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N-BROMOACETYL-β-D-GLUCOPYRANOSYLAMINE AS AFFINITY LABEL FOR β-D-GLUCOSIDASE FROM *ASPERGILLUS CARBONARIUS*

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ÖSSZEFOGLALÁS

***ASPERGILLUS CARBONARIUS* ÁLTAL TERMELT β-D-GLÜKOZIDÁZ AFFIN JELÖLÉSE N-BRÓMOACETIL-β-D-GLÜKOPIRANOZILAMINNAL**

A fungális β-D-glükozidázok az enzimek igen népes és fontos csoportját képviselik, melyeknek a szénhidrát alapú biomassza lebontásában van fontos szerepük. Ezért ezen enzimek működési mechanizmus vizsgálata fontos kutatási területté vált az utóbbi időkben. Az *Aspergillus carbonarius* által termelt β-D-glükozidáz működési mechanizmus vizsgálata során az aktív centrum katalitikus oldalláncainak vizsgálatára egy speciális affin jelölőt, az N-brómacetil-β-D-glükopiranozilamint alkalmaztuk. Az inaktivátor irreverzibilisen alkilezi az enzim aktív centrumában található nukleofil oldalláncot, ami a szubsztrát hasításában vesz részt. Kísérleteink során meghatároztuk az inaktiváció egyensúlyi és sebességi állandóját, bizonyítottuk, hogy az inaktivátor valóban az aktív centrumban levő oldalláncokat módosítja. A látszólagos sebességi állandó pH függéséből megállapítottuk, hogy az inaktivátor a katalítikus nukleofil oldalláncot alkilezi.

INTRODUCTION

β-D-glucosidases (β-D-glucoside glucohydrolases, EC 3.2.1.21) catalyse the hydrolysis of alkil- and aryl-β-glucosides, diglucosides and oligoglucosides. β-D-glucosidases are a very important group of enzymes because of their potential uses in biotechnological processes such as biomass (cellulose) degradation, ethanol production, detoxication processes, flavor industry and synthesis of useful β-glucosides.

In the Institute of Biochemistry of University of Debrecen β-D-glycosidases are widely examined with respect to their mechanism of action. As a part of this project β-D-glucosidase from *Aspergillus carbonarius* is also investigated. The highly cellulolytic fungus *Trichoderma reesei* produces a very active cellulase enzyme complex but deficient in β-D-glucosidase activity. β-D-glucosidases hydrolyse cellobiose which inhibits other enzymes in the cellulase enzyme complex. Fungi belonging to the genus *Aspergillus* produce β-D-glucosidases in high yield which are compatible with *T. reesei* cellulases and promote saccharification of cellulose.

Specific inactivators (or affinity labels) are widely used in the investigation of the mechanism of action of glycosidases. First of all in the identification of catalytic groups in the enzymatic active center. The affinity labeling techniqe uses such compounds which are reactive substrate analogues that bind specifically into the active center. In the active site either they are activated by the catalytic mechanism of the enzyme and finally form covalent bond with catalytic or reacitve groups or they have a reactive group in the aglycone which reacts with the proton donor or the nuleoplhile in the active center of the enzyme [1].

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CONCLUSION

The effect of N-bromoacetyl β-D-glucosylamine on β-D-glucosidase from *Aspergillus carbonarius* was investigated.

The enzyme was completely inactivated by the inactivator. This is a characteristic of active-site directed modifying agents. These inactivators work by the following scheme:

$$E+I \rightarrow E\,I \rightarrow E\text{-}I$$

The kinetic of the inactivation can be described with the following equation:

$$\ln(A_0/A)=(k_i\ t)/(1+K_i/I)=k_{app}\ t$$

where V_0 is the relative initial reaction rate
V is the reaction rate in the given time
K_i is the apparent inhibition constant
k_i first order rate constant of the inactivation
k_{app} is the apperent first-order rate constant

The apparent rate constant represents the velocity of the alkylation of the nucleophile in the active centre. With the transformation of the 2nd equation we can see the dependence of the apperent rate constant on the inactivator concentration:

$$1/kapp=1/k_i + (K_i/k_i)\cdot\ 1/I$$

In the case of the different inactivator concentrations the apparent first order rate constants (k_{app}) were determined from the slopes of the lines fitted to the logarithmic plot of the experimental relative rate values (A_0/A) against time.

