6. INTRODUCTION AND AIMS

β–D–Glucosidases catalyze the hydrolysis of aryl– and alkyl–β–D–glucosides as well as glucosides containing a glycosyl moiety as aglycon (eg., cellobiose). Current research on β–glucosidases has significant scientific, medical and economic implications. β–Glucosidases play central role in numerous biological processes. In human therapeutics the deficiencies of β–glucosidase encoding genes are responsible for many hereditary diseases. The specific inhibitors of glycoside hydrolysis can serve as potential drugs in the case of diabetes or virus infections. Plant β–glucosidases have been implicated in a variety of growth, productivity, defense and food toxicity related reactions such as cyanogenesis.

Cellulose, the most abundant renewable resource, forms the highest proportion of agricultural residues and municipal garbage. In fungi and bacteria β–glucosidases are involved in cellulose and cellobiose catabolism as part of the cellulase complex. Cellulases break down cellulose to cellobiose and β–glucosidases hydrolyze cellobiose to two glucose molecules. β–Glucosidase is inhibited by its end product, glucose; the substrate cellobiose accumulates and in turn inhibits the cellulase complex. If the rate–limiting step catalyzed by β–glucosidase in cellulose hydrolysis can be overcome, glucose production from cellulose by enzymatic means should become economically feasible. Thus, fungal and bacterial β–glucosidases appear as ideal candidates for engineering a β–glucosidase to be used as part of the cellulase complex in the industrial–scale conversion of cellulose to glucose.

It has already been shown that the *Aspergilli* are powerful producers of β–glucosidases and supplementation of cellulases with exogenously added *Aspergillus* β–glucosidase increases the rate of cellulose hydrolysis. Extending the research of new enzyme sources we investigated the β–glucosidase of *Aspergillus carbonarius*. The main aim of our research was to investigate the mechanism of action of this enzyme because this fungus and its β–glucosidase have not been examined yet.

The aims of the present study were as follows:

– isolation of the β–glucosidase from *Aspergillus carbonarius*, comparing the β–glucosidase production of *A. carbonarius* to that of other well known *Aspergilli*, characterization of the β–glucosidase and comparing its properties to other β–glucosidases isolated from the different *Aspergillus* strains

– purification of the β–glucosidase from *A. carbonarius*

– investigation the mechanism of action of the β–glucosidase from *A. carbonarius*. It included the examination of the substrate binding site by using different inhibitors. We also planned to

investigate the catalytic amino acid side chains in the active center of the enzyme by kinetic analysises and using chemical modification reagents. The synthesis of *N*-bromoacetyl β-D-glucopyranosylamine, an active site specific inactivator molecule, and using it for affinity labeling was also part of our aims.

7. MATERIALS AND METHODS

The β-D-glucosidases isolated from the different *Aspergilli* (*A. carbonarius*, *A. niger* and *A. phoenicis*) were produced on two kinds of fermentation media; a liquid medium containing glucose as carbon source and a solid state medium containing wheat bran as carbon source were used. For the purification of the enzymes modern chromatographic methods were applied including hydrophobic exchange chromatography, size exclusion chromatography and chromatofocusing. The purity of the final enzyme preparations was checked by SDS-polyacrylamid gel electrophoresis. Molecular weight of the *A carbonarius* β-glucosidase was determined by SDS-PAGE.

The β-glucosidase activity was measured by spectrofotometric method using a synthetic chromogen substrate and cellobiose. Kinetic parameters were determined by nonlinear fitting of the classic Michaelis-Menten rate equation to the data sets using the software GraFit. Molecular acid dissociation constants of the enzyme molecule (pK_{E1}, pK_{E2}) and of the enzyme-substrate complex (pK_{ES1}, pK_{ES2}) as well as pH-independent kinetic constants were determined on the basis of diprotic enzyme modell by nonlinear fitting. The acid dissociation constants of the chemically modified amino acid residues were also determined by nonlinear fitting using the GraFit program.

To determine the type of the catalitically active amino acid residues and to examine their role in the enzyme action we applied selective chemical modifying reagents and analysed the kinetics of the modification reactions. The active site modifying character of the inactivators were proved by applying competitive inhibitors.

