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Full Length Research Paper

Extracellular leucine aminopeptidase produced by *Aspergillus oryzae* LL1 and LL2

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In a screen of 175 fungal strains, *Aspergillus oryzae* LL1 and LL2 were identified as having the highest extracellular leucine aminopeptidase (LAP) activity. LAP activity was optimal when *A. oryzae* LL1 was subjected to submerged fermentation with an inoculum size of 10^5 spores per ml and an agitation of 100 rpm at 30°C in media containing defatted soybean and rice husk with a pH of 4.5 for 72 h. Partial characterization of *A. oryzae* LL1 LAP revealed that it could be greatly inhibited by 2 mmol L^{-1} of Pb^{2+}, Cu^{2+}, or Fe^{2+}. In contrast, 2 mmol L^{-1} Zn^{2+} stimulated LAP activity about one-fold compared to adding 30 μmol L^{-1} zinc increased D_H to 33.3% for LAP LL1 during a piolot plant scale experiment hydrolysis of chopped chicken breast meat (Lin et al., 2008; Eur Food Res. Technol., 2008) . To determine the stability of *A. oryzae* LL1 LAP, the samples were stored at -80, -20, 4 and 25°C for eight weeks with residual activities being reduced to 84, 72, 61 and 58%, respectively. Taken together, our data suggest that *A. oryzae*, LL1 LAP has tremendous potential for use in the food industry.

Key words: leucine aminopeptidase, *Aspergillus oryzae*, culture conditions, fermentation, Zn^{2+}.

INTRODUCTION

Aminopeptidases catalyze the cleavage of amino acid residues from the N-terminus of peptides and proteins. Aminopeptidases comprise a large group of enzymes that exist in animal and plant tissues as well as in micro organisms. Despite substantial knowledge about bacterial and mammalian aminopeptidases, the function of amino-peptidases remains largely unclear. Leucine aminopepti-dase (LAP, EC 3.4.11.1) metabolizes L-leucylglycine and other peptides with N-terminal leucine. It also hydrolyzes a large variety of substrates, which do not contain leucyl residues (Smith and Hill, 1960) . LAPs are exopeptidases belonging to a class of zinc-requiring metalloproteases. However, a few enzymes fermentation require a cobalt ion for full enzyme activity. LAPs have been isolated from a variety of tissues and organs, and it exhibits a variety of physico-chemical properties de-pending on the enzyme source (Taylor, 1993; Wart and Lin, 1981). Microbial sources of LAP include *Escherichia coli* (Vogt, 1970), *Aeromonas proteolytica* (Prescott and Wilkes, 1976), marine *pseudomonads* (Merkel et al., 1981), and *Streptomyces griseus* (Spungin and Blumberg, 1989). LAP produced by the latter has been particularly well characterized. In lactic bacteria of biotechnological interest, such as *Lactobacillus sanfrancisco* CB1, amino-peptidases generate the characteristic texture and taste of sourdough, meats and cheese (Gobbetti et al., 1996).

It has been reported that the bitterness of petides is proportional to the number of hydrophobic amino accids in its N -terminus (Ney and Retzlaff, 1985). The exopepti-dases, such as LAPs that can cleave hydrophobic acids are valuable for preparation of debittered protein hydro-lysate (Rao et al., 1998). Except for the above function, during the storage of meat, amino acids are released from peptides by the action of neutral aminopeptidases, such as LAP (Joseph and Sanders, 1996), aminope-ptidases B, C and H (Ishiura et al., 1987; Nishimuraet al., 1992; Rhyu et al., 1992), DapII (Parsons and Pennington, 1976), and several dipeptidases (Okitani et al., 1981), resulting in an improved meat flavor. Aminopeptidases from *A. niger* are used in the production of cheese, for baking, and for the preparation of soy hydrolysates. Furthermore, recent studies demonstrated that bacterial

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Abbreviations: LAP; leucine aminopeptidase, **PDA;** potato dextrose agar, **SmF;** submerged fermentation, **SSF;** solid-state

APs are valuable for the preparation of a debittered protein hydrolysate in the food industry (Arima et al., 2006), because of their broad substrate specificity and functions as amidases and esterases (Bienvenue et al., 2002).

Exopeptidases from *Aspergillus oryzae* and *Aspergillus sojae*, such as LAP, are known to play an important role in the digestion of soybean protein to produce free amino acids. *Aspergillus* cultures have a long history of safe use in producing enzymes for protein hydrolysis such as in the production of traditional soy sauce. Cultures such as *A. oryzae* are used as high yield expression systems for the production of industrial enzymes by heterologous methods. Some enzymatic properties of carboxypeptidase I, II, III and LAP I, II, III, VI were well reported (Nakadai et al., 1972 a, b, c; Nakadai et al., 1973 a, b, c; Nakadai et al., 1977). The filamentous fungus *A. oryzae* has been used to produce traditional fermented foods such as rice wine, soybean paste and soy sauce for more than 1,000 years. Because of its long use in food production, *A. oryzae* is listed as GRAS (that is, generally regarded as safe) status by the U.S. Food and Drug Administration. The use of *Aspergillus* as a host for the production of industrial proteins has led to detailed studies on its proteolytic spectra. Although the endopeptidase spectrum is thoroughly studied, the aminopeptidase spectrum has yet to be analyzed (Rao et al., 1998).

