α-L-Arabinofuranosidases from *Aspergillus terreus* with Potential Application in Enology: Induction, Purification, and Characterization

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In the presence of L-arabitol as sole carbon source, *Aspergillus terreus* CECT 2663 produces three α-L-arabinofuranosidases (ABFs) named ABF1, ABF2, and ABF3, with molecular masses of 90 000, 82 000, and 78 500 Da, respectively. The synthesis of these enzymes is under carbon catabolite repression. Western blotting revealed that ABF2 is immunologically related to the α-L-arabinofuranosidase B previously isolated from *Aspergillus niger*. The three *A. terreus* proteins have been purified to homogeneity. They are acidic proteins with optimal pHs of 5.0 for ABF1 and ABF2 and 5.5 for ABF3 and optimal temperatures of 50, 60, and 65 °C, respectively. Kinetic constants for the purified enzymes on *p*-nitrophenyl α-L-arabinofuranoside (*p*NPA) as substrate have been determined. The three enzymes maintain elevated activities in the presence of ethanol or glucose at those concentrations normally present in must or wine.

Keywords: *Aspergillus terreus; α-L-arabinofuranosidase; L-arabitol; enzyme purification; wine aroma*

INTRODUCTION

It is now well established that certain monoterpenes of grapes contribute significantly to wine flavor (Gunata *et al.*, 1986). These compounds are present partly as free volatile forms and partly as glycosidically bound nonvolatile precursors. The latter fraction is made up of disaccharide glycosides, namely α-L-arabinofuranosyl *β*-D-glucopyranoside or α-L-rhamnofuranosyl *β*-D-glucopyranoside, the aglycons of which can be terpenol, terpenediol, 2-phenylethanol, or benzyl alcohol. These glycosidically bound nonvolatiles can be released by enzymatic hydrolysis. In a first step, and depending on the precursor, the glycosidic linkage is cleaved by either an α-L-arabinofuranosidase (ABF), an α-L-rhamnosidase, or a *β*-D-apiosidase, and then a *β*-D-glucosidase liberates the monoterpenols.

Because one of the terminal sugars in these glycosides is α-L-arabinofuranose, we decided to focus our attention on the purification of ABF, with the subsequent aim to clone the corresponding genes. This class of hemicellulases has been isolated from a number of bacteria and fungi such as *Bacillus subtilis*, *Erwinia carotovora*, *Streptomyces purpurascens*, *Aspergillus nidulans*, *Aspergillus terreus*, *Aspergillus niger*, *Sclerotinia fructigena*, and *Sclerotinia sclerotiorum*. In our search for the ABF activity, *A. terreus* was chosen as an efficient producer. Recently, three alkaline *A. terreus* ABFs from strain VTT-D-82208 have been isolated by Luonteri *et al.* (1995). To start with cloning by reverse genetics of their corresponding genes, we decided to obtain the pure proteins from a different *A. terreus* strain (CECT 2663). Surprisingly, we obtained a different spectrum of ABF activities. In this paper, we describe the induction of the synthesis of ABF activity in cultures of *A. terreus* CECT 2663 strain, as well as the purification and characterization of three ABFs which, surprisingly, are different from those previously purified by Luonteri *et al.* (1995). The effect of some enological parameters on the enzyme activities is also reported.

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MATERIALS AND METHODS

Organism and Cultivation. *A. terreus* CECT 2663 was obtained from the Spanish Type Culture Collection and maintained at 4 °C by subculturing on potato dextrose agar (Difco Laboratories, Detroit, MI). Growth for enzyme induction experiments was carried out on *Aspergillus* minimal medium (AMM) as described previously (Ramón *et al.*, 1993). For enzyme purification, the mycelium was transferred to a 5 L Erlenmeyer flask with 2 L of the AMM containing 1% of L-arabitol and incubated at 37 °C on an orbital shaker (200 rpm). After a 24 h incubation, the mycelium was first removed by filtration through nylon membrane and then by centrifugation (8000*g*, 30 min, 5 °C). Supernatant (1.5 L) was concentrated to 200 mL in a Minitan ultrafiltration system (Millipore Corp., Bedford, MA) using a 10 000 molecular weight cutoff polysulfone filter.

