Purification and properties of an extracellular aminopeptidase from *Streptomyces lividans* 1326

JAYANT S. APHALE† and WILLIAM R. STROHL*

Department of Microbiology, The Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43210, USA

(Received 4 September 1992; revised 19 October 1992; accepted 26 October 1992)

Streptomyces lividans was investigated for extracellular proteinases. The major extracellular proteinase activity detected under all conditions tested was L-leucine aminopeptidase activity. Slight extracellular L-proline aminopeptidase activity was also detected. No clear evidence for the presence of serine proteinases in *S. lividans* culture broths was found using several different methods. The major extracellular proteinase of *S. lividans*, i.e. the L-leucine aminopeptidase, was purified 33-fold to homogeneity. The purified enzyme was found by SDS-PAGE to have an M_r of 34000. The purified enzyme had a final specific activity of 3.6 units mg^{-1} , a K_m for L-leucine-*p*-nitroanilide of 300 μM and a $V_{\max}$ of 4.2 $\mu\text{mol min}^{-1} \text{mg}^{-1}$. The pure enzyme did not exhibit proteolytic activity on azocasein or L-proline-*p*-nitroanilide, substrates for other proteinase activities observed in crude extracts of *S. lividans*.

Introduction

There has been significant interest in the use of *Streptomyces lividans*, the primary streptomycete recombinant DNA host strain, as a host strain for secretion of heterologous recombinant proteins into the culture broth (Lichenstein *et al.*, 1988; Taguchi *et al.*, 1989; Bender *et al.*, 1990; Malek *et al.*, 1990). Thus, it is important to elucidate the complement of extracellular proteinases which may degrade potentially valuable secreted recombinant products produced in fermentations with this strain. Aretz *et al.* (1989) indicated that *S. lividans* contains both aminopeptidase and chymotrypsin-like serine proteinase activities. Their data suggested that the

serine proteinase activity is strong rather than weak (Aretz *et al.*, 1989). We have also previously noted that *S. lividans* contains a significant proteolytic activity against azocasein, although the source of that activity was not characterized (Gibb *et al.*, 1989).

In a recent investigation of a recombinant neutral proteinase from *Streptomyces* sp. C5 expressed in *S. lividans*, we noticed that the major extracellular proteinase activity contributed by the host strain, *S. lividans* 1326, was an aminopeptidase (Lampel *et al.*, 1992). Thus, we have examined *S. lividans* for its extracellular proteinase activities, and have purified and characterized the major extracellular proteinase of *S. lividans*, a leucine aminopeptidase (LAP; EC 3.4.11.-).

Methods

Organisms and fermentation conditions. The strain used in this study, *S. lividans* 66 (John Innes strain 1326), was obtained from D. A. Hopwood (John Innes, Norwich, UK). This strain has been maintained on TSA (trypticase soy agar; Becton Dickinson) plates. For inoculum preparation, spores were aseptically scraped from a TSA plate and inoculated into a 250 ml flask containing 50 ml of MB (milk broth) medium, which contained (g l^{-1}): MOPS buffer, 1.49; Nutrient Broth (Difco), 1.0; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.735; KH_2PO_4 , 0.5; K_2HPO_4 , 0.5; and fructose, 0.25. The pH was adjusted to 7.2 with 1 M-NaOH prior to autoclaving. Powdered dry milk (Carnation Co.) was autoclaved separately as a 20% (w/v) solution in double-distilled water and added to the medium after cooling to make a final concentration of 20 g l^{-1} . Conditions used for growth and analysis of the primary and secondary seed cultures and 10 litre fermentation cultures of *S. lividans* are described elsewhere (Lampel *et al.*, 1992).

* Author for correspondence. Tel. (614) 292-1919; fax (614) 292-1538; e-mail: wstrohl@magnus.acs.ohio-state.edu.

† Present address: Cangene Corporation, Mississauga, Ontario, Canada L4V 1T4.

Abbreviations: ala-pNA, L-alanine-*p*-nitroanilide; BAPNA, *N*- α -benzoyl-L-arginine *p*-nitroanilide; CBZ-GGL-pNA, carbobenzoxy-glycyl-glycyl-leucine *p*-nitroanilide; N-CBZ-PLG-hydroxamate, *N*-carbobenzyloxy-prolyl-leucyl-glycine hydroxamate; DIFP, diisopropylfluorophosphate; FAGLA, *N*-[3-(2-furyl)acryloyl]-glycyl-leucine amide; FALGPA, *N*-[3-(2-furyl)acryloyl]-leucyl-glycyl-prolyl-alanine; gly-pNA, L-glycine-*p*-nitroanilide; LAP, L-leucine aminopeptidase; leu-pNA, L-leucine-*p*-nitroanilide; lys-pNA, L-lysine-*p*-nitroanilide; NTA, nitrilotriacetic acid; SAAPF-pNA, *N*-succinyl-alanyl-alanyl-prolyl-phenylalanine *p*-nitroanilide; TAME, *N*- α -*p*-tosyl-L-arginine methyl ester.

