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# Effect of Brassinolide on Tyrosine Phosphorylation of Pea Leaf Proteins

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**Abstract**—Brassinosteroid-induced phosphorylation of tyrosine residues in proteins was studied. Proteins of crude extract of pea leaves were analyzed by one- and two-dimensional electrophoresis followed by Western blotting with monoclonal antibodies PY20 to phosphotyrosine proteins. One- and two-dimensional electrophoresis revealed 7 and 13 tyrosine-phosphorylated proteins, respectively. Brassinolide increased the phosphorylation level of most of these proteins. With inhibitors of tyrosine protein phosphatases, such as phenylarsine oxide and orthovanadate, the level of tyrosine phosphorylation of these proteins increased.

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*Key words*: brassinolide, tyrosine phosphorylation of proteins, *Pisum sativum*, tyrosine protein phosphatases, tyrosine protein kinases

Brassinosteroids (BS) are hormones of plants with the structure similar to that of steroid hormones of animals [1]. Exogenous BS increased the plant resistance to cold, salinity, drought, and pathogen-induced infections. Brassinosteroids are important regulators of plant growth and development: mutants in biosynthesis of BS and response to them display dwarfism, florescence delay, and decreased fruiting [2, 3].

At present, many researchers study molecular mechanism of signal transduction from BS [4-7]. Receptors of BS have been found and characterized [8, 9], and they can be transphosphorylated by Ser/Thr [10]. Transduction of the BS signal involves many proteins [11-13]. However, information on the involvement of particular signaling systems in the plant response to BS is very insufficient. The transduction of BS signal involves $Ca^{2+}$-dependent protein kinases, mitogen-activated kinases [14, 15], BIN2 kinase [16], and also the lipoxygenase cascade [17].

The crucial role of protein phosphorylation/dephosphorylation in transformation and transduction of information into the genetic apparatus of the cell is well known [18]. Residues of serine, threonine, histidine, aspartate, and tyrosine are mainly phosphorylated. At present, considerable attention is given to tyrosine phosphorylation, notwithstanding its low contribution to the total phosphorylation of proteins. In the cells of vertebrates, tyrosine phosphorylation plays a determining role in the regulation of growth, proliferation, phases of the cell cycle, and integration of the cytoskeleton in response to different influences [19]. There is much less information about the role of tyrosine phosphorylation of proteins in plants [20]. There are data on protein tyrosine kinase activity and tyrosine phosphorylated proteins [21] including the cytoskeleton proteins profilin [22] and actin [23], on involvement of the protein tyrosine phosphorylation in response to elicitor [24], its role in somatic embryogenesis [25, 26], and ion transport [27]. We have not found data on the effect of BS on tyrosine phosphorylation of proteins. Therefore, the purpose of our work was to study tyrosine phosphorylation of plant proteins and the effect of brassinolide (BL) on it.

## MATERIALS AND METHODS

**Plant material.** Pea plants (*Pisum sativum* L.) were grown for nine days under conditions of 10-h photoperiod on Hoagland–Arnon I nutrition medium. The shoots were separated from the roots and placed into the growth

*Abbreviations*: BL) brassinolide; BS) brassinosteroids; ECL) enhanced chemiluminescence; PAO) phenylarsine oxide.

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medium (control) or into medium containing effectors (0.1 μM BL, 100 μM orthovanadate, and 50 μM phenylarsine oxide (PAO)).

**Preparing samples for analysis.** Leaves were frozen in liquid nitrogen and homogenized in medium (1 : 2) containing 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA, 0.1 mM orthovanadate, 1 mM theophylline, and 3% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 16,000*g* for 10 min at 5°C. The supernatant was immediately used to prepare both the sample for one-dimensional (1D) electrophoresis and the protein precipitate for two-dimensional (2D) electrophoresis. The sample for 1D electrophoresis was prepared as described in [28]. The proteins for 2D electrophoresis were precipitated with cold 80% (w/v) acetone, the precipitate was washed thrice in cold acetone, and 100 μg protein was dissolved in 150 μl of buffer for isoelectrofocusing (8 M urea, 2 M thiourea, 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% (v/v) ampholyte (pH 3-10), 3 mM DTT, and 20 mM Tris).

**Tyrosine phosphorylation of soluble leaf proteins.** Tyrosine phosphorylation of proteins was determined by one- or two-dimensional electrophoresis followed by immunoblotting with PY20 monoclonal antibodies to phosphotyrosine.

