1,2-α-D-Mannosidase from *Penicillium citrinum*: molecular and enzymic properties of two isoenzymes

Takashi YOSHIDA, Takashi INOUE and Eiji ICHISHIMA

Laboratory of Molecular Enzymology, Department of Applied Biochemistry, Faculty of Agriculture, Tohoku University, 1-1, Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981, Japan

Two isoforms of acidic 1,2-α-D-mannosidases have been isolated from culture filtrate of *Penicillium citrinum*. The pI values of the two forms, designated 1,2-α-mannosidase Ia and Ib, were 4.6 and 4.7 respectively. Isoenzymes Ia and Ib exhibited the same molecular mass which was determined to be 53 kDa by SDS/PAGE and 54 kDa by gel-permeation chromatography. Enzymes Ia and Ib hydrolysed yeast mannan and 1,2-α-linked mannooligosaccharides, but did not hydrolyse *p*-nitrophenyl α-D-mannoside. The optimal pH for the hydrolysis of Man(α1 → 2)Man was 5.0 for both isoenzymes. Similar kinetic parameters were determined for the two forms. Activation energy was a little lower for Ia than Ib. There was little difference between the enzymes with regard to their performance at acidic or alkaline pH. The N-terminal amino acid sequences of the two enzymes were identical. Analysis of C-terminal peptides, which were prepared by tryptic digestion and anhydrotrypsin–agarose chromatography, showed that Ia and Ib had the same amino acid sequences in the C-terminal region. Tryptic digestion revealed a slight difference between the isoenzymes in the pattern of cleaved peptides on SDS/PAGE.

INTRODUCTION

The study of the biological function of glycoprotein glycans is very attractive in the field of cell biology [1]. α-Mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) is known to play an important role in the processing of mannose-containing glycans *in vivo*, since a deficiency in this enzyme results in the lethal disease, mannosidosis, a hereditary disease reported in humans [2] and cattle [3–5]. α-Mannosidases have also been employed in the analysis of mannose-containing glycans, for example, high-mannose-type sugar chains of glycoproteins [6]. During a course of study on the culture filtrate of *Penicillium citrinum*, we found 1,2-α-mannosidase activity which hydrolyses yeast mannan. The activity consists of two isoenzymes. So far, the existence of isoenzymes has been reported for α-mannosidases such as rat liver Golgi mannosidase [7], rat liver membrane-bound endoplasmic reticulum mannosidase [8] and yeast vacuolar mannosidase [9]. These are all multimeric enzymes and yeast vacuolar α-mannosidase is not specific for the 1,2-α-mannosidic linkage. We feel that *Penicillium* is worthy of investigation since the existence of isoenzymes for a monomeric 1,2-α-mannosidase has not previously been shown. The trivial name *Penicillium* 1,2-α-D-mannosidase is suggested for the enzyme. Here, we describe purification and characterization of two forms of 1,2-α-D-mannosidase from *P. citrinum*.

MATERIALS AND METHODS

Materials

A commercial product, 'Protease B', from *P. citrinum* was provided by Amano Pharmaceutical Co., Nagoya, Japan. Baker's yeast mannan, 1-deoxymannojirimycin, Man(α1 → 3)Man, Man(α1 → 2)Man-OMe, swainsonine, trypsin (EC 3.4.21.4) and α-chymotrypsin (EC 3.4.21.1) were purchased from Sigma, St. Louis, MO, U.S.A. Before use, Man(α1 → 3)Man was treated with *Aspergillus saitoi* 1,2-α-mannosidase [10] for 27 h at 30 °C and at pH 5.0 to digest contaminating Man(α1 → 2)Man. The mixture was then loaded on to a gel-permeation column (TSKgel G2000PW) and the peak of mannobiose was recovered and lyophilized. Man(α1 → 2)Man was prepared by controlled acetolysis of baker's yeast mannan, followed by gel filtration of the deacetylated products on a Bio-Gel P4 column [11]. Man(α1 → 6)Man, Man(α1 → 2)Man(α1 → 2)Man, Man(α1 → 2)Man(α1 → 2)Man-ol and Man(α1 → 2)Man(α1 → 2)Man(α1 → 2)Man were generously given by Dr. T. Nakajima of this laboratory. Man(α1 → 2)Man-ol and Man(α1 → 2)Man(α1 → 2)Man(α1 → 2)Man-ol were prepared by reduction of the corresponding mannobiose or mannotetraose with $NaBH_4$ [12]. Glycopeptidase F [EC 3.5.1.52; peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase] and anhydrotrypsin–agarose were purchased from Takara Shuzo Co., Kyoto, Japan. *p*-Nitrophenyl α-D-mannoside and other reagents were from Nacalai Tesque Co., Kyoto, Japan. *Aspergillus saitoi* carboxypeptidase (EC 3.4.16.1) was generously given by Dr. Y. Chiba of this laboratory.