Enzyme Purification. The concentrate (200 mL), after desalting, was further concentrated to 50 mL and buffered with 20 mM MOPS buffer, pH 7.0, by ultrafiltration in a Filtron stirred cell using a 10 000 molecular weight cutoff Omega membrane (Filtron Technology Corp., Northborough, MA).

Step 1. The 50 mL concentrate was applied to an anion-exchange column with DEAE Bio-Gel A pre-equilibrated with 20 mM MOPS, pH 7.0. Elution at 30 mL/h was carried out with a NaCl linear gradient from 0 to 500 mM. The ABF-containing fractions were pooled, dialyzed, and concentrated by ultrafiltration (Filtron 10 000 Da membrane).

Step 2. The concentrate was applied to a Resource Q ion-exchange column pre-equilibrated with the same buffer as above. Elution at 1 mL/min of adsorbed protein was completed with a nonlinear gradient of NaCI (0−300 mM). The active fractions of ABF, eluting in two peaks during the gradient, were combined in two separate pools (in the following designated as pool I and pool II). Both pools were desalted and concentrated 10-fold by ultrafiltration (Filtron 10 000 Da membrane).

Step 3. Pool I was applied to the same Resource Q column pre-equilibrated as above with 20 mM MOPS, pH 7.0. Elution

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of bound proteins with a nonlinear gradient of NaCl in this buffer led to two distinct active fractions.

Step 4. The first fraction of the preceding step was poured onto a chromatofocusing Mono P column previously equilibrated with 25 mM methylpiperazine, pH 5.7. Elution was performed at 0.5 mL/min with a pH gradient from 5.7 to 4.0 in polybuffer 74, pH 4.0 (Pharmacia, Uppsala, Sweden).

Step 5. Pool II from step 2 was loaded onto the Resource Q ion-exchange column pre-equilibrated with 20 mM Bis-Tris, pH 6.2, and the protein was eluted with a nonlinear gradient of NaCl in the same buffer.

Enzyme and Chemical Assays. ABF activity was determined by incubating for 15 min at 50 °C a reaction mixture containing 250 μL of 1 mM *p*-nitrophenyl α-L-arabinofuranoside (*p*NPA) in 50 mM succinate buffer, pH 5.5, and 250 μL of suitably diluted enzyme in the same buffer. The reaction was stopped by adding 1 mL of 2 M Na_2CO_3, after which the absorbance was measured at 400 nm and activity calculated using the molar extinction coefficient of 13 700 M^{-1} cm^{-1} (Rombouts *et al*., 1988). One unit of ABF activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute. Endoarabinase activity was determined according to van der Veen *et al*. (1991) using sugar beet arabinan as a substrate.

Soluble protein was estimated by the Coomassie dye-binding assay (Bradford, 1976) using bovine serum albumin (fraction V) as standard. The presence of a protein in effluents of column chromatography was detected by the absorbance at 280 nm. Reducing sugars were quantified by the Nelson−Somogyi method (Somogyi, 1952). Analyses were made in duplicate.

Kinetics. The Michaelis−Menten constants, K_M and V_{max}, were determined on *p*NPA at concentrations ranging from 0.1 to 5.0 mM at the optimal temperature and pH of the corresponding enzymes. The values of these constants were calculated from Hanes plots, which gave the best fittings.

Electrophoresis, Isoelectric Focusing, and Western Analysis. SDS−PAGE was carried out according to Smith (1984) in 10% (w/v) polyacrylamide gel slabs at pH 8.6 using 25 mM Tris base buffer containing 0.1% SDS. The gel was stained with silver as described by Merril *et al*. (1981). The low molecular mass calibration mixture from Pharmacia was used as standard. Analytical isoelectric focusing (IEF) was performed on precast IEF polyacrylamide gels containing Pharmalyte carrier ampholytes in the pH range 3.5−9.0. Samples were focused using a Multiphor II System (Pharmacia). Protein bands were stained with Coomassie Brilliant Blue R 250 and their isoelectric points determined by comparison with protein standards of known pI (Sigma).