Proteinase assays and protein determination. LAP activity in cell-free fermentation broth was determined by monitoring the release of *p*-nitroaniline ($\epsilon_{410} = 8480 \text{ l mol}^{-1} \text{ cm}^{-1}$; Vinci, 1988) from leu-pNA at 410 nm using a single-beam spectrophotometer (Beckman model DU-64). Fermentation samples (500 μl) collected at time intervals were centrifuged at 10000 r.p.m. for 10 min in a microcentrifuge to pellet the mycelia. A 100 μl sample of the supernatant was added to 880 μl of a buffer which was composed of 100 mM-HEPES (pH 8.0), 10 mM-MgCl₂ and 10 mM-CaCl₂ (HCM buffer), and held at 37 °C for 15 min. A resulting uncharacterized precipitate was removed by centrifugation at 10000 r.p.m. for 15 min in a microcentrifuge. To the clarified cell free broth, 20 μl of a 100 mM-solution of leu-pNA in 95% (v/v) ethanol was added to begin the assay, which was carried out at ambient temperature (about 22 °C).

The following *p*-nitroanilide proteinase substrates were also assayed in HCM buffer as described above: SAAPF-pNA, a substrate for chymotrypsin-like proteinases (Delmar *et al.*, 1979); BAPNA, a substrate for trypsin-like proteinases (Erlanger *et al.*, 1961); and other potential aminopeptidase substrates, ala-pNA, gly-pNA, lys-pNA and pro-pNA. Stock solutions of 5.0 mM were used for each synthetic substrate to yield a final concentration in the assay of 0.25 mM. Leu-pNA was dissolved in 95% ethanol, BAPNA was dissolved in DMSO, and SAAPF-pNA was dissolved in 100 mM-HEPES buffer (pH 7.0).

Thermolysin-like neutral proteinase activity was measured by the hydrolysis of FAGLA as described by Feder (1968) and esterase activity was assayed by the hydrolysis of TAME as described by Roberts & Elmore (1974). The enzymic hydrolysis of azocasein was assayed as described previously (Gibb *et al.*, 1989). The milk hydrolysis activity of the proteinase was determined by the spectrophotometric assay described by Lampel *et al.* (1992).

Proteinase assays were also performed on cell extracts of *S. lividans* obtained after breakage. Mycelia from 300 ml of fermentation broth were harvested by centrifugation at 16000 *g* for 15 min at 4 °C. The mycelial pellet was rinsed once with ice-cold HCM buffer and resuspended in 5 ml of the same buffer. The resuspended mycelia were disrupted in a French pressure cell (American Instrument Co.) at 16000 lbf in⁻² (about 110 MPa). Unbroken mycelia and cellular debris were pelleted by centrifugation at 20000 *g* for 30 min (4 °C) and the supernatant was used for intracellular proteinase assays. Protein concentration was determined by the dye-binding method of Bradford (1976) using a commercial protein assay reagent (Bio-Rad) and bovine serum albumin as the standard.

Electrophoresis procedures. SDS-PAGE was carried out as described by Lampel *et al.* (1992). Proteins in the gels were fixed with 50% (v/v) methanol for 2 h and stained with 2% (w/v) Coomassie brilliant blue-R for 2 h. The gels were destained for 2 h by diffusion into an aqueous solution of 7.5% (v/v) acetic acid and 50% (v/v) methanol.

[³H]DIFP-binding assay. The presence of serine proteinases in the culture broths of *S. lividans* 1326 and other streptomycetes, grown in GYE medium (Gibb *et al.*, 1989), was determined by [³H]DIFP binding for 30 min at 37 °C using the procedure described by Coletta & Miller (1986). [³H]DIFP [Dupont-NEN; 4.4 Ci mmol⁻¹, 1 $\mu\text{Ci } \mu\text{l}^{-1}$ (163 GBq mmol⁻¹, 37 kBq μl^{-1})] was added to each sample to achieve a final concentration of 100 μM and the solutions were incubated for 30 min at 37 °C. After incubation, the proteins were precipitated with ice-cold 10% (w/v) TCA for 20 min. The precipitated proteins were recovered by centrifugation in a microcentrifuge at 10000 r.p.m. for 15 min and dried under vacuum. The precipitate was then resuspended in 14 μl of sample buffer and the proteins were separated by SDS-PAGE according to Dreyfuss *et al.* (1984). The gels were dehydrated by soaking them twice for 20 min each in 20 vols DMSO, after which they were impregnated with PPO by immersion for 1 h in 4 vols 22.2% (w/v) PPO in DMSO. Excess PPO in the gel was precipitated *in situ* by

soaking the gels for 1 h in double-distilled water. The PPO-impregnated gel was then dried under vacuum for 1 h using a mini slab gel drier (Hoefel Scientific Instruments). The DIFP-binding proteins in the gel were visualized by autoradiography for 48 to 72 h on X-ray film (Kodak XAR).

Purification of *S. lividans* LAP. Mycelia from 3 litres of culture broth were harvested after 120 h of fermentation by tangential flow filtration using a Pellicon filtration system (Millipore) equipped with 0.45 μm membranes (2 ft² of membrane area). The cell-free culture broth (2800 ml) was chilled to 4 °C by holding on ice and was concentrated to 200 ml using the Pellicon filtration unit equipped with an *M_r* 10000 cut-off membrane (5 ft² of membrane area). The retentate, which contained all the LAP activity, was used for further purification work and was lyophilized and stored as a powder at -20 °C when not in use.

