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Pectolytic Enzymes of the Industrial Fungus *Aspergillus kawachii*

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Abstract *Aspergillus kawachii* extracellular pectinases were screened in liquid cultures with different carbon sources. The fungus grown on citrus pectin or lemon pomace produced at least one of these inducible pectinases: acidic polygalacturonase, pectin lyase, pectin methylesterase, α-L-arabinofuranosidase, α-1,5-endoarabinase, β-D-galactosidase/exogalactanase, and β-1,4-endogalactanase. The lemon-pomace filtrates also contained significant α-L-rhamnosidase and β-D-fucosidase activities. Most of the screened pectinases were active at pH 2.0-2.5, indicating that the *A. kawachii* enzymes were acidophilic. Under the culture conditions employed we could not detect enzymatic degradation of soybean rhamnogalacturonan. The *A. kawachii* pectinase-production-related regulatory phenomena of induction-repression resemble those described for other *Aspergillus* sp.

Keywords: *Aspergillus kawachii*, pectolytic enzymes, carbon source

Introduction

The white mold *Aspergillus kawachii* has been widely used in the Japanese and Korean food industry for the preparation of *koji* and *nuruk*, the fermentation starters used for the elaboration of the traditional alcoholic beverages *soju* and *makgeoli*, respectively (1,2). During fermentation the fungus releases extracellular enzymes that dissolve and saccharify the raw material (rice, barley, and sweet potato) of the *soju* and *makgeoli* mash. Because of the fermentation mash's strong acidity (about pH 3.0) some of these extracellular enzymes are acidophilic; with protein, cellulose, xylan, and starch being the substrates for the best characterized *A. kawachii* hydrolases (3-8). Pectin -a complex heteropolysaccharide in plant primary cell walls and middle lamellae- may represent a complementary carbon source (C-source) for fungal growth, some of whose degradation products can potentiate the induction of enzymes involved in flavor development. Except for the polygalacturonases (PGases) (9-12), little information is available regarding *A. kawachii* pectin-degrading (pectolytic enzymes or pectinases) enzymes. Thus, a characterization of the pectinases of this fungus may help both elucidate the processes of grain maceration and fermentation used to prepare fermented beverages and enable the discovery of novel acidophilic enzymes.

Therefore cell-free fungal-culture filtrates were screened for those pectinases that either act on the pectin backbone or degrade the side chains. The fungus was grown on media containing pectin or lemon pomace as C-sources since most *Aspergillus* genes expressing cell-wall-degrading enzymes are induced by cell-wall polymers or molecules derived from them (13). A reference medium with glucose was also used to test for constitutive production of the screened pectinases (11). It was found that *A. kawachii* produced a complex pool of mostly inducible pectin-degrading enzymes, although we could not detect rhamnogalacturonase.

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Materials and Methods

Chemicals and solutions Polygalacturonic acid (PGA), citrus pectin (degree of methylation [DM], 53%), carboxymethylcellulose sodium salt (CMC), xylan from birchwood (XYL), *p*-nitrophenyl-α-L-arabinofuranoside, *p*-nitrophenyl-β-D-fucopyranoside, *O*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-α-L-rhamnopyranoside, and *p*-nitrophenyl-β-D-xylopyranoside were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soybean rhamnogalacturonan (RG), sugar-beet araban (ARA), and potato galactan (GAL) were from Megazyme (Wicklow, Ireland). Lemon (*Citrus limon* Burm) pomace (LP) was obtained from Citrinor (Tucumán, Argentina). After the separation of seeds from the raw material, the peels were shredded with a food processor, sieved and a 80 to 100 mesh fraction used. LP composition (%, w/w on a w.b.) was: water, 8.2; galacturonic acid, 29; neutral sugars, 36 (glucose 50%, arabinose 20.5%, galactose 14%, xylose 7.0%, rhamnose 1.7%, and fucose 0.8%); and protein, 6.7. Olivex® was from Novozyme (Bagsvaerd, Denmark). This commercial product is an uncharacterized enzymic mélange of many different plant-cell-wall-degrading enzymes and was used as a positive control for rhamnogalacturonase activity. The enzymatic product was desalted with a PD-10 column equilibrated with 20 mM sodium acetate buffer (AcB), pH 5.0. Chemicals and equipment for column chromatography were from Amersham Pharmacia Biotech (Uppsala, Sweden). Unless otherwise stated, CPB contained 50 mM citric acid/25 mM Na_2HPO_4.