We have conducted a screen of 175 fungi strains from the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan) and have identified two strains of *A. oryzae*, LL1 and LL2, which exhibit high extracellular LAP activities. In previous paper (Lin et al., 2008), we described the complement of extracellular LAP produced from a submerged fermentation of *A. oryzae* LL1 using chicken meat. We also compared these activities to other commercial enzymes: LAP LL1's LAP specific activity was 0.054 U mg^{-1}, the enzyme substrate hydrolysis characteristics were comparable to Flavourzyme, a typical fungal complex of exopeptidases and endoproteases are used in industrial food processing.

The protease specific activity of LAP LL1, as also compared to other commercial products such as Alcalase, Esprose, Nutrase and Protamex typically used as proteases for food processing. Additionally, we performed a pilot-plant protein hydrolysis test using LAP LL1 on 54 kg of chicken meat.

Here we describe the optimal culture conditions for these strains as well as the partial characteristics of each, and we would also try to describe the complement of extracellular LAP which produced with *A. oryzae* LL1 using synthetic substrate. Moreover, *A. oryzae* LL1 LAP could be greatly inhibited by divalent cations such as Pb^{2+}, Cu^{2+}, or Fe^{2+} and Zn^{2+} can stimulate LAP activity about one-fold. Finally we demonstrate that the stability of *A.*
oryzae LL1 LAP, samples without added any adjuvant were stored at various temperatures (-80, -20, 4 and 25°C), these retained at least 58% of its activity when stored at for eight weeks.

MATERIALS AND METHODS

Materials

A total of 175 strains of fungi (including 47 *Aspergillus* sp. strains) were obtained from the BCRC of Taiwan. *Aspergillus* strains included *A. niger*, *A. carbonarius*, *A. tamarii*, *A. foetidus*, *A. wentii*, *A. awamori*, *A. flavus*, *A. versicolor*, *A. oryzae* var. *effuscus*, *A. phoenicis, A. japonicus*, *A. niger var. intermidius*, *A. niger var. nunus*. Other fungi strains included *Emericell*a sp., *Amylomyces* sp., *Penicillium* sp., *Rhizopus* sp., *Mucor* sp., *Rhizomucor* sp., *Antinomucor* sp., *Monascus* sp. and *Moniliell*a sp. Wheat bran, rice husk and defatted soybean were purchased from a local market. The synthetic aminopeptidase substrate, *N*-succiny-L-Leu-*p*-nitroanilide, was obtained from Sigma-Aldrich. Casein was purchased from Merck. Other chemicals were of the highest commercially available grade.

Culture

All cultures were maintained in BS medium (Ichishima et al., 1973). Media was prepared by combining 0.3 g wheat bran, 0.45 g defatted soybean, 0.75 ml distilled H_2O and 7.5 µl conc. HCl and autoclaving the mixture for one hour (151 lb/in^2, 121°C). After sterilization, 49.25 ml distilled H_2O was added to the mixture and the pH was adjusted to 5.5. The solution was then re-autoclaved. To examine the effect of carbon and nitrogen sources on LAP production, media was supplemented with different ratios of various agricultural residues such as wheat bran, rice husk and defatted soybean.

Activation of strains

Lyophilized *A. oryzae* LL1 cultures were transferred into PDA slants and inoculated at 25°C for 7 - 14 days until sporation. One loop of spores was incubated in 50 ml BS medium, gently shaking, at 30°C for 4 days. Cultures were then centrifuged at 12,000 rpm for 10 min, and supernatants were measured for leucine aminopeptidase activity.

Spore counting

Spores were harvested from PDA slant cultures, which had been incubated at 25°C for 7 - 14 days with 9 ml of 0.1% (w/v) Tween 80. Spores were directly counted from diluted samples using a counting chamber (Bright-line® Hemacytometer, Fisher Scientific).

Submerged fermentation (SmF)

Rice husk and defatted soybean (0.525 g of each) were suspended in 25 ml H_2O containing 0.125 ml 1N HCl (pH 5.5), and the solution was autoclaved for 20 min. *A. oryzae* LL1 cultures containing 10^6 spores were transferred to the solution and allowed to incubate at 30°C with shaking (100 rpm) for two to six days.

Solid-state fermentation (SSF)

A. oryzae LL1 cultures containing 10^6 spores were transferred to glass plates containing 5 g of substrate (that is, wheat bran or defatted soybean) and incubated at 30°C without shaking for four days. Cultures were washed with sterilized water, and the broth was

centrifuged to obtain starting seed (supernatant). The starting seed (1 ml) was inoculated onto 5 g of substrate (containing wheat bran or defatted soybean) and incubated at 30°C for six days. One flask containing fermented material was diluted with 50 ml of distilled H_2O (~0.1 g of fermented material/ml), stirred for 40 min, filtered under vacuum, and centrifuged. The supernatant was used as a crude enzyme solution.

5 L Fermentation conditions

A. oryzae LL1 was grown in BS medium (10^5 spores ml^{-1}) shaking at 150 rpm, at 30°C for 17 h. Either 2 or 3% of this seed culture was transferred to 5 L fermenter (BioFlo 3000 Bench-top fermenter; New Brunswick Scientific Co., Inc., Edison, New Jersey, USA) containing 2 or 3 L of BS medium, respectively, and was fermentation was performed under various conditions at 30°C.

