

## ANTIOXIDANT ACTIVITY OF PEPTIDES OBTAINED FROM WHEAT AND COTTONSEED PROTEINS

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### Abstract

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Enzymatic hydrolysis of various wheat proteins and cottonseed with acid (Prolive PAC 30L “EnzymeBioProduct” Ltd, Russia) and neutral proteases (*Neutrase*, “Novozymes”, Denmark) was studied. The antioxidant properties of the produced peptides depended on the properties of the proteins and enzymes used. It was shown that when using acid protease peptides, derived from wheat albumin possess 10-12 times higher reducing power than peptides derived with neutral protease. When using cottonseed albumin, a different relationship was established. Reducing power of peptides, produced with neutral protease was 2-3 times higher than peptides processed using acid proteases. When hydrolyzing cottonseed globulin, the reducing power of the resulting peptides is higher, when acid protease is used.

In a model system, the highest inhibition rate of oxidation of (+)-catechin is observed when peptides derived from cottonseed and wheat proteins with neutral proteases were used. In all cases, peptides derived with acid protease reduce the rate of oxidation of (+)-catechin to a smaller degree. Apart from having antioxidant activity, the peptides can also affect the activity of antioxidant enzymes. Activating effect is observed at low degrees of hydrolysis of albumin and it depends on the type of the enzyme and the substrate protein.

*Key words:* wheat, cottonseed, protein, protease, hydrolysis, peptides, antioxidant properties, (+)-catechin oxidation

### Introduction

It is known that spoilage of food raw materials and finished products is the result of complex physical, chemical and microbiological processes: such as hydrolysis, oxidation and development

of microbial flora, which are closely linked. The occurrence and speed of these processes are determined by many factors: composition and state of food system, moisture, pH, and activity of enzymes, storage and processing of raw materials, the presence of antimicrobial, antioxidant and pre-

servative substances (Nechaev, 2007).

Various natural and synthetic preservatives and antioxidants are used for extending the storage period of end products. Great attention is also paid to the use of natural antioxidants based on polyphenols and peptides derived from plant materials (Lagouri and Nisteropoulou, 2009; Beermann et al., 2009 and Jacobsena et al., 2008)

Recently, among the many antioxidants of various origins used in food industry (Andrea et al. 2010), peptide antioxidants attract great interest (Davalos et al., 2004; Chen et al., 1995; Hernandez-Ledesma et al., 2005 and Pihlanto, 2006).

Many studies have shown that peptide antioxidants can be produced by enzymatic hydrolysis of various proteins. Some active peptide antioxidants and peptides that can utilize free radicals are identified in various hydrolysates of proteins such as ovalbumin (Davalos et al. 2004), soybean protein (Chen et al., 1995), milk proteins such as  $\alpha$ -laktalbumin and  $\beta$ -lactoglobulin (Hernandez-Ledesma et al., 2005 and Pihlanto, 2006) etc.

By using different enzymes, peptides with different properties can be obtained from a single source of protein.

One study that aims production of antioxidant peptides (Jean-Yu Hwang et al., 2010) focuses on the effect of various enzyme preparations on the enzymatic hydrolysis of defatted peanut kernels. Hydrolysates obtained by esperase possess higher antioxidant than the ones, produced with neutrase, pepsin, protease A and protease N. Antioxidant activity is measured kinetically, using linoleic acid. The molecular weight of the peptides derived with esperase ranged from 3 to 5 kDa. Antioxidant activity was 3 times higher than that of ascorbic acid.

Enzymatic hydrolysis of soybean proteins with trypsin, chymotrypsin and subsequent ultra filtration and fractionation lead to production of 9 peptides with antioxidant properties. Molecular weight of these peptides is less than 1 kDa and they are abundant in aromatic amino acid residues (Beer-

mann et al. 2009). Peptides from soybean protein, as the authors point out, have antioxidant properties that could be associated with the presence of tyrosine. These results provide new aspects of their application in food, cosmetics and pharmaceuticals.

The aim of this work is a comparative study of the antioxidant activity of peptides derived from proteins of cereals and cottonseeds.

