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POSSIBILITY OF APPLYING FERULOYL ESTERASE FROM *ASPERGILLUS NIGER* A.n.8 FOR DEGRADATION OF A CELL WALL COMPLEX IN SELECTED CEREALS

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A preparation of feruloyl esterase was obtained from post culture medium of *Aspergillus niger* A.n.8. 5-day culture. The preparation demonstrated the maximal activity at pH 4.3 and a temperature of 55°C; molecular weight of the investigated enzyme was 30 000 Da. The preparation released ferulic acid from natural cereal arabinoxylanes.

Synergy between the activity of the preparation obtained and xylanase activity was proved. The addition of the feruloyl esterase preparation significantly increased the amount of reducing saccharides, released by xylanase from a substrates containing natural cereal arabinoxylanes.

INTRODUCTION

The utilisation of by-products of plant raw material processing is a serious technological and economical problem in many branches of industry. Total or partial processing of by-products, leading to a further usage of biomass residues, is connected with the necessity of degrading the most resistant structures that form cell walls of plants. Results of researches carried out over the last twenty years have supported the view that the integrity, durability, and resistance to degradation (including enzymatic action) of cell walls are, to the great extent, connected with the presence of phenolic acids in molecules of structural polysaccharides of hemicelluloses. These acids esterify xylane- and arabinoxylane-composing saccharides; moreover, they form interpolysaccharide bonds and cross links between polysaccharides and lignin molecules. The physiological function of phenolic acids, present in the wall structures of primary plant cells, consists in enhancing the mechanical and thermal resistance of cells, inhibiting cell growth, and increasing tissue resistance to biodegradation and invasion of pathogenic microorganisms [Abdel-Aal, 2001; Faulds & Williamson, 1999; Grabber *et al.*, 2000; Ishii, 1997; Tharanathan, 2002; Waldron *et al.*, 1997]. Numerous investigations have shown that the amount of phenolic acids, mainly ferulic and *p*-coumaric acids and, to some extent, of caffeic and sinapic acids, incorporated into cell wall structures, can be considerable. It is particularly significant in plants from the *Gramineae* family where the *contribution* of arabinoxylanes to the cell wall structures amounts to around 45%. For example, up to 1000–18 000 μg of ferulic acid and 500–650 μg of coumaric acid have been detected in 1 gram of plant cereals dry mass [Renger & Steinhart, 2000; Sun *et al.*, 2001]. Significant amounts of ferulic acid have been found in sugar beets where this acid esterifies pectin-incorporated saccharides. Detailed investigations have shown that beet pulp contains even up to 8 mg of ferulic acid per 1 g dry mass [Bonnin *et al.*, 2001]. Ferulic acid has been detected in hardwood [Sun *et al.*, 2001] and, although to a lesser degree, in *Gymnospermae* plants [Carnachan & Harris, 2000].

Investigations have indicated that a complete degradation of cell wall structures co-forming xylanes and arabinoxylanes, besides activities for hydrolysis of glycoside bonds, requires the activity of appropriate esterases: acetylxylane esterase and feruloyl esterase which, in respect of their function, are referred to as xylanolysis accessory enzymes. Overview of references confirms that feruloyl esterases release ferulic acid from polysaccharides esterified by phenolic acid, contained in plant materials, including cereal grains [Bonnin *et al.*, 2001], various grass straws [Bartolome *et al.*, 1997; Bonnin *et al.*, 2001; Borneman *et al.*, 1990; McCrae *et al.*, 1994], by-products of the cereal industry [Bonnin *et al.*, 2001; Donaghy *et al.*, 2000; Dzedzyulya *et al.*, 1999; Fauld & Williamson, 1995; Garcia-Conesa *et al.*, 1999; Yu *et al.*, 2002], and sugar beet pulp [Kroon & Williamson., 1996; de Vries *et al.*, 1997; Dzedzyulya *et al.*, 1999]. It was found that enzymatic removal of phenolic acids facilitates the enzymatic degradation of structural polysaccharides and improves the digestibility of many fodders [Williamson *et al.*, 1998; Yu *et al.*, 2002].