A pre-packed Pharmacia Superose-6 FPLC column (HR 10/30) equilibrated with 50 mM-Tris/HCl (pH 6.5) containing 150 mM-NaCl was used for gel-filtration chromatography. Approximately 700 μg of protein were loaded onto the matrix. The flow rate of buffer was 0.5 ml min⁻¹ and 250 μl fractions were collected for analysis. Ion-exchange chromatography was performed using prepacked Mono-Q FPLC columns (HR 10/10, 1 $\times$ 10 cm; HR 5/5, 0.5 $\times$ 5 cm), pre-equilibrated with 5 vols 20 mM-Tris/HCl (pH 8.0), on a Pharmacia FPLC apparatus. The flow rate was adjusted to 1 ml min⁻¹ and bound proteins were eluted from the resin by application of a linear 0 to 1.0 M-NaCl gradient in buffer. Fractions of 1 ml were collected for analysis.

Determination of kinetic constants. *K_m* and *V_{max}* values for leu-pNA were determined from an Eadie-Hofstee plot generated from the initial reaction velocities obtained with leu-pNA concentrations between 0.1 and 3.0 mM and 550 ng of the purified LAP per assay. Each assay was carried out at 22 °C in 20 mM-Tris/HCl (pH 8.0) and the change in absorbance at 410 nm was monitored continuously using a Beckman DU-64 spectrophotometer for each substrate concentration for 5 min.

Effects of inhibitors and influence of metal ions. Purified LAP was dialysed against 10 mM-HEPES (8.0) to remove any contaminating cations and was then incubated separately with each inhibitor for 1 h at room temperature before residual enzyme activity was measured. Inhibitor concentrations were 3×10^{-3} M for DIFP and 1×10^{-3} M for the others (*o*-phenanthroline, Na₂-EDTA, bestatin, NTA, EGTA, pepstatin, phosphoramidon, chymostatin, aprotinin and N-CBZ-PLG-hydroxamate).

Various cations were added as chloride or acetate salts to the purified enzyme to assess their effect on enzyme activity. The ability of these cations to restore activity to enzyme previously inactivated with *o*-phenanthroline was also determined. Enzyme inactivated with 10 mM-*o*-phenanthroline was incubated with each cation preparation for 5 min at ambient temperature (22 °C) before measuring restored activity.

Effect of temperature and pH on enzyme activity. Leu-pNA hydrolysis by purified LAP was assayed at different temperatures using a temperature-controlled cuvette holder attached to a Haake recirculating water bath. The cuvette was precalibrated using a temperature microprobe (YSI model 42SC tele-thermometer) to determine actual reaction temperatures. For each assay, the buffer was prewarmed for 5 min prior to addition of enzyme and substrate.

The effect of pH on leu-pNA hydrolysis was determined using the following buffers at 100 mM, supplemented with 10 mM-CaCl₂ and 10 mM-MgCl₂: MES (pH 6.4-7.0); HEPES (pH 7.0-8.2); and Tris/HCl (pH 8.0-8.8). The assays for optimal pH were carried out at ambient temperature (about 22 °C).

Reproducibility of results. Unless otherwise stated, all experiments were carried out at least in duplicate and the results given are means of

those data. For purification, all steps were performed at least twice. The data given are for a representative purification scheme.

Results and Discussion

Proteolytic activities of *S. lividans*

Aretz *et al.* (1989) reported that *S. lividans* TK24 contained significant extracellular chymotrypsin-like and aminopeptidase proteinase activities, and we found that *S. lividans* 1326 contains significant azocasein hydrolysis activity (Gibb *et al.*, 1989). Recently, a small, extracellular, neutral proteinase of *S. lividans* has been identified through cloning experiments (Lichenstein *et al.*, 1992; Butler *et al.*, 1992). Under normal conditions, however, this milk-hydrolysing proteinase has very little activity (Butler *et al.*, 1992; Lampel *et al.*, 1992). Additionally, a gene encoding an intracellular aminopeptidase P has been isolated from *S. lividans* and sequenced (Butler *et al.*, 1991). Moreover, *S. lividans* has been used as a recipient for cloning proteinase genes from other streptomycetes because it lacks significant milk hydrolysis activity (Lampel *et al.*, 1992). Thus, we have re-investigated the proteinase activities of *S. lividans* to clarify the types of proteinase activities present. As shown in Table 1, the major extracellular proteinase activity of *S. lividans* is a LAP activity. Azocasein hydrolysis activity was also observed, but no milk hydrolysis activity was found. *S. lividans* culture broths also displayed a low level of extracellular proline

aminopeptidase activity. Significant intracellular proline aminopeptidase activity was observed, probably due to the aminopeptidase P (Butler *et al.*, 1991).

Only background levels of proteinase activity on SAAPF-pNA (for chymotrypsin-like proteinases) or BAPNA (for trypsin-like proteinases) were found in the culture supernatants of *S. lividans* 1326. Although the reported values are for *S. lividans* 1326 grown in MB medium, we also observed the same results when *S. lividans* 1326 was grown in TSB (trypticase soy broth) and GYE (glucose yeast extract) media (data not shown). Both the azocasein- and leu-pNA hydrolysing activities of *S. lividans* culture broths were inhibited by the metalloproteinase inhibitors *o*-phenanthroline and EGTA, but not by the serine proteinase inhibitors DIFP and PMSF (data not shown). These data suggest that if any enzymes possessing serine proteinase activity are being produced by *S. lividans*, they probably constitute a negligible portion of the total proteinase activity.

