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Purification and Characterization of an α-Mannosidase from the Tropical Fruit Babaco (*Vasconcellea* × *Heilbornii* Cv. Babaco)

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An α-mannosidase (EC 3.2.1.24) present in the lyophilized latex of babaco (*Vasconcellea heilbornii*) has been purified to apparent homogeneity by native PAGE. The purification involves a three-step procedure with successive anion exchange with Q Sepharose HP, lectin affinity chromatography using ConA Sepharose 4B, and gel filtration using Superdex 200 prep grade. The molecular mass was determined to be in the range of 260−280 kDa by Superdex 200 prep grade gel filtration, and isoelectric focusing showed a p*I* range between 5.85 and 6.55, suggesting different glycosylated isoforms. The optimal temperature for the α-mannosidase was determined to lie between 50 and 60 °C, and the optimal pH was 4.5 at 50 °C. The K_m value for *p*-nitrophenyl α-mannopyranoside (pNPM) was found to be 1.25 mM and the V_{max}, 2.4 μkat mg^{-1} at 50 °C and 1.94 μkat mg^{-1} at 40 °C. The pure α-mannosidase was specific for mannose and did not display activity for any other tested synthetic substrates.

KEYWORDS: α-Mannosidase; babaco; latex; purification; characterization; subunit composition

INTRODUCTION

Babaco (*Vasconcellea heilbornii*) is a fruit native to Ecuador, which is grown extensively and widely appreciated for its fresh taste and pleasant aroma. High production in combination with being used mainly as a fresh fruit or for juice production has led to market saturation. The present work is a part of a project to increase the understanding of the enzymes present in babaco and to investigate enzymes that could be of scientific and/or commercial importance. This study was initiated as a collaboration between the Department of Physical and Analytical Chemistry, Surface Biotechnology, Uppsala University, Sweden, and the Food Science and Biotechnology Department, Escuela Politécnica Nacional, Ecuador. During the course of the initial studies it was found that babaco exhibited considerable α-manosidase activity. It has been shown earlier that the latex of green fruits of papaya, a close relative to babaco and well-known for its high content of proteases (*1, 2*), also contain significant levels of some glycosidases, notably β-glucosisdase, β-galactosidase, and α-mannosidase (*3, 4*). Papaya is, to our knowledge, the only relative for which an α-mannosidase has been described. Babaco and its closest relatives have recently been rehabilitated into their own genus of *Vasconcellea* and are thus no longer considered to be a section of the *Carica* (*5*−*7*) genus. The close relationship between *Carica* and the *Vasconcellea* genera, as seen by the similarities in terms of the enzymes studied so far, can provide useful clues of information with regard to enzymes yet to be investigated. A recent example of such similarities can be seen in a study of the cysteine proteases present in babaco (*8*).

Sugar-degrading enzymes, *glycosidases*, play key roles in many biological processes (*9, 10*). Plant α-mannosidases are believed, together with other glycosylases, to take part in the degradation of polysaccharides, glycoproteins, and glycoconjugates, both in seed germination as well as in fruit development. There are not a large number of plant α-mannosidases isolated and characterized to date, even though the *Canavalia ensiformis* (jack bean) α-mannosidase has been extensively studied (*11*−*14*). The identification of jack bean α-mannosidase as a zinc metalloenzyme requiring Zn^{2+} for full activity (*12*) has proven to be true also for other studied plant α-mannosidases. As part of our work in studying the enzymes of the babaco fruit we here describe the purification and characterization of an α-mannosidase present in the latex of the fruit.

MATERIALS AND METHODS

Latex Collection and Extractions. Lyophilized latex was obtained from Escuela Politecnica Nacional, Departemento de Ciencia de los Alimentos y Biotecnologica, Quito, Ecuador. The latex was collected from unripe fruits, between 4 and 7 months old, originating from a greenhouse plantation south of Quito. A superficial cut was made along the five faces of the fruit with a stainless steel blade, as described earlier (*15*), and the drops of latex were collected in a sterilized container. Collected latex was lyophilized and frozen until use. Extract was prepared from shaking 13.5 g of lyophilized latex together with 450 mL of 20 mM Tris-HCl (pH 7.5) (30 mg/mL) at room temperature for

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1−2 h followed by shaking in a cold room for at least 8 h. Particulate material was separated by centrifugation at 11000*g* for 20 min followed by filtering of the supernatant through glass wool. Finally, the extract solution was sterile filtered using a 0.2 μm filter (Sarstedt, Nümbrecht, Germany) and stored at 4 °C until use.

Determination of the Protein Content. The protein content of extractions and the different purification steps including the final α-mannosidase were determined using the Bio-Rad Protein Micro assay (Bio-Rad, Hercules, CA) following the Microtiter Plate Protocol.

