Acta Biochimica Polonica

Vol. 43 No. 3/1996

515–520

QUARTERLY

Endoproteinase activities in wheat leaves upon water deficit*

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Received: 3 July, 1996

Key words: acclimation, endoproteinases, proteolysis, water deficit, wheat

In wheat leaves acclimated and non-acclimated to water deficit the azocaseinolytic activities of endoproteinases were increased about 7-fold under drought conditions. Under such conditions both the pH dependence profile and the endoproteinase pattern were also changed. The predominant contribution of serine proteinase (about 50% of total endoproteinase activity) remains unaltered in the drought stressed leaves. Cysteine proteinase was induced to the same extent in the drought-stressed leaves irrespective of the acclimation pretreatment, while the contribution of aspartic proteinase was reduced upon water deficit but in the acclimated stressed leaves was as high as in the non-stressed leaves. These changes in the pattern of endoproteinases seem to imply that the water deficiency affects endogenous proteolysis.

Plants respond to water deficit by reduction of protein synthesis [1–3] and substantial remodelling of protein components but at the same time synthesis of specific stress proteins, such as dehydrins, osmotin and others, takes place [4–6]. For this reorganization of plant metabolism proteolysis is a prerequisite. Mechanisms and control of protein turnover in plants are, however, poorly understood. Recent evidences indicate that plant proteolysis requires energy [7] and that this process is highly energy consuming. ATP-dependent hydrolysis of ^{125}I-lysozyme in mature wheat leaves constituted about 1/2 of total proteolytic activity (unpublished). The energy used for ATP-dependent proteolysis amounts to about one half of that required for protein synthesis in wheat leaves and increases to about 75% under water deficiency [8].

The aim of the present paper was to evaluate the activity of ATP-independent endoproteinases in wheat leaves under water deficiency. We have also taken into consideration the process of acclimation which is decisive for plant tolerance to drought.

MATERIALS AND METHODS

Plant material. Plants of spring wheat (*Triticum aestivum* L. var. milturum) were grown in soil in a growth chamber and were watered daily. Control (non-stressed) plants were grown under these conditions throughout the whole experimental period. At the stage of third leaf, a part of plants were drought-pretreated (drought acclimated) by cessation of watering during a period sufficiently long to cause a critical water saturation deficit (WSD, causing 50% tissue injuries) of the third leaf. After this treatment the plants were rewatered and then subjected together with non-pre-

*This paper was supported by a grant No. 5 P06A 013 10 from the State Committee for Scientific Research.

Abbreviation: PMSF, phenylmethanesulfonyl fluoride; PVP, polyvinylpolypyrrolidone; WSD, water saturation deficit.

treated plants to soil drought conditions leading to 50% WSD of the fully mature, non-senescent fifth leaves. To eliminate the indirect effect of drought on plant development, all measurements were carried out at the same developmental stage (developmental control). Also, to eliminate a possible effect of leaf age, control leaves (non-acclimated and acclimated) were analyzed at the beginning and at the end of drought treatment. Water saturation deficit in the studied leaves, calculated according to Stocker [9], was used to characterize the plant state under soil drought conditions. The average WSD of leaves from both non-acclimated and acclimated plants taken for the analyses was 50%.

Extraction and measurement of endoproteinase activity. Fully expanded leaves of the plants were ground in liquid nitrogen and about 1 g of tissue was extracted with 5 ml of cold extraction medium containing 0.2 g PVP (polyvinylpolypyrrolidone) and 5% mercaptoethanol in 50 mM Tris/HCl, pH 7.5. The homogenate was filtered and centrifuged at 15000 *g* for 10 min. The supernatant was used as the crude enzyme preparation. The effect of pH on endoproteinase activity was studied with the following buffers all at 0.25 M concentration: glycine/HCl (pH 3.0), citrate/phosphate (pH 4.0 and 5.0), Mes/KOH (pH 6.0 and 7.0), Tris/HCl (pH 8.0) and bicine/HCl (pH 9.0 and 10.0). The reaction mixture contained 0.1 ml crude extract, 0.3 ml of 0.5% azocasein and 0.6 ml of buffer. After 3 h at 37°C the reaction was stopped by adding 2 ml of 12% trichloroacetic acid, and acid-soluble products were determined spectrophotometrically at 340 nm. One unit of azocaseinolytic activity was defined as the amount of protein causing a 0.01 increase in A_{340} referring to the 0 time values (the reaction stopped immediately after starting). To characterize the endoproteinase composition, the selective inhibitors of serine (2.5 mM phenylmethanesulfonyl fluoride; PMSF), cysteine (1.0 mM iodoacetate) aspartic (5 μg/ml of pepstatin) and metallo-proteinases (10 mM EDTA) were used. The inhibitors were added to the enzyme extracts and the mixtures were preincubated during 1 h prior to the addition of the substrate.