## Materials and Methods

Neutral proteolytic enzyme preparation from *Bacillus amyloliquefaciens* (Neutrase, "Novozymes", Denmark) and acid proteolytic enzyme preparation from fungus – *Aspergillus niger* (Prolive PAC 30L "EnzymeBioProduct" Ltd, Russia) were used. Proteins isolated from high-grade wheat flour and defatted cottonseed kernels were used as substrate. Substrates were water-soluble, salt-soluble (10% NaCl), alcohol-soluble (80% ethanol) and alkaline soluble (0.2% NaOH). Horseradish peroxidase was isolated in laboratory conditions, according to previously described protocol (Ermarkova, 1972).

### *Enzymatic hydrolysis of proteins*

0.5-1% solution of the corresponding protein in 0.1M universal buffer, pH 4.2 (in the case of acid protease) and pH 7.0 (in the case of "neutrase") were prepared and a 0.1% protease solution was added. The mixture was stirred and kept for some time in a thermostat at 30°C, then 2 ml TCA (trichloroacetic acid) was added to 2 ml of the sample in order to stop enzymatic reaction. Then the settled solution was passed through a paper filter and to 1 ml of filtrate 5 ml of 0.5M solution of sodium carbonate was added. While stirring, 1 ml of working solution of Folin was added. The intensity of the blue coloring was measured with a photoelectric colorimeter at 670 nm against the control sample in a 10 mm cuvette (GOST 20264.2-85, 1985). The content of hydrolysis products (*P*) was determined by a calibration curve, obtained using tyrosine.

### **Sample preparation**

5 ml fractions of the reaction mixture were collected at 0, 1<sup>st</sup>, 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup>, and 8<sup>th</sup> hour. Sample fractions were heated in a water bath for 5-10 min to inactivate the enzyme and passed through a paper filter. Their inhibitory and activating effects on peroxidase were studied.

The reducing power of peptides and the effect of peptides in the samples on the rate of oxidation of (+)-catechin were determined in model system, as well.

### **Determination of peroxidase activity**

Peroxidase activity was determined by measuring the rate of oxidation of benzidine with horseradish peroxidase in the presence of hydrogen peroxide (Ermakova, 1972). 2 ml of benzidine (5 mM), 2 ml enzyme solution, 2 ml of water (quantity of the products of protein hydrolysis added in the water – 0.5 ml) and 2 ml of 3% hydrogen peroxide were prepared in 0.1N acetate buffer. The rate of oxidation of benzidine was measured in a 20 mm cuvette at 670 nm.

### **Reducing power assay**

The reducing power was estimated according to the method described by Hwang et al. (2009). The hydrolysate aliquot (0.5 ml) was mixed with 0.5 ml 0.2 M phosphate buffer (pH 6.6) and 0.5 ml 1.0 % potassium ferricyanide and then incubated in a water bath at 50°C for 20 min. After cooling down, 0.5 ml of 10% trichloroacetic acid was added to the mixture to stop the reaction, and then the mixture was centrifuged at 1500 g for 10 min. The supernatant (1 ml) was mixed with 1 ml distilled water and 0.2 ml 0.1% FeCl<sub>3</sub>, and the absorbance was measured at 670 nm after setting in the dark at room temperature for 10 min.

### **Incubation Conditions**

(+)-Catechin (4 mM) was used as an internal standard for quantification. It was dissolved in acetate buffer (0.1 M, pH 4.2) containing ethanol (20%, v). The rather high ethanol concentration was chosen in order to avoid microbial develop-

ment during storage of the solutions. Ferrous chloride was added to give final iron concentrations of 10 mg/L. The content of hydrolysis products of protein in 10 ml incubation medium was 0.2 ml (Oszmianski et al., 1996).

### **Browning Measurements**

Browning of the solutions (40°C) was estimated by measuring the increase in absorbance in a 10 mm cuvette at 440 nm using a KFK-2-YXL 4.2 photoelectric colorimeter (Russia).

