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Extraction and immobilization in one step of two β-glucosidases released from a yeast strain of *Debaryomyces hansenii*

Paolo Riccio,* Rocco Rossano,* Mara Vinella,† Paola Domizio,† Francesco Zito, Francesco Sansevrino,** Assunta D'Elia,* and Iolanda Rosi†**

**Dipartimento di Biologia, Difesa e Biotecnologie Agro-Forestali, University of Basilicata, Potenza, Italy, †Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, University of Firenze, Firenze, Italy, **Dipartimento di Biochimica e Biologia Molecolare, University of Bari, Bari, Italy*

An extracellular, constitutive, and nonglucose repressed β-glucosidase from a yeast strain of Debaryomyces hansenii *was purified and immobilized using a one-step procedure on hydroxyapatite (HTP). Analysis of purified enzyme gave two bands both on SDS gel electrophoresis, native gel electrophoresis, and capillary electrophoresis. The two bands on SDS gels were positive for carbohydrate staining. Their apparent molecular mass was estimated to be 122 and 96 kDa with carbohydrates, and 109 and 81 kDa after carbohydrate removal, respectively. Amino acid analysis of electroblotted bands revealed that the N-terminus was blocked in both cases. Gel slices corresponding to the two bands, as obtained after native gel electrophoresis, were found to be reactive when incubated separately with* p-*nitro-phenyl-*β-D-*glucopyranoside (*pNPG*) as substrate. The* K_m *of the two forms coeluted from HTP in the same fractions was 3.68 ± 0.06 mM. The optimum pH was 5. The immobilized enzyme exhibited a lower activity than the purified free enzymes, but both were much more stable than the enzymes in cell-free supernatant. The two enzyme isoforms in the mixture were only active against few glycosides with β-linkage configuration. Since the HTP-bound enzyme was found to be active, stable, easily separable from the substrate, and reusable, it could be potentially used in its immobilized form for the release of specific-bound aroma in wine and fruit juices.* © *1999 Elsevier Science Inc.*

Keywords: Yeast β-glucosidases; purification; immobilization; hydroxyapatite; aroma enhancement; wine; fruit juices

Introduction

Terpenes are a class of compounds responsible for the varietal aroma of many fruits and their fermenting products (juice, wine). Among the terpenes, the monoterpenols (linalool, nerol, geraniol, α-terpineol, and citronellol) are the most active from an olfactory point of view due to their low sensory threshold; however, most of monoterpenols in fruits are bound to β-glucopyranose which is linked to sugars such as α-L-rhamnopyranose or α-arabinofuranose or β-apiofuranose to give flavorless glycosidic complexes.[1,2] This aromatic potential is released naturally during fruit maturation by endogenous β-glycosidases.[3] As these enzymes are unable to release all the bound terpenols, many attempts have been made to use exogenous β-glucosidases mainly from plants (grape, sweet almond) and from microorganisms (molds and yeasts).

Plant-produced β-glucosidases were characterized by a restricted specificity with respect to aglycon, they were not very active between pH 3–4, and they were inhibited by a

Address reprint requests to Prof. Iolanda Rosi, Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Via Donizetti, 6, University of Firenze, 50144 Firenze, Italy
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glucose concentration over 1%.[4,5] Similar inhibition was observed for β-glucosidase of fungal origin.[6] The β-glucosidases produced by the yeasts (*Candida molischiana*, *Candida wickeramii*, and *Saccharomyces cerevisiae*) were less sensitive to glucose and had a wider specificity for aglycon.[7,8]

In previous studies[9,10] performed to study the β-glucosidase activity in yeasts of oenological origin, we have found a strain of *Debaryomyces hansenii* producing an extracellular, constitutive, nonglucose repressed β-glucosidase. The activity of this enzyme was not inhibited by high ethanol and glucose concentrations and was not greatly influenced by acidic pH and low temperatures. The enzyme was able to produce a significant amount of monoterpenols and benzyl- and 2-phenylethyl alcohol from wines containing glycosidic precursors.[11]

The present paper describes the one-step purification and biochemical characterization of a β-glucosidase activity released from a yeast strain of *D. hansenii*. This activity was due to two glycosylated isoforms of the enzyme showing similar activity but different molecular mass. These forms were not separated but since the purification procedure also makes their mixture available in immobilized form, they could represent as such a useful tool for the enhancement of wine and fruit juice aroma.

