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POSSIBILITIES FOR IMPROVING ANTIOXIDANT ACTIVITY OF SOYBEAN (*GLICINE MAX* L.) SEEDS AND PLANT PRODUCTIVITY AFTER TREATMENT WITH CONFORMATION CHANGED LISOZYME (THERMODASE)

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Summary. This work contains results from the studies under field conditions for the effect of thermodase (0.1 %, pH 6.8) on improving the antioxidant activity of seeds from two soybean varieties and their productivity. The trial was performed without applying mineral fertilizers and herbicides. The obtained results showed that the antioxidant activity FRAP activity and productivity increased in both varieties compared to the control plants. The results can be used in the breeding practice (stem height, seed yield and antiradical activity, the last one as a breeding marker).

Key words: thermodase, Hen Egg White Lysozyme (HEWL), productivity, antioxidant activity.

Abbreviations: FRAP- ferric reducing antioxidant power; HEWL - Hen Egg White Lysozyme; PP- polyphenol compounds.

INTRODUCTION

Thermodase is a modified Hen Egg White Lysozyme (HEWL) (Fig. 1) through heat denaturation and definite pH, at which the enzyme acquires a new conformation with changed vital activity.

The changed and strengthened vital activity is a consequence of the part of denaturation. The folding and unfolding of such proteins is a subject of study at the molecular level by the scientists, since their biological activity predetermines functional changes in the living organisms with modified physiological

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#Part of this investigation was performed in the Acad. M. Popov Institute of Plant Physiology – Bulgarian Academy of Sciences, when the author has been working at institute.

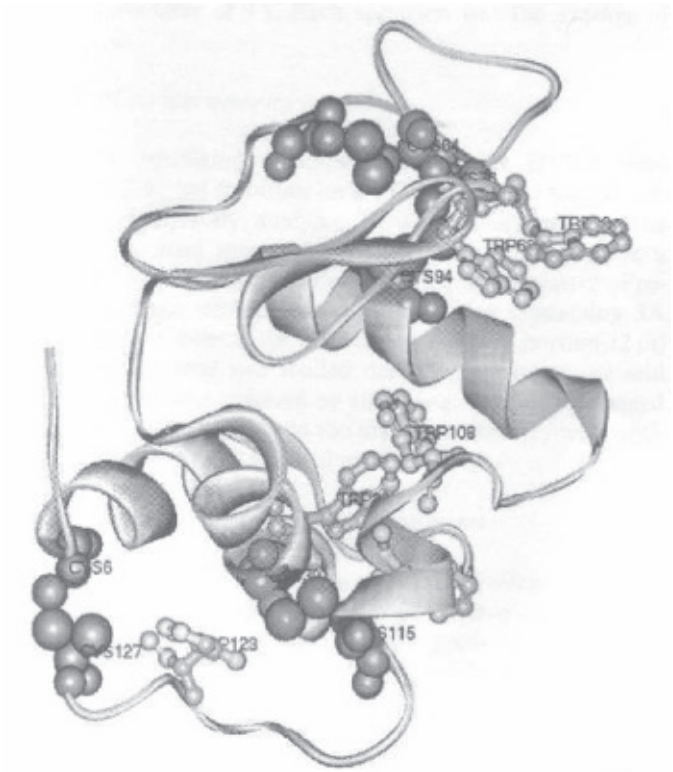

Fig. 1. Structure of HEWL composed of two domains of global protein. The alpha domain is composed of three alpha helices and $C3_{10}$-terminal helix and beta domain is composed of one antiparallel three-stranded beta-sheet and one loop. (Protein Sci 3, 1994, p. 883).

properties (Atassi et al., 1971). In medical practice there are molecular diseases caused by partial denaturation of proteins, such as Alzheimer (increased concentration and conformation change of human lysozyme), Parkinson, diabetes of type 2, etc. (Steven et al., 2007). The bacterial cell wall is a substrate of thermodase. Through hydrolysis, the thermodase breaks 1,4-ß bonds between N-acetyl muramic acid (NAM) and N-acetyl glucose amine residues (Fig. 2) in the cell wall of the bacteria and injures it. The thermodase shows also the properties of lectins (Arnaudov, 1990). Phytochemicals described in literature as various classes of phenol compounds (PP) and more specifically phenolic acids, flavanoids, isoflavonoids, procyanins and tannins are constituents of soybean seeds (Malenic et al., 2007). These compounds manifest antioxidant activity and are in the focus of many researchers. Being antioxidants, these compounds take place in preparation of diet foods against many diseases (Yong et al., 2001). Some of these classes are also related to other physiological processes in plants as acceleration of growth, assimilation of mineral elements, plant-microbial interactions (isoflavons) (Ucheda and Syano, 1982). Depending on their application purpose, methods for improving (accelerating) their antioxidant capacity and ferric reducing antioxidant power are applied. Here should be mentioned the microbial fermentation as one of the methods for