**One- and two-dimensional electrophoresis.** 1D electrophoresis was conducted in 6-20% polyacrylamide gel gradient according to [28]. Twenty-five micrograms of protein was applied onto each track. For 2D electrophoresis, sample-carrying gel strips to be isofocused with immobilized pH gradient (pH 3-10, 7 cm) were rehydrated at 20°C for 12 h in isofocusing chambers under the following conditions: 15 min at 250 V, then the voltage was linearly increased to 4000 V during 2 h, and then the sample was isofocused for 2 h at the same voltage. The total quantity of volt-hours was 24,000 at 20°C and 50 μA per strip. Then the strips were kept for 15 min in equilibrating buffer 1 (6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 50 mM Tris-HCl, traces of Bromophenol Blue, and 2% (w/v) DTT) and in equilibrating buffer 2 (6 M urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl, 0.005% Bromophenol Blue, and 2.5% (w/v) iodoacetamide). Both ends (0.5 cm each) of the strip were cut off, and the remaining part was applied onto a 2D mini-gel (Tris-glycine, 1 mm in thickness, 6-20% polyacrylamide gel). 2D electrophoresis was conducted using electrode buffer containing 25 mM Tris-HCl, 192 mM glycine, and 0.1% SDS at 5 mA per gel for 20 min, and then at 10 mA for 120-150 min.

**Immunoblotting.** To study tyrosine phosphorylated proteins, after 1D or 2D electrophoresis they were transferred onto poly(vinyl difluoride) membranes for 90 min at 150 mA, according to a standard protocol, using a device for half-dry blotting. Then the membranes were blocked for 1 h at 5°C with 1% (w/v) bovine serum albumin (BSA) dissolved in 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM NaCl and 0.05% Tween-20. Then the blots were incubated for 1 h at room temperature with PY20 anti-phosphotyrosine horseradish peroxidase conjugate diluted 1 : 1000 in the same buffer. To visualize tyrosine phosphorylation of proteins, the blots were incubated for 1 min in a chemiluminescent (ECL) reagent (ECL, enhanced chemiluminescence) and exposed on an X-ray film; the proteins were stained with 0.1% Coomassie R-250 for 10 min and then washed with 50% ethanol.

**Analysis of the results.** Because the shoots were exposed to brassinolide and vanadate for a relatively short time, the protein content was virtually unchanged, and, consequently, the intensity of chemiluminescence was sufficiently informative about the tyrosine phosphorylation level of proteins. However, comparing phosphorylation of individual proteins, it was necessary to take into account that the intensity of chemiluminescence (ECL) depended on the protein contents. Therefore, we determined the ratio of chemiluminescence intensity to content of one or another protein. To calculate the specific tyrosine phosphorylation of the electrophoretically separated proteins, the membranes stained with Coomassie R-250 and radioautographic films with phosphorylated proteins were scanned using an Epson Perfection 3170 Photo. The scanned image of the 1D electrophoresis data were changed to numerical values of optical density with the Image Master 1D program (England), and the data of 2D electrophoresis were processed with the Flicker program (http://open2dprot.sourceforge.net/Flicker) (USA). The specific tyrosine phosphorylation was expressed as the ratio of optical density of phosphorylated protein (chemiluminescence) identified on the radioautography film to optical density of the same protein identified on the membrane stained with Coomassie R-250. The specific tyrosine phosphorylation was evaluated in relative units.

**Quantitative determination of protein.** The protein quantity was determined by the Bradford method [29] with BSA as the standard.

**Reagents.** The following reagents were used: Tris-HCl, glycine, DTT, sodium orthovanadate, urea, thiourea, CHAPS, iodoacetamide, ampholytes, strips for isofocusing, polyvinylpyrrolidone, SDS, N,N,N′,N′-tetramethylethylenediamine (TEMED), glycine, Bromophenol Blue, ammonium persulfate, acrylamide, Coomassie R-250 and G-250, and also methylene-*bis*-acrylamide (BioRad, USA). Monoclonal antibodies to tyrosine phosphorylated proteins (PY20 clone), poly(vinyl difluoride) membranes, ECL-reagent, Tween 20, and BSA were from Amersham Pharmacia Biotech (England). PMSF, EDTA, and theophylline were from Sigma (USA). X-Ray film, NaCl, and glycerol were of domestic production. Brassinolide was synthesized in the

