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Purification and Properties of Three Cellobiases from *Aspergillus niger* A20

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Abstract

Three cellobiases, here called cellobiase A, B, and C, from the culture filtrate of *Aspergillus niger* A20, were purified by precipitation with ammonium sulphate, gel filtration through Sephadex G-75, and column chromatography of DEAE-cellulose. The purified enzymes were homogeneous on polyacrylamide disk electrophoresis. The mol wt of the purified enzymes were estimated by SDS-gel electrophoresis to be 88,000, 80,000, and 71,000 for cellobiases A, B, and C, respectively. The enzymes were active at pH 4.5 and 55–60°C. The pattern of their amino acid compositions showed high contents of aspartic acid, glutamic acid, threonine, serine, and glycine. The apparent K_m values for cellobiose were 0.9, 1.63, and 1.0 m*M* for cellobiases A, B, and C, respectively. Calcium ions stimulated cellobiases B and C, and Co^{2+} and Mg^{2+} ions stimulated cellobiase A. The purified enzymes hydrolyzed cellobiose and aryl-β-D-glucosides, but they had no action on sucrose, maltose, and cellulose. The three cellobiases catalyzed transglycosylase reaction, and the major product formed from cellobiose was tetramer of glucose.

Index Entries: *Aspergillus niger*; cellobiase; purification; properties.

Introduction

The enzymatic conversion of native cellulose to glucose is catalyzed by multiple enzyme system *(1)*. It includes Endo-β-1,4-glucanase (E.C. 3.2.1.4), exo-β-1,4-glucanase (E.C. 3.2.1.91), and cellobiase (β-gluosidase, β-D-glucosidase glucohydrolase, E.C. 3.2.1.21). Although cellobiase does not directly participate in cellulose degradation, it plays an important role in practical saccharification to relieve the inhibition of cellobiohydrolase by cellobiose.

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Trichoderma species seem to be the best sources of extracellular cellulases that can solubilize highly ordered cellulose. However, they produce low levels of cellobiase, and, to increase the saccharification rate, supplementation of external cellobiase is often recommended *(2)*.

Cellobiase is produced and purified to homogeneous components by a variety of microorganisms, such as *Penicillium funiculosum (3), Aspergillus terreus (4), Sporotrichum thermophile (5), Sulfolobus solfataricus (6), Phanerochaete chrysosporium (7), Fusarium oxysporum (8),* and *Bifidobacterium adolescentis (9)*.

Earlier work showed the potential of *Aspergillus niger* A20 for the production of cellobiase *(10)*. The fascinating aspect of cellobiase produced by *A. niger* A20 is its higher yield, which is about 46 times that of *Trichoderma reesei* QM9414 *(1)*. This article deals with the purification and properties of cellobiase from *A. niger* A20.

Materials and Methods

Microorganism

The fungal culture used in the present work was obtained from the culture collection of the Centre of Culture of the National Research Centre, Cairo, Egypt. It was maintained in potato dextrose agar (PDA) at 5°C.

Enzyme Production

The culture medium for enzyme production has the following composition (g/L): 15.0 cellulose; 2.0 lactose; 10 wheat bran; 2.5 KH_2PO_4; 0.3 $CaCl_2 \cdot 2H_2O$; 0.3 $MgSO_4 \cdot 7H_2O$; 1.5 urea; 0.7 $(NH_4)_2SO_4$; 0.5 yeast extract; 0.25 peptone; 0.5 L (–) sorbose; 0.005 Fe $SO_4 \cdot 7H_2O$; 0.0014 $ZnSO_4 \cdot 7H_2O$; 0.0022 $MnSO_4$; 0.002 $CoCl_2$; and 2 mL/L Tween-80. The pH was maintained at pH 4.0 during the fermentation. For preparation of the inoculum, 1 mL spore suspension from 7-d-old culture on PDA (8×10^7 spores/mL) was transferred to 50 mL of growth medium in a 250-mL Erlenmeyer flask and incubated in rotary shaker (100 *g*) at 30°C for 5 d. Cultivation was also made in 250-mL Erlenmeyer flasks, each containing 50 mL sterile medium. One mL of inoculum (2%, v/v) was transferred to the growth medium, and the flasks were incubated at 30°C on a rotary shaker (100 *g*) for 8 d.