The reactive substrate analogue affinity labelling molecule was synthetized applying the methods of preparative organic chemistry. Thin layer chromatography was applied for following the reactions and checking the purity of the end product molecule. The structure of the products were proved by NMR spectroscopy.

8. RESULTS AND DISCUSSION

8.1. Comparing the β–glucosidase production of *A. carbonarius* to that of different *Aspergillus* strains

The β–glucosidase production of *A. carbonarius* was compared to that of two well characterized *Aspergillus* species, *A. niger* and *A. phoenicis*, respectively. For enzyme production two different growth conditions were used; the liquid fermentation medium contained glucose as carbon source while the solid state medium contained wheat bran carbon source. Each *Aspergillus* strain produced extracellular β–glucosidase. The enzyme production reached the highest value on the 10^{th} day on wheat bran and on the 9^{th} day on glucose carbon source in the case of all *Aspergillus* strains examined. In the case of the submerged fermentation the fungi started to produce β–glucosidase only when all the glucose was consumed from the fermentation broth, this refers to catabolit repression. The amount of β–glucosidase increased continuously until the last day of fermentation in both media. Comparing the β–glucosidase production of the shaken flask cultures on the glucose carbon source, *A. phoenicis* achieved the highest enzyme activity, yield and productivity values whilst *A. niger* had the highest specific activity value. On solid state medium *A. niger* proved to be the best enzyme producer.

8.2. Purification and characterization of β–glucosidase from *A. carbonarius*

A rapid and efficient procedure was developed for the purification of the β–glucosidases, which involved three steps; hydrophobic interaction chromatography (HIC), size exclusion chromatography and chromatofocusing. The *A. carbonarius* β–glucosidase was purified 102-fold with an overall yield of 60 % and a specific activity of 715 nkatal/mg protein towards *p*–nitrophenyl β–D–glucopyranoside (*p*NP-Glc). The β–glucosidases were purified to homogeneity and the purified enzyme showed a single protein band on SDS-PAGE. In the purification procedure pigments, polisaccharides and contaminating proteins had to be eliminated from the crude preparations. The fermentation broth contained other glycosid hydrolases, such as β-D-galactosidase, β-D-xylosidase and α-L-arabinosidase, respectively.

All enzymes examined showed only β–glucosidase activity. In order to characterize the enzymes from different sources the isoelectric points of the enzymes were determined by chromatofocusing and the effect of temperature and pH on enzyme activity and stability was also investigated using both *p*NP-Glc and cellobiose as substrates. The β–glucosidase isolated

from *A. carbonarius* was an acidic protein with pI value of pH 4.2. The temperature optima of the enzyme was 60 ^{o}C and after two hours of incubation it remained stable up to 50 ^{o}C for both *p*NP-Glc and cellobiose as substrates. The pH optima of the *A. carbonarius* β–glucosidase was pH 5.0 with *p*NP-Glc and pH 4.0-4.5 with cellobiose substrate and was stable in the pH range of 4.0-8.0. The β–glucosidases isolated from *A. phoenicis* and *A. niger* had similar properties and also similar results were obtained for the enzymes produced on glucose and wheat bran carbon sources.

The kinetic parameters of the β–glucosidases were determined by non-linear fitting of the classic Michaelis-Menten rate equation to the experimental data. The kinetic parameters of the *A. carbonarius* β–glucosidase were K_m=0.67 mM and V_{max}=224 mkatal/kg for *p*NP-Glc, K_m=3 mM and V_{max}=243 mkatal/kg for cellobiose as substrate. There was no significant difference between the K_m values of the enzymes from different origins. The K_m values were 3.4-6.2 times higher for cellobiose than for *p*NP-Glc in all cases. The V_{max}/K_m values for cellobiose were 24-65 % of those for *p*NP-Glc. These facts indicate that the enzymes have higher affinity towards *p*NP-Glc and hydrolyse it more efficiently than cellobiose (Table 2.). The molecular weight of the *A. carbonarius* β–glucosidase was determined by SDS-PAGE. The molecular weight of the enzyme is 108 kDa.