Enzyme activity measurements

Cellulase

Cellulase activity was measured using a modified version of an assay described elsewhere (Sardjono and Weiger, 1998). Approximately 1 g of fermented culture was extracted with 10 ml of 0.1 % (w/v) Tween 80 for 30 min. After centrifugation at 3,000 rpm for 10 min, the resulting supernatant was diluted in 0.05 mol L^{-1} citric acid buffer (pH 4.8). Diluted sample (0.5 ml), along with a 1 x 6 cm strip of Whatman No.1 filter paper curled around a glass rod, were then added to a test tube containing 1 ml of the citric acid buffer. The reaction mixture was incubated at 50°C for 60 min. The reaction was terminated by addition of 3 ml dinitrosalicylic acid solution (DNS). After mixing, the tubes were transferred to a boiling water bath for 5 min and cooled in ice. Twenty milliliters of distilled water was subsequently added to the mixture. The contents were then mixed and allowed to sit at room temperature for 20 min to let the pulp settle. The color that had developed was quantified in a spectrophotometer at 540 nm. Enzyme blank, reagent blank, and glucose standard solutions were treated in the same way. The absorbance of all experimental samples was corrected for the absorbance of the enzyme and reagent blanks. Cellulase activity, or milligrams of glucose produced per gram of fermented culture, was extrapolated from the glucose standard curve.

Amylase

Extracts used for the cellulase assay were also used for amylase measurement (Sardjono and Weiger, 1998). Briefly, 0.5 ml of supernatant was incubated with 0.5 ml of 2.0% (w/v) soluble starch in 0.05 mol L^{-1} citric acid buffer (pH 4.8) at 50°C for 20 min. The reaction was terminated with DNS. Activities were extrapolated from glucose standards and corrected for activities found in blanks with substrate and blanks without enzyme. Activity was expressed in units (U), with 1 U being defined as 1 μmol of glucose released per minute at 50°C.

Protease

Casein proteolytic activity was assayed at 40°C as described else where (Kunitz, 1946, 1947). One unit of activity was defined as the quantity of protease required to increase the absorbance at 280 nm by 0.002/min after trichloroacetic acid precipitation of the reaction mixture.

Aminopeptidases

Aminopeptidase activity was assayed by spectrophotometric determination of *N*-succiny-L-Leu-*p*-nitroanilide release, as described previously (Arora and Lee, 1990). One unit of activity was defined as one μmol of *p*-nitroaniline formed per minute. To examine the effects of various metal cations on aminopeptidase activity, *A. oryzae* LL1 broth was pre-incubated with indicated metal cations in various concentrations (2 - 50 mmol L^{-1}) at room temperature for 30 min, and enzyme activity was measured. Each experiment was performed in triplicate.

Protein concentration

Protein concentrations were determined with Bio- Rad protein assay reagent (Bio-Rad laboratories) using bovine serum albumin standards, according to the manufacturer's protocol.

Storage stability

A. oryzae LL1 broth was divided into small aliquots and stored at various temperatures (that is, -80, -20, 4 and 25°C) for eight weeks. For the stability testing, residual enzyme activity was measured from each stored sample at the indicated times. Each stability experiment was performed in triplicate. All data from sample stability studies were further subjected to Analysis of Variance (ANOVA) using a Randomized Block Design procedure.

RESULTS AND DISCUSSION

Screening of fungal strains for LAP activity

Screening of 175 fungal strains, including 47 *Aspergillus* strains, revealed that LAP production varied among the *Aspergillus* strains. *A. niger*, *A. carbonarius*, *A. tamarii*, *A. foetidus*, *A. wentii*, *A. awamori*, *A. flavus*, *A. versicolor*, *A. oryzae* var. *effuscus*, *A. phoenicis, A. japonicus, A. niger* var. *intermidius* and *A. niger* var. *nunus* exhibited very low LAP activity (data not shown). Notably, *A. oryzae* LL1 and LL2 produced high extracellular LAP activities (0.385 and 0.250 U mL^{-1}, respectively).

Aspergillus cultures have been used for nonspecific aminopeptidase production (Blinkovsky et al., 2000). *A. oryzae*, in particular, has the ability to secrete large amounts of a wide-range of enzymes into the extracellular environment, and this characteristic has been exploited for the commercial production of both recom-binant and endogenous enzymes. Consistent with our findings, Nampoothiri and colleagues have previously screened 28 *Aspergillus* strains and have identified two *A. sojae* strains and one *A. oryzae* strain as being superior LAP producers (Nampoothiri et al., 2005). The LAP activities for the majority of strains examined here were between 0 and 0.10 U mL^{-1} (138 strains). Only twelve strains exhibited LAP activities higher than 0.10 U mL^{-1}. Interestingly, even a few *A. oryzae* strains were good *oryzae* LL1 and LL2 are the most suitable for further industrial and commercial applications.

Table 1. The effect of various ratios of agricultural residues on LAP production by *Aspergillus oryzae* LL1.