The aim of the presented research work was to investigate the possibility of applying feruloyl esterase from *Aspergillus niger* A.n.8 for the improvement of xylanase activity. In this work, the conditions of obtaining a preparation of the mentioned esterase and its preliminary purification for characterization purposes are described. The activity of preparation of feruloyl esterase from *A. niger* A.n.8 towards natural substrates was investigated, from the point of view of its participation in cytolysis processes.

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MATERIALS AND METHODS

Cell strain and culture conditions. The source of feruloyl esterase was post culture medium from culture of strain *Aspergillus niger* A.n.8., deposited in the Collection of the Industrial Microorganisms at the Institute of Agricultural and Food Biotechnology in Warsaw. Cultures were carried out on a rotary shaker at a temperature of 37°C for 5 days in the liquid medium containing in 1 L: KH_2PO_4 – 2 g; $(NH_4)_2SO_4$ – 1.4 g; $CO(NH_2)_2$ – 0.3 g; $MgSO_4$ x $7H_2O$ – 0.3 g; $CaCl_2$ – 0.3 g; Tween 80 – 0.1 mL; and wheat bran – 30 g.

Initial purification of feruloyl esterase prepration. Post culture liquid medium, after separation from mycelium and nutrient solid remnants, was filtered through MNWL 10000 membrane. The ultra-concentrate obtained was analysed using hydrophobic interactions chromatography (HIC); the fraction with esterase activity was dialysed against water and lyophilised.

HIC was performed on Phenyl Sepharose CL 4B column (0.9 cm x 15 cm) washed with 1 mol/L $(NH_4)_2SO_4$ in 0.05 mol/L phosphate buffer at pH 7. The column was eluted at a liquid flow of 1 mL/min subsequently with: 1 mol/L $(NH_4)_2SO_4$ in 0.05 mol/L phosphate buffer at pH 7, in eluent gradient 1 mol/L – 0 mol/L $(NH_4)_2SO_4$ in 50 mL of the same buffer, in buffer and in water. Fractions active towards *p*-nitrophenol acetate (*p*-NPA) were collected.

Determination of molecular weight of feruloyl esterase from *A. niger* A.n.8

Gel filtration. Filtration was carried out on a Sephacryl S-100 HR column (0.9 x 60 cm) and elution with 0.1 mol/L NaCl solution in 0.1 mol/L Tris-CH_3COOH buffer at a flow rate of 1 mL/min. The following protein standards were used for column calibration: ribonuclease, chymotrypsinogen A, ovalbumin, and bovine serum albumin.

Investigation of the influence of pH and medium temperature on feruloyl esterase formulation activity. The influence of pH was investigated in 0.05 mol/L citrate buffers in the range of pH 3.0–6.5; influence of temperature in 0.05 mol/L citrate buffer pH 5.0, respectively.

Obtaining cell wall preparations from rye and malt. Meal extracted from finely ground rye grain or malt was boiled for 1 h in 80% ethanol under reflux condenser. The product was air dried, subsequently ground and washed in water at a temperature 50°C, followed by four times washing in hot water (65°C, 30 min), at 10 g product: 160 mL water ratio. Next, the product was washed in ethanol and ether, dried at room temperature and ground.