Microorganisms, culture conditions, and biomass separation The fungal strain was *Aspergillus kawachii* IFO 4308 kindly provided by Prof. Takuo Sakai (Osaka Prefecture University, Osaka, Japan). A stock culture was

prepared in potato dextrose agar (PDA) and kept under mineral oil at 4°C. For strain cultivation, a liquid medium (11) with some modifications was used whose composition was (g/L): K_2HPO_4, 1.0; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; tryptone, 5.0; and one of 3 C-sources [glucose (Glu), citrus pectin (Pec), and LP]. Culture conditions and sample processing for the determination of biomass and enzyme activities have been described (11). Here, the freeze-dried filtrate, when required, was dissolved in water to give a 10 times concentrated solution compared to the original culture filtrate; centrifuged at 10,000×g for 10 min; desalted with a PD-10 column; and stored at 5°C.

Enzyme activity assays PGase, celulase (CMCase), and xylanase activities were determined with 0.5%(w/v) substrate solutions in CPB (PGA, CMC, and XYL, respectively) at the indicated pH by measuring the generation of reducing groups by the Somogyi-Nelson method (14). Rhamnogalacturonase (RGase), arabinase (ARAase), galactanase (GALase), and arabinogalactanase (AGase) activities were measured in the same way but with 0.2% substrate solutions (RG, ARA, GAL, and AG, respectively). Pectin lyase (PeLase) and pectate-lyase activities were assayed spectrophotometrically at 235 nm (ε_{235}=4,600/M·cm) with 0.5%(w/v) citrus pectin or PGA, respectively, in CPB at the indicated pH (15). For pectate lyase measurement, 1.0 mM $CaCl_2$ was added to the reaction mixture. Pectin methylesterase (PMEase) was assayed by measuring released methanol by the alcohol-oxidase method (16) in a reaction mixture containing 0.5%(w/v) citrus pectin in CPB. α-L-Arabinofuranosidase (ARFase), β-D-fucosidase (β-fuc), β-D-glucosidase, α-L-rhamnosidase (α-Rham), β-D-xylosidase, and β-D-galactosidase (β-gal) activities were determined with their specific *p*(*O*)-nitrophenyl glycosides. Assays were performed in CPB (1/4 strength) with 1.0 mM substrate in a final volume of 200 µL. The reaction was stopped by adding 400 µL of 0.4 M Na_2CO_3. Substrate hydrolysis was calculated from the amount of *p*-nitrophenol (ε_{405}=18,500/M·cm) or *O*-nitrophenol (ε_{420}=4,500/M·cm) released. All enzyme activities were determined at 37°C in duplicate with a blank run for every substrate solution. One unit of enzyme activity was defined as the production of 1 µmol of reducing groups, methanol, or *p*(*O*)-nitrophenol per min.

Biochemical techniques Anion exchange chromatography of PGases and other pectinases was performed with a Resource-Q column equilibrated with 20 mM AcB (pH 5.0). After sample addition, the column was washed with 5 volumes of the equilibrating buffer and then eluted with a linear gradient of NaCl (0-500 mM in buffer). The flow rate was 3.0 mL/min and the fraction volume 1.0 mL.