Enzymatic Activity Assays. All assays for α-mannosidase activity in extracts, fractions, and pure α-mannosidase were performed in triplicates in the same manner unless otherwise stated, using *p*-nitrophenyl-α-D-mannopyranoside (pNPMα). Pure α-mannosidase was also investigated for activity toward *p*-nitrophenyl-α-D-arabinopyranoside, *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-fucopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, *o*-nitrophenyl-β-D-galactopyranoside, *o*-nitrophenyl-*N*-acetyl-β-*N*-galactosaminide, *p*-nitrophenyl-*N*-acetyl-β-*N*-galactosaminide, *p*-nitrophenyl-*N*-acetyl-β-*N*-glucosaminide, *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitrophenyl-α-D-xylopyranoside, and *p*-nitrophenyl-β-D-xylopyranoside at 5 mM, using the same assay conditions (Sigma-Aldrich, St. Louis, MO). One unit of α-mannosidase activity was expressed as micromoles of *p*-nitrophenol liberated per milliliter per minute under the assay conditions using a molar extinction coefficient of 18.4 μM^{-1} cm^{-1} at A_{405}. Results of activity are given in katals (kat; kat = mol/s, where 1 unit = 1 μmol/min ≈ 16.67 nkat). The standard assay was performed by incubation of 10 μL of sample with 100 μL of 5 mM substrate in 100 mM NaAc (pH 4.5) for 30 min at 50 °C in a microtiter plate well (Sarstedt). The reaction was stopped by the addition of 100 μL of 1 M Na_2CO_3, and the absorbance was read at 405 nm using a Labsystem Multiskan MS microplate spectrophotometer (Thermo Labsystems, Waltham, MA). Subtraction was made to correct for background absorbance from substrate and sample. All dilutions of enzyme for glycosidase activity assays were performed with 0.25 mg/mL BSA in 20 mM Tris-HCl (pH 7.5).

Purification of α-Mannosidase. All chromatographic equipment, gels, and standards were supplied by GE Healthcare, Uppsala, Sweden. Chromatography was performed using a FPLC system at room temperature and absorbance monitoring at 280 nm. All fractions were stored at 4 °C after collection.

(a) Anion Exchange Chromatography. A volume of 450 mL of Babaco latex extract of 30 mg/mL latex in 20 mM Tris-HCl (pH 7.5) was batch adsorbed to approximately 30 mL of Q Sepharose HP gel previously equilibrated with 20 mM Tris-HCl (pH 7.5). The extract and gel suspension were incubated at 25 °C for 1 h with shaking on a rotary shaker, after which the gel was separated from unadsorbed material by suction filtering. The gel was washed carefully with approximately 5 column volumes of 20 mM Tris-HCl (pH 7.5) and packed in an XK26/20 column (2.6 × 20 cm). Additional washing with 100 mL of 20 mM Tris-HCl (pH 7.5) was performed at 3 mL/min before a stepwise gradient of 1 M NaCl in the same Tris-HCl buffer was applied. Unwanted material was washed out in two steps using 115 mL of 0.1 M NaCl and 60 mL of 0.2 M NaCl, respectively, before elution of the α-mannosidase-containing material by a total of 90 mL of 0.3 M NaCl. Throughout the experiment 14 mL fractions were collected. Fractions with high α-mannosidase activity were pooled for further purification.

(b) ConA Affinity Chromatography. A volume of 36 mL, from the 42 mL Q Sepharose HP fraction pool, was applied at 0.5 mL/min to a ConA Sepharose 4B column (1.0 × 5.0 cm) previoulsy equilibrated with 20 mM Tris-HCI (pH 7.5) containing 0.5 M NaCl. The column was washed with equilibration buffer to baseline. The bound α-mannosidase was eluted with 0.5 M α-methyl-D-mannopyranoside in the same buffer at 0.1 mL/min. The elution flow was immediately halted as soon as an increase in absorbance at 280 nm was observed. A pause for 6 h was performed to increase the efficiency of α-mannosidase displacement by α-methyl-D-mannopyranoside. Elution at 0.5 mL/min was resumed, and a 2 mL fraction was collected. Additional incubations with stopped flow were performed followed by elution at 0.5 mL/min. The total duration of the elution lasted for 9 h. Fractions of 2 mL were collected from each pause, and the four fractions of highest enzyme activity were pooled (approximately 8 mL) and concentrated with a Millipore Centriplus YM-30 centrifugal filter unit (Millipore, Billerica, MA) to a volume of 0.9 mL.