## **Results and Discussion**

### **Enzymatic hydrolysis of various plant proteins with acid and neutral proteases**

Proteins, derived from wheat flour and cottonseed undergo hydrolysis at different rates, depending on the used enzymes. Figure 1 shows the hydrolysis of various wheat proteins with acid and neutral protease. The presented data shows that, the rate of proteolysis depends on the source of protein and the used enzyme. Alkaline-soluble proteins have the highest velocity of enzymatic hydrolysis when an acid protease is used (Figure 1a and 1b, curve 4). Formation of hydrolysis products of alkaline-soluble proteins was 1.6 μmol/ml for 6- hour hydrolysis when an acid protease was used, and 1 μmol/ml when neutral protease was used. Enzymatic hydrolysis of albumin, globulin and prolamins, has much lower hydrolysis rate than the one of alkaline-soluble proteins. In all cases, acid proteases have high activity when used with different wheat proteins.

Enzymatic hydrolysis of water-soluble proteins has the lowest rates. Rate of formation of hydrolysis products, when using acid and neutral protease was 0.4 and 0.2 μmol/ml, respectively.

Similar experiments were performed with proteins extracted from defatted cottonseed kernels (Figure 2). The presented data shows that the initial rate of hydrolysis of albumin and globulin with acid proteases follow the same pattern. In the case

of globulin, increasing hydrolysis time leads to formation of higher quantity of hydrolysis products (Figure 1a, b, curves 1). During the initial period (1 h) of the reaction, the accumulation of acid protease hydrolysis products of albumin and globulin was  $0.56 \mu\text{mol/ml}$ . After 8 hours of hydrolysis, the content of the products of hydrolysis in the case of albumin and globulin is  $1.16$  and  $1.4 \mu\text{mol/ml}$ , respectively. In all cases, the rate of formation of hydrolysis products was lower when neutral protease was used (Figure 1a, b, curves 2). Hydrolysis of albumin with neutral protease is carried out at a high speed and the formation of hydrolysis product for 8 hours incubation is  $0.842 \mu\text{mol/ml}$ , and in the case of globulin is  $0.51 \mu\text{mol/ml}$ .

#### *Effects of peptides on antioxidant enzymes*

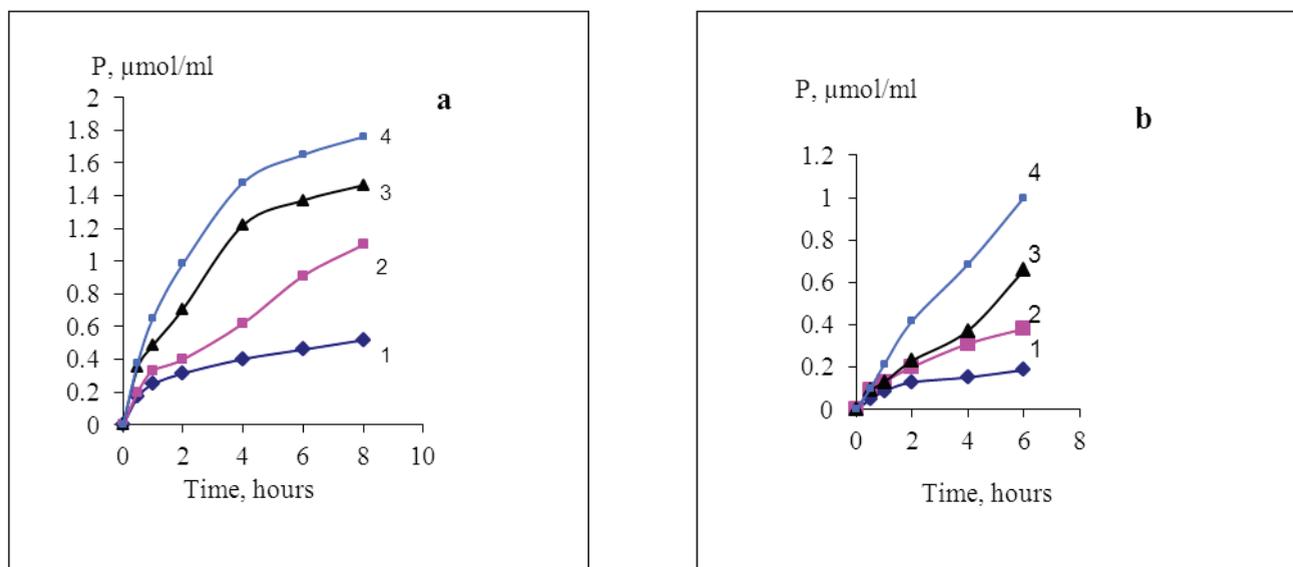
Hydrolysis products of different proteins affect on the activity of antioxidant enzymes in different ways. Figure 3 shows the effect of hydrolysis products of proteins in wheat on activity of peroxidase. Figure 3a shows that peptides obtained through enzymatic hydrolysis of various wheat proteins with acid protease did not significantly affect the activ-

