INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Purification and characterization of host and recombinant proteinases from *Streptomyces lividans*

Aphale, Jayant Shrihari, Ph.D.

The Ohio State University, 1992
PURIFICATION AND CHARACTERIZATION OF HOST AND RECOMBINANT PROTEINASES FROM *Streptomyces lividans*

A Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By

Jayant Shrihari Aphale, B.S., M.S.

****

The Ohio State University

1992

Dissertation Committee:

Dr. C. J. Daniels
Dr. Aldis Darzins
Dr. K. E. Kendrick
Dr. W. R. Strohl

Approved by

William R. Strohl
Advisor
Department of Microbiology
Copyright by
Jayant Shrihari Aphale
1992
ACKNOWLEDGMENTS

This is to express sincere appreciation to Dr. William R. Strohl for his insight and guidance throughout my graduate research career. Thanks go to Drs. Kathleen Kendrick and Samuel Black for generously permitting use of their laboratory equipment and for many helpful interactions. Thanks also go to the other members of my dissertation committee, Drs. Charles Daniels and Aldis Darzins for their suggestions and comments. I am indebted to Donald Ordaz for his invaluable help in fermentations and downstream processing. Assistance offered unhesitatingly on various occasions by Dr. Robert Woodman and by Yun Li is gratefully acknowledged. Technical assistance by Cynthia Miller, during metal analysis experiments is noted. I would like to thank Gary Kleman, Mike Dickens and Dr. Richard Plater for their friendship and encouragements. To Prarthana, my friend and my wife, I offer sincere thanks for your emotional support and your willingness to endure with me the hardships of graduate life. To my parents, thank you for your patience, understanding and faith in me.
VITA

October 21, 1960 ...................... Born - Bombay, India

1981 ............................... B. S., University of Pune India.
1983 ............................... M. S., University of Bombay India.
1983-1985 ......................... Lecturer, University of Bombay India.
1986-present ..................... Graduate Teaching Associate,
                                The Ohio State University,
                                Columbus, Ohio.

PUBLICATIONS


FIELD OF STUDY

Major Field: Microbiology.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS .......................................................... ii

VITA .................................................................................................... iii

TABLE OF CONTENTS ................................................................. v

LIST OF FIGURES ........................................................................... x

LIST OF TABLES ................................................................................ xiii

LIST OF ABBREVIATIONS .............................................................. xv

CHAPTER I ......................................................................................... 1

LITERATURE REVIEW ................................................................. 1

*Streptomyces* .................................................................................... 1
  Ecology .......................................................................................... 1
  Ultrastructure and genetics ......................................................... 4
  Significance .................................................................................... 4

Extracellular enzymes ........................................................................ 7
  Uses of extracellular enzymes .................................................... 8
  Enzyme secretion ........................................................................... 9

Proteinases ....................................................................................... 11
  Classification of proteinases ....................................................... 13
  Secretion, processing and activation of bacterial extracellular
  proteinases .................................................................................. 20
  Mechanism of action of bovine carboxypeptidase A .................. 21
  Mechanism of action of bacterial thermolysin ......................... 26
  Proteinases in peptide synthesis ................................................. 30
  Streptomycte proteinases .......................................................... 32

Research objectives .......................................................................... 37
CHAPTER II ................................................................. 40

Purification and characterization of the proteinase encoded by
Streptomyces sp. C5 SnpA ........................................... 40

A) Introduction ......................................................... 40

B) Subcloning and analysis of the snpA gene and its upstream
sequences .............................................................. 43

Materials and Methods .............................................. 43

Bacterial strains, maintenance, and cultivation
conditions ................................................................. 43
DNA cloning procedures and genetic manipulations .......... 43
Restriction enzyme mapping of plasmid pANT42 .......... 43
Design and radiolabelling of synthetic oligonucleotide
fragments ................................................................. 55
Radiolabelling of large DNA fragments ......................... 55
DNA-DNA hybridizations .......................................... 56

Results ................................................................. 58

Subcloning of pANT21 .............................................. 58
Localization of the snpA gene in pANT42 .................... 59
Hybridization of insert DNA to DNA from other
streptomycetes ......................................................... 59
Activity of the sequences upstream of snpA .................. 64

C) Purification, biochemical characterization and preliminary studies
in the physiological regulation of the Streptomyces C5
proteinase SnpA from Streptomyces lividans(pANT42) ....... 69

Materials and Methods .............................................. 69

Bacterial strain, maintenance, and cultivation
conditions ................................................................. 69
Chemicals ................................................................. 69
Shake flask and fermentation cultures ............................ 70
Milk hydrolysis assay and protein determination ............ 71
Purification of the milk-hydrolyzing proteinase from
S. lividans(pANT42) ................................................. 72
Purification of the milk-hydrolyzing proteinase from
S. galilaeus(pANT42) ................................................. 74
Electrophoresis procedures ........................................ 74
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate specificity of the recombinant proteinase</td>
<td>75</td>
</tr>
<tr>
<td>Casein as substrate of the milk-hydrolyzing proteinase</td>
<td>77</td>
</tr>
<tr>
<td>N-terminal amino acid analysis of the major cleavage products of β-casein</td>
<td>77</td>
</tr>
<tr>
<td>Effect of inhibitors and influence of metal ions</td>
<td>78</td>
</tr>
<tr>
<td>Metal ion determination</td>
<td>79</td>
</tr>
<tr>
<td>Quantification of the metal ligand of SnpA</td>
<td>79</td>
</tr>
<tr>
<td>Effect of temperature and pH on enzyme activity</td>
<td>80</td>
</tr>
<tr>
<td>N-terminal sequencing of the purified neutral proteinase</td>
<td>80</td>
</tr>
<tr>
<td>Amino acid composition of the purified neutral proteinase</td>
<td>80</td>
</tr>
<tr>
<td>Physiological regulation of the snpA gene expression in S. lividans(pANT42)</td>
<td>81</td>
</tr>
<tr>
<td>Growth media and shake flask cultivation of S. lividans(pANT42)</td>
<td>81</td>
</tr>
<tr>
<td>Isolation and purification of RNA from S. lividans(pANT42)</td>
<td>82</td>
</tr>
<tr>
<td>RNA slot blot procedures</td>
<td>82</td>
</tr>
<tr>
<td>RNA-DNA hybridization</td>
<td>83</td>
</tr>
<tr>
<td>Results</td>
<td>84</td>
</tr>
<tr>
<td>Expression of snpA in fermentation cultures of S. lividans(pANT42)</td>
<td>84</td>
</tr>
<tr>
<td>Purification of neutral proteinase from S. lividans(pANT42) and S. galilaeus(pANT42)</td>
<td>87</td>
</tr>
<tr>
<td>Physical properties of the milk-hydrolyzing proteinase</td>
<td>92</td>
</tr>
<tr>
<td>N-terminal amino acid sequence of SnpA</td>
<td>92</td>
</tr>
<tr>
<td>Amino acid content of purified proteinase</td>
<td>92</td>
</tr>
<tr>
<td>Effect of inhibitors and addition of metals on proteinase activity</td>
<td>99</td>
</tr>
<tr>
<td>Determination of metal ligand</td>
<td>99</td>
</tr>
<tr>
<td>Substrate specificity</td>
<td>103</td>
</tr>
<tr>
<td>Effect of medium composition on expression of snpA</td>
<td>107</td>
</tr>
<tr>
<td>Analysis of RNA-DNA hybridizations</td>
<td>107</td>
</tr>
<tr>
<td>D) Discussion</td>
<td>113</td>
</tr>
<tr>
<td>Subcloning of a gene from Streptomyces sp. C5 encoding milk-hydrolyzing proteinase</td>
<td>113</td>
</tr>
<tr>
<td>Activity of the upstream sequences</td>
<td>114</td>
</tr>
<tr>
<td>Purification and characterization of SnpA</td>
<td>116</td>
</tr>
<tr>
<td>Physiological regulation of SnpA</td>
<td>131</td>
</tr>
</tbody>
</table>
CHAPTER III  ........................................... 134

Studies on the proteinases of *Streptomyces lividans* 1326 .............................. 134

A) Introduction ........................................... 134

B) Purification and characterization of the extracellular leucine aminopeptidase (LapA) from *S. lividans* 1326 ............................ 136

Materials and Methods ........................................... 136

Bacterial strain, maintainence and fermentation conditions ........................................... 136
Preparation of cell-free extracts ........................................... 137
Proteinase assays and protein determination ........................................... 138
3H-DIFP-binding assay ........................................... 139
Preparation of crude enzyme ........................................... 141
Protein purification ........................................... 141
1. Free solution isoelectric focusing (FS-IEF) ........................................... 141
   Preparation of the crude enzyme sample for FS-IEF ........................................... 141
   Electrofocusing of proteins in the crude enzyme sample ........................................... 142
2. Gel filtration chromatography ........................................... 142
   Low pressure Sephadex G-75 gel filtration ........................................... 142
   FPLC Superose-6 gel filtration ........................................... 143
3. Ion exchange chromatography (IEX) ........................................... 143
   Low pressure IEX ........................................... 143
   FPLC IEX ........................................... 144
Effects of inhibitors and influence of metal ions on enzyme activity ........................................... 144
Polyacrylamide gel electrophoresis ........................................... 145
Effect of temperature on enzyme activity ........................................... 145
pH optimum ........................................... 145
Determination of kinetic constants ........................................... 146

Results ........................................... 147

Proteinolytic activities of *S. lividans* ........................................... 147
Physiological regulation of the leucine aminopeptidase type of extracellular proteinase activity (LapA) from *S. lividans* 1326 ........................................... 147
Fermentation ........................................... 152
Enzyme purification ........................................... 152
Enzyme characterization ........................................... 166
D) Discussion ..................................................................................................... 179
   Determination that S. lividans possesses only one major extracellular leucine aminopeptidase activity ................. 179
   Physiological regulation of LapA .................................................. 181
   Purification and characterization of LapA ......................... 183
   Comparison of S. lividans LapA with other streptomycete leucine aminopeptidases ................. 185

CHAPTER IV ................................................................. 188
Summary and future experiments ................................................. 188
BIBLIOGRAPHY ............................................................. 191
LIST OF FIGURES

Figure 1. Life cycle of a typical streptomycte. ......................................................... 3
Figure 2. Schematic illustration of the interaction of a proteinase with its substrate. ................................................................. 14
Figure 3. Bovine carboxypeptidase A ................................................................. 23
Figure 4. Bacterial thermolysin ................................................................. 27
Figure 5. Subcloning of the neutral proteinase gene (snpA) ................. 50
Figure 6. Restriction map of plasmid pANT42 .................................................... 53
Figure 7. Southern hybridization of the γ-32P dATP end-labelled synthetic oligonucleotide probe with pANT42 that had been digested with PvuII and with Sphi-PvuII ........................................ 60
Figure 8. Southern hybridization of the α-32P dCTP randomly-labelled 624 bp Sphi-PvuII DNA fragment of pANT42 from C5-snpA to restriction endonuclease-digested DNA from various streptomycetes .......... 62
Figure 9. Southern hybridization of the α-32P dCTP randomly-labeled 624 bp Sphi-PvuII DNA fragment of pANT42 from C5-snpA to restriction endonuclease-digested DNA from Streptomyces sp. C5 and S. lividans 1326 ........................................... 65
Figure 10. Comparison of the milk hydrolysis activities of various recombinant streptomycetes ......................................................... 67
Figure 11. Fermentation profile of S. lividans(pANT42) grown in a MB medium .................................................................................. 85
Figure 12. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) of SnpA ............................................................................... 90
Figure 13. HPLC gel filtration chromatography of SnpA .......................... 93
Figure 14. Determination of pH for optimal SnpA activity ......................... 95
Figure 15. Determination of temperature for optimal SnpA activity ............ 97
Figure 16. Energy-dispersive X-ray fluorescence spectrometry (EDXFS) of SnpA .......................................................... 104
Figure 17. SDS-PAGE analysis of the degradation of α-casein and β-casein ................................................................. 108
Figure 18. RNA slot blot analysis using the end-labelled synthetic probe .......................................................... 111
Figure 19. Alignment of the N terminal amino acid sequence of SnpA with the deduced product of the snpA gene ....................... 117
Figure 20. Comparison between the deduced amino acid sequence of the C5-SnpA proteinase and the SL-SnpA/prt ......................... 127
Figure 21. 3H-DIFP binding to proteins in culture supernatents of various streptomycetes .......................................................... 149
Figure 22. Fermentation profile of S. lividans ........................................ 153
Figure 23. Mono-Q (HR 10/10) ion exchange chromatography ............ 157
Figure 24. Superose-6 gel filtration chromatography .......................... 159
Figure 25. Mono-Q (HR 5/5) ion exchange chromatography ................ 161
Figure 26. SDS-PAGE of the purified 34,000 Mr leucine aminopeptidase . 164
Figure 27. Temperature optimum for activity of the 34,000 Mr leucine aminopeptidase . ............................................. 171
Figure 28. pH optimum for activity of the 34,000 Mr leucine aminopeptidase ......................................................... 173
Figure 29. Lineweaver-Burk plot of the initial rate of hydrolysis of L-leu-pNA by 550 ng of the pure leucine aminopeptidase against various concentrations of L-leu-pNA (0.1 mM to 3 mM) . 175
Figure 30. Eadie-Hofstee plot of the initial rate of hydrolysis of L-leu-pNA by 550 ng of the pure leucine aminopeptidase against various concentrations of L-leu-pNA (0.1 mM to 3 mM).
LIST OF TABLES

Table 1. Families of proteinolytic enzymes ................................................................. 16

Table 2. Bacterial strains and plasmids used in this research work. ...................... 44

Table 3. Scheme for the purification of *Streptomyces* sp. C5 milk-hydrolyzing proteinase ............................................................................................................. 88

Table 4. Amino acid composition of the purified SnpA. ........................................ 100

Table 5. Effects of inhibitors on proteinase encoded by *Streptomyces* sp. C5 *snpA* gene .................................................................................................................. 101

Table 6. Summary of the characteristics of the purified SnpA proteinase ............... 102

Table 7. Effect of medium components on activity of SnpA. .................................. 110

Table 8. Zinc binding sites of metalloproteinases ................................................... 130

Table 9. Proteinolytic activities of *S. lividans*. ..................................................... 148

Table 10. Effect of medium composition on the expression of the total leucine aminopeptidase activity of *S. lividans* 1326. ............................................... 151

Table 11. Scheme for the purification of the 34,000 Mr aminopeptidase of *S. lividans*. ....................................................................................................................... 163

Table 12. Substrate specificity of the 34,000 Mr aminopeptidase of *S. lividans*. ............................................................................................................................. 167

Table 13. Effect of divalent cations on the purified 34,000 Mr aminopeptidase of *S. lividans* .............................................................................................................. 168

Table 14. Effects of inhibitors on the purified 34,000 Mr aminopeptidase of *S. lividans*. ............................................................................................................... 169

xiii
Table 15. Reactivation of the inactivated 34,000 M_\text{r} aminopeptidase by divalent cations ................. 170

Table 16. Leucine aminopeptidases from *Streptomyces* spp. .............. 187
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BAPNA</td>
<td>L-Benzoyl-arginyl-paranitroanilide</td>
</tr>
<tr>
<td>BRL</td>
<td>Bethesda Research Laboratories</td>
</tr>
<tr>
<td>CAPS</td>
<td>3-[cyclohexylamino]-1-propanesulfonic acid</td>
</tr>
<tr>
<td>CBZ-GGL-pNA</td>
<td>N-carbobenzoxy-Gly-Gly-Leu-paranitroanilide</td>
</tr>
<tr>
<td>CBZ-PLG-hydroxymate</td>
<td>N-carbobenzoxy-Pro-Leu-Gly-hydroxymate</td>
</tr>
<tr>
<td>CDW</td>
<td>cell dry weight</td>
</tr>
<tr>
<td>CFB</td>
<td>cell free broth</td>
</tr>
<tr>
<td>CM</td>
<td>carboxymethyl</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DIFP</td>
<td>diisopropylfluorophosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EDXFS</td>
<td>energy-dispersive X-ray fluorescence spectrophotometry</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis-(ß-amino ethyl ester) N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>FAGLA</td>
<td>N-(3-[2-furyl]acryloyl)-gly-leu amide</td>
</tr>
<tr>
<td>FALGPA</td>
<td>N-(3-[2-Furyl] Acryloyl)-leu-gly-pro-ala</td>
</tr>
<tr>
<td>FCRC</td>
<td>Fredrick Cancer Research Facility</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast process liquid chromatography</td>
</tr>
<tr>
<td>FS-IEF</td>
<td>free solution isoelectric focusing</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>IEX</td>
<td>ion exchange chromatography</td>
</tr>
<tr>
<td>IMRU</td>
<td>Institute of Microbiology, Rutgers University</td>
</tr>
<tr>
<td>IoA</td>
<td>iodoacetic acid</td>
</tr>
</tbody>
</table>
kbp, kb : kilobase pairs

Leu-pNA : L-leucyl-p-nitroanilide

MA : milk agar
MAT : milk agar containing thiostrepton
MB : milk broth
MBT : milk broth containing thiostrepton
MES : 2-[N-morpholino]ethanesulfonic acid
MNBN : minimal base medium with nutrient broth and milk
MOPS : 3-[N-morpholino]propanesulfonic acid
Mr : relative molecular mass
MW : molecular weight

NTA : nitrilotriacetic acid

PAGE : polyacrylamide gel electrophoresis
PHEN : 1,10-phenanthroline
pI : isoelectric point
PMSF : phenylmethylsulfonylfluoride
PPO : 2,5-diphenyloxazole
PPLGPA : 4-phenylazobenzyloxycarbonyl-prolyl-leucyl-glycyl
prolyl-D-arginine
psi : pounds per square inch
PTH : phenylthiohydantoin
PVDF : polyvinylidene difluoride

rDNA : recombinant DNA

SAAPF-pNA : N-succinyl-alanyl-alanyl-prolyl-phenylalanine
p-nitroanilide
SDS : sodium dodecyl sulfate
SSC : standard saline citrate
SSPE : standard saline phosphate EDTA

TAME : N-α-p-tosyl-L-arginine methyl ester
TCA : trichloroacetic acid
TLCK : N-α-p-tosyl-L-lysine chloromethyl ketone
TPCK : N-tosyl-L-phenylalanine chloromethyl ketone
Tris : Tris(hydroxymethyl)aminomethane
TSA : trypticase soy agar
TSB : trypticase soy broth
CHAPTER I

LITERATURE REVIEW

A)  *Streptomyces.*

Ecology. *Streptomyces* strains are widely distributed in nature [Lechevalier, 1981]. While their primary niche is the soil ecosystem [Williams, 1978], they are also known to occur in freshwater and marine environments [Cross, 1981], salt marsh areas [Hunter *et al.*, 1981], fodders [Lacey, 1978], and in the air [Lloyd, 1969], predominantly in the form of spores. Streptomycetes appear late in the natural microbial successions that colonize and degrade organic matter, which results in a fairly restricted spectrum of nutrients available for growth. Such nutrients are mostly high molecular weight polymers such as chitin [Williams and Robinson, 1981], starch [Williams, 1978], pectin [Kaiser, 1971], and certain hemicelluloses [Waksman and Diehm, 1931]. Streptomycetes are obligate aerobes that prefer soils of moderate moisture content [Williams *et al.*, 1972], and most grow optimally in a mesophilic temperature range. They can be grouped into two general categories based on pH requirements for growth [Kutzner, 1981]. Acidophilic streptomycetes grow in the pH
range of 3.5 to 6.5, with optimum growth at a pH of 5.0 [Williams et al., 1971], while neutrophilic streptomycetes grow from pH 5.0 to 9.0 with an optimum pH for growth at pH 7.0. Some streptomycetes have been isolated from soils with a pH greater than 9.0 [Taber, 1960], but they appear to occur only rarely in such alkaline conditions. Nutritionally, streptomycetes are classified as chemoheterotrophs and in general they do not exhibit any special nutritional requirements. Streptomycetes produce a variety of proteinolytic exoenzymes [Morihara et al., 1967; Narahashi et al., 1968; Pokorny et al., 1979; Laluce and Molinari, 1979; Chandrasekaran and Dhar, 1987] and readily grow on complex proteins. A few streptomycetes are pathogenic to certain plants, being the causative agents of scab disease of potato and sugar beet [Lapwood, 1973] and soil rot of sweet potato [Person and Martin, 1940].

The life cycle of streptomycetes [Fig. 1] growing on solid substrates involves the formation of two different types of mycelia: a "substrate mycelium" grows on and into the culture substratum and, as the colony matures, a second type of mycelium, the "aerial mycelium" emanates upward from the substrate mycelium. The latter type of mycelium eventually differentiates to form spores. Thus, the ability of the substrate mycelium to grow firmly attached to its substrate allows full utilization of the nutrients present in the substratum. The aerial mycelia are covered with a hydrophobic sheath, probably to prevent desiccation [Coleman and Ensign, 1982], and their role appears primarily to be reproductive. It has been postulated that the substrate mycelium serves as the source of nutrients for the growth of aerial
grey pigmentation, maturation & release of spores

rounding up of immature spores

wall thickening & rounding off at junctions of spore compartments

sporulation septation

completion of coiling

germination & outgrowth

free spore

substrate mycelium

growth of aerial hyphae with fibrous sheath

aerial hypha

initiation of coiling

Figure 1. Life cycle of a typical streptomycete.
[from Hopwood, 1988, with modifications]
mycelium [Mendez et al., 1985]. Hyphal growth, both in liquid [Brana et al., 1982] and on solid medium, is confined to the hyphal tips.

**Ultrastructure and genetics.** Streptomycetes possess a prokaryotic cellular ultrastructure typical of a Gram-positive bacterium [Williams et al., 1973]. Their cell wall consists of a peptidoglycan network cross-linked by peptide subunits of L-alanine, L-glutamic acid, and L,L-diaminopimelic acid joined by glycine bridges [Nakamura et al., 1977] and the cytoplasmic membrane shows a typical unit membrane structure.

Streptomycetes have coenocytic mycelia, i.e., cross walls within the mycelia are typically absent. The genetic material is situated centrally within the mycelium and is distributed in discreet packages along the entire length of the hyphae [Chen, 1966]. The genome of the members of the genus *Streptomyces* consists of a single circular chromosome of size 8,000 kb [Hopwood and Merrick, 1977], and which has a high guanosine plus cytosine (G+C) content of 68-78 mol% [Goodfellow and Cross, 1984]. Genetic exchange between streptomycetes in the environment is thought to be accomplished primarily through conjugation [Hopwood et al., 1973].

**Significance.** Streptomycetes have proven to be extremely important organisms to modern biotechnology. A wide variety of economically significant and/or biologically active compounds, including antibiotics, chemotherapeutic agents, ionophores, immuno-modulators, hydrolytic enzymes, and enzyme inhibitors have been obtained from various *Streptomyces* species. More than 6000 different antibiotics have been
discovered to date. Antibiotics are members of a class of compounds called secondary metabolites. A secondary metabolite is defined as "a naturally produced substance which does not play an explicit role in the internal economy of the organism that produces it" and is hypothesized to be produced due to the selective advantage that it can provide the producer organism [Stone and Williams, 1992]. Secondary metabolites are generally produced during stationary phase and are typically repressed by growth-promoting substrates. In addition to the antibiotics, *Streptomyces* species produce metabolites that are useful as herbicides such as bialaphos [Imai *et al*., 1984], insecticides such as L-alanosine [Matsumoto *et al*., 1984], antiparasitic agents such as ivermectin [Chabala *et al*., 1980], plant growth regulators such as gaugerotin [Murao and Hayashi, 1983], and antithrombotic agents such as staurosporine [Oka *et al*., 1986], to name a few. In the industrial waste management area, the breakdown of lignocellulose to a soluble reactive polymer called acid precipitable polymeric lignin (APPL) by *Streptomyces* is thought to have economic potential [Pettey and Crawford, 1985].

Secreted proteins are desired for both down-stream processing purposes and for the absence of folding requirements during and after purification. Streptomycetes are also potentially attractive hosts for the expression of heterologous proteins because: (i) they are Gram-positive and therefore they export correctly folded proteins through only a single membrane; (ii) since streptomycetes are Gram-positive, they produce no endotoxin, a cell envelope component of Gram-negative bacteria which must be carefully removed during downstream processing of *Escherichia coli*-
produced recombinant proteins, adding cost to the processes; (iii) industrial fermentation of streptomycetes is very well known due to their historical use as producers of antibiotics; and (iv) the cloning vectors for streptomycetes are now extensively described and are rapidly becoming better understood.

The production of heterologous recombinant proteins in streptomycetes, however, has certain limitations which are being encountered by those industries that are attempting to utilize them. These limitations currently include: (i) limited knowledge about promoter activity in streptomycetes as compared to other better known organisms such as *E. coli* and *B. subtilis* (Strohl, 1992); (ii) limited knowledge about secretion mechanisms in streptomycetes (Brawner *et al.*, 1992); (iii) limited knowledge about the fermentation conditions required for optimal production of recombinant proteins in streptomycetes; and (iv) the production by streptomycetes of extracellular proteinases, which can potentially hydrolyze secreted recombinant protein products.