Materials and methods

Organism and condition of growth

The yeast used was *D. hansenii* DBVPG 4025.[9] The culture was maintained at 4°C on a slope of yeast malt agar (YM) which contained (g l^{-1} in distilled water): Malt extract, 3; yeast extract, 3; peptone, 5; glucose, 10; and agar, 20. This was subcultered once a year. The yeast was grown on a medium previously defined[10] and containing 6.7 g l^{-1} yeast nitrogen base (YNB, Difco, Detroit, MI); 20 g l^{-1} glucose; 20 g l^{-1} K_2HPO_4, and 0.25 g l^{-1} Tween 80. This was buffered at pH 5 with phosphate-tartrate (25 mM tartaric acid, 50 mM K_2HPO_4). The medium was sterilized by filtration (Millipore membrane HA, 0.22 μm, 47 mm). Erlenmeyer flasks (1-l) filled to 10% of their volume were inoculated with a 20-h-old culture grown on the above medium to give an initial OD_{600} of 0.2. The aerobic culture was incubated at 25°C and shaken at 150 rpm on a gyratory shaker for 22 h.

Preparation of samples for enzyme purification

The culture broth was centrifuged at 8,000 rpm for 10 min at 4°C. The supernatant (2 l) was filtered on 0.22 μm cellulose acetate filters (Millipore, Eschborn, Germany) and then used as the source of extracellular enzyme for the one-step purification on hydroxyapatite.

Enzyme assays

β-glucosidase activity was assayed by measuring the amount of *p*-nitrophenol (*p*NP) released from *p*-nitrophenyl(*p*NP)-β-D-glucopyranoside (*p*NPG, Sigma, St. Louis, MO) as substrate according to the method previously described.[9] The reaction mixture of 0.4 ml contained 0.2 ml of enzymatic solution and 0.2 ml of 15 mM *p*NPG in 100 mM citrate/phosphate buffer at pH 5. The reaction was performed for 30 min at 30°C and stopped by adding 1.2 ml of 50 mM Na_2CO_3/$NaHCO_3$ buffer (pH 10.2). The released *p*-nitrophenol was measured spectrophotometrically at 400 nm using $\epsilon = 14.13$ mM^{-1} cm^{-1} as calculated at the experimental conditions at pH 8.6.

β-fucosidase, β-galactosidase, α-rhamnosidase, α-arabinosidase, α-glucosidase, β-lactosidase, β-xylosidase, and β-cellobiosidase activities were assayed by measuring the *p*NP released by the enzyme from *p*NP-β-D-fucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-α-L-rhamnopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-α-L-arabinopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-α-D-lactopyranoside, *p*NP-β-D-xylopyranoside, and *p*NP-β-D-cellobioside as substrates (Sigma), respectively. Each substrate was used at 15 mM. The enzyme assay conditions were the same as described for β-glucosidase. One unit (U) of enzyme activity is that which released 1 μmol *p*-nitrophenol min^{-1}. Specific activity was expressed as units of activity mg^{-1} protein.

Protein assay

Protein content was determined using the Bio-Rad Bradford reagent in the microassay procedure. BSA was used as a standard.

One-step purification on hydroxyapatite

Dry hydroxyapatite powder (Bio-Gel HTP, Bio-Rad Laboratories, Hercules, CA, catalogue No. 130-0420) was added (1 g/50 UI) to the filtered supernatant (2 l) stirring for 20 min at 20°C. The enzyme adsorbed on the hydroxyapatite (HTP-E) was eluted washing the precipitate obtained after centrifugation at 12,000 rpm at 4°C with 400 mM phosphate buffer pH 7.

SDS polyacrylamide gel electrophoresis

Denaturing polyacrylamide gel electrophoresis was performed on discontinuous gels (stacking gel: 4% T, 3% C; running gel: 6% T, 3% C) according to Schägger & von Jagow.[12] Samples were prepared by resuspending the lyophilized eluted enzyme in a medium containing 4% SDS, 2% β-mercaptoethanol, 12% glycerol, 0.01% blue bromophenol, and 50 mM Tris-HCl pH 6.8.

