

**Monika Prendecka¹, Jerzy Rogalski², Anna Olszewska¹,
Katarzyna Skórzyńska-Dziduszo¹, Krystyna Lupa-Zatwarnicka¹**

¹Human Physiology Department, Medical University of Lublin

²Department of Biochemistry, Maria Curie-Skłodowska University

**A potential dietary supplement - properties of purified,
extracellular b-glucosidase isolated from fungus
phlebia radiata – preliminary study
Potencjalny suplement diety - właściwości oczyszczonej,
zewnętrzkomórkowej b-glukozydazy
z grzyba phlebia radiata – badania wstępne**

Summary

The civilization of the twentieth and twenty-first century brought a real revolution in nutrition and food preparation. Highly industrialized communities opted for food production efficiency, low cost and ease of preparing meals because of, that is what the busy and fast living modern man expect. As a result we eat highly performed, nearly sterile, devoid of nutritional value meal products instead of variety of healthy foods, such as fruits, vegetables, lean meats and fish, not be getting all the micronutrients that body needs.

In this context dietary supplement is a vitamin, mineral, or herb that one takes to improve health or wellness. It is clear that, in such a case, these supplements are generally not intended to cure or treat diseases or medical conditions, but only help to keep the person in good condition or to improve it to some extent. Vitamins, minerals and other biological active substances, also called micronutrients, nourish the body and help to keep it healthy. Therefore, it is obvious that one can get most of micronutrients by eating a variety of foods in a daily diet. Unfortunately, it is quite difficult to achieve, because of the low availability of unprocessed, organic foods. Consequently, the next step of modern man seems to be searching for a substances that can improve the quality of the food we eat or to improve valuable nutrients bioavailability. One of the components that could in the future be used as a biologically active food ingredient is enzyme - b-1,4-glucosidase. In this context, particular attention should be paid fungal glucosidase, because of their broad substrate specificity, very high stability and optimum activity over a wide range of pH and temperature.

The purpose of this work was isolation, purification and characterization fungal b-glucosidase from culture filtrates of *Phlebia radiata* in preliminary study to determine the possibility of using this type of preparation as a dietary supplement in the future.

Phlebia radiata was found to secrete b-glucosidase when it was grown in liquid culture with wheat bran as the carbon source. Highly purified b-glucosidase (BGS) was obtained by means of the column chromatography on DEAE-Sephadex, and chromatofocussing on Polybuffer Exchanger PBE-94. It was found that b-glucosidase (BGS) was optimally active at 50°C and pH 5,0.

Key words: Nutrition, supplementation,, b-glucosidase, substrates, deglycosylation, purification, biodegradation

Streszczenie

Cywilizacja dwudziestego i dwudziestego pierwszego wieku przyniosła prawdziwą rewolucję w żywieniu człowieka i przetwarzaniu produktów żywnościowych. Społeczeństwa wysoko uprzemysłowione naciskały na efektywną produkcję żywności, niskie koszty i uzyskanie łatwych w przygotowaniu półproduktów żywnościowych, ponieważ to jest oczekiwaniem współczesnego, zapracowanego i szybko żyjącego człowieka. W efekcie zamiast zróżnicowanego, zdrowego jedzenia takiego jak owoce, warzywa, chude mięso i ryby spożywamy wysoko przetworzone, niemal jałowe, pozbawione wartości odżywczych pokarmy, przez co nie przyswajamy niezbędnych składników pokarmowych.

Dlatego też, w tym kontekście, suplementami diety będą witaminy, minerały, zioła, które przyjmujemy w celu polepszenia naszego zdrowia i samopoczucia. Jasne jest zatem, suplementy nie są substancjami przeznaczonymi do leczenia konkretnych schorzeń ale w celu utrzymania dobrego stanu zdrowia lub jego polepszenia ale jedynie w ograniczonym zakresie. Witaminy, minerały i inne substancje biologicznie czynne odżywiają ciało człowieka i utrzymują je w dobrym zdrowiu. Stąd, oczywistym jest, że powinniśmy przyjmować jak najwięcej tych cennych składników w naszej codziennej diecie. Niestety jest to dość trudne do osiągnięcia z uwagi na niską dostępność do nieprzetworzonych, naturalnych produktów żywnościowych. Zatem kolejnym krokiem współczesnego człowieka powinno stać się poszukiwanie substancji, które mogą poprawić jakość żywności, przez nas spożywanej, lub mogących polepszyć biodostępność cennych składników pokarmowych. Jednym z takich czynników, które mogłyby w przyszłości być użyte jako biologicznie czynne składniki pokarmowe jest enzym - b-1,4-glukozydaza. Szczególna uwaga należy się tutaj grzybowym glukozydazom, z racji ich szerokiej specyficzności substratowej, wysokiego stopnia stabilności oraz szerokiemu zakresowi działania pH i temperatury.