The enzymatic degradation of RG and ARA was qualitatively analyzed by using an high performance liquid chromatography (HPLC, Waters Associated, Milford, MA, USA). The sample (20 µL) was applied to a Shodex SC1011 carbohydrate column (6.5×300 mm, Shoko Co., Ltd., Tokyo, Japan) coupled to a refractive index detector (RID 2414). Elution was carried out with HPLC grade water at 80°C at a flow rate of 0.8-1.0 mL/min. GAL degradation products were analyzed by thin layer chromatography (TLC) analysis. Samples (15 µL) were spotted on aluminium sheets (silicagel 60 F254; Merck, Damstadt, Germany) and the chromatography performed 4 times by the ascending method with *n*-butanol:acetic acid:water (6:4:3, v/v/v) as the solvent system. For visualization, the dried plate was sprayed with 3%(w/v) phosphomolybdic acid in 10% sulphuric acid-ethanol followed by heating at 105°C for 5 min. Glucose was measured with the glucose oxidase-peroxidase reagent (Wiener Laboratorios, Rosario, Argentina) and galacturonic acid by the *m*-hydroxydiphenyl-sulfuric acid technique (17). In LP culture total carbohydrates were determined by the phenol sulphuric reagent with glucose as standard (14) and the glucosamine content by the Ride and Drisdale method (18). Mycelial growth was calculated through the relationship between glucosamine content and biomass dry weight, estimated by using the mycelium collected at 30 hr from Glu medium, and was found to be about 30 mg glucosamine/g dry mycelium.

Results and Discussion

Growth of *A. kawachii* in media with different C-sources The time course of *A. kawachii* growth in media containing glucose, pectin, or LP as C-sources is shown in Fig. 1. The fungus could utilize pectic substances as C-sources, as judged by the consumption of galacturonic acid (about 100%) in Pec and LP media, or by the neutral sugars released from LP. In all media the fungal growth pattern was in the shape of pellets, with the maximum biomass being reached within 30-35 hr cultivation when the C-source became exhausted. A less dynamic abrupt in LP medium was probably because of changes in the mycelial chitin content upon entering stationary growth phase. Growth yields based on the consumption of the C-source were in the range of 0.40-0.60 (g/g). The change in culture pH depended on the carbohydrate source and decreased progressively in Glu medium during glucose consumption, thereafter increasing sharply. Acidification is attributable mainly to fungal citric acid production. Similar patterns were observed in LP and Pec media, but there acidification was less during the growth phase and alkalinization started with about 30% of the C-source already remaining in the culture medium. Ammonia was absent from these cultures during alkalinization. At the end of the growth phase (about 30 hr) the culture pHs were 2.8, 4.2, and 6.7 and the dry biomass concentrations (g/L) 7.5, 4.2, 2.9, for Glu, Pec, and LP media, respectively.

Screening of pectinases These results demonstrate the ability of *A. kawachii* to grow on pectin and thus to produce enzymes for degrading this complex C-source. Culture filtrates were therefore screened for enzyme activities in samples from the end of the growth phase (about 30 hr) using assays carried out at pH 5.0 as well as 2.0 in order to detect acidophilic enzymes. Two kinds of pectinases were screened, those acting on the homogalacturonan (HG) or rhamnogalacturonan I (RG I) backbone and those known as *accessory enzymes*, which debranch the pectin hairy regions (13) (Table 1). Enzyme production was normalized to fungal-growth biomass (mU/g biomass), rather than expressed per unit volume of culture. It was previously reported the production of *A. kawachii* PGases in Glu medium. In this study AGase was the only