Assay for Cellobiase Activity

The cellobiase activity was determined according to the method reported by Berghem and Pettersson *(11)*, as follows: To 1 mL 0.4% (w/v) cellobiose dissolved in 0.05 citrate–phosphate buffer (pH 4.5), 0.5 mL diluted enzyme solution was added. The reaction was incubated in a water bath for 30 min at 50°C. The reaction was stopped by heating the reaction mixture in a boiling water bath for 10 min. The glucose released was determined by glucose oxidase–peroxidase reagent *(12)*. One unit (U) of the enzyme activity was defined as the amount of the enzyme that release 1 µmol glucose from cellobiose per min under the assay conditions.

Ultrafiltration

Ultrafiltration was carried out by using Millipore Pellicon Cassette System, polysulphone membrane, porosity of mol wt 10,000. Filtration rate was 10 mL/min.

Enzyme Purification

Gel Filtration

The enzyme sample obtained by $(NH_4)_2SO_4$ precipitation at 80% was dissolved in a minimum volume of citrate–phosphate buffer (0.05 *M*, pH 4.5). The enzyme sample was chromatographed on a Sephadex G-75 (Sigma, St. Louis, MO) column (2.0 × 80.0 cm), and was eluted with the same buffer at 8°C. The fraction volume was 40 mL, with a flow rate of 50 mL/h. The fractions containing cellobiase activity were pooled. An equal volume of chilled absolute ethanol was slowly added to the pooled fractions. The precipitates were collected by centrifugation and then dissolved in a minimum quantity of 0.01 *M* citrate–phosphate buffer (pH 4.5). The enzyme solution was centrifuged (3500 *g*) for 20 min at 5°C to remove the insoluble materials, and used directly as starting material for the ion-exchange chromatography.

Ion-Exchange Chromatography

A column of DEAE-cellulose DE-52 (2.0 × 80.0) equilibrated with 0.01 *M* citrate–phosphate buffer (pH 4.5) was used for chromatographic purification. Elution was performed at 8°C with citrate–phosphate buffer (pH 4.5), with stepwise-increasing molarity *(13)*. The flow rate was 40 mL/h, using peristaltic pump (ELMED 340) and fraction collector (LKB 2112). Ten mL fractions were collected.

Electrophoresis

Disk gel electrophoresis of protein samples was done according to the method of Davis *(14)*.

Determination of Molecular Weight

This was done by the method of Weber and Osborn *(15)* using phos-phorylase b (97,400), bovine serum albumin (67,000), egg albumin (45,000), pepsin (35,000), and lysozyme (14,400) as standard proteins.

Amino Acid Analysis

About 500 µg purified protein was hydrolyzed in a sealed tube for 24 h at 110°C with 6 *M* HCl. The hydrolyzate was analyzed by Beckman 120-C Amino Acid Auto Analyzer.

Substrate Specificity

One mL of enzyme solution was incubated with 1 mL of each substrate in citrate–phosphate buffer (0.05 *M*, pH 4.5) at the optimum temperature of

each enzyme. The sugars obtained from carboxymethylcellulose (CMC), xylan, starch, dextrin, and cellulose were determined (as glucose) by the method of Somgyi *(16)*. The glucose released from lactose sucrose, maltose, and cellobiose was determined by glucose oxidase–peroxidase reagent *(12)*. ρ-nitrophenyl released from ρ-nitrophenyl-β-D-glucosoide (PNPG), ρ-nitropheny-β-D-galactoside (PNOGa), and ρ-nitrophenyl-β-D-xyloside (PNPX) was measured by the addition of 2 mL of 0.2 *M* Na_2CO_3, and the developed color was measured at 400 nm. One U of enzyme activity was defined as the amount of the enzyme that released 1 µmol glucose or ρ-nitrophenol/min.