Celem niniejszej pracy były badania wstępne, izolacja, oczyszczanie i charakterystyka grzybowej b-glukozydazy z płynów pohodowlanych *Phlebia radiata* pod kątem oceny jej przyszłego zastosowania jako suplementu diety.

Stwierdzono, że *Phlebia radiata* wydziela b-glukozydazę podczas wzrostu na podłożu płynnym zawierającym otręby pszenne jako źródło węgla. Wysoki stopień oczyszczenia b-glukozydazy (BGS) został osiągnięty dzięki zastosowaniu chromatografii kolumnowej na DEAE-Sefarozie i chromatooogniskowaniu z użyciem PBE-94. Otrzymano enzym, którego optimum działania znajdowało się w temperaturze 50°C i pH 5,0.

Słowa kluczowe: Żywienie, suplementacja, b-glukozydaza, substraty, deglikozylacja, oczyszczanie, biodegradacja

Introduction

b-Glucosidases are heterogenous group of enzymes, widely distributed in nature, from microorganisms to vertebrates, which hydrolyze O-glycosidic bonds at the terminal, nonreducing end of complex carbohydrates with retention of anomeric configuration. The retaining mechanism involves acidic catalysis that protonates the substrate and leads to formation of the transition state oxocarbenium ion.

b-Glucosidases can be divided into three groups with respect to their ability to hydrolyze different substrates: enzymes that exhibit high specificity towards aryl-b-D-glucosides, other b-glucosidases preferentially hydrolyse cellobiose and cellooligosaccharides and are also called cellobiases and with broad-specificity, cleaving both types of substrates to the same extent (Decker et al. 2000).

β -Glucosidases have potentially board application. The use of β -glucosidase in the wine industry seems to be very interesting. Moreover β -glucosidase can find application in process of bioconversion of phenolic compounds from cranberries pomace (Zheng, Shetty, 2000). Free fenolics and phenolic acids can be used as natural food ingradients as antioxidants (Bocco et al. 1998), nutraceuticals (Shetty, 1997). Cranberry pomace contains many phenolic compounds which occur in bound forms of fenolic acids conjugated with glucose or other sugars. And this is why enzymatic hydrolysis of phenolic glycosides by for example β -glucosidase appears to be an attractive mean of increasing the free fenolic acids in wine or fruit juice to enrich taste, aroma and potentially increasing nutraceutical value (Shetty, 1997).

Increase in β -glucosidase activity could potentially be regarded as beneficial due to hydrolysis products of plant glucosides, some of which have more antimutagenic, antioxidative, anticarcinogenic and immune stimulatory properties than the respective glucosides. Therefore, its relevance for animal and human health will also depend upon the nature of dietary plant glucosides (Hijová et al. 2012).

The activity of these enzymes with toxicological importance could be altered by the diet, ultimately potentially decreasing the risk of carcinogenesis (Nalini et al. 2004; Manju et al. 2006).

Another possible direction of β -glucosidase use in food supplementation seems to be bioconversion of isoflavones. Isoflavones are non-steroidal phytoestrogenic and antioxidative polyphenolic molecules with the potential to protect against hormone-dependent diseases due to their partial agonist and antagonist estrogen effects (Kano et al. 2006). Izumi et al. (2000) demonstrated that aglycones are absorbed faster and in higher amount than glucosides in human. However, approximately 80–95% of isoflavones in raw and unfermented soybean exist as glucosides conjugates that are less bioactive and non-bioavailable. Interestingly, past studies have demonstrated that β -glucosidase-producing probiotics could biotransform the isoflavone glucosides to biologically potent aglycones (Tsangalis et al. 2002). Past epidemiological studies have shown that soy isoflavones could impart hormone associated therapeutic effects. However, most soy isoflavones are present in the forms of glucosides, which are less bioactive compared with the aglycone counterparts.