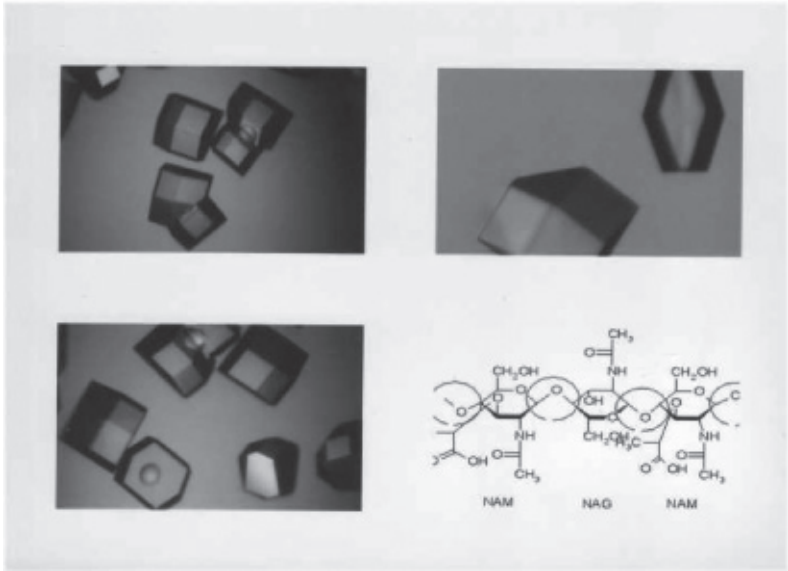

Fig. 2. Crystalline structure of HEWL and the enclosed in circle β-1,4 bonds between NAM and NAG that the thermodase degrades in the bacterial cell wall.

improving the antioxidant activity of isoflavons in the soybean flour. Pure cultures of *Bacillus subtillis* strain MR10 and TK8, *B. natto* and *Rhizopus olygosporus* (Wangputtisin et al., 1997) were used. The fermentation method is used also for tea extract (green, black and oolong) for reducing the content of peroxide species of linoleic acid (Yen and Chen, 1995). The ferric reducing antioxidant power of these extracts correlates with the content of PP and with the number of $OH^{\cdot}$ groups and aromatic rings, the concentration of extracts and conjugation level of the compounds. This phenomenon of structural formations is confirmed for epicatechin gallate (Benzie and Szeto, 1999; Lunder, 1992). The compounds in these extracts are electron donors and can react with free radicals to stable products and termination of the radical chain reaction. The conditions for seeds storage and the proper selection of geographic regions for soybean cultivation can also contribute to improving the antioxidant and FRAP activity (M. Swanson, M. Stoll et al., 2004). Higher antioxidant activity of PP and physiological pH has been registered. The polyphenol compounds have a negative redox potential at higher pH and attack the peroxide and hydroxyl radicals which have a positive redox potential at pH 7 (1000 to 2300 mV) (Simic and Jovanovic, 1994). The capability of phenol compounds to scavenge free radicals depends on the dose and increases with increasing the antioxidant concentration and depends on their structure (Ucheda, et al., 1987). Treatment of soybean seeds with yellow husk with water vapor under pressure results also in higher DPPH and FRAP activity and TPC, tannins and radical scavenging capacity (Xu and Chan, 2008). The application of bioorganic fertilizers leads to enhancement of the antioxidant capacity of soybean seeds and improves the rhizosphere around the soybean roots (Hanan et al., 2008).

The increase in the productivity of soybean plants can be influenced also by the products of second metabolites and by the intensified photosynthesis and nitrogen fixation, as it has been reported in our previous investigation (Kolev et al., 2003; Kolev and Aleksieva, 2009). No data are available in literature on the effect of thermodase (conformation changed HEWL) on the physiological processes in plants except for our studies on nitrogen fixation (Kolev et al. 2003) and content of polyphenolic compounds in *Glycine max* (Kolev and Aleksieva, 2009). The aim of the present study was to improve the antioxidant activity of soybean (*G. max* L.) seeds and plant productivity by applying conformation changed lysozime (thermodase).