Antibodies against the purified *A. niger* ABFA, ABFB, and endoarabinase (van der Veen *et al*., 1991) were kindly provided by Dr. J. Visser (Section of Genetics of Industrial Microorganisms, Wageningen Agricultural University, The Netherlands). Cross-reactivity between the antisera and the samples was tested by western blotting. Incubation of nitrocellulose blot with the specific antisera followed by staining with alkaline phosphatase-labeled goat anti-mouse IgG was done as described by the manufacturer (Boehringer, Mannheim, Germany).

Effects of pH and Temperature on Enzyme Activity and Stability. The optimum pH for the purified enzymes was determined by incubating the enzyme preparations with *p*NPA in the Teorell and Stenhagen universal buffer (Stauffer, 1989) at pHs ranging from 2.0 to 9.0. For determination of pH stability, the purified enzymes were preincubated at 25 °C in the same universal buffer as above at pH 3.0, 4.0, and 4.4, and at intervals the remaining activity was measured under standard conditions.

The optimum temperature was determined by measuring the activity of the enzymes at different temperatures in the range 24−75 °C. The thermal stability of the protein was determined by incubating the enzyme preparation at 20, 40, 50, 60, 65, and 70 °C and measuring the remaining activity at various times using the standard procedure.

Influence on Activity of Some Effectors and Compounds. The effects of various reagents and metal ions on the purified ABFs were assayed by measuring enzyme activities under standard conditions after preincubating the enzymes for 30 min in 50 mM succinate buffer, pH 5.5, containing the effectors under study at 2 mM concentration. Considering the potential application of the *A. terreus* ABFs in wine flavor enhancement, the influence of glucose and ethanol on activity of those enzymes was investigated.

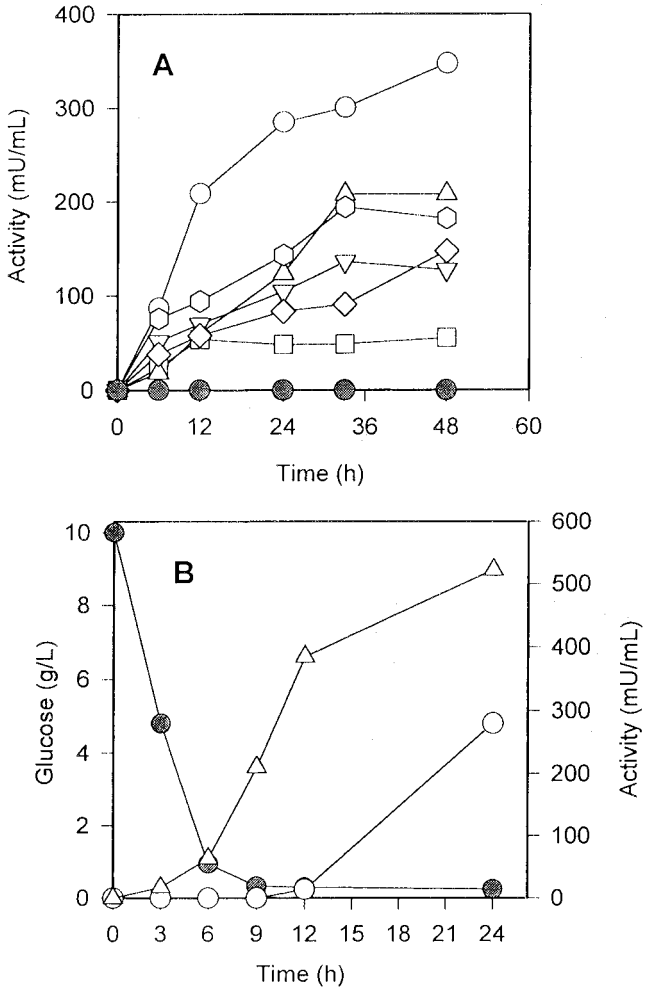

Figure 1. (A) Time course of ABF production by *A. terreus* CECT 2663 grown on AMM supplemented with L-arabitol (○), D-arabinose (□), L-arabinose (⬡), 1,5-α-L-arabinan (△), araban (◇), arabinoxylan (▽), or D-glucose (●). (B) The effect of D-glucose on ABF production under induction conditions in *A. terreus* grown on 2% (w/v) L-arabitol (△) and on 2% (w/v) L-arabitol + 1% (w/v) D-glucose (○). Consumption of D-glucose (●).