Flavonoids are polyphenolic compounds found abundantly in plants, which may play a dietary role in reducing the risk from chronic diseases such as cardiovascular disease and cancer. The flavonoids exist in nature almost exclusively as β -glycosides.

The aglycone forms, daidzein, glycitein and genistein are developed by the hydrolytic action of the enzyme β -glucosidase on the glucosidic isoflavone precursors. Some studies have reported the effects of genistein in preventing and inhibiting prostate cancer, as well as its anticancer effect, similar to tamoxifen the anti-estrogenic drug utilized in breast cancer therapy. Biological activity depends on the presence or absence of the glycoside residue. The position and nature of the sugar residue may increase the uptake of the compound in the small intestine. However, the aglycone is likely to have a greater biological effect than the glycoside, so deglycosylation via a β -glucosidase activity would be an important step in metabolism. Additionally, the enzyme β -glucosidase is required for the hydrolysis of the β -glycosidic bonds of glucosides prior to the production of aglycones (Siok-Koon Yeo, Min-Tze Liong, 2010).

The aim of the study

The purpose of this work was to isolate, purify and characterize the β -glucosidase from culture filtrates of *P. radiata* in preliminary study to determine the possibility of using this type of preparation as a dietary supplement in the future.

Materials and methods

ORGANISM AND CULTURE CONDITIONS: Inoculum from *P. radiata* Fr. 79 (ATCC 64658) isolated in the Department of Microbiology of Helsinki University, Finland, was maintained on 2% (wt/vol) malt agar slants. The mycelial mats were collected and homogenized in a waring blender. After inoculation with 4% (vol/vol) of the homogenates, 100-ml conical flasks, each containing 10 ml of ADMS-LN medium supplemented with 1% wheat bran (Helsingin Mylly Oy, Helsinki, Finland) as a carbon source, were incubated at 28°C.

ENZYME ASSAY.

β -Glucosidase activity was measured in accordance with Wood, T.M., and Bhat, K.M. (1988) using 1% cellobiose as a substrate and liberated glucose content was measured using Lloyd and Whelan method (Lloyd J.B. and Whelan W.J. 1969).

ASSAYS. The protein content in the examined enzyme preparation was determined by means of the Bradford method (Bradford, 1976). The saccharide content in protein molecules was determined as in (Dubois et al. 1956).

CHROMATOGRAPHY AND CHROMATOFOCUSSING. Columns for ion-exchange chromatography packed with DEAE-Sepharose fast flow (1,5 ´ 25 cm equilibrated with 50 mM Tris-HCl buffer, pH 6,5); and chromatofocussing (1 ´ 30 cm; packed with Polybuffer Exchanger PBE-94; eluted in the pH range 3,4-7,4) were attached to the BIO-RAD Econo System.

ESTIMATION OF MOLECULAR MASS. The molecular weight determination was made using gel permeation chromatography on Protein-Pak 300 SW column connected to the Shimadzu HPLC system composed of LC-9A Chromatography pump, the SPD-M6A diode array detector and the 7125 model sampling valve (Rheodyne, Berkeley, USA) with 100 ml loop. The mobile phase 0.1 M acetate buffer (pH 5.0) was run at the flow rate 0.5 ml/min. The column was calibrated by the linear least square method employing Quattro Pro software (Borland, USA) on IBM PC using the MS II Gel-filtration Standard Set (Serwa, Heidelberg, Germany).

KINETIC STUDY. The kinetic parameters of the purified β -glucosidase were carried out by the use either salicin, cellobiose or pNPG as substrates in concentrations 0,05% - 1,3% for salicin and cellobiose and 0,25mM - 3,0mM for pNPG. The kinetic constants (K_m and V_{max}) were calculated on IBM PC computer running the Wilmann 4 (1985; M.S.U., MN) software.