Native polyacrylamide gel electrophoresis

Nondenaturing polyacrylamide gel electrophoresis was performed on discontinuous gels (stacking gel: 4% T, 3% C; running gel: 6% T, 3% C) according to Schägger & von Jagow.[13] Samples were prepared by resuspending the lyophilized eluted enzyme in a medium containing 12% glycerol, 0.01% blue bromophenol, and 50 mM Tris-HCl pH 6.8.

Transfer on PVDF membrane and amino acid analysis

For protein sequencing, the peptides separated by SDS gel electrophoresis were transferred on polyvinilidene difluoride (PVDF, Millipore) membranes[14] and stained for 1 min with 0.2% Coomassie Brilliant Blue R-250/0.05% and Coomassie Brilliant Blue G-250 in methanol/acetic acid/H_2O (4:1:4, v/v). After destaining of the membranes with methanol/acetic acid/H_2O (4:1:4, v/v), membrane slices corresponding to the peptide bands were analyzed for Edman degradation with the apparatus 491A of Applied Biosystem.

Capillary electrophoresis

The sample for the capillary electrophoresis was prepared by diluting 1:1 the eluted enzyme with the running buffer (100 mM phosphate pH 2.5) after dialysis performed under stirring for 3 h at 4°C against 2 l of 50 mM Tris-HCl pH 7.4 using a Spectropor membrane with a cut-off of 3,500 Da. All separations were performed using the BioFocus 2000 Capillary Electrophoresis

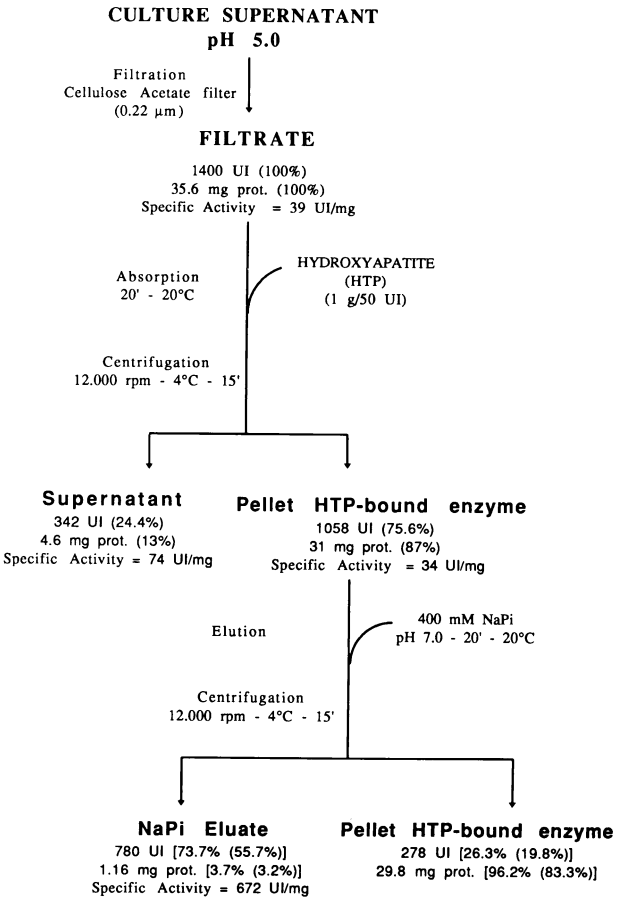

Figure 1 Flowsheet of the one-step procedure applied for the purification of β-glucosidase isoforms

system (Bio-Rad Laboratories) and a fused silica capillary (24 mm × 25 μm) internally coated with a covalently bonded linear hydrophilic polymer. Samples were loaded by injection at 40 psi*sec and separated under a 10 KV constant voltage using +→− polarity. Detection was at 280 nm.