ity of peroxidase. The hydrolysis products of albumin slightly activate peroxidase. Peptides derived from alcohol-soluble proteins and alkaline-soluble almost did not affect the activity of peroxidase.

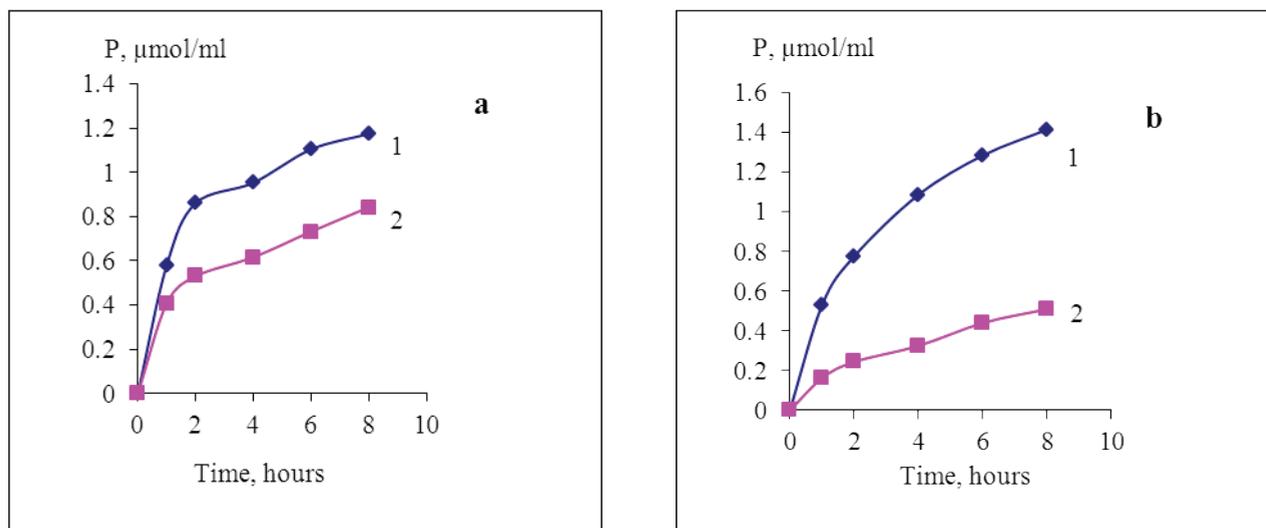
Activating effect of peptides on peroxidase was observed when neutral proteinase was used as proteolytic enzyme (Figure 3b). Studies have shown that the hydrolysis products of albumin increase peroxidase activity. In this case, the degree of hydrolysis of proteins is important. At low degrees of hydrolysis, the activating effect of hydrolysis products of protein was higher. The hydrolysis products of albumin for 1 hour can increase the activity of peroxidase 3.5-4 times (Figure 3b, curve 1). Increasing time of hydrolysis of proteins, i.e., increasing the degree of hydrolysis of proteins leads to decrease in activating effect of peptides.

Peptides obtained by hydrolysis of salt-soluble, alcohol-soluble and alkaline-soluble wheat proteins did not affect the activity of peroxidase.