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## RESULTS

The majority of pea shoot polypeptides had molecular weights of 15 and 47 kD. Very likely, these polypeptides were small and large rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) subunits (Fig. 1b), as 50% of leaf soluble proteins are known to be chloroplast proteins, mainly represented by small and large rubisco subunits. The set of polypeptides was not changed under treatment with BL; therefore, we do not present the data. Antibodies to phosphotyrosine were noticeably cross-reactive with seven soluble polypeptides with molecular weights of 15, 19, 22, 26, 31, 36, and 47 kD, and tyrosine phosphorylation levels of the 15-, 19-, 36-, and 47-kD polypeptides were the highest (Fig. 1c). However, the 36- and 22-kD polypeptides had the highest specific tyrosine phosphorylation, the specific phosphorylation of the 15-, 19-, 26-, 31-, and 47-kD polypeptides being significantly lower (Fig. 2).

In the control, the level of tyrosine phosphorylation (chemiluminescence) (Fig. 3, a-c) of each polypeptide insignificantly depended on the time after excision of the shoots (5, 20, 30 min). This suggested that the shoot excision procedure (mechanical damage of the tissues, changes in the hydrostatic pressure in the stem vessels, and other consequences associated with the shoot separation) did not significantly affect the protein phosphorylation within the time of observation. However, such changes could occur on shorter or longer exposures. Thus, the specific tyrosine phosphorylation of 19-, 31-, and 36-kD polypeptides was slightly increased in the control shoots 30 min after excision (Fig. 3c). Brassinolide increased the specific tyrosine phosphorylation of the 19- and 31-kD polypeptides at all exposure times and also of the 22-, 36-, and 47-kD polypeptides at the exposures for 20 and 30 min (Fig. 3, b and c).

Changes in the protein phosphorylation levels depend on activities of the oppositely acting enzymes—protein kinases and protein phosphatases. Therefore, we have compared the effects of 20-min incubation with BL and the protein phosphatase inhibitor orthovanadate on tyrosine phosphorylation of polypeptides in the separated pea shoots. The orthovanadate concentration was suffi-

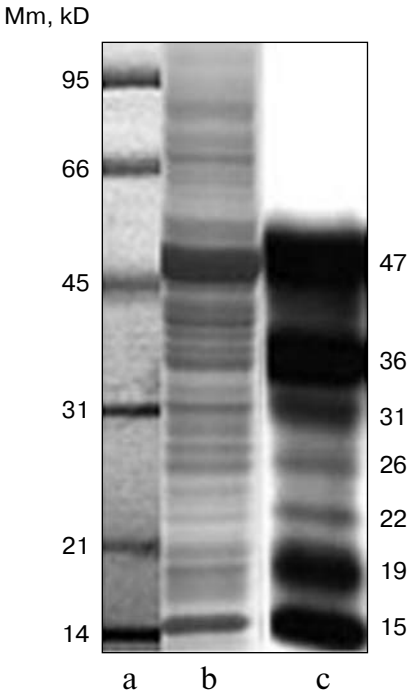


**Fig. 1.** Tyrosine phosphorylation of soluble proteins from leaves of 9-day-old pea seedlings: a) markers; b) spectrum of polypeptides stained with Coomassie R-250; c) tyrosine phosphorylation of polypeptides. Twenty-five micrograms of protein was applied to each track.