Substrates	Wheat bran: rice husk		Defatted soybean: wheat bran		Defatted soybean: rice husk	
Strains	*A. oryzae* LL1	*A. oryzae* LL2	*A. oryzae* LL1	*A. oryzae* LL2	*A. oryzae* LL1	*A. oryzae* LL2
Ratio (by gram)	LAP (U ml^{-1})		LAP (U ml^{-1})		LAP (U ml^{-1})	
1.5 : 0	0.032 ± 0.012	0.026 ± 0.009	0.054 ± 0.021	0.066 ± 0.019	0.017 ± 0.007	0.070 ± 0.022
0 : 1.5	0.008 ± 0.002	0.016 ± 0.008	0.038 ± 0.011	0.047 ± 0.021	0.052 ± 0.009	0.014 ± 0.009
1.2 : 0.3	0.024 ± 0.008	0.056 ± 0.012	0.131 ± 0.021	0.156 ± 0.041	0.117 ± 0.026	0.021 ± 0.007
0.3 : 1.2	0.009 ± 0.001	0.048 ± 0.007	0.120 ± 0.015	0.183 ± 0.033	0.156 ± 0.035	0.093 ± 0.031
1 : 0.5	0.025 ± 0.002	0.058 ± 0.014	0.223 ± 0.035	0.213 ± 0.027	0.259 ± 0.046	0.116 ± 0.047
0.5 : 1.0	0.018 ± 0.007	0.056 ± 0.005	0.217 ± 0.029	0.239 ± 0.036	0.174 ± 0.062	0.148 ± 0.039
0.9 : 0.6	0.023 ± 0.011	0.046 ± 0.004	0.224 ± 0.017	0.194 ± 0.028	0.211 ± 0.033	0.134 ± 0.025
0.6 : 0.9	0.013 ± 0.003	0.118 ± 0.032	0.219 ± 0.027	0.234 ± 0.035	0.202 ± 0.043	0.163 ± 0.036
0.75 : 0.75	0.014 ± 0.004	0.042 ± 0.013	0.227 ± 0.023	0.161 ± 0.012	0.230 ± 0.037	0.127 ± 0.028
2.0 : 2.0	0.026 ± 0.009	0.147 ± 0.024	0.170 ± 0.016	0.157 ± 0.017	0.069 ± 0.027	0.059 ± 0.013
1.2 : 0.9	0.025 ± 0.007	0.072 ± 0.017	0.226 ± 0.035	0.133 ± 0.025	0.360 ± 0.062	0.142 ± 0.031
0.9 : 1.2	0.013 ± 0.003	0.078 ± 0.009	0.265 ± 0.029	0.152 ± 0.031	0.380 ± 0.045	0.150 ± 0.043
1.0 : 1.0	0.016 ± 0.004	0.073 ± 0.015	0.286 ± 0.020	0.175 ± 0.021	0.364 ± 0.038	0.099 ± 0.028
0.5 : 0.5	0.012 ± 0.005	0.023 ± 0.010	0.172 ± 0.018	0.102 ± 0.015	0.178 ± 0.028	0.125 ± 0.042

Agricultural residues: nitrogen source (defatted soybean); carbon sources (wheat bran and rice husk). Results are means ± S.D. for triplicate experiments.

Effect of carbon and nitrogen sources on LAP production

First, we examined the effects of different carbon sources on LAP productivity of both *A. oryzae* LL1 and LL2 strains. To investigate the optimal basal media for further fermentation experiments, *A. oryzae* LL1 and LL2 were cultured for six days in the presence of various proportions (1.5:0 to 2:2) to any two of three material (wheat bran, rice husk and defatted soybean). The results of the fermentation were shown in Table 1, the LAP production of both strains varied with the particular substrate used. Both strains displayed poor LAP production when cultured in various proportions of wheat bran and rice husk, indicating the defatted soybean is an important nitrogen source for LAP production. In general, *A. oryzae* LL1 preferred rice husk over wheat bran as its carbon source. Maximal LAP activities were observed in cultures containing 0.9:1.2 defatted soybean and rice husk (0.380 U mL^{-1}). Thus, media containing the highest protein (defatted soybean) and nutrient concentration (rice husk) resulted in higher enzyme production. In the case of *A. oryzae* LL2, LAP production was more favorable in the presence of wheat bran, as compared to rice husk. Maximal LAP activity occurred in the presence of 0.5:1.0 defatted soybean and wheat bran (0.239 U mL^{-1}). Of note, LAP production was decreased in the presence of only wheat bean (0.032 and 0.026 U mL^{-1} for *A. oryzae* LL1 and LL2, respectively), rice husk (0.008 and 0.016 U mL^{-1} for *A. oryzae* LL1 and LL2, respectively), or defatted soybean (0.054 and 0.066 U mL^{-1} for *A. oryzae* LL1 and LL2, respectively). Moreover, when only one of the three agricultural residues was used as a nutrient source, growth of *A. oryzae* LL1 or LL2 was depressed. This finding indicates that both carbon and nitrogen sources are important for the growth of the strains and for LAP production. Interestingly, the addition of casein and peptone as nitrogen sources had no significant effect on LAP production (data not shown).

Optimal culture conditions

Growth curve

To investigate the ability of *A. oryzae* LL1 to produce LAP, the strain was grown on BS media in shake flasks at 30°C. LAP activity was assayed by spectrophotometric determination of *N*-succiny-L-Leu-*p*-nitroanilinerelease, as described in “Materials and methods”. Figure 1 shows the time course of LAP secretion and growth of *A. oryzae* LL1. During 20 to 40 h after inoculation, the cell weight increased significantly as the cells started to grow. Cell weight then decreased between 40 to 50 h and leveled off thereafter. The reason for this plateau in cell weight is not clear. LAP secretion commenced during the early logarithmic phase of growth, and there was a linear increase in secretion from the middle logarithmic phase of growth until cell weight began to decrease. Maximal LAP secretion (0.385 U mL^{-1}) was maintained for at least 40 hr. In summary, the largest mass of fungi and the maximal LAP activity occurred at approximately 40 h post inoculation although the full time course of growth curve was set to six days in this experiment.