(c) Superdex 200 Prep Grade Gel Filtration Chromatography. Gel filtration of concentrated fractions with α-mannosidase activity from the previous ConA Sepharose 4B step was performed with a HiLoad 16/60 Superdex 200 prep grade gel filtration column (1.6 × 60 cm) equilibrated in 20 mM Tris-HCl (pH 7.5) with 150 mM NaCl. The column was loaded with 0.5 mL of concentrate at 0.5 mL/min, and 2 mL fractions were collected. Fractions of high enzyme activity were pooled (8 mL) and concentrated using a Millipore Centriplus YM-30 centrifugal filter unit to a volume of 30 μL. The molecular weight was determined after calibration by use of calibration proteins of both low and high molecular weight ranges from 6500 to 75000 (Gel Filtration LMW Calibration Kit) and 43000 to 669000 (Gel Filtration HMW Calibration Kit), respectively, with Blue Dextran 2000 as void volume marker.

Electrophoresis. All electrophoretic equipment and reagents were supplied by GE Healthcare, Uppsala, Sweden. Pure α-mannosidase and samples from purification steps were analyzed using native PAGE and SDS-PAGE with 8−25 gradient gels using a PHAST system and staining with Coomassie Brilliant Blue. SDS-PAGE of the final product was performed under both nonreducing (2.5% SDS) and reducing (2.5% SDS with 5% β-mercaptoethanol) conditions.

The molecular weights of the obtained bands were determined by comparing their relative migration distance (Rf) to those of standards of known molecular weight. Protein standards in the range of 94000−14400 from the LMW-SDS Marker Kit were used to plot Rf against the logarithms of their molecular weights. Determination of the position responsible for the α-mannosidase activity in native gels was identified by cutting the lanes of a gel run with eight identical samples into pieces and performing activity tests. Each piece was incubated in an Eppendorf tube together with 500 μL of 5 mM of pNPMα in 200 mM NaAc (pH 4.5) for 2 h at room temperature followed by 50 °C for 30 min. One hundred microliters of incubation solution and 100 μL of 1 M Na_2CO_3 were mixed in a microtiter plate, after which the absorbance at 405 nm was determined. Gel lanes not used for activity tests were stained with Coomassie Brilliant Blue. By comparing the part of the gel that showed the highest activity with the stained gel, the position of the enzyme was identified in the gel. The p*I* of the pure α-mannosidase was determined by isoelectric focusing (IEF) using a PhastGel IEF 3−9 gel with the Broad pI Kit from GE Healthcare, Uppsala, Sweden. The gel was stained with Coomassie Brilliant Blue.

Effect of Temperature and Thermal Stability. Determination of optimum temperature for the α-mannosidase was performed with 5 mM pNPM in 100 mM NaAc−HAc (pH 5.0) using incubation temperatures in the interval from 20 to 70 °C. Thermal stability was investigated after incubation of the enzyme at 4, 21, and 50 °C during 24 h, 72 h, and several weeks (4 °C). Detection of loss in α-mannosidase activity was performed using standard assay conditions.

Effect of pH. The optimum pH for α-mannosidase activity was determined using 5 mM pNPM in 200 mM NaAc−HAc with pH ranging from pH 3.5 to 6.00 under standard assay conditions.

Kinetic Parameters. The kinetic behavior was examined using standard assay conditions with pNPM in 100 mM NaAc−HAc (pH 4.5) except for the inclusion of 1 mM Zn in the 5 mM pNPM stock solution. All dilutions of enzyme were performed with a solution of 0.25 mg/mL BSA. The linearity of the α-mannosidase activity at fixed substrate and enzyme concentration was tested using reaction times up to 90 min. Lineweaver−Burk plots were established with substrate concentrations ranging from 0.05 to 15 mM pNPM at 40 and 50 °C.

Effect of EDTA, Metal Ions, and Mannose. A volume of 750 μL of α-mannosidase in 20 mM Tris-HCl (pH 7.5) was diluted with 0.25 mg/mL BSA and incubated for 2 h with 250 μL of 0.2 M EDTA containing 0.5 M NaCl (pH 7.5). An incubation control was made with 50 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl instead of EDTA. The EDTA-treated α-mannosidase was desalted using PD10 columns (GE Healthcare) previously equilibrated with 100 NaAc−HAc (pH 4.5). Elution was performed with 2 mL of equilibration buffer. The effect

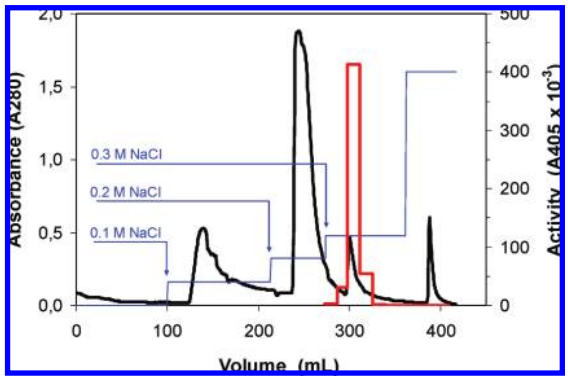

Figure 1. Q Sepharose HP chromatography after batch adsorption of babaco latex extract (black). Activity of α-mannosidas was measured in collected fractions (red). The salt gradient from 0 to 1 M NaCl (thin blue line) shows stepwise elution with active fractions being eluted at 0.3 M NaCl.