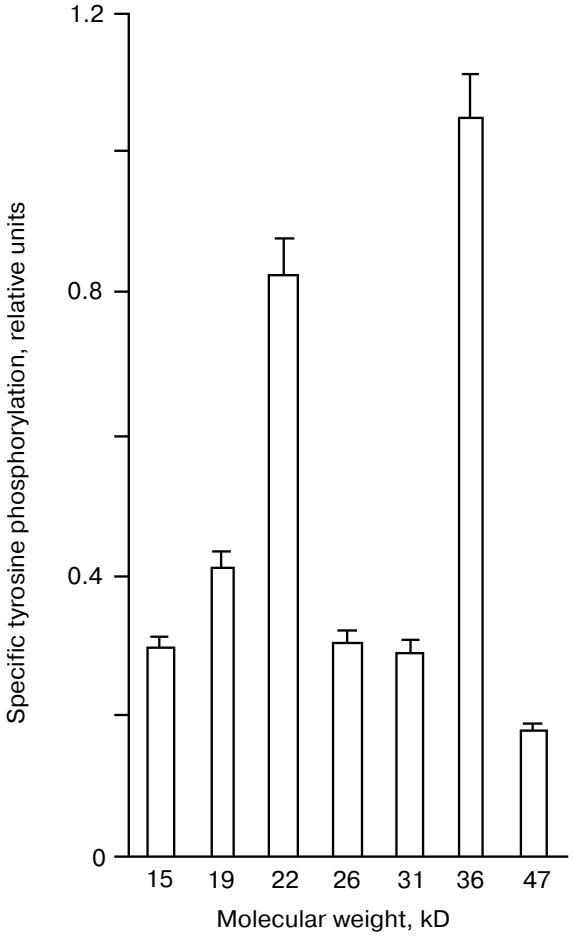


**Fig. 2.** Specific tyrosine phosphorylation of polypeptides from 9-day-old pea leaves. The polypeptides were separated by 1D electrophoresis.

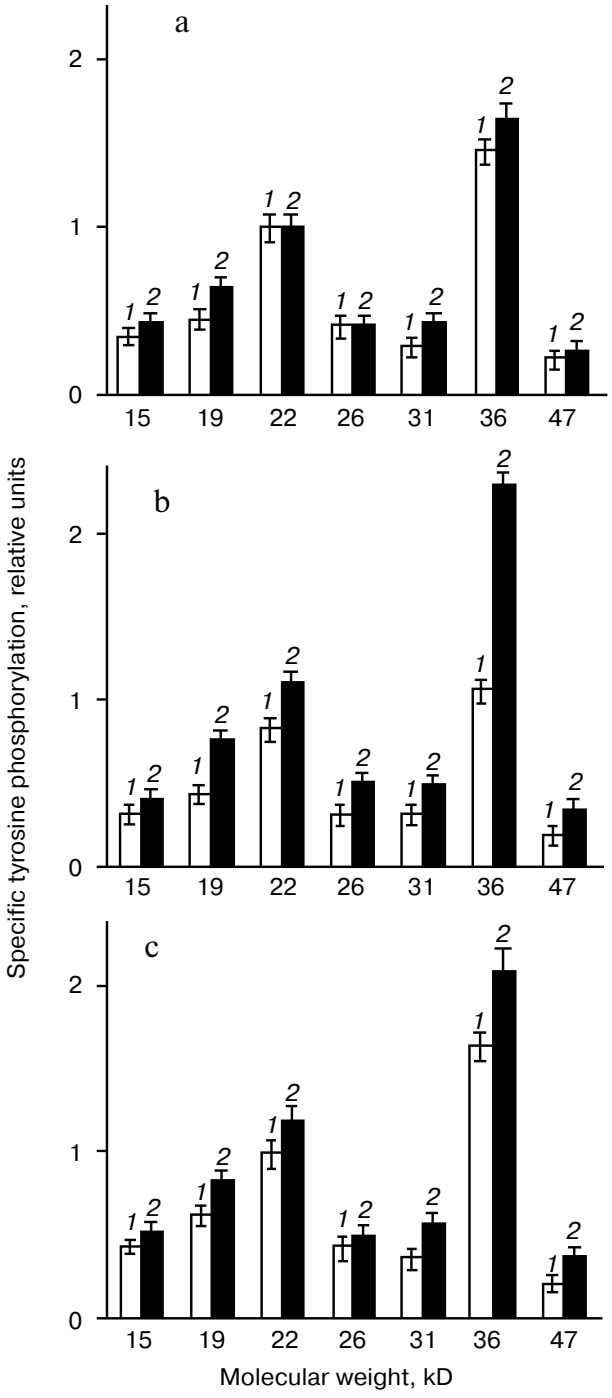


**Fig. 3.** Changes in specific tyrosine phosphorylation of polypeptides from leaves of 9-day-old pea seedlings: a) 5 min; b) 20 min; c) 30 min. *1*) Control; *2*) brassinolide (0.1 μM). The polypeptides were separated by 1D electrophoresis.