Enzyme assays and protein determination

p-Nitrophenyl α-D-mannosidase activity was determined by the published method [13] at 30 °C and pH 5.0. For α-mannosidase determination, 100 μl of 2% baker's yeast mannan dissolved in 0.1 M sodium acetate/acetic acid buffer, pH 5.0, was added to 50 μl of enzyme solution. The incubation was carried out at 30 °C and the reaction was stopped by heating to 100 °C for 2 min. For a qualititative analysis, 15 μl of the reaction mixture was applied as a spot to Toyo no. 51 filter paper. For paper chromatography, the following solvent was used: butan-1-ol/pyridine/water (6:4:3, by vol.) Sugar spots were detected with the silver/NaOH dip reagent [14]. In a quantitative assay, released reducing sugar was determined by the method of Somogyi-Nelson [15]. One katal of 1,2-α-D-mannosidase was defined as the amount of enzyme required to liberate 1 mol of mannose from baker's yeast mannan per s at 30 °C and pH 5.0. For the study of optimal conditions and stability of the enzyme,

Abbreviations used: Man, α-D-mannopyranoside; Man-ol, mannitol; Man-OMe, methyl α-D-mannopyranoside; PVDF, poly(vinylidene difluoride).

reaction mixtures containing 50 mM sodium acetate/acetic acid buffer, pH 5.0, 20 μg of Man(α1 → 2)Man and 1 μg of enzyme in a total volume of 30 μl were incubated for 30 min at 30 °C. The reaction was arrested by heating to 100 °C for 2 min, and released mannose was determined by the method of Park and Johnson [16]. In these cases, mixtures that contained mannobiose but not enzyme were always used for blanks. When reaction products were analysed by gel-permeation h.p.l.c., samples were loaded on to a TSKgel G2000PW column (0.75 cm × 60 cm) which was run in water at the rate of 0.7 ml/min, and eluates were monitored with a differential refractometer (R401-type, Waters). Protein concentrations were measured by the method of Lowry et al. [17], with BSA as standard.

Purification of 1,2-α-D-mannosidase

All operations were carried out at below 4 °C unless otherwise indicated. Crude enzyme preparation (30 g) obtained from a commercial product, Protease B, from *P. citrinum* was dissolved in 300 ml of 10 mM sodium acetate/acetic acid buffer, pH 5.0. After the pH of the solution had been adjusted to 5.0 with 1 M HCl, 60–90 %-satd. $(NH_4)_2SO_4$ precipitate was collected by centrifugation for 30 min at 10000 *g*. The precipitate was dissolved in 200 ml of 10 mM sodium acetate/acetic acid buffer, pH 5.0, and enzymes in the solution were precipitated by adding methanol up to 65 % concentration. The precipitate was collected by centrifugation for 20 min at 10000 *g* and dissolved in 100 ml of 10 mM sodium acetate/acetic acid buffer, pH 5.0, followed by dialysis against the same buffer. The dialysed solution was applied to a column (5 cm × 25 cm) of DEAE-TOYOPEARL 650M equilibrated with 10 mM sodium acetate/acetic acid buffer, pH 5.0, and the column was eluted with a linear gradient of 0–0.25 M NaCl in 10 mM sodium acetate/acetic acid buffer, pH 5.0. Active fractions were pooled, dialysed against 20 mM sodium acetate/acetic acid buffer, pH 4.0, and applied to a column (2.2 cm × 17.5 cm) of SP-TOYOPEARL 650M equilibrated with the same buffer at the same pH, and the column was eluted with a linear gradient of pH from 4.0 to 5.5 in 20 mM sodium acetate/acetic acid buffer. Active fractions were pooled and applied to a column (1.2 cm × 31 cm) of DEAE-TOYOPEARL 650S equilibrated with 20 mM sodium acetate/acetic acid buffer, pH 5.0. Elution was performed in 20 mM sodium acetate/acetic acid buffer, pH 4.0, and fractions containing isoenzyme Ia or Ib were collected separately. Both of the effluents were dialysed against 20 mM sodium acetate/acetic acid buffer, pH 5.0, and kept at 4 °C until use.

To determine molecular mass by h.p.l.c., samples were loaded on to a gel-permeation column (Shodex protein WS-803F) which was run in 10 mM Tris/HCl buffer (pH 8.3) containing 0.3 M Na_2SO_4 at the rate of 0.5 ml/min, and eluates were monitored at 215 nm.

PAGE

PAGE was carried out by the method of Davis [18], and SDS/PAGE by the method of Laemmli [19]. After electrophoresis, gels were stained with Coomassie Brilliant Blue. Isoelectric focusing was performed by the method of Garfin [20] using a Bio-Rad Model 111 Mini isoelectric focusing cell. In the case where the protein was recovered from the gel after PAGE, gel was soaked in a solution containing 0.05 % Serva Blue G and 5 % acetic acid for 15 min at 4 °C. The stained band was sliced off and homogenized by the method of Kobayashi et al. [21], then extracted overnight in 20 mM sodium acetate/acetic acid buffer, pH 5.0, at 4 °C. The suspension was filtered through glass-wool and the filtrate was assayed for 1,2-α-D-mannosidase activity with mannobiose and yeast mannan as substrates.

Glycopeptidase F digestion

The isoenzyme Ia or Ib (approx. 5 μg each of protein) was boiled for 5 min in a solution containing 10 mM Tris/glycine, pH 8.3, and 0.04 % of SDS in a total volume of 52 μl, then glycopeptidase F (0.75 unit in 3 μl) was added and incubated for 24 h at 37 °C. The reaction was stopped by boiling for 2 min and the mixture was analysed by SDS/PAGE (15 % polyacrylamide gel).