Glycoprotein detection and deglycosylation procedure

The carbohydrate moieties bound to the proteins were detected by dying SDS-PAGE gels according to the PAS method.[15]

Proteins were deglycosylated using the enzymatic deglycosylation kit from Bio-Rad Labs. (Cat. no. 170-6500) and following the denaturing protocol described below. Briefly, protein fractions eluted from HTP with 400 mM phosphate pH 7.0 were dialyzed versus 50 mM Tris-HCl pH 7.4 at 4°C for 3 h. The enzymes NANase II and O glycosidase DS were then added to the proteins and incubated at 37°C for 1 h. After denaturation with SDS and β-mercaptoethanol, the enzyme PNGase F was added to the mixture and left to incubate for 3 h at 37°C. Proteins were then applied for SDS gel electrophoresis.

Results

Enzyme purification

Figure 1 schematically shows an example of the procedure followed for the purification of β-glucosidase from the yeast *D. hansenii* DBVPG 4025. This procedure was based on the batchwise use of hydroxyapatite to extract, concentrate, and purify the enzyme in one step. Most of the enzyme present in culture supernatant was bound to HTP. The enzyme was not released from HTP up to 100 mM phosphate. Elution with 400 mM phosphate allowed us to obtain the free enzyme with a purity degree 17-fold higher than that in the culture supernatant where it was in fact already relatively high. The enzyme was further concentrated 12-fold by ultrafiltration on an Amicon YM5 membrane. As an alternative XM50 membranes were used without loss of the enzyme.

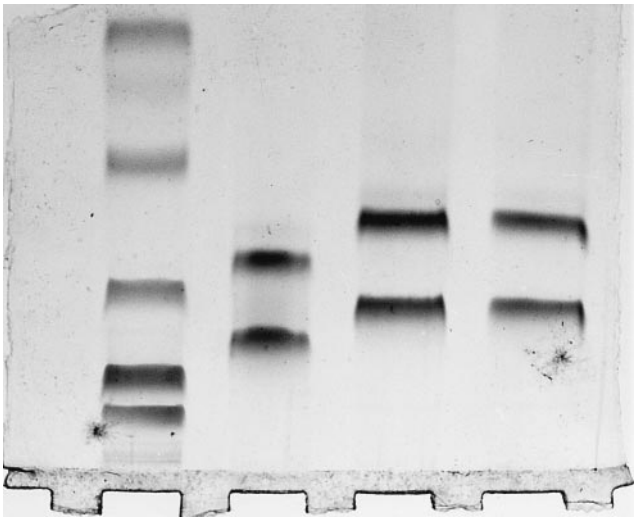

Figure 2 SDS-PAGE on 6% acrylamide gel. From left to right: Standard proteins (Pharmacia Biotech): myosin, 212 kDa; α_2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; transferrin, 76 kDa; glutamic-dehydrogenase, 53 kDa. Second lane: Fraction eluted from hydroxyapatite with phosphate. Other two lanes: deglycosylated proteins

Polyacrylamide gel electrophoresis and capillary electrophoresis

Figure 2 shows the SDS-PAGE of the HTP-eluted enzyme fraction. Two protein bands were observed with an apparent molecular mass of 122 ± 4 and 96 ± 4 kDa (2nd lane from right). The presence of bound carbohydrates was demonstrated by using the PAS reagent on SDS gels (not shown). After deglycosylation, the molecular mass of the two bands was reduced to 109 ± 3 and 81 ± 3, respectively (other two lanes in *Figure 2*).

Two bands were observed again in the native PAGE (*Figure 3*). This result was further confirmed as two peaks were observed carrying out capillary electrophoresis on the eluted enzyme (*Figure 4*).

The β-glucosidase activity was tested after cutting unstained gel slices corresponding to the two bands from native PAGE. In both cases, enzyme activity was observed using the synthetic substrate *p*NPG. In particular, the higher MW band showed an enzyme activity fourfold higher than the lower MW band.