Table 2. Kinetic parameters of the β–glucosidases from different *Aspergillus* strains*

Strain	***p*NP-Glc**			**cellobiose**		
	K_m **(mM)**	V_{max} **(mkatal/kg)**	V_{max}/K_m **(kat/kg·M)**	K_m **(mM)**	V_{max} **(mkatal/kg)**	V_{max}/K_m **(kat/kg·M)**
A. carbonarius	0.67	224	335	2.99	243	81
A. niger	0.48	372	775	2.97	595	200
A. phoenicis	0.58	267	461	2.0	404	202

*The table shows data for the enzymes produced on wheat bran carbon source.

8.3. Investigation of the mechanism of action of the β–glucosidase from *A. carbonarius*

8.3.1. Investigation of the substrate binding site

The β–glucosidase isolated from *A. carbonarius* has narrow substrate specificity because it only hydrolyses β-glucosidic bonds. It means that the orientation of the hydroxyl groups of the pyranoside ring of the substrate is very important possibly because of establishing essential hydrogen bonds on the substrate binding.

Inhibition studies were carried out to obtain information about the substrate binding site of the β–glucosidase. The inhibition experiments with glucose and glucono(1-5)lacton provided information about the interactions between the substrate binding site and the carbohydrate moiety of the substrate. The good inhibitory effect of the glucose reveals that, in contrast to other β–glucosidases examined earlier in our department, the interaction of the carbohydrate moiety of the substrate is as important as the one with the hydrophobic aglycon. Glucono(1-5)lacton is a transition state analogue inhibitor and its strong inhibitory effect proves that in the transition state the substrate is distorted into half chair conformation. 1-Thio-glucoside inhibitors were used to obtain further information on the structure of the active centre with respect to the interactions with the aglycon part of the substrate. Our results indicated that the aglycon binding site has hydrophobic character.

8.3.2. pH dependence of reaction kinetic parameters

According to the results of the investigation of the effect of pH on the kinetic parameters, the hydrolysis of the substrate can be described on the basis of the diprotic enzyme modell, that is, two ionizable amino acid side chains takes part in the catalysis; one of them must be ionized while the other must be protonated. The pH dependence of the V_{max} gives information about the amino acid side chains hydrolysing the substrate and the V_{max}/K_m reveals for the enzyme efficiency and substrate binding. At the pH dependence of the V_{max}/K_m for *p*NP-Glc the values of the molecular acid dissociation constants of the free enzyme were pK_{E1}=2.8 and pK_{E2}=5.93. These values permit us to conclude that besides an ionized carboxylate group, a protonated carboxyl group is involved in the formation of the ES complex. The very low p*K* value of the ionized carboxylate suggests that this is an extremely acidic group. In the case of the pH dependence of the maximal velocity for *p*NP-Glc hydrolysis the acid dissociation constants of the amino acid side chains involved in catalysis were pK_{ES1}=2.24 and pK_{ES2}=6.14. These groups are similar to those of the free enzyme. The acidity of these groups in the free enzyme changed on binding of the substrate in a way that the anionic group became more acidic and the proton donor became weaker acidic which is favourable for the efficient catalysis.

8.3.3. Chemical modification of carboxylate groups

On the basis of the pH dependence studies we wanted to investigate the possibility whether carboxylate groups are the essential ionizable groups in *A. carbonarius* β-glucosidase. For this purpose Woodward reagent K and a water soluble carbodiimide (1-ethyl-