Transferase Activity

The transferase activity of the purified cellobiases was done as described by Wood and McCrae *(17)*. The products were detected by TLC on silica gel plates (Kieselgel G) by the method of Funaguma et al. *(18)*.

Protein Determination

This was achieved by the method of Lowry et al. *(19)*.

Determination of Total Sugars

Sugar content of the enzyme was determined by the method of Dubois et al. *(20)* as glucose.

Effect of Metal Ions

In separate experiments, 1 mL of each purified enzyme solution was preincubated with 1 mL of the tested metal ion solution at the required molarity at 25°C for 60 min. The residual activity was measured under standard assay conditions.

Results and Discussion

Purification of Aspergillus niger *A20 Cellobiase*

The culture broth (500 mL) was concentrated by ultrafiltration and fractionated by 80% saturation of ammonium sulphate. The resulting precipitate was dissolved in citrate–phosphate buffer (0.05 *M*, pH 4.5) and dialyzed against the same buffer. The enzyme sample was applied to Sephadex G-75 column, equilibrated, and eluted with citrate–phosphate buffer (0.05 *M*, pH 4.5). The protein fractions that showed cellobiase activity were collected and precipitated with 50% ethanol. The precipitate obtained was dissolved in citrate–phosphate buffer (0.01 *M*, pH 4.5) and chromatographed on a column of DEAE-cellulose DE- 52. Elution was carried out using citrate–phosphate buffer, with stepwise increasing molarity (Fig. 1). Elution with 0.025 *M* citrate–phosphate buffer (fractions 8–20) afforded a pure cellobiase component (cellobiase A), which contributed