Effect of temperature and pH

The effect of temperature on LAP yield was examined by

Figure 1. Time course of A. oryzae LL1 growth and LAP production. A. oryzae LL1 was cultured in in BS medium over a period of 96 hr. Extracellular LAP activity ( ) and dry cell weight ( ) were determined at regular intervals over 96 h. The graph reflects data from experiments performed in triplicate.

culturing *A. oryzae* LL1 and LL2 strains in basal media supplemented with 0.6% (w/v) wheat bran and 0.9% (w/v) defatted soybean (pH 5.5) at 25, 30 , 35 and 40°C. It exhibited temperature-dependent LAP production (data not shown). *A. oryzae* LL1 and LL2 cultures yielded maximal LAP production at 30°C. At 25, 35 and 40°C, extracellular LAP activity in *A. oryzae* LL1 cultures was decreased to 58.5, 8.5 and 1.4% of the maximal activity, respectively. In case of *A. oryzae* LL2, the LAP activity was reduced to 88.6, 26.2 and 2.3% of maximal activity, when grown at 25, 35 and 40°C, respectively. The effect of initial pH of the medium on LAP yield was examined by using media of pH 4.0 - 7.0. The results shows LAP production by both strains was also dependent on pH. As shown in Table 2, significant LAP activity was observed in the pH range 4.0 - 7.0. The optimum initial pH for mycelial biomass and LAP production of *A. oryzae* LL1 was between 4.5 and 5.5. Although, the optimum initial pH for LAP production of *A. oryzae* LL2 was between 5.5 and 7.0, however, the LAP yield tended to decline significantly when pH was below 4.5. Optimum initial pH of media for both *A. oryzae* LL1 and *A. oryzae* LL2 was 5.5. The final pH of media after 96 h of culture was variable, ranging from 5.8 to 8.7.

Effect of inoculum size and rotation rate

Inoculum size is reportedly another important factor influencing mycelial growth and enzyme production (Elinbaum et al., 2002). LAP production of strains *A. oryzae* LL1 and *A. oryzae* LL2 were performed in basal media containing 0.6% (w/v) wheat bran and 0.9% (w/v) defatted soybean (pH 5.5) at 30 °C for 96 h did not exhibit any significant, variable LAP production at different inoculum levels (10^3 − 10^7 spores) . Under these conditions, inocula of 10^5 spores yielded the better results for both *A. oryzae* LL1 and LL2. The results have shown that, in our other screened *Aspergillus* spp., inocula between 10^6 − 10^7 spores per ml result in optimal LAP production. The discrepancy between these findings may be attributable to the fact that *A. oryzae* LL1 and LL2 grow more rapidly than other strains (data not shown). Optimal growth times and rotation rates were also examined. Both *A. oryzae* LL1 and LL2 exhibit maximal LAP activities at 72 h post inoculation and at a rotation rate of 100 rpm (data not shown).

The effect of SmF and SSF on the production of extracellular LAP, cellulase, amylase and casein by A. oryzae LL1

Extracellular LAP, cellulase, amylase and casein activities were measured after SmF (Figure 2A) and SSF (Figure 2B). SmF was associated with a higher level of LAP activity as compared to SSF (*E*max 0.38 U mL^{-1} vs. 0.05 U mL^{-1}). It must also be mentioned that the production

Table 2. The effect of pH on LAP production by *Aspergillus oryzae* LL1 and LL2.

Strain	*A. oryzae* LL1	*A. oryzae* LL2
pH	LAP (U ml^{-1})	LAP (U ml^{-1})
4.0	0.265 ± 0.031	0.034 ± 0.012
4.5	0.322 ± 0.085	0.067 ± 0.014
5.0	0.290 ± 0.062	0.116 ± 0.025
5.5	0.350 ± 0.083	0.211 ± 0.058
6.0	0.231 ± 0.072	0.183 ± 0.037
6.5	0.235 ± 0.053	0.150 ± 0.022
7.0	0.214 ± 0.044	0.146 ± 0.041

Results are means ± S.D. for triplicate experiments.

of LAP decreased significantly two days after SSF for unknown reasons. Many studies have demonstrated that protein production differs under SSF and SmF culture conditions (Acuna-Arguelles et al., 1995; Hashimoto et al., 1999). In many cases, the production of these proteins in SSF culture exceeds that in SmF cultures. Oda and coworkers have shown that, in the case of *A. oryzae*, the total amounts of protein secreted in SSF cultures are four to six fold greater than in SmF cultures (Oda et al., 2006). Under the culture conditions here, which used either SSF or SmF with agricultural residues as substrates, the reverse was true for LAP secretion. In the case of amylase and cellulose, activities were similar under SSF and SmF conditions. However, extracellular casein proteolytic activity was markedly increased under SmF conditions compared with SSF conditions (*E*max 1,680 U mL^{-1} vs. 502 U mL^{-1}). It has been reported that the majority of proteins secreted from filamentous fungi are highly glycosylated (Peberdy, 1994). In *Aspergillus kawachii*, about 80% of β-glucosidase was localized in the cell wall in submerged culture, while about 80% of it was secreted in solid-state culture (Iwashita et al., 1999). Together, *A. oryzae* drastically alters the manner of protein secretion in response to the submerged or solid-state culture (Oda et al., 2006). This is attributable to the fact that fungal cultures adopt different growth patterns when cultivated in liquid and solid substrates. Under SmF conditions, they are exposed to hydrodynamic forces, while in solid-state systems growth is restricted to the surface of the solid matrix. In the latter, microbial growth and product formation occurs on the surfaces of solid substrates in the near absence of free water. Thus, fungi are the most commonly used microorganisms in solid-state bioprocessing due to the low amount of available water (Pandey et al., 2001).