Continued development of new, useful streptomycete phage and plasmid cloning vectors has enabled a wide variety of genes to be cloned and expressed in streptomycetes over the past several years [Hopwood *et al.*, 1985a]. Complete antibiotic pathways have been cloned [Malpartida and Hopwood, 1984; Binnie *et al.*, 1989; Malpartida *et al.*, 1990], and novel hybrid antibiotics have been made *via* interspecies cloning of streptomycete DNA [Hopwood *et al.*, 1985b]. Several antibiotic resistance genes have also been cloned [e.g. Mitchell *et al.*, 1990; Li *et al.*, 1990; Plater and Robinson, 1992]. Heterologous proteins such as bovine growth
hormone [Gray et al., 1984], interleukin-2 [Munoz et al., 1985], *Salmonella typhimurium* β-lactamase [Ali and Dale, 1986], interleukin-1β [Lichenstein et al., 1988], tumor necrosis factor [Chang and Chang, 1988], interferon-α1 [Noack et al., 1988], *E. coli* galactokinase [Lichenstein et al., 1988], *Flavobacterium* sp. phosphotriesterase [Steiert et al., 1989], a sweet tasting plant protein thaumatin [Illingsworth et al., 1989], a bacterial peptide pheromone [Taguchi et al., 1989], a granulocyte macrophage-colony stimulating factor (GM-CSF) [Malek et al., 1990], and a lipase from *Pseudomonas cepacia* [Joergensen et al., 1991], to name a few examples, have been produced from genes cloned into streptomycetes.

B) **Extracellular enzymes.** Microorganisms are ubiquitous in nature and are responsible for the recycling of much of the complex organic material in the environment. Many of the natural macromolecules can serve as a major food source for heterotrophic microorganisms, but since most of the macromolecules are of a relatively large size, they cannot be readily transported into the cells in their native state for catabolism. Microorganisms liberate enzymes out of the cell into the environment to degrade polymeric materials to low molecular weight compounds which then can be assimilated. Among the bacteria, bacilli, clostridia, pseudomonads, myxobacteria, cytophagas, and many actinomycetes, especially streptomycetes, are prolific producers of extracellular hydrolases which act on high molecular weight substrates. A few extracellular hydrolytic enzymes have low molecular weight
substrates, a notable example being penicillinase (β-lactamase), which hydrolyses the β-lactam ring of the antibiotic penicillin to render it harmless.

Uses of extracellular enzymes. Enzymes have been used throughout history in processes such as leather tanning, cheese making, in the preparation of malted barley for beer brewing, and in the leavening of bread. Such processes typically used enzymes in the form of animal and plant tissues or whole microorganisms. The first instance of a partially purified enzyme from living cells being utilized for commercial purposes occurred in 1894. In this process, *Aspergillus oryzae* was grown on moist rice or wheat bran and the secreted amylase was extracted with water or salt to be used as a digestive aid [Miura, 1958]. The usefulness of extracellular enzymes is greatly appreciated as these are usually easier to recover and purify than their cytoplasmic counterparts. Cell breakage is unnecessary and problems involving removal of nucleic acid are absent. It is also easier to obtain very high yields of extracellular enzymes because the yield of these proteins is typically not restricted by the biomass obtainable. Growth of the enzyme industry in the 1960's was dramatically amplified by the introduction of laundry detergents containing an alkaline proteinase from *Bacillus licheniformis*. The use of microbially produced rennins for cheese manufacture [Law, 1984] and the enzymatic conversion of sucrose into a mixture of glucose and fructose [Satoh *et al.*, 1976] as a food sweetener have since been developed and represent two recent growth areas of industrial enzyme usage. The bulk of the enzyme market comprises of proteinases and carbohydratases which
together account for 90% of the total; the remainder includes technical and pharmaceutical products. The trend over the past 30 years has been away from animal and plant sources and towards microorganisms as the source for production of industrial enzymes. Most new enzyme products are derived from bacteria or fungi. There are several reasons for this: (i) the sources of animal and plant enzymes are often variable and unpredictable; (ii) microorganisms grow more rapidly (i.e., they have a higher productivity) and are ideal for intense cultivation; (iii) medium constituents are cheap and available in bulk quantities; and (iv) the choice of producer microorganisms and enzymes formed is wide and can be improved by relatively straightforward genetic manipulations.

**Enzyme secretion.** In fungi and Gram-positive bacteria, some enzymes are transported through the cytoplasmic membrane, diffuse through the cell wall and accumulate in the environment. In Gram-negative bacteria, the cell wall comprises two unit membranes separated by the periplasm containing a thin peptidoglycan layer. Several major species of proteins are located in this periplasm, including the binding proteins that function in the transport of small molecules and in chemotaxis (e.g., arabinose binding protein, maltose binding protein), "scavenging" enzymes that function in breakdown of complex molecules to simple precursors or those enzymes which function in cell wall biosynthesis (e.g., alkaline phosphatase, UDP-glucose hydrolase), detoxifying enzymes (e.g., β-lactamase, aminoglycoside 3’ phosphotransferase II), and other metabolically important proteins that do not fall
in these groups (e.g., cytochrome C, nitrite reductase). The key feature of all these proteins is that they have crossed the cytoplasmic membrane and are therefore exocytoplasmic.

Proteins that traverse membranes in Gram-positive bacteria typically possess an amino terminal extension of about 25-30 amino acid residues, known as the signal peptide. All signal peptides contain three particular domains, an amino terminal hydrophilic segment, a central stretch of predominantly hydrophobic amino acids, and a more polar carboxy terminal region [von Heijne, 1985]. The hydrophilic region is positively charged and can be of variable length, generally between one and seven amino acids. The central hydrophobic core of the signal sequence plays a crucial role in secretion [Emr and Silhavy, 1983]. It appears that the ability of the hydrophobic core to form two areas of α-helix, split by a central polar residue, is critical for protein export [Emr and Silhavy, 1983]. The signal peptide terminates before the cleavage site with an amino acid that has a short side chain (usually alanine or glycine). When the signal peptide emerges from the ribosome, it interacts with the inner membrane of the cytoplasmic membrane in bacteria. As the polypeptide is elongated in the translational process, it passes through the membrane and a membrane-bound processing enzyme, signal peptidase, removes the signal peptide on the outer side of the membrane. This allows the protein to assume its normal configuration on the outside of the membrane. Recognition of the site for cleavage of the signal sequence from the mature protein seems to be dependent on the amino acids present in positions -3 and -1 relative to the cleavage site [von Heijne, 1983].
The secretion of proteins is remarkably similar in eukaryotic and prokaryotic cells to the extent that eukaryotic signal sequences are recognized and processed by prokaryotic cells and *vice versa* [Randall *et al.*, 1987].

C) **Proteinases.** Proteolytic enzymes are ubiquitous in microorganisms, and extracellular proteinases are probably the most widespread of all microbial secreted enzymes. These enzymes are simple to detect and are often synthesized in high yield.

The reactions catalyzed by proteolytic enzymes fall into five categories: (i) hydrolysis of peptide bonds; (ii) hydrolysis of amides of amino acids; (iii) peptide bond synthesis and transpeptidation reactions; (iv) hydrolysis of esters of amino acids; and (v) exchange of oxygen between water and the carboxyl group of amino acids [Mihalyi, 1972]. Alteration of a carbon-nitrogen bond is involved in the first three and a carbon-oxygen bond in the last two of these reactions. Of these five types of reactions, the hydrolysis of peptide bonds is the natural function of most of these enzymes *in situ*.

Proteinases have been implicated in a wide variety of biological functions and mechanisms. These range from random digestion, as an initial step in metabolizing proteinaceous substrates, to the more specific role of processing precursors, as part of an activation or inactivation mechanism [Holzer and Heirrich, 1980; Neurath, 1984]. Proteinases that digest substrates randomly are generally of lower specificity, e.g., only one exposed amino acid such as Lys for *Achromobacter* proteinase I [Masaki *et al.*, 1981] or Glu for *Staphylococcus aureus* endoproteinase [Houmard and

The term "proteinase" is synonymous with the term "peptide hydrolase"; these terms include all enzymes that cleave peptide bonds. Proteinases are further subdivided into exopeptidases, enzymes that cleave peptide bonds at the amino or carboxy-terminus, and endopeptidases, those that cleave peptide bonds internally and randomly within a polypeptide. Dipeptidases [Ito et al., 1983; Ota, 1986; Dasarathy et al., 1989] are a class by themselves as they require the presence of both a charged α-amino and a charged α-carboxyl group in the immediate vicinity of the peptide bond. This requirement can be satisfied only by dipeptides, and hence the absolute specificity of these enzymes.

The specificity of a proteinase is expressed towards a portion of the polypeptide substrate which contains the peptide bond to be cleaved preferentially (Fig. 2). Historically, studies with synthetic substrates [Bergmann and Fruton, 1941] have shown that the specificity of a proteinase depends on presence or absence of charged groups in the vicinity of the susceptible peptide bond. The notation proposed by Schecter and Berger [1967] is widely used to designate the amino acid residues flanking the scissile peptide bond (Fig. 2).
Classification of proteinases. Proteinases have traditionally been classified according to molecular size, charge, or substrate specificity. With regard to origin, these enzymes may be classified into animal, plant, and microbial proteinases. A more rational system is now based on a comparison of active sites, mechanism of action, three-dimensional structure and amino acid sequence homologies. Four general mechanistic classes have been recognized by the International Union of Biochemistry (Table 1), and within these classes, six families of proteinases are recognized to date (two groups of serine proteinases, two groups of metalloproteinases, acid [aspartic] proteinases, and sulfhydryl [cysteine] proteinases) [Neurath, 1984]. A recent addition to the proteinase classification scheme is the bacterial ATP-dependent group of proteinases, which up to now contains Lon, Alp, Clp, and an unknown proteinase from *Escherichia coli* [Maurizi, 1992]. Members of each family of proteinases have a characteristic, highly conserved sequence of amino acid residues arranged in a particular configuration to form an active site (Table 1). The serine and sulfhydryl proteinases form covalent enzyme-substrate complexes and have amino acids at their catalytic site that participate directly in nucleophilic attack on the scissile peptide bond. The metalloproteinases and the acid proteinases do not form covalent enzyme-substrate complexes and catalyze the hydrolysis of peptide bonds without nucleophilic attack by a functional group of the enzyme. Members of each family are believed to have descended from a common ancestor by divergent evolution [Neurath, 1984].

The most thoroughly studied bacterial proteinases are the serine proteinases of the chymotrypsin and trypsin families. The "class" of serine proteinases includes
Figure 2. Schematic illustration of the interaction of a proteinase with its substrate. The proteinase is represented as the dark area. $P_1 - P_7$ are the side chains of six amino acids, and $S_1 - S_7$ of the substrate are the corresponding subsites on the enzyme. [from Schecter and Berger, 1967].
Figure 2

Scissile bond
Table 1. Families of proteinolytic enzymes. [from Neurath, 1984, with modifications]

<table>
<thead>
<tr>
<th>Family</th>
<th>Representative proteinases</th>
<th>Characteristic active site residues$^{a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine proteinase I</td>
<td>Chymotrypsin</td>
<td>Asp$^{102}$, Ser$^{196}$, His$^{27}$</td>
</tr>
<tr>
<td></td>
<td>Trypsin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Elastase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pancreatic kallikrein</td>
<td></td>
</tr>
<tr>
<td>Serine proteinase II</td>
<td>Subtilisin</td>
<td>Asp$^{47}$, Ser$^{202}$, His$^{64}$</td>
</tr>
<tr>
<td>ATP-dependent proteinases</td>
<td>Clp</td>
<td>Ser$^{111}$, His$^{134}$</td>
</tr>
<tr>
<td></td>
<td>Lon</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Alp</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>unknown function</td>
<td>-</td>
</tr>
<tr>
<td>Cysteine proteinases</td>
<td>Papain</td>
<td>Cys$^{53}$, His$^{318}$, Asp$^{354}$</td>
</tr>
<tr>
<td></td>
<td>Actinidin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rat liver cathepsins B and H</td>
<td></td>
</tr>
<tr>
<td>Aspartic proteinases</td>
<td>Penicillopepsin</td>
<td>Asp$^{33}$, Asp$^{233}$</td>
</tr>
<tr>
<td></td>
<td>Rhizopus chinesis acid proteinase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Renin</td>
<td></td>
</tr>
<tr>
<td>Metallo-proteinase I</td>
<td>Bovine carboxypeptidase A</td>
<td>Zn$^{3}$, Glu$^{29}$, Tyr$^{246}$</td>
</tr>
<tr>
<td>Metallo-proteinase II</td>
<td>Thermolysin</td>
<td>Zn$^{3}$, Glu$^{14}$, His$^{231}$</td>
</tr>
</tbody>
</table>

$^{a}$The number of each residue corresponds to the position of that particular amino acid in the amino acid sequence of the specific enzymes listed in bold.

$^{b}$Metal ligand
two distinct families: the trypsin/chymotrypsin-like family and the subtilisin-like family of proteinases. These differ from each other in amino acid sequence and three-dimensional structure, despite a common active site geometry and catalytic mechanism. These proteinases are sometimes referred to as "alkaline proteinases", because they generally have a pH optimum for activity of 9-11. These proteinases typically possess a broad range of peptidolytic activities as well as esterase activity. While these alkaline proteinases are unaffected by metal-chelating agents, they are inhibited by diisopropylfluorophosphate (DIFP) which binds irreversibly to the serine residue in the active site of the enzyme. Those serine proteinases that require Ca++ for stability or activity [Krier et al., 1983] are affected by metal chelating agents.

There are some distinct exceptions to the pH optima described above. Certain serine proteinases have isoelectric points of 3.5-4.5 and correspondingly low pH optima of 4-6 [Palubinskas et al., 1984].

A recently described class of proteinases is the ATP-dependent proteinases [Maurizi, 1992]. Even though such proteinases have been identified in both animal tissues and prokaryotes, E. coli is the only prokaryote from which two such proteinases have been purified. The Lon proteinase was the first to be identified by Chung and Goldberg in 1981 and now is known to play a critical role in degradation of specific native regulatory proteins as well as in the degradation of abnormal proteins in E. coli [Gottesman, 1989]. Katayama-Fujiyama et al. [1987] identified the second ATP-dependent proteinase from E. coli and named it the Clp proteinase. The Clp proteinase, also known as Ti proteinase [Hwang et al., 1987], degrades casein
and is a two component (ClpA and ClpP) complex. ClpA has a Mr of 81,000 and has intrinsic ATPase activity [Katayama et al., 1988]. ClpP has a native Mr of approximately 240,000 and is made up of two hexameric rings superimposed on each another (a total of 12 monomeric units), with a central cavity [Maurizi et al., 1990a]. ClpP contains the proteinolytic activity and represents a unique family of serine proteases whose active site is made up of Ser111 and His136 [Maurizi et al., 1990b].

The clpP gene has been demonstrated to be a heat shock gene whose product was identified as the previously unidentified heat shock protein F21.5 [Kroh and Simon, 1990]. The product of the alp gene of E. coli, which may be an ATP-dependent proteinase or a regulator of an ATP-dependent proteinase, was shown be responsible for degrading several Lon substrates in vivo [Trempy and Gottesman, 1989]. More recently Kroh and Simon [1991] have indicated the presence of an unknown ATP-dependent proteinolytic activity that is σ32 independent and negatively regulated by DnaK. This proteinolytic activity was proven by these authors to be different from that associated with the Lon, Clp or Alp proteinases.

Sulfhydryl proteinases, with cysteine in the active site, are widely distributed in nature and can be found in plants, bacteria, and mammals. The plant enzymes papain (from papaya), ficin (from figs), bromelain (from pineapple), and actinidin (from kiwi fruit) represent a structurally homologous family. Bacterial sulfhydryl proteinases include clostripain (from Clostridium histolyticum) and staphylococcal proteinase (from hemolytic streptococci), while the mammalian versions include cathepsin A and B [Fersht, 1977].
Acid proteinases (optimally active in the pH range of 2-3), with glutamate or aspartate in their active site, include penicillopepsin (from *Penicillium janthinellum*), mammalian pepsin, renin, and certain fungal proteinases (*Rhizopus*-pepsin) [Fersht, 1977]. The specificity of these proteinases is directed towards the peptide bonds located between large hydrophobic residues, and these enzymes are inhibited by pepstatin, a hexapeptide that, in the transition state, resembles normal substrates [Umezawa, 1976].

The metalloproteinases primarily fall into one of two families: those proteinases similar to mammalian pancreatic carboxypeptidase A and those similar to thermolysin from *Bacillus thermoproteolyticus* [Hofmann, 1985]. Pancreatic carboxypeptidase A differs from bacterial thermolysin-like enzymes in tertiary structure even though both are zinc metalloenzymes and have somewhat similar active site configurations. These proteinases generally have maximal activity in the pH range of 7.0-8.0 and are inhibited by metal-chelating agents. Other metalloproteinases that require Zn$^{++}$ for activity are collagenase, angiotensin-converting enzyme, the Zn$^{++}$ G proteinase from *Streptomyces albus*, the D,D-carboxypeptidases involved in cell wall metabolism, and the aminopeptidases [Fersht, 1977].

Several proteinases have not yet been assigned to any one of the above mentioned classes. It is unclear whether some of them, e.g., human insulin-degrading proteinase [Affholter et al., 1988], may represent new types of catalytic mechanisms. The proteinase from the crayfish *Astacus*, formerly thought to belong to none of the
known classes of proteinases [Zwilling et al., 1981; Titani et al., 1987] has now been identified as a zinc metalloenzyme [Stocker et al., 1988].

Secretion, processing and activation of bacterial extracellular proteinases. Bacteria synthesize a large variety of intracellular and extracellular proteinases. Intracellular proteinases are highly specific and are involved in several biological processes such as removal of signal peptides from newly synthesized proteins, activation of inactive precursors, inactivation of regulatory proteins, and the breakdown of abnormal or foreign proteins. Many bacteria also secrete proteinases in the extracellular medium. Some of these proteinases are toxins or factors involved in virulence, while others (i.e. nutritional enzymes) show low substrate specificity and degrade proteins to produce small peptides and amino acids which can be transported into the cell and utilized.

In Gram-positive bacteria, extracellular proteins are made initially as precursors containing an amino-terminal extension, the signal peptide, which is removed during export to generate the mature extracellular protein [Pugsley and Schwartz, 1985]. In Gram-negative bacteria, two distinct mechanisms for protein secretion have been described. Some proteins are translocated through the cytoplasmic membrane by a signal-peptide-dependent step and then are transported out of the cell by means of specific secretion proteins located in the cell envelope. Other types of extracellular proteins do not have a signal peptide and are secreted via a separate pathway [Pugsley, 1988].
A special, unifying feature of the various proteinase secretion pathways is that these enzymes (i.e., extracellular proteinases) represent a potential danger for the cells, especially if they have broad substrate specificity. There are two general mechanisms for protecting the host cell against unwanted proteolysis. First, bacteria synthesize inactive precursors (zymogens) which are later activated by limited proteinolysis [Neurath, 1986]. Second, proteinases are inactivated by interaction with proteinase inhibitors [Laskowski and Kato, 1980]. All extracellular proteinases studied so far are synthesized as inactive precursors with an additional polypeptide segment, the propeptide, that is removed to form the mature secreted protein. Propeptides have various lengths and locations in the precursor and, besides maintaining the proteinase in the inactive form, may promote the correct folding of the proteinase, alter proteinase specificity, act as a membrane anchor, or play a role in the secretion process by acting in a chaperone-like capacity [Winther and Sorensen, 1991]. Interestingly, such propeptides are rarely found in exoproteins other than proteinases.

Mechanism of action of bovine carboxypeptidase A. As mentioned previously, the metalloproteinases do not form covalent intermediates with their substrates. Rather than rely on hydrogen bonding via an "oxyanion hole", as do serine proteinases [Henderson, 1970], the metalloproteinases utilize coordination to a metal ion to exert the catalytic effect [Powers and Harper, 1988]. This metal ion is usually Zn$$^{++}$$, although in some cases other transition metal ions such as Co$$^{++}$$ or Cu$$^{++}$$ can
substitute for the zinc. Most of the data describing the mechanism of action of metalloproteinases and involvement of the metal ion required for their activity is derived from the studies on bovine carboxypeptidase A. This enzyme was first isolated by Waldschmidt-Leitz in 1929 and later crystallized by Anson in 1937 [described in Hartsuck and Lipscomb, 1971]. Carboxypeptidase A has a $M_r$ of 35,472 as determined from its amino acid sequence [Bradshaw et al., 1969] and contains one zinc atom per molecule. The presence of metal ions in these enzymes was initially deduced from the inhibition of the enzyme activity by reagents which specifically chelate or combine with these ions. Zinc was subsequently identified by emission spectroscopy [Vallee and Neurath, 1955]. The stereochemical structure of carboxypeptidase A has been determined by X-ray diffraction [Quirocho and Lipscomb, 1971]. The molecule has an extended, twisted, pleated sheet structure which divides it into two segments (Fig. 3). One zinc atom and ten water molecules are trapped inside this molecule. The ligands of the Zn$^{++}$ atom have been identified as His69, Glu72, and His196, with the fourth position being occupied by a water molecule. More recent studies [Rees et al., 1981] at the 1.75 Å level have indicated that the zinc atom is actually in a pentavalent coordination, its fifth ligand being the second oxygen of Glu72. The active site forms a pocket and a groove [Lipscomb et al., 1968]. The pocket, which accomodates the C-terminal side chain of the bound peptide substrate, is filled with water molecules in the unoccupied enzyme. Most of these are expelled when substrates or inhibitors are bound. The active site does not have any charged groups and hence shows a preference for uncharged terminal residues in the
Figure 3. Bovine carboxypeptidase A [from Lipscomb et al., 1969]. The zinc atom is shown in the center as a filled dark circle.
substrate. An ionic bond between the terminal carboxyl group of the uncleaved peptide and Arg145 of carboxypeptidase A is formed after the terminal side chain of the substrate has been positioned in the active site. The formation of the ionic bond rotates Tyr248 so that it can form hydrogen bonds with both the terminal and penultimate peptide nitrogen atoms of the substrate. After this conformational change has taken place, the peptide bond to be cleaved is now surrounded by the Zn\(^{++}\) atom and residues Arg145, Glu270, and Tyr248. With the substrate positioned in this way, the carbonyl group of the scissile peptide bond faces towards the zinc atom while its oxygen replaces the water molecule which previously occupied the fourth coordination place of the metal. At the same time, the two hydrogen bonds to Tyr248 on either side of this group introduce considerable strain on the peptide bond about to be cleaved. Although chemical modifications have established the crucial role of Glu270 in the catalysis [Petra and Neurath, 1971] and in spite of the availability of a high resolution structure of carboxypeptidase A at 1.75 Å [Rees et al., 1981], it is still unclear as to whether the catalysis proceeds via a general base mechanism or via a nucleophilic attack by a group from the enzyme on the scissile peptide bond. It is hypothesized that the cleavage itself could be accomplished by Tyr248 donating a proton to the nitrogen atom of the scissile peptide bond, and Glu270 performing a nucleophilic attack on the carbon atom of the scissile peptide bond. Alternatively, it is thought that after the Tyr248 has donated the proton, a general base catalysis takes place in which attack by the oxygen atom of a water molecule, on the carbon atom of the scissile peptide bond, is promoted by its
hydrogen bonding to Glu270. In either case, an acid anhydride would be formed between Glu270 and the carboxyl group of the cleaved peptide bond, which could then be readily hydrolyzed by water to the free acid [Mihalyi, 1972].

Mechanism of action of bacterial thermolysin. Thermolysin is an extracellular proteinase that has been isolated from the culture medium of the thermophilic microorganism *Bacillus thermoproteolyticus* [Endo, 1962]. Thermolysin is an endopeptidase with a $M_r$ of 34,000 and contains one zinc atom and four calcium atoms per molecule of enzyme [Hofmann, 1985]. While thermolysin resembles pancreatic carboxypeptidase A in its molecular size and the presence of zinc in the catalytic site, there is no evidence of any sequence homology between these two enzymes [Titani *et al.*, 1972]. The overall tertiary structure of thermolysin has been described [Titani *et al.*, 1972]. Thermolysin does not contain any disulfide bridges. A cleft seen across the center (Fig. 4) accommodates the zinc, while a pocket next to the $\text{Zn}^{++}$ determines specificity [Matthews *et al.*, 1974]. The zinc atom is in a tetrahedral arrangement to the nitrogens of His142 and His146, to one of the carboxyl oxygens of Glu166, and to a water molecule [Matthews *et al.*, 1974]. The bound $\text{Ca}^{++}$ ions contribute to the overall stability of the molecule [Feder *et al.*, 1971] and may also protect the surface loops of the enzymes from autolysis [Matthews *et al.*, 1974].
Figure 4. Bacterial thermolysin [from Colman et al., 1972]. The zinc atom is shown in the center as a stippled circle. The four calcium atoms responsible for heat stability are drawn as solid circles.
Figure 4
The reactions catalyzed by thermolysin can be explained by a single mechanism [Pangburn and Walsh, 1975; Kester and Matthews, 1977]. Involved in catalysis are the Zn\(^{++}\) ion, Glu143, His231, and a water molecule. Briefly, the substrate binds to the hydrophobic pocket of thermolysin via the carbonyl group of the scissile peptide bond and displaces a water molecule from the Zn\(^{++}\) ion. The Zn\(^{++}\) ion in turn binds to this incoming carbonyl group and polarizes the peptide bond. Following substrate binding, Glu143 encourages the nucleophilic attack of a water molecule on the carbonyl carbon of the scissile peptide bond. At the same time, His231 donates a proton to the nitrogen of the scissile peptide bond, facilitating the hydrolysis of the carbon-nitrogen bond of the intermediate to release the two product peptides.