The protein content of leaf extracts was determined according to Bradford [10].

RESULTS

The content of soluble protein was reduced by half in the mature wheat leaves (Table 1) upon water deficit. Transient dehydration (acclimation) led to a small but significant increase in the protein content and, despite its reduction upon water deficit, the protein level was by about 20% significantly higher in the stressed acclimated leaves than in the non-acclimated ones.

As can be seen from Fig. 1, the maximum azocaseinolytic activity in all crude leaf extracts was observed at pH 5.0. In the control acclimated and non-acclimated leaves the activity at pH 5 was about 1.5-fold higher than at pH 4 and about 1.6-fold higher than at pH 6. The transient water deficit (acclimated leaves) practically did not affect the pH dependence of endoproteinase activity, however, some slight but significant increase of the activity at pH 7.0 was observed. Water deficit increased about seven-fold the proteolytic activities at pH 5.0 in both acclimated and non-acclimated leaves. Although induction was the same irrespective of the acclimation pretreatment, some differences in the pH curve can be clearly seen. The endoproteinase activities at low and high pH were enhanced: e.g. as compared to the respective control leaves, in acclimated stressed leaves an about 9-fold increase was observed at pH 6 and 7, whereas in non-acclimated ones at the same pH values the increase was about 6-fold and 25-fold, respectively. Similarly, at pH 4.0 water

Table 1

Effect of water deficit on the soluble protein content (mg/g dry weight) in non-acclimated and acclimated wheat leaves.

In brackets the protein content in percentage of that in control non-acclimated leaves. Values in the columns without common letters are significally different ($P = 0.05$, Tukey's HSD test).

Leaves		Protein content
Non-acclimated	control	92.5 a (100)
	stressed	53.0 b (57)
Acclimated	control	128.4 c (139)
	stressed	71.6 d (77)

deficit caused an about 7-fold increase in acclimated leaves whereas in non-acclimated ones only a 5-fold increase.

These differences are related to changes in the activities of particular endoproteinases, assayed with the use of specific inhibitors (Table 2). Since drought resulted in some (about 15%) decrease in dry weight but in about 50% reduction of protein level, enzymatic activities were expressed both per mg protein or g dry weight. Also one should realize that specificity of the inhibitors used is not absolute and the sum of the activities of individual endoproteinases is higher than the total azocaseinolytic activity. This concerns mainly the effect of PMSF, since it affects some sulfhydryl enzymes, e.g. cysteine proteinases. Therefore, one can expect an overestimation of the activity of serine proteinases. However, large differences between the acclimated and non-acclimated leaves upon water stress irrespective of the way of expression of the results enable to draw conclusions as to the function of particular endoproteinases.

In the mature non-senescent wheat leaf, PMSF, an inhibitor of serine enzymes, exerted the most pronounced effect (57%). Iodoacetate, a cysteine-enzyme inhibitor and pepstatin, an inhibitor of aspartic enzymes, modified the proteolytic activity to a similar extent (29 and 35%, respectively). Inhibition by EDTA, referring to metallo-proteinases, did not exceed 10%. Water deficiency pretreatment (acclimated control) had no influence on the enzyme susceptibility to PMSF and iodoacetate but decreased its sensitivity to pepstatin. Drought (50% WSD) influenced the proteinase pattern but in a different way: it decreased almost by a half the participation of aspartic proteinases in total non-inhibited proteolysis in non-acclimated leaves, but increased it in acclimated leaves to the level observed in non-acclimated control ones. Irrespective of acclimation, participation of cysteine proteinase increased almost twofold and the contribution of serine proteinase remained at the level observed for control leaves.