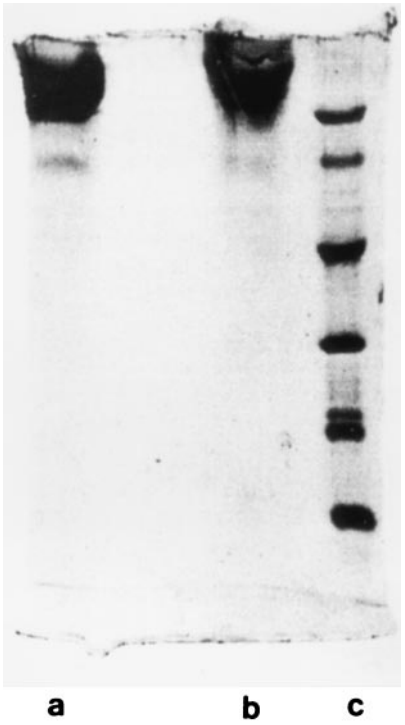

Figure 3 Native gel electrophoresis on 6% acrylamide gel. Standard proteins (lane c), as in *Figure 2.* Lanes (a and b): hydroxyapatite eluate without SDS

Protein sequencing

In order to determine the amino acid sequence of the N-terminus of the protein, the SDS-polyacrylamide gel was electroblotted onto a polyvinylidene difluoride membrane (*Figure 5*) and then protein bands were subjected to automatic protein sequencing. For both bands, it appeared that the N-terminus was blocked.

Enzymatic activity as a function of pH

Enzymatic activity of β-glucosidase was determined by spectrophotometric assay on the eluted enzyme using the synthetic substrate *p*NPG (15 mM) in phosphate-citrate buffer with a pH range from 3–7. Optimal activity was observed at pH 5 (*Figure 6*).

Enzyme kinetic

The enzyme kinetic was followed for 5 min at various substrate concentrations ([*p*NPG] = 1.0, 3.0, 5.0, 8.0, 10.0 mM) in 100 mM phosphate-citrate buffer at pH 5 and 30°C. The Michaelis-Menten constants, calculated by the Lineweaver-Burk plot, were $K_m = 3.68 \pm 0.06$ mM and $V_{max} = 98.6 \pm 4.9$ mM min^{-1}.

Substrate specificity

The ability of purified β-glucosidase to hydrolyze various substrates with α and β configurations was determined. The

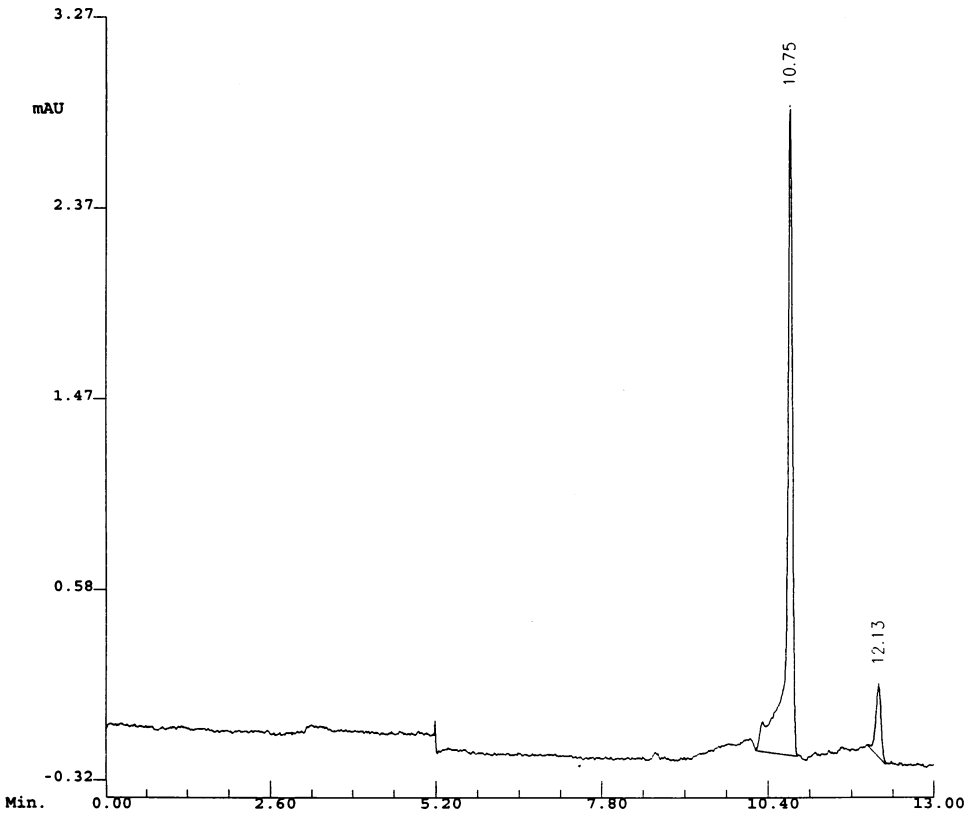

Figure 4 Electropherogram of purified β-glucosidase after capillary electrophoresis using a 24 cm × 25 μm coated capillary with phosphate buffer (pH 2.5)