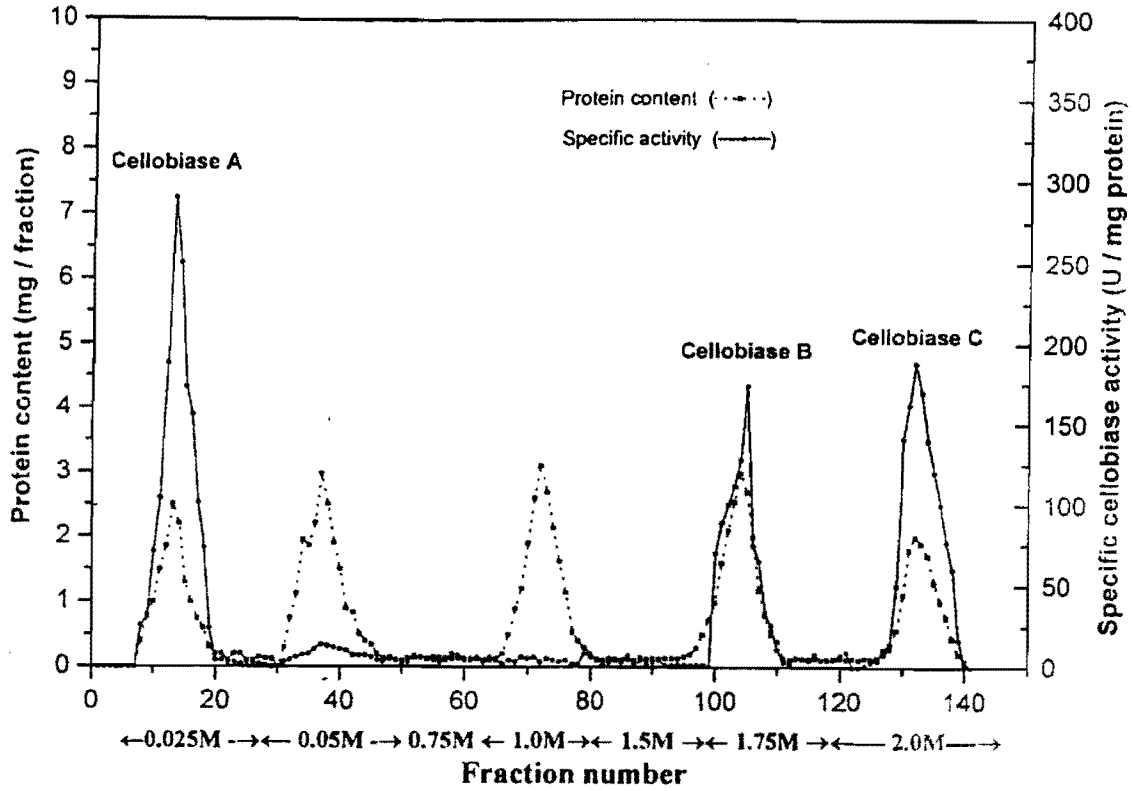

Fig. 1. Elution profile of cellobiases from *A. niger* A20 on DEAE-cellulose DE-52 using ion exchange chromatography. The elution was carried out stepwise, by increasing the molarity of citrate–phosphate buffer (pH 4.5), as shown. The cellobiase peaks obtained are indicated with the letters A, B, and C.

20.05% of the applied activity. Elution with 1.75 *M* of citrate–phosphate buffer yielded a protein component that contributed 16.1% of the original activity (cellobiase B). The third cellobiase component (cellobiase C) was eluted by 2.0 *M* citrate–phosphate buffer, and showed 13.3% of the total activity.

The purification steps of *A. niger* A20 cellobiase are summarized in Table 1. The purified cellobiases contributed 20.05, 16.1, and 13.3% of the applied crude enzyme activity, and showed 23, 14.9, and 17.5-fold purification for cellobiases A, B, and C, respectively. Each purified cellobiase gave a single protein band on disk gel electrophoresis (Fig. 2). This multiplicity of fungal cellobiase is in accord with the results reported for other fungi. Thus, two cellobiase components were detected in *Trichoderma koningii (17)*, three in *P. chrysosporium (7)*, and four in *Sclerotium rolfsii (21)*.

Properties of A. niger *A20 Cellobiases*

Specific Activity

The specific activities of the pure cellobiases were 184.1, 119.32, and 140.35 U/mg protein for cellobiase A, B, and C, respectively. These values are higher than those reported by Chirico and Brown Jr. *(22)* for *T. reesei* (52 U/mg protein) and by Bhat et al. *(5)* for *S. thermophile* (89 U/mg protein).

Table 1
Purification Steps of *Apergillus niger* A20 Cellobiase

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification (-fold)	Recovery (%)
Culture filtrate	1629.56	13036.48	8.00	1.00	100.00
Ultrafiltrate	1152.70	10950.65	9.50	1.19	84.00
Ammonium sulphate saturation (80%)	376.42	8432.00	22.40	2.80	64.68
Gel filtration (Sephadex G-75)	197.50	7979.63	40.40	5.05	61.21
Ethanol fractionation (50%)	130.00	6552.00	50.40	6.30	50.25
Ion exchange chromatography (DEAE-Cellulose DE-52)					
Cellobiase A (fractions 8–21)	14.20	2614.22	184.10	23.00	20.05
Cellobiase B (fractions 99–109)	17.60	2100.00	119.32	14.92	16.10
Cellobiase C (fractions 127–137)	12.38	1737.62	140.35	17.54	13.33