Additionally, we tested the culture broth of *S. lividans* with [³H]DIFP, followed by SDS-PAGE and autoradiography, to determine if serine proteinases might be present that were not detected with our enzyme assays. The concentrated *S. lividans* 1326 culture broth apparently does not have proteins that bind [³H]DIFP (Fig. 1). On the other hand, proteins from the culture broths of *Streptomyces* sp. strain C5-A13, *S. coelicolor* A3(2) and *S. rimosus*, organisms known to produce serine proteinases (Vinci, 1988; Gibb *et al.*, 1989), strongly bound [³H]DIFP (Fig. 1). These results suggest the absence of serine proteinases in the culture broth of *S. lividans*, since serine proteinases are known to bind strongly and irreversibly to DIFP. Additionally, the DNA of several streptomycetes has been probed with a mixed, consensus oligonucleotide probe deduced from the active site of serine proteinases (Vinci, 1988). Whereas DNA isolated from *S. griseus*, *S. coelicolor*, *S. longisporus* and *Streptomyces* sp. strain C5 contained several *Bam*HI DNA fragments which hybridized strongly with the oligonucleotide probe at high stringencies, no DNA fragments from *S. lividans* hybridized strongly with the serine proteinase active site probe, even at lowered stringencies (Vinci, 1988). Taken altogether, the cumulative data indicate that *S. lividans* probably does not contain serine proteinases, or at least not significant serine proteinase activity, contrary to what was previously indicated (Aretz *et al.*, 1989). Similarly, *Streptomyces clavuligerus* apparently has a neutral proteinase as its only major extracellular proteinase activity (Bascaran *et al.*, 1990). This is rather unusual, however, since most streptomycetes produce multiple types of proteinases, including serine and metalloproteinase endopeptidases and amino- and carboxypeptidases (Narahashi, 1970; Pokorny *et al.*, 1979; Chandrasekaran & Dhar, 1987).

Table 1. Proteinolysis activities detected in cell extracts and culture supernatants of *S. lividans* 1326

One unit of activity for hydrolysis of the following substrates is defined as: azocasein hydrolysis, increase in A_{340} of 0.001 A min^{-1} ; 2% milk hydrolysis, decrease in A_{580} of 0.001 A min^{-1} ; FAGLA hydrolysis, decrease in A_{314} of 0.001 A min^{-1} ; TAME hydrolysis, increase in A_{247} of 0.001 A min^{-1} ; and FALGPA hydrolysis, increase in A_{345} of 0.001 A min^{-1} . Units of activity for the *p*-nitroanilide substrates are reported as $\mu\text{mol min}^{-1}$. All data are means of at least duplicate samples.

Substrate tested	Type of proteinase activity detected using given substrate	Proteinase activity [units (mg protein) ⁻¹]	
		Intracellular	Extracellular
Azocasein	General	Not done	12
2% Milk	General	0	0
BAPNA	Trypsin-like	2	2
SAAPF-pNA	Chymotrypsin-like	3	3
TAME	Esterase	0	0
FAGLA	Thermolysin-like	0	0
FALGPA	Collagenase	0	0
leu-pNA	Aminopeptidase (LAP)	13	60
pro-pNA	Aminopeptidase	100	15
ala-pNA	Aminopeptidase	0	0
lys-pNA	Aminopeptidase	0	0
gly-pNA	Aminopeptidase	0	0

Fig. 1. Autoradiogram of concentrated culture broths of streptomycetes grown in GYE medium (Gibb *et al.*, 1989) and treated with [3 H]DIFP, which binds irreversibly to the active sites of serine proteinases, followed by separation of the proteins through 12% (w/v, acrylamide) SDS-PAGE and autoradiography to show the [3 H]DIFP-binding proteins. Approximately 10 μ g of protein were added per lane. Lanes: 1, *S. lividans* 1326 culture broth; 2, DIFP-binding proteins from *Streptomyces* sp. C5-A13 culture broth (Gibb *et al.*, 1989); 3, DIFP-binding proteins from *S. coelicolor* A3(2) culture broth (Vinci, 1988); and 4, DIFP-binding proteins from *S. rimosus* culture broth (Pokorny *et al.*, 1979).

Fig. 2. Fermentation profile of *S. lividans* cultivated in MB medium. The dry weight of the mycelium (■) and activity of LAP (●) are shown.