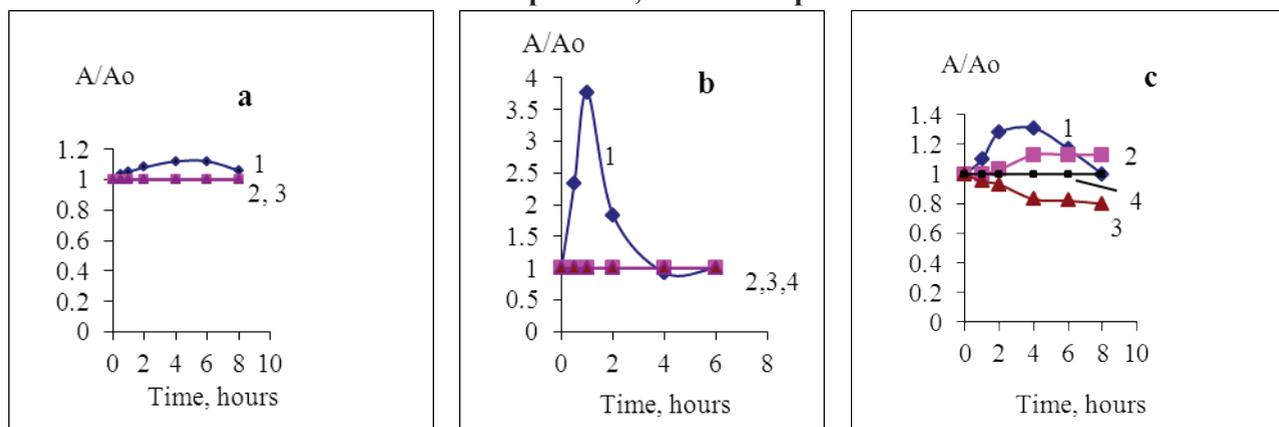
Peptides derived from cottonseed proteins influence peroxidase activity differently (Figure 3c). The hydrolysis products of albumin with acid and neutral protease (Figure 3c, curves 1 and 2)



**Fig. 1. Enzymatic hydrolysis of various proteins of wheat with an acidic (a) and neutral protease (b):**  
 1 – water-soluble proteins, 2 – salt –soluble proteins, 3-alcohol-soluble proteins, 4-alkaline-soluble proteins



**Fig. 2. Enzymatic hydrolysis albumin (a) and globulin (b) of cottonseeds with proteolytic enzymes: 1 - acidic protease, 2 - neutral protease**



**Fig. 3. Influence of degree of hydrolysis of various wheat proteins (a, b) and cottonseed (c), over the activity of horseradish peroxidase: a - peptides derived from water-soluble (1), alcohol-soluble (2) and alkaline-soluble protein of wheat with an acid protease; b - peptides derived from water-soluble (1), alcohol-soluble (2) and alkaline-soluble protein of wheat with a neutral protease; c - peptides derived from albumin with acid (1), neutral protease (2) and from globulin with acid (3) and neutral (4) protease**

slightly increase peroxidase activity. The hydrolysis products obtained from salt-soluble proteins with acid protease have inhibitory properties (Figure 3c, curve 3). Hydrolysis products of globulin with a neutral protease did not affect the activity of peroxidase (Figure 3a, curve 4).

#### **Reducing power of the peptides**

Researchers have shown that peptides have reducing powers. Our studies show that the reducing

power of peptides derived from different proteins differs significantly.

Figure 4 shows the reducing power of various peptides from wheat proteins. It should be noted that the peptides obtained from albumin by hydrolysis with acid protease have a low reducing power (Figure 4, curve 1) compared to peptides derived from alcohol-soluble and alkaline-soluble proteins (Figure 4, curves 2 and 3).

Peptides derived from water-soluble proteins with neutral protease have a high reducing power (Figure 4b, curve 1). The reducing power of peptides derived from alcohol-soluble and alkaline-soluble proteins has intermediate value.