Western blotting

Isoenzymes Ia and Ib (0.08 and 0.1 μg of protein respectively) were separated by PAGE at pH 9.4 and transferred to poly-(vinylidine difluoride) (PVDF) membrane using a semi-dry electroblotter (Trans-Blot SD, Bio-Rad, Richmond, CA, U.S.A.). The membrane was then soaked in T-PBS [10 mM sodium phosphate-buffered saline, pH 7.2, with 0.05 % (v/v) Tween 20] containing 3 % (w/v) skimmed milk for 1 h at 37 °C. After three washes in T-PBS, the membrane was soaked in PBS (10 mM sodium phosphate-buffered saline, pH 7.2) containing 1:200 dilution of antibody raised in rabbit against purified *Penicillium* 1,2-α-mannosidase Ib for 30 min at room temperature. After three more washes in T-PBS, the antigen–antibody complex was detected using goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Seikagaku Co., Tokyo, Japan) with 3,3′-diaminobenzidine and H_2O_2 as substrates in 50 mM Tris/HCl, pH 7.2.

N-Terminal sequence analysis

Purified enzyme was applied to a gel-permeation column (Shodex protein WS-803F) and h.p.l.c. was performed in 0.3 M ammonia/acetic acid buffer, pH 6.9, at the rate of 0.5 ml/min. Eluates were monitored at 225 nm, and the fraction containing the peak of protein was recovered and concentrated to approx. 30 μl, and then spotted on to a small piece of PVDF membrane with gentle aspiration from the rear. The membrane was then rinsed in 10 % methanol/water several times and dried. N-Terminal amino acid analysis was performed on the membrane using an Applied Biosystems (ABi) 473A gas–liquid–solid-phase protein sequenator with an Applied Biosystems 610A data-analysis system.

Analysis of a C-terminus-containing peptide

Preparation of a C-terminus-containing peptide was performed fundamentally as described by Ishii and Yokosawa [22,23]. Isoenzyme Ia, Ib or Ia and Ib (approx. 75 μg, 132 μg or 120 μg of protein respectively) was boiled for 5 min in a solution containing 40 mM Tris/HCl, pH 8.0, 2 mM $CaCl_2$, 0.05 % SDS and 2.5 % 2-mercaptoethanol in a total volume of 50 μl. Then *N*-Tos-Phe-CH_2Cl (TPCK)-treated trypsin (1.1 μg of protein in 1 μl) was added and the mixture incubated for 72 h at 4 °C. After the addition of 5 μl of 1 M sodium acetate/acetic acid buffer, pH 5.0, the mixture was applied to a minicolumn of anhydrotrypsin–agarose (100 μl of gel in a yellow tip) which had been equilibrated in 50 mM sodium acetate/acetic acid buffer, pH 5.0. After the mixture had completely soaked into the gel, the column was washed in 100 μl of the same buffer. At this pH the gel will absorb peptides made by trypsin digestion, with arginine or lysine residues at the C-terminus. As a result, a peptide containing the C-terminal residue of the original protein will flow through

the gel if the C-terminal amino acid is not arginine or lysine. The first fraction eluted was recovered and analysed by h.p.l.c. using a Lichrospher 100 CN (5 μm) column (125 mm × 4 mm) with a linear gradient (0.8–64 % in 80 min) of acetonitrile in 0.1 % trifluoroacetic acid at a flow rate of 0.6 ml/min. Eluates were monitored at 215 nm. Eluted peptide was recovered, dried and analysed on an Applied Biosystems (ABi) 473A gas–liquid–solid-phase protein sequenator.

Peptide mapping

Isoenzyme Ia or Ib (approx. 11 μg each of protein) was boiled for 5 min in a solution containing 40 mM Tris/HCl, pH 8.0, 4 mM $CaCl_2$ and 0.04 % SDS in a total volume of 23 μl; then trypsin (1.02 units in 1 μl) was added and the mixture incubated for 1 h at 37 °C. The digestion was stopped by the addition of 5 μl of 10 % SDS solution followed by boiling for 2 min. Digested samples were analysed by SDS/PAGE (18 % polyacrylamide gel). In this experiment, the amount of trypsin was below the level detectable by Coomassie Brilliant Blue staining. In the case where a peptide fragment was recovered after PAGE, gel was stained with Coomassie Brilliant Blue and the specific band sliced out. Extraction of the peptide from the gel was performed as described by Tsugita et al. [24]. A piece of gel was soaked in a solution of 70 % formic acid for one night at room temperature. The solution was concentrated and spotted on to a small piece of PVDF membrane which was then washed in 10 % methanol/water several times. After drying, the membrane was used for amino acid sequencing by an Applied Biosystems (ABi) 473A gas–liquid–solid-phase protein sequenator.

RESULTS

Purification of *Penicillium* 1,2-α-D-mannosidase

1,2-α-D-Mannosidase activity could be separated from *p*-nitrophenyl α-D-mannosidase by the first DEAE-TOYOPEARL 650M chromatographic step. When the enzyme preparation from the first DEAE-TOYOPEARL 650M chromatography was analysed by PAGE at pH 9.4, two protein bands were observed close together with many contaminating proteins. After SP-TOYOPEARL 650M chromatography, the enzyme fraction contained the two bands as major components. To discover which protein band is responsible for the activity, we made a preliminary characterization of the enzyme at this step. Each band was cut separately from the gel after PAGE (recovery of each protein was 33 % for the lower band and 30 % for the upper one), and the protein extracts were examined for α-mannosidase activity with yeast mannan as substrate. Release of mannose was observed for each protein (results not shown). In this experiment, we preliminarily checked that neither enzyme preparation produced a spot of mannose without the presence of mannan on paper chromatography.