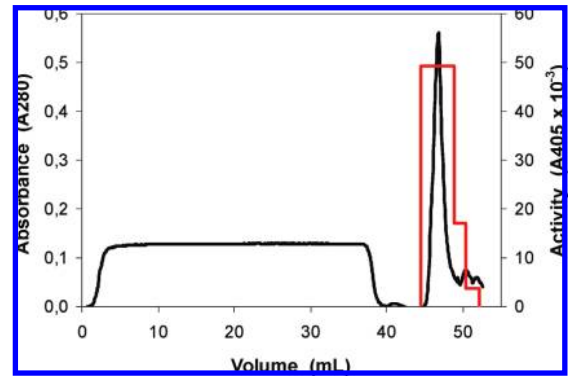

Figure 2. ConA Sepharose 4B chromatography (black) of active, pooled and concentrated fractions from Q Sepharose. Eluted fractions were tested for activity (red), and active fractions were subsequently pooled and concentrated.

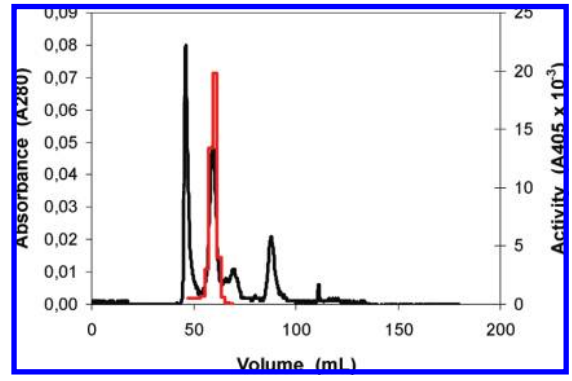

Figure 3. Superdex 200 prep grade gel filtration (black) of concentrated pooled fractions containing α-mannosidase from ConA Sepharose 4B chromatography. Fractions were measured for activity (red). Active fractions were pooled and concentrated.

of metal ions was studied by incubating 100 μL of desalted sample at room temperature with 10 μL of both 1 and 10 mM concentrations of the following salts: $AgNO_3$, $CaCl_2$, $CuSO_4$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$, respectively. The α-mannosidase activity from each incubation was determined using standard assay conditions. The effect of D-mannose was investigated at concentrations ranging from 1 to 500 mM.

Amino Acid Composition. The amino acid composition of the purified α-mannosidase, with the exception of tryptophan, was determined at the Amino Acid Analysis Center at the Biomedical Centre of Uppsala University, Uppsala, Sweden. The content of glucosamine was also determined. Lyophilized latex was included as a control of starting material.

RESULTS AND DISCUSSION

Extraction of α-mannosidase from lyophilized latex of babaco was performed in 20 mM Tris-HCl (pH 7.5). Protease inhibitors were not used. Preliminary experiments with crude extracts showed not only high activity of α-mannosidase but also significant activity of β-glucosidase, acetyl β-glucosaminidase, acetyl β-galactosaminidase, and cysteine proteases (results not shown). Even though babaco, as recently described (*8*), has a high content of cysteine proteases in its latex, the α-mannosidase in the extract from the lyophilized latex showed no sign of degradation due to protease activity. Initial investigations showed that the α-mannosidase was stable at room temperature for several days with little loss in activity. Extractions for purification were performed with a rotary shaker for 2 h at room temperature to increase the solubilization of the lyophilized latex before the extraction was placed in the cold room for overnight shaking.