Holmes and Matthews [1981] have proposed that burial of the charged Glu143 and Zn\(^{++}\) in a nonpolar environment after substrate binding might be one of the forces behind the catalytic mechanism. The subsequent neutralization of Glu143 and the Zn\(^{++}\) ion has been thought to provide the free energy needed to form the tetrahedral transition state [Holmes and Matthews, 1981]. These authors have complexed hydroxymic acid derivatives, known to be potent inhibitors of thermolysin [Nishino and Powers, 1978], with thermolysin and have determined by X-ray analysis that Zn\(^{++}\) in such complexes is in a pentacoordinate state. The Zn\(^{++}\) was shown to be liganded to His142, His146 and Glu166 from thermolysin, and to two positions on the hydroxymic acid derivative. The authors have proposed that during actual catalysis, the Zn\(^{++}\) becomes temporarily pentacoordinate by interacting with both the
water molecule and the incoming substrate. The Zn\(^{2+}\) is then thought to serve a dual role of polarizing the substrate carbonyl bond as well as correctly aligning the carbonyl bond and the attacking nucleophile.

Hangauer *et al.* [1984] have utilized the power of interactive computer graphics on enzyme-substrate and enzyme-inhibitor complexes to modify and extend the previously proposed mechanism of the action of thermolysin. While preserving the general features of the previous mechanisms of the action of thermolysin, these authors have proposed further modifications. According to their experimental observations, the substrate initially penetrates the active site only partially and does not coordinate with the Zn\(^{2+}\) ion. A tetrahedral intermediate is attained simultaneously with full penetration of the substrate into the active site. The zinc-bound water molecule is then thought to move towards Glu143 which in addition to functioning as a general base may shuttle two protons from the nucleophilic water to the scissile peptide bond. This could then lead to release of product [Hangauer *et al.*, 1984].

**Proteinases in peptide synthesis.** The reactions catalyzed by the proteolytic enzymes are reversible under appropriate conditions. A particular case of this reversal is the exchange of partners between two peptide bonds. Even though proteinases of all four mechanistic groups, except for the ATP-dependent proteinases, have been used for peptide synthesis [Fruton, 1982], these transpeptidation reactions are more frequent with some enzymes, such as papain and pepsin, than with others.
The main attraction of using proteinases lies in their capacity to effect peptide bond formation stereospecifically without the need for side chain protection. In proteinase-mediated peptide synthesis, the enzymatic specificity prevents the formation of undesired by-products often formed in the course of conventional chemical synthesis. The equilibrium of the hydrolysis of the peptide bond mostly lies to the side of the cleavage products and the opposite reaction, which is the synthesis of peptide bonds, occurs only in the presence of a large excess of the normal reaction products. The first reported instance of such a reaction was the amide bond formation between an acylamino acid and an amino acid anilide in the presence of papain, a plant sulfhydryl proteinase [Bergmann and Fraenkel-Conrat, 1937].

A variety of proteinases is now used in the synthesis of peptide hormones [Andersen et al., 1986], neuropeptides [Kullmann, 1982], peptides used as sweeteners, such as aspartame [Oyama et al., 1981], and in protein modifications such as the semisynthesis of human insulin [Morihara et al., 1979; Morihara, 1987]. A proteinase from Streptomyces cellulosae has been reported [Muro et al., 1987] to catalyze the condensation reaction forming oligopeptides from different dipeptides. Peptide-bond synthesis is also exploited by nature. In the initial translation product of concanavalin A, the order of the N- and C-terminal half molecules is reversed. Proteinolytic cleavage and subsequent resynthesis of the correct polypeptide chain are accomplished by some unknown proteinase which is specific for the asparagine at the P₁ residue [Bowles et al., 1986].
Streptomyces proteinases. *Streptomyces* spp. are known to contain a wide variety of proteinase activities. Since the mid-1960s, *Streptomyces griseus* strain K-1 has been used commercially to obtain a mixture of several proteinolytic enzymes, called Pronase. Pronase is a partially purified preparation containing aminopeptidase, carboxypeptidase, neutral proteinase, and alkaline proteinase activities [Awad et al., 1972]. The amino- and carboxy- peptidases and the neutral proteinase are metalloproteinases, while at least three serine proteinases are responsible for the alkaline proteinase activity.

Apart from the commercial use of Pronase and a few reports describing the presence of extracellular proteinases, characterization of streptomycete proteinases has only recently begun. Renko et al. [1981] purified a serine proteinase from *S. rimosus* that has a $M_r$ of 22,000, an isoelectric point of 4.9, a temperature optimum of 50°C, and a broad pH range (4-11) in which proteinolytic activity against hemoglobin could be detected. An extracellular subtilisin-like serine proteinase was purified and characterized from *S. spheroides* strain 35 [Krier et al., 1983]. This enzyme was shown to have a $M_r$ of 28,000, an isoelectric point of >10, an optimum pH for activity of 10.5, an optimum temperature for activity of 50°C, and an apparent requirement for Ca$^{++}$ as a stabilizing cation. This proteinase was reportedly similar to the C and D proteinases of *S. griseus* Pronase. A similar study characterized the extracellular serine-type proteinase A of *S. rutgersensis* [Kaluger et al., 1983]. This proteinase also has a pH optimum of 9-10, a $M_r$ of 18,000-20,000, and since it was related to the proteinases A and B of *S. griseus* pronase, it was
assigned to the chymotrypsin family. An interesting trypsin-like extracellular proteinase with a Mr of 31,500 was purified from *Streptomyces* sp. 771 [Palubinskas *et al.*, 1984]. This proteinase was different from other reported streptomycete proteinases in having a relatively high proportion of the amino acids glutamate and aspartate, reflecting a low isoelectric point of 3.75. The authors compared this enzyme with other trypsin-like proteinases from streptomycetes and found that the trypsin-like proteinases all had 299-302 amino acid residues and could be split into two isoelectric point classes, those with a pI of about 3.75 to 4.0 (*Streptomyces* sp. 771 and *Saccharopolyspora erythraea* proteinases) or those with a pI of about 9 (*S. fradiae* and *S. griseus* proteinases). Muro *et al.* [1987] purified an extracellular serine proteinase from *S. cellulosae* that has a Mr of 22,000 to 26,500, an optimum pH of 6.5 to 7.0, and an optimum temperature for activity of 55°C. Proteinases with high isoelectric points are more typical of trypsin-like proteinases from the bacilli and other organisms. While the unifying feature of such enzymes is their classification as serine proteinases, having single subunits with Mr of ca. 18,000 to 36,000, high temperature optima (ca. 50°C for most of them), sensitivity to DIFP (where tested), stimulation by Ca²⁺ (where tested), and similar amino acid compositions, their major diversity lies in substrate specificity, pI, and optimal pH for activity.

*Streptomyces* spp. can also produce a wide variety of neutral proteinases. A low molecular weight neutral proteinase (Mr of 15,000) has been isolated from *S. caespiotus* that has activity against casein [Yokote and Noguchi, 1969]. This seems to be the only recorded streptomycete neutral proteinase with such a low molecular
weight. Three neutral proteinases produced by *S. kinoluteus* [Nakamura et al., 1969a, b], two neutral proteinases produced by *S. verticillatus* var. *zymogenes* [Nakamura et al., 1970a], one neutral proteinase produced by *S. griseolus* [Nakamura et al., 1970b], and two neutral proteinases from *S. cacaoi* var. *asoensis* [Nakamura et al., 1970c] have been isolated and characterized. These particular proteinases share a common feature of possessing strong anti-inflammatory activities. The molecular masses of these proteinases were not reported by the authors. A neutral proteinase from *S. naraensis* was purified and shown to be a glycoprotein having a Mr of 37,100, a pI of 4.2 and containing 1 atom of zinc per mole of the enzyme [Hiramatsu and Ouchi, 1972]. N-carbobenzoxy-L-phenylalanyl-aminohexyl-Sepharose was an effective affinity adsorbent for this neutral proteinase during investigations by Hiramatsu [1982]. Even though the thermal stability of this enzyme was shown to be increased with the addition of Ca$^{++}$ ions, enzyme inactivated with EDTA could be reactivated only after the addition of Zn$^{++}$ ions, suggesting the necessity of Zn$^{++}$ for activity. Recently, Chang et al. [1990] cloned the extracellular neutral metalloproteinase gene (*npr*) of *S. cacaoi* into *S. lividans*. The purified proteinase has a Mr of 35,000 and contains a conserved Zn$^{++}$ ligand binding site similar to those found in other neutral proteinases [Jongeneel et al., 1989]. Furthermore Zn$^{++}$ was found to restore more than 98% of the original activity of the proteinase that was lost upon EDTA treatment [Chang et al., 1990]. These are some examples of the diversity and unusual characteristics of extracellular proteinases from streptomycetes.
Streptomyces extracellular proteinases are generally considered to be nutritional enzymes that function to degrade protein substrates to oligomeric molecules that can be assimilated. These proteinases would fall into two categories: (i) those enzymes produced during exponential growth phase which are responsible for hydrolysis of proteins for the nutrition of rapidly-growing mycelia; and (ii) those enzymes produced during stationary phase (or antibiotic production phase) which are thought to be responsible in situ for hydrolysis of substrate mycelial proteins to provide nutrients for the development of aerial mycelia and spores [Chater, 1984; Mendez et al., 1985; Gibb and Strohl, 1988]. The coincidence of extracellular proteinase production and autolysis of Streptomyces peucetius mycelia [Gibb and Strohl, 1988] has extended this hypothesis to suggest that the proteinases provide peptide nutrients to the differentiating cells by hydrolyzing endogenous proteins made by the vegetative mycelium at an earlier stage of their life cycle.

Not much is actually known about the molecular basis of regulation of Streptomyces proteinases. Spontaneous mutants of Streptomyces lactamdurans have been isolated that had lost the ability to sporulate, produce cephamycin C, and excrete serine proteinases [Ginther, 1979]. A common regulatory process has been hypothesized by the author in this case.

Although extracellular proteinases may allow streptomycetes to grow well in complex fermentation media, these proteinases could also possess the potential to degrade secreted, foreign proteins produced from recombinant DNA in those organisms. Deletion of such extracellular proteinase genes, while increasing the
stability of the recombinant protein due to decreased proteinolysis, may also reduce the ability of the host organism to grow in standard industrial media. The proteins in such ingredients are usually semi-hydrolyzed in order to keep the media costs down, and host proteinases may be required to hydrolyze these proteins further in order to provide energy for growth. Hence, a comprehensive study on the types of proteinases, the roles each proteinase plays, the regulation of proteinase production, and the ability of each proteinase to degrade the desired recombinant proteins is critical to producing heterologous proteins in recombinant streptomycete fermentations. In relation to the use of streptomycetes as hosts for rDNA, knowledge of the presence or absence of proteinases, along with their regulation, is of crucial importance to the stability of heterologous protein products.
Research objectives

The main objective of my research during the initial stages of this project was to purify the SnpA proteinase, a protein encoded by the snpA gene of Streptomyces C5 that had been cloned into S. lividans 1326 on plasmid pANT21. Since this plasmid contained the snpA gene on a relatively unstable 6.56 kb DNA insert, I initially decided to execute a variety of subcloning experiments to determine the location of this gene on the insert DNA. Preliminary subcloning experiments resulted in the generation of plasmids pANT22 and pANT23. Subsequent transformation of S. lividans with these plasmids and the resulting phenotypic assessment of milk hydrolyzing activity on Milk Agar plates indicated that plasmid pANT23 was carrying the snpA gene. Further subcloning experiments resulted in the development of a recombinant plasmid, designated pANT42, that stably carried the snpA gene. The resulting strain, S. lividans(pANT42), was then used as the source of the SnpA. After reproducible purification of the SnpA proteinase, I made it my priority to characterize this proteinase biochemically by performing substrate screening experiments, analysis of cation requirements and inhibition and reactivation studies. The SnpA proteinase was further characterized physically by investigating its temperature stability, pH optimum, molecular mass, subunit composition and finally by the identification and quantification of the metal ligand necessary for its activity.
Since the recombinant plasmid, pANT42, still contained a relatively large fragment of the cloned *Streptomyces C5* DNA (ca. 6.56 kbp), it was necessary to localize the actual proteinase gene (*snpA*) so that it could be sequenced and characterized genetically by collaborators. Restriction endonuclease mapping of pANT42 was undertaken as the first step towards reaching this goal. Later, the strategy involved design and synthesis of a mixed 17 base pair oligonucleotide probe (2 degeneracies) from the partial N-terminal amino acid sequence of the purified SnpA. Southern hybridization analysis of endonuclease-digested pANT42 with this probe was then utilized to determine the exact location of the *SnpA* gene on plasmid pANT42. This gene was subsequently sequenced by collaborators of this laboratory and was proven to be located as I had determined.

During initial substrate screening experiments with *S. lividans* carrying the recombinant plasmid pANT42, an extracellular leucine aminopeptidase activity was discovered in the culture broth. As the purified recombinant proteinase, SnpA, did not exhibit any activity against L-leu-pNA (the substrate for leucine aminopeptidase), the aminopeptidase activity was concluded to be from the host, *S. lividans*. The research goal at this point in time then shifted towards further investigating this L-leu-pNA hydrolyzing proteinase activity and also identifying other potential proteinases that could be secreted by *S. lividans*. This was accomplished by testing the concentrated culture broth of *S. lividans* for activity against a variety of both natural and synthetic proteinase substrates. Since the leucine aminopeptidase was the only major extracellular proteinolytic activity associated with the host *S. lividans* strain,
attempts to purify and characterize the protein(s) responsible for this activity were undertaken as the next logical step involved in the studies of *Streptomyces* proteinases.
CHAPTER II

Purification and characterization of the proteinase encoded by *Streptomyces* C5 *snpA*

A) Introduction

Streptomycetes are known to contain a wide variety of both serine and neutral proteinases with varied substrate specificities [Pokorny et al., 1979; Chandrasekaran and Dhar, 1987]. Most of the investigations of streptomycete proteinases, however, have been restricted to dealing with the structures and cleavage mechanisms of the various proteinases of *Streptomyces griseus* which constitute "pronase" [Narahashi, 1970]. Little is actually known about the regulation of proteinase expression in streptomycetes, largely because only a few genes encoding streptomycete proteinases have been cloned and sequenced. Henderson et al. [1987] have reported that colonies of *Streptomyces lividans* 1326 did not produce significant clearing zones when grown on milk-agar plates. These authors have used this property to assist their cloning of two serine proteinases (SGPA and SGPB) from *S. griseus* into *S. lividans*. Recently a gene from *Streptomyces cacaoi* encoding a neutral proteinase with a
deduced Mr of 35,000 was isolated and sequenced [Chang et al., 1990]. The deduced amino acid sequence of the S. cacaoi neutral proteinase indicated that it was significantly different from the thermolysin-like neutral proteinases which are common to Bacillus strains.

Streptomyces sp. C5, a mutagenized strain isolated as an overproducer of anthracycline antibiotics [White and Stroshane, 1984], produces negligible extracellular proteinase activity against azocasein but has strong extracellular proteinolytic activity against the proteins in Carnation non-fat dry milk [Gibb et al., 1987; Gibb et al., 1989]. S. lividans 1326 possesses negligible activity against dry milk [Lampel, 1988]. The respective differences in proteinolytic profiles of Streptomyces sp. C5 and S. lividans 1326 were utilized to clone the gene encoding a milk protein-degrading neutral proteinase from the former strain into the latter [Lampel, 1988]. The initial clone containing the snpA gene, pANT21, has been further characterized by subcloning procedures by me. The protein encoded by the snpA gene, C5-SnpA, has been purified to homogeneity and characterized biochemically. Interestingly, this proteinase in its cloned state seems to be expressed early in the growth cycle of S. lividans 1326, as demonstrated by biochemical assays and slot-blot hybridization analyses.

The combination of its small size, substrate specificity, inhibition profile, and the time of expression during fermentation indicates that this enzyme, named C5-SnpA, is a novel neutral proteinase which may comprise a new subclass of neutral proteinases. A second gene of Streptomyces sp. C5, potentially affecting proteinase
gene expression, was discovered during subcloning procedures. This gene, which lies upstream of the \textit{snpA} gene on plasmid pANT42, is transcribed divergently to \textit{snpA}. This gene has been tentatively named \textit{snpR} as its product has a similarity to the LysR family of regulatory proteins and is thought to control positively the expression of a proteinase gene from \textit{S. lividans}. 
B) Subcloning and analysis of the snpA gene and its upstream sequences.

Materials and Methods

Bacterial strains, maintenance, and cultivation conditions. The bacterial strains used in this study are described in Table 2. These strains were maintained in the laboratory as described previously [Dekleva et al., 1985].

R2YE medium described by Hopwood et al. [1985a] and trypticase soy agar (Becton Dikinson, Cockeysville, MD) were typically used for routine maintenance of the strains. Generalized milk-protein hydrolyzing activity was detected by screening for zones of hydrolysis around colonies growing on milk-agar (MA) plates, which consisted of the following components (in g/L): MOPS buffer, 1.49; Nutrient Broth (Difco, Detroit, MI), 1.0; KH2PO4, 0.5; K2HPO4, 0.5; fructose, 0.25; CaCl2·2H2O, 0.735; and agar, 15. The pH was adjusted to 7.2 with 1 N sodium hydroxide prior to autoclaving. Powdered dry milk (Carnation Co., Los Angeles, CA) was autoclaved separately as a 20% solution (w/v) in double distilled water and added to the medium after cooling to make a final concentration of 20 g/L. Thioestreptone at 50 μg/ml was typically included in media that were used to cultivate plasmid-carrying strains.

DNA cloning procedures and genetic manipulations. Procedures used for the preparation of Streptomyces plasmid and chromosomal DNA were as previously
Table 2. Bacterial strains and plasmids used in this research work.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. C5</td>
<td>milk protein-hydrolyzing proteinase producer</td>
<td>FCRC*</td>
</tr>
<tr>
<td><em>S. gallinarum</em> 31133</td>
<td>lacks milk protein-hydrolyzing activity</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>S. peucetius</em> 29050</td>
<td>lacks milk protein-hydrolyzing activity</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>S. lividans</em> 1326</td>
<td>minor, post-exponential growth phase milk protein-hydrolyzing activity</td>
<td>D.A. Hopwood</td>
</tr>
<tr>
<td><em>E. coli</em> DH50F'</td>
<td>$\phi 80$dICZ$\Delta$(lacZYA-argF) U169 deoR recA1 endA1 hsdR17(r$^+$ m$^+$) supE44 $\Delta$ thi1 gyrA96 relA1</td>
<td>BRL</td>
</tr>
<tr>
<td><strong>Plasmids:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pIJ702</td>
<td>5.69 kbp; derivative of pIJ350; HC, Thio', Mel*</td>
<td>D.A. Hopwood</td>
</tr>
<tr>
<td>pKC505</td>
<td>18.7 kbp; <em>Streptomyces</em>-<em>E. coli</em> shuttle vector; LC, Apr*</td>
<td>Richardson et al., 1987</td>
</tr>
<tr>
<td>pUC19</td>
<td>2.688 kbp; Amp*</td>
<td>J.N. Reeve</td>
</tr>
<tr>
<td>pANT21</td>
<td>12.25 kbp; pIJ702 with 6.56 kbp SstI cloned DNA fragment from <em>Streptomyces</em> sp. C5; unstable; confers strong milk protein-hydrolyzing activity to <em>S. lividans</em></td>
<td>Lampel, 1988</td>
</tr>
<tr>
<td>pANT22</td>
<td>23.5 kbp; pKC505 with 4.8 kbp BamHI fragment from pANT21; does not confer milk protein-cleaving activity to <em>S. lividans</em></td>
<td>this work</td>
</tr>
<tr>
<td>pANT23</td>
<td>26.15 kbp; pKC505 with 7.45 kbp BamHI fragment from pANT21; confers moderate, early milk protein-hydrolyzing activity to <em>S. lividans</em>; Thio*</td>
<td>this work</td>
</tr>
<tr>
<td>pANT42*</td>
<td>8.76 kbp; pIJ702 with 3.31 kbp BamHI-SstI fragment from pANT21; stably maintained in host; confers strong, early milk protein-cleaving activity to <em>S. lividans</em></td>
<td>this work</td>
</tr>
<tr>
<td>pANT54</td>
<td>3.9 kbp; pUC19 with 1.2 kbp KpnI-SphI fragment from pANT42</td>
<td>this work</td>
</tr>
<tr>
<td>pANT55*</td>
<td>6.9 kbp; pIJ702 with 1.2 kbp SstI (from polylinker of pUC19)-SphI fragment from pANT42; confers moderate, slow milk protein-hydrolyzing activity to <em>S. lividans</em></td>
<td>this work</td>
</tr>
<tr>
<td>pANT60</td>
<td>3.79 kbp; pIJ702 with 1.1 kbp SphI-SstI fragment from pANT42; confers strong milk protein-hydrolyzing activity on <em>S. lividans</em></td>
<td>this work</td>
</tr>
</tbody>
</table>
"Table 2 (continued),"

Abbreviations: ATCC, American Type Culture Collection; BRL, Bethesda Research Laboratories; FCRC, Fredrick Cancer Research Center; HC, high copy number plasmid; LC, low copy number plasmid; Thior, thiostrepton resistance; Apr, apramycin resistance; Amp, ampicillin resistance; Mel+, production of melanin pigment; kbp, kilo-base pairs.

b Also confers strong, early milk protein-hydrolyzing activity to *S. galilaeus* 31133.

c Also confers moderate, slow milk protein-hydrolyzing activity to *S. galilaeus* 31133.
described [Lampel and Strohl, 1986]. Plasmid pANT21 (pIJ702 containing a 6.56 kbp SstI insert from *Streptomyces* sp. C5), which had earlier been isolated by Lampel [1988], was used to subclone the *snpA* gene. Plasmid pIJ702 is a high copy number *Streptomyces* vector which carries the thiostrepton resistance (*tsr*) gene from *Streptomyces azureus* as a selectable marker and the tyrosinase (*melC2*) gene from *Streptomyces antibioticus* as a nonselectable phenotypic marker [Katz *et al.*, 1983]. To localize the milk hydrolysis-conferring gene within the 6.56 kb SstI fragment, pANT21 (which contains two BamHI sites; one in pIJ702, and one in the cloned DNA) was digested with BamHI and ligated into the BamHI site of pKC505, an 18.7 kb, low-copy, *E. coli-Streptomyces* shuttle vector, to make plasmids pANT22 (23.5 kb) and pANT23 (26.15 kb) in *E. coli* DH5α (Table 2). Plasmids pANT22 and pANT23 were then each introduced by transformation into *S. lividans* protoplasts, and apramycin-resistant transformants were transferred to milk agar plates and screened for milk protein-hydrolyzing activity. *S. lividans*(pANT23) displayed milk-hydrolyzing activity on milk plates containing apramycin whereas *S. lividans*(pANT22) did not (Table 2), indicating that *snpA* was located on the 3.31 kb DNA fragment (data not shown). The milk-hydrolysis zones displayed by colonies of *S. lividans*(pANT23) on milk agar plates were smaller and took slightly longer to develop than those produced by *S. lividans*(pANT21). Nevertheless, the zones of hydrolysis were still significantly larger and were produced earlier than the background zones produced by untransformed *S. lividans* or by *S. lividans*(pIJ702). The 3.5 kb SstI-BamHI insert from pANT23 was purified by electroelution from a 1% (w/v) agarose gel. This fragment was
ligated into the BglII-SstI sites of pIJ702 to produce pANT42. Plasmid pIJ702 that had previously been linearized by digestion with BglII and SstI was treated with calf intestinal alkaline phosphatase to remove 5' phosphates in order to prevent self ligation. The ligated DNA was introduced by transformation into protoplasts of S. lividans 1326 by standard methods described previously [Lampel and Strohl, 1986]. After 16 h of growth at 30°C, the protoplasts were challenged with 30 μg of thiostrepton per ml of medium and regenerated as described previously [Lampel and Strohl, 1986]. The transformants were allowed to sporulate, typically for 3-5 days at 30°C, and then were replica-plated using sterile velvet pads to plates containing MA plus 50 μg/ml of thiostrepton (MAT plates). After 24 to 36 h of incubation, the plates were screened for colonies producing cleared zones of hydrolysis. Milk-protein-hydrolyzing transformants were picked over to fresh MAT plates to verify their proteinase phenotype. Other subclones were constructed from the original plasmid conferring milk protein-hydrolyzing activity, pANT21, and screened by similar procedures (Table 2; Fig. 5). The SphI-KpnI fragment of ca. 1.35 kb from pANT23 was subcloned in pIJ702. This was achieved by initial cloning of this fragment into the SphI and KpnI sites of plasmid pUC18. The resulting plasmid (pANT54) was then digested with SphI and SstI. The SphI-SstI fragment of ca. 1.35 kb from pANT54 was recovered by electroelution from a 1% agarose gel and ligated into pIJ702 which had been previously digested with SphI and SstI, to yield pANT55. This procedure had to be followed because the KpnI site of pIJ702 cannot be used
for cloning purposes as it lies in the region essential for replication of this vector [Hopwood et al., 1985a].