When each protein was incubated with Man(α1 → 2)Man, Man(α1 → 3)Man or Man(α1 → 6)Man for 2 h at 30 °C and the mixture was analysed by gel-permeation h.p.l.c., only Man(α1 → 2)Man was shown to be cleaved by each protein. We labelled the faster moving protein 1,2-α-D-mannosidase Ia and the slower moving one 1,2-α-D-mannosidase Ib. Then a preparation containing the two forms was applied to the second DEAE-TOYOPEARL 650S column, which was eluted in 20 mM sodium acetate/acetic acid buffer, pH 4.0. The two forms were separated by this chromatographic step: enzyme Ib was eluted faster than enzyme Ia, and at this step each enzyme showed a single band on PAGE under native conditions (Figure 1a) and on SDS/PAGE (Figure 1b). Recoveries of protein and activity were a little higher for enzyme Ib than Ia (Table 1).

Enzyme characterization

The optimum pH for the hydrolysis of Man(α1 → 2)Man was determined to be 5.0 for both enzymes. At an acidic pH, enzyme Ia showed approx. 20 % of the activity at pH 3.0 and no activity at pH 2.0, whereas enzyme Ib showed approx. 70 % and 30 % of the activity at pH 3.0 and pH 2.0 respectively. At a neutral pH, both enzymes showed approx. 50 % of the activity at pH 6.0 and no activity at pH 7.0. After incubation for 2 h at 30 °C, both enzymes were most stable in a range of pH from 6.0 to 7.0. At pH 3, enzymes Ia and Ib retained approx. 60 % and 30 % of the activity respectively. At pH 10, enzyme Ia retained approx. 40 % of the activity but Ib had no activity. Thermal characteristics were almost identical for the two enzymes: the highest activity was observed at 50 °C. Both enzymes lost approx. 60 % of activity after preheating to 60 °C for 10 min.

Both of the enzymes were partially inhibited with 10 mM $CuSO_4$ (70), $CoCl_2$ (30), $FeSO_4$ (60), $ZnCl_2$ (75) and $AgNO_3$ (60) (the values in parentheses are the percentage inhibition). Addition of 10 mM $CaCl_2$ or $MgCl_2$ to the reaction mixture had no effect on the activities. EDTA at 1 mM concentration had a slight

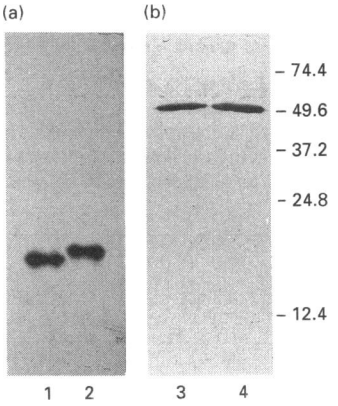

Figure 1 PAGE and SDS/PAGE of the two forms of 1,2-α-mannosidase from *P. citrinum*

Purified enzymes Ia and Ib after the second DEAE-TOYOPEARL chromatography were analysed by PAGE at pH 9.4 under native conditions (**a**) or by SDS/PAGE (**b**). Lanes 1 and 3, enzyme Ia; lanes 2 and 4, enzyme Ib. In (**b**) the positions of molecular-mass markers (cross-linked cytochrome *c* proteins) are indicated in kDa at the right side of the gel. To each lane, 3.3 μg (lanes 1–3) or 3.6 μg (lane 4) of protein was applied.

Table 1 Purification of 1,2-α-D-mannosidase from *P. citrinum*

Enzyme activity (with yeast mannan as substrate) and protein concentrations were determined as described in the Materials and methods section.

Step	Total activity (nkat)	Total protein (mg)	Specific activity (mkat/kg)	Purification (fold)	Yield (%)
Crude extract	92.2	635	0.1	1	100
DEAE-TOYOPEARL 650M	16.5	59.6	0.3	3	18
SP-TOYOPEARL 650M	6.4	2.5	2.6	26	7
DEAE-TOYOPEARL 650S					
Isoform Ia (pI 4.6)	1.1	0.3	4.0	40	1
Isoform Ib (pI 4.7)	2.8	0.9	3.1	31	3

Table 2 Hydrolysis of various manno-oligosaccharides

Reaction mixtures that contained 50 mM sodium acetate/acetic acid buffer, pH 5.0, manno-oligosaccharides (10 μg each) and 0.4 μg of enzyme in a total volume of 30 μl were incubated for 40 min at 30 °C. The reaction was stopped by heating to 100 °C for 2 min, and released mannose was determined by the method of Park and Johnson [16]. Man(α1 → 3)Man was pretreated with *Aspergillus saitoi* 1,2-α-mannosidase to eliminate contaminating Man(α1 → 2)Man as described in the Materials and methods section. Assays were performed in duplicate at least and the mean values are given. In all assays, mixtures that contained manno-oligosaccharide but not enzyme were used for blanks.