tides (Fig. 4). Theoretically, BL could increase the protein phosphorylation level in five cases: first, increasing the activities of tyrosine protein kinases; second, decreasing the activities of tyrosine protein phosphatases; third, combining the first and second effects; fourth, increasing the activities of tyrosine protein kinases more strongly than those of tyrosine protein phosphatases; and, fifth, decreasing the activities of tyrosine protein kinases less than those of tyrosine protein phosphatases. It is difficult to suppose BL affecting tyrosine protein phosphatases as strongly as their specific and nonspecific inhibitors. More likely, the hormone acts on the enzymes responsible for phosphorylation/dephosphorylation of proteins via some mediators. It should be noted that any BL-induced increase in the orthovanadate-increased level of protein phosphorylation (on the combined exposure to these effectors) suggested that the phytohormone significantly increased the activities of protein kinases (exceeding the decrease in the activ-

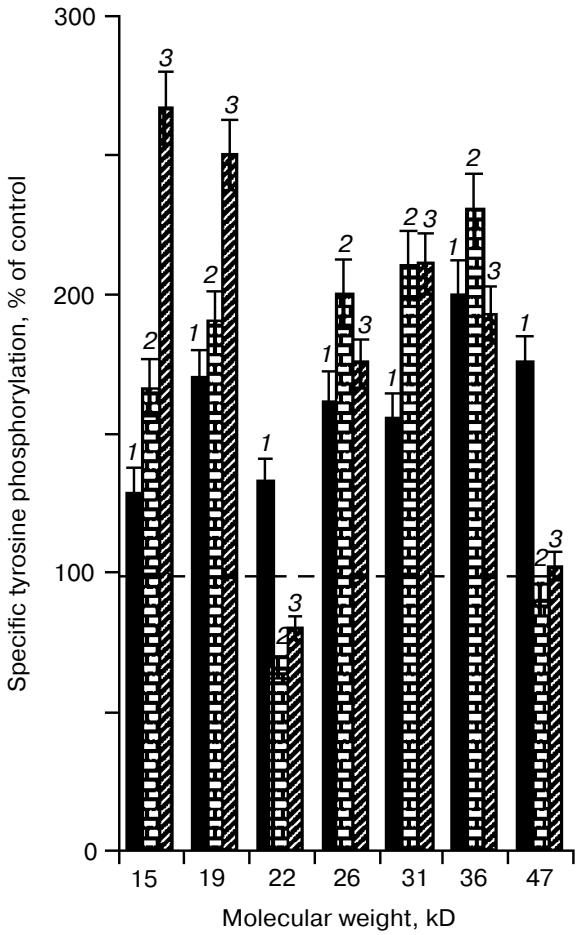


**Fig. 4.** Effects of brassinolide (BL) and orthovanadate on specific tyrosine phosphorylation of polypeptides from pea leaves: *1*) BL (0.1 μM); *2*) orthovanadate (100 μM); *3*) BL + orthovanadate. Dashed line, control. The exposition time to the effectors was 20 min. The polypeptides were separated by 1D electrophoresis.

ciently high to virtually inhibit the enzyme activity and, as a result, increase the level of protein phosphorylation. BL increased the specific tyrosine phosphorylation of all polypeptides, orthovanadate increased it in most cases, but decreased it in the case of the 22- and 47-kD polypep-

ities of tyrosine protein phosphatases by orthovanadate). This was recorded for the 15- and 19-kD polypeptides.

The inhibitor of tyrosine phosphoprotein phosphatases, PAO, increased specific tyrosine phosphorylation of all polypeptides (Fig. 5), and this confirmed the tendency observed on the treatment with orthovanadate. However, PAO markedly increased tyrosine phosphorylation of the 22-kD polypeptide (Fig. 5), whereas orthovanadate decreased it (Fig. 4); PAO also did not decrease specific tyrosine phosphorylation of the 47-kD polypeptide. In this experiment, BL also increased the levels of specific tyrosine phosphorylation of all polypeptides (Fig. 5). The combined effect of PAO and BL on specific tyrosine phosphorylation of polypeptides was unlike the combined effect of orthovanadate and BL. The differences seemed to indicate different specificity of orthovanadate and PAO relative to different tyrosine protein phosphatases [30] and different polypeptides.

It is impossible to separate polypeptides with the same molecular weight but different in p*I* by one-dimensional electrophoresis. Therefore, we used two-dimensional electrophoresis. Figure 6 presents data of 2D electrophoresis of tyrosine phosphorylated proteins from pea leaves in the control and after *in situ* exposure to BL for 20 min. In the leaves of the control plants, the polypeptides with the same molecular weights were tyrosine phosphorylated (Fig. 6a), as was shown by 1D electrophoresis. Some of these polypeptides were individual proteins and others were groups of polypeptides with the same molecular weight but different p*I*.