Small scale plasmid preparations from *Escherichia coli* suitable for restriction and ligation were performed as described previously [Birnboim and Doly, 1979]. Overnight cultures of *E. coli* were transformed by standard techniques as described by Maniatis et al. [1982]. The cells were incubated for 60 min at 37°C before plating to permit expression of antibiotic resistance determinants. When pKC505 [Richardson et al., 1987] or its derivatives were used, the plates were incubated at 31°C for expression of the apramycin resistance gene.

**Restriction enzyme mapping of plasmid pANT42.** Restriction endonuclease mapping of pANT42 (Fig. 6) was carried out after single and double endonuclease cleavage using standard procedures. Fragments generated by cleavage with restriction enzymes were separated by electrophoresis at 80 V through a 1% agarose gel. Electrophoresis was terminated after 45 min or after the blue marker dye (bromophenol blue) had reached approximately two-thirds of the length of the gel. The gel was then soaked in an aqueous solution of 2% (w/v) ethidium bromide for 15 min and destained by diffusion in water for 3 min. The gel which was illuminated from the bottom with a long wavelength ultraviolet light source (Fotodyne, New Berlin, WI) was photographed with a Polaroid land camera using a black and white instant film (Type 667; Sigma Chemical Co., St. Louis, MO) and a red-orange filter.
Lambda DNA that had been digested with \textit{Hind}III was used as the molecular size standard.
Plasmid pANT21, which was isolated from a milk-hydrolyzing colony of recombinant *S. lividans* 1326 was used for subcloning purposes. Plasmid pANT22 was constructed by subcloning the 4.8 kb *BamHI* fragment from pANT21 into pKC505. Plasmid pANT23 was constructed by subcloning the 7.45 kb *BamHI* fragment from pANT21 into pKC505. Plasmid pANT42 was constructed by subcloning the 3.31 kb *BamHI-SstI* fragment from pANT21 into *BglII-SstI* digested pIJ702 (the 236 bp *BglII-SstI* DNA fragment from pIJ702 was lost in the construction of pANT42). Plasmid pANT55 was constructed by subcloning the *SphI-KpnI* fragment from pANT42 into pIJ702. This was accomplished by initial subcloning of this fragment into the multiple cloning site of plasmid pUC19, to form plasmid pANT54, and subsequent recovery of a *SphI-SstI* fragment from pANT54. Plasmid pANT60 was constructed by subcloning the 1.1 kb *SphI-SstI* fragment from pANT42 into pIJ702.
Figure 5
"Figure 5 (continued)"
Figure 6. Restriction map of plasmid pANT42.

Location of the inserts in pANT55 (pIJ702 containing the 1.2 kb SstI-SphI fragment from pANT54, "snpR") and pANT60 (pIJ702 containing the 1.1 kbp SphI-SstI fragment, snpA) are shown. Plasmid pANT42 was digested with multiple combinations of restriction endonucleases (Gibco/BRL) as per manufacturers' instructions. The resulting DNA fragments were separated by gel electrophoresis through a 1% agarose gel. The size of each of the DNA fragments was determined by comparison to known standards which had been resolved in a separate lane on the same gel.
Figure 6
Design and radiolabelling of synthetic oligonucleotide fragments. A mixed synthetic oligonucleotide (5'-TTCGCC(G)ACC(G)CAGATCGC-3'; 17-mer, 2 degeneracies) was designed from the amino acid sequence of the N-terminus of the purified SnpA (AAAVTVYNA). This oligonucleotide was synthesized at the Biochemical Instrumentation Center at The Ohio State University. Two hundred pmole of this oligonucleotide were mixed with 150 μCi of γ-32P-ATP (7000 Ci/mmol, ICN Biomedicals, Costa Mesa, CA). The end-labelling reaction was initiated by addition of 20 units of T4 polynucleotide kinase (Gibco BRL, Gaithersburg, MD) and was allowed to proceed for a period of 30 min at 37°C. Unincorporated nucleotides were removed by centrifugation through a Sephadex G-50 column at 3,000 rpm for 6 min [Maniatis et al., 1982]. About 28% of the radioactive label was routinely incorporated into the synthetic oligonucleotide using this procedure.

Radiolabelling of large DNA fragments. Linear DNA fragments (larger than 500 base pairs) were isolated from 1% agarose gels as described previously. These were then labeled by the 32P-labeled random primer procedure described by Feinberg and Vogelstein [1983]. Briefly, the double stranded target DNA was boiled for 10 min in a boiling water bath to separate the DNA strands and immediately chilled on ice for 10 min to prevent reassociation of the separated strands. Fifty μCi of α-32P-dCTP (3000 Ci/mmol, NEN, Boston, MA) were then incubated with 200 pmole of the single stranded DNA fragment. After addition of 1 μg of random primers (Promega, Madison, WI), 6 units of the large fragment (Klenow) of DNA polymerase I (Gibco
BRL, Gaithersburg, MD) were added to initiate formation of the complementary strand of the single stranded target DNA. The reaction was allowed to proceed for 8-10 h at room temperature, and unincorporated nucleotides were removed by centrifugation through a Sephadex G-50 column at 3,000 rpm for 6 min [Maniatis et al., 1982]. About 45% of the radioactive label was routinely incorporated in the target DNA fragment using this procedure.

**DNA-DNA hybridizations.** For hybridization analyses, samples of chromosomal DNA or plasmid DNA, purified from the streptomycetes as described previously [Lampel and Strohl, 1986] were completely digested with restriction endonucleases according to manufacturers' directions. DNA was transferred from agarose gels to BA-85 nitrocellulose filters (0.2 μ pore size, Schleicher and Schuell, Inc., Keene, NH) by the method of Southern [1975] and fixed to the nitrocellulose by baking for 2 h under vacuum at 85°C. Filters were prehybridized at 42°C for a minimum of 4 h in a buffer composed of 5X SSC (1X SSC is 8.77 g/L NaCl; 4.41 g/L sodium citrate, pH 7.0), 50% formamide, 0.1% SDS, 5X Denhardt solution [Hopwood et al., 1985a], and 300 μg of denatured salmon sperm DNA per ml (Sigma Chemical Co., St. Louis, MO). The radioactively labelled DNA probe was added and allowed to hybridize to the DNA on the filter for a period of 10-12 h at 42°C. The filters were then washed twice at room temperature in 0.2X SSC and 0.01% SDS. The 5X SSC was substituted by 6X SSPE (1X SSPE is 8.77 g/L NaCl; 1.38 g/L sodium phosphate, monobasic; 0.373 g/L disodium EDTA, pH 7.4) in all steps when hybridizations were
performed using the end-labelled oligonucleotide. Filters containing the radioactive DNA were subjected to autoradiography using Kodak X-OMAT AR-5 X-ray film, which was exposed at -70°C for 4 to 48 h, as required, and DuPont Cronex intensifying screens were used to increase the signal.
Results

Subcloning of pANT21. Since the initial subcloning experiments involving construction of plasmids pANT22 and pANT23 indicated that the gene coding for the milk-hydrolyzing proteinase was located on the 3.31 kb BamHI-SstI fragment of pANT21, this fragment was ligated with BglII- and SstI-digested pIJ702 to construct plasmid pANT42 (Table 2; Fig. 5). Deletion of the 3.25 kb BamHI-SstI Streptomyces sp. C5 DNA fragment from pANT21 removed the instability that had been encountered during transformation experiments performed by a previous investigator [Lampel, 1988]. Thus, pANT42 could be stably maintained in and conferred strong milk-hydrolyzing activity on S. lividans and S. galilaeus 31133. Further subcloning of the ca. 1.1 kb SphI-SstI DNA fragment of pANT42 into SphI- and SstI-digested pIJ702 resulted in pANT60 (Table 2; Fig. 5), which also conferred stable, strong milk-hydrolyzing activity on S. lividans 1326. The 1.2 kb SstI-SphI fragment located immediately upstream of the Streptomyces sp. C5 DNA in pANT60 was subcloned from pANT54 (Table 2) into pIJ702 to make pANT55. Interestingly, this plasmid also conferred a milk hydrolysis activity on S. lividans, although this activity was never as strong as nor observed as early as that produced by cultures of S. lividans(pANT42) or S. lividans(pANT60). The difference in the size and timing of milk hydrolysis zones produced by S. lividans(pANT21) versus S. lividans(pANT23), which led to initial localization of the snpA gene, is probably due to a gene dosage (copy number) effect.
Localization of the \textit{snpA} gene in pANT42. Unique restriction enzyme cleavage sites within the insert DNA in plasmid pANT42 were determined by digesting pANT42 with multiple restriction enzymes (Fig. 6). Data obtained after probing with the N-terminus specific end-labelled synthetic oligonucleotide indicated that the \textit{snpA} gene in pANT42 is localized within the \textit{PvuII-SphI} fragment of ca. 1.1 kb (Fig. 7).

Hybridization of insert DNA to DNA from other streptomycetes. Once the sequence of the \textit{snpA} gene was determined [Lampel \textit{et al.}, 1992], a Southern blot experiment was performed using the \textit{SphI-PvuII} fragment internal to the \textit{CS-snpA} gene as a probe to determine if homologues of \textit{snpA} were present in other streptomycetes. Using prehybridization and hybridization conditions of 50% formamide, 5X SSC, and 0.1% SDS and 42°C, followed by two successive washes at 65°C with 0.2X SSC, 0.01% SDS, no hybridization of the \textit{C5-snpA} probe to DNA isolated from \textit{S. peucetius} ATCC 29050, \textit{S. galilaeus} ATCC 31133, \textit{S. coelicolor} A3(2), or \textit{S. griseus} IMRU 3499 was observed, although a ca. 6.6 kb \textit{SstI} fragment from \textit{Streptomyces} sp. C5 hybridized to the probe (Fig. 8). However, a single faint band indicative of DNA fragments homologous to \textit{C5-snpA} in \textit{S. lividans} 1326 was observed on the autoradiograph [data not shown]. Thus, DNA from \textit{S. lividans} 1326 was hybridized with the probe and washed at lowered stringencies until the bands were sufficiently visible for photography (Fig. 9).
Figure 7. Southern hybridization of the $\gamma^{\text{32P}}$ dATP end-labelled synthetic oligonucleotide probe with pANT42 that had been digested with $Pvu$II and with $Pvu$II-$Sph$I and resolved by gel electrophoresis through a 1% agarose gel. The intensely hybridizing bands in lanes 1 and 2 (duplicate gel loadings) represent the 0.6 kb $Pvu$II-$Sph$I fragment from pANT42 (Fig. 6) while the intensely hybridizing bands in lanes 3 and 4 (duplicate gel loadings) represent the 1.1 kb $Pvu$II fragment from pANT42 (Fig. 6). The hybridization was carried out in 6X SSPE buffer at 42°C and as described in the methods section.
Figure 8. Southern hybridization of the $\alpha^{-\text{32P}}$ dCTP random-labelled 624 bp $SphI$-$PvuII$ DNA fragment of pANT42 from C5-$snpA$ to restriction endonuclease digested DNA from various streptomycetes. Approximately 30 $\mu$g of the chromosomal DNA from each of the streptomycete tested was digested with 1 unit/$\mu$g DNA of $SstI$ for 4 h at $37^\circ$C before resolving the DNA fragments by gel electrophoresis through a 1% agarose gel. Lane 1: lambda-DNA digested with $HindIII$; Lane 2: pIJ702 digested with $SstI$; Lane 3: pANT42 (ca. 2 $\mu$g) digested with $SphI$-$PvuII$; Lane 4: pANT60 (ca. 2 $\mu$g) digested with $SphI$-$PvuII$; Lane 5: Streptomyces C5; Lane 6: S. lividans 1326; Lane 7: S. coelicolor A(3)2; Lane 8: S. griseus; Lane 9: S. galilaeus ATCC 31133; Lane 10: S. peucetius.

A ca. 6.6 kb DNA fragment from Streptomyces C5 to which the radiolabelled probe could hybridize is indicated by an arrow. Note the absence of hybridization of this probe to the chromosomal DNA isolated from other streptomycetes while hybridizing strongly to the ca. 0.6 kb fragment from both pANT42 and pANT60.
Activity of the sequences upstream of snpA. The SphI-KpnI fragment of ca. 1.35 kb from pANT21 was subcloned in pIJ702 to generate plasmid pANT55 (Fig. 5), which was then used to transform S. lividans 1326 by standard procedures [Hopwood et al., 1985a]. The resulting transformant, S. lividans(pANT55), showed weak zones of hydrolysis on MA plates after ca. 22 h or more of growth. The zones of hydrolysis on MA plates were significantly larger than untransformed S. lividans or S. lividans transformed with pIJ702 (data not shown), but smaller than those produced by S. lividans(pANT42) or S. lividans(pANT60) (Fig. 10).
Figure 9. Southern hybridization of the $\alpha-^{32}$P dCTP random-labeled 624 bp SphI-PvuII DNA fragment, of pANT42, from C5-snpA to restriction endonuclease digested DNA from *Streptomyces* sp. C5 and *S. lividans* 1326. Lanes: 1, pIJ702 (ca. 2 $\mu$g) digested with SstI; 2, pANT42 (ca. 2 $\mu$g) digested with SphI and PvuII (to give a hybridizing fragment the same size as the probe); 3, 5, 7, *Streptomyces* sp. C5 DNA (ca. 15 $\mu$g) digested with BamHI, XhoI, and SstI, respectively; 4, 6, 8, *S. lividans* 1326 (ca. $\mu$g) DNA digested with BamHI, XhoI, and SstI, respectively. The DNA fragments to which arrows are drawn are given in kilobase-pairs (kbp). The hybridizations were carried out in 5X SSC at 42°C and as described in the methods section. The nitrocellulose membrane was washed twice at room temperature in 2X SSC containing 0.1% SDS. Each wash was carried out for a period of 10 min.
Figure 9
Figure 10. Comparison of the milk hydrolysis activities of various recombinant streptomycetes harboring the plasmid containing the C5-snpA gene (pANT42) and the C5-snpR gene (pANT55). These streptomycetes were grown on a MAT plate. (A): *S. lividans*(pANT42); (B): *S. lividans*(pANT55); (C): *S. galilaeus*(pANT42); (D): *S. galilaeus*(pANT55); (E): *S. galilaeus*(pANT60). The dark area around the *S. galilaeus* colonies is due to production of a colored pigment. Areas of milk hydrolysis on the milk agar plate can be observed as clear zones around the colonies against a white background.
Figure 10

S. lividans pANT55

S. galilaeus pANT55

S. galilaeus pANT60

S. lividans pANT42

S. lividans pANT60
C) Purification, biochemical characterization and preliminary studies in the physiological regulation of the *Streptomyces* C5 proteinase SnpA from *Streptomyces lividans* (pANT42).

Materials and Methods

**Bacterial strain, maintenance, and cultivation conditions.** The cultures used in this study include *Streptomyces* sp. C5, *Streptomyces lividans* 1326, and *Streptomyces galilaeus* ATCC 31133, which are described in the previous chapter (Table 2). *S. lividans* 1326(pANT42) and *S. galilaeus* (pANT42), which are the recombinant strains carrying the neutral proteinase gene from *Streptomyces* sp. C5, were used for the purification studies. These strains were cultivated in media which have been described in the previous chapter. Media were substituted with 50 μg/ml of thiostrepton when recombinant strains were being used.

**Chemicals.** Reagent grade acetone was from Mallinckrodt. Sephadex G-75, CM-Sepharose, proteinase substrates and inhibitors, buffers, salts, the electrophoresis calibration kit and sodium dodecyl sulfate were from Sigma Chemical Co. Acrylamide and *bis*-acrylamide were obtained from the Fisher Scientific Co.
Shake flask and fermentation cultures. For shake-flask cultures, spores from an entire plate of MAT medium were aseptically scraped off with a sterile nichrome wire loop and used to inoculate a 250 mL flask containing 50 ml of MB. The liquid medium was supplemented with 15 μg/ml of thiostrepton (MBT; MAT lacking agar) and a coiled spring was included to facilitate mycelial dispersion [Dekleva et al., 1985]. After incubation for 24 h at 30°C with rotary shaking (250 rpm), the entire culture (primary seed culture) was used to inoculate a 2-liter flask containing 950 ml of MBT (ca. 5% inoculum, v/v) and a coiled spring. After further incubation for 36 h, the entire amount of the secondary seed was inoculated into 9 L of MBT (ca. 10% inoculum, v/v) contained in a 14 L glass fermentor (New Brunswick Scientific). Five ml of undiluted antifoam (Mazu DF-60P; Mazer Chemical Co., Gurnee, IL) were added to the medium prior to autoclaving to prevent foaming. Agitation in the fermentor was due to a triple bladed stainless steel impeller rotating at 400 rpm. The air flow through the medium was maintained at 1 vol of air per vol of medium per min (vvm) using in-house pressurized air (15 psi). The air was first passed through a presterilized glass wool filter cartridge (3 cm x 15 cm) to maintain medium sterility. The fermentation medium temperature was maintained at 30°C by utilization of a water jacket internal to the fermentation vessel. The pH of the medium was adjusted to 7.2 with 5 N sodium hydroxide prior to autoclaving. The pH was monitored but not controlled once the fermentation was in progress. Fermentation was allowed to proceed for a period of 26 to 28 h. Fifty ml of the fermentation broth were
aseptically collected at predetermined intervals into sterile 50 ml plastic tubes (114 mm x 29 mm, Sarstedt).

The growth of fermentation cultures of streptomycetes was determined by measuring the dry weight of the streptomycete mycelium as previously described [Dekleva et al., 1985]. Mycelia from 50 ml of the sample collected at each time point in the course of fermentation were pelleted at 16,000 x g for 10 min at 4°C using a Sorvall RC2-B centrifuge. The supernatant was discarded and the mycelia were rinsed once with sterile physiological saline solution and resuspended in 5 ml of the same solution. Resuspended mycelia were poured into preweighed aluminium pans and put in a dry air oven to dry at 90°C-100°C. After 24 h, pans were removed from the oven, cooled under vacuum in a desiccator and reweighed. The difference in weights was recorded as the mycelial dry weight. A blank of sterile medium was included as control. The pans were handled with a clean pair of forceps at all times during this procedure. Aliquots from periodic samples were checked for purity by plating onto MAT plates and also by observation under a phase contrast microscope (Spencer, PhaseStar) at 1000x magnification.

**Milk hydrolysis assay and protein determination.** The milk hydrolysis activity of the proteinase encoded by *snpA* was determined in a spectrophotometric assay using 750 μl of 50 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer (pH 7.0) and 200 μl of an autoclaved solution containing 20 mg/ml of nonfat dry milk (Carnation Co., Los Angeles, CA) in the same buffer. The substrate solution and the
buffer were premixed in a cuvette. To start the reaction, 50 μl of the enzyme preparation were added to the cuvette containing the substrate-buffer solution to give a final volume of 1000 μl. The decrease in absorbance at 580 nm was continuously monitored for 2 to 5 min using a dual beam spectrophotometer (Bausch and Lomb Spectronic 2000). One unit of milk-hydrolyzing enzyme activity is defined as the activity required to decrease the absorbance of the milk solution at 580 nm by 0.001 A/min.

In preliminary experiments it was necessary to determine if the activity was due to combined activities of multiple proteinases (including potential serine proteinases). Thus, the enzyme solution was pretreated with 3 mM diisopropylfluorophosphate (DIFP) for 60 min at ambient temperature prior to assaying for the milk hydrolysis activity. Since the proteinase encoded by snpA is not sensitive to DIFP, this inhibitor was routinely added to assay mixtures.

Protein was determined by the method described by Bradford [1976] using a commercial protein assay reagent (Bio-Rad, Richmond, CA) and bovine serum albumin as the standard.

**Purification of the milk-hydrolyzing proteinase from *S. lividans*(pANT42).** All protein purification steps were carried out at 4°C unless otherwise stated. Mycelia were harvested after 24 h of fermentation by cross flow filtration using a Millipore Pellicon cassette system equipped with 0.45 μm membranes (2 ft² of membrane area). The mycelium-free broth was chilled to 4°C and was concentrated using the Millipore
filtration unit equipped with a 10,000 molecular weight cutoff membrane (5 ft² of membrane area). The retentate, which contained all of the milk-hydrolyzing proteinase activity, was used for further purification and was stored without any further treatment at -20°C when not in use. Further fractionation of enzyme activity was achieved by gently adding acetone, chilled to -70°C, to the enzyme preparation and stirring slowly. The enzyme preparation was held in an ethanol:ice bath during the acetone fractionation procedures. Acetone was added to a final concentration of 55% v/v and the resulting precipitate was recovered by centrifugation for 20 min at 16,000 x g. The supernatant from this step, which contained less than 1% of the milk-hydrolyzing activity, was discarded. After removal of residual acetone from the pellet under a vacuum, the precipitate was resuspended in a minimal volume of 50 mM HEPES buffer (pH 6.5), placed into dialysis tubing (Sigma, average flat width of 35 mm), and dialyzed for 24 h against a 1000-fold volume of the same buffer. The dialyzed enzyme solution was applied to a carboxymethyl-Sepharose (CM-Sepharose) column (2.5 cm x 10 cm) equilibrated with the same buffer. The flow rate was adjusted to 150 ml/h and 5 ml fractions were collected. Active fractions were pooled and lyophilized using a Savant vacuum centrifuge equipped with a refrigerated condensation trap. The dry powder from the pooled active fractions was resuspended in 1.5 ml of 50 mM HEPES (pH 7.0) and applied to a Sephadex G-75 column (1 cm x 90 cm) which had previously been equilibrated with the same buffer. The flow rate was adjusted to 6 ml/h, and 2 ml fractions were collected. Fractions containing milk-hydrolyzing activity were pooled, lyophilized as described above, and resuspended in
1 ml of 50 mM HEPES (pH 7.0). The size of the purified, non-denatured proteinase was analyzed by gel filtration on a TSK-G2000XL HPLC column using an elution buffer containing 50 mM HEPES (pH 7.0) plus 50 mM NaCl. The approximate size of the purified undenatured protein was determined by linear regression of the purified proteinase in comparison to known size standards. Relative molecular mass standards used were (Mr given in parentheses): bovine serum albumin (66,000), rabbit glyceraldehyde 3-phosphate dehydrogenase (36,000), bovine carbonic anhydrase (29,000), and bovine trypsinogen (24,000).

**Purification of the milk-hydrolyzing proteinase from S. galilaeus(pANT42).** Milk hydrolyzing proteinase, SnpA, was purified from the culture broth of *S. galilaeus*(pANT42) by a scaled down procedure. Cells from 50 ml of the culture broth were harvested by centrifugation (16,000 × g, 10 min, 4°C) after 28 h of growth. Cell free broth was concentrated by ultrafiltration through a low adsorption, hydrophilic YM membrane (Centriprep, Amicon, Danvers, MA). The remainder of the purification protocol was as described above.