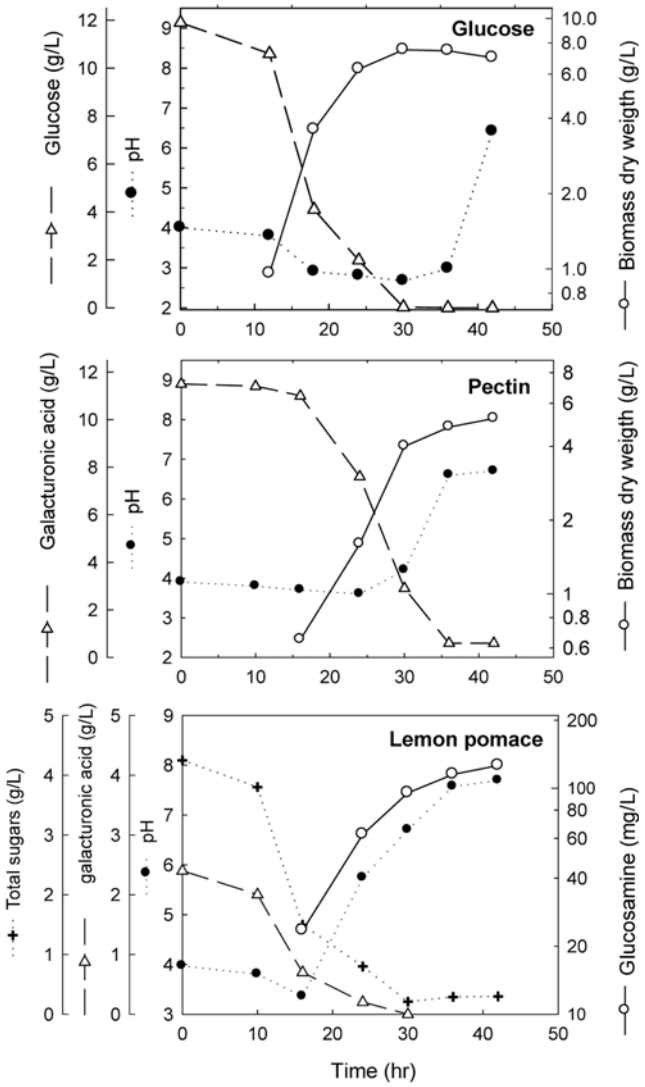

Fig. 1. Growth of *A. kawachii* in media with different C-sources. The fungus was grown in 1-L shaker flasks containing 100 mL of medium in a rotary shaker at 200 rpm and 30°C. Spore inoculum was 10^6/mL. The response of galacturonic acid in the phenol-sulphuric method was 62% of that with glucose as standard. Therefore, a corrected value of the initial total soluble carbohydrates in LP medium was 5.0 g/L. According to the glucosamine values, the biomass produced at 30 hr in this culture was about 2.9 g/L.

additional pectinase activity detected in Glu medium. Nevertheless, the production of both enzymes in Pec or LP cultures was markedly enhanced over their production in Glu medium, indicating a stimulation by pectin, and probably other constituent(s) of LP, on their synthesis. Moreover, Pec- and LP-grown cultures contained most of the other pectinases tested with positive results being obtained for PeLase, PMEase, ARAase, ARFase, GALase, and β-gal, as well as for α-Rham and β-fuc activities- these last two particularly in the LP cultures. The ARFase activity expressed by this strain is particularly high when compared to the other glycosidic activities. Pectate lyase activity was not detected in any medium.

LP was the best C-source for pectinase production in general; and with the exception of PeLase, all enzyme activities were positive when assayed at pH 2.0 and 5.0, thus indicating the presence of acidophilic enzymes in the pectinase pool. The present results show that the C-source clearly influences the induction of pectinases in *A. kawachii*. Galacturonic acid, a general inducer of pectinolytic enzymes in *A. niger*, may play the same role for the *A. kawachii* pectinases (13); but other compounds as well may enhance the expression of genes encoding pectolytic enzymes since LP was a better C-source for pectinase production than citrus pectin. Moreover, a pH-regulated gene expression may also produce differences in the pectinase-pool composition since the pH differs significantly between both media. Kojima *et al.* (10) suggest the presence of a pH-regulated expression system for the PGases in *A. kawachii*; accordingly, a similar system may be present for the other pectolytic enzymes.