Ferulic acid release by feruloyl esterase preparation and the influence of this preparation on the release of reducing saccharides from natural substrates. The following substrates were used for the investigation of ferulic acid release and feruloyl esterase and xylanase synergism: a preparation of cell walls from rye and malt, wheat bran, xylan from oat spelts (Sigma). A 5% suspension of the substrates in 0.05 mol/L citrate buffer (pH 5.0) with feruloyl esterase preparation from *A. niger* A.n.8 FE-4 (0.34 U/mL), with xylanase preparation (1.4 β-xylanxylanhydrolase E.C.3.2.1.8. from *Trichoderma viride*, Sigma, containing: <0.2 cellulase, < 0.02 β-glucosidase, and < 0.002 % β-xylosidase) 0.3 XU/mL, or with both the enzymes, was incubated at a temperature of 40°C with gentle mixing. Control samples were samples of substrates incubated under the same conditions without the addition of enzymes. After 16-h incubation the samples selected for the determination of ferulic acid were mixed with ethanol at 1:1 ratio and cooled down; the samples selected for the determination of saccharides were thermally inactivated in boiling water for 5 min. The samples were centrifuged before determinations. Ferulic acid was quantified using HPLC technique, saccharides were determined chromatographically and by the standard Nelson-Samogyi method, applying xylose solution as a standard. Saccharide chromatography was performed at a temperature of 80°C using a Shodex KS-802 column equipped with guard precolumn and refractometric detector. The column was eluted at a flow rate of 1 mL/min with reverse osmosis and redestillation water of 1 μS cm^{-1} conductivity.

Determination of enzyme activity. The activity against methyl ferulate and methyl coumarate was determined using 5 mmol/L solution of these esters in reaction mixture. Both esters were synthesised according to the previously described procedure [Borneman *et al.*, 1990]. Reaction mixture contained 0.9 mL of substrate solution and 0.1 mL of appropriately diluted enzyme solution. Substrate solution was prepared by mixing ester diluted in methanol and 0.05 mol/L citrate buffer (pH 5.0) to get the final methanol concentration of 4% in the reaction mixture. Incubation was conducted at a temperature of 40°C for 20 min. Enzymatic reaction was subsequently stopped by mixing samples with methanol at 1:1 (v/v) ratio and cooling down. The released ferulic acid and coumaric acid, respectively, were determined by high performance liquid chromatography HPLC, using a Bondapak C_{18} column (2 x 300 mm) with guard precolumn and UV detector. Samples of 10 μL volume were injected into the column equilibrated with methanol: 0.1% orthophosphoric acid (45:55) solution. Elution was conducted at a flow rate of 0.5 mL/min, using stepwise increasing methanol in 0.1% orthophosphoric acid gradient 45% to 65% for 10 min and 65% to 100% for 1 min. Detection was performed at a wavelength of λ=280 nm. Ferulic acid and coumaric acid contents were calculated using a respective internal standard and applying Millenium 32 software. International unit (U) was accepted as the measure of feruloyl esterase activity, corresponding to the amount of enzyme which releases 1 μmol of ferulic acid in 1 minute under reaction conditions.

The activity against *p*-nitrophenol acetate (*p*NPA) was determined using 5 mmol/L substrate concentration. Reaction mixture consisted of 1.4 mL of substrate and 0.1 mL of appropriately diluted enzyme solution. Substrate solution was prepared immediately before use by mixing *p*NPA solved in isopropanol and 0.05 mol/L citrate buffer pH (5.0) to get final isopropanol concentration of 8% in the reaction mixture. Incubation was carried out at a temperature of 40°C for 15 min. The released *p*-nitrophenol was determined spectrophotometrically at a wavelength of λ=400 nm after mixing the samples with 1.5 mL of

0.5 mol/L phosphate buffer at pH 7.1. The activity unit (AU) corresponded to the release of 1 μmol of *p*-nitrophenol in 1 min under the described conditions.

Xylanase activity was evaluated by the determination of reducing saccharides, released from 1% xylane from wheat oat (Sigma) suspension in 0.05 mol/L citrate buffer (pH 5.0), using standard method of Nelson-Samogyi [Ghose & Bisaria, 1987].

Protein was determined using the Bradford's method [1976].