MATERIALS AND METHODS

The thermodase was obtained according to the methodology described by Kolev et al. (2003). The antioxidant activity of seeds was determined by the method of Brand et al. (1995) with the free stable radical DPPH, which is purple (violet) coloured when dissolved in methanol. Applied to a medium with antioxidants, it decreases the colouration intensity, which is an indicator of the activity of the natural antioxidant measured at 515 nm. The reduction reaction of DPPH by one antioxidant (AO) or radical ($R^{\cdot}$) proceeds according to the following equation:

$$DPPH^{\cdot} + AH \rightarrow DPPH^{\cdot} - H + A^{\cdot}$$
$$DPPH^{\cdot} + R \rightarrow DPPH^{\cdot} - R$$

The ferric reducing antioxidant power (FRAP) was determined using the 2,4,6 tripiridin-*s*-triazine. The method is based on the reduction of the blue-colored Fe^{3+} ion in this complex to the colorless Fe^{2+}. 2 g of the samples were taken and placed in 10 ml 80% ethanol. Then the material was ground and centrifuged for 15–20 min at 15000 x *g*. The total PP in the supernatant absorb at 765 nm. The DPPH activity was measured at 515 nm and the FRAP – at 593 nm, according to the procedures described in literature. The field trial was carried out in 2004–2006 in the experimental field at the Institute of Forage Crops Pleven – Pavlikeni branch after the Shanin method of 'long plots' as shown in Fig. 3 with two soybean varieties – early Canadian variety Korada and mid-early Bulgarian variety Srebrina. The variety seeds were sown at a 0.7 m space between the rows and 4 cm space in the rows at optimum for the crops terms. The trial was laid out in four replications under conditions of natural wetting, without use of herbicides and mineral fertilizers. The test trials for each variety included: control (non-treated plants); plants treated with 0.1%, (pH 6.8) solution of thermodase enzyme in phase V_2 (first ternate leaf); plants treated with 0.1% enzyme solution in phase R_2 (flowering). The effect of the enzyme on productivity parameters as plant height (cm), the number of nods and weight of seed per plant (g) by biometric analysis on a mean sample of 35 plants for each variant was studied. The obtained results were statistically processed by variation analysis. The differences between the means were proved by the value of the coefficient of significance, according to the Student's *t*-test (Zapryanov and Marinkova, 1978).

Fig.3. The field trial photographed at the end of flowering. There was a difference in the status of the control (left) and thermodase-treated plants (right).

RESULTS AND DISCUSSION

In living organisms free radicals, the so-called ROS (O_2^-, H_2O_2, $^{\cdot}OH$) are released in response to external or internal factors. Their inactivation is performed by antioxidants that degrade ROS (the oxidant) and they are known in literature as an oxidant stress provoking degenerative processes in the living organisms. Our studies are concentrated on the non-enzyme systems, the antioxidant activity of which is shown in Tables 1 and 2. The results revealed an increase in the DPPH and FRAP activities and total polyphenols content. These data are in agreement with the results of other authors (Yen and Chen, 1995). For the Korada variety the values for DPPH$^{\cdot}$ and FRAP activities were comparable. It could be supposed that during seed development the concentration of AOS decreases as a result of their participation in the early embryogenesis as signal molecules taking part in the growth processes. AOS can have also a regulatory function in the changes of gene expression during seed development, germination and latency (Bailli, 2004). For that reason, the probability for cell structures injury is reduced. An equilibrium in the oxidation-reduction process between the antioxidant and oxidant is established (Table 1). This could be due to the activation of the enzyme by the antioxidant to suppress the AOS generation, or under the effect of thermodase on the free radicals sources (mitochondria, peroxysomes and chloroplasts). The class of total polyphenols (16%) that exerted antioxidant activity will be an object of further research. Some of the total polyphenols which do not participate in the oxidation-reduction processes may take part in other physiological processes or synthesis of similar compounds. There are data in literature revealing

Table 1. Content of total polyphenols, antioxidant and FRAP activity in soybean seeds varieties Korada and Srebrina (2004).

Variants	Natural polyphenols [ppm]	% of Control	DPPH· [μm/g]	% of Control	FRAP [μm/g]	% of Control
Korada – Control	2358±6.810	100.0	0.372±0.003	100.0	17.98±0.003	100.0
Korada – Experiment	2757±7.640*	16.0	0.392±0.002*	5.0	18.96±0.250*	5.0
Srebrina – Control	2367±7.640	100.0	0.350±0.003	100.0	16.42±0.100	100.0
Srebrina - Experiment	2513±6.810*	6.0	0.392±0.003*	12.0	16.85±0.190*	26.0

*Differences significant at P=0.95% after Origin.

Table 2. Content of total polyphenols, antioxidant and FRAP activity in soybean seeds varieties Korada and Srebrina (2005).