The RG I backbone degradation is carried out by specific hydrolases and lyases. There was an attempt to detect enzymes able to degrade the RG I main chain by using the commercial products: the soybean RG preparation as substrate and the enzymatic mixture in Olivex as a positive enzymatic control. Incubation of a 0.2%(w/v) RG solution with a 1:50 dilution of Olivex for 1 hr at pH 5.0 showed a positive increment in the reducing power of the substrate solution corresponding to about 180 mU/mL of RGase activity. This degradation of RG by Olivex was confirmed by HPLC, where the elution pattern further suggested that the RG had been degraded by endo-acting enzymes (not shown). In contrast, neither did the reducing power of the RG solution increase even after 12 hr of incubation with a 10× concentrate 30 hr filtrate from Pec or LP media, nor was RG degradation detected by HPLC analysis. It was therefore concluded that *A. kawachii* did not produce an enzyme that degraded soybean RG under the culture conditions employed. Moreover, the induction of accessory and HG-degrading enzymes in the absence of RGase activity were also observed when other agricultural wastes such as sugar beet, mango peel, or apple pomace were used as C-sources instead of LP (data not shown). Besides pectinases *A. kawachii* produces in LP medium several enzyme activities involved in cellulose and hemicellulose degradation: CMCase (92 mU/mL), xylanase (106 mU/mL), β-D-xylosidase (1.28 mU/mL), and β-D-glucosidase (156 mU/mL). In conclusion, neither these enzymes nor the pectinases tested degrade RG, thus confirming the requirement for specific rhamnogalacturonases in the degradation of the RG I backbone, despite their accompanying weak ARFase activity (19). Enzymes able to cleave the main chain of rhamnogalacturonan have been described in several aspergilli (13). It is currently being investigated if *A. kawachii* produces rhamnogalacturonases under other cultivation conditions or in solid state fermentation.

Partial characterization of pectinases Samples from Glu (for PGase only) and LP filtrates were applied to a Resource Q column and the different pectolytic activities in the chromatography fractions were assayed (Fig. 2 and 3). With the exception of PGases, the elution profiles of the pectinases were equivalent when enzyme activities were measured at either pH 5.0 or 2.0. Therefore, for the sake of clarity only the results at the former pH are shown.

***PGases*:** In previous experiments we found that *A.*

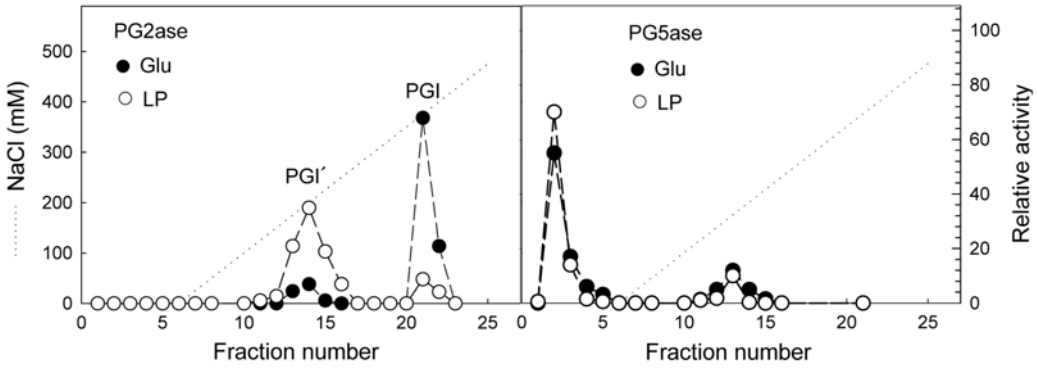

Fig. 2. Anion-exchange chromatograms of acidic (PG2ase) and nonacidic (PG5ase) PGases produced by *A. kawachii* in Glu and LP media. The sample was 300 μL of a 10× concentrated 30 hr Glu or LP filtrate. PG2ase and PG5ase: PGase activity measured at pH 2.0 and 5.0, respectively. The activity of the fractions is expressed as a percent of the total PG2ase or PG5ase recovered.