**Electrophoresis procedures.** Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% (w/v) resolving gel and a 3% (w/v) stacking gel as described by Laemmli [1970]. The final concentration of SDS in both gels was 0.1% (w/v). Samples were prepared by mixing 5 to 10 μg of protein in a sample buffer containing 125 mM Tris-HCl (pH 6.8), 5%
(v/v) β-mercaptoethanol, 10% (w/v) glycerol, 0.1% (w/v) SDS, and a few crystals of bromphenol blue. A constant amperage of 20 mA was applied until the dye front had reached the bottom edge of the gel. For routine screening of proteins and for the α- and β-casein degradation assays, the gels were stained with a solution containing 50% (v/v) methanol, 7.5% (v/v) acetic acid, and 0.2% (w/v) coomassie brilliant blue R, and then destained by diffusion into a solution containing 50% (v/v) methanol and 7.5% (v/v) acetic acid. For the assay of purified proteinases, the proteins in the SDS-PAGE gels were detected using silver nitrate as described by Merrill et al. [1983]. The Mr of the denatured proteinase was determined by linear regression in comparison to known size standards after separation by SDS-PAGE. The standards used were (Mr, given in parentheses): bovine albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (subunit; 36,000), carbonic anhydrase (29,000), bovine trypsinogen (24,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

Substrate specificity of the recombinant proteinase. The purified milk-hydrolyzing enzyme was tested for its ability to cleave several synthetic amino acyl p-nitroanilides: N-succinyl-alanyl-alanyl-prolyl-phenylalanyl p-nitroanilide (SAAPF-pNA), a substrate for chymotrypsin-like proteinases [Delmar et al., 1979]; N-α-benzoyl-L-arginine p-nitroanilide (BAPNA), a substrate for trypsin-like proteinases [Erlanger et al., 1961]; benzyloxycarbonyl-glycyl-glycyl-leucyl p-nitroanilide (CBZ-GGL-pNA), a substrate for subtilisin-like proteinases [Lyublinskaya et al., 1974]; and L-leucyl p-
nitroanilide (Leu-pNA), a substrate for leucine aminopeptidases [Aretz et al., 1989]. Stock solutions of 5.0 mM were used for each synthetic substrate to yield a final concentration in the assay of 0.25 mM except for L-leu-pNA which was used at a final concentration of 2.5 mM. The BAPNA and CBZ-GGL-pNA were dissolved in dimethylsulfoxide, SAAPF-pNA was dissolved in 100 mM HEPES buffer (pH 7.0) and L-leu-pNA was dissolved in 95% ethanol. The reactions were carried out in 100 mM HEPES (pH 7.0) in a final volume of 1.0 ml. The absorbance of the p-nitroaniline released by hydrolysis of all of the amino acyl-p-nitroanilides was measured continuously at 410 nm using a spectrophotometer (Bausch and Lomb Spectronic 2000). The molar extinction coefficient of p-nitroaniline in this buffer is 8480 M⁻¹·cm⁻¹ [Vinci, 1988]. Additional assays were carried out as above but at a pH of 8.0, and similar assays were carried out in the presence or absence of 10 mM CaCl₂. Thermolysin-like neutral proteinase activity was measured by the hydrolysis of N-(3-{2-furyl}acryloyl)-gly-leu amide (FAGLA) as described by Feder [1968], and esterase activity was assayed by the hydrolysis of Nα-p-tosyl-L-arginine methyl ester (TAME), as described by Roberts and Elmore [1974]. The enzymatic hydrolysis of the specific collagenase substrates, N-(3-{2-furyl}acryloyl)-leucyl-glycyl-prolyl-alanine (FALGPA) and 4-phenylazobenzyloxy-carbonyl-prolyl-leucyl-glycyl-prolyl-D-arginine, were measured as described by Van Wart and Steinbrink [1981] and Wünsch and Heidrich [1963], respectively. The enzymatic hydrolysis of azocasein was assayed as described previously [Gibb et al., 1987, Gibb and Strohl, 1988], and the hydrolysis of azocoll was carried out essentially as described by Rufo et al. [1990].
Casein as substrate of the milk-hydrolyzing proteinase. The cleavage of α- or β-casein was determined by incubating purified enzyme (5 μg) with a solution containing 25 μg of the protein substrate in distilled water. The reactions were allowed to incubate for time intervals ranging from 5 to 60 sec and were stopped by the addition of SDS-PAGE sample buffer, followed by immediate immersion of the solution in a boiling water bath for 3 min. The cleavage products were separated by SDS-PAGE as described above. The proteinase inhibitors 1,10-phenanthroline (10 mM) or phenylmethylsulfonylfluoride (PMSF; 10 mM) were included in certain of these assays to determine the ability of these compounds to inhibit α- and β-casein hydrolysis. The major cleavage products of β-casein were subjected to N-terminal amino acid analysis as described below.

N-terminal amino acid analysis of the major cleavage products of β-casein. After electrophoresis, coomassie-blue staining and destaining, the gel was soaked in cold transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, pH 11.0) for 15 min. During this time a polyvinylidene difluoride membrane (PVDF; Immobilon P, Millipore, Inc., Boston, MA) was rinsed briefly in 100% methanol, soaked in deionized water for 5 min and stored in transfer buffer. The gel, which was sandwiched between the PVDF membrane and several sheets of blotting paper (Whatman 3mm), was assembled into a blotting apparatus (Trans-Blot, Bio-Rad). Protein transfer was carried out at 4°C at a constant voltage of 90 V (200 mA) for 150 min with the PVDF membrane towards the cathode. The PVDF membrane was
then rinsed in deionized water for 5-10 min, air dried, and stored at -20°C. The electro-blotted peptide was subjected to an N-terminal sequence analysis on an Applied Biosystems Model 470A protein sequencer equipped with an on-line 120A phenylthiohydantoin (PTH) analyzer and a 900A control/data analysis module.

**Effect of inhibitors and influence of metal ions.** The following proteinase inhibitors [Salvesen and Nagase, 1989] were assessed for their ability to inhibit the milk-hydrolysis activity of the purified enzyme: diisopropyl fluorophosphate (DIFP); ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis (β-amino ethyl ester) N,N,N',N'-tetraacetic acid (EGTA), 1,10-phenanthroline (PHEN); N-tosyl-L-phenylalanine chloromethyl ketone (TPCK); N-p-tosyl-L-lysine chloromethyl ketone (TLCK); N-(carbobenzoxy)-Pro-Leu-Gly hydroxymate (CBZ-PLG-hydroxymate) [Moore and Spilburg, 1986]; aprotinin; bestatin; phosphoramidon [Holmquist, 1977; Komiyama et al., 1975]; pepstatin; dithiothreitol (DTT); iodoacetate; and HgCl₂. All inhibitors were tested in the range of 1-10 mM concentration against the milk-hydrolyzing activity of the purified proteinase. Additionally, some of the inhibitors were also tested for inhibition of the azocoll-hydrolysis activity of the purified proteinase. For these assays, the enzyme was preincubated with the inhibitor for 1 h at ambient temperature before residual enzyme activity was measured. Controls included incubation of the enzyme in an equal volume of buffer for the same period and incubation of the enzyme in solvents in which the inhibitors were dissolved. Additionally, various cations were added to
the purified enzyme to determine if they could counteract the effects of certain inhibitors on enzyme activity, and the ability of these cations to restore activity to previously inactivated enzyme was also determined. Inactivated enzyme was incubated with each cation preparation for 15 min at ambient temperature before measuring residual activity.

**Metal ion determination.** The purified protein was dialyzed at 4°C for 24 h against a 1000-fold vol of double distilled water with two changes of the water before performing metal ion determinations. The presence of heavy metal associated with the proteinase was determined by energy-dispersive X-ray fluorescence spectrometry (EDXFS) using a SpecTrace 4050 spectrometer (Tracor X-ray, Inc., Mountainview, CA).

**Quantification of the metal ligand of SnpA.** Zinc, determined by EDXFS to be the major heavy metal in the pure proteinase, was quantified in a preparation of extensively-dialyzed, pure proteinase by atomic absorption spectrometry at 213.8 nm using a Perkin-Elmer (Norwalk, CT) Model 403 atomic absorption spectrometer. The content of zinc per molecule of dialyzed proteinase was calculated by dividing the moles of zinc in the purified proteinase preparation by the moles of proteinase, based on a M, of 15,500 for the enzyme.
Effect of temperature and pH on enzyme activity. The purified enzyme was assayed at various temperatures using a temperature controlled cuvette holder attached to a Haake recirculating water bath. The temperature inside the cuvette was measured using a temperature microprobe (YSI model 42SC tele-thermometer) to determine the actual reaction temperature. The thermostability of the enzyme was determined by incubating enzyme at appropriate temperatures for periods of time, during which samples were assayed for residual enzyme activity. The effect of pH on enzyme activity was determined by performing the standard milk-hydrolysis assay in 100 mM HEPES buffer at various pH values between 6.5 and 8.5.

N-terminal sequencing of the purified neutral proteinase. Ten picomoles of purified neutral proteinase were adsorbed onto a pre-wetted PVDF membrane as described above and the N-terminal amino acid sequence was determined by Edman degradation using an automated gas-phase protein sequencer (Model 470A; Applied Biosystems, Inc., Foster City, CA) and an on-line PTH analyzer (Model 120A; Applied Biosystems, Inc.).

Amino acid composition of the purified neutral proteinase. The pure proteinase was subjected to SDS-PAGE and then was transferred onto a PVDF membrane by the method described above. The amino acid composition of the protein was determined by the Pico-Tag amino acid analysis system (Millipore Corp., Milford, MA). A parallel amino acid analysis was performed on performic acid-treated protein in order
to determine cysteine content by standard methods [Cohen et al., 1989]. Tryptophan was determined separately after hydrolysis of an identical sample of the proteinase with 4 M methanesulfonic acid [Cohen et al., 1989].

Physiological regulation of *snpA* gene expression in *S. lividans*(pANT42).

**A) Growth media and shake flask cultivation of *S. lividans*(pANT42).** *S. lividans*(pANT42) was maintained on R2YE medium containing 50 μg/ml thiostrepton. The following media (each designated by a capital letter) were used: 

- **T**, pancreatic digest of casein, 17 g/L; papaic digest of soybean meal, 3 g/L; sodium chloride, 5 g/L; dipotassium phosphate, 2.5 g/L; dextrose, 2.5 g/L; pH 7.2;  
- **G**, Modified T medium - (pancreatic digest of casein, 17 g/L; dextrose, 8.5 g/L; sodium chloride, 5 g/L; papaic digest of soybean meal, 3 g/L; dipotassium phosphate, 2.5 g/L; yeast extract, 2 g/L; pH 7.2);  
- **M**, minimal base medium with nutrient broth and milk (MNBM) - (powdered milk, 20 g/L; nutrient broth, 1 g/L; calcium chloride dihydrate, 0.74 g/L; potassium phosphate dibasic trihydrate, 0.6 g/L; potassium phosphate monobasic, 0.5 g/L; fructose, 0.05 g/L; pH 7.2. The milk was autoclaved in minimal volume of water and added separately to the rest of the medium after cooling);  
- **J**, Modified MNBM - powdered milk, 20 g/L; yeast extract, 1 g/L; sodium nitrate, 0.86 g/L; potassium phosphate dibasic trihydrate, 0.6 g/L; fructose, 0.05 g/L; 10X trace metal solution, 2 ml/L; pH 7.2); and  
- **K**, similar to J medium with the exception that milk was omitted from this preparation.
Spores from a R2YE plate were harvested by a sterile platinum wire loop and inoculated aseptically into 50 ml of each of the sterile T, G, M, J and K media. Mycelia were grown by agitation of the flasks for 42 h on a rotary shaker at 250 rpm and 30°C. Measurement of mycelial dry weight, extracellular proteinase assay and determination of extracellular protein was carried out as described in the previous section.

B) Isolation and purification of RNA from S. lividans (pANT42). Ten milliliter aliquots from each broth sample obtained at pre-determined time intervals from a 10 L fermentation culture were used for isolation of RNA. The cells were washed twice with sterile 10% (v/v) glycerol before proceeding with the RNA isolation protocol. Total RNA was isolated from the washed cells using standard protocols [Hopwood et al., 1985a], and the solution of purified RNA in diethyl pyrocarbonate (DEPC)-treated double distilled water was stored as an approximately 50% (v/v) suspension in isopropanol at -70°C.

C) RNA slot blot procedures. Ten micrograms of total RNA from each of the samples were adsorbed by vacuum suction onto a prewetted nitrocellulose membrane (0.45 μm, Bio-Rad) using the Bio-Dot SF Microfiltration apparatus (Bio-Rad, Richmond, CA) and by following instructions provided. Each slot of the apparatus was rinsed under vacuum with 500 μl of 6X SSPE before disassembling the filtration apparatus. The nitrocellulose membrane was allowed to air-dry for 30 min and was
stored at ambient temperature, between two clean sheets of Whatmann 3M filter paper, prior to use in hybridization experiments.

D) RNA-DNA hybridization. Pre-hybridization and hybridization of the RNA on the nitrocellulose membrane with the end labelled synthetic oligonucleotide probe was carried out at 42°C in 6X SSPE buffer. The protocol followed was essentially similar to that used previously for DNA-DNA hybridizations.
Results

Expression of snpA in fermentation cultures of S. lividans(pANT42). As described in the previous chapter, Streptomyces lividans 1326 did not contain significant proteolytic activity for the hydrolysis of milk (Table 2). On plates of MA medium, minimal zones of hydrolysis around colonies of S. lividans were observed, but only after 5 to 7 days of incubation. Also, fermentation cultures of S. lividans 1326 in MB medium reproducibly did not produce any significant milk-cleaving proteolytic activity over a period of 120 h (data not shown). Once the snpA gene was cloned in S. lividans as pANT42, the activity of the milk-hydrolyzing proteinase was measured as a function of time in fermentation cultures of the recombinant strain without apparent interference from other proteinases. When cultures of S. lividans(pANT42) were grown in stirred tank fermentors, the milk-hydrolyzing proteinase was expressed during exponential growth of the culture with optimal activity at ca. 16 to 20 h (approximately mid-exponential phase) of culture age (Fig. 11). This result was expected since transfer of S. lividans(pANT21), S. lividans(pANT42), or S. lividans(pANT60) mycelia to fresh MAT plates results in rapid formation of hydrolysis zones (within 4 to 8 h; Table 2). A L-leucine aminopeptidase activity (measured at pH 8.0; sensitive to EDTA but not to DIFP) also was produced by S. lividans 1326, whether it was untransformed or transformed with pANT42. This L-leucine aminopeptidase activity, which has been noted previously [Aretz et al., 1989], was not significant until after the culture had entered stationary phase (Fig. 11).
Figure 11. Fermentation profile of *S. lividans*(pANT42) grown in a MB medium. The dry weight of the mycelium (●), the activity of recombinant milk-hydrolyzing enzyme (▲) during exponential growth phase, and the activity of the host L-leucine aminopeptidase (■) occurring in stationary phase are shown. The milk hydrolysis activity was measured as described in the methods section, except that the cell-free culture broth samples were treated for 15 min with 3 mM DIFP prior to the assay to ensure that no serine proteinase activity contributed to the milk-hydrolysis activity measured. The L-leucine aminopeptidase activity was measured by release of p-nitroaniline from L-Leu-pNA.
Figure 11

Dry wt (g/L)

Protease Activity

Time (Hours)
There was no difficulty in separating these proteinase activities, as the milk-hydrolyzing enzyme encoded by *snpA* does not cleave Leu-pNA, and the L-leucine aminopeptidase of *S. lividans* does not have the ability to hydrolyze milk protein.

**Purification of neutral proteinase from *S. lividans*(pANT42) and *S. galilaeus*(pANT42).** The milk-hydrolyzing enzyme encoded by *snpA* was purified from a 24 h fermentation culture of *S. lividans*(pANT42). The spectrophotometric milk hydrolysis assay was used to quantify the activity of the neutral proteinase, since, at the time of purification, the enzyme did not hydrolyze any of the other substrates tested. All of the milk-hydrolyzing activity was in the culture filtrate. Two "purification" steps were used to concentrate this proteinase activity, ultrafiltration through a 10,000 MW cut-off membrane and acetone precipitation. Essentially all of the milk-hydrolyzing proteinase activity from the 0.45 \( \mu \text{m} \) cut-off fraction was retained by the 10,000 MW cut-off membrane (Table 3). After ultrafiltration, some of the milk degrading activity was observed in the filtrate. This activity was assumed to be due to leakage of the active enzyme (15,500 M\(_r\)) during filtration through the 10,000 MW cutoff membrane. Although a 5-fold and 7-fold loss in yield was obtained for the filtration and acetone-precipitation steps, respectively, the enzyme was concentrated about 12-fold in these steps to volumes that could be used for chromatographic separations. Attempts to use alternative methods for concentration of the enzyme, including precipitation with either ammonium sulfate or ethanol, resulted in lower yields than shown in Table 3. The acetone-precipitated protein was
Table 3. Scheme for the purification of *Streptomyces* sp. C5 milk-hydrolyzing proteinase from fermentation culture broths of *S. lividans* (pANT42).

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (units*)</th>
<th>S. A.⁵</th>
<th>P. F.⁶</th>
<th>% Rec.⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell free broth</td>
<td>5000</td>
<td>1910</td>
<td>2.13 x 10⁶</td>
<td>115</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Filtrate (&gt;10K)</td>
<td>750</td>
<td>375</td>
<td>4.50 x 10⁵</td>
<td>1200</td>
<td>1.1</td>
<td>21</td>
</tr>
<tr>
<td>70% acetone</td>
<td>40</td>
<td>88</td>
<td>5.87 x 10⁴</td>
<td>667</td>
<td>0.6</td>
<td>2.7</td>
</tr>
<tr>
<td>Dialysis</td>
<td>33</td>
<td>59</td>
<td>4.60 x 10⁴</td>
<td>780</td>
<td>0.7</td>
<td>2.0</td>
</tr>
<tr>
<td>CM-Sepharose</td>
<td>4</td>
<td>4.7</td>
<td>8.64 x 10³</td>
<td>1846</td>
<td>1.7</td>
<td>0.4</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>3.6</td>
<td>1.3</td>
<td>5.09 x 10³</td>
<td>5085</td>
<td>4.6</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*One unit of activity is defined as the decrease in absorbance at 580 nm of 0.001/min.

⁵S. A.: Specific activity

⁶P. F.: Purification factor.

⁷% Rec.: Percent recovery.
dialyzed against a 1000-fold volume of buffer, which slightly increased the specific activity of the proteinase, and was applied to a CM-Sepharose column. The milk-hydrolyzing enzyme was eluted in the "flow-through" volume of the CM-Sepharose ion exchange step, which afforded a ca. 2.4-fold purification over the previous step. Most of the contaminating proteins remained on this column, providing a reasonable purification of the enzyme (Table 3). Moreover, this step also removed an uncharacterized brown pigment which partially interfered with other purification steps attempted during early stages of this project. No milk-hydrolyzing activity was eluted from the column after a 0 to 1 M NaCl gradient was applied, indicating that all of the milk-hydrolyzing activity was due to the single proteinase which eluted in the "flow-through" fractions. The active "flow-through" fractions from the CM-Sepharose column were pooled, lyophilized, and applied to a Sephadex G-75 column. The milk-hydrolyzing enzyme was eluted from the Sephadex G-75 column in a single, sharp peak which yielded a pure protein as demonstrated by SDS-PAGE analysis (Fig. 12A). The final specific activity indicated that the pure protein was about 5-fold purified, but with only a 0.3% yield (Table 3). Using a scaled-down procedure of the one described above, the milk-hydrolyzing proteinase was also purified to electrophoretic homogeneity from shake-flask cultures of *S. galilaeus* ATCC 31133(pANT42) as shown in Figure 12B. *S. galilaeus* ATCC 31133 was used as an alternative host for expression of the proteinase gene because it also did not produce a milk-protein degrading activity. *S. galilaeus*(pANT42) was not chosen as the primary host due to accumulation of significant levels of a dark brown pigment in the
Figure 12. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) of SnpA.

(A). Lane 1 - Low molecular weight standards. The standards are (M_r given in parentheses): bovine albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (subunit; 36,000), carbonic anhydrase (29,000), bovine trypsinogen (24,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). Lane 2 - Proteinase, encoded by *Streptomyces* sp. C5 *snpA* gene, purified from a 10-liter culture broth of *S. lividans* 1326(pANT42). M_r of the purified denatured proteinase was estimated by linear regression calculation.

(B). Lane 1 - Protein standards as described above, and Lane 2 - proteinase purified from 24 h shake flask culture of *S. galilaeus* 31133(pANT42). The proteins in both A and B were stained with silver nitrate.
fermentation broth during growth. Such pigments are known to affect protein purification procedures in an adverse way [Gibb, 1987].

**Physical properties of the milk-hydrolyzing proteinase.** The purified proteinase from cultures of either *S. lividans*(pANT42) or *S. galilaeus*(pANT42) was a monomeric protein with a Mr of ca. 15,500 as demonstrated by SDS-PAGE (Fig. 12) and 16,000 by gel filtration using a TSK-G2000XL HPLC column (Fig. 13). The optimum pH for activity of the proteinase is 7.0, with approximately half the amount of activity still present at pH 8.0 (Fig. 14). The optimal temperature for maximal activity of the neutral proteinase was 55°C, with 50%, 73%, and 89% of optimal activity obtained at 24°C, 37°C, and 44°C, respectively (Fig. 15). The activity also dropped off very quickly above 55°C; only ca. 88% and 52% of optimal activity were obtained after incubations at 60°C and 65°C, respectively (Fig. 15). The activity of the cloned milk-hydrolyzing proteinase was completely lost after incubation at 80°C for 30 min.

**N-terminal amino acid sequence of SnpA.** The N-terminal amino acid sequence of SnpA, purified from the culture broth of *S. lividans*(pANT42), was determined to be AAVTVVYNASGAPSFATQIA.

**Amino acid content of purified proteinase.** The amino acid content of the purified proteinase was determined after acid hydrolysis (Table 4). Based on the analysis of all of the amino acids and a Mr of 15,500 as described above, the neutral proteinase
Figure 13. HPLC gel filtration chromatography of SnpA. Calculation of the approximate apparent molecular mass of non-denatured C5-SnpA using an HPLC TSK-G2000XL column. The column chromatography was performed at ambient temperature and the flow rate of the buffer (50 mM HEPES, pH 7.0 plus 50 mM NaCl) was adjusted to 0.5 ml/min. The proteins eluting from the column were analyzed by their absorption at 280 nm. The peak determined to signify C5-SnpA was confirmed by the ability of the protein in those fractions to hydrolyze milk. Relative molecular mass standards used were (molecular weight given in parentheses): bovine serum albumin (66,000), rabbit glyceraldehyde 3-phosphate dehydrogenase (36,000), bovine carbonic anhydrase (29,000), and bovine trypsinogen (24,000). The retention time of the proteinase from *Streptomyces* sp. C5 (24.2 min; ▲) suggests a Mr value of ca. 16,000 for the non-denatured enzyme.
HPLC GEL FILTRATION
TSK-G2000XL

Log Molecular Mass x 1000

Retention Time (min)

66K
36K
29K
24K
SnpA

Figure 13
Figure 14. Determination of pH for optimal SnpA activity. Effect of pH on SnpA activity was determined by incubating enzyme in 100 mM HEPES that had been preadjusted to appropriate pH values before addition of the substrate. The enzyme activity is reported as Δ OD_{580}/5 min. The enzyme had been preincubated with 3 mM DIFP for 60 min at ambient temperature before starting the assay reaction. All determinations were carried out at ambient temperature.
Figure 14
Figure 15. Determination of temperature for optimal SnpA activity. Effect of temperature on activity of SnpA was determined by performing the enzyme assay at appropriate temperatures. The enzyme activity is reported as $\Delta \text{OD}_{590}/5 \text{ min}$. The enzyme was incubated with 3 mM DIFP for 60 min at ambient temperature. Assay buffer (100 mM HEPES) at pH 7.0 was pre-warmed to each assay temperature for 5 min prior to addition of the enzyme and the substrate. The assay was carried out and monitored in a quartz cuvette placed in temperature controlled cuvette holder. The cuvette holder had been pre-calibrated earlier with a temperature microprobe.
Figure 15
had an amino acid composition which was very comparable to the composition, with the exception of the glycine concentration, of the deduced amino acid sequence encoded by *Streptomyces* sp. C5 *snpA* gene [Lampel *et al.*, 1992].

**Effect of inhibitors and addition of metals on proteinase activity.** The milk-hydrolyzing activity of the proteinase encoded by *Streptomyces* sp. C5 was not inhibited by DIFP, TPCK, TLCK, aprotinin, bestatin, phosphoramidon, or disodium EDTA, but was completely inhibited by 10 mM 1,10-phenanthroline (Tables 5 and 6), indicating that this enzyme probably requires a metal for either stability or activity. After complete inactivation of the enzyme by 1,10-phenanthroline, the milk-hydrolyzing activity of the proteinase could be reactivated 100% or 120% by the addition of 10 mM Zn++ or 10 mM Co++, respectively (Table 5), but activity was not restored by addition of 10 mM Ca++ (data not shown). The specific inhibitor of collagenase, CBZ-PLG-hydroxymate, did not inhibit the azocoll-hydrolyzing activity of the purified proteinase (Table 5).

**Determination of metal ligand.** The purified enzyme was extensively dialyzed against 10 mM HEPES buffer (pH 7.0) or double distilled water to determine if its putative metal cofactor could be removed easily. Even after extensive dialysis against either buffer or water, the enzyme remained essentially 100% active, indicating that the presumed metal ligand was tightly bound to the enzyme. The metal content in both undialyzed and extensively dialyzed preparations of the enzyme was determined by
Table 4. Amino acid composition of the purified SnpA.

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Determined by analysis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Determined from DNA sequence&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>Cys&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Asp + Asn</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Glu + Gln</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>Phe</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Gly</td>
<td>32</td>
<td>17</td>
</tr>
<tr>
<td>His</td>
<td>4</td>
<td>43</td>
</tr>
<tr>
<td>Lys</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Leu</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Met</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Pro</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Arg</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Ser</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>Thr</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Val</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Trp&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Tyr</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Done by Pico-Tag method as described in methods section; the number of amino acid residues are calculated as percent of protein with a Mr of 15,740; all numbers are rounded off to the nearest integer.

<sup>b</sup>Deduced from DNA sequence of mature SnpA proteinase [Lampel et al., 1992].

<sup>c</sup>Cysteine was determined after hydrolysis with performic acid [Cohen et al., 1989].

<sup>d</sup>Tryptophan was determined after hydrolysis with 4 M methanesulfonic acid [Cohen et al., 1989].
Table 5. Effects of inhibitors on proteinase encoded by *Streptomyces* sp. C5 snpA gene.

