plasmatic membrane from unembryonated seeds and of whole seeds

№	Variant	Activity of ATP-ase of plasmatic membrane with Mg^{2+} mkM Pi /(mg protein in 1 hour)		Activity of ATP-ase of plasmatic membrane with Ca^{2+} mkM Pi /(mg protein in 1 hour)	
		- CSH	+ CSH	- CSH	+ CSH
1.	Unembryonated seeds	180	280	140	250
2.	Whole seeds	200	0	160	400

Table 2

The effects of cell-free extracts from wheat embryos treated by CSH & from the brain on activity of ATP-ase of plasmatic membrane which isolated from unembryonated wheat seeds

№	Variant	Activity of ATP-ase with Mg^{2+} mkM Pi /(mg protein in 1 hour)	Activity of ATP-ase with Ca^{2+} mkM Pi /(mg protein in 1 hour)
1.	CSH	0	0
2.	cell-free extracts from brain or embryos	0	0
3.	CSH + cell-free extracts from brain	70	140
4.	CSH + cell-free extracts from wheat embryos	20	810

After 2 hours of soaking the embryo parts of wheat seeds were grinded in porcelain mortar with 0.05 M tris-HCl buffer pH 7.4 in ratio 1:4 (w/v). Then homogenate was centrifuged 2000 x g 15 min. The cell-free extract containing 14-3-3 proteins was used for the further experiments. Also it is well known that brain contains large quantity of 14-3-3 proteins. We also took the cell-free extract from sheep brain as described above.

For the experiment unembryonated wheat seeds were taken. They were soaked on 3 hours in both cell-free extracts separately. The results of the experiment are represented at the table 2.

As shown from this table the cell-free extracts from wheat embryos after acting of CSH and brain extract together with CSH indeed activate Ca^{2+}-dependent ATP-ase of plasmatic membrane from unembryonated wheat seeds.

As shown by us this ATP-ase is inhibited by vanadate. Now we know the biochemical mechanism of increasing of the level of the cytosolic Ca^{2+} are caused by mutual action of CSH and 14-3-3 proteins on Ca^{2+}-dependent ATP-ase of plasmatic membrane from aleurone layer.

Also we carried out the experiments with effect of CSH on whole wheat seeds. In this case CSH stimulate the formation of 14-3-3 proteins and they together act to aleuron layer. After 3 hours of treatment of whole wheat seeds by solution containing 0.23μM CSH we cut the embryos part of seeds.

Thus our results undoubtedly speaks that CSH and 14-3-3 proteins indeed switch on the mechanism of increasing of cytosolic Ca^{2+} by activation of Ca^{2+} dependent ATP-ase.

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Резюме

Цитокининнің екінші деңгейлі гормоны және 14-3-3 протеиндер плазматикалық мембрананың Ca^{2+} тәуелді АТФ-азасын активтендіреді. Осы фермент ванадатпен тежеледі. Ca^{2+} тәуелді АТФ-аза цитозольді кальцийдің мөлшерін көбейтеді.

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