Optimized 5 L fermentation conditions

Fermentation conditions are known to affect enzyme production by filamentous fungi (Pandey et al., 1999). The factors that influence the morphology of filamentous fungi as well as their productivity in submerged culture include mechanical parameters, which are simply referred to as agitation (Kelly et al., 2004), the composition of the culture medium (Grimm et al., 2005), as well as the viscosity of the medium (Yang et al., 2003). As shown in Table 3, various Smf fermentation conditions were altered to determine the optimal conditions for *A. oryzae* LL1 LAP production. Working volumes of 2 or 3 L were not associated with appreciably different LAP activity. Similarly, LAP activity did not differ as a result of inocula of 10^4 or 10^5 spores per ml, in a working volume 2 L. Our results revealed that the optimal culture conditions, which yielded a LAP activity of 0.18 U mL^{-1}, consisted of a 3 L working volume, inoculae of 3% hyphae (by inoculum of filamentous cell), a 400 rpm rotation rate, and a 46 h fermentation time. The changes in fungal morphology during SmF have been well investigated for a number of industrially important fermentations (Papagianni, 1995; Papagiann et al., 2001).

In liquid environments, fungi grow as pellets or free mycelia, and each form has its own characteristics, which greatly affect the yields (Rogalski et al., 2006). A study of *A. niger* demonstrated that β-fructofuranosidase production under SmF conditions was enhanced by supplementing fermentation medium with sucrose, urea, yeast extract and minerals such as $(NH_4)_2SO_4$ (Ashokkumar et al., 2001). Additional experiments will be required to determine whether supplementation with sucrose, urea, yeast extract and minerals may accelerate LAP production by *A. oryzae* LL1.

The effect of metal ions on LAP activity

Mg^{2+}, Ca^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+}, or Pb^{2+} (2 mmol L^{-1} final concentration) was added to a crude enzyme solution obtained from *A. oryzae* LL1 cultures, and LAP activity was measured. As shown in Table 4, Pb^{2+}, Cu^{2+}, Fe^{2+} markedly decreased LAP activity. The effects of Cu^{2+} and Pb^{2+} were particularly strong, with LAP activity being