Substrate	Mannose released by *Penicillium* 1,2-α-D-mannosidase (mmol/s per kg of protein)	
	Ia	Ib
Man(α1 → 2)Man	29.5	30.9
Man(α1 → 3)Man	0.0	0.0
Man(α1 → 6)Man	0.0	0.0
Man(α1 → 2)Man(α1 → 2)Man	30.1	32.9
Man(α1 → 2)Man(α1 → 2)Man(α1 → 2)Man	22.3	24.9
Man(α1 → 2)Man-OMe	30.4	32.4
Man(α1 → 2)Man-ol	0.0	0.0
Man(α1 → 2)Man(α1 → 2)Man-ol	7.9	–
Man(α1 → 2)Man(α1 → 2)Man(α1 → 2)Man-ol	17.0	–

Table 3 Kinetic parameters toward Man(α1-2)Man-OMe

Reaction mixtures that contained 50 mM sodium acetate/acetic acid buffer, pH 5.0, up to 32 μg of Man(α1 → 2)Man-OMe and 0.4 μg of enzyme in a total volume of 30 μl were incubated for 10 min at 20 °C, 30 °C, or 40 °C. The reaction was stopped by adding 70 μl of 0.2 M Na_2CO_3 and chilling in an iced bath. Released mannose was determined by the method of Park and Johnson [16]. Assays were performed twice in triplicate and the values are given as means ± S.D.

Enzyme	Temperature (°C)	$10^4 \times K_m$ (M)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	E_a (kJ · mol^{-1})
Ia	20	4.7 ± 0.2	1.6 ± < 0.1	3.4	
	30	5.2 ± 0.2	2.5 ± < 0.1	4.9	34 (20–30 °C)
	40	5.2 ± 0.1	3.3 ± < 0.1	6.4	22 (30–40 °C)
Ib	20	5.7 ± 0.2	1.9 ± < 0.1	3.4	
	30	6.0 ± 0.2	3.1 ± < 0.1	5.2	36 (20–30 °C)
	40	7.4 ± 0.2	4.3 ± < 0.1	5.9	26 (30–40 °C)

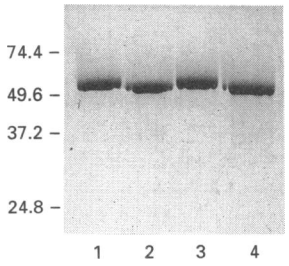

Figure 2 Changes in the electrophoretic mobility of 1,2-α-mannosidases from *P. citrinum* caused by glycopeptidase F treatment

Purified proteins of enzymes Ia and Ib were treated with glycopeptidase F and analysed by SDS/PAGE as described in the Materials and methods section. Lane 1, untreated enzyme Ia; lane 2, glycopeptidase-treated enzyme Ia; lane 3, untreated enzyme Ib; lane 4, glycopeptidase-treated enzyme Ib. The positions of molecular-mass markers (cross-linked cytochrome *c* proteins) are indicated in kDa at the left side of the gel. To each lane, approx. 2.5 μg of protein was applied.

inhibitory effect (approx. 10% inhibition) on the enzymes. Swainsonine, a potent inhibitor of rat liver mannosidase II [25], did not inhibit *Penicillium* enzymes Ia and Ib even at a concentration as high as 3 mM. Both enzymes were inhibited by 1-deoxymannojirimycin, an inhibitor of rat liver Golgi mannosidase IA [26], with an IC_{50} of approx. 200 μM. This was a fairly high concentration compared with that for rat Golgi mannosidase IA for which IC_{50} was 1 μM [26]. In the case of the *Penicillium* enzymes, inhibition by the drug was not linear with its concentration, and approx. 10% of the activity was observed in the presence of a concentration of the drug of 1 mM.

Specificity and kinetic studies

Enzymes Ia and Ib hydrolysed baker's yeast mannan, producing free mannose, but did not act on *p*-nitrophenyl α-D-mannoside at all. Mannobioses, mannotrioses, mannotetraoses and derivatives were also examined for hydrolysis by both enzymes, and both showed a specificity toward the 1,2-α-D-mannosidic linkage (Table 2). As to the derivatives, Man(α1 → 2)Man-OMe was cleaved a little more efficiently than Man(α1 → 2)Man, whereas Man(α1 → 2)Man-ol was not a substrate (Table 2).

To compare the activation energy (E_a) between enzymes Ia and Ib, kinetic parameters for the hydrolysis of Man(α1 → 2)Man-OMe were determined at 20 °C, 30 °C and 40 °C (Table 3). We observed no great difference in any parameter between the two enzymic forms, although Ia had somewhat lower K_m values than Ib, and Ib had somewhat higher $k_{cat.}$ values than Ia at each temperature. E_a was always a little lower for Ia than for Ib at each range of temperature.

Molecular properties

Enzymes Ia and Ib showed slightly different mobilities for each other during PAGE performed at pH 9.4 (Figure 1a), although they were indistinguishable when PAGE was performed at pH 4.3. The pI values were determined by isoelectric focusing to be 4.6 for Ia and 4.7 for Ib. The two forms of the enzyme showed the same electrophoretic mobilities on SDS/PAGE (Figure 1b), from which the molecular mass was deduced to be 53 kDa. On h.p.l.c. analysis using a Shodex protein WS-803F gel-permeation column, each form of the enzyme showed a single peak at the same retention time, which corresponded to a molecular mass of 54 kDa. When a preparation containing both Ia and Ib was loaded on to the column, only a single peak was observed. These results caused us to assume that the molecular masses of the two forms were almost the same. Enzymes Ia and Ib could be distinguished only by PAGE performed under native conditions at pH 9.4.