Batch adsorption of babaco latex extract to Q Sepharose HP ion exchanger provided a very efficient capture of the α-mannosidase from the bulk of the proteins. The proteins that failed to bind to the resin could be easily washed away with the 20 mM Tris-HCl (pH 7.5) equilibration buffer. After packing of the gel in the column (2.6 × 20 cm), washing commenced until the baseline was below 0.05 absorbance unit. Elution of unwanted material was accomplished by stepwise elution in two elution steps of 0.1 and 0.2 M NaCl before elution of the α-mannosidase with 0.3 M NaCl (**Figure 1**). The activity yield from elution was very high, with >96% of the α-mannosidase activity maintained in the eluate pool. In the second purification step 36 mL of the 42 mL pooled Q Sepharose HP fractions showing α-mannosidase activity was loaded to ConA Sepharose 4B. Again the majority of proteins did not bind to the gel, whereas the α-mannosidase was strongly bound. Elution was performed using 0.5 M methyl mannoside in 20 mM Tris-HCl (pH 7.5) with 0.5 M NaCl. Earlier reports of ConA affinity chromatography of α-mannosidase has resulted in low yields due to the tight binding to the matrix (*16, 17*). There are also records of successful purification using the same method with very high yield, although, interestingly, this separation does not involve the sugar binding site of the lectin (*18*). To improve the yields in these experiments, enhanced incubation times with elution buffer for >8 h were utilized, resulting in a yield from this step of >69% (**Figure 2**). Fractions showing α-mannosidase activity were pooled and concentrated using a Centriplus YM-30 centrifugal filter unit (Millipore). In the final chromatographic step 0.5 mL of 0.9 mL of the α-mannosidase containing concentrate was loaded to a Superdex 200 prep grade gel filtration column previously equilibrated with 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl (**Figure 3**). Resulting fractions of α-mannosidase were pooled and concentrated using a Microcon YM-30 centrifugal filter unit (Millipore) for further studies. The final yield from the purification was 49.5%, which is one of the higher noted from plant, even though at least up to 75% has been reported (*18*). The high final yield of α-mannosidase is likely attributed to the selective initial capture ion exchange step, in combination with the prolonged incubation times during elution from ConA Sepharose. The use of batch adsorption, even though not a novel method in itself, has not, to our knowledge, been reported before for an α-mannosidase. A summary of the purification of the babaco α-mannosidase is shown in **Table 1**.

The purity of the α-mannosidase was verified by native PAGE (Coomassie Brilliant Blue staining) and showed one homoge-

Table 1. Purification of Babaco α-Mannosidase

step	mL	activity[a] (nkat mL^{-1})	protein concn (mg mL^{-1})	total activity (nkat)	total protein (mg)	specific activity (μkat mg^{-1})	purification factor	yield (%)
crude extract	214.00	3.4	17.48	719.8	3740.626	1.924×10^{-4}	1	100.0
Q Sepharose	20.00	34.9	0.28	698.7	22.373	3.123×10^{-2}	162	97.1
ConA concentrate	0.50	1005.1	1.69	502.6	0.846	5.943×10^{-1}	3088	69.8
Superdex 200 prep grade concentrate	0.03	11904.9	5.21	357.1	0.156	2.283	11863	49.6

[a] Results of activity are given in kat (kat = mol/s, where 1 unit = 1 μmol/min ≈ 16.67 nkat).