Variants	Natural polyphenols [ppm]	% of Control	DPPH· [μm/g]	% of Control	FRAP [μm/g]	% of Control
Korada – Control	2364±2.080	100.0	0.323±0.002	100.0	16.70±0.110	100.0
Korada – Experiment	2605±6.500*	10.0	0.352±0.009*	9.3	18.18±0.250*	8.8
Srebrina – Control	2265±1.450	100.0	0.298±0.002	100.0	16.14±0.180	100.0
Srebrina - Experiment	2443±2.080*	7.8	0.342±0.002*	15.0	17.71±0.040*	9.6

*Differences significant at P= 0.95% after Origin.

that one and the same class of PP can have either higher or lower antioxidant activity depending on the genotype of the plants (Malenic et al., 2007). The second variety Srebrina was characterized by higher antiradical and FRAP activities which was in accordance with literature data. Here should be pointed out that the antioxidant activity was lower than the ferric reducing antioxidant power, thus suggesting a change in the redox potential and the occurrence of reduction processes. Thus, reducing substances accumulate in the seed on account of the antiradical activity, which was also confirmed by the tendency to lower PP content. The data in Table 2 demonstrate a typical antiradical activity of the two varieties. The values of the antioxidant activity were higher than those for the ferric reducing power of the antioxidants. Wang and Meya (2005) reported that during the protein hydrolysis the soybean protein structures were changed and more active amino acid groups were exposed. For that reason the soybean

peptides could have higher activity than the intact protein. It could be assumed that the fractional composition of the protein structure of the soybean seeds in the investigated seeds was changed as a result of the treatment with thermodase. The results on soybean productivity are shown in Tables 3 and 4. Regardless of

Table 3. Thermodase influence on structural parameters of productivity in Korada cultivar for the period 2004 – 2006.

Korada cv.	Phenological stage 'first ternate leaf'				
	Parameters of productivity				
	Plant height [cm]	Branches per plant [No]	Pods per plant [No]	Seeds per plant [No]	Seed weight per plant [g]
Check	50±0.5	2±0.1	38±1.2	81±2.1	14.1±0.3
Treated with 0.1%	56±0.5***	2±0.1^{ns}	42±0.9***	89±1.0**	16.7±0.3***
Korada cv.	Phenological stage 'flowering'				
	Parameters of productivity				
	Plant Height [cm]	Branches per plant [No]	Pods per plant [No]	Seeds per plant [No]	Seed weight per plant [g]
Check	50±0.5	2±0.1	38±1.2	81±2.1	14.1±0.3
Treated with 0.1%	62±0.9***	2±0.2^{ns}	49±0.7***	105±1.9***	20.1±0.5***

Differences significant at **P=1% and ***P=0.1%; ^{ns} – non-proved differences.

Table 4. Thermodase influence on structural parameters of productivity in Srebrina cultivar for the period 2004 – 2006.

Srebrina cv.	Phenological stage 'first ternate leaf'				
	Parameters of productivity				
	Plant height [cm]	Branches per plant [No]	Pods per plant [No]	Seeds per plant [No]	Seed weight per plant [g]
Check	82±1.1	3±0.2	82±1.3	170±5.3	25.4±0.2
Treated with 0.1%	89±1.4***	4±0.2***	87±1.2**	180±2.0^{ns}	26.7±0.3***
Srebrina cv.	Phenological stage 'Flowering'				
	Parameters of productivity				
	Plant height [cm]	Branches per plant [No]	Pods per plant [No]	Seeds per plant [No]	Seed weight per plant [g]
Check	82±1.1	3±0.2	87±1.3	170±5.3	25.4±0.2
Treated with 0.1%	95±1.4***	4±0.2***	96±3.4***	213±7.8***	31.7±0.9***

Differences significant at **P = 1% and ***P= 0.1%; ^{ns} – non-proved differences.

the phase of treatment, the productivity of both varieties was increased. The effect of the enzyme was more strongly expressed during the flowering phase. The differences with respect to the control variant concerned the plant height, number of pods and seeds, seed weight of one plant. The increased productivity can be also affected by the intensified nitrogen fixation and photosynthesis as described in our previous studies under the same experimental conditions (Kolev et al., 2003; Kolev et al., 2009).

CONCLUSIONS

1. An increase in the antioxidant activity ($DPPH^{\cdot}$), ferric reducing antioxidant power FRAP and total polyphenols content was established.
2. A change in the reduction status of the seeds from the varieties tested from the exerted antiradical effect to its reduction and equalizing the ferric reducing power of the antioxidant with its antiradical activity was registered.
3. Treatment of soybean plants of both varieties with the enzyme thermodase during the flowering phase caused a statistically significant positive effect on productivity compared with controls.

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