The apparent inhibition constant (K_i) and the first-order rate constant (k_i) were determined from the double reciprocal replot of the k_{app} values versus inactivator concentration; K_i=0.89 mM, k_i=0.0173 1/M min.

On the logarithmic plot of the apparent first-order rate constant versus the logarithm of inactivator concentration the slope of the line was around 1, indicating that 1 molecule inactivator reacted with 1 molecule enzyme.

Giving a competitiv inhibitor, namely *p*-nitrothiophenyl β-D-glucopyranoside, we found that the enzyme was protected by the inhibitor against the inactivation. The protection proved the active site directed character of the inactivator.
From the pH dependence of the apparent rate constant we found that the rate of the inactivation was the highest on the optimum pH value, pH 4.0. In this pH the nucleophile in the active site is deprotonated having a negative charge. It shows that the inactivator reacts with the catalytic nucleophile.
It is in opposite with the case of the cassava β-glucosidase, which was examined earlier in the Institute of Biochemistry where the optimum of the apparent rate constant was at pH 8. In that case the acid catalytic group is deprotonated and the inactivator modifies that group. In lower pH values inactivation did not occur, or only with lower rate showing that the inactivator did not react with the catalytic nucleophile [1].
Experiments are in progress to identify this nucleophile.

METHODS

Culture conditions: For enzyme production *A. carb.* was grown on solid medium containing 20 g wheat bran and 100 ml distilled water. The culture was incubated at 27 °C for 10 days and was harvested by filtration.

Purification: A rapid and efficient purification procedure was developed to get pure enzyme solution in which hydrophobic interaction chromatography, gel filtration and chromatofocusing were used.

Enzyme assay: β-D-glucosidase activity was assayed by using a 1 ml reaction mixture containing 100 µl 5 mM *p*-nitrophenyl β-D-glucopyranoside (*p*NPG), 0.02 M Na-acetate buffer (pH=4) and an appropriate dilution of enzyme solution at 50 °C for 10 minutes. The reaction was stopped by 2 ml of 0.2 M Na-borate buffer (pH=10) and the *p*-nitrophenol release was monitored at A_{400}.
One enzyme unit was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol min^{-1} under these conditions.

Inactivation studies with N-bromoacetyl β-D-glucopyranosylamine: The apparent inhibition constant (K_i) and the first-order rate constant (k_i) were determined by incubating the enzyme in 0.2 M Na-acetate buffer, pH 4.0, using varying inactivator concentrations (1-3 mM) at 50°C. Samples were taken at intervals to determine remaining β-D-glucosidase activity. Pseudo-first-order rate constants (k_{app}) were obtained from the slopes of the lines fitted to the plot of the natural logarithm of the inverse of the residual activity against time. K_i and k_i were computed from the plots of the inverse of the pseudo-first-order rate constants against the inverse of the inactivator concetration, using the kinetic software GraFit.
Protection of β-D-glucosidase from inactivation was accomplished using *p*-nitro-thiophenyl β-D-glucopyranoside (*p*NTPG) as a competitive inhibitor. Inactivation mixtures containing 3 mM NBAGA were incubated in the presence and absence of *p*NTPG (8 mM = 4 K_i). Aliquots were removed at appropriate intervals and assayed for residual activity against *p*NPG. The degree of protection was determined from the ratio between the pseudo-first-order rate constant in the presence and the absence of *p*NTPG.
pH-dependence of the inactivation process was studied in the range of pH 3.0-7.5. Pseudo-first-order rate constants, determined as described above were plotted against pH and the titration curve was fitted using GraFit.

REFERENCE

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