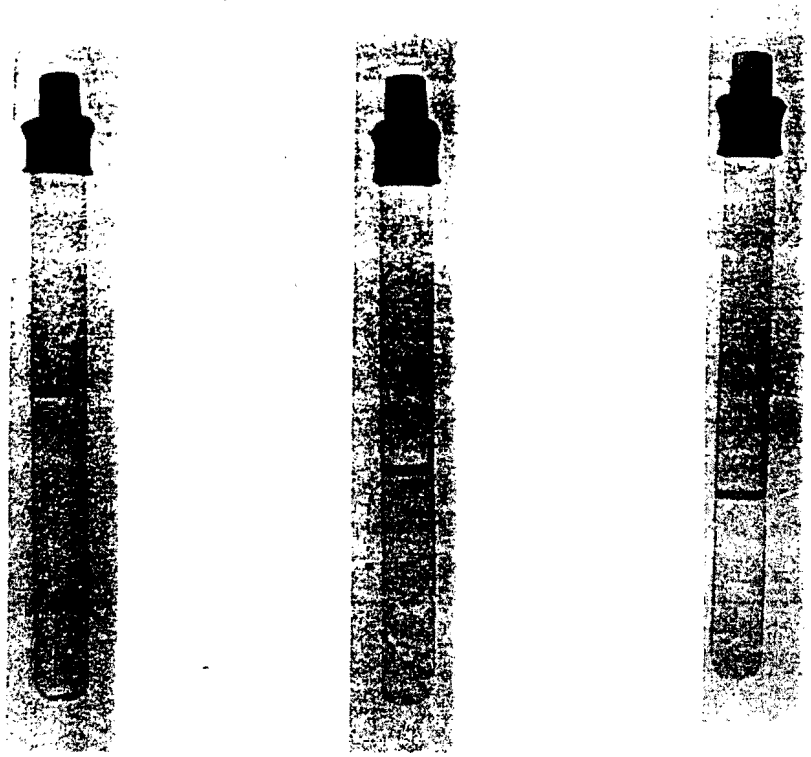

Fig. 2. Polyacrylamide gel disk electrophoresis of the purified cellobiases A, B, and C. The enzymes were electrophoresed at 3 mA/tube in Tris-glycine buffer (pH 8.3). The anode is at the bottom.

Molecular Weight

The mol wt, as determined by SDS-polyacrylamide gel electrophoresis, were estimated to be 88×10^3, 80×10^3, and 71×10^3 for cellobiases A, B, and C, respectively (Table 2). These values are favorably comparable to those reported by Chirico and Brown Jr. *(22)* for *T. reesei* (81×10^3), Alconada and Martinez *(8)* for *F. oxysporum* (66×10^3), and Lymar et al. *(7)* for *P. chrysosporium* (96×10^3). The mol wt of *A. niger* A20 cellobiases, however, were much smaller than those of *P. funiculosum* (230×10^3) *(3)*, and *S. thermophile* (240×10^3) *(5)*.

Carbohydrate Content

The three cellobiases proved to be glycoproteins. The carbohydrate content of the pure cellobiases were estimated to be 8.8, 9.4, and 7.2% (w/w) for cellobiases A, B, and C, respectively (Table 2). The carbohydrate content of *Trichoderma viride* β-glucosidase was 0.7% (w/w) *(22)*; that of *T. koningii* β-glucosidase contains 2% (w/w) carbohydrate *(17)*. Furthermore, Workman and Day *(4)* reported that *A. terreus* β-glucosidase contains 65% (w/w) carbohydrate. On the other hand, Desai et al. *(13)* did not find any carbohydrate in *Scytalidium lignicola* β-glucosidases I and II. These results may reflect the variation in the chemical structure of β-glucosidase from different strains.

Table 2
Properties of Pure *Aspergillus niger* A20 Cellobiases

Property	Cellobiase A	Cellobiase B	Cellobiase C
Specific activity (U/mg protein)	184.1	119.32	140.35
Molecular weight	88×10^3	80×10^3	71×10^3
Carbohydrate content (%)	8.00	9.40	7.20
Optimum pH	4.5	4.5	4.5
Optimum temperature (°C)	55	55	60
Half-life at optimum pH and temperature (min)	27.0	32.25	45.0
Deactivation rate constant at 60°C ($\times 10^3$/min)	11.1	9.30	6.60
K_m (m*M* cellobiose)	0.90	1.63	1.00
V_{max} (μmol glucose/mg protein/min)	333.30	212.20	144.00

Effect of pH

The three cellobiases were optimally active at pH 4.5 (Table 2). Similar pH optima have been reported for *A. japonicus (23)* and *T. reesei (22)*. The optimal pH of the three cellobiases were close to the optimal pH of the widely used *Trichoderma* cellulase (optimal pH, 4.8), particularly *T. reesei (1)*. This may have economical advantage for enriching these cellulase complex with *A. niger* A20 cellobiases.