RESULTS AND DISCUSSION

Preparation and preliminary characteristics of feruloyl esterase preparation

After initial purification, using ultrafiltration and hydrophobic interaction chromatography, and after dialysis and lyophilisation, the feruloyl esterase preparation was obtained from *A. niger* A.n.8. post culture medium, containing 40% of protein and demonstrating feruloyl esterase activities. The preparation was able to hydrolyse: methyl ferulate, with a good activity, and methyl coumarate, although with considerably less activity (Table 1). Results of experiments described in literature have proved that feruloyl esterases release also acetic acid, bounded with xylanes by ester bonds [Faulds & Williamson, 1993; Tenkanen, 1998]. In the present investigations, the preparation from *A. niger* A.n.8. was found to effectively hydrolyse *p*-nitrophenol acetate (*p*NPA), an artificial substrate suitable for investigations of esterases. The preparation demonstrated a high activity, more than 85% of the maximal one, in the pH range of 4.0–5.8 with the maximum at pH 4.3. It was stable at a temperature of 40°C and lost half of its activity at a temperature of 60°C after 18 min. *Ca*. 90% of enzyme activity was lost at a temperature of 70°C after 10 min of incubation. The molecular weight of an active fraction of the investigated preparation, determined using gel filtration, was 30 000 Da.

TABLE 1. Characteristics of feruloyl esterase preparation from *A. niger* A.n.8.

Activity		(U/mg protein)	K_m (mmol)
Substrate:	Methyl ferulate	12.01	0.85
	Methyl coumarate	1.42	1.70
	p-Nitrophenol acetate	52.2	3.33
pH optimum		4.3	
Temperature optimum		55°C	
Molecular weight of active fraction:			
Gel filtration	Sephacryl S-100	30 000 Da	

Activity of feruloyl esterase formulation from *A. niger* A.n.8. towards natural substrates

The arabinoxylane-rich preparations of cereal origin, including preparations of rye and malt cell walls and wheat bran, were selected for the investigation of the activity of feruloyl esterase preparations from *A. niger* A.n.8. towards natural substrates. The activity of the investigated enzymatic preparation towards xylane was also tested. The composition of saccharides present in hydrolysates of selected substrates pointed out to the significant content of arabinoxylanes. All hydrolysates contained also glucose (Table 2).

TABLE 2. Content of selected saccharides in hydrolysates of substrates used in the study.

Substrate	Saccharides (mg/g)		
	Xylose	Arabinose	Glucose
Cell wall preparation from rye	125	60	810
Cell wall preparation from malt	160	30	400
Wheat bran	51	26	207
Xylan from oat spelts	641	116	77

Many references have provided information that plant cereals contain significant amounts of ferulic acid in their tissues. Even 1170 μg of ferulic acid [Andreasen *et al.*, 2000] in rye grains and up to 2443 μg [Lampereur *et al.*, 1997] in wheat, per 1 g dry mass, have been detected. Respectively, in 1 g dry mass of barley up to 600–624 μg and 260 μg *p*-coumaric acid have been found [Hernanz *et al.*, 2001; Zupfer *et al.*, 1998]. As mentioned in the Introduction, ferulic acid forms structures particularly resistant to enzymatic degradation. Regarding this, the content of this compound in the cereal industry waste products is as a rule even higher. The content of ferulic acid has been determined to be equal to 31 mg/g dry mass in maize bran [Bonnin *et al.*, 2001], 4 mg/g dry mass in oat hulls [Yu *et al.*, 2002], whereas in spent grains the content of the acid discussed has been about fivefold higher than in the unprocessed substrate [Hernanz *et al.*, 2001].

The investigations carried out demonstrated that feruloyl esterase from *A. niger* A.n.8. releases ferulic acid from preparations of rye and malt cell walls and, although to the much less extent, from wheat bran. The release of ferulic acid from oat xylane was not confirmed (Figure 1). The presence of xylanolytic activity in the medium was necessary for revealing the activity of feruloyl esterase. An analogous regularity was signalized in the descriptions of other feruloyl esterases of microbiological origin. Previously investigated esterases demonstrated the highest affinity to estrified arabino-xylooligomers and their activity was evidently dependent on the detachment of side branches of xylane chain by endoxylanase [Fillingham *et al.*, 1999; Ralet *et al.*, 1994; de Vries *et al.*, 2000]. The release of ferulic acid from arabinoxylanes in the medium where the xylanolytic activity was not displayed, was not detected or was at a trace level [Bartolome *et al.*, 1997; Faulds & Williamson, 1995; Sancho *et al.*, 2002; de Vries *et al.*, 2000].