Chemicals and Substrates. L-Arabitol, D-arabinose, L-arabinose, and *p*NPA were obtained from Sigma Chemical Co. (St. Louis, MO). 1,5-α-L-Arabinan and arabinoxylan were supplied by Megazyme Pty. Ltd. (Sydney, Australia). Beet arabinan was kindly provided by Dr. J. Visser. Alkaline phosphatase-labeled goat anti-mouse IgG and alkaline phosphatase-labelled goat anti-rabbit IgG were purchased from Boehringer. Acrylamide was from Bio-Rad (Hercules, CA). All other chemicals used were commercially available reagent grade.

RESULTS AND DISCUSSION

Induction and Repression of the Synthesis of ABF Activity in *A. terreus*. ABF production was carried out by growing *A. terreus* in the presence of various sugars and polysaccharides as potential inducers. All the assayed carbon sources, with the exception of glucose, induced ABF production (Figure 1A). The highest level of ABF activity (348 munits/mL) was

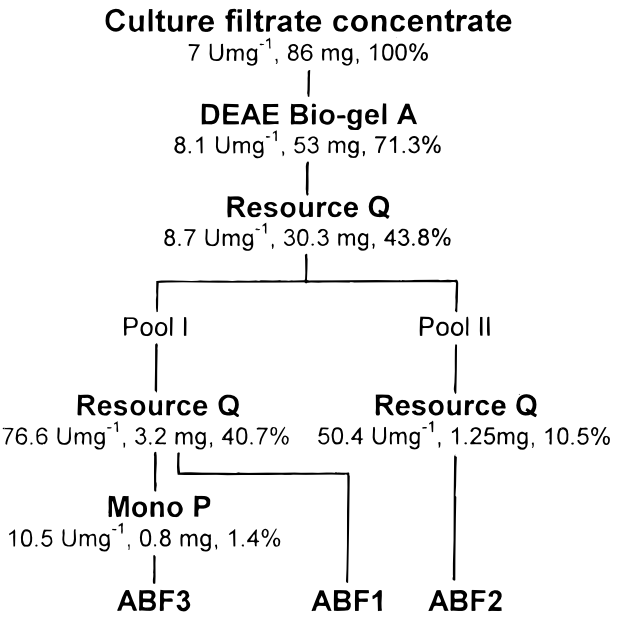

Figure 2. Results of the purification procedure for the three ABFs secreted by *A. terreus*. Specific activity (units/mg), protein content (mg), and activity yield (%) are reported for the various steps.

obtained on L-arabitol. Moreover, while induction levels attained by the other sugars appeared to be maximal by 33 h of cultivation, a continuous increase in enzyme production was observed from that time in the medium including L-arabitol. Also, in other filamentous fungal species, like *A. nidulans* (Ramón *et al.*, 1993) and *A. niger* (van der Veen *et al.*, 1993), L-arabitol is the best inducer for arabinase production. In a further experiment, it was found that the optimal L-arabitol concentration for ABF production was 10 g/L (data not shown), and for this reason the next experiments were carried out using these culture conditions.

To study carbon catabolite repression of ABF synthesis, the time course of enzyme production was followed in transfer experiments to medium containing L-arabitol as inducer in the presence (repression) or absence (induction) of 1% (w/v) D-glucose. In the presence of this sugar, induction by L-arabitol was delayed approximately 12 h (Figure 1B). As soon as the D-glucose concentration in the medium decreased to 0.033% (w/v), ABF repression was overcome (Figure 1B). To confirm this result, a transfer experiment to medium with L-arabitol and 0.033% D-glucose was carried out. In these culture conditions, no carbon catabolite repression took place (data not shown).

To detect the presence in *A. terreus* of ABFs similar to those previously isolated from *A. niger* (van der Veen *et al.*, 1993), western blots were made using specific antisera against the *A. niger* ABFA, ABFB, and endoarabinase enzymes (data not shown). An 80 000 Da *A. terreus* protein related to the *A. niger* ABFB enzyme was detected. To the contrary, even when using highly concentrated samples, cross-reactivity with the other *A. niger* antibodies was not detected (results not shown). The absence of a protein related to the *A. niger* endoarabinase was further confirmed by the failure to detect endoarabinase activity in the culture filtrate.