Treatment of the enzymes with glycopeptidase F in a denatured condition caused a slight change in mobility on SDS/PAGE (Figure 2). Although we could not determine it exactly, the decrease in molecular mass was less than 1000 Da for both forms. Both enzymes were suggested to have a short chain of asparagine-linked oligosaccharide.

Native proteins of Ia and Ib were tested for susceptibility to proteolysis. Treatment with trypsin (specific for lysine and arginine, proteinase/protein ratio 1:10, w/w) for 36 h at pH 8.0 and at 30 °C, with α-chymotrypsin (specific for aromatic amino acids, proteinase/protein ratio 1:5, w/w) for 48 h at pH 8.0 and at 30 °C or with *Aspergillus saitoi* carboxypeptidase (wide range of specificity toward C-terminal amino acids [27], proteinase/protein ratio 1:5, w/w) for 48 h at pH 3.0 and at 30 °C did not change the electrophoretic mobility of enzymes Ia and Ib on PAGE (results not shown). We do not know the reason, but

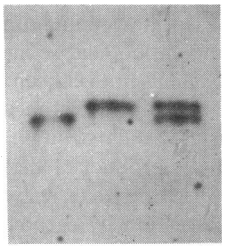

Figure 3 Immunoblot analysis of *Penicillium* 1,2-α-mannosidase Ia and Ib

The enzymes were electrophoresed on polyacrylamide gel at pH 9.4 and transferred to PVDF membrane, which was treated with anti-(enzyme Ib) serum as described in the Materials and methods section. Lane 1, enzyme Ia; lane 2, enzyme Ib; lane 3, enzymes Ia and Ib.

native proteins of Ia and Ib were supposed to be somewhat resistant to proteolytic digestion.

Antibody test

Immunological cross-reactivity was tested by using specific antibody raised in rabbit against purified *Penicillium* 1,2-α-mannosidase Ib. When Western-blotting analysis was performed using the serum, anti-(enzyme Ib) reacted with enzyme Ib as well as enzyme Ia (Figure 3). In another analysis by the Ouchterlony double-diffusion technique [28], the serum cross-reacted with both enzymes. The precipitation lines of each enzyme fused completely. These results suggest that the two isoenzymes of *Penicillium* 1,2-α-mannosidase can be regarded as being immunologically homologous.

N-Terminal analysis

It was difficult to prepare enzymes Ia and Ib for amino acid sequence analysis using reversed-phase h.p.l.c.: only slight recovery could be achieved from an octadecyl-silyl or octyl-silyl silica-gel column even at high concentration of organic solvent. Electroblotting of enzymes after SDS/PAGE on to PVDF membrane is known to be a good method, but in our case the amount of blotted enzymes was insufficient to obtain clear data. By using a gel-permeation column for h.p.l.c. and PVDF membrane instead, we were able to determine the N-terminal amino acid residues up to the 14th cycle of sequencing (Figure 4). The two forms of the enzyme had the same amino acid at the N-terminus, which was determined to be serine, and the following thirteen amino acid residues were also identical.

Analysis of a C-terminus-containing peptide

Proteins of enzymes Ia and Ib were completely digested by prolonged digestion with trypsin at low temperature. We found that these conditions gave reproducible and efficient fragmentation. When a preparation of enzyme Ia or Ib after tryptic digestion was loaded on to an anhydrotrypsin–agarose column and the flow-through fraction was analysed by h.p.l.c., a single peak was observed in each case on a gradient of acetonitrile at the retention time (R_t) of 33 min. We had preliminarily checked that autodigested trypsin did not give any peaks around that time on h.p.l.c. For each preparation of Ia and Ib, a peak of R_t 33 min was recovered, dried and analysed by protein sequenator. The two peptides showed the same sequence (Figure 4). In both cases, peaks of amino acid appeared and could be identified up to the 11th cycle of sequencing (tyrosine) and no peak was detected after the 12th cycle. When a preparation containing

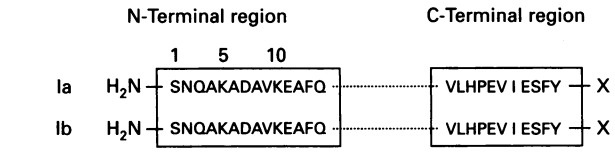

Figure 4 Amino acid sequences of the N-terminal and C-terminal regions of 1,2-α-mannosidases Ia and Ib from *P. citrinum*

Rectangles indicate identity. X means C-terminal amino acid residue.