Many streptomycetes, most notably *S. griseus*, even produce multiple serine proteinases (Narahashi, 1970). Thus, it appears that *S. lividans* 1326 and *S. clavuligerus*

Fig. 3. Chromatographic steps used in the purification of LAP from *S. lividans* 1326. For each step, protein (A_{280} ; —) and enzyme activity (●; $\Delta A_{410} \text{ min}^{-1}$) were measured as described in Methods. The dashed lines (---) in (a) and (c) indicate the NaCl gradients. (a) Mono-Q (HR 10/10) FPLC ion-exchange chromatography; the enzyme eluted in two broad peaks, the first in the wash-through (pool 1) and the second in a broad area between 250 and 550 mM-NaCl (pool 2). (b) Superose-6 (HR 10/30) FPLC gel-filtration chromatography; the enzyme from either pool 1 or pool 2 of (a) eluted from the column at 35.5 min. (c) Mono-Q (HR 5/5) FPLC ion-exchange chromatography; the enzyme from either pool 1 or pool 2 from (a) eluted at 200 mM-NaCl.

are unusual with respect to most streptomycetes in their complement of extracellular proteinases.

Fermentation profile and purification of *S. lividans* LAP

LAP activity was first detected after 18 h of culture growth but began to increase significantly only after the onset of stationary phase (Fig. 2), indicating that the

Table 2. Scheme for the purification of the M_r 34000 LAP from *S. lividans* culture supernatantOne unit of activity equals 1.0 μmol *p*-nitroaniline released min^{-1} .

Purification step	Total vol. (ml)	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Purification factor (-fold)	Percentage recovery
Cell-free broth	2800	2296	253	0.11	1.0	100
> M_r 10000 retentate	200	430	94	0.22	2.0	37
Lyophilization*	30	425	119	0.28	2.5	47
Mono-Q HR 10/10	8	53	7.6	0.14	1.3	3
Superose-6	4	1.7	1.2	0.70	6.5	0.5
Mono-Q HR 5/5	1	0.011	0.04	3.64	33	0.02

* Followed by resolubilization of the proteins in 20 mM-Tris/HCl buffer (pH 8.0).

aminopeptidase is a post-growth associated activity. This was observed in a variety of fermentations using different media (data not shown). Therefore, fermentation broths from cultures 120 h old were used for purification of *S. lividans* LAP.

Several different protein purification procedures were used in attempts to purify the extracellular LAP from *S. lividans* 1326. The major difficulty was the presence, in the supernatant fluids, of an uncharacterized brown-pigmented substance that bound to the aminopeptidase, clogged several of the columns, and generally interfered with the purification procedures. The final purification scheme involved successive Mono-Q and Superose-6 chromatography steps. After lyophilization to concentrate the protein, the resuspended mixture was fractionated into two large pools using a Mono-Q HR 10/10 column. Two peaks of LAP activity were obtained by this step, both containing some pigment. The first peak of activity eluted in the wash-through and the second eluted in a broad region from 250 to about 550 mM-NaCl (Fig. 3a). A 2-fold loss in the purification of the enzyme was noted at this step (Table 2). LAP concentrated from the active fractions of pool 1, however, eluted only in a sharp peak at 200 to 250 mM on the second Mono-Q 5/5 column (data not shown). Thus, it appears that the Mono-Q 10/10 column was apparently 'overloaded'. Since the column was designated to handle considerably more protein than was loaded, we surmise that the 'overloading effect' was due to interaction of the column and proteins with the interfering brown pigment.

Superose-6 gel-filtration chromatography of pool 2 from the large Mono-Q resin at about 250 mM-NaCl helped in removing more of the brown pigment from the enzyme preparation (Fig. 3b), while offering an additional 5-fold purification (Table 2). The protein concentrated from the Superose-6 step was loaded onto a smaller Mono-Q ion exchanger (HR 5/5), which gave a further 5-fold purification of the enzyme (Table 2). All

the pigment was separated from the active LAP which eluted at approximately 200 mM-NaCl (Fig. 3c), while the pigment eluted from the column at about 550 mM-NaCl in this step. While this protocol resulted in a 33-fold final purification of the LAP, only 0.02% of the active enzyme could be recovered (Table 2). This tremendous loss was largely due to the problems associated with the contaminating brown pigment.

SDS-PAGE analysis of the most active fraction from this Mono-Q step revealed a homogeneous polypeptide of M_r 34000 (Fig. 4). This same protein eluted from a calibrated Superose-6 column at 35.5 min, which corresponds to an M_r of 19000 (data not shown). This Superose-6 elution profile occurred even in the presence of 0.15 M-NaCl and was reproducible with several different preparations of partially and completely purified protein. The large difference in calculated M_r from SDS-PAGE and Superose-6 may result from an incomplete protein denaturation or binding of SDS, or an atypical retardation of the enzyme on the gel-filtration support column (Superose-6). Interestingly, the two aminopeptidases from *S. griseus* displayed nearly identical anomalous elution profiles from Superose-12 gel filtration as compared to their M_r values determined from SDS-PAGE, as did *S. lividans* LAP (Spungin & Blumberg, 1989).

LAP purified from pool 1 of the Mono-Q HR 10/10 column also eluted from Superose-6 at 35.5 min and from Mono-Q HR 5/5 at 200 mM-NaCl (data not shown). The resulting preparation had a major protein of M_r 34000 (by SDS-PAGE), but LAP purified from pool 1 was not homogeneous.