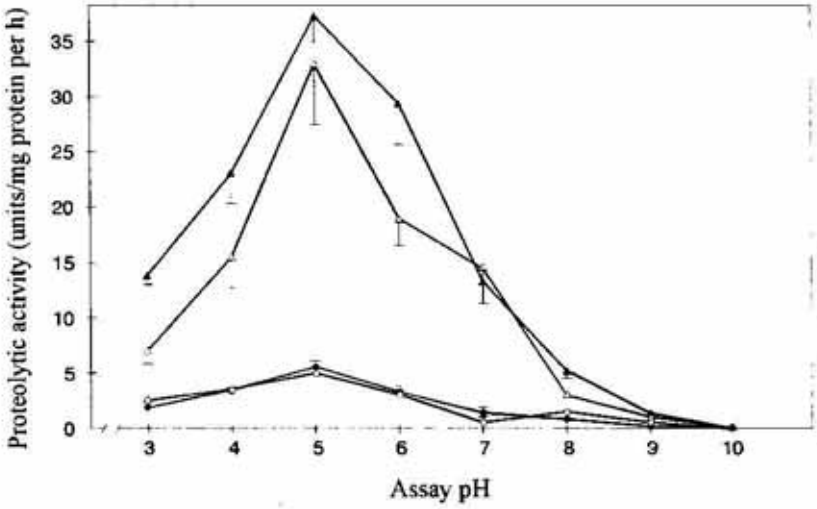

Fig. 1. Effect of water deficit on the pH profile of azocaseinolytic activities in extracts from control (circles) and stressed (triangles) leaves of non-acclimated (open symbols) and acclimated (closed symbols) plants.
Data presented are means of three separate experiments ± SD.

Table 2

Changes in the activities (units/mg protein per h) of different types of endoproteinases in the total azocaseinolytic activities at pH 5.0 in leaf extracts from control and dehydrated leaves of non-acclimated and acclimated plants

Leaves		Serine proteinase		Cysteine proteinase		Aspartic proteinase	
Non-acclimated	control	2.78	(257)[a]	1.39	(129)	1.71	(158)
	stressed	17.71	(939)	17.38	(920)	6.65	(352)
Acclimated	control	2.96	(380)	1.61	(206)	1.43	(188)
	stressed	20.49	(1457)	18.29	(1306)	11.33	(809)

[a]In brackets data in units/g dry weight per hour.

DISCUSSION

The results on the endoproteinase activities in wheat leaves under water deficiency clearly indicate participation of ATP-independent proteolysis in plant metabolism. The abolishing effect of cycloheximide on the increase in proteolysis during dehydration in the PEG (polyethylene glycol)-treated leaf segments [11] and in glucose-starved maize roots [12] provide evidence for the synthesis of proteolytic enzymes. Acclimation had no effect on the quantitative induction of proteolysis under water stress conditions. However, differences observed in the pH-activity dependence and changes in the endoproteinase pattern clearly indicate that water deficit stress essentially modifies proteolysis. Inhibition by PMSF, referring to serine endoproteinase, increased proportionally to the total induction of proteolysis. In contrast, a pronounced induction of cysteine endoproteinase under water stress, similarly as in temperature stresses [13] and in senescent leaves [14], points to the important role of this particular endoproteinase under any stress in plant. Possibly in connection with the increased function of cysteine proteinase is a large energy expenditure for maintenance of sulfhydryl groups under stress; this concerns mainly protein-SH including, thus, cysteine endoproteinase [15]. However, induction of cysteine proteinase was the same in the acclimated stressed leaves. The third enzyme studied, aspartic proteinase responded to stress conditions in a different way. The contribution of this enzyme was reduced on water deficit, but in the acclimated stressed leaves was as high as in the non-stressed leaves. This might be reflected in a higher proteolytic activity at lower pH observed in stressed acclimated leaves. The recovery of this activity in the acclimated stressed leaves to the control values is noteworthy.

Precise evaluation of the function of individual endoproteinases is impossible since specificity of their substrates both *in vitro* and *in vivo* remains unknown. The mechanism of their functioning in protein degradation in the leaf cells is also unclear. Endoproteinases are mainly localized in vacuoles [16, 17] while the bulk of protein in plastids [18, 19], therefore integrity of membranes under stress seems to be of utmost importance. Rubisco constituting the bulk of leaf protein is preferentially hydrolysed at pH 5.0 [20, 21]. Electron micrographs of mesophyll protoplasts in senescing wheat leaves revealed changes in association of chloroplasts and vacuoles and imply even a phagocytic-type of this association [17]. A comparison of the endoproteinase pattern of senescing barley leaves [22] and that of drought-stressed leaves shows differences except for the increased role of cysteine proteinase in both barley and wheat leaves. The question can be also posed whether very high proteolysis of leaf proteins under drought or other stresses is advantageous for the plant, helping in reorganization of cellular proteins, or, being very extensive, leads to disintegration of plant cell. However, after a transient water deficit and total leaf recovery the proteolytic activity drops to the level observed for non-stressed leaves. In irreversibly senescent leaves, e.g. at 70% WSD, the proteolytic activity was reduced almost by half as compared to the leaves with 50% WSD (unpublished). This would imply that proteolysis observed at 50% WSD (acclimation conditions) should be attributed to the process of protein remodelling.

We would like to thank Professor Konstancja Raczyńska-Bojanowska for many stimulating discussions and critical reading of the manuscript.

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