Similar experiments were performed with albumin and globulin of cottonseed. The results are shown in Figure 5. The data presented shows that the hydrolysis products of albumin produced, using neutral protease (Figure 5a, curve 2) have high reducing power, compared to the samples obtained with acid proteases (Figure 5a, curve 1). In the case of globulin, peptides derived with acid protease have high reducing power (Figure 5b, curve 1).

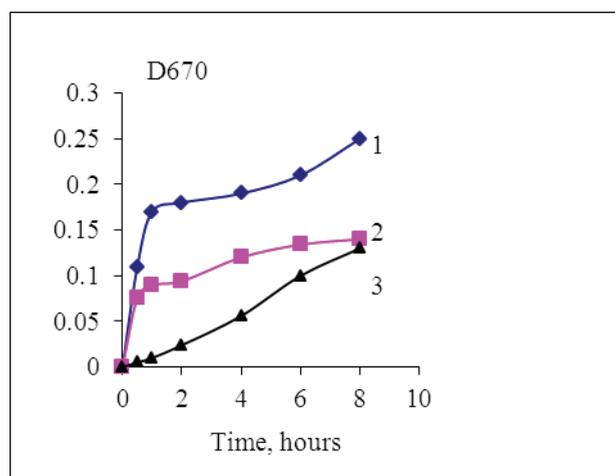
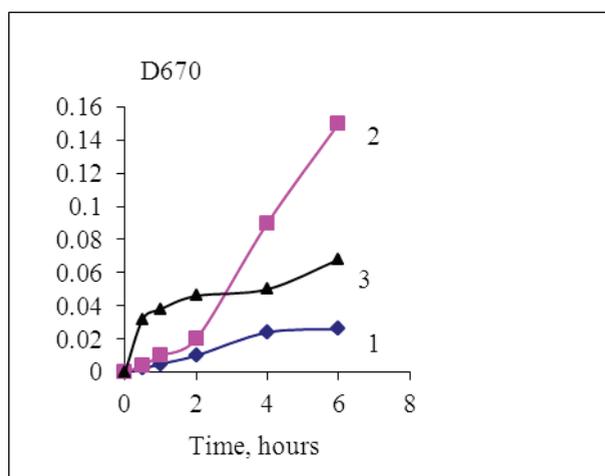
#### ***Effect of peptides on the rate of oxidation of (+)-catechin in model system***

The effect of peptides derived from different wheat proteins with acid and neutral proteases on the rate of oxidation of (+)-catechin is shown in Figure 6. A decrease in the rate of oxidation of (+)-catechin is observed in all samples of peptides. Hydrolysis products of alcohol-soluble proteins and alkaline-soluble proteins with acid and neutral proteases affect the rate of oxidation of (+)-catechin (Figure 6a and b, curves 3, 4) almost identically. In the case of peptides derived from albumin

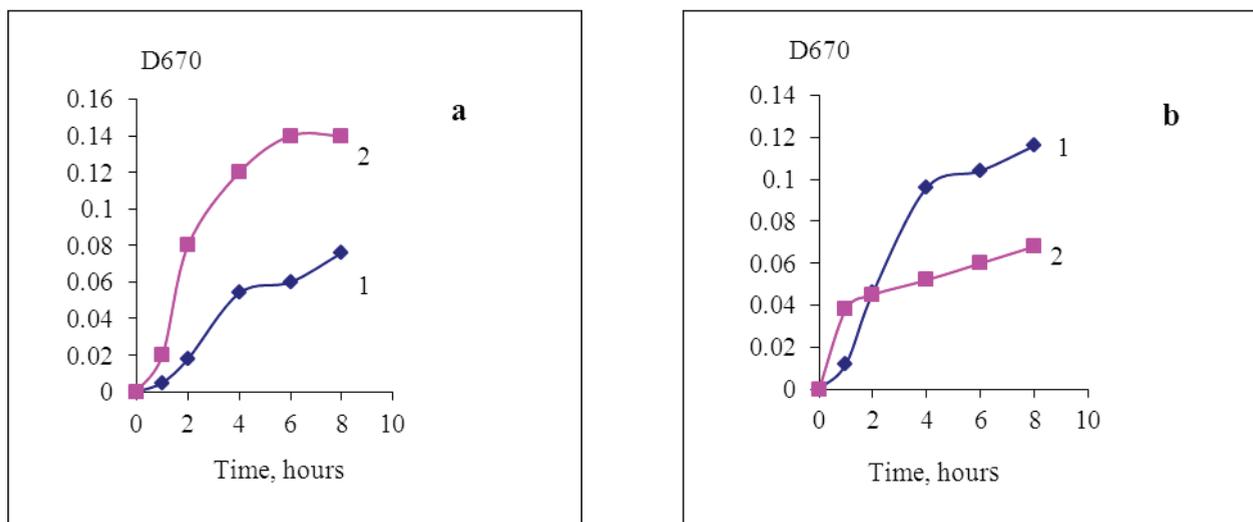
by hydrolysis it can be seen significant difference. The hydrolysis products of albumin, produced using acid proteases, have a lower antioxidant activity and the rate of oxidation of (+)-catechin in model system decreases slightly (Figure 6a, curve 2) compared to control samples. In the case of albumin, the highest antioxidant activity of the peptides is demonstrated, when using neutral protease (Figure 7b, curve 2). The data presented shows that the decrease in the rate of oxidation of (+)-catechin is relatively lower, when compared with the peptides derived from albumin with acid protease.