<table>
<thead>
<tr>
<th>Inhibitor (concentration)</th>
<th>Type of proteinase inhibited</th>
<th>Milk hydrolysis (%) inhibition</th>
<th>Azocoll hydrolysis (%) inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF (10 mM)</td>
<td>Serine proteinase</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DIFP (3.5 mM)</td>
<td>Serine proteinase</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phosphoramidon (5 mM)</td>
<td>Metalloproteinase</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CBZ-PLG-hydroxymate (10 mM)</td>
<td>Collagenase</td>
<td>nd*</td>
<td>0</td>
</tr>
<tr>
<td>Aprotinin (10 mM)</td>
<td>Metalloproteinase</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>Bestatin (10 mM)</td>
<td>Metalloproteinase</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>TPCK (10 mM)</td>
<td>Serine proteinase</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>TLCK (10 mM)</td>
<td>Serine proteinase</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>EDTA (10 mM)</td>
<td>Serine proteinase</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>EGTA (10 mM)</td>
<td>Serine proteinase</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>PHEN (1, 3, 5, 10 mM respectively)</td>
<td>Metalloproteinase</td>
<td>7, 44, 56, 100</td>
<td>100 (@ 10 mM PHEN)</td>
</tr>
<tr>
<td>PHEN (10 mM) + Zn**+** (10 mM)</td>
<td>Metalloproteinase</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>PHEN (10 mM) + Co**+**</td>
<td>Metalloproteinase</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>Dithiothreitol (5, 10, 20 mM respectively)</td>
<td>Thiol proteinase</td>
<td>15, 21, 28</td>
<td>nd</td>
</tr>
<tr>
<td>Iodoacetate (20 and 30 mM respectively)</td>
<td>Thiol proteinase</td>
<td>15, 20</td>
<td>nd</td>
</tr>
<tr>
<td>HgCl₂ (1, 5, 10 mM respectively)</td>
<td>Thiol proteinase</td>
<td>85, 100, 100</td>
<td>nd</td>
</tr>
</tbody>
</table>

*Abbreviations used: nd, not done; CBZ-PLG-hydroxymate, N-(carbobenzoxy)-Pro-Leu-Gly-hydroxymate; DIFP, diisopropylfluorophosphate; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis-(β-amino ethyl ester) N,N,N',N'-tetraacetic acid; PHEN, 1,10-phenanthroline; TLCK, N-p-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.

bAdded as zinc and cobalt acetate.
### Table 6. Summary of the characteristics of the purified SnpA proteinase

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Known substrates</td>
<td>milk, α-casein, β-casein, azocoll</td>
</tr>
<tr>
<td>No activity on*</td>
<td>BAPNA, FAGLA, TAME, SAAFP-pNA</td>
</tr>
<tr>
<td></td>
<td>CBZ-GGL-pNA, LEU-pNA, FALGPA, PPLGPA</td>
</tr>
<tr>
<td>Relative molecular mass (Non-denatured)</td>
<td>16,000</td>
</tr>
<tr>
<td>Subunit size (SDS-PAGE)</td>
<td>15,500</td>
</tr>
<tr>
<td>Size deduced from gene sequencec</td>
<td>15,740</td>
</tr>
<tr>
<td>Appearance in growth phased</td>
<td>exponential phase</td>
</tr>
<tr>
<td>Optimum pH for activity</td>
<td>7.0</td>
</tr>
<tr>
<td>Optimum temperature for activity</td>
<td>55°C</td>
</tr>
<tr>
<td>Optimum [Zn++]</td>
<td>20 mM</td>
</tr>
<tr>
<td>Inhibited by*:</td>
<td>PHEN, DTT, IOA, HgCl₂</td>
</tr>
<tr>
<td>Not inhibited by*:</td>
<td>PMSF, DIFP, TPCK, TLCK, EDTA, EGTA, Aprotinin, Bestatin, Phosphoramidon, N-CBZ-PLG-hydroxamate</td>
</tr>
</tbody>
</table>

*aAbbreviations (all substrates tested were at 0.25 mM): BAPNA, benzoyl-arginine-p-nitroanilide; CBZ-GGL-pNA, benzyl-oxy carbonyl-glycyl-glycyl-leu-p-nitroanilide; N-CBZ-PLG-hydroxymate, N-CBZ-Pro-Leu-Gly-hydroxymate; FAGLA, N-(3-[2-furyl]acyr yloyl)-gly-leu amide; FALGPA, N(3-[2-Furyl]Acryloyl)-Leu-Gly-Pro-Ala; SAAPF-pNA, succinyl-alanyl-alanyl-prolyl-pe-nitroanilide; TAME, Nα-p-tosyl-L-arginine-methyl ester.

*bAbbreviations: DIFP, diisopropylfluorophosphate; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis-(β-amino ethyl ester) N,N,N',N'-tetraacetic acid; IOA, iodoacetamide; PHEN, 1,10-phenanthroline; PMSF, phenylmethylsulfonylfluoride; TLCK, N-p-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; PPLGPA, 4-phenylazobenzyloxy carbonyl-prolyl-leucyl-glycyl-prolyl-D-arginine.

*cLampel *et al.* [1992].

*dIn the recombinant strain *S. lividans*(pANT42).
X-ray analysis using a Tracor X-ray Dispersive Analysis Instrument. The dialyzed sample of the enzyme contained significant levels of zinc and trace levels of copper (Fig. 16), suggesting that zinc was likely to be the metal cofactor in this neutral proteinase. Atomic absorption spectroscopy analysis showed that 0.166±0.004 mg (average ± standard deviation of 3 protein determinations) of the purified proteinase contained 0.401±0.004 μg (average ± difference of actual values of 2 determinations) of zinc, indicating that the enzyme contains 0.58 moles of zinc per each mole of extensively dialyzed, pure enzyme.

**Substrate specificity.** The neutral proteinase encoded by C5-snpA was found to have the milk-hydrolyzing activity described earlier in this thesis, which was not inhibited by DIFP but was completely inhibited by 10 mM 1,10-phenanthroline (Table 5). The enzyme showed no activity with the synthetic substrates L-leucine p-nitroanilide, BAPNA, SAAPF-pNA, TAME, or FAGLA, either at a pH of 7.0 or 8.0. The purified enzyme also did not hydrolyze azocasein, but had relatively strong activity against azocoll (Table 6). Since azocoll is a standard substrate used by collagenases, the enzyme was tested for its ability to hydrolyze the specific collagenase substrates, FALGPA [Van Wart and Steinbrink, 1981] and 4-phenylazobenzyloxy carbonyl-prolyl-leucyl-glycyl-prolyl-D-arginine [PPLGPA, Wunsch and Heidrich, 1963]. No activity of the purified neutral proteinase was found on these collagenase substrates (Table 6).

Since the milk hydrolysis assay measured a decrease in absorbance at 580 nm which was an indication of SnpA activity, it was assumed that the predominant milk
Figure 16. Energy-dispersive X-ray fluorescence spectrometry (EDXFS) of SnpA.

A) EDXFS was performed on double distilled water
B) EDXFS was performed on purified SnpA in double distilled water.

Zinc was determined as the predominant metal in the pure enzyme preparation after comparing spectrum "A" to spectrum "B". The calcium and zinc peaks in both spectrums are indicated by arrows numbered 1 and 2 respectively. The Ca++ peak in both spectrums is due to the calcium present in the double distilled water, as confirmed by spectrum "A". The moderately sized peak on the left of the Ca++ peak (peak #2) in spectrum B indicates Cu++. The positions of the Zn++, Ca++ and Cu++ peaks on the spectrums were confirmed by comparing to known standards.
proteins, α- and β-casein, were being hydrolyzed. Thus this enzyme was incubated with pure α-casein and β-casein (both from Sigma Chemical Co., MO) for short time periods (e.g. 5 to 30 sec) and the proteinolytic degradation products were separated by SDS-PAGE. Figure 17A shows the degradation, after a 15 sec incubation, of α- and β-casein by the purified neutral proteinase. Moreover, the data shown in Figure 17A demonstrate the inability of 10 mM PMSF and the ability of 10 mM 1,10-phenanthroline to inhibit the proteolytic activity on α- and β-casein of the purified neutral proteinase. As can be seen in Figure 17A, β-casein was degraded preferentially over α-casein after the 15 sec incubation. In an attempt to determine cleavage site specificity, β-casein (25 μg) was incubated with 5 μg of the neutral proteinase for 10 sec, after which the hydrolytic products were separated by SDS-PAGE, and their N-terminal amino acid sequences were analyzed. The first and second most prominent proteolytic fragments had N-terminal amino acid sequences of FAQT and FTES, respectively. The sites in β-casein that these would represent, shown in Figure 17B, are KIHP↓FAQT and PVQP↓FTES, suggesting that the proteinase preferentially cleaves the available peptide bond between proline and phenylalanine. After 25 to 30 sec incubations of α- or β-casein with the neutral proteinase, however, no remaining casein proteolytic products were observed on the gels (data not shown), indicating that several cleavage sites for C5-SnpA are present in both α- and β-casein. That β-casein was observed to be cleaved faster than α-casein could potentially be due to the fact that there are more exposed proline residues in β-casein.
Effect of medium composition on expression of snpA. The effect of medium composition on production of the SnpA proteinase is shown in Table 7. This proteinase was maximally produced in the K medium. T medium, which is routinely used to cultivate streptomycetes (Dr. M. J. Butler, personal communication), yielded the least amount of SnpA activity. While the G medium was found to support the best growth amongst these five media, on the basis of final cell dry weights, it nevertheless supported only about half the specific production of the SnpA activity as compared to the K medium (Table 7).

Analysis of RNA-DNA hybridizations. Adequate amounts of the total RNA could be obtained from S. lividans(pANT42) cells harvested at designated time intervals during cultivation in the M medium. The amount of transcript to which the end labelled synthetic DNA probe hybridized was maximal at about 18 h of cultivation after which the steady state amount of transcript specific to the probe decreased (Fig. 18). No transcript could be observed after 36 h that hybridized to the DNA probe.
Figure 17. SDS-PAGE analysis of the degradation of α-casein and β-casein.

(A). SDS-PAGE showing hydrolysis of milk proteins by *Streptomyces* sp. C5 neutral proteinase. All proteins in this gel were stained with coomassie blue R. Lanes: 1, molecular weight standards (LMV-7, Sigma Chemical Co., St. Louis, MO); 2, 5 µg of C5-SnpA alone; 3, 25 µg of α-casein; 4, 25 µg of α-casein incubated with 5 µg of C5-SnpA for 15 sec; 5, the same as lane 4 but preincubated for 15 min with 10 mM 1,10-phenanthroline; 6, the same as lane 4 but preincubated for 15 min with 10 mM PMSF; 7, 25 µg of β-casein; 8, 25 µg of β-casein incubated with 5 µg of C5-SnpA for 15 sec; 9, the same as lane 8 but preincubated for 15 min with 10 mM 1,10-phenanthroline; 10, the same as lane 8 but preincubated for 15 min with 10 mM PMSF.

(B). The amino acid sequence of β-casein, with arrows indicating the cleavage sites represented by the N-terminal amino acid sequences of the most prominent early cleavage products after 10 sec incubation of 5 µg of C5-SnpA with 25 µg of β-casein.
Figure 17

A

kD
66
45
36
29
24

1 2 3 4 5 6 7 8 9 10

B

1 REL E E L N V P G E I V E S L S S S E E S I T R I N K K I
31EK F Q S E E Q Q T E D L E D Q D K I H P F Q A T Q S L V V
61P F P G P I P N S L P Q N I P L T Q T P V V V P P F L Q P
121E S Q S L T L T D V E N L H L M P L L L Q S W M H Q P H Q P
151L P P T V M F P P Q S V L S L S Q S K V L P V V E K A V P Y
181P Q R D M P I Q A F L L Y Q Q P V L G P V R G P F P I I V
Table 7. Effect of medium components on activity of SnpA.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Volumetric Production (units/L)</th>
<th>CDW&lt;sup&gt;b&lt;/sup&gt; (g/L)</th>
<th>Specific Production (units/g CDW&lt;sup&gt;b&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>54</td>
<td>4.0</td>
<td>13.5</td>
</tr>
<tr>
<td>G</td>
<td>284</td>
<td>5.5</td>
<td>52</td>
</tr>
<tr>
<td>M</td>
<td>245</td>
<td>2.4</td>
<td>102</td>
</tr>
<tr>
<td>J</td>
<td>305</td>
<td>2.1</td>
<td>145</td>
</tr>
<tr>
<td>K</td>
<td>472</td>
<td>1.4</td>
<td>337</td>
</tr>
</tbody>
</table>

Medium composition is mentioned in text.

<sup>a</sup>unit : Δ<sub>580</sub>/min of 0.001 = 1 unit.

<sup>b</sup>CDW : Cell dry weight
Figure 18. RNA slot blot analysis using the end-labelled synthetic probe. Approximately 5 μg of total RNA (Lane A) and ca. 25 μg of total RNA (Lane B) from *S. lividans* (pANT42) cells, harvested at pre-determined time intervals during a fermentation in MB medium, similar to that in Fig. 11, were adsorbed onto a nitrocellulose membrane by vacuum filtration through a slot-blot apparatus (Bio Rad, Richmond, CA). About 200 pmoles of the radioactively labelled synthetic oligonucleotide probe were hybridized to the RNA on the nitrocellulose by performing a 20 h incubation, in hybridization buffer, at 42°C. The numbers beside each of the hybridizing bands indicate the duration of cell cultivation (in hours) at which total RNA was isolated from the cells.
Figure 18
D) Discussion.

Subcloning of a gene from Streptomyces sp. C5 encoding milk-hydrolyzing proteinase.

In a survey of streptomycetes for their ability to hydrolyze powdered milk and azocasein, I found that while Streptomyces C5 could produce large zones of hydrolysis on MA medium, Streptomyces peucetius ATCC 29050 and Streptomyces galilaeus ATCC 31133 produced essentially no zones, and Streptomyces lividans 1326 produced very weak zones of hydrolysis on powdered milk. Moreover, the zones of milk hydrolysis from S. lividans cultures were only visible after several days of incubation on milk agar plates. Streptomyces lividans 1326 has added advantages for being chosen as the host for cloning purposes since it can be transformed with recombinant plasmid at high efficiency [Lampel and Strohl, 1986]; it behaves phenotypically as a restrictionless host [Thompson et al., 1980]; availability and ease of handling of the high copy number plasmid vector pIJ702 and similar cloning vectors; and negligible levels of serine proteinase activity were detected [see chapter III]. Even though Streptomyces sp. C5 produced large zones on powdered milk plates, this strain did not significantly hydrolyze azocasein [Gibb et al., 1987, Gibb et al., 1989]. The other streptomycete strains tested are known to hydrolyze azocasein strongly [Gibb et al., 1989]. This led our laboratory to attempt the cloning of the milk-hydrolyzing proteinase gene, now called snpA, of Streptomyces sp. C5 using S. lividans as the host.
[Lampel, 1988; Lampel et al., 1992]. The initial cloning experiments by Lampel [1988] had led to the construction of plasmid pANT21, which suffered a severe structural integrity problem as seen by a high frequency of loss of the milk protein-degrading phenotype on retransformation of wild type S. lividans with pANT21. This was accompanied by deletions in the Streptomyces sp. CS DNA inserted in the plasmid [Lampel, 1988]. The subcloning experiments that I performed resulted in plasmid pANT42 which is stably maintained in both S. lividans and S. galilaeus without spontaneous deletions occurring. These data suggest that the instability problems encountered with pANT21 may be due to the piece of insert DNA in pANT21 upstream of the piece of insert DNA that had been subcloned into pIJ702 to construct pANT42. During initial subcloning experiments plasmids pANT22 and pANT23 were constructed. While S. lividans transformed with pANT22 did not exhibit any milk hydrolysis activity on a milk agar plate, S. lividans that had been transformed with pANT23 did display early milk hydrolysis activity, but only as weak zones of clearing on a milk agar plate. The weak milk protein-hydrolyzing activity associated with S. lividans(pANT23) may be due to the low copy number of the plasmid pANT23.

Activity of the upstream sequences. Experiments by Chang et al. [1990] have led to cloning of a neutral proteinase gene from S. cacaoi into S. lividans. During subcloning analysis of the cloned gene, the authors discovered that deletion of an SphI fragment upstream of the npr gene resulted in a three-fold enhancement of Npr
activity. However, they have not offered an explanation for this phenomenon. Recently, both Butler et al. [1992] and Lichenstein et al. [1992] have independently shown that directly upstream from SL-snpA/prt is a divergently transcribed gene. Butler et al. [1992] named this gene snpR, and Lichenstein et al. [1992] named the gene "orfI". Butler et al. [1992] and Lichenstein et al. [1992] have both demonstrated, by the use of deletion analyses, that the snpR/orfI gene product is necessary for expression of the snpA/prt proteinase gene. Both authors also have utilized data base searches to verify that this gene encodes a protein that has a relatively high sequence similarity with CatR [Rothmel et al., 1990], a member of the LysR family of bacterial activator proteins [Henikoff et al., 1988].

During my early attempts to determine the exact location of snpA, I had constructed pANT55, a derivative of pIJ702 containing the 1.2 kb KpnI-SphI DNA fragment representing the region directly upstream of C5-snpA (Fig. 6). Transformation of S. lividans 1326 with pANT55 reproducibly resulted in the formation of a slow (visible only after 24-36 h of incubation) but distinguishable, milk-hydrolysis activity above background levels. Transformation of S. galilaeus with pANT55, on the other hand, resulted in no proteinolytic activity whatsoever, even after 48 to 54 h of incubation (Fig. 10). This upstream region (e.g., the pANT55 insert) has been sequenced [Lampel et al., 1992; K. Lampel and J. Lampel, personal communication] and exhibits a very high degree of identity to the snpR/orfI gene of S. lividans 1326. Since S. lividans contains an snpA/prt gene (probably silent) and S. galilaeus apparently does not (as determined by Southern hybridization data, Fig. 8),
it seems probable that the transformation of *S. lividans* with pANT55, a plasmid carrying an *snpR/orf1*-equivalent gene, could have activated the normally silent SL-*snpA/prt* gene which was visualized phenotypically as a slow milk-hydrolyzing activity.

**Purification and characterization of SnpA.** As shown in Figure 12, the milk-hydrolyzing proteinase encoded by C5-*snpA* has a *M*ₚ of 15,500 (as determined by SDS-PAGE analyses) whether purified from the culture broth of *S. lividans*(pANT42) or *S. galilaeus*(pANT42). The sequence of the first 20 amino acids at the N-terminus of this pure protein was determined and matched exactly the amino acid sequence deduced from the DNA sequence of *Streptomyces* sp. C5 *snpA* (Fig. 19). The deduced amino acid sequence of the mature protein yields a calculated *M*ₚ of 15,740 [Lampel *et al.*, 1992], which agrees with the physical data obtained herein. The amino acid composition determined from the purified protein and the amino acid composition deduced from the cloned gene are similar (Table 7), except for the high glycine content in the analysis of the purified protein. This is not entirely unanticipated, as glycine yields much higher than expected are routinely obtained during amino acid analysis [Dr. John Lowbridge, Biochemical Instrumentation Center, Ohio State University, personal communication]. All of these determinations indicate that the protein purified and characterized in this work was encoded by the *snpA* gene from *Streptomyces* sp. C5. The similarity in sizes of the C5-*snpA* gene product from both *S. galilaeus* and *S. lividans* also indicates that these two organisms
Figure 19. Alignment of the N terminal amino acid sequence of SnpA with the nucleotide sequence of *snpA* (the DNA sequencing of *snpA* was carried out by J. Lampel and K. Lampel) and the deduced amino-acid sequence of SnpA. Both the deduced amino acid sequence identical to the N-terminus of the mature protein and the conserved Zn$^{++}$ binding amino acid motif are doubly underlined [from Lampel et al., 1992]. The first thick arrow from the 5' end of the nucleotide sequence indicates a predicted protein processing site as per von Heijne's rules [von Heijne, 1983] while the following thick arrow indicates the processing site as determined by the sequenced N-terminus of the protein.
Figure 19
recognize and cleave off the leader peptide from mature C5-SnpA in an analogous manner.

As mentioned previously, microbial endolytic proteinases fall into four major groups, including the serine or alkaline proteinases, which have a serine residue at the active site and which are inhibited by hydroxyl-reactive organofluorides such as DIFP and PMSF [Salvesen and Nagase, 1989]; the sulfhydryl proteinases, which contain a cysteine at the active site and are markedly stimulated by the presence of thiol reagents such as DTT [North, 1989]; the acid proteinases, which typically have an aspartic acid residue at the active site and are active under low pH conditions [Hofmann, 1985]; and the neutral proteinases, which are active at neutral pH, typically contain a metal ligand as part of the active site, and are inhibited by chelating agents such as EDTA, EGTA, and 1,10-phenanthroline [Hofmann, 1985; Salvesen and Nagase, 1989]. The major characteristics of *Streptomyces* sp. C5 SnpA are summarized in Table 6. This small proteinase has optimal activity at a pH of 7.0 and it contains a zinc ligand which can be chelated from the enzyme by relatively high concentrations of 1,10-phenanthroline but not by EDTA or EGTA. C5-SnpA is not inhibited by DIFP or PMSF, indicating that it is not a serine proteinase, and DTT inhibits rather than stimulates activity of the enzyme, indicating that it is not a sulfhydryl proteinase. Furthermore, high concentrations (20 and 30 mM) of iodoacetate have only a minimal inhibitory effect on the proteinase, further indicating it is not a sulfhydryl proteinase [Salvesen and Nagase, 1989]. Thus, C5-SnpA has characteristics typical of neutral proteinases. The small size (ca. 15,500 daltons) of
the mature recombinant SnpA proteinase expressed in *S. lividans*, however, is highly unusual. Nearly all the neutral proteinases that have been isolated from bacteria thus far have ranged in molecular weight from 28,000 to 57,000 daltons. The only exception to this is the neutral proteinase of M, 15,000 that has been purified and crystallized from *S. caespitosus* [Yokote and Naguchi, 1969]. These authors demonstrated the *S. caespitosus* proteinase to be other than a carboxypeptidase or an aminopeptidase by means of incubation with synthetic substrates. The proteinase that they crystallized shows a marked preference for the carboxyl side of phenylalanine and tyrosine residues in the substrates tested. Their inhibition studies indicated a total inhibition of proteinolytic activity by 5 mM DIFP and 50 mM 2-mercaptoethanol, making the *S. caespitosus* proteinase a somewhat unusual type of neutral proteinase and clearly different from SnpA. Zn++, at 20 mM, inhibited the *S. caespitosus* proteinase activity by 18%, while 20 mM each of Sn++, Ag++, Pb++, and 50 mM of EDTA inhibited activity totally. Thus, even though the *S. caespitosus* proteinase appears to be the only reported proteinase that is similar in its physical size to the *Streptomyces* sp. C5 SnpA, it exhibits dramatically different biochemical characteristics from the SnpA proteinase.

FAGLA, a substrate designed for measuring the activity of thermolysin-like neutral proteinases of *Bacillus* spp. [Feder, 1968], is not a substrate for C5-SnpA (Table 6). Additionally, phosphoramidon, a relatively specific inhibitor for thermolysin-like neutral proteinases [Holmquist, 1977; Komiyama et al., 1975], did not inhibit C5-SnpA, even at very high concentrations (Tables 5, 6). Phosphoramidon at
5 μM and 10 μM concentrations inhibited clostridial collagenase completely in control experiments (data not shown) whereas these concentrations of phosphoramidon had no effect on C5-SnpA. In fact, a 10 mM concentration of phosphoramidon also had no effect on the activity of the C5-SnpA. Other than the primary zinc binding domain (HExxH) found in a variety of zinc-containing proteinases [Jongeneel et al., 1989], additional conserved amino acid sequences normally associated with zinc-binding and active sites of thermolysin-like neutral proteinases were not found in the deduced amino acid sequence of C5-SnpA [Lampel et al., 1992]. Calcium ions, which are normally associated with thermolysin and several of the proteinases like it [Hofmann, 1985], did not stimulate activity or restore activity of 1,10-phenanthroline-treated C5-SnpA. This further indicates the difference between this proteinase and thermolysin-like proteinases. Likewise, C5-SnpA has no similarity to carboxypeptidase A-like neutral proteinases [Bradshaw et al., 1969]. Although azocoll was found to be a good substrate for C5-SnpA, the proteinase had no activity on two specific collagenase substrates (Table 6). The azocoll-hydrolyzing activity of C5-SnpA was not inhibited by the specific collagenase inhibitor, CBZ-PLG-hydroxymate (Table 5). This indicates that C5-SnpA is not an unusual collagenase. Thus, C5-SnpA appears to be a highly unusual, low molecular weight neutral proteinase, different both from the low molecular weight neutral proteinase isolated from S. caesptiosus and from other reported streptomycete neutral proteinases, as well as neutral proteinases in general.
The molar ratio of zinc to proteinase for the purified C5-SnpA was approximately 0.6:1. On the basis of data obtained with other neutral proteinases [Hofmann, 1985] and the presence of a single zinc-binding domain in the enzyme, a value somewhat closer to a 1:1 ratio was expected. The low value obtained may be due to loss of zinc during dialysis, especially since secondary zinc-binding domains are not present in the proteinase. Cobalt could substitute for zinc (and even yielded a slightly better activity), but calcium did not stabilize the enzyme or increase the specific activity of the enzyme against milk proteins. Whether or not the zinc is involved in the actual catalytic mechanism is unknown at this time.