Results

The great source of enzymes that belong to the class of glucose hydrolases are white rot fungi. The enzymes that can be obtained from that organisms have specific features like optimum pH in slightly acidic range, high temperature tolerance and significant stability. *Phlebia radiata* is efficient producer of extracellular enzymes (including b-glucosidase) that degrade complex macromolecules like e.g. cellooligosaccharides (Rogalski, J., et al., 1991).

The first step of the study was to examine the dynamics of b-glucosidase secretion during 14 days of growth on the media containing 1% of wheat bran as the carbon source.

Fig. 1 represents changes in b-glucosidase activities during 14 days of cultivation on the media containing 1% wheat bran as a carbon source. Maximal level of activity was observed in fifth day of cultivation.

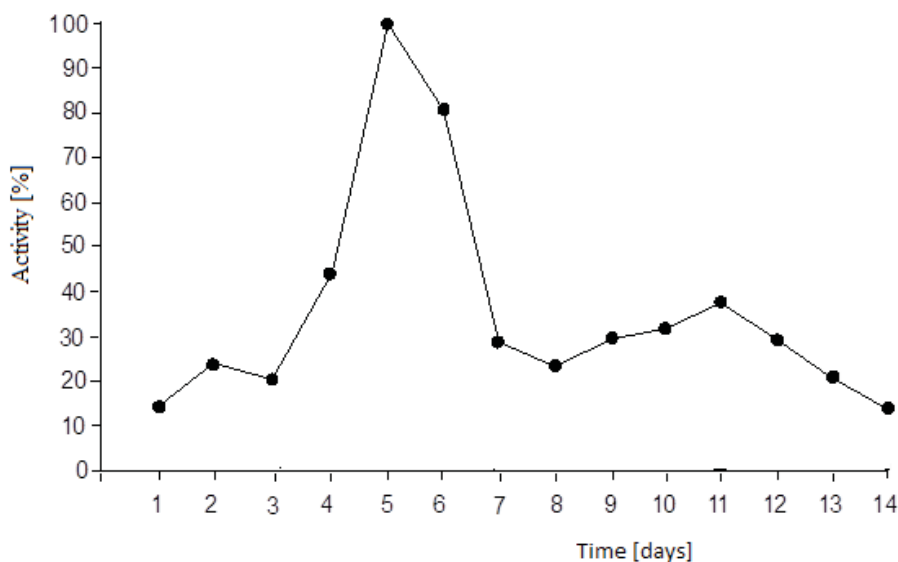


Fig. 1. Changes of b-glucosidase activity during growth on ADMS-LN medium supplemented with 1% wheat bran.

The next step was to determine the optimum of temperature and pH for best activities of b-glucosidase. For this purpose, the activity of the enzyme was examined in the range of temperature between 4 - 70°C and pH 3,5 □ 6,5. It was estimated that maximum activity of b-glucosidase was in pH 5,0 (Fig. 2. A) and in temperature 50 °C (Fig. 2. B).