Properties of *S. lividans* LAP

The purified LAP had a specific activity on leu-pNA of 3.6 $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$. No other hydrolysis activity was observed on any other substrates tested,

Fig. 4. Determination of M_r by SDS-PAGE of the purified *S. lividans* LAP. Lane 1, Size standards; bovine serum albumin (M_r 66000), egg albumin (45000), glyceraldehyde 3-phosphate dehydrogenase (subunit: 36000), carbonic anhydrase (29000), bovine trypsinogen (24000), soybean trypsin inhibitor (20100) and bovine α -lactalbumin (14200); lane 2, proteins from the $> M_r$ 10000 ultrafiltration step; lane 3, proteins from the large Mono Q (10/10) step; lane 4, proteins from the Superose-6 step; and lane 5, purified M_r 34000 LAP from the small Mono Q (5/5) step. Proteins in the gel were stained with Commassie brilliant blue-R.

Table 3. Effect of divalent cations on *S. lividans* LAP activity

The purified enzyme was first dialysed against 100 mM-HEPES (pH 8.0) for 24 h at 4 °C. Divalent cations were added to a final concentration of 10 mM and incubated with the LAP at 37 °C for 5 min before assaying for activity against leu-pNA. The specific activity of the dialysed enzyme (control value) to which no metal was added was $3.64 \mu\text{mol min}^{-1} \text{mg}^{-1}$. The values reported are means of duplicate assays.

Divalent cation (added as:)	Percentage of control activity
Control (no metal added)	100
CoCl_2	177
$\text{Ca}(\text{CH}_3\text{COO})_2$	100
$\text{Zn}(\text{CH}_3\text{COO})_2$	100
SrCl_2	100
NiCl_2	83
BaCl_2	77
MnCl_2	73
MgCl_2	70
StCl_2	39
CdCl_2	24
CuCl_2	13
CrCl_2	7
HgCl_2	6

including 2% (w/v) milk, azocasein, BAPNA, SAAPF-pNA, pro-pNA, gly-pNA, ala-pNA or lys-pNA (data not shown).

The effect of various metals on the activity of extensively dialysed, purified *S. lividans* LAP is shown in Table 3. Dialysed *S. lividans* LAP retains 100% of the activity of undialysed enzyme, indicating that the metal required for activity of *S. lividans* LAP (probably Zn^{2+}) was not removed from the enzyme by dialysis alone.

Table 4. Effects of inhibitors on *S. lividans* LAP

All inhibitors except DIPF were at 10 mM final concentration. DIPF was at 3.0 mM final concentration. Activity was measured against leu-pNA as the substrate. Activity of the untreated enzyme was $3.6 \mu\text{mol min}^{-1} \text{mg}^{-1}$ and was taken as 100%. The values reported are means of duplicate assays.

Inhibitor	Percentage inhibition
<i>o</i> -Phenanthroline	93
$\text{Na}_2\text{-EDTA}$	77
Bestatin	64
NTA	60
EGTA	46
Pepstatin	0
Phosphoramidon	0
Chymostatin	0
Aprotinin	0
N-CBZ-PLG-hydroxamate	0
DIPF	0

Treatment with Co^{2+} stimulated activity of the dialysed enzyme, while treatment with Ca^{2+} , Zn^{2+} or Sr^{2+} yielded the same activity as untreated enzyme. The stimulation of aminopeptidase activity by Co^{2+} has been observed previously (Wagner *et al.*, 1979; Nakamura *et al.*, 1984). All of the other divalent metals or metalloids tested inhibited LAP activity to various degrees.

S. lividans LAP is sensitive to chelating agents and bestatin, but not to known inhibitors of thermolysin-like metalloproteinases, serine proteinases or acid proteinases (Table 4). *o*-Phenanthroline was the most effective inhibitor of *S. lividans* LAP. After treatment with *o*-phenanthroline, however, activity was reproducibly

restored 100% by addition of excess Co^{2+} and was 70% restored by addition of excess Zn^{2+} (data not shown). Excess Ca^{2+} did not restore activity of *o*-phenanthroline-treated *S. lividans* LAP, indicating that it is not required for activity (data not shown). Enzymic activity of LAP diminished significantly, however, when the enzyme was stored at 4 °C in the absence of Ca^{2+} for more than 7 d. On the other hand, enzyme preparations stored in buffers containing Ca^{2+} did not lose significant leu-pNA-hydrolysing activity even after a month at 4 °C. The combined data indicate that Ca^{2+} is required for stability of *S. lividans* LAP but is not necessary for its activity against leu-pNA.

The K_m and $V_{\max}$ values for leu-pNA hydrolysis by *S. lividans* LAP (pH 8.0, 22 °C) are 3.0×10^{-4} M and $4.24 \mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively (data not shown). K_{cat} for hydrolysis of leu-pNA is 2.3 s^{-1} , and K_{cat}/K_m is 7.7×10^3 . These values are similar to those obtained by Vitale *et al.* (1986) for the *S. rimosus* LAP.

The optimum temperature for activity of *S. lividans* LAP is 46 °C, with 85% of the activity remaining at 36 °C and 55 °C, and 45% of the activity remaining at 20 °C and 62 °C (data not shown). The optimum pH for activity is 8.0, with about 70% of the activity remaining at pH values of 7.2 and 8.2. Less than 50% of the maximal activity was obtained at pH values of 7.0 and 8.4 (data not shown).