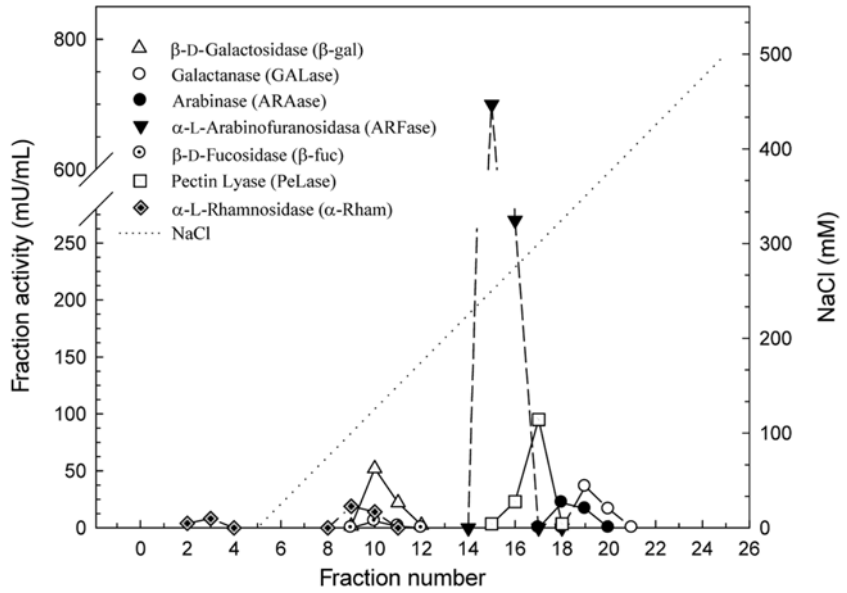

Fig. 3. Anion-exchange chromatograms of some pectolytic activities produced by *A. kawachii* in LP medium. The sample was 500 μL of a 10× concentrated 30 hr LP filtrate. ARAase and GALase activities were determined by measuring the generation of reducing groups by the Somogyi-Nelson method after the incubation of 950 μL of the substrate solution [0.2%(w/v) ARA or GAL in 5 mM AcB (pH 5.0)] with 50 μL of the collected fraction for 2 hr at 37°C.

kawachii grown in Glu medium produced 2 types of PGases judging from the ability to hydrolyze PGA at pH 2.0 and 5.0 (11,12): the acidic enzymes active at pH 2.0 but inactive at pH 5.0 (PG2ase activity) and the nonacidic species with the reverse specificity (PG5ase activity). Although in Glu and LP filtrates the same PG2ase (I and I') and PG5ase (II and III) peaks were resolved, indicating that similar enzymes were produced in both media, the

Table 1. Pectolytic activities produced by *A. kawachii* IFO 4308 in media with different C-sources[1)]

Carbon source	Assay pH[2)]	Enzyme activity (mU/g biomass)									
		PGase	PeLase	PMEase	ARFase	β-Gal	β-Fuc	α-Rham	AGase	ARAase	GALase
Glucose	2.0	3.7	0[3)]	0	0	-	0	0	1.4	0	0
	5.0	42.9	0	0	0	<0.1	0	0	1.7	0	0
Pectin	5.0	154	2.6	-	13.3	13.5	0.15	<0.1	1.2	-	-
Lemon Pomace	2.0	25.9	0	4.0	2.9	9.3	-	4.8	2.0	0.35	0.7
	5.0	380	13.4	11.5	10.6	14.5	1.03	12.4	2.51	2.5	4.8

[1)]Samples for enzyme-activity screening were taken from each medium at 30 hr of culture.
[2)]pH at which the enzyme activity was measured.
[3)]0, not detected under the assay conditions; -, not determined; Activities are expressed as the mean value for 3 independent cultures with a variation coefficient of 10-15%; Pectate lyase and rhamnogalacturonase were not detected in any medium.