Purification of the *A. terreus* ABFs Induced by L-Arabitol. Figure 2 summarizes the results and the scheme of the purification procedure. Anion exchange on DEAE Bio-Gel A of the culture filtrate concentrate yielded a unique broad peak of ABF activity, eluted by 0.3 M NaCl, which contained 71.3% of original activity and 61.6% of the applied proteins (purification factor 1.15). Also, pigments of phenolic nature were removed in this step. The subsequent application of the combined, dialyzed, and concentrated active fractions to a Resource Q anion-exchange column led to the fractionation of ABF into two peaks (pools I and II) containing, as a whole, 43.8% of the total ABF activity and 35.2% of the total protein. Purification attained was 1.25-fold.

By isoelectric focusing and zymography of these two fractions after desalting and concentrating, it was found that pool I showed on IEF gel a broad active band at pH around 4.5, whereas Pool II showed an active band at pH 3.8. Pool I was subjected to anion exchange using the same Resource Q column. Two ABF peaks were observed which, as a whole, contained only 4.2% of the initial protein. The purification factor was 10.9. The peak eluting at 0.2 M NaCl contained a protein named ABF1 which, as shown by electrophoresis, appeared homogeneous. Its specific activity (27.5 units/mg) is 2.75 times that shown by the first peak.

Combined fractions of the other peak (elution at 0.1 M NaCl) were adsorbed onto a Mono P chromatofocusing column which eluted in a single, sharp protein peak at pH 4.6, where the ABF activity was located. This peak had approximately 0.9% of the starting protein and an activity yield of 1.4%. On SDS−PAGE, the protein appeared as a single band. The protein of this peak was named ABF3, and its purification factor was 1.5.

In a final step, the purification of the ABF activity contained in pool II was achieved by repeating chromatography on this same column but pre-equilibrated at a lower pH (6.2) with 20 mM Bis-Tris. SDS−PAGE revealed the existence of one band named ABF2. The overall purification factor attained for this enzyme was 7.2, the activity being only 10.5% of that of the initial material.

To identify which of the three purified *A. terreus* ABFs was related to the *A. niger* ABFA and ABFB enzymes, samples of the pure proteins were analyzed by western blotting using specific antisera. Only a strong specific reactive band appeared with the ABF2 when using ABFB antiserum, indicating that these two proteins are immunologically related.

Table 1. Properties of the Three Purified α-L-Arabinofuranosidases from *A. terreus*

	ABF1	ABF2	ABF3
M_r	90 000	82 000	78 5000
p*I*	4.2	3.8	4.6
pH optimum	5.0	5.0	5.5
T optimum (°C)	50	60	65
activation energy (kcal/mol)	29.1	36.6	41.0
K_M[a] (mM)	0.35	0.47	0.31
V_{max}[a] (units/mg of protein)	133	175	75
V_{max}/K_M	381	372	242

[a] Michaelis constants on *p*NPA.

Biochemical Identification of the Pure Enzymes. The physicochemical properties of the purified enzymes are listed in Table 1. The three enzymes had acidic p*I* values of 4.2, 3.8, and 4.6 for ABF1, ABF2, and ABF3, respectively. These are higher than the 3.3 reported for *A. nidulans* (Ramon *et al.*, 1993) and 3.3 and 3.5 for two *A. niger* ABFs (van der Veen *et al.*, 1993) and clearly different from the basic values (7.5, 8.3, and 8.5) for the proteins purified from the VTT-D-82209 strain (Luonteri *et al.*, 1995). Each of the purified ABFs showed a single silver-stained protein band on SDS−PAGE which, on the basis of their mobilities, had