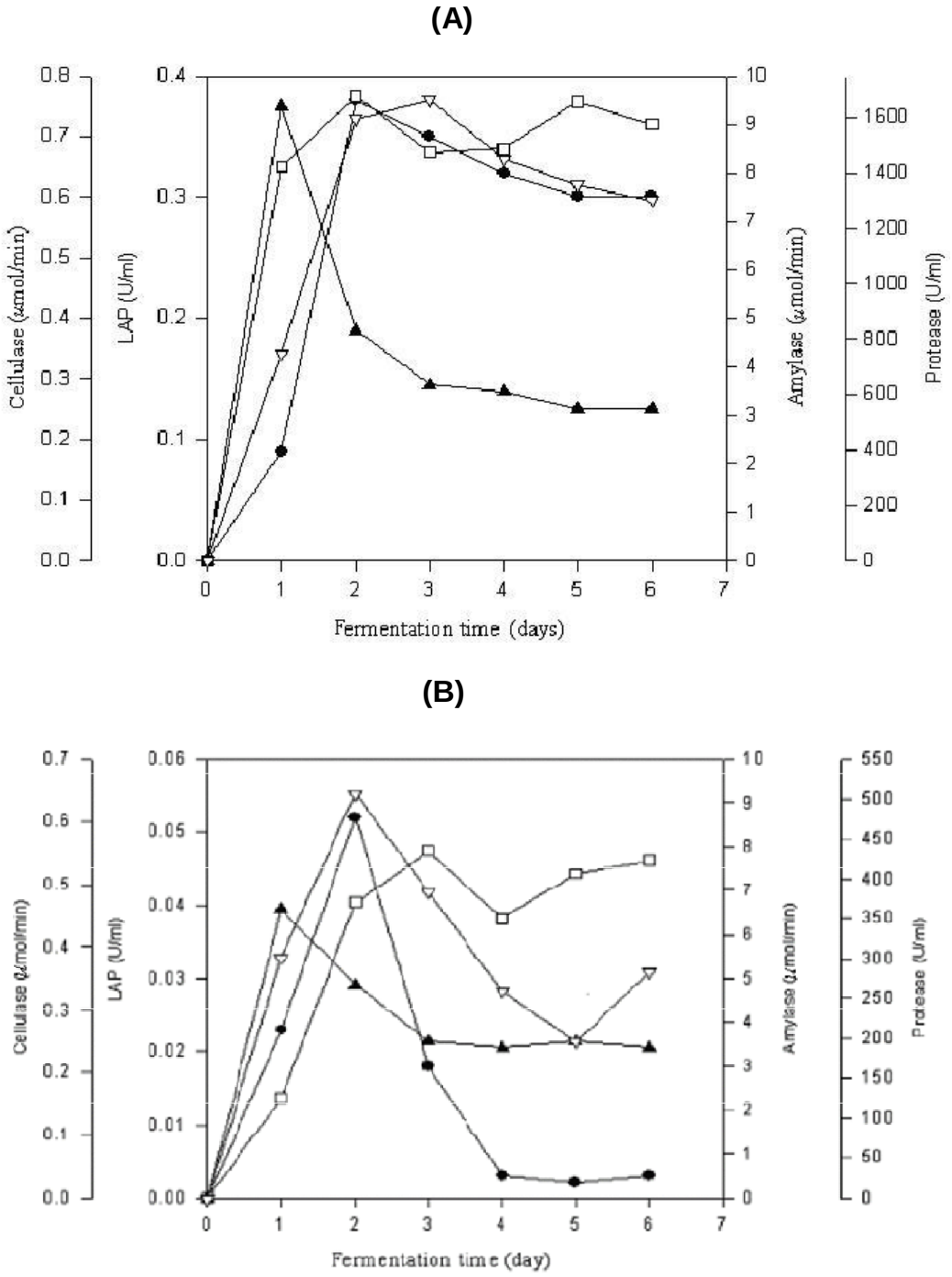

Figure 2. A. oryzae LL1 enzyme activity profiles during submerged (SmF) and solid state fermentation (SSF). Extracellular LAP ( ), cellulase ( ), amylase ( ), and protease ( ) were assayed under SmF (A) and SSF (B) conditions at the indicated times.

decreased to 5.9 and 4.5% of control, respectively. Fe^{2+} has previously been shown to increase the relative activity *Bacillus kaustophilus* LAP to 168.5% (Lin et al., 2004). Here, Fe^{2+} decreased LAP activity to 37.9% of control. In contrast, Zn^{2+} increased enzyme activity by approximately one-fold. The magnitude of this effect was similar over a wide-range of Zn^{2+} concentrations (0.1 − 50 mmol L^{-1}; data not shown). Metalloaminopeptidases have been show to exhibit a broad range of metal-ion dependence. For example, *S. griseus* aminopeptidase

Table 3. Optimization of 5 L fermentation conditions for *A. oryzae* LL1 LAP production.

Experimental conditions	A			B		C		D	E	F
Working volume (L)	2			2		2		2	3	3
Inoculum	10^4 spore			10^5 spore		2% hyphae		3%hyphae	2% hyphae	3% hyphae
Rotation rate (rpm)	200	300	400	400	600	400	500	400	400	400
pH	7.3	7.4	8.1	7.6	8.5	8.6	8.6	8.7	8.6	7.5
DO (%)	1.3	6.2	98	77	101	98	116	109	85.9	100
Fermentation time (hr)	48	67	48	53	53	51	48	50	52	46
LAP LL1(U ml^{-1})	0.01	0.07	0.13	0.11	0.06	0.13	0.15	0.17	0.16	0.18

Table 4. Effect of metal ions on LAP LL1 activity.

Metal ion (2 mmol L^{-1})	Relative activity (%)
None	100.0 ± 0.2
Mg^{2+}	101.6 ± 2.8
Ca^{2+}	100.6 ± 3.5
Cu^{2+}	4.5 ± 2.1
Co^{2+}	91.6 ± 3.8
Fe^{2+}	37.9 ± 1.9
Zn^{2+}	184.3 ± 3.2
Mn^{2+}	82.9 ± 2.4
Pb^{2+}	5.9 ± 1.3

Results are means ± S.D. for triplicate experiments.

requires Zn^{2+} for activity (Greenblatt et al., 1997), while other aminopeptidases are activated by Co^{2+}, Mn^{2+} and Ca^{2+} (Herrera-Camacho et al., 2000; Cottrell et al., 2000; Ando et al., 1999). The majority of aminopeptidases, including *S. griseus* aminopeptidase, belong to the M1 family of peptidases. These metalloenzymes contain a zinc-binding motif HEXXH (Hooper, 1994) and require zinc for enzymatic activity. Our findings suggest that *A. oryzae* LL1 LAP belongs to M1 family of aminopeptidases. Conserved regions within the M1 family of enzymes have been used to clone a zinc aminopeptidase from the industrially used fungus, *A. niger* (Basten et al., 2001). Analysis of LAP derived from bovine lens and from tomato has revealed the presence of two zinc-binding sites. Site 1 readily exchanges Zn^{2+} for other divalent metal cations including and Co^{2+}. Site 2 has a higher affinity for Zn^{2+}, allowing it to retain Zn^{2+} under conditions that allow for exchange of the Zn^{2+} in site 1.

Mg^{2+}, Ca^{2+} and Mn^{2+} had no stimulatory effect on LAP amidolytic activity. Interestingly, neither did Co^{2+}. Zn^{2+} as well as Co^{2+} has been shown to restore activity of LAP from an *A. sojae* strain (ATCC 42249) that had been inhibited by metal-chelating agents such as EDTA and dipicolinic acid (Chien et al., 2002). In addition, cobalt-dependent aminopeptidases such as *Saccharomyces cerevisiae* aminopeptidase yscCo-II have been reported (Herrera-Camacho et al., 2000). Lin and colleagues have suggested that Co^{2+}, which has rich spectroscopic properties, is an ideal substitute for native Zn^{2+} (Lin et al., 1997). We found Co^{2+} was able to enhance the thermostability of *A. oryzae* LL1 LAP. That is, 50 mmol L^{-1} Co^{2+} resulted in a residual LAP activity of 80% compared to 66% in control samples, after heating to 60°C for 60 min (data not shown). High concentrations of Zn^{2+} (10 and 50 mmol L^{-1}), on the other hand, enhanced the loss of LAP activity caused by heating samples to 60°C for 10 min (data not shown). Residual LAP activity was found to be 32% in Zn^{2+}-treated samples compared to 90% in control samples.