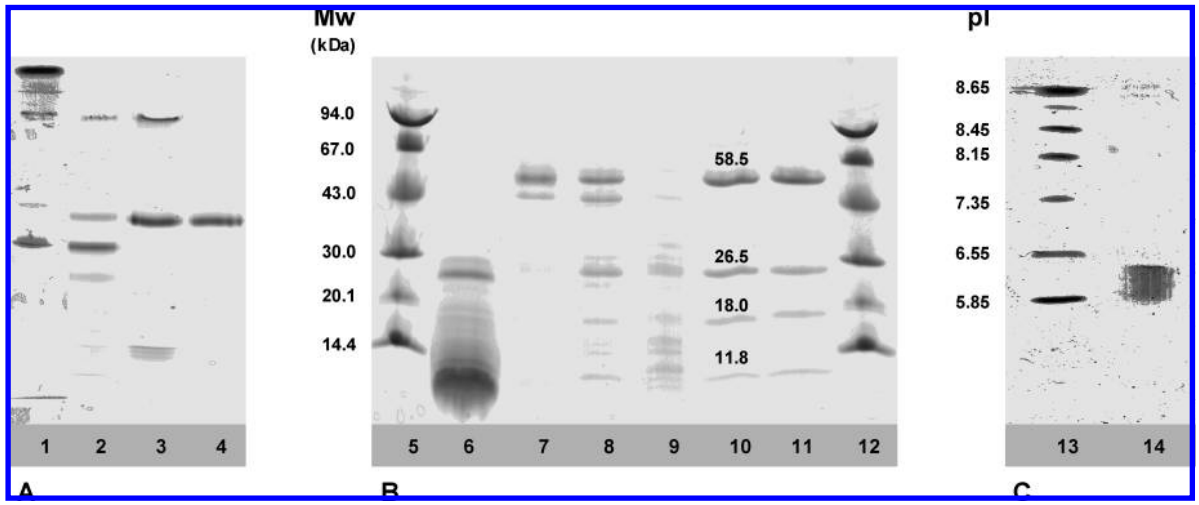

Figure 4. Electrophoretic analysis of α-mannosidase. (**A**) Native PAGE stained with Coomassie Brilliant Blue displaying the steps of α-mannosidase purification: Babaco latex extract start material (lane 1); concentrated pooled fractions eluted after batch adsorption to Q Sepharose HP (lane 2); concentrated pooled fractions from ConA Sepharose 4B (lane 3); pure α-mannosidase from concentrated Superdex 200 prep grade fractions (lane 4). (**B**) SDS-PAGE stained with Coomassie Brilliant Blue: LMW Calibration Kit Proteins (lane 5 and 12); reduced SDS of Babaco latex extract start material (lane 6); reduced SDS of concentrated pooled fractions from Q Sepharose HP (lane 7); unreduced SDS of concentrated pooled fractions from ConA Sepharose 4B (lane 8); reduced SDS of concentrated pooled fractions from ConA Sepharose 4B (lane 9); unreduced SDS of pure α-mannosidase from concentrated Superdex 200 prep grade fractions (lane 10); reduced SDS of pure α-mannosidase from concentrated Superdex 200 prep grade fractions (lane 11). (**C**) Isoelectric focusing with PhastGel IEF 5-8 stained with Coomassie Brilliant Blue: broad pI Kit (lane 13); pure α-mannosidase from concentrated Superdex 200 prep grade fractions (lane 14).

neous band (**Figure 4**). The enzymatic identity of this band was verified by cutting identical unstained lanes of the same gel, equivalent to lane 4 in **Figure 4**, into different pieces and performing activity assays on each piece. By relating the presence of activity with the respective position on the gel, the band responsible for the α-mannosidase activity could be identified (results not shown). Investigation with both unreduced and reduced SDS-PAGE gave identical results, revealing four separate bands. The molecular weights of the subunits were determined according to their relative migration distance compared to reference proteins from calibration using the LMW Calibration Kit Proteins (GE Healthcare). The two largest and most pronounced bands corresponded to estimated molecular masses of 58.5 and 26.5 kDa, respectively, whereas the two smaller bands correspond to molecular masses of 18 and 12 kDa. This four-band pattern shows similarities to that of *Prunus serotina* Erh (*16*) and *Medicago sativa* (*20*). The subunit stochiometry could not be determined on the basis of the detected bands due to the uncertain multiplicity. The α-mannosidase is likely to be a heterogenic oligomer, similar to the composition found in *Hordeum vulgare* and *M. sativa* (*17, 20*). At a multiplicity of two subunits per subunit, the total molecular mass would be approximately 230 kDa, which is well in the range of several other plant α-mannosidases (*13, 14, 18, 20*). On the other hand, a higher molecular mass of between 260 and 280 kDa was obtained from gel filtration with Superdex 200 prep grade calibrated with protein standards in the range of 14–660 kDa. This value is closer to the value of 260 kDa earlier described for papaya (*4*). Molecular weight determination by gel filtration and SDS-PAGE are both approximate methods. Results from these techniques will thus always suffer from a level of inaccuracy, and it is therefore difficult to say whether this difference is significant. The data from the p*I* by isoelectric focusing also indicated different isomers of glycosylation, as seen by the diffuse band in the pH range of 5.85−6.55. The degree of glycosylation can greatly influence the behavior electrophoretic mobility of subunits during electrophoresis. On this basis, the α-mannosidase should have a molecular mass in the range between 230 and 280 kDa, depending on the stoichiometry. It can be concluded that the subunits of the α-mannosidase are not bound to each other by disulfide bridges, as seen by the identical appearance of the lanes with the pure α-mannosidase run under denaturing conditions with and without the presence of mercaptoethanol (**Figure 3B**). This is in accordance with a similar investigation of α-mannosidases of *Phaseolus vulgaris*, which utilized dissociation of subunits by guanidine hydrochloride followed by sedimentation equilibrium analysis (*19*). The combined information from size exclusion chromatography together with the subunit molecular weights from denaturing PAGE still does not clearly point to a specific subunit structure. By assuming a combination of paired subunits, several different heteromeric octamer compositions seem plausible, all resulting in a molecular mass between 230

Table 2. Amino Acid Composition of Babaco α-Mannosidase

residue	babaco (%)	*Canvalia ensiformis*[a] (%)	*Phaseolus vulgaris* I[b] (%)	*Phaseolus vulgaris* II[b] (%)
Ala	6.95	7.65	7.03	7.02
Arg	5.55	4.56	4.49	4.88
Asx	11.96	11.55	12.02	12.30
Cys	trace	nd	1.02	0.96
Glx	10.65	11.60	11.64	8.33
Gly	6.92	7.37	7.35	7.37
His	2.10	3.25	2.04	2.25
Ile	5.77	3.02	5.31	5.61
Leu	8.64	8.96	9.29	9.59
Lys	5.78	7.65	8.27	8.31
Met	1.08	2.86	trace	trace
Phe	4.51	5.11	4.70	4.75
Pro	4.41	2.75	3.98	4.08
Ser	7.71	8.36	7.66	9.07
Thr	4.68	6.00	5.82	6.10
Trp[c]	xx	xx	xx	xx
Tyr	5.11	4.90	1.43	1.28
Val	8.17	4.40	7.97	8.10
glucosamine[d]	2.0	–	–	–