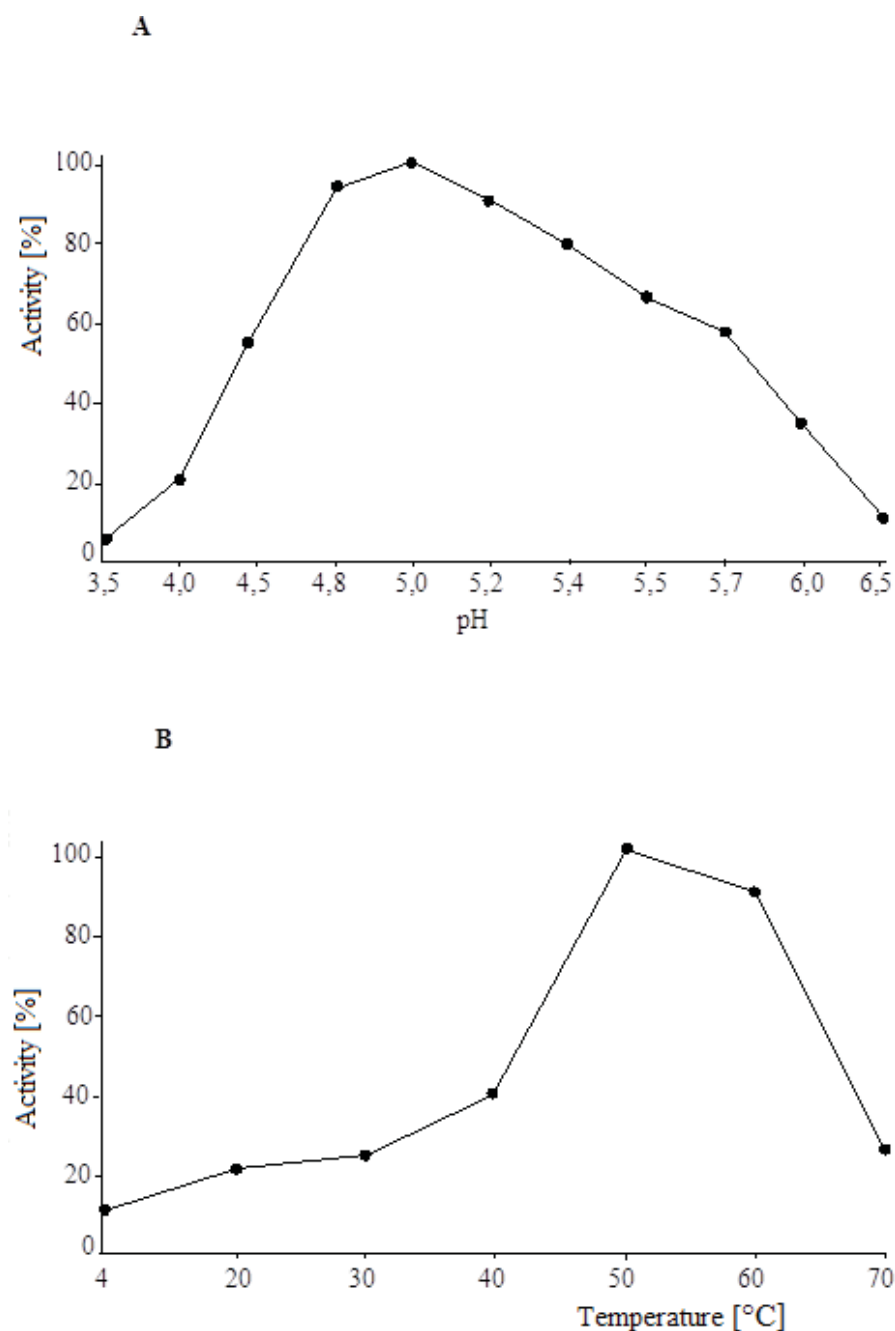


Fig. 2. The effect of pH (A) and temperature (B) on the activity of b-glucosidase from *Phlebia radiata* during growth on ADMS-LN medium supplemented with 1% wheat bran.

Purification of b-glucosidase involved the following steps: ion-exchange chromatography on DEAE-Sephacel fast flow and chromatofocussing on PBE 94. The results representative purification are shown in table 1 and figures 3-4. After the ion exchange chromatography on DEAE-Sephacel, several protein fractions were observed but only one contained b-glucosidase activity corresponding to the peak fraction eluted in 0,23 – 0,30 M NaCl (Fig. 3). After this step the specific activity was 4292,17 mU/mg. This step resulted in nearly 10-fold purification with the recovery of 89% (table 1).