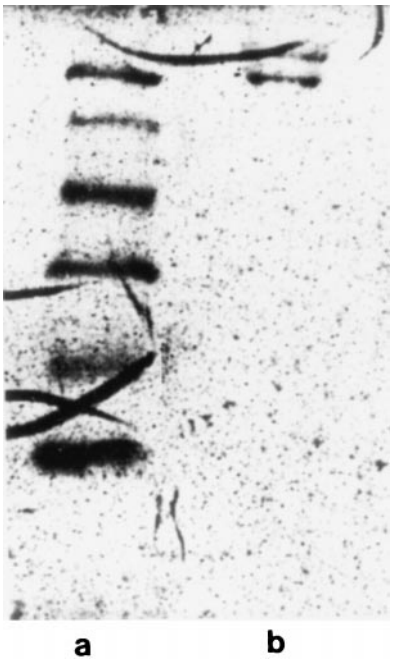

Figure 5 Blotting for amino acid sequence analysis. Lane a: Standard proteins as in *Figure 3* and lane (b): β-glucosidase

enzyme rapidly hydrolyzed *p*NP-β-D-glucopyranoside and *p*NP-β-D-fucopyranoside (*Table 1*). The enzyme had low activity on *p*NP-β-D-galactopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-β-D-xylopyranoside, and *p*NP-β-D-cellobioside. The enzyme failed to hydrolyze the other substrates.

Activity of the immobilized enzyme

The enzyme was extracted from culture supernatant by HTP and the activity was calculated as the difference between activity values in the culture supernatant before and after adsorption on hydroxyapatite. The value of bound HTP enzyme activity was 38.6 IU (80%). As calculated by the difference between activity values in the culture supernatant before and after adsorption on hydroxyapatite, 38.6 IU (80%) were bound to HTP. The HTP-bound enzyme was resuspended in 100 mM acetate buffer pH 5 and incubated with 15 mM pNPG at different enzyme concentrations as described in MATERIALS AND METHODS. The enzyme was found to be active also in bound form and therefore suitable for using in immobilized form although only 48% (18.5 IU) of the expected activity was found. Acetate buffer was here preferred to the phosphate-citrate buffer to avoid the release of the enzyme from HTP since enzyme elution can indeed occur at low (20 mM) citrate concentration. No change was observed when measuring activity of the free enzyme in acetate instead of citrate-phosphate buffer.

Storage and stability

The purified enzyme stored at pH 5 either at +4°C or −21°C retained about 90% of its activity after 55 days despite the temperature chosen. The HTP-bound enzyme was as stable as the free enzyme. Using the same conditions, the enzyme in the cultural supernatant retained only 10% of its activity.

Discussion

In this paper, we have shown the extraction, immobilization, and characterization of two β-glucosidases from the yeast *D. hansenii*. A major problem was the very low concentration of enzymatic activity in culture supernatants. Concentration of the enzyme was first attempted by ultrafiltration, salting out with ammonium sulfate, or adding either ethanol or polyethylene glycol. Dry gel polymers made of acrylamide and agarose were also used.

The key step for the extraction and concentration of the enzyme was its strong adsorption on hydroxyapatite (HTP), a form of calcium phosphate, a mixed bed ion-exchanger. As we have reported elsewhere[16] HTP is not expensive if produced in one's own laboratory, it is not toxic, and it is very useful for the purification of a variety of proteins also by one-step procedures.[17] The chromatographic behavior of