In the control, we have revealed 13 tyrosine phosphorylated polypeptides (Fig. 6); seven of them had more or less similarly high levels of tyrosine phosphorylation (Nos. 2, 3, 6, 7, 9, 10) and six had lower levels (Nos. 1, 4, 5, 11, 12, 13). Comparison of the results of 1D and 2D electrophoresis suggested that the 15-kD polypeptide consisted of two phosphorylated polypeptides (Nos. 1, 2), the 19-kD polypeptide of two polypeptides (Nos. 3, 4), the 31-kD polypeptide of two polypeptides (Nos. 6, 7), and the 36-kD polypeptide contained three polypeptides (Nos. 8, 9, 10). Tyrosine phosphorylation of the 22-kD polypeptide in most cases was not detected by 2D electrophoresis.

The specific tyrosine phosphorylation of proteins separated by 2D electrophoresis was calculated, and BL was shown to increase it for the majority of polypeptides, especially of Nos. 8-11 (Fig. 7). The levels of specific tyrosine phosphorylation of polypeptides Nos. 3-5 were not calculated because these proteins could not be detected on membranes.

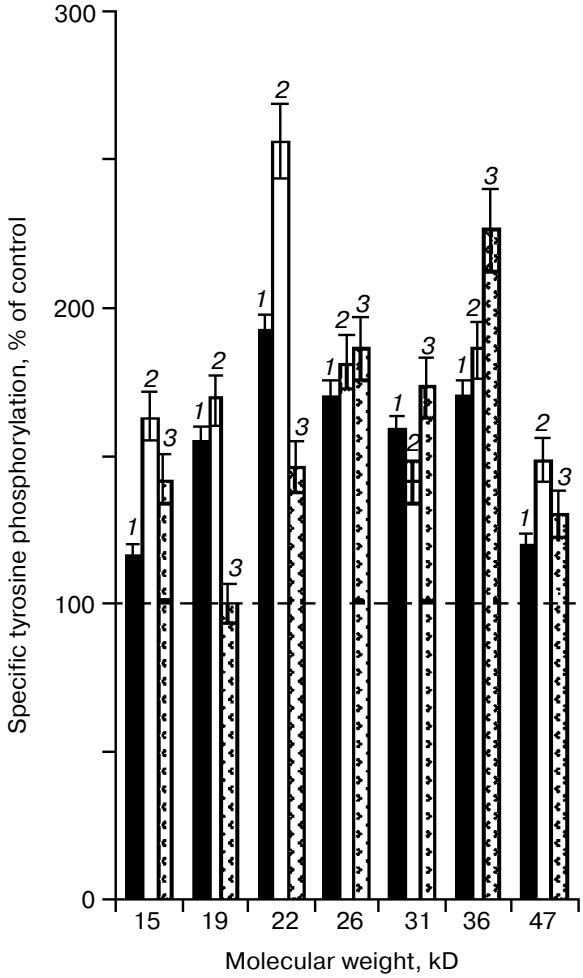


**Fig. 5.** Effects of brassinolide (BL) and phenylarsine oxide (PAO) on specific tyrosine phosphorylation of pea polypeptides: *1*) BL (0.1 μM); *2*) PAO (50 μM); *3*) BL + PAO. Dashed line, control. The time of exposure to the effectors was 20 min. The polypeptides were separated by 1D electrophoresis.

## DISCUSSION

Although activities of tyrosine protein phosphatases are shown to be significantly higher than activities of tyrosine protein kinases [19], it is difficult to conclude such a situation to exist *in situ* because in this case no phosphorylated proteins would be detected. Moreover, the abundance of data on the role of various protein kinases in the signaling systems of cells suggests very effective phosphorylation of proteins. On the other hand, this phosphorylation has to be transitory, which is impossible without the high but regulated activities of protein kinases. One of the most important factors regulating activities of the enzymes responsible for phosphorylation/dephosphorylation of proteins can be a rapid increase in the concentrations of cytoplasmic $H^+$ and $Ca^{2+}$ observed under the influence of various stress agents and stress phytohormones [18].