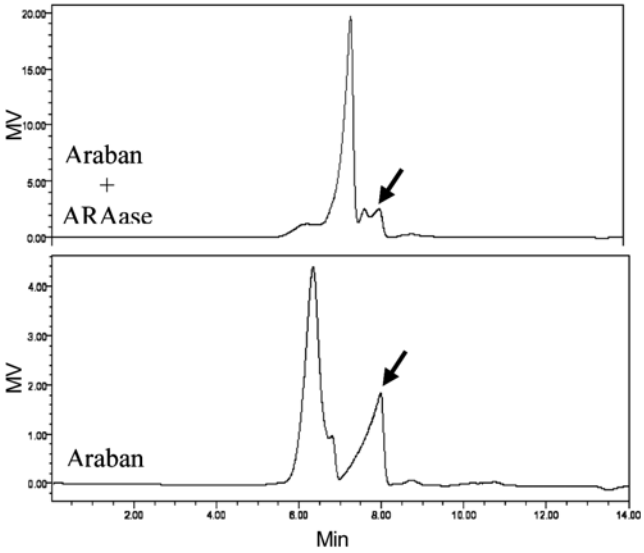

Fig. 4. HPLC analysis of araban after incubation with a chromatographic fraction containing ARAase activity. Of 0.2%(w/v) araban, 1.0 mL were incubated with 1.0 mU of ARAase (fraction 18, Fig. 3) in 5.0 mM AcB (pH 5.0) for 2 hr at 37°C. The estimated araban hydrolysis was 1.0 %. The peaks with a RT of about 8 min corresponds to acetate (arrows). The RT of arabinose was 12.25 min (not shown).

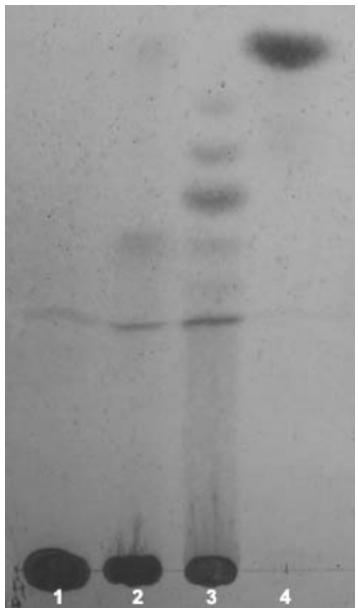

Fig. 5. TLC analysis of galactan reaction products. Of 0.2%(w/v) galactan in 5.0 mM AcB (pH 5.0), 1.0 mL was incubated at 37°C for 2 hr with 4.0 mU GALase (fraction 20, Fig. 3) or 8 hr with 2.0 mU β-gal (fraction 10, Fig. 3). The estimated galactan hydrolysis was about 4.0 and 1.0%, respectively. Lane 1, substrate blank (2- or 8-hr incubation); lane 2, galactan incubated with β-gal; lane 3, galactan incubated with GALase; and lane 4, galactose (1 g/L).

distribution of the PG2ase activity between the 2 peaks varied markedly with the C-source. Whereas in Glu medium the predominant peak (>95% of the PG2ase activity) corresponded to PG2ase I (11), in the LP filtrate the main peak (80% of the total PG2ase activity), recovered in the void volume, was PG2ase I'. The increased PG2ase activity found in LP medium, as compared to Glu medium, may therefore be attributed to the induction of an acidic PGase (the PG2ase I'; Table 1). By contrast, the relative activities of the 2 PG5ase peaks did not change with the C-source. Previous results indicated that the predominant peak (>95% of the PG5ase activity in the chromatography fractions) and the lesser peak correspond to PG5ase II and PG5ase III respectively (12). The synthesis of these 2 nonacidic endo-PGases moreover seems to be enhanced by pectin components.

PeLase: Only 1 peak with PeLase activity was detected, indicated by viscosimetric and pH activity assays as an endo-pectin lyase, with an optimum at pH about 5.0 and exhibiting only 8 and 23% maximum activities at pH 2.5 and 5.5, respectively.