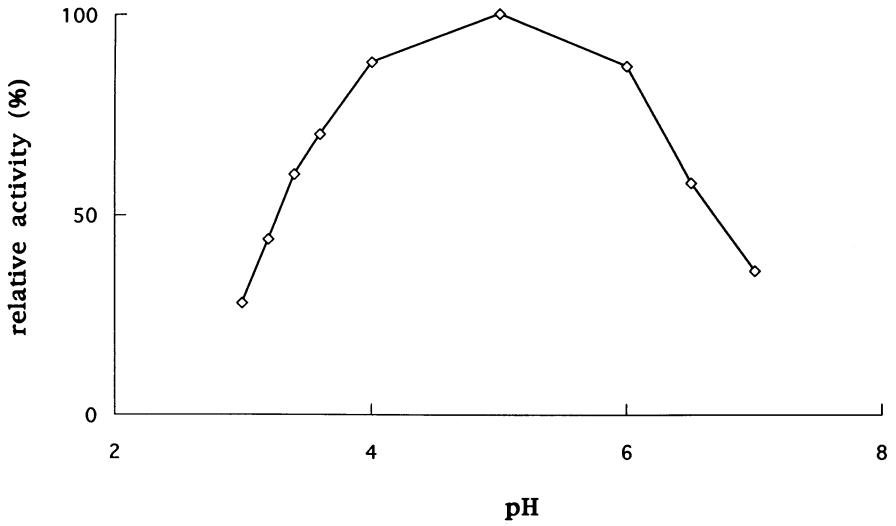

Figure 6 Assessment of optimum pH for β-glucosidase activity

Table 1 Substrate specificity of purified β-glucosidase

Substrate[a]	% Enzymatic activity
*p*NP-α-L-rhamnopyranoside	0
*p*NP-α-L-arabinopyranoside	0.15
*p*NP-α-L-arabinofuranoside	0
*p*NP-α-D-glucopyranoside	2
*p*NP-β-D-fucopyranoside	15
*p*NP-β-D-lactopyranoside	0
*p*NP-β-D-cellobioside	1.5
*p*NP-β-D-xylopyranoside	1.5
*p*NP-β-D-galactopyranoside	1.5
*p*NP-β-D-glucopyranoside	100

The substrate concentration was 15 mM. The conditions for the enzyme assay are given in MATERIALS AND METHODS

the proteins can give valuable information on their basic or acidic nature. Here, the observation that the enzyme could be specifically eluted by 20 mM citrate, which has a higher affinity for calcium than phosphate, clearly indicates that the enzyme has an acidic nature.

HTP was fundamental not only to concentrate and easily purify the enzyme in a single step, but also it was useful to obtain the enzyme in an immobilized form. The advantages of this procedure are: i) High stability of enzymatic preparation as shown in a recent study[18] on the influence of some process variables of winemaking (pH, temperature, glucose, ethanol, and sulfur dioxide concentration) on the activity of HTP-immobilized β-glucosidase; ii) simplicity, rapidity, and reproducibility of the immobilization step to a nontoxic matrix; and iii) stability of the enzymatic preparation over time and repetitive usage after separation of the substrate.

Our enzyme clearly shows two different glycosylated forms and not a diffuse heterogeneity. The two forms appear to have reproducible molecular masses in different preparations. One has an apparent MW similar to that reported by Vasserot *et al.*[19] (only one band of 94 + 4 kDa), but our mixture possesses less affinity for β-*p*NPG. When compared with the β-glucosidases reported by Gueguen *et al*,[20] our enzyme does not appear to be split into subunits since it is also active after native electrophoresis showing similar molecular masses to that found with the SDS-PAGE. Moreover, removal of carbohydrate clearly shows that the two bands correspond to two different isoforms having similar carbohydrate content.

At the present, it is not yet clear whether the lower activity of the enzyme in HTP-bound form is due to steric hindrance or to different behavior of the two isoforms when bound to the HTP matrix.

The finding that our purified β-glucosidase is very specific in hydrolyzing β-linkages could have interesting application for the development of the varietal character of wines and fruit juices. It is conceivable that the use of other enzymes in addition to the β-glucosidase reported here may be required for the complete release of the bound aromatic compounds that can be present in many fruits and their fermenting products. To enhance the release of aroma-active compounds, tests are being performed to prepare a HTP-immobilized multienzymatic hydrolytic system showing simultaneous α-rhamnosidase, α-arabinosidase, and β-glucosidase activity. At the present, we are trying to identify the components of a multienzymatic system found in the culture supernatant of a different yeast strain.

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