Similar experiments were performed with the cottonseed proteins (Figure 7). Hydrolysis products of globulin obtained with acid and neutral protease significantly differed in antioxidant activity. Figure 7 presents the dependence of the rate of oxidation of (+)-catechin in the presence of different peptides from cottonseed proteins.

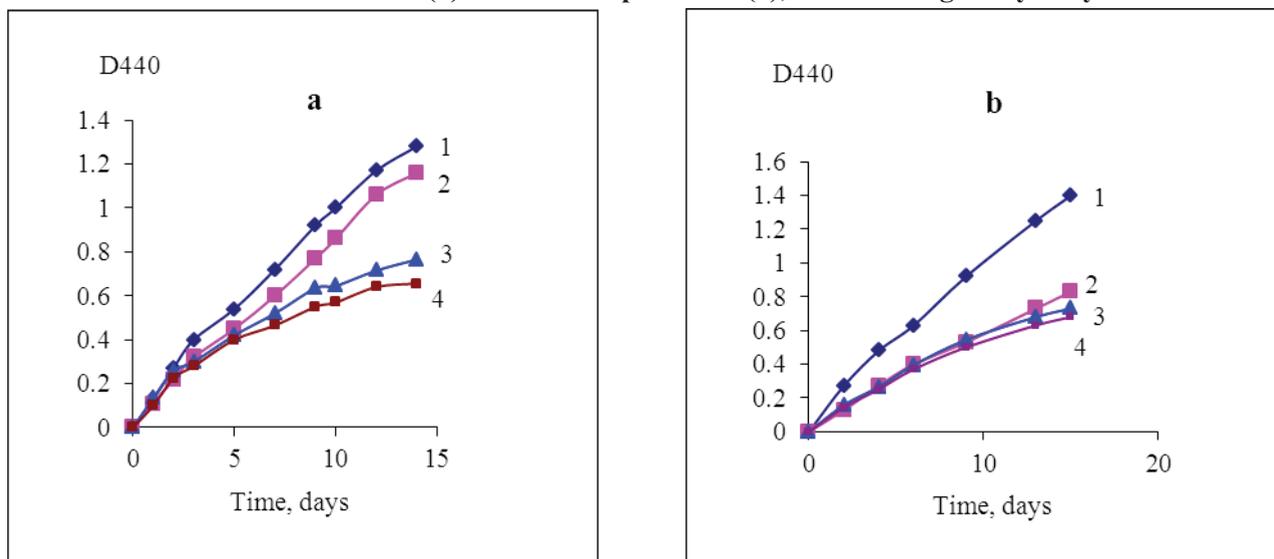
The data presented Figure 7 shows that peptides derived from globulin of cottonseed with different proteolytic enzymes have different antioxidant activity. The change in the rate of darkening of catechin solutions was low when present peptides derived with neutral proteases were used in the media (Figure 7, curve 3). The change in optical density