3-(3-dimethylaminopropyl)carbodiimide, EDAC) in the presence of nucleophile glycin methyl ester were used as selective modifying agents for these functional residues. The enzyme was inactivated by both reagents. While the WRK inactivated the enzyme according to pseudo-first order kinetics, in the case of the EDAC we observed complex kinetics where the inactivation of the enzyme could be resolved into two first order processes. The difference in the kinetics of the inactivations can be explained by the different modification mechanism of the reagents. In the case of the WRK the inactivation is more specific for the carboxyl groups located in the active site due to the reagent's hydrophobic character and structural similarity to the substrate. In the case of the EDAC mediated inactivation the glycin methyl ester (which is activated by the EDAC) makes a nucleophile attack modifying not only the active site carboxylates but other carboxylates, too which are easily accesible for the reagent. Kinetic analysis of the modifications suggested that one EDAC or WRK molecule reacted with one enzyme molecule. These results further supported that at least one essential carboxyl residue is present in the *A. carbonarius* β-glucosidase. The rate of inactivations were also measured in the presence of a competitive inhibitor (*p*-nitro-thiophenyl β-D-glucopyranoside, *p*NTP-Glc). In both cases the enzyme was partially protected against inactivation by the inhibitor demonstrating that the catalytically active group is involved in the active center of the enzyme and may play catalytic role. From the pH dependence of the EDAC-mediated inactivation the acid dissociation constant of the modified group was pK_A=4.61, supporting the hypothesis that a carboxylate residue of an Asp or Glu plays catalytic role in the active center of the β-glucosidase. Considering that the rate of inactivation was the highest at the enzyme's optimum pH value (pH 4.5-5.5), and that in this pH the nucleophile is deprotonated and the acid catalyst group is protonated we can assume that the EDAC modified the catalytic nucleophile. Similar result was obtained with WRK, which inactivated the enzyme at pH optimum, which is pH 5.0. The effect of modifying the essential carboxylate on the kinetic constant of the enzyme was also investigated. After incubation with WRK V_{max} decreased to the half of that of the unmodified enzyme, while K_m did not changed significantly. These results clearly indicate that the carboxylate group plays role in the substrate cleavage and not binding.

8.3.4. Affinity labeling of the carboxyl residues in the active site

As it was shown by the inactivation with specific chemical modifications (WRK, EDAC and glycin-methyl-ester) it can be suggested that a carboxylate nucleophile is involved in thje catalytic process. To obtain further support to this idea an affinity label, *N*-

bromoacetyl-β-D-glucopyranosylamine (NBAGA) was used, whose affinity labeling character has been already proved in the case of other β-glucosidases. NBAGA was synthesized in our laboratory in two steps; to obtain bromoacetic anhydride, bromoacetic acid was treated with *N*,*N*'-dicyclohexylcarbodiimide and than with the reaction of the bromoacetic anhydride and β-D-glucopyranosylamine NBAGA was obtained.

The enzyme was effectively inactivated by NBAGA according to pseudo-first order kinetics. Protection by *p*NTP-Glc against inactivation demonstrated the active site specific (affinity labeling) character of the NBAGA. The rate of inactivation was the highest at the optimum pH value of the enzyme suggesting that the catalytic nucleophile was modified by the NBAGA similarly to the case of the EDAC-mediated modification. Also, the acid dissociation constant of the modified group (pK_A=4.5) was very similar to that of the residue modified by EDAC. These facts permit us to conclude that modification by NBAGA could be directed toward the carboxyl group acting as the catalytic nucleophile in the mechanism of action of this enzyme. This is in good agreement with earlier observations where in the case of different glycoside hydrolases the catalytic nucleophile was modified by *N*-bromoacetyl-β-D-glycopyranosylamines. However in some cases the modification of the acid catalyst proton donor group was reported. According to our results Figure 2. shows the proposed mechanism of inactivation of *A. carbonarius* β –glucosidase by NBAGA.