Effect of Temperature

The optimum temperature was investigated at pH 4.5. Cellobiase A and B were optimally active at 55°C, whereas cellobiase C showed optimal activity at 60°C (Table 2). Temperature optima of 55–60°C were reported for *Aspergillus japonicus (23)* and *F. oxysporum (8)*.

Thermal Stability

The thermal stability was estimated after preincubation of the enzyme solutions in citrate–phosphate buffer (0.05 *M*, pH 4.5) at their optimal temperatures (i.e., 55°C for cellobiases A and B, and 60°C for cellobiase C) for 60 min. The results (Table 2) showed that the calculated half-life of cellobiases A, B, and C for thermal deactivation were 27.0, 32.25, and 45.0 min. Comparatively, cellobiase C was the most thermostable. The deactivation rate constants at 60°C for cellobiases A, B, and C were 11.1×10^{-3}, 9.3×10^{-3}, and 6.6×10^{-3}/min, respectively. Cellobiase C seemed to be more thermostable than other cellobiases from *T. koningii (17)* and *Neurospora crassa (24)*.

K_m and V_{max}

The calculated values of the kinetic parameter, K_m (Michaelis constant), for cellobiose were 0.9, 1.36, and 1.0 m*M* for cellobiases A, B, and C, respectively (Table 2). The low K_m values recorded for the pure cellobiases

Table 3
Amino Acid Composition of Pure *Aspergillus niger* A20 Cellobiases

	Molar ratio (%)		
Amino acid	Cellobiase A	Cellobiase B	Cellobiase C
Aspartic acid + asparagine	18.40	19.70	18.63
Threonine	13.90	16.20	17.30
Serine	14.33	13.70	12.70
Glutamic acid + glutamine	10.44	9.84	11.60
Glycine	8.40	7.90	10.32
Alanine	6.90	5.12	6.40
Valine	5.22	4.80	6.70
Cystine	0.77	0.54	0.80
Methionine	1.33	1.24	0.93
Isoleucine	2.14	1.19	0.84
Leucine	4.80	5.30	4.90
Tyrosine	3.40	4.80	2.12
Phenylalanine	4.22	3.84	3.90
Lysine	1.14	1.71	0.29
Histidine	1.20	1.22	0.26
Arginine	1.22	1.00	0.71
Proline	1.80	1.30	0.97
Tryptophan	ND[a]	ND	ND

[a]ND, not determined.

may reflect the affinities of *A. niger* cellobiases for cellobiose hydrolysis. These values were favorably comparable to those displayed by two β-glucosidases from *T. koningii* (K_m values = 1.18 and 0.86 m*M*, cellobiose) *(17)*, and *S. thermophile* (K_m value = 0.83 m*M* cellobiose) *(5)*. In addition, the K_m values of *A. niger* A20 cellobiases were lower than those reported for *S. lignicola* cellobiases I and II (K_m values = 2.85 and 5.0 m*M* cellobiose) *(18)*, and *T. viride* (K_m value = 2.5 m*M* cellobiose) *(25)*.

Amino Acid Composition

The three cellobiases were similar in being rich in aspartic acid, threonine, serine, and glutamic acid (Table 3). These amino acids contributed 57.0, 59.44, and 60.22% of the total amino acids of cellobiase A, B, and C, respectively, and were the predominant amino acids in the protein portion of β-glucosidase from *T. reesei (22)* and *P. funiculosum (3)*. The three cellobiases were poor in sulfur-containing amino acids; however, they differed significantly in their amino acid patterns.

Effect of Some Metal Ions

The effects of some metal ions on the activity of *A. niger* A20 cellobiases are summarized in Table 4. Of the metal ions investigated, Ca^{2+} stimulated the activity of cellobiase B and C, and Co^{2+} and Mg^{2+} stimulated cellobiase A. However, Hg^{2+} and iodine completely inhibited the three cellobiases.