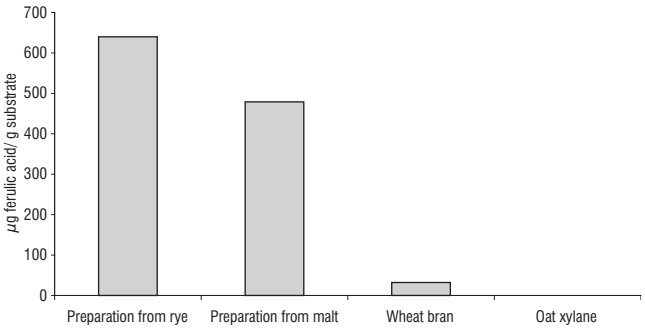

FIGURE 1. Amount of ferulic acid released from 1 g of different substrates by feruloyl esterase from *A. niger* A.n.8. during 16 h of incubation at 40°C in 0.05 mol/L citrate buffer (pH 5) medium.

Synergy between feruloyl esterase activity and activity of selected cytolytic enzymes

Our investigations showed that the introduction of the preparation of feruloyl esterase from *A. niger* A.n.8., besides xylanase, to the suspension of natural arabinoxylanes significantly increases the effectiveness of reducing saccharides release (Figure 2). Such effect was also observed for xylane suspension from oat spelts where the release of ferulic acid by the investigated enzymatic preparation was not registered previously (Figure 1). It can be supposed that a part of phenolic acids, bound to oat spelts xylane, could be removed in the course of preparation of xylane fraction from this material. Nevertheless, the action of xylanase was significantly improved as a result of the removal of a small (not exceeding the detection threshold of the method applied in the above described investigation) residue of ferulic acid by feruloyl esterase preparation.

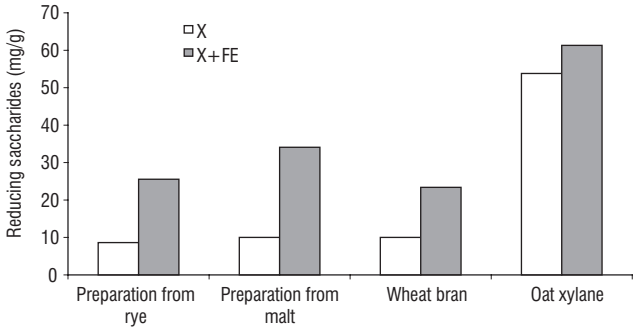

FIGURE 2. Release of reducing saccharides from 1 g of cereal substrates by xylanase (X) or xylanase and feruloyl esterase from *A. niger* A.n.8. (X+FE) during 16 h of incubation at 40°C in 0.05 mol/L citrate buffer (pH 5) medium.

It was demonstrated, using the HPLC technique, that the introduction of feruloyl esterase preparation to the reaction mixture significantly increases the release of free pentoses from arabinoxylane-containing substrates. The amount of xylose and arabinose released by xylanase significantly increased after the addition of feruloyl esterase preparation to preparations of rye cell walls, wheat bran and oat xylane. Besides pentoses, the investigated preparation from *A. niger* A.n.8. released glucose from all applied substrates (Figure 3). Minor amounts of glucose were released even from oat xylane where the content of this saccharide did not exceed 10% (Table 2).

The synergy between feruloyl esterases and xylanases was already mentioned in publications with the experimental data on cereals or cereal product substrates [Faulds *et al.*, 2002; Fillingham *et al.*, 1999; Garcia *et al.*, 1998; de Vries *et al.*, 2000; Yu *et al.*, 2003]. The synergy between feruloyl esterase and α-glucuronidase activity in degradation of beet pectins [de Vries *et al.*, 2000] and between feruloyl esterase and cellulase in the release of reducing saccharides from oat hulls was confirmed. This last observation formed grounds for the statement that the removal of ferulic acid from polysaccharide complex facilitates the action of cellulolytic enzymes [Yu *et al.*, 2003]. In other investigations, it was demonstrated that feruloyl esterases catalyze dissolution of β-glucans and pentosanes from barley endosperm [Moore *et al.*, 1996], whereas the release of ferulic acid from barley spent grains is significantly increased by the presence of