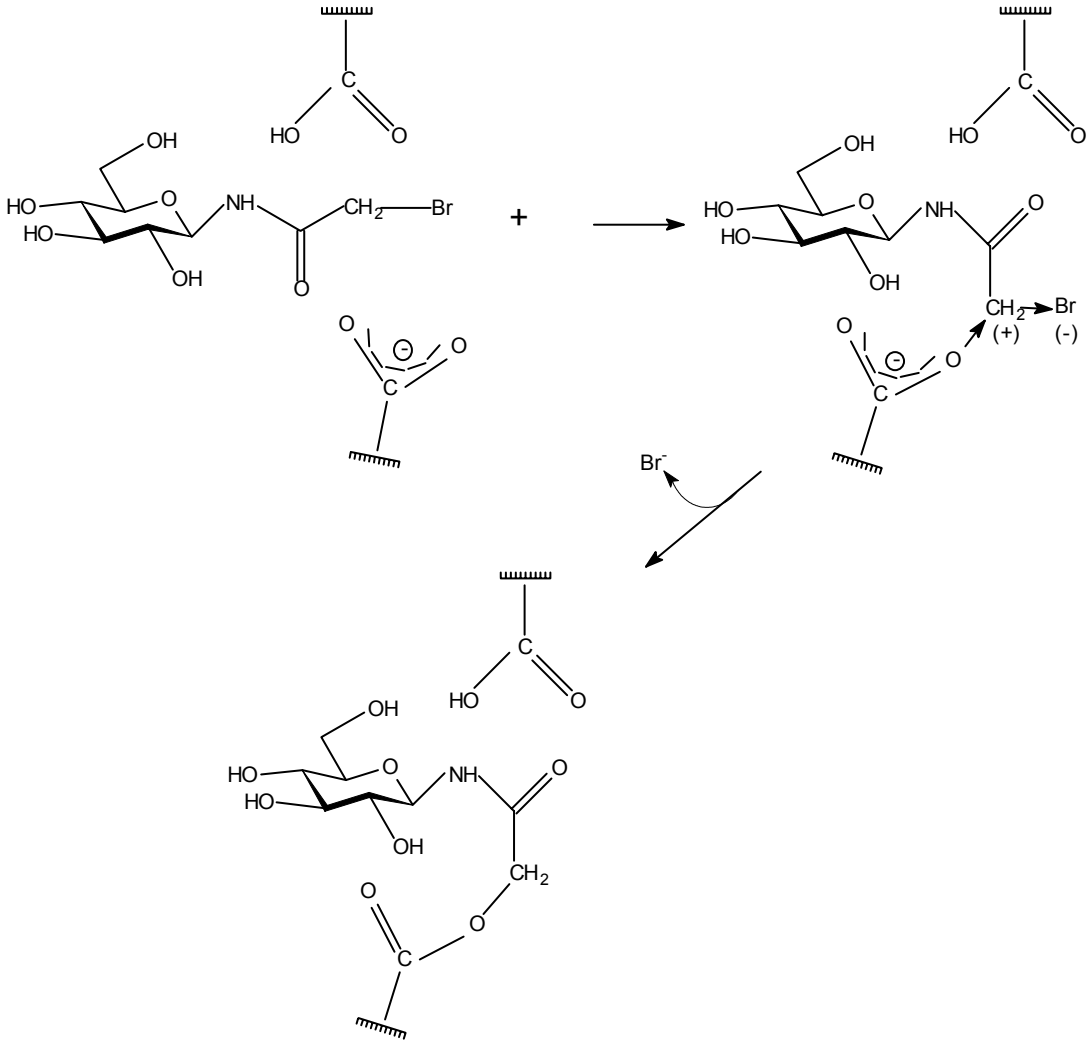

Figure 2. Proposed mechanism of inactivation of *A. carbonarius* β–glucosidase by *N*-bromoacetyl-β-D-glucopyranosylamine

9. POSSIBILE UTILISATION OF THE RESULTS

There are many possibilities for applying the β-glucosidase produced by *Aspergillus carbonarius* in practice:

β-Glucosidases can be used in various biotechnological processes. The β-glucosidase from *A. carbonarius* also can be applied first of all in biomass degradation and in bioethanol production from cellulosic materials.

Identification of the catalytic amino acid residues is possible by using the affinity labeling technique, which was applied for the investigation the mechanism of action of the β-glucosidase, and changing these residues by site directed mutagenesis can increase the efficiency of cellulose degradation. Knowledge of the mechanism of action can be exploited in the identification of catalytic groups in other β-glucosidases.

Changing the catalytic residues by site directed mutagenesis can alter the hydrolitic activity of the enzymes by increasing the transferase activity. It provides the application of the altered enzymes in the synthesis of complex oligosaccharides, which can be found in glycoproteins. In the chemo-enzymatic synthesis of oligosaccharides the enzyme's specifity can be exploited instead of the difficult application of protective groups and regio- and chemo-selective reagents.