Our findings of a relatively fast and significant increase in the level of protein phosphorylation under the influence of orthovanadate and PAO (Figs. 4 and 5) suggested high activity of tyrosine protein phosphatases in

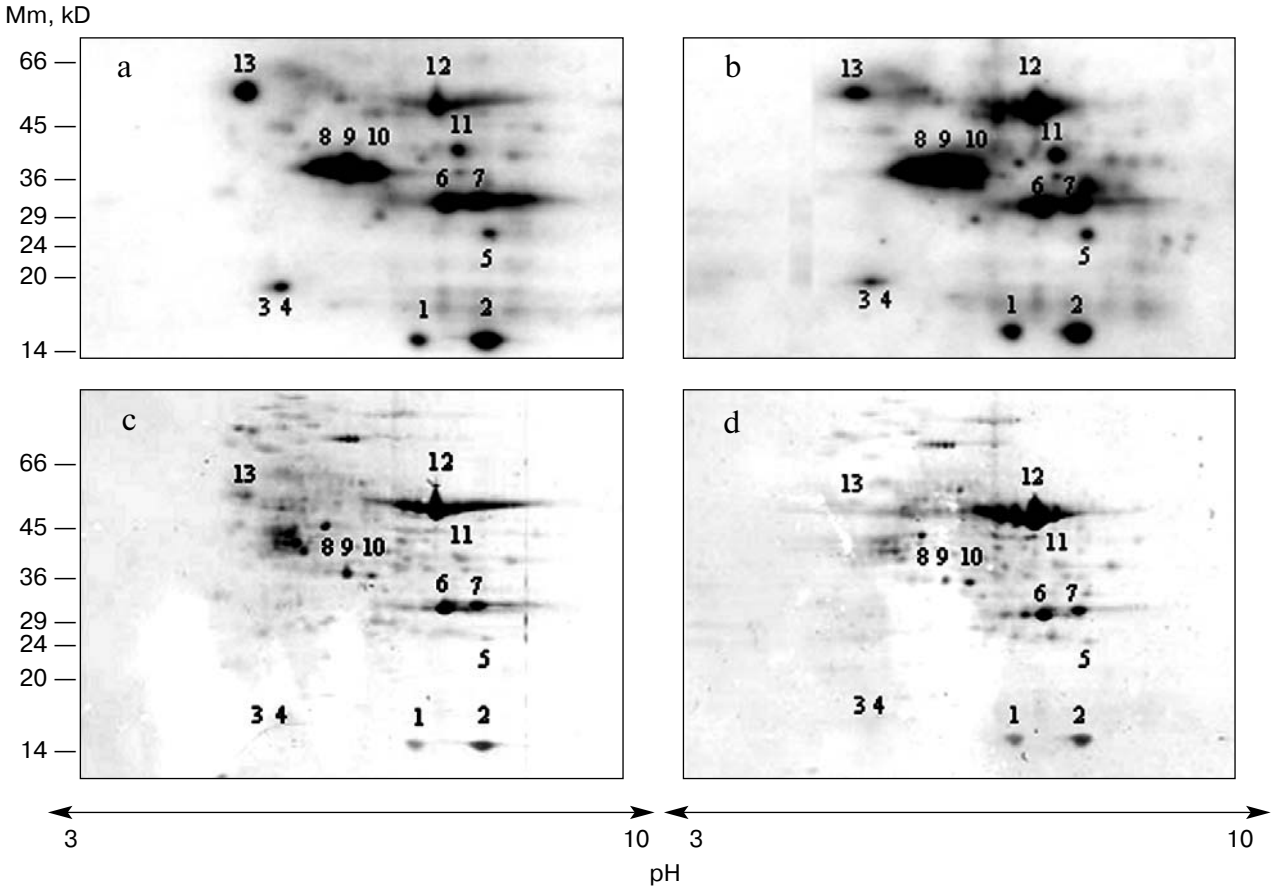


**Fig. 6.** 2D electrophoresis of polypeptides from 9-day-old pea leaves. Tyrosine phosphorylated proteins: a) control; b) brassinolide (BL) (0.1 μM). Proteins stained by Coomassie R-250: c) control; d) BL. The time of exposure to BL was 20 min.