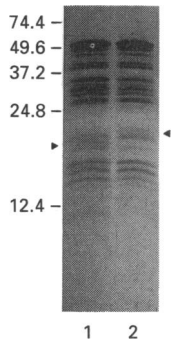

Figure 5 Tryptic patterns of 1,2-α-mannosidases Ia and Ib on SDS/PAGE

Enzymes Ia and Ib were digested with trypsin and analysed by SDS/PAGE as described in the Materials and methods section. Lane 1, digested enzyme Ia; lane 2, digested enzyme Ib. The positions of molecular-mass markers (cross-linked cytochrome *c* proteins) are indicated in kDa at the left side of the gel. Arrowheads indicate 20 kDa fragment (lane 1) and 22 kDa fragment (lane 2).

both Ia and Ib was digested by trypsin, loaded on to the anhydrotrypsin column and the flow-through fraction analysed by h.p.l.c., a single peak appeared at R_t 33 min. On amino acid sequencing of the peptide, we confirmed that the first residue was valine and the following ten residues were identical with those of Ia or Ib. In this case also, there was no detectable peak after the 12th cycle of sequencing. These results suggest that enzymes Ia and Ib have the same amino acid sequences in the C-terminal region.

Peptide mapping

When denatured proteins of Ia and Ib were partially digested by trypsin and analysed by SDS/PAGE, all but a few of the cleaved peptides showed a similar pattern on SDS/PAGE (Figure 5). A few unique fragments were observed for each form of the enzyme: for example, a 20 kDa fragment was observed only in Ia and a 22 kDa fragment was observed only in Ib. The 22 kDa peptide could be recovered from the gel and analysed on the amino acid sequenator. We determined the partial amino acid sequence of the peptide as follows: NGWGAXAVDALXTAV-IMG (X means an unidentified residue). We have not yet succeeded in sequencing the 20 kDa fragment. Digestion of enzymes Ia and Ib with other proteinases, *Achromobacter lyticus* proteinase I (EC 3.4.21.50, specific for lysine), α-chymotrypsin and *Staphylococcus aureus* V8 proteinase (EC 3.4.21.19, specific for glutamate and aspartate), was examined, but no clear and reproducible differences in the patterns of fragments produced for the two isoenzymes were observed on SDS/PAGE.

DISCUSSION

We purified two forms of 1,2-α-mannosidase from culture filtrate of *P. citrinum*. These enzymes showed only a few but definite differences from most other mammalian 1,2-α-mannosidases

[26,29,30]. The *Penicillium* enzymes did not require detergents for maintenance of activity, were not activated by Ca^{2+} and showed greater resistance to 1-deoxymannojirimycin. 1-Deoxymannojirimycin is a mannose analogue known to inhibit mammalian 1,2-α-mannosidases [31]. Whereas rabbit liver 1.2-α-mannosidase is inhibited by 1-deoxymannojirimycin with a K_i of 7 μM [32], the *Penicillium* enzymes Ia and Ib are only inhibited by such high concentrations of the drug that they can actually be viewed as being resistant to it.

When various manno-oligosaccharides were examined for hydrolysis by the *Penicillium* enzymes Ia and Ib, both forms of the enzyme cleaved specifically 1,2-α-linked manno-oligosaccharides. The rate of hydrolysis was shown to be affected by the terminal structure at the reducing side of the substrate. For instance, Man(α1 → 2)Man-OMe was cleaved a little more efficiently than Man(α1 → 2)Man, whereas Man(α1 → 2)Man-ol was not cleaved by the enzymes at all. On the other hand, the hydrolysis of Man(α1 → 2)Man was not affected at all by the presence of Man(α1 → 2)Man-ol even at the same concentration (results not shown). When Man(α1→2)Man(α1 → 2)Man-ol and Man(α1 → 2)Man(α1 → 2)Man(α1 → 2)Man-ol were examined for cleavage, more mannose was released as the sugar chain became longer, although in these cases the rates of hydrolysis were still slower than for non-reduced oligomannoses. These results suggest that Man(α1 → 2)Man-ol has far less affinity for the enzyme than Man(α1 → 2)Man, and at least a two-pyranose-ring structure is required for *Penicillium* 1,2-α-mannosidases to accept manno-oligosaccharides at the catalytic sites.

Several types of isoenzyme have been reported for multimeric α-mannosidases to date [7–9]. Rat liver Golgi mannosidases IA and IB have been reported to show different behaviour during purification and on gel-permeation chromatography [26], suggesting that the two proteins have different pI values and molecular mass. In the case of the soluble form of rat liver mannosidase and membrane-bound endoplasmic reticulum α-mannosidase, the soluble form of the enzyme is supposed to be derived from endoplasmic reticulum membrane α-mannosidase by proteolysis [8]. In yeast α-mannosidase, each isoenzyme has a different number of subunits (107 kDa, 73 kDa and 31 kDa) making up the multimeric enzyme [9]. In this case, the 107 kDa subunit was supposed to give 73 kDa and 31 kDa subunits by proteolysis [9]. Compared with these cases, it may be unique that *Penicillium* 1,2-α-mannosidases Ia and Ib exhibited the same molecular mass, and they differed from each other in pI by only 0.1. We could not detect a difference in molecular mass between the two by SDS/PAGE and gel-permeation h.p.l.c. Many of the properties of enzymes Ia and Ib were similar: both showed maximum activity at pH 5.0 with the same substrate specificity, did not require Ca^{2+} for their activities, shared a similar sensitivity for glycosidase inhibitors and showed immunological identity. Their kinetic parameters with regard to Man(α1 → 2)Man-OMe were slightly different. Enzymes Ia and Ib showed a difference in their performances at acidic and alkaline pH.