[a] Data obtained from ref *11*. [b] Data obtained from ref *19*. [c] Tryptophan content was not determined for the pure α-mannosidase; thus, the total amino acid compositions for *C. ensiformis* and *P. vulgaris* have been corrected with the tryptophan value omitted (xx). [d] Percentage of glucosamine is determined as part of the total amino acid composition including glucosamine. Posts where no residues were detected are marked "nd". A dash (–) indicates posts for which data were not supplied.

and 290 kDa. The upper part of this range, around 260−280 kDa, seems to be most probable on the basis of gel filtration data.

The determined *amino acid composition* of the whole α-mannosidase (**Table 2**) was compared to results from previously published plant α-mannosidases from two isoforms from kidney bean, *P. vulgaris* (*19*), and from jack bean, *C. ensiformis* (*13*). All four have very similar compositions with only minor deviations, showing high amounts of glutamic acid and aspartic acid, valine, serine, and leucine. The babaco α-mannosidase has a somewhat lesser content of lysine and threonine. However, differences also exist between the two other studied α-mannosidases from kidney bean and jack bean. The presence of 2% glucosamine points to the degree of glycosylation of the enzyme, which in part could explain the polydisperse pattern found from p*I* analysis with electrophoresis.

The established amino acid composition of the α-mannosidase was used for a search with the AACompIdent tool at ExPASy Home page in the Swiss-Prot and TrEMBL databases for proteins. No similarity was found in Swiss-Prot, but with TrEMBL the closest entries were found to be those of different clones of α-mannosidase from *Arabidopsis thaliana*, another family of the Brassicales branch and thus more closely related to babaco than kidney bean and jack bean. Investigations of the thermal stability for the α-mannosidase showed no loss in activity after storage at 21 °C for 24 h in 20 mM Tris-HCl (pH 7.5) with 150 mM NaCl. No loss in activity was observed even after 72 h. However, storage at 50 °C for 24 h resulted in >90% loss in enzyme activity (results not shown). Stability from storage at 4 °C provided no detectable loss of activity during the tested time period of >2 months. The temperature optimum for the babaco latex α-mannosidase was found to lie between 50 and 60 °C (**Figure 5**). The relative α-mannosidase activity was significantly lowered already at 40 °C, leaving only 60% of the optimal, whereas 75% of the activity remained at 70 °C.

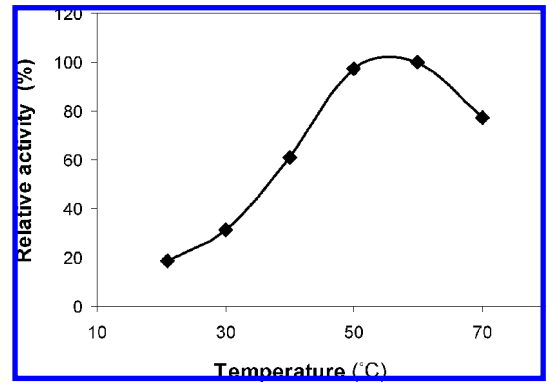

Figure 5. Temperature dependence of α-mannosidase activity. The test was conducted using 5 mM pNPG in 100 mM NaAc (pH 4.5) and incubation for 30 min at six different temperatures.

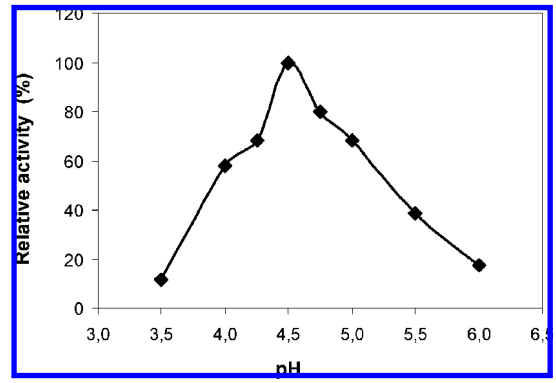

Figure 6. pH dependence of α-mannosidase activity. The test was performed at 50 °C and using 5 mM pNPG in 100 mM NaAc set to the appropriate pH and incubated for 30 min.