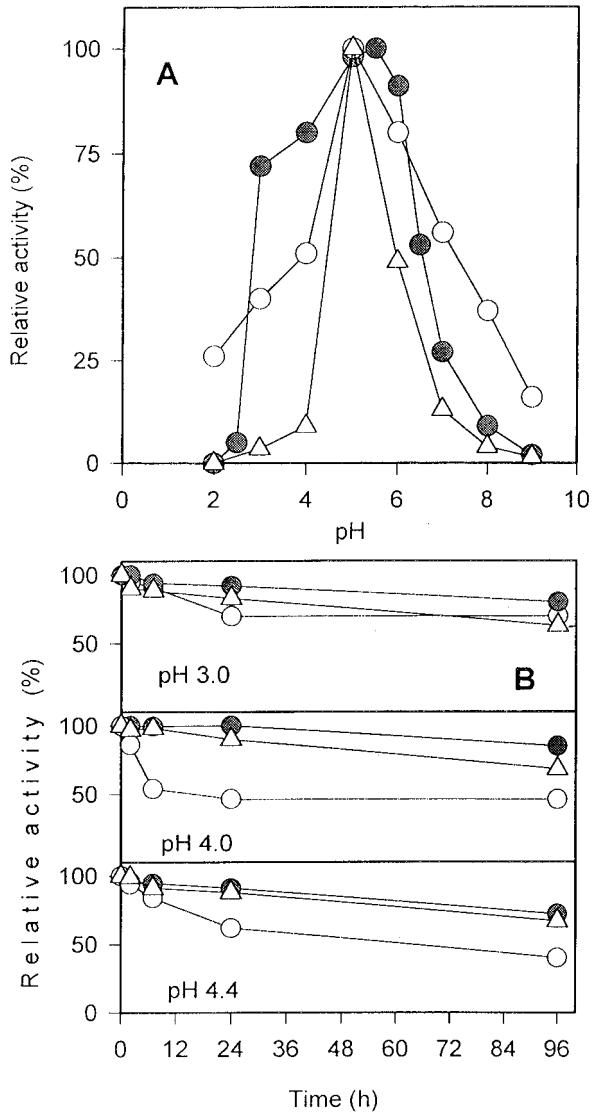

Figure 3. Effect of pH on activity (A) and stability (B) of ABF1 (●), ABF2 (○), and ABF3 (△).

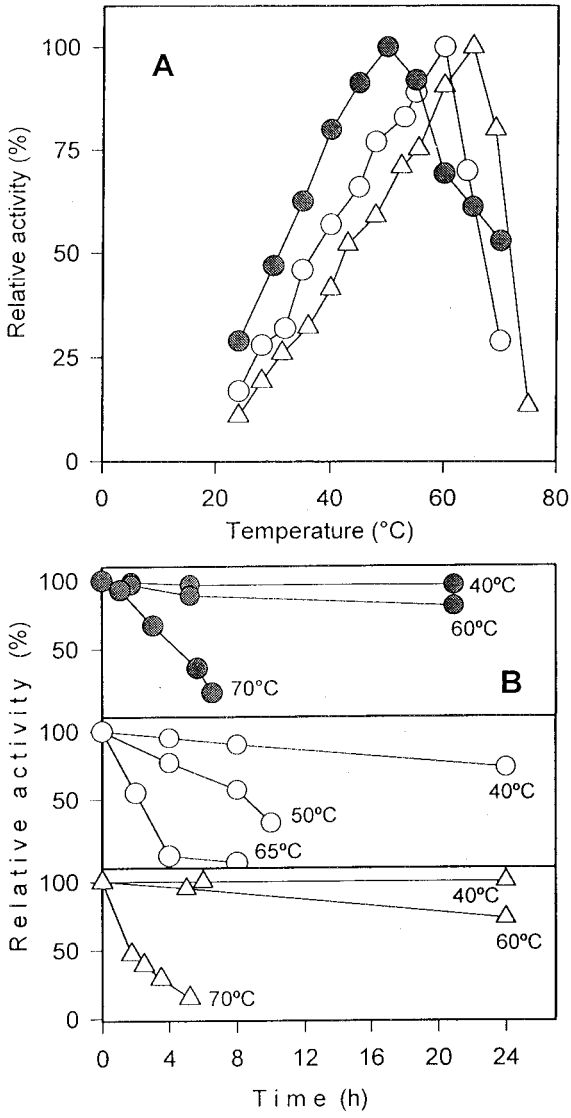

Figure 4. Effect of temperaure on activity (A) and stability (B) of ABF1 (●), ABF2 (○), and ABF3 (△).

apparent molecular masses of 90 000, 82 000, and 78 500 Da for ABF1, ABF2, and ABF3, respectively. These estimated molecular masses were greater than those observed for other fungal ABFs, which ranged from 43 000 to 80 000 Da (Ramón *et al.*, 1993; van der Veen *et al.*,1993; Luonteri *et al.*, 1995).