At present, we do not know the exact mechanism that gives rise to isoenzymes of a specific α-mannosidase having a similar nature in this mould, but analysis of some parts of their structures has given clues for speculation. First, N-terminal amino acid analysis has revealed that enzymes Ia and Ib have the same sequences at the N-terminus. Secondly, by combined usage of anhydrotrypsin–agarose, h.p.l.c. and amino acid sequenator, we have obtained information on the structures of the C-terminal regions of Ia and Ib. C-Terminus-containing peptides from Ia and Ib exhibited the same amino acid sequences. Although the C-terminal amino acid itself has not yet been determined, the two peptides appear to have the same amino acid sequences next to the C-terminal residue. These results strongly suggest that isoform Ia or Ib is not a proteolytic product of the other form. This is consistent with the observation that enzymes Ia and Ib are resistant to proteolytic digestion by carboxypeptidase. Peptide mapping after tryptic digestion showed a few unique fragments for each enzyme, suggesting the presence of a few differences in amino acid sequence within the polypeptides. The existence of duplicated genes for isoenzymes is known for Taka-amylase A in *Aspergillus oryzae* [33]. In the case of *Penicillium* 1,2-α-mannosidase, further analysis of the protein and gene structures should provide a clear answer to the mechanism causing the two isoenzymes.

This study was supported in part by a grant from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1 Olden, K., Bernard, B. A., Humphries, M. J., Yeo, Tet-K., Yeo, Kiang-T., White, S. L., Sheila, A. N., Bauer, H. C. and Parent, J. B. (1985) Trends Biochem. Sci. **10**, 78–82
2 Ockerman, P. A. (1967) Lancet **ii**, 239–241
3 Phillips, N. C., Robinson, D., Winchester, B. C. and Jolly, R. D. (1974) Biochem. J. **137**, 363–371
4 Norden, N. C., Lundblad, A., Ockerman, P. A. and Jolly, R. D. (1973) FEBS Lett. **35**, 209–212
5 Burditt, L. J., Phillips, N. C., Robinson, D., Winchester, B. G., Van De Water, N. S. and Jolly, R. D. (1978) Biochem. J. **175**, 1013–1022
6 Bischoff, J., Liscum, L. and Kornfeld, R. (1986) J. Biol. Chem. **261**, 4766–4774
7 Tulsiani, D. R. P., Hubbard, S. C., Robbins, P. W. and Touster, O. (1982) J. Biol. Chem. **257**, 3660–3668
8 Bischoff, J. and Kornfeld, R. (1986) J. Biol. Chem. **261**, 4758–4765
9 Yoshihisa, T., Ohsumi, Y. and Anraku, Y. (1988) J. Biol. Chem. **263**, 5158–5163
10 Ichishima, E., Arai, M., Shigematsu, Y., Kumagai, H. and Sumida-Tanaka, R. (1981) Biochim. Biophys. Acta **658**, 45–53
11 Stewart, T. S., Mendershausen, P. B. and Ballou, C. E. (1968) Biochemistry **7**, 1843–1854
12 Takahashi S. and Kobata, A. (1974) J. Biochem. **76**, 783–788
13 Matta, K. L. and Bahl, Om. P. (1972) J. Biol. Chem. **247**, 1780–1787
14 Anet, E. F. L. J. and Reynold, T. M. (1954) Nature (London) **174**, 930
15 Somogyi-Nelson, M. (1952) J. Biol. Chem. **195**, 19–23
16 Park, J. T. and Johnson, M. G. (1949) J. Biol. Chem. **181**, 149–151
17 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
18 Davis, B. J. (1964) Ann. N. Y. Acad. Sci. **121**, 404–427
19 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
20 Garfin, D. E. (1990) Methods Enzymol. **182**, 459–477
21 Kobayashi, M., Hiura, N. and Matsuda, K. (1985) Anal. Biochem. **145**, 351–353
22 Ishii, S., Yokosawa, H., Shibata, S. and Kasai, K. (1979) Adv. Exp. Med. Biol. **120A**, 15–27
23 Yokosawa, H. and Ishii, S. (1979) Anal. Biochem. **98**, 198–203
24 Tsugita, A., Ataka, T. and Uchida, T. (1987) J. Protein Chem. **6**, 121
25 Tulsiani, D. R. T., Harris, T. M. and Touster, O. (1982) J. Biol. Chem. **257**, 7936–7939
26 Tulsiani, D. R. T. and Touster, O. (1988) J. Biol. Chem. **263**, 5408–5417
27 Ichishima, E. (1991) Comments Agric. Food Chem. **2**, 279–298
28 Ouchterlony, O. and Nilson, L. A. (1973) in Handbook of Experimental Immunology (Weir, O. M., ed.), 2nd edn., vol. 1, pp. 19.1–19.39, Blackwell, Oxford
29 Forsee, W. T., Palmer, C. F. and Schutzbach, J. S. (1989) J. Biol. Chem. **264**, 3869–3876
30 Schweden, J., Legler, G. and Bause, E. (1986) Eur. J. Biochem. **157**, 563–570
31 Elbein, A. (1987) Annu. Rev. Biochem. **56**, 497–534
32 Schutzbach, J. S. and Forsee, W. T. (1990) J. Biol. Chem. **265**, 2546–2549
33 Tsukagoshi, N., Furukawa, M., Nagaba, H., Kirita, N., Tsuboi, A. and Udaka, S. (1989) Gene **84**, 319–327

Received 26 June 1992/25 August 1992; accepted 7 September 1992