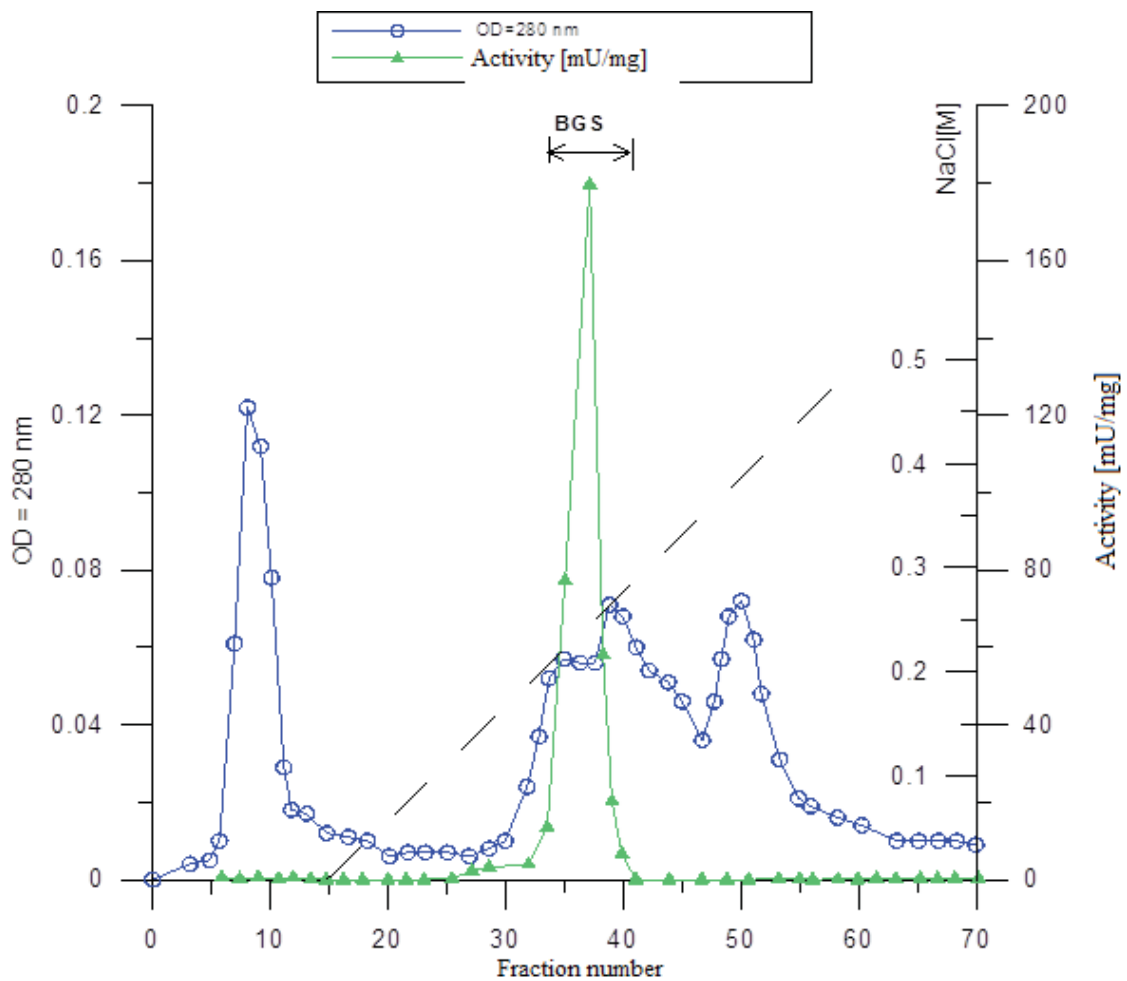


Fig. 3. The ion-exchange chromatography on DEAE-Sepharose (fast flow) of crude b-glucosidase from *P. radiata* post culture liquid with 1% wheat bran as the carbon source.

The main active fraction was pooled and further fractionated by chromatofocussing on Polybuffer exchanger PBE-94. The chromatographic pattern is depicted on figure 4. b-Glucosidase activity was concentrated in one narrow peak. The isoelectric point was estimated to be 4,98. The purified b-glucosidase showed a high level of specific activity (about 39273,61 mU/mg protein).