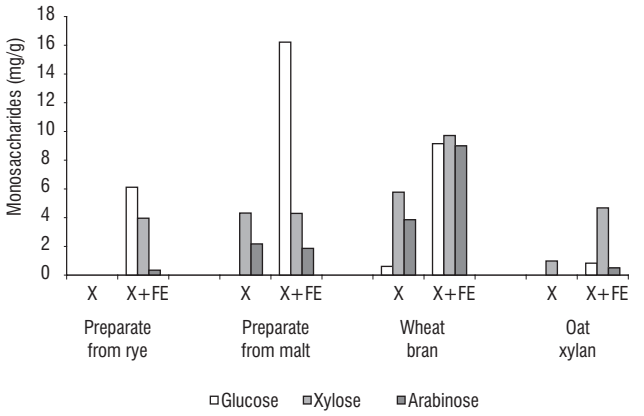

FIGURE 3. Monosaccharides released from 1 g of cereal substrates by xylanase (X) or xylanase and feruloyl esterase from *A. niger* A.n.8. (X+FE) during 16 h of incubation at 40°C in 0.05 mol/L citrate buffer (pH 5.0) medium.

β-glucanase in the medium [Faulds *et al.*, 2002]. The cited relationships support the supposition that ferulic acid is bound not only to arabinoxylanes but to other polysaccharides as well. The release of glucose from preparations of cereal origin by feruloyl esterase preparation, observed in our investigations, points to the importance of the bonds created by ferulic acid for the stability of structures which incorporate glucose. The results obtained confirm the model of construction of cereal cell wall [Bamfort & Kanauchi, 2001] in which the external layer of cell wall is built of xylose, arabinose and ferulic acid complex.

CONCLUSIONS

1. The preparation of feruloyl esterase was obtained from post culture medium of *A. niger* A.n.8. It demonstrated a high activity in the pH range of 4.0–5.8 with the maximum at 4.3. The optimal temperature for the obtained formulation was 55°C. The molecular weight of the investigated enzyme amounted to *ca*. 30 000 Da.

2. The investigated enzymatic preparation demonstrated activity towards substrates containing natural arabinoxylanes. It released free ferulic acid, in xylanase-containing medium, from selected substrates of cereal origin.

3. Synergy between the activity of feruloyl esterase preparation from *A. niger* A.n.8. and xylanase activity was confirmed. The addition of the described enzyme increased the effectiveness of release of reducing saccharides by xylanase from natural arabinoxylane-containing substrates. In the presence of feruloyl esterase preparation from *A. niger* A.n.8. xylanase released more free pentoses from natural arabinoxylanes.

4. Synergy between xylanase activity and activity of feruloyl esterase offers the possibility of using a preparation from *A. niger* A.n.8. to facilitate degradation of natural structural polysaccharides, containing incorporated phenolic acids.

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MOŻLIWOŚCI WYKORZYSTANIA ESTERAZY FERULANOWEJ Z *ASPERGILLUS NIGER* A.n.8 DO DEGRADACJI KOMPLEKSU ŚCIAN KOMÓRKOWYCH WYBRANYCH ZBÓŻ

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Preparat esterazy ferulanowej otrzymano z cieczy pohodowlanej 5 dobowej hodowli *Aspergillus niger* A.n.8. Preparat wykazywał maksymalną aktywność w pH 4,3 i w temperaturze 55°C; masa cząsteczkowa badanego enzymu wynosiła 30 000 Da. Z naturalnych arabanoksylanów zbożowych preparat uwalniał kwas ferulowy.

Wykazano współdziałanie otrzymanego preparatu z ksylanazą. Dodatek preparatu esterazy ferulanowej zwiększa istotnie ilość uwalnianych przez ksylanazę cukrów redukujących z substratów zawierających naturalne arabinoksylany zbożowe.