**Fig. 4.** The increase of reducing power of hydrolysis products of various proteins of wheat with an acidic (a) and neutral protease (b), depending on the degree of hydrolysis: 1-water-soluble protein, 2-alcohol-soluble protein, 3-alkaline-soluble protein



**Fig. 5. Dependence of reducing power of hydrolysis products of albumin (a) and globulin (b) in cottonseed with acid (1) and neutral proteases (2), from the degree hydrolysis**



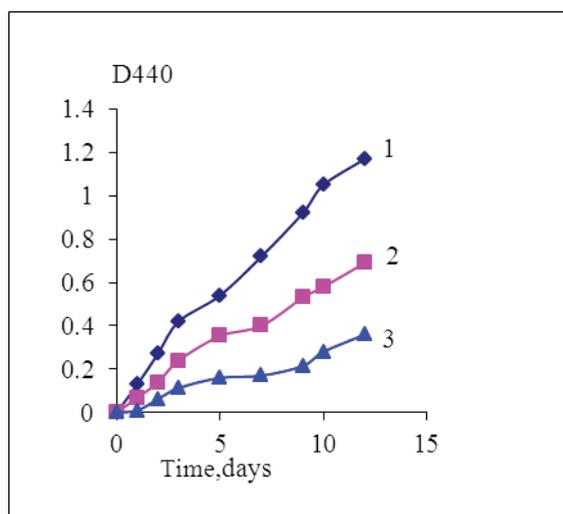
**Fig. 6. Effect of peptides derived from different wheat proteins with acid (a) and neutral protease (b) on the rate of oxidation of (+)-catechin: 1 - control, 2 - in the presence of peptides derived from water-soluble proteins, 3 - alcohol-soluble proteins, 4 – alkaline-soluble proteins**

of solutions of catechin for 12 days of incubation was measured at 440 nm was 1.170 in the control sample, 0.690 in the presence of peptides derived with an acid protease and 0.360 in the presence of peptides derived with a neutral protease.

Thus, the cottonseed proteins have a high antioxidant activity and these proteins could be used as a source of antioxidant peptides.

### Conclusion

Enzymatic hydrolysis of wheat flour and cottonseed proteins with acid and neutral proteases leads to production of peptides with different antioxidant properties. Peptides derived from wheat albumin with acid proteases possess 10-12 times higher reducing power than the peptides derived



**Fig. 7.** Effect of peptides derived from globulin of cottonseeds on the rate of oxidation of (+)-catechin

with neutral proteases. In the case of cottonseed albumin, reducing power of peptides, produced with neutral proteases was 2-3 times higher than the one of peptides derived with the acid proteases. In the case of cottonseed globulin, the reducing power was higher, when the hydrolysis of proteins was carried out with acid proteases.

It was shown that antioxidant activity of peptides depends on protein source and type of proteolytic enzyme. In a model system, peptides derived from wheat albumin with acid protease significantly reduced the rate of oxidation of (+)-catechin. The strongest inhibition effect on the oxidation of (+)-catechin is demonstrated by peptides derived from alcohol-soluble, alkaline-soluble wheat proteins and salt-soluble cottonseed proteins with neutral protease. In all cases, peptides, produced, using acid protease reduced the rate of oxidation of (+)-catechin to a smaller degree.

Peptides do not only have antioxidant activity, but they can also affect the activity of antioxidant enzymes. Depending on the degree of hydrolysis and enzyme that are used, inhibitory and activating effects of the peptides are different. Peptides derived from wheat albumin with neutral protease

increase peroxidase activity. The increase in hydrolysis degree of protein, leads to disappear activating effect of peptides. Activating effect is observed at low degree of hydrolysis of albumin. Peptides derived from salt-soluble, alcohol-soluble and alkaline-soluble wheat proteins do not affect the peroxidase activity.

Peptides derived from cottonseed albumin with acid and neutral protease, increase peroxidase activity, and the peptides derived from globulin, significantly inhibit peroxidase activity.

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