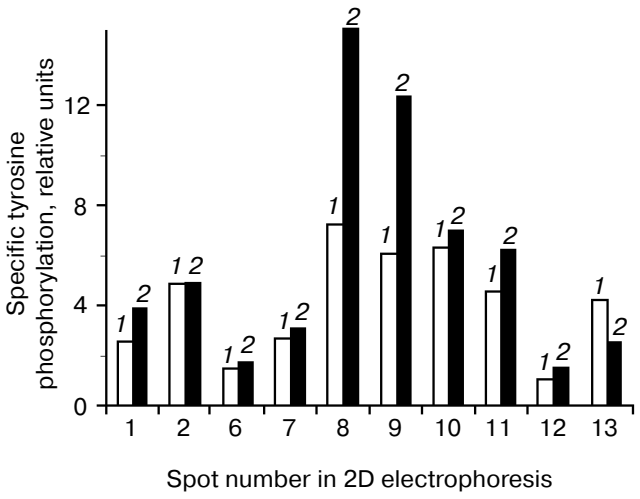


**Fig. 7.** Effect of brassinolide (BL) on specific tyrosine phosphorylation of polypeptides from leaves of 9-day-old pea seedlings: *1*) control; *2*) BL (0.1 μM). The time of exposure to the effectors was 20 min. The polypeptides were separated by 2D electrophoresis. The levels of specific phosphorylation of polypeptides Nos. 3-5 were not calculated because these proteins could not be detected on the membranes because of their low content in the gel.

the leaves of the control pea plants and their regulation by BL. Orthovanadate, a nonspecific inhibitor of tyrosine protein phosphatases, was earlier shown to inhibit all such enzymes in the animal cells [31]. Plants were also shown to possess tyrosine phosphoproteins, which could be dephosphorylated by authentic tyrosine protein phosphatases and protein phosphatases of dual specificity, the activities of which were suppressed by orthovanadate [20].

Although the effects of BL, orthovanadate, and PAO on tyrosine phosphorylation of proteins are more or less similar (Figs. 4 and 5), it is still unclear if only the BL-induced inhibition of dephosphorylation of tyrosine residues in proteins is the cause of the increase in their phosphorylation. On a strong inhibition of tyrosine protein kinases by orthovanadate and PAO, the level of protein phosphorylation during the concurrent exposure to BL could mainly depend on changes in the activities of protein kinases under the phytohormone treatment. The validity of this explanation is supported by the combined effect of BL and orthovanadate on specific tyrosine phosphorylation of the 15- and 19-kD polypeptides, which exceeds the activating effect of each of them. However, on

the exposure to the combination of BL and orthovanadate, the first agent could not considerably change the effect of the other on specific tyrosine phosphorylation of the 22- and 47-kD polypeptides (Fig. 4). Data presented in Figs. 4 and 5 show that different tyrosine protein kinases and tyrosine protein phosphatases and individual proteins (with attack on phosphate on tyrosine residues and their dephosphorylation) specifically react to activators and inhibitors of tyrosine protein kinases and tyrosine protein phosphatases. The different direction of the effects of orthovanadate + BL and PAO + BL (Figs. 4 and 5) may be also explained by addition of the effects of the agents influencing the polypeptides with the same molecular weight, especially as 2D electrophoresis has shown some apparent polypeptides to be groups of polypeptides with the same molecular weight but different p*I*. However, if two agents have a similar effect only on the same one of two enzymes with opposite action (either tyrosine protein kinases or tyrosine protein phosphatases), their effects have, as a rule, to be additive. But if the agents act on different enzymes, we shall observe different responses, as it is shown in Figs. 4 and 5 under the exposure to a combination of the agents.

Changes in the phosphorylation level of polypeptides in response to orthovanadate or PAO can also depend on the turnover rate of phosphorylation/dephosphorylation of individual proteins. The higher is the rate of such turnover, the faster the level of their phosphorylation will increase on the inhibition of phosphatases. The higher is the turnover rate of phosphorylation/dephosphorylation of a protein, the *a priori* greater role has it to play in the operative regulation of metabolism. On consideration of BL-induced changes in tyrosine phosphorylation, this, first of all, can be assigned to protein components of BL-dependent signaling systems which cause dramatic changes in the metabolism by reprogramming the expression of various genes producing components of the subsequent adaptive responses.

The preliminary comparison of pea and *Arabidopsis* 2D electrophoregrams (presented on the site www.expasy.org) suggests that polypeptides Nos. 1 and 2 are small subunits, and polypeptide No. 12 is a large subunit of rubisco. However, to precisely identify the revealed tyrosine phosphorylated proteins remains for our future studies.

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