Since the cloned milk-hydrolyzing proteinase contained two cysteine residues (on the basis of the deduced sequence of the mature enzyme; Table 4), it is possible that a disulfide bond-linked pair of cysteinyl residues may be present in the native protein. The inhibition of the milk-hydrolyzing activity of the purified proteinase by DTT (Table 5) further suggests the presence of disulfide bridges in the native protein which may be required for the active conformation. I have noted that pure preparations of this enzyme are stable at 4°C for the period of a year, indicating that this enzyme is very stable in solution. Similarly, the enzyme is equally stable and active when suspended in distilled water or buffer. The presence of a disulfide linkage could be a possible explanation of the enhanced stability of this enzyme. SL-SnpA/Prt, a similar proteinase from S. lividans, also appears to contain two cysteine residues [Butler et al., 1992; Lichenstein et al., 1992]. Rufo et al. [1990] have purified a thermostable neutral proteinase from Bacillus subtilis (Mpr) that appears to contain
disulfide bridges. Recently, Tatsumi et al. [1991] have purified a small, highly thermostable, neutral proteinase (NpII) from *A. oryzae*. The authors have shown the presence of six cysteinyl residues in this proteinase and postulate the formation of disulfide bridges as a possible explanation for the marked thermostability of this enzyme.

The actual substrate for C5-SnpA is not known at this time. The primary proteinolytic cleavage sites in β-casein involve the peptide bond between a proline residue on the amino side of the cleavage and a phenylalanine residue on the carboxyl side of the cleavage. Thermolysin preferentially cleaves on the amino-terminal side of most amino acids except cysteine, lysine, or arginine (Hofmann, 1985), and thermolysin-like proteinases typically are characterized by their ability to cleave on the amino side of hydrophobic amino acid residues, particularly phenylalanine and leucine [Morihara, 1967]. This specificity is thought to reside in the hydrophobicity of the catalytic pocket dictated by the amino acids surrounding the HExxH-zinc binding domain [Hofmann, 1985; Jongeneel et al., 1989]. The deduced amino acid sequence of C5-SnpA also contains relatively hydrophobic amino acids in the region of the HExxH-zinc binding domain [Lampel et al., 1992]. It is possible that C5-SnpA has a preference for the amino terminal side of hydrophobic amino acids as do other neutral proteinases. Moreover, the P-F bonds of β-casein that were preferentially cleaved could have been exposed on the surface of the substrate, thus making them more accessible to the enzyme. Incubation of C5-SnpA with either α- or β-casein for more than 25 sec resulted in absence of detectable protein bands on
the gel after denaturing polyacrylamide gel electrophoresis. It is likely that there are several cleavage sites in α- and β-casein which are recognized by this proteinase.

The sequence of the first 20 amino acids from the N-terminus of the *Streptomyces* sp. C5 neutral proteinase, purified from culture broths of *S. lividans* 1326(pANT42), was identical to a region of the deduced amino acid sequence of the cloned gene as shown in Figure 19. The deduced amino acid sequence of the mature proteinase includes 148 amino acids which could theoretically encode a protein with a M_r of 15,740 [Lampel et al., 1992]. The M_r of purified C5-SnpA was found by SDS-PAGE and gel filtration to be ca. 15,500 and 16,000 respectively, indicating good agreement between the deduced amino acid sequence and the actual size of the proteinase.

Both Butler et al. [1992] and Lichenstein et al. [1992] have simultaneously and independently discovered an identical gene from *S. lividans*. Butler et al. [1992] have denoted this gene as snpA, while Lichenstein et al. [1992] have named this gene prt. I will be referring to this gene from *S. lividans* as snpA/prt in order to prevent confusion regarding these separate notations for an identical gene. The snpA/prt gene and its surrounding DNA sequence, described by both Butler et al. [1992] (SL-snpA) and Lichenstein et al. [1992] (prt), has a ca. 71% homology with C5-snpA and its adjoining sequences [Lampel et al., 1992]. Autoradiographic analyses using the radiolabelled 642 bp *PvuII/SphI* fragment from pANT42 initially did not indicate *S. lividans* 1326 chromosomal DNA fragments demonstrating hybridization intensities similar to those obtained with *Streptomyces* sp. C5 DNA fragments. This was
surprising as signal intensities were expected to be the same when the considerable DNA sequence identity (ca. 70% overall) between SL-snpA/prt and C5-snpA is taken into consideration. This could potentially be due to the degradation of *S. lividans* 1326 DNA under certain conditions as described elsewhere [Zhou *et al.*, 1988]. The C5-SnpA and SL-SnpA/prt differ in the amino acid sequences at the N-terminus of the mature C5-SnpA [Fig. 20; W. R. Strohl, personal communication]. This is important because the N-terminal amino acid sequence obtained from purified SnpA confirmed that I had isolated the protein encoded by the recombinant *Streptomyces* sp. C5 snpA gene and not the protein encoded by the host *S. lividans* snpA/prt gene. I have not observed in any of my experiments a larger protein product of C5-snpA which appears to be cleaved to the final size of ca. 15,500. Furthermore, while *S. galilaeus* is lacking an inherent neutral proteinase activity, I have been successful in isolating a neutral proteinase from *S. galilaeus*(pANT42) with the same size and activity as the one isolated from *S. lividans*(pANT42) (Fig. 12), further confirming that the purified SnpA is the cloned gene product.

The conserved zinc-binding motif found in the deduced SnpA amino acid sequence (Table 8) is characteristic of a thermolysin-like neutral proteinase [Jongeneel *et al.*, 1989]. Moreover, metalloproteinases of the thermolysin superfamily also have additional secondary ligand sites including a sequence conserved with INEAISD, of which the glutamic acid residue is the zinc ligand [Hofmann, 1985], and DNGGVHINSG, of which the histidine is at the active site [Hofmann, 1985]. C5-SnpA does not contain amino acid sequences resembling these additional ligand-
binding and active sites [Lampel et al., 1992] indicating that it is considerably different from the thermolysin-like metalloproteinases. The difference in molecular weights between thermolysin and C5-SnpA further substantiates this fact.

Tatsumi et al. [1991] have recently described the sequence of proteinase NpII, a protein with a M, of 19,000, from Aspergillus oryzae. This proteinase also contains a single zinc binding site but lacks the second zinc ligand Glu residue and the active site His residue. Nevertheless, the deduced amino acid sequence of this proteinase (NpII) shows little similarity with that of C5-SnpA (W. R. Strohl, personal communication). Moreover, NpII has very different substrate specificities than does C5-SnpA [Tatsumi et al., 1991], and NpII is markedly thermostable [Tatsumi et al., 1991] whereas C5-SnpA loses activity rapidly at 80 °C, indicating that NpII is very different from the proteinase encoded by C5-snpA.

The difference between SnpA and other neutral proteinases is not necessarily surprising, given the difference in size, substrate specificity, and sensitivity to inhibitors between C5-SnpA and these other neutral proteinases. Thus, it appears that C5-SnpA is significantly different from all previously published neutral (metallo-) proteinases.

Butler et al. [1992] have presented data indicating the mature form of the proteinase encoded by the S. lividans 1326 snpA/prt to be about 24,000 M, with smaller species also formed, while Lichenstein et al. [1992] contend that only a single 22,000 M, polypeptide species is formed. The C5-snpA gene encodes a neutral
Figure 20. Comparison between the deduced amino acid sequence of the C5-SnpA proteinase and the SL-SnpA/prt. The double dots indicate amino acid identity between the two sequences and the single dots indicate conservative substitutions of amino acids. Considerable similarity (ca. 71% amino acid identity) can be measured between these two amino acid sequences. The amino acid sequence of the N-terminus of the mature *Streptomyces* sp. C5 protein is doubly-underlined and the conserved Zn$^{++}$ ligand binding site is singly-underlined. Amino acids "upstream" of the N-terminus of the mature C5-SnpA would be part of the putative pre-pro leader sequence [Lampel et al., 1992].
<table>
<thead>
<tr>
<th></th>
<th>C5-SnpA</th>
<th>SL-SnpA</th>
</tr>
</thead>
<tbody>
<tr>
<td>59</td>
<td>MRHPLSVLTAAGLSLATLGLGTAGPASATPTAEAGAPVVAYDGSPAGSPADAKA一一AAN</td>
<td>56</td>
</tr>
<tr>
<td>56</td>
<td>RAFEAVLRSVEKRAANPKSTAVTVVYHAGAEPFATQLRTGQIWNSSSVSNVRLQAG</td>
<td>119</td>
</tr>
<tr>
<td>119</td>
<td>ASSGVDFTYREGNDPRGSYASTDGHGRYIFLDYRQNYTDSTVTAHEGTVLQGPDHYS</td>
<td>179</td>
</tr>
<tr>
<td>179</td>
<td>SSGVDFTYREGNDPRGSYASTDGHGRYIFLDYRQNYTDSTVTAHEGTVLQGPDHYS</td>
<td>176</td>
</tr>
<tr>
<td>229</td>
<td>GPCSELMSGGPGPSCTNAYPNSAERSRVNQLMWANGFAAAMDKAEEK-SAR</td>
<td>227</td>
</tr>
</tbody>
</table>

**Figure 20**
proteinase in both *S. lividans* 1326 and *S. galilaeus* ATCC 31133, which upon isolation from the culture broths has a *M*<sub>r</sub> of 15,500 on analysis by electrophoresis through denaturing polyacrylamide gels. This suggests that the processing of the mature C5-SnpA neutral proteinase in both strains is similar. The deduced amino acid sequences of the leader peptides from C5-*snpA* and SL-*snpA/prt* are somewhat different (Fig. 20) and may be responsible for the difference in the sizes of the mature products. Furthermore, the multiple forms of SL-SnpA (20,000 to 24,000 *M*<sub>r</sub>) appear to have activity against azocasein [M. J. Butler, personal communication], whereas the 15,500 *M*<sub>r</sub>, C5-SnpA proteinase definitely does not hydrolyze azocasein (Table 6). Speculatively, it could be possible that the different activities of these different forms of *snpA* gene products may be due to the presence or absence of additional N-terminal amino acids. Enzymes of multiple sizes are known to be encoded by the same gene. A gene of *Streptomyces griseus* IMRU 3570 encodes two α-amylases of 50,000 and 57,000 *M*<sub>r</sub>, each with the same N-terminal sequences and cross-reactive with antibodies made to the larger protein [Garcia-Gonzalez *et al.*, 1991].

**Physiological regulation of SnpA.** The cloned *Streptomyces* sp. C5-*snpA* gene was expressed during exponential growth phase in *S. lividans*. One might debate that this initial proteinase activity could be due to the residual extracellular neutral proteinase that had been carried over to the 10 L fermentor from the seed cultures. Subsequent slot blot experiments have indicated that the mRNA for *snpA*, both in *Streptomyces*
Table 8. Zinc binding sites of metalloproteinases  
[W. R. Strohl, personal communication]

<table>
<thead>
<tr>
<th>Source</th>
<th>Protease</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces C5</em></td>
<td>SnpA</td>
<td>VT AHE TGH VLG L</td>
</tr>
<tr>
<td><em>S. cacaoi</em></td>
<td>Metalloproteinase</td>
<td>TVH EAGH SLM S</td>
</tr>
<tr>
<td><em>Serratia</em></td>
<td>Metalloproteinase</td>
<td>TF THE IGH ALGL</td>
</tr>
<tr>
<td><em>Enterococcus</em></td>
<td>Gelatinase</td>
<td>V VGHE MTH GVT E</td>
</tr>
<tr>
<td><em>Erwinia B374</em></td>
<td>ProB proteinase</td>
<td>SF THE IGH ALGL</td>
</tr>
<tr>
<td><em>Erwinia EC16</em></td>
<td>Metalloproteinase</td>
<td>TL THE IGH ALGL</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Neutral proteinase</td>
<td>VT AHE MTH GVT Q</td>
</tr>
<tr>
<td><em>B. amyloficiens</em></td>
<td>Metalloproteinase</td>
<td>VT AHE LTH GVT Q</td>
</tr>
<tr>
<td><em>B. stearothermophilus</em></td>
<td>Thermolysin</td>
<td>V VGHE LTH AVTD</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>Metalloproteinase</td>
<td>V IGHELTH AVTE</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Peptidase</td>
<td>V IGHE YFHN WT</td>
</tr>
<tr>
<td><em>Leishmania</em></td>
<td>Surface proteinase</td>
<td>V VTHE MAHALG</td>
</tr>
<tr>
<td><em>Listeria</em></td>
<td>Mp1 proteinase</td>
<td>I VGHELTH AVIQ</td>
</tr>
<tr>
<td><em>Ps. aeruginosa</em></td>
<td>Elastase</td>
<td>V AAHE VSH GFD</td>
</tr>
<tr>
<td><em>Crayfish</em></td>
<td>Digestive proteinase</td>
<td>TII HELMHAIG</td>
</tr>
<tr>
<td><em>Rat</em></td>
<td>Stromelysin</td>
<td>V AAHE LAH SLG</td>
</tr>
<tr>
<td><em>Human</em></td>
<td>Stromelysin</td>
<td>V AAHE IGH SLG</td>
</tr>
<tr>
<td><em>Human</em></td>
<td>Fibroblast collagenase</td>
<td>V AAHE LGHS LG</td>
</tr>
<tr>
<td><em>Human</em></td>
<td>Gelatinase</td>
<td>V AAHE FGH AMG</td>
</tr>
<tr>
<td><em>Human</em></td>
<td>Endopeptidase 24.11</td>
<td>V IGHE I THGF D</td>
</tr>
<tr>
<td><em>Human</em></td>
<td>Aminopeptidase</td>
<td>V IAHE LAH QWF</td>
</tr>
</tbody>
</table>
sp. C5 (data not shown) and *S. lividans*(pANT42), was most abundant during the exponential growth phase of the cultures. This indicates that the proteinase activity detectable during the exponential growth phase is indeed encoded by the C5-*snpA* gene. The proteinase encoded by *S. lividans snpA/prt* was also observed early in the exponential phase of growth after the gene had been cloned onto a high copy number plasmid [Butler *et al.*, 1992]. Such growth-phase-related proteinase activities are uncommon for the streptomycete proteinases studied to date, since most streptomycete proteinase activities are observed after exponential growth has ceased [Gibb and Strohl, 1988]. Thus, it is possible that the *in situ* role of SnpA in *Streptomyces* sp. C5 may be as a primary nutritional hydrolase, i.e., to hydrolyze exogenous protein substrates as carbon and energy sources for exponential growth.

Preliminary experiments at the shake flask level indicated the SnpA proteinase to be expressed most strongly when cultivated in K medium, which is devoid of any externally added milk (Table 7). Gibb [1987] has hypothesized that proteinases are generally induced by the substrates that they hydrolyze. This does not seem to be the case here as I have found milk to be the only substrate (amongst the substrates tested) that can be hydrolyzed by this enzyme. Addition of milk to the medium supporting the strongest expression of SnpA (K medium), to give the J medium, actually decreased the volumetric production of SnpA by 35% (Table 7) while the specific production of SnpA decreased by approximately one-half (Table 7). On further analysis of the medium components, I observed that casein alone does not seem to be the factor controlling the expression of SnpA. Even if the T and G media
(Table 7) possess similar levels of other media components, the G medium supports a 5.25-fold enhanced volumetric production of SnpA over the T medium. The only difference in these media is the presence of yeast extract in the G medium. This could potentially indicate that some unknown component, possibly a small peptide from the yeast extract that could be taken up readily by the cell, is activating SnpA expression by an unknown mechanism. Also, conceivably there could be an unknown peptide in the T medium that could be inhibiting the activity of C5-SnpA. The M medium does not contain any yeast extract, but as it is nutritionally less rich than the G medium and it supported less growth as evident by low cell dry weights. Similar observations in which proteinase production is augmented by nutrient starvation have been reported for *S. clavuligerus* [Bascaran et al., 1990]. These authors have also noted that proteinase production from *S. clavuligerus* was stimulated by low levels of yeast extract. Thus the absence of yeast extract in the M medium could conceivably be counteracted by the poorer growth, leading to SnpA expression levels comparable to those obtained in G medium (Table 7). Although the J medium contains yeast extract and also supports less growth (as reflected by the cell dry weights obtained) it does not seem to possess significantly higher levels of SnpA. This may possibly be due to the repressive effects of the milk added to the medium. When milk was omitted from this medium, to give the K medium, the yield of SnpA increased significantly. The pH of all the five media at the termination of cultivation, at 42 h, was between 8.3-8.6, thus effectively eliminating a role for medium pH in the
differences in production of the SnpA. Although these observations seem significant, it should be noted that they are from a single set of experiments.
CHAPTER III

Studies on the proteinases of *Streptomyces lividans* 1326

A) Introduction

Although many proteinases with broad substrate specificities have been isolated from the culture broths of a variety of *Streptomyces* species [Narahashi, 1970; Pokorny *et al.*, 1979; Chandrasekaran and Dhar, 1987], interest in proteins secreted into the medium by *S. lividans* has begun only recently. *S. lividans* has been shown to possess various extracellular enzymatic activities such as cellulase [Kluepfel *et al.*, 1986], xylanase [Morosoli *et al.*, 1986], β-galactosidase [Eckhardt *et al.*, 1987], endoglucanase [Shareck *et al.*, 1987], proteinases [Aretz *et al.*, 1989], β-glucosidase [Mihoc and Kluepfel, 1990], and xylanase B [Kluepfel *et al.*, 1990].

*S. lividans* is the primary streptomycete host strain used in recombinant DNA experiments, and there are several examples of the use of *S. lividans* as a host for secretion of heterologous recombinant proteins into the culture broth [Noack *et al.*, 1988; Lichenstein *et al.*, 1988; Chang and Chang 1988; Taguchi *et al.*, 1989; Bender
et al., 1990]. Therefore, it is important to know the ensemble of extracellular proteinases which may degrade potentially valuable secreted recombinant products produced in fermentations using this strain.

During the studies on production of SnpA by *S. lividans* (pANT42), an extracellular leucine aminopeptidase type of activity from *S. lividans* was identified. Only in *Streptomyces clavuligerus* has it been shown that under conditions of maximal proteinase production, the extracellular proteinase activity against azocasein seemed to be represented by a single metalloproteinase [Bascaran et al., 1990]. From my investigations, it seems that *S. lividans* 1326 has one major extracellular proteinolytic activity, and this is of the leucine aminopeptidase type. Described here is the purification and biochemical characterization of this leucine aminopeptidase from the culture broth of *S. lividans* 1326 using ion exchange chromatography and gel filtration techniques. Evidence is presented which indicates that *S. lividans* 1326 lacks substantial serine proteinase activities.
B) **Purification and characterization of the extracellular leucine aminopeptidase (LapA) from *S. lividans* 1326.**

**Materials and Methods.**

**Bacterial strain, maintainence and fermentation conditions.** The strain used in this study was *S. lividans* 1326. This strain was used as the source for the extracellular leucine aminopeptidase and was maintained on Trypticase Soy Agar (TSA) plates (TSB {Beckton Dickinson, Cockeysville, MD} supplemented with 20 g/L of Bacto agar {Difco, Detroit, MI}). For inoculum preparation, spores were aseptically scraped from a TSA plate and inoculated into a 250 ml flask containing 50 ml of MB medium (MB medium is essentially the same as MBT medium described in the previous chapter, but without any thiostrepton supplementation) and a coiled spring for mycelial dispersion [Dekleva *et al.*, 1985]. The culture was grown for 32 h at 30°C by agitation on an orbital shaker at 250 rpm. The entire contents of this primary seed culture were then used to inoculate a 2 L flask containing 950 ml of MB medium (ca. 5% inoculum, v/v) and a coiled spring. Growth was allowed to proceed for 24 h, to provide the secondary seed. The entire contents of the secondary seed were used to inoculate a 14 L glass fermentor (New Brunswick Scientific) containing 9 L of MB medium (ca. 10% inoculum, v/v). Five ml of undiluted antifoam (Mazu
DF-60P; Mazer Chemical Co., Gurnee, IL) were added to the medium prior to autoclaving to prevent foaming. Agitation in the fermentor was at 400 rpm, temperature at 30°C, and air flow at 1 vvm as described previously. The pH of the medium was adjusted to 7.2 with 5 N sodium hydroxide prior to autoclaving. The pH was monitored but not controlled once the fermentation was in progress. Fermentation was allowed to proceed for 120 h. Fifty ml samples of the fermentation broth were collected aseptically in sterile plastic tubes (Sarstedt) at regular time intervals.

For regulation studies, spores from *S. lividans* 1326 maintained on TSA medium were aseptically harvested by a sterile platinum wire loop and inoculated into 50 ml of each of the sterile T, G, M, J, and K media (composition of these media has been mentioned earlier in Chapter II, p. 81).

**Preparation of cell-free extracts.** Mycelia from 300 ml of fermentation broth were harvested by centrifugation at 16,000 x g for 15 min at 4°C and the supernatant was saved for further analyses. The mycelial pellet was rinsed once with cold HCM buffer and resuspended in 5 ml of the same on ice. The resuspended mycelia were disrupted in a French pressure cell (American Instrument Co., Urbana, II) at 16,000 psi. Unbroken mycelia and cellular debris were pelleted by centrifugation at 20,000 x g for 30 min. The supernatant from this step was designated as intracellular material and was saved for further analyses.
Proteinase assays and protein determination. Proteinolytic activity in cell free fermentation broth was determined by monitoring the release of p-nitroaniline from the synthetic substrate L-leu-pNA ($\epsilon = 8480 \text{l/mole-cm}$) at 410 nm using a single-beam spectrophotometer (Model DU-64, Beckman Instruments, Fullerton, CA). Mycelia from 500 μl of each of the fermentation broth samples, collected at predesignated time intervals, were pelleted by centrifugation at 10,000 rpm for 10 min in a microcentrifuge. The cell free broth (100 μl) was added to 880 μl of a buffer which was composed of 100 mM HEPES (pH 8.0), 10 mM MgCl$_2$, and 10 mM CaCl$_2$ (HCM), and held at 37°C for 15 min. The resulting uncharacterized precipitate was removed by centrifugation at 10,000 rpm for 15 min in a microcentrifuge. To the clarified cell free broth, 20 μl of a stock solution of 100 mM L-leu-pNA in 95% ethanol were added. The release of paranitroaniline from the L-leu-pNA, signifying proteinase activity, was monitored continuously for a period of 3 min as mentioned above. The following were also used as substrates: N-succinyl-alanyl-alanyl-prolyl-phenylalanine p-nitroanilide (SAAPF-pNA), N-$\alpha$-benzoyl-L-arginine p-nitroanilide (BAPNA), L-ala-pNA, L-gly-pNA, L-pro-pNA, L-lys-pNA, N-(3-[2-Furyl]Acryloyl)-leu-gly-pro-ala (FALGPA), N-(3-[2-furyl]acryloyl)-gly-leu amide (FAGLA), and N$\alpha$-p-tosyl-L-arginine methyl ester (TAME). Increase in absorbance at 410 nm due to release of para-nitroaniline indicated proteinase activity. In the case of FAGLA, a decrease in absorbance at 314 nm would indicate cleavage of the dipeptide substrate, thus indicating proteinolytic activity [Feder, 1968]. When TAME was used as a substrate, an increase in absorbance at 247 nm would indicate proteinolytic activity.
An increase in absorbance at 345 nm would indicate proteinolytic activity when using FAGLPA as the substrate [Van Wart and Steinbrink, 1981]. Similar proteinase assays were also performed on the intracellular contents obtained after mycelial breakage as described above.

Protein concentration of the cell free broth and the intracellular contents was determined by the dye-binding method of Bradford [1976] using a commercial protein assay reagent (BIO-RAD, Richmond, CA) and bovine serum albumin as the standard. Cell growth determination was carried out as described in Chapter II.