PMEase: Only 1 peak of PMEase activity was observed (fraction 10-12, not shown), whose middle fraction, nearly devoid of PGase activities, showed a PMEase optimum with pectin DM 53 at pH 4.5 and retained 25% of the maximum activity at pH 2.0.

ARAase and ARFase: In LP cultures *A. kawachii* produced at least 2 enzymes able to hydrolyze arabinosyl linkages (Fig. 3) since peaks containing ARAase and ARFase activities were separated. Because the peak containing the ARFase activity did not show ARAase activity even after prolonged incubation with araban (8 hr), an exo-acting mechanism of the ARFase on this substrate could be theoretically ruled out. Other arabinose-containing substrates were not tested. Multiple forms of ARFase detected in the culture broth of different fungi differ in their substrate specificity (20). Koseki *et al.* (21) purified 2 ARFases (AkAbfA and AkAbfB) from *A. kawachii* wheat bran cultures using chromatographic conditions similar to those employed here. The optimum pH of both enzymes with pnp-Ara$_f$ was 4.0 (the optimum pH for the ARFase activity with the same substrate was 3.5-4.0). AkAbfA and AkAbfB acted synergistically with xylanase in the degradation of arabinoxylan. The exact role of the ARFase induced in LP culture with respect to pectin and xylan degradation and the relationship of this ARFase to AkAbfA and AkAbfB need to be established. By contrast, the HPLC results obtained (Fig. 4) indicate the absence of free arabinose and a dramatic decrease in the molecular weight of araban after incubation with ARAase (from fraction 18, Fig. 3), thus suggesting the presence of an α-1,5-endoarabinase (13) in this fraction

GALase and β-gal: Figure 3 shows that the galactan-degrading activity, though partially coeluting with the ARAase, was separated from the β-gal activity. Analysis by TLC (Fig. 5) of the galactanase-galactan degradation products showed the absence of galactose along with oligomer formation, indicating the presence of a β-1,4 endogalactanase in the fraction assayed (13). Likewise, after prolonged incubation (8 hr) of galactan with β-gal (fraction 10), a net increase in reducing sugars could be observed, and TLC analysis showed a weak galactose spot together with a galacto-oligosaccharide product. It was concluded that the enzyme carrying out the β-gal activity also display an exo-acting activity towards galactan.

α-Rham and β-D-fucosidase: These 2 enzymes are not

usually classified as pectinases, although they might hydrolyze the rhamnose and fucose residues present in the side chains of certain pectin regions, such as RG II (13,22). One active fraction for β-fuc (eluting together with β-gal) and 2 active fractions for α-Rham were detected, one in the washing buffer (Rham II) and the other eluting elsewhere (Rham I). Rham I represented more than 95% of the total α-Rham activity recovered and is the main α-Rham produced by *A. kawachii* in LP medium. Further purification of this enzyme showed that Rham I was free from β-D-glucosidase activity (i.e., it was not a naringinase enzyme complex); has an pH optimum for pnp-Rha_p in the range of 4.0-5.0, with 10% maximum activity retained at pH 2.0; and is able to hydrolyze naringin and hesperidin, but not quercitin. Rham I seems to be related to the recently purified and characterized α-L-rhamnosidase isolated from a rhamnose-grown culture of the same *A. kawachii* strain (23).

Arabinogalactanase: The AGase activity expressed in Glu and LP media could not be detected in the chromatography fractions although AG may be hydrolyzed by the same enzymes that act on ARA and GAL. The reason for this result is at present not clear.

In conclusion *A. kawachii* is an excellent source of pectolytic enzymes. The establishment of the role of pectinases in *soju* or *makgeoli* brewing awaits their complete purification and characterization. Meanwhile, the crude enzymatic pool of *A. kawachii*, and especially certain of the constitutent enzymes, would appear promising candidates for technological applications under highly acidic conditions.

Acknowledgments

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