Storage stability

In general, enzymes undergo irreversible denaturation (that is, conformational alteration entailing a loss of biological activity) during frozen storage, even at temperatures of -80°C or less. To determine the stability of *A. oryzae* LL1 LAP, samples were stored at -80, -20, 4 and 25°C for eight weeks, and LAP activity was measured at regular intervals over this time. As shown in Figure 3, LAP activity was markedly reduced after the first three weeks of storage at all temperatures. At three weeks, residual LAP activities were inversely proportional to their associated storage temperatures, with activity being reduced to 84,

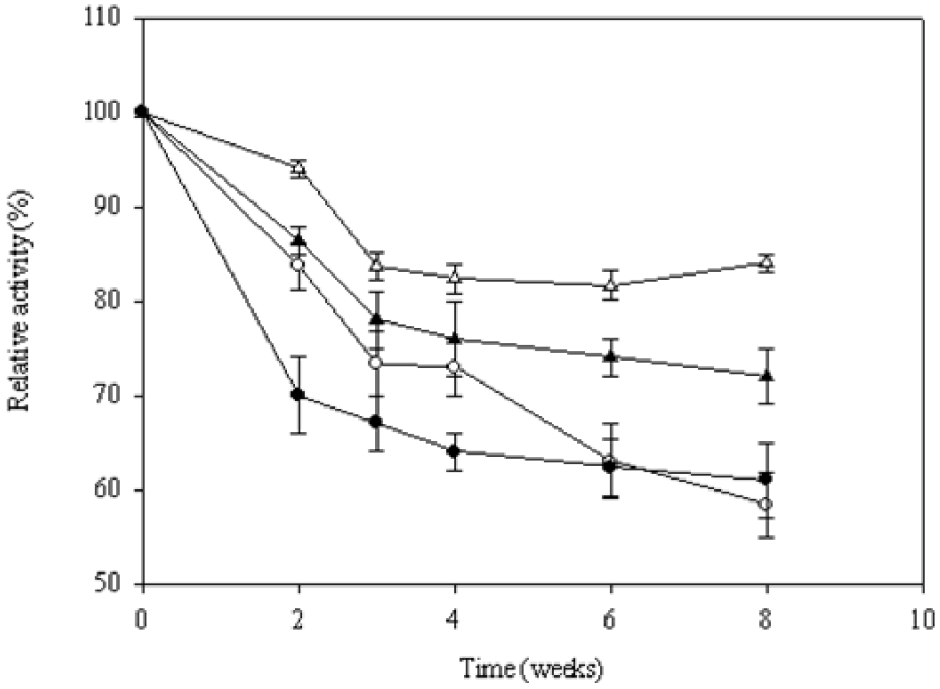

Figure 3. A. oryzae LL1 LAP storage stability. Culture supernatants were stored at -80 C ( ),-20 C ( ),4 C ( ), or 25 C ( ), and LAP activity was assayed at regular intervals over eight weeks. The graph reflects data from experiments performed in triplicate.

77, 75 and 64% at temperatures of -80, -20, 4 and 25°C, respectively. LAP activity remained relatively stable from 4 - 8 weeks at temperatures of -80, -20 and 25°C. In contrast, samples stored at 4ëC exhibited an additional ~ 10% decreased in LAP activity during this time. The finding that the LAP denaturation of occurred quickly after storage and then leveled off was expected. However, the finding that LAP activity continued to decrease during 4 - 6 weeks of storage at 4°C was not. This result is most probably attributable to assay variation or other unknown factors and can be discounted. Although our results reveal that freezing samples at -80 and -20°C has only a minor effect on *A. oryzae* LL1 LAP activity, further studies will be required to assess the long-term stability of the enzyme.

Conclusions

We have isolated *A. oryzae* LL1 and LL2 as having the highest extracellular LAP activity from 175 screened fungi strains that were collected by BCRC Taiwan. In previous paper, enzyme substrate hydrolysis characteristics of LAP LL1 as compared to other commercial enzymes typically used as food enzymes for food processing or even more better results were received (Lin et al., 2008). In this study, extracellular LAP and casein activities were compared after SmF and SSF. However, SmF was associated with a higher level of LAP activity as compared to SSF, and extracellular casein proteolytic activity was increased to about three-fold under SmF conditions compared with SSF conditions. Additionally, *A. oryzae* LL1 LAP revealed that it could be greatly inhibited by 2 mmol L^{-1} of Pb^{2+}, Cu^{2+} and Fe^{2+}, but 2 mmol L^{-1} Zn^{2+} increased LAP activity about one-fold on the synthetic peptide substrate, *N*-succiny-L-Leu-*p*-nitroaniline compared to enhance meat hydrolysis, which is relevant to the food industry. Interestingly, high concentrations of Zn^{2+} (50 mmol L^{-1}) affected the thermostability of LAP LL1, whereas equivalent concentrations of Co^{2+} could enhance it's thermostability. Finally, LAP LL1 retained at least 75% of its activity when stored for eight weeks at or below 4°C, it seemed plausible that LAP LL1 is stable. Moreover, the thermostability of *A. oryzae* LL1 LAP, coupled with its ability to selectively release certain N-terminal amino acids, will make it an interesting tool for controlling the degree of hydrolysis and flavor develop-ment in a wide range of substrates. Taken together, this study reveals that *A. oryzae* LL1 LAP has properties that make it an excellent candidate for use and application in the food industry.

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