10. LIST OF PUBLICATIONS

Publications in the field of the dissertation:

1. **Jäger, Sz.**, Brumbauer, A., Fehér, E., Réczey, K. and Kiss, L.
   Production of β-glucosidases from different *Aspergillus* strains.
   Acta Biologica Debrecina **22** (2000), 135-139
2. **Jäger, Sz.** and Kiss, L.
   N-Bromoacetyl-β-D-glucopyranosylamine as affinity label for β-D-glucosidase from *Aspergillus carbonarius*.
   Acta Biologica Debrecina **23** (2001) 1-3.
3. **Jäger, Sz.**, Brumbauer, A., Fehér, E., Réczey, K. and Kiss, L.
   Production and characterization of β-glucosidases from different *Aspergillus* strains.
   World Journal of Microbiology and Biotechnology **17** (2001), 455-461.
4. **Jäger, Sz.** and Kiss, L.
   Investigation of the active site of the extracellular β-D-glucosidase from *Aspergillus carbonarius*.
   Archives of Biochemistry and Biophysics (2003) Submitted for publication.

Publications in other fields:

5. Agócs, A., Herczegh, P., **Jäger, Sz**., Kiss, L. and Batta, Gy.
   Synthesis of 3-oxagranatane-type alkaloid analogs from carbohydrates.
   Tetrahedron **57** (2001) 253-239.
6. Beki, E., Nagy, L., Vanderleyden, J., **Jäger, Sz**., Kiss, L., Fülöp, L., Hornok, L. and Kukolya, J.
   Cloning and heterologous expression of a β-D-mannosidase encoding gene from *Thermobifida fusca* TM51.
   Applied and Environmental Microbiology **69** (2003) 9-15.

Lectures and posters:

1. **Jäger, Sz**. és Kiss, L.
   β-Glükozidáz izolálása és tisztítása *Aspergillus carbonarius*-ból.
   Magyar Poliszacharidkémiai Munkabizottsági Ülés, 1999, Budapest
2. **Jäger, Sz**. and Kiss, L.
   Investigation of the mechanism of action of β-glucosidase enzymes.
   International Student Conference of Vasile Goldis University, 1999, Arad, Románia
3. **Jäger, Sz**., and Kiss, L.
   Purification and characterization of extracellular β-glucosidase from *Aspergillus carbonarius*
   Annual Meeting of the Committee of Carbohydrate Chemistry of the Hungarian Academy of Sciences, 1999, Mátrafüred
4. **Jäger, Sz**., and Kiss, L.
   Purification and characterization of extracellular β-glucosidase from *Aspergillus carbonarius*
   26th Meeting of the Federation of European Biochemical Societies 1999, Nizza, Franciaország
5. **Jäger, Sz**., and Kiss, L.
   N-Bromoacetyl-β-D-glucopyranosylamine as affinity label for β-glucosidase from *Aspergillus carbonarius.*
   Annual Meeting of the Committee of Carbohydrate Chemistry of the Hungarian Academy of Sciences, 2000, Mátrafüred

6. **Jäger, Sz**., Brumbauer, A., Fehér, E., Réczey, K., és Kiss, L.

   Különböző *Aspergillus* fajok által termelt β-glükozidáz enzimek fermentációja és jellemzése.

   IX. Fermentációs kollokvium, MBKE Biotechnológiai Szakosztály és MTA Biomérnöki Munkabizottság, 2000, Debrecen

7. **Jäger, Sz**., and Kiss, L.

   N-Bromoacetyl-β-D-glucopyranosylamine as affinity label for β-glucosidase from *Aspergillus carbonarius*.

   27th Meeting of the Federation of European Biochemical Societies 2001, Lisszabon, Portugália

8. **Jäger, Sz**., és Kiss, L.

   Aspergillus carbonarius β-glükozidázának izolálása, tisztítása és működési mechanizmusának vizsgálata.

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9. Béki, E., Kukolya, J., Posta, K., **Jäger, Sz**., Kiss, L., Hornok, L.

   A *Thermobifida fusca* TM51 törzsből származó β-mannozidáz (EC 3.2.1.25) gén klónozása és jellemzése.

   Az 50 éves Magyar Mikrobiológiai Társaság 2001 évi Jubileumi Nagygyűlése, 2001, Balatonfüred

10. **Jäger, Sz**., Kiss, L.

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    DE TTK Biológus Napok 2001, Debrecen

11. **Jäger, Sz**., és Kiss, L.

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    Debreceni Tudományos Napok, a Biológiai Tanszékcsoport Napja, 2002, Debrecen