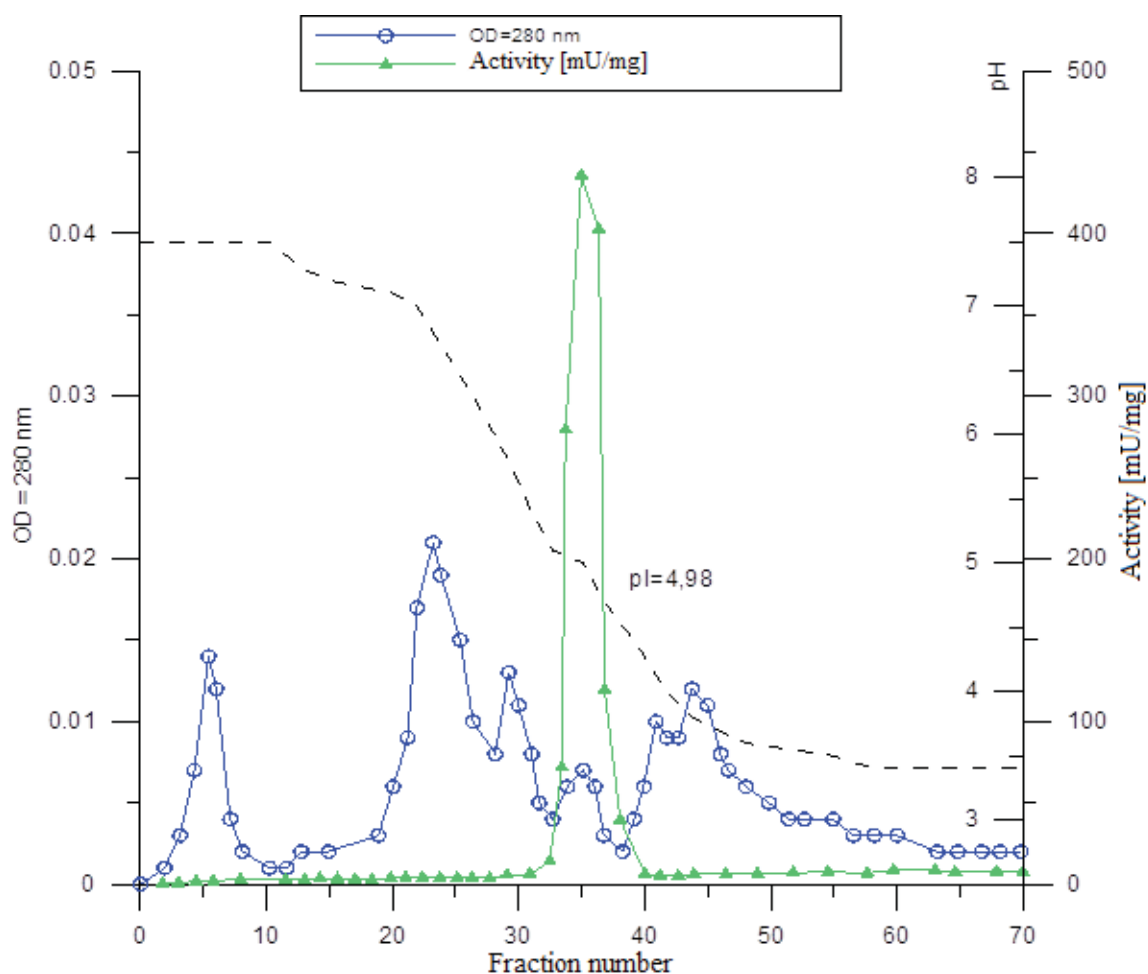


Fig. 4. Chromatofocussing pattern for the preparation of DEAE-Sepharose b-glucosidase on PBE-94.

Tab. 1. Purification of *P. radiata* b-glucosidase from after culture liquid containing 1% wheat bran as the only carbon source.

Preparation	Total protein [mg]	Specific activity [mU/mg]	Purification [-fold]	Yield [%]
Supernatant 21000 $\square$ g	43,17	460,04	1,00	100,00
DEAE-Sepharose	4,15	4292,17	9,33	89,73
PBE94 pI = 4,98	0,36	39273,61	85,37	71,69

Homogeneity of the b-glucosidase fraction was examined by size-exclusion high-performance liquid chromatography where the final purified enzymatic preparations appears as a single chromatographic peak.

The method of Dubois (1956) made it possible to determine the carbohydrate content in purified form of b-glucosidase. The enzyme contain the carbohydrate in its structure 15,22%. That indicates b-glucosidase from *P. radiata* is a glycoproteine.

The last point was to determine the kinetic constants of purified enzyme. The kinetic constants were estimated for three substrates: salicine, cellobiose and low molecular weight, syntetic substrate – p-nitrophenyloglucopyranoside (pNPG). Obtained results indicate that b-glucosidase from *P. radiata* has high substrate specificity for cellobiose ($K_m = 0,43$ mM) and for syntetic substrate pNPG ($K_m = 0,54$ mM) (table 2).