Since purified *S. lividans* LAP is not inhibited by DIFP, aprotinin or chymostatin, it is not a serine proteinase. This enzyme is also not inhibited by N-CBZ-PLG-hydroxamate and does not hydrolyse FAGPA, indicating that it does not have collagenase activity. The inability of the *S. lividans* LAP to hydrolyse FAGLA and the inability of phosphoramidon to inhibit leu-pNA hydrolysis indicate that the enzyme does not belong to the thermolysin-family of neutral proteinases. Nevertheless, *S. lividans* LAP was inhibited by *o*-phenanthroline, $\text{Na}_2\text{-EDTA}$, NTA and EGTA, indicating that it is a metalloproteinase. Furthermore, the enzyme was partially inhibited by bestatin, a known inhibitor of aminopeptidases (Vitale *et al.*, 1986). The combined data indicate that *S. lividans* LAP is a metallo-enzyme, produced during stationary phase, that has a rather narrow range of substrate specificities.

Comparison of *S. lividans* LAP with other streptomycete LAPs

Morihara (1967) demonstrated the presence of a LAP from *Streptomyces fradiae* that requires Ca^{2+} for its activity. Uwajima *et al.* (1973) purified a M_r 19000 LAP from culture supernatants of *Streptomyces peptidofaciens* that was inhibited by EDTA and *o*-phenanthroline. Their EDTA-inactivated enzyme was successfully reactivated

by addition of Ca^{2+} , indicating a probable requirement of Ca^{2+} for enzymic activity. Another Ca^{2+} -requiring LAP was purified from *Streptomyces* sp. 771 by Krest'yanova *et al.* (1983). Vitale *et al.* (1986) purified an extracellular LAP from *S. rimosus* and characterized its properties in detail. This enzyme is stable over a broad pH range, has an M_r of 27000, a pI of 7.3, requires Ca^{2+} for its LAP activity, and is inhibited by Zn^{2+} . Two aminopeptidases (API and APII) purified from *S. griseus* were originally shown to require Ca^{2+} for their enzymic activity (Vosbeck *et al.*, 1973). More recently, however, these LAPs were proved to be Zn^{2+} -containing enzymes that are activated by calcium (Spungin & Blumberg, 1989). Our data suggest that the M_r 34000 LAP from *S. lividans* is a zinc metalloproteinase which is stabilized, but not necessarily activated, by calcium. The *S. lividans* LAP, therefore, is very different from the LAPs of *S. peptidofaciens*, *S. fradiae* and *S. rimosus*. On the other hand, *S. lividans* LAP appears to be very similar, but apparently not identical, to the *S. griseus* LAPs, API and APII.

We thank Don Ordaz and the Ohio State University Fermentation Facility for assistance with the fermentations. We also thank Dr Kathleen Kendrick and her laboratory group for use of their FPLC during these investigations.

References

- ARETZ, W., KOLLER, K. P. & RIESS, G. (1989). Proteolytic enzymes from recombinant *Streptomyces lividans* TK24. *FEMS Microbiology Letters* **65**, 31–36.
- BASCARAN, V., HARDISSON, C. & BRAÑA, A. (1990). Regulation of extracellular protease production in *Streptomyces clavuligerus*. *Applied Microbiology and Biotechnology* **34**, 208–213.
- BENDER, E., VOGEL, R., KOLLER, K.-P. & ENGLER, J. (1990). Synthesis and secretion of hirudin by *Streptomyces lividans*. *Journal of Bacteriology* **34**, 203–207.
- BRADFORD, M. M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- BUTLER, M. J., BERGERON, A., KRYGSMAN, P., SOOSTMEYER, G., ZIMNY, T. & MALEK, L. T. (1991). Cloning and characterization of an aminopeptidase P gene from *S. lividans* 66. *Abstracts, 1991 International Symposium on Biology of Actinomycetes*, P1–123.
- BUTLER, M. J., DAVEY, C. C., KRYGSMAN, P., WALCZYK, E. & MALEK, L. T. (1992). Cloning of genetic loci involved in endoprotease activity in *Streptomyces lividans* 66: a novel neutral protease gene with an adjacent divergent putative regulatory gene. *Canadian Journal of Microbiology* **38**, 912–920.
- CHANDRASEKARAN, S. & DHAR, S. C. (1987). Multiple proteases from *Streptomyces moderatus*. I. Isolation and purification of five extracellular proteases. *Archives of Biochemistry and Biophysics* **257**, 395–401.
- COLLETTA, P. L. & MILLER, P. G. G. (1986). The extracellular proteases of *Myxococcus xanthus*. *FEMS Microbiology Letters* **37**, 203–207.
- DELMAR, E. G., LARGMAN, C., BRODRICK, J. W. & GEOKAS, M. C. (1979). A sensitive new substrate for chymotrypsin. *Analytical Biochemistry* **99**, 316–320.
- DREYFUSS, O., ADAM, S. A. & CHOI, Y. D. (1984). Physical change in cytoplasmic messenger ribonucleoproteins in cells treated with inhibitors of mRNA transcription. *Molecular and Cell Biology* **4**, 415–423.