Reports have noted optimal temperatures below 40 °C (*16, 21*), but the present results indicate a higher temperature preference, similar to those found in the more distantly related Solanaceae family (*22, 23*). The determined pH optimum of the α-mannosidase agrees with the majority of earlier published results (*3, 13, 14, 16*), even though it is one of the higher ones noted, with a sharp peak at pH 4.5 and remaining activity at pH 5.0 still higher than at pH 4.0 (**Figure 6**). The K_m was determined to be 1.25 mM using a substrate range from 0.05 to 15 mM of *p*-nitrophenyl-α-mannopyranoside in 100 mM NaAc-HAc (pH 4.5) and two different temperatures, 40 and 50 °C. The V_{max} was found to be 2.4 μkat mg^{-1} at 50 °C and 1.94 μkat mg^{-1} at 40 °C. The values are of the same order of magnitude as those from other α-mannosidases, but a direct comparison of results is difficult to perform due to the variety in use of test temperatures and other test conditions (*11, 13, 20*).

The effect of different metal ions on α-mannosidase activity was tested both with and without removal of preexisting ions by EDTA treatment (**Table 3**). Addition of 1 mM Cu^{2+} and Mn^{2+} ions clearly lowered the activity compared to the untreated α-mannosidase sample. Addition of 1 mM of the other tested ions had little effect, although a slight increase in activity was observed for Zn^{2+}. Addition of 10 mM salt resulted in a drastic drop of activity, down to 6%, for Cu^{2+}. Decreased activity was also observed for Ca^{2+} and Mn^{2+} at this concentration. On the other hand, addition of 10 mM Zn^{2+} increased the activity by 28%. The presence of EDTA strongly reduced the enzyme activity for all ions. However, the addition of Zn^{2+} ions after exposure to EDTA was able to fully restore the activity. These results agree well with the requirement of α-mannosidase for Zn^{2+}, as it is a

Table 3. Effect of Metal Ions and EDTA on the Activity of α-Mannosidase

	% relative activity[a]			
	non EDTA treated		EDTA treated	
addition	1 mM	10 mM	1 mM	10 mM
without ion	100	100	36	36
Ca^{2+}	101	69	40	39
Cu^{2+}	48	6	39	3
Mg^{2+}	107	109	41	37
Mn^{2+}	71	62	41	71
Zn^{2+}	112	128	106	134

[a] Relative activity of pure α-mannosidase after preincubation for 1 h at 25 °C with different concentrations of metal ions. The activity was measured under standard conditions and is expressed relative to a sample not treated or exposed to EDTA and ions.

Table 4. Relative Activity of Babaco α-Mannosidase with Various Substrates and Substrate Inhibition by Mannose

substrate[a]	relative α-mannosidase activity[b,c,d] (%)
pNP-α-arabinopyranoside	0.05
pNP-β-cellobioside	0.02
pNP-β-glucopyranoside	0.07
pNP-β-fucopyranoside	0.13
pNP-α-galactopyranoside	0.13
oNP-β-galactopyranoside	0.16
oNP-*N*-acetyl-β-galactosaminide	0.41
pNP-*N*-acetyl-β-galactosaminide	0.22
pNP-*N*-acetyl-β-glucosaminide	0.64
pNP-α-mannopyranoside	100.00
pNP-β-mannopyranoside	0.20
pNP-α-xylopyranoside	1.43
pNP-β-xylopyranoside	0.13
α-mannopyranoside, 1 mM	106
α-mannopyranoside, 10 mM	94
α-mannopyranoside, 50 mM	70
α-mannopyranoside, 100 mM	51
α-mannopyranoside, 200 mM	33
α-mannopyranoside, 500 mM	9

[a] Activity was measured under standard conditions with a 5 mM concentration of the respective substrate. [b] Activity is expressed as absorbance units measured at 405 nm. [c] Relative activity of pure α-mannosidase for different substrates. Related to pNP-α-mannopyranoside activity (100%) measured under standard conditions with a 5 mM concentration of the respective substrate. [d] Relative activity of pure α-mannosidase in the presence of different concentrations of mannose measured under standard conditions and expressed relative to activity in the absence of mannose.

known Zn-dependent enzyme (*12*). Substrate inhibition studies were performed in the presence of different concentrations of mannose. Inhibition was observed above 10 mM with 50% activity at 100 mM and <10% activity at 500 mM (**Table 4**). The α-mannosidase was shown to be very specific to *p*-nitrophenol-α-mannopyranoside, and specificity toward all other synthetic *p*-nitrophenol glycoside substrates was below 2%. In summary, an α-mannosidase was purified with an approximate yield of 50% by use of batch adsorption anion exchange, followed by selective binding to ConA and gel filtration. The pure enzyme displays typical attributes similar to other known and characterized plant α-mannosidases. However, the suggested octameric subunit structure of the enzyme is unusual, whereas most other described plant α-mannosidases display a dimeric or tetrameric structure.

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