ABF1 showed maximum activity at pH 5.5, but, unlike the other two enzymes, more than 70% of its initial activity was present at pH 3.0 (Figure 3A). To the contrary, ABF2 and ABF3, which exhibited maximum activity at pH 5.0, showed a rapid decrease of activity below this value (Figure 3A). It is important to note that the ABFs obtained from the VTT-D-82208 strain have optimal activity at pH 4.5 (Luonteri *et al.*, 1995). The activities of the three enzymes declined rapidly at pH values above their optima, entering the alkaline range, an unusual situation for arabinases of fungal origin (Kaji, 1984). These pH optima are higher than those previously reported for other fungal ABFs, which lie in the 3.5−4.5 range (Baker *et al.*, 1979; Poutanen, 1988; van der Veen *et al.*, 1991; Ramón *et al.*, 1993). As can be seen in Figure 3B, ABF3 was rather stable in the pH 3.0−4.4 range, as no more than 25% of its initial activity was lost after 96 h of incubation at room temperature. ABF1 exhibited a very similar behavior. Under the same conditions, ABF2 retained only around a 40% of its initial activity when incubated for 96 h at pH 4.0 and 4.4.

Optimal temperatures of activity were 50, 60, and 65 °C for ABF1, ABF2, and ABF3, respectively (Figure 4A). While for ABF1 and ABF2 these optima were similar to those of other fungal ABFs (Rombouts *et al.*, 1988; Gunata *et al.*, 1990), ABF3 showed jointly with an ABF from *A. nidulans* (Ramón *et al.*, 1993) the highest temperature optimum, 65 °C, described to date. Thermal denaturation of the purified ABFs followed first-order inactivation kinetics, which reinforces the fact that the enzymes are pure (Figure 4B). At pH 5.5, the enzymes lost less than 20% of their activity after 24 h at 40 °C. At 60 °C, ABF1 and ABF3 seemed to be quite stable, and half-lives of these two enzymes at 70 °C were 2 and 5 h, respectively. The ABF2 had the worst stability, and its activity was only 7% after 4 h at 65 °C.

Kinetic Constants. Apparent Michaelis−Menten constants K_M (Table 1) for ABF1, ABF2, and ABF3 were 0.35, 0.47, and 0.31 mM, respectively. Substrate specificity, as the V_{max}/K_M value shows, was higher for ABF3. Assuming only one active site, the maximum number of bonds cleaved per enzyme molecule per minute was in the range $(4-7) \times 10^2$.

Influence of Some Effectors and Compounds of

Enological Relevance. Heavy metal ions, such as Hg^{2+} and Cu^{2+} at 2 mM concentration, decreased drastically the activity of the three purified ABFs. While ABF2 was especially susceptible to inhibition by Zn^{2+} and Cd^{2+} (inactivation of 96%), the other two enzymes showed inhibitions of only around 40%. Co^{2+}, Ca^{2+}, and EDTA (EDTA at 2 mM and 10 mM concentrations) did not affect the activities. The reducing agent dithiothreitol had no effect on any of the three ABFs, suggesting that sulfhydryl groups play a minor role in these enzyme activities.

Glucose at 21.2% (w/v) concentration, a level of this sugar usually found in unfermented grape juice, reduced by 20% the original activity of ABF2 and ABF3. Somewhat higher reduction (36%) was exhibited by ABF1. Inhibitions of 66%, 38%, and 20% were observed for ABF1, ABF2, and ABF3, respectively, when activity was measured in the presence of ethanol at 12% (w/v) concentration.

In conclusion, three *A. terreus* ABFs have been purified and partially characterized. The enzymes are different from those previously purified by Luonteri and co-workers (1995) using a different *A. terreus* strain. Studies of the effect of these pure enzymes on microvinification processes and the cloning of their encoding genes are now in progress.

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