- ERLANGER, B. F., KOKOWSKI, N. & COHEN, W. (1961). The preparation and properties of two new chromogenic substrates of trypsin. *Archives of Biochemistry and Biophysics* **95**, 271–278.
- FEDER, J. (1968). A spectrophotometric assay for neutral protease. *Biochemical and Biophysical Research Communications* **32**, 326–332.
- GIBB, G. D., ORDAZ, D. E. & STROHL, W. R. (1989). Overproduction of extracellular proteinase activity by *Streptomyces* C5-A13 in fed-batch fermentations. *Applied Microbiology and Biotechnology* **31**, 119–124.
- KREST'YANOVA, I. N., VASIL'eva, L. I., BARTOSHEVICH, I. E., AKPAROV, V. K. & NAKHAPETIAN, L. A. (1983). Isoelectric focusing of a preparation of proteolytic enzymes from *Streptomyces* 771. *Prikladnaya Biokhimiya i Mikrobiologiya* **19**, 217–225. (English translation: *Prikladnaya Biokhimiya i Mikrobiologiya* **19**, 175–183).
- LAMPEL, J. S., APHALE, J. S., LAMPEL, K. A. & STROHL, W. R. (1992). Cloning and sequencing of a gene encoding a novel extracellular neutral proteinase from *Streptomyces* sp. strain C5 and expression of the gene in *Streptomyces lividans* 1326. *Journal of Bacteriology* **174**, 2797–2808.
- LICHENSTEIN, H., BRAWNER, M. E., MILES, L. M., MYERS, C. A., YOUNG, P. R., SIMON, P. L. & ECKARDT, T. (1988). Secretion of interleukin-1 β and *Escherichia coli* galactokinase by *Streptomyces lividans*. *Journal of Bacteriology* **170**, 3924–3929.
- LICHENSTEIN, H. S., BUSSE, L. A., SMITH, G. A., NARHI, L. O., MCGINLEY, M. O., ROHDE, M. F., KATZOWITZ, J. L. & ZUKOWSKI, M. M. (1992). Cloning and characterization of a gene encoding extracellular metalloprotease from *Streptomyces lividans*. *Gene* **111**, 125–130.
- MALEK, L. T., SOOSTMEYER, G., DAVEY, C., KRYGSMAN, P., COMPTON, J., GRAY, J., ZIMNY, T. & STEWART, D. (1990). Secretion of granulocyte macrophage-colony stimulating factor (GM-CSF) in *Streptomyces lividans*. *Journal of Cell Biochemistry, Supplement* **14A**, 127.
- MORIHARA, K., OKA, T. & TSUZUKI, H. (1967). Multiple proteolytic enzymes of *Streptomyces fradiae*. *Biochimica et Biophysica Acta* **139**, 382–387.
- NAKAMURA, T., KIUCHI, N. & FUJIYAMA, S. (1984). Purification and partial characterization of leucine aminopeptidase from *Actinomyces viscosus*. *Matsumoto Shigaku* **10**, 130–135.
- NARAHASHI, Y. (1970). Pronase. *Methods in Enzymology* **19**, 651–664.
- POKORNY, M., VITALE, L. J., TURK, V., RENKO, M. & ZUVANIC, J. (1979). *Streptomyces rimosus* extracellular proteinases. 1. Characterization and evaluation of various crude preparations. *European Journal of Applied Microbiology and Biotechnology* **8**, 81–90.
- ROBERTS, D. V. & ELMORE, D. T. (1974). Kinetics and mechanism of catalysis by proteolytic enzymes. *Biochemical Journal* **141**, 545–554.
- SPUNGIN, A. & BLUMBERG, S. (1989). *Streptomyces griseus* aminopeptidase is a calcium-activated zinc metalloprotein. Purification and properties of the enzyme. *European Journal of Biochemistry* **183**, 471–477.
- TAGUCHI, S., KUMAGAI, I., NAKAYAMA, J., SUZUKI, A. & MIURA, K. (1989). Efficient extracellular expression of a foreign protein in *Streptomyces* using secretory proteinase inhibitor (SSI) gene fusions. *Bio/Technology* **7**, 1063–1066.
- UWAJIMA, T., YOSHIKAWA, N. & TERADA, O. (1973). A crystalline aminopeptidase from *Streptomyces peptidofaciens*: physio-chemical properties and characteristics as a Ca-metalloprotease. *Agricultural and Biological Chemistry* **37**, 2727–2733.
- VINCI, V. A. (1988). *Biochemical and genetic analysis of serine proteases of Streptomyces spp.* PhD Dissertation, The Ohio State University, Columbus, USA.
- VITALE, L. J., RENKO, M., LENARCIC, B., TURK, V. & POKORNY, M. (1986). *Streptomyces rimosus* extracellular proteinases. 3. Isolation and characterization of leucine aminopeptidase. *Applied Microbiology and Biotechnology* **23**, 449–455.
- VOSBECK, K. D., CHOW, K. & AWAD, W. M. (1973). The proteolytic enzymes of the K-1 strain of *Streptomyces griseus* obtained from a commercial preparation (pronase). *Journal of Biological Chemistry* **248**, 6029–6034.
- WAGNER, F. W., RAY, L. E., AJABNOOR, M. A., ZIEMBA, P. L. & HALL, R. L. (1979). *Bacillus subtilis* aminopeptidase: purification, characterization and some enzymatic properties. *Archives of Biochemistry and Biophysics* **197**, 63–72.