Tab. 2. Physico-chemical and kinetic data of purified b-glucosidase from *P. radiata*.

β -glucosidase	Molecular weight [kDa]		Isoelectric point [pH]		Carbohydrate content [%]	
BGS	45,9		4,98 $\pm$ 0,14		15,22 $\pm$ 4,28	

β -gluco- sidase	salicin		cellobiose		pNPG	
	K_m [mM]	V_{max} [μ M/ min*mg]	K_m [mM]	V_{max} [μ M/ min*mg]	K_m [mM]	V_{max} [μ M/ min*mg]
BGS	25,01 $\pm$ 1,53	0,08 $\pm$ 0,02	0,43 $\pm$ 0,02	0,24 $\pm$ 0,01	0,54 $\pm$ 0,02	13,11 $\pm$ 0,08

Discussion

Maximal level of b-glucosidases activity was observed in fifth day of cultivation on the media containing 1% wheat bran as the only carbon source. That results are not surprising from note that wheat bran contain complex hemicelluloses with residues of acethyl, arabinosyl, 4-O-glucuronyl and fenolic acids attached to main chain.

In next step the optimum pH and temperature was estimated. As it is shown at fig. 2 (A, B) optimal pH and temperature for b-glucosidase activity are 5,0 and 50°C. That data correspond with that obtained for other fungal glucosidases. Analogous results were obtained for b-glucosidase from *Trichoderma reesei* and *Phlebia radiata* during cultivating on the cellulose containing media (Rogalski et al. 1991), or b-glucosidase from *Chalara paradoxa* (Lucas et al. 2000). pH 4,5 for optimal b-glucosidase activity is common for enzymes isolated from *Aspergillus niger* (Wotanabe et al. 1992) or *Aureobasidium pullans* (Saha et al. 1994).

b-Glucosidases from *Phlebia radiata* have identical optimal temperature for their activity as described for *Trichoderma reesei*, *Phlebia radiata* (Rogalski et al.1991) or *Aspergillus oryzae* (Riou et al. 1998), but there are some differences like for example b-glucosidase isolated from *Chalara paradoxa* optimal temperature is 45°C (Lucas et al. 2000). Purified b-glucosidase was chromatofocussed on the column packed with PBE 94, and isoelectric point ($pI = 4,98$) was determined by this way. The purified enzyme was acidic protein. Different described before b-glucosidase has an isoelectric point in acidic pH like glucosidases isolated from *Aspergillus japonicus* ($pI = 4,7$) or *Aspergillus niger* ($pI = 4,1$) (Decker et al. 2000).

By the use of high performance, size exclusion chromatography on the Protein-Pack™ column the molecular mass of obtained b-glucosidase from *Phlebia radiata* was determined. The molecular mass of the purified protein was 45,9 kDa that is characteristic for extracellular enzymes.

The content of the carbohydrate component was determined by the usage of Duboise method and obtained the value 15,22%. This indicates that b-glucosidase from *P. radiata* is a glycoprotein which is characteristic of secretory proteins.

Determination of kinetic constants allowed to state that purified glucosidase is an enzyme with high substrate specificity towards cellobiose ($K_m = 0,43$ mM) and synthetic substrate pNPG ($K_m = 0,54$ mM). Similar values were obtained for glucosidase from *Chalara paradoxa* (raw starch digesting fungus), respectively $K_m = 0,58$ mM and $K_m = 0,52$ mM (Lucas et al. 2000).

The results in this paper can become a starting point for more detailed research in the future to allow the use of b-1,4-glucosidase in food and drug industry.

Conclusions

1. Highly purified b-glucosidase (BGS) from *Phlebia radiata* has maximum activity in pH 5,0 and temperature in 50°C what suggest that this fungus can be a good source of the potentially applicable enzyme.
2. According to results obtained from kinetic study b-glucosidase hydrolyze different substrates like salicin, cellobiose or even synthetic pNPG therefore, it can be concluded that the aglycone forms, daidzein, glycitein and genistein can be developed by the hydrolytic action of the fungal β -glucosidase on the glucosidic isoflavone precursors.

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