Table 4
Effect of Various Chemicals
on Activity of Pure *Aspergillus niger* A20 Cellobiases

Reagent	Final molarity (m*M*)	Relative activity (%)		
		Cellobiase A	Cellobiase B	Cellobiase C
None		100.00	100.00	100.00
$CaCl_2$	5	95.73	112.2	104.32
	10	93.24	124.32	109.52
$CoCl_2$	5	107.32	98.32	88.0
	10	101.24	96.54	84.0
$FeSO_4$	5	99.83	97.36	96.3
	10	90.84	91.84	90.32
$CuSO_4$	5	43.40	36.24	43.05
	10	39.23	30.42	42.5
$CdSO_4$	5	64.93	53.74	52.22
	10	48.61	44.68	50.00
$MgSO_4$	5	109.30	97.63	112.63
	10	105.65	94.25	106.32
$ZnSO_4$	5	58.33	56.12	52.77
	10	55.55	48.07	48.61
Iodine	5	0.00	0.00	0.00
$HgCl_2$	5	0.00	0.00	0.00
$BaCl_2$	5	67.01	57.36	55.83
	10	51.70	54.64	55.50
EDTA[a]	5	100.30	60.90	55.00
	10	101.30	60.38	52.22
Cystein	5	98.30	98.70	96.80
	10	97.50	98.20	96.30
Cystine	5	96.30	98.10	94.00
	10	96.00	96.30	91.30

[a]Ethylenediaminetetraacetic acid.

On the other hand, Cu^{2+}, Cd^{2+}, Zn^{2+}, and Ba^{2+} showed a moderate inhibition of the three cellobiases. In general, *A. niger* A20 cellobiases react with these reagents in a manner very similar to other cellobiases from *A. terreus (4)*, *A. foetidieus (26)*, and *Evernia prunastri (27)*.

Activity Toward Various Substrates

The activities of the three purified cellobiases toward various substrates are presented in Table 5. No activity was detected against cellulose, maltose, and sucrose. Feeble or negligible activity was detected on lactose, xylan, CMC, and starch. *A. niger* cellobiases could hydrolyze both aryl-β-D-glucosides and cellobiose, which is a typical feature of most cellobiases *(3,17,26)*. However, the relative activities toward cellobiose and aryl β-D-glucosides depended on the source of the enzyme *(17)*. In general, the high specificity of *A. niger* A20 cellobiases justifies its suitability for enriching cellulolytic complexes defective in cellobiase, especially that of *Trichoderma*.

Table 5
Substrate Specificity of *Aspergillus niger* A20 Pure Cellobiases

Substrate	Substrate concentration	Specific activity (U/mg protein)		
		Cellobiase A	Cellobiase B	Cellobiase C
Cellobiose	12 mM	198.62	137.70	140.35
PNPG	12 m*M*	294.8	265.98	283.9
PNPGa	12 m*M*	5.33	9.47	8.18
PNPX	12 m*M*	6.36	5.23	12.8
Lactose	12 m*M*	2.80	2.40	4.50
Maltose	12 m*M*	0.00	0.00	0.00
Sucrose	12 m*M*	0.00	0.00	0.00
Xylan	1.0% (w/v)	0.36	0.24	0.24
CMC	1.0% (w/v)	1.36	1.00	0.94
Starch	1.0% (w/v)	1.16	0.21	0.04
Dextran	1.0% (w/v)	0.66	0.24	0.73
Cellulose (Avicell)	1.0% (w/v)	0.00	0.00	0.00

Transferase Activity

Transferase activity of *A. niger* A20 pure cellobiases was investigated at high concentration of cellobiose (5%, w/v), and the products were analyzed by TLC. The analyses showed large amounts of a component with the same mobility as cellotetraose, with traces traveling at the same speed as authentic cellotriose. *A. niger* cellobiases were similar to other fungal β-glucosidases in catalytransglycosylase reaction *(5,17,28)*. However, the principal product formed from cellobiose was a tetramer, rather than trimer, as in *T. koningii (17)*.

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