**3H-DIFP-binding assay.** *S. lividans* was cultivated with agitation (250 rpm, orbital shaker) in 50 ml MB for 72 h at 30°C. Thirty ml of the cell free broth were dried to powder by lyophilization under vacuum for 8 h using a LabConco (FreezeDryer 3) lyophilizer. The entire amount of the lyophilized powder (500 mg) was resuspended in 1 ml of sterile distilled water and dialyzed against 1000 vol of 20 mM Tris-HCl (pH 8.0). The protein concentration of the sample to be analyzed for serine proteinase activity was adjusted to 1 µg of protein per µl of 20 mM Tris-Cl. 3H-DIFP (NEN, 4.4 Ci/mmol, 1 µCi/µl) was added to each sample to achieve a final DIFP concentration of 100 µM in a reaction volume of 100 µl. The radiolabelled DIFP was allowed to bind to potential serine proteinases in each sample by incubation for 30 min at 37°C. At the end of the incubation period, 250 µl of an ice-cold 10% (w/v) solution of trichloroacetic acid (TCA) were added to each sample and the samples were stored on ice for 20 min to ensure complete precipitation of the
proteins. The precipitated proteins were recovered by centrifugation in a microcentrifuge at 10,000 rpm for 15 min. The supernatant was discarded into a solution of 5 N sodium hydroxide to neutralize the DIFP and the precipitate was dried under vacuum and resuspended in 14 μl of SDS-PAGE sample buffer. At this point the solution turned yellow due to presence of residual TCA in the precipitate. One μl of 5N sodium hydroxide was added to neutralize the solution, which resulted in a blue colored solution. The samples were incubated at 100°C for 3 min in the neutralized sample buffer and cooled to room temperature. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12% (w/v) vertical slab gels as described previously. After completion of electrophoresis, proteins in the gels were fixed in 50% (v/v) methanol for 2 h and stained by incubation with 0.2% (w/v) Coomassie brilliant blue-R for 2 h as described previously. The gel was destained by diffusion into an aqueous solution of 7.5% (v/v) acetic acid and 50% (v/v) methanol for 2 h. The gels were then dehydrated by soaking them twice for 20 min each in 20 vol of dimethyl sulfoxide (DMSO), after which they were impregnated with 2,5-diphenyloxazole (PPO) by immersing in 4 vol of 22.2% (w/v) of PPO in DMSO for 1 h. Excess PPO in the gel was precipitated in situ by soaking the gels in double distilled water for 1 h. This step also helped to remove the DMSO from the gel which otherwise leads to artefactual blackening of the X-ray film [Bonner and Laskey, 1974]. The PPO impregnated gel was then dried under vacuum on a mini slab gel drier (Dry-gel Jr., Hoefer Scientific Instruments, San Francisco, CA) for 1 h. The DIFP-binding proteinases in the gel were visualized by placing the dried gel in
contact with X-ray film (Kodak XAR) at -70°C and developing the film after 48 to 72 h.

**Preparation of crude enzyme.** Mycelia from 3 L of culture broth were harvested after 120 h of fermentation by tangential flow filtration using a Pellicon filtration system (Millipore, Danvers, MA) equipped with 0.45 µm membranes (2 ft² of membrane area). The cell-free broth (2,800 ml) was chilled to 4°C by holding on ice and was concentrated to 200 ml using the Pellicon filtration unit equipped with a 10,000 MW cutoff membrane (5 ft² of membrane area). The retentate, which contained all the leucine aminopeptidase activity, was the source of enzyme for further purification and was lyophilized and stored as a powder at -20°C when not in use.

**Protein purification:**

1. **Free solution isoelectric focusing (FS-IEF).**

   **Preparation of the crude enzyme sample for FS-IEF.** The lyophilized powder containing the crude enzyme was resuspended in a minimal volume of double distilled water and dialyzed against two changes of 1000 vol of double distilled water at 4°C. The enzyme sample was clarified after termination of dialysis by centrifugation for 15 min at 12,000 x g and 4°C in a refrigerated centrifuge. The clarified supernatant was diluted to 55 ml with cold double distilled water and adjusted to 0.8% (v/v) 3-10
Ampholine (Bio Rad, Richmond, CA) concentration so as to generate a pH gradient from pH 3.0 to 10.0 at the end of the isoelectric focusing.

**Electrofocusing of proteins in the crude enzyme sample.** The entire 55 ml of the crude enzyme sample containing the ampholytes was loaded into a horizontal free solution electrophoresis apparatus (Rotofor Cell, Bio Rad, Richmond, CA). The enzymes in the crude preparation were fractionated by isoelectric focusing for 150 min at a constant power setting of 12 W according to manufacturer's instructions. Heat generated during isoelectric focusing was dissipated by cooling the electrofocusing chamber internally by means of a hollow ceramic cooling finger through which pre-chilled water (2°C) was circulated continuously.

2. Gel filtration chromatography.

**Low pressure Sephadex G-75 gel filtration.** Fractions from the isoelectric focusing procedure that showed activity against L-leu-pNA were pooled. The active enzyme was separated from ampholytes and other proteins in this preparation by passage through a Sephadex G-75 gel filtration column (100 cm x 1.5 cm). The gel filtration column was developed with a buffer composed of 50 mM HEPES (pH 7.0) and 50 mM NaCl. The flow of buffer through the column was maintained at a constant rate of 12 ml/h and 2.5 ml fractions were collected. Active fractions were pooled and concentrated by ultrafiltration through a hydrophilic, low adsorption YM membrane (Centriprep 10, Amicon, Danvers, MA) with a capacity to retain molecules larger than 10,000 MW.
FPLC Superose-6 gel filtration. Gel filtration chromatography was also performed using a Superose-6 (Pharmacia, Uppasala, Sweden) matrix contained in a 300 mm x 10 mm glass column and using the Pharmacia FPLC system. The proteins were separated according to size by passage through this matrix at a flow rate of 0.5 ml/min in an elution buffer composed of 50 mM Tris·HCl (pH 6.5) and 150 mM NaCl. Approximately 700 μg of protein were loaded and 250 μl fractions were collected for analysis of proteinase activity.

3. Ion exchange chromatography (IEX).

Low pressure IEX. DEAE-Sepharose (Sigma Chemical Co., St. Louis, MO) contained in a 10 cm x 2.5 cm glass column was pre-equilibrated at pH 6.0, pH 7.0 or pH 8.0 values (in different experiments) by passing 10 vol of 20 mM Tris·HCl, adjusted to the appropriate pH values through the matrix at 25 ml/h. The enzyme preparation to be fractionated was first dialyzed in 20 mM Tris·HCl that had been pre-adjusted to the appropriate pH value prior to loading onto the matrix. After sample application, the column was washed with two column vol of buffer and proteins bound to the matrix were eluted by generating a linear gradient of 0 to 1.0 M NaCl in buffer. Fractions of 5 ml were collected for analysis of proteinase activity.

CM-Sepharose chromatography was performed the same way as described above for the DEAE-Sepharose matrix using 20 mM Tris·HCl at pH 6.0, pH 7.0, or pH 8.0. Approximately 100 mg of the crude enzyme preparation were loaded on each of the matrices.
FPLC IEX. Ion exchange chromatography was performed using prepacked Mono-Q columns (HR 5/5, 0.5 cm x 5 cm or HR 10/10, 1 cm x 10 cm), pre-equilibrated with 5 vol of 20 mM Tris-HCl (pH 8.0), on a Pharmacia FPLC apparatus (Pharmacia, Uppasala, Sweden). The flow rate was adjusted to 1 ml/min when the Mono-Q (HR 5/5) was being used and to 2 ml/min when the Mono-Q (HR 10/10) was being used, and bound proteins were eluted away from the resin by application of a linear 0 to 1.0 M NaCl gradient in buffer. Fractions of 1 ml were collected (2 ml when Mono-Q HR 10/10 was used) in pre-sterilized 1 ml microcentrifuge tubes (Bio Rad, Richmond, CA) and frozen at -70°C for later analyses.

Effects of inhibitors and influence of metal ions on enzyme activity. The following proteinase inhibitors were assessed for their effect on ability of the purified proteinase to hydrolyze L-leucine-pNA: DIFP; NTA; Na₂-EDTA; EGTA; 1,10-phenanthroline; phosphoramidon; chymostatin; aprotinin; bestatin; pepstatin; and N-CBZ-PLG-hydroxymate (all purchased from Sigma). Inhibitor concentration was 3 x 10⁻³ M for DIFP and 1 x 10⁻³ M for the rest. The enzyme was dialyzed against 10 mM HEPES (8.0) to remove any contaminating cations and was preincubated separately with each inhibitor for 1 h at room temperature before residual enzyme activity was measured. 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 1,10-phenanthroline was also
determined. Enzyme inactivated with 10 mM 1,10-phenanthroline was incubated with each cation preparation for 5 min at 37°C before measuring restored activity.

**Polyacrylamide gel electrophoresis.** Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% resolving gel and a 3% stacking gel as described by Laemmli [1970]. The final concentration of SDS in both the gels was 0.1% (w/v). Electrophoretic conditions for separation of proteins through the gel, visualization of proteins in the gel by silver staining [Merrill et al., 1983], and calculation of the apparent molecular size by comparison to known standards were essentially identical to the procedure described in Chapter II.

**Effect of temperature on enzyme activity.** The enzyme assay was performed at various temperatures using a temperature-controlled cuvette holder attached to a Haake recirculating water bath. The interior of the cuvette holder was precalibrated using a temperature microprobe (YSI model 42SC tele-thermometer) so as to reflect the actual reaction temperature. The buffer (100 mM HEPES, pH 8.0) was prewarmed for 5 min at each of the assay temperatures prior to addition of the enzyme and the substrate.

**pH optimum.** The effect of pH on enzyme activity was determined by testing for activity against L-leu-pNA in each of the following buffers: MES (pH 6.4-7.0);
HEPES (pH 7.0-8.2); and Tris (pH 8.0-8.8) supplemented with 10 mM CaCl$_2$ and 10 mM MgCl$_2$. The concentration of each buffer was 100 mM and the enzymatic assay was carried out at ambient temperature.

**Determination of kinetic constants.** The efficiency of binding and hydrolysis of the synthetic substrate L-leu-pNA by the pure leucine aminopeptidase was investigated by standard methods. The $K_m$ value for L-leu-pNA was determined by generating a Lineweaver-Burk plot and an Eadie-Hofstee plot of the initial reaction velocities using a substrate range of 0.1 mM to 3 mM L-leu-pNA and 550 ng of the purified leucine aminopeptidase per assay. Each assay was carried out at ambient temperature in 20 mM Tris-Cl (pH 8.0) and the change in absorbance at 410 nm was monitored continuously (DU-64 spectrophotometer, Beckman Instruments, CA) for each substrate concentration for 5 min. Since the rate of hydrolysis of L-leu-pNA by the purified leucine aminopeptidase over time deviated from linearity after 5 min, change in absorbance values after 5 min were not taken into account while determining kinetic constants.
Results

Proteinolytic activities of *S. lividans*. The presence of both intracellular and extracellular proteinase activities was investigated by measuring hydrolysis of a variety of synthetic p-nitroanilide substrates. The results of these proteinase assays indicate that the major extracellular proteinase activity in the *S. lividans* culture extract is of the aminopeptidase type with a preference for leucine on the amino side of the cleaved peptide bond (Table 9). While the cell free broth of *S. lividans* exhibited some pro-pNA hydrolyzing activity, a strong intracellular proteinase activity which readily hydrolyzed pro-pNA was noted (Table 9). Essentially no intracellular or extracellular serine proteinase activity could be detected under the assay conditions utilized. Subsequent autoradiographic analyses involving $^3$H-DIFP binding of proteins in the extracellular broth indicated a single, very weak band on the developed X-ray film, suggesting the possible presence of a single serine proteinase (Fig. 21).

Physiological regulation of the leucine aminopeptidase type of extracellular proteinase activity (LapA) from *S. lividans* 1326. The total extracellular leucine aminopeptidase activity from *S. lividans* 1326 was maximum when the cells were cultivated in J medium which contained powdered milk, yeast extract, nitrate, phosphate and fructose along with trace metal elements (Table 10). This observation agreed well when both the volumetric production and specific production values were compared to each other and to values obtained from growth in other media.
Table 9. Proteinolytic activities of *S. lividans*.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Type of proteinase detected</th>
<th>Proteinase Activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intracellular</td>
</tr>
<tr>
<td>Azocasein</td>
<td>general</td>
<td>nd</td>
</tr>
<tr>
<td>2% Milk</td>
<td>general</td>
<td>0</td>
</tr>
<tr>
<td>L-leu-pNA</td>
<td>aminopeptidase</td>
<td>13</td>
</tr>
<tr>
<td>BAPNA</td>
<td>trypsin</td>
<td>2</td>
</tr>
<tr>
<td>N-CBZ-SAAPF-pNA</td>
<td>chymotrypsin</td>
<td>3</td>
</tr>
<tr>
<td>pro-pNA</td>
<td>aminopeptidase</td>
<td>100</td>
</tr>
<tr>
<td>ala-pNA</td>
<td>aminopeptidase</td>
<td>0</td>
</tr>
<tr>
<td>lys-pNA</td>
<td>aminopeptidase</td>
<td>0</td>
</tr>
<tr>
<td>gly-pNA</td>
<td>aminopeptidase</td>
<td>0</td>
</tr>
<tr>
<td>FAGLA</td>
<td>thermolysin</td>
<td>0</td>
</tr>
<tr>
<td>TAME</td>
<td>trypsin</td>
<td>0</td>
</tr>
<tr>
<td>FALGPA</td>
<td>collagenase</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* One unit of activity in the case of azocasein hydrolysis was the increase in absorbance at 340 nm of 0.001 A/min. One unit of activity in the case of 2% Milk hydrolysis is the decrease in absorbance at 580 nm of 0.001 A/min. Activity in the case of the p-NA substrates is reported as μmol/min. One unit of activity in the case of FAGLA is the decrease in absorbance at 314 nm of 0.001 A/min. In the case of TAME, one unit of activity is represented by an increase in absorbance at 247 nm of 0.001 A/min. In the case of FALGPA one unit of activity is the increase in absorbance at 345 nm of 0.001 A/min.

Figure 21. $^3$H-DIFP binding to proteins in culture supernatents of various streptomycetes. An autoradiogram showing the binding of $^3$H-DIFP to potential serine proteinases present in the cell free broth (CFB) of various streptomycete fermentations. Approximately 10 µg of protein per lane were fractionated by electrophoresis through a denaturing 12% polyacrylamide gel at a constant current of 20 mA. Lane 1: *S. lividans* 1326 CFB; Lane 2: *S. C5-A13* CFB; Lane 3: *S. rimosus* CFB; Lane 4: *S. coelicolor* CFB.
Table 10.  Effect of medium composition on the expression of the total leucine aminopeptidase activity of *S. lividans* 1326.

<table>
<thead>
<tr>
<th>Medium</th>
<th>S. lividans 1326 leucine aminopeptidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volumetric Production (Unitsa/L)</td>
</tr>
<tr>
<td>T</td>
<td>43</td>
</tr>
<tr>
<td>G</td>
<td>150</td>
</tr>
<tr>
<td>M</td>
<td>116</td>
</tr>
<tr>
<td>J</td>
<td>759</td>
</tr>
<tr>
<td>K</td>
<td>69</td>
</tr>
</tbody>
</table>

Medium composition is mentioned in text.

aOne unit = One μmole of p-nitroaniline released per min.

bCDW : Cell dry weight
The leucine aminopeptidase activity was lowest in the T medium. The J medium was best suited for expression of the extracellular proteinolytic activity, reflecting an approximately 18-fold increase in volumetric production and an approximately 18-fold increase in specific production of the enzyme over that from the T medium (Table 10). The G medium could sustain an approximately 3.5-fold increase in volumetric production of the leucine aminopeptidase activity as compared to the T medium while only about a 2.7-fold and a 1.6-fold increase in volumetric production was observed when the M and the K media respectively were used for cultivation of the cells (Table 10). The pH of the culture broths at the cessation of fermentation in all of these media was between 8.4 and 8.7.

**Fermentation.** Over the course of a 120 h fermentation, growth, pH and extracellular aminopeptidase activity were monitored at intervals of about 4 h during the exponential phase of growth and about 12 h during the stationary growth phase. The leucine aminopeptidase activity was first detected after 18 h of culture growth but began to increase significantly only after the onset of stationary phase (Fig. 22).

**Enzyme purification.** Cross-flow filtration of the fermentation broth proved to be an efficient and rapid procedure for harvesting the mycelia and for concentration of the proteinase from the cell free broth. Free solution isoelectric focusing sequestered a large amount of an unknown, contaminating brown pigment and other proteins away from the active enzyme. This method also helped to concentrate the active enzyme
Figure 22. Fermentation profile of *S. lividans* cultivated in MB medium. The dry weight of the mycelium (♦), and the activity of the L-leucine aminopeptidase (●) occurring in stationary phase are shown. The L-leucine aminopeptidase activity was measured by release of p-nitroaniline from Leu-pNA and is reported as Δ O.D.410/5 min.
Figure 22

![Graph showing the relationship between DRY WT (g/L) and TIME (HOURS).]
in conveniently workable volumes in a single step procedure. Much of the pigment still remaining in the IEF fractions that showed leucine aminopeptidase activity could be partially separated away from the enzyme by low pressure gel filtration chromatography through a Sephadex G-75 column (data not shown). The contaminating pigment in the semi-pure preparation of the enzyme at this stage could be separated further from the active enzyme by anion exchange chromatography at pH 8.0 using the Mono-Q resin. The leucine aminopeptidase activity initially eluted from the resin as two sharp peaks; one as a "wash through" peak and the other at approximately 350 mM NaCl concentration, while most of the pigment eluted out from the resin at a much higher NaCl concentration of about 500 mM (data not shown). Gel filtration of fractions pooled from the bound activity peak, and, which still contained traces of the brown pigment, through a Superose-6 FPLC column, yielded a single protein of ca. 34,000 M, (data not shown), which actively hydrolyzed L-leu-pNA. The enzymatic activity against L-leu-pNA that eluted in the wash-through fractions was fractionated by passing through a Superose-6 gel filtration matrix and was also represented by a single protein species of ca. 34,000 M, after analysis by denaturing polyacrylamide gel electrophoresis (data not shown). The 34,000 M, leucine aminopeptidase was purified ca. 35-fold by adhering to the protocol described above (data not shown). However, the brown pigment could never be completely disengaged from the active leucine aminopeptidase by this protocol.

During later stages of the research, an alternative, improved purification scheme was developed which involved successive Mono-Q and Superose-6
chromatography steps. The initial anion exchange step of the resuspended lyophilized cell free broth involved the use of the larger Mono-Q column (HR 10/10) where the leucine aminopeptidase activity along with some pigment eluted at approximately 350 mM NaCl concentration (Fig. 23). A 2-fold loss in the purification of the enzyme was noted at this step (Table 11). A wash-through peak of leucine aminopeptidase activity was also observed, which on fractionation through a Superose-6 gel filtration matrix and analysis by SDS-PAGE yielded a protein of 34,000 Mr (data not shown). Superose-6 gel filtration chromatography of the pooled active fractions that eluted from the Mono-Q resin at ca. 350 mM NaCl concentration removed more of the brown pigment from the enzyme preparation (Fig. 24), while providing an additional 5-fold enzyme purification (Table 11). A smaller Mono-Q ion exchanger (HR 5/5) used after this gel filtration step gave a further 20-fold purification of the enzyme (Table 11). All the pigment was separated away from the active leucine aminopeptidase which eluted at approximately 200 mM NaCl concentration (Fig. 25) while the pigment was eluted from the column at ca. 550 mM NaCl concentration. SDS-PAGE analysis of an aliquot of the fraction showing strong leucine aminopeptidase activity from this Mono-Q step revealed a polypeptide of a relative molecular mass of 34,000 daltons (Fig. 26). No wash-through peak of leucine aminopeptidase activity could be detected when the smaller Mono-Q ion exchanger was used as the last stage of the purification protocol (Fig. 25). While this protocol resulted in a 33-fold final purification of the leucine aminopeptidase, only 0.06% of
Figure 23. Mono-Q (HR 10/10) ion exchange chromatography. A pre-packed (HR 10/10) Mono-Q column was equilibrated with 20 ml of 20 mM Tris-HCl (pH 8.0) and developed with a 40 ml linear gradient of 0 to 1.0 M NaCl in 20 mM Tris-C. The flow rate of the buffer was maintained at 2 ml/min and fractions of 2 ml were collected and assayed for enzyme activity. The thick line indicates enzymatic activity against L-leu-pNA and the thin line indicates a continuous $A_{280}$ trace of the material passing through the column. Pool 1 is the wash-through peak of activity and Pool 2 contains the activity that bound to the ion exchanger. The sloping dotted line indicates the linear NaCl gradient.
Figure 23
Figure 24. Superose-6 gel filtration chromatography. A pre-packed Pharmacia Superose-6 column (HR 10/30) was equilibrated with 50 mM Tris-HCl (pH 6.5) containing 150 mM NaCl. The flow rate of buffer was at 0.5 ml/min and 250 μl fractions were collected for analysis. The thick line indicates enzymatic activity against L-leu-pNA and the thin line indicates a continuous A$_{280}$ trace of the material passing through the column.
Figure 25. Mono-Q (HR 5/5) ion exchange chromatography. A pre-packed (HR 5/5) Mono-Q column was equilibrated with 20 mM Tris·HCl (pH 8.0) and developed with a 25 ml linear gradient of 0 to 1.0 M NaCl in buffer. The flow rate of the buffer was maintained at 1 ml/min and fractions of 1 ml were collected and assayed for enzyme activity. The thick line indicates enzymatic activity against L-leu-pNA and the thin line indicates a continuous $A_{280}$ trace of the material passing through the column. The sloping dotted line indicates the linear NaCl gradient.
Figure 25
Table 11. Scheme for the purification of the 34,000 M<sub>r</sub> leucine aminopeptidase of <i>S. lividans</i>.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (mL)</th>
<th>Total protein (mg)</th>
<th>Activity (units)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Specific activity (units/mg)</th>
<th>Purification (-fold)</th>
<th>Recovery (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFB&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2800</td>
<td>2296</td>
<td>253</td>
<td>0.11</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>&gt;10 kDa retentate</td>
<td>200</td>
<td>430</td>
<td>94</td>
<td>0.22</td>
<td>2.0</td>
<td>37</td>
</tr>
<tr>
<td>Concentration by lyophilization</td>
<td>30</td>
<td>425</td>
<td>119</td>
<td>0.28</td>
<td>2.5</td>
<td>47</td>
</tr>
<tr>
<td>Mono-Q&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8</td>
<td>53.1</td>
<td>7.6</td>
<td>0.14</td>
<td>1.3</td>
<td>3</td>
</tr>
<tr>
<td>Superose-6</td>
<td>4</td>
<td>1.7</td>
<td>1.2</td>
<td>0.71</td>
<td>6.5</td>
<td>0.47</td>
</tr>
<tr>
<td>Mono-Q&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
<td>0.011</td>
<td>0.04</td>
<td>3.64</td>
<td>33</td>
<td>0.016</td>
</tr>
</tbody>
</table>

<sup>*</sup>Cell free broth  
<sup>b</sup>Mono-Q HR 10/10  
<sup>c</sup>Mono-Q HR 5/5  
<sup>d</sup>One unit = 1.0 µmol of p-nitoaniline released per minute
Figure 26. SDS-PAGE of the purified 34,000 M₅ leucine aminopeptidase of S. lividans. The proteins were separated by electrophoresis through a 12% vertical denaturing polyacrylamide gel at a constant current setting of 20 mA. The proteins in the gel were stained by silver nitrate. Lane 1, molecular weight standards (LMV-7, Sigma). The standards are (molecular weight given in parentheses): bovine albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (subunit; 36,000), carbonic anhydrase (29,000), bovine trypsinogen (24,000); and Lane 2, purified 34,000 M₅, leucine aminopeptidase.
the active enzyme could be recovered, indicating a large loss of enzyme activity during the purification protocol (Table 11).

**Enzyme characterization.** Table 12 shows the substrates which can be cleaved by the purified 34,000 Mₗ, *S. lividans* leucine aminopeptidase indicating that this enzyme possesses a narrow substrate specificity. Tables 13, 14, and 15 show the effect of metal ions on dialyzed enzyme, effect of inhibitors, and reactivation of the dialyzed enzyme which had been inactivated with 1,10-phenanthroline, respectively. Among the divalent cations tested, cobalt increased activity the most, followed by zinc (Table 13). The leucine aminopeptidase of *S. lividans* is nearly totally inactivated by 1,10-phenanthroline and partially inactivated by NTA (Table 14). Inactivated proteinase was fully reactivated by incubation with cobalt, but only partly by zinc (Table 15). The inactivated enzyme could not be reactivated by calcium (Table 15) and EDTA inhibited the leucine aminopeptidase activity only partially (Table 14). The Mₗ of this proteinase was found to be about 34,000 ± 1000 as indicated by SDS-PAGE (Fig. 26). The optimum temperature for activity for this enzyme is about 46⁰C (Fig. 27), the optimum pH for activity is 8.0 (Fig. 28), and the Kₘ value for L-leu-pNA is 300 μM as determined by the Lineweaver-Burke method is (Fig. 29) and 304 μM as determined by the Eadie-Hofstee method (Fig. 30). The theorotical Vₘₐₓ value for this enzyme was calculated to be 4.24 μM/min/mg and the Kₑₐₙ value for L-leu-pNA is 2.27/sec.
Table 12. Substrate specificity of the purified 34,000 M_r aminopeptidase of *S. lividans*.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Type of proteinase detected</th>
<th>Proteinase activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azocasein</td>
<td>General</td>
<td>0</td>
</tr>
<tr>
<td>2% Milk</td>
<td>General</td>
<td>0</td>
</tr>
<tr>
<td>L-leu-pNA</td>
<td>aminopeptidase</td>
<td>3.64</td>
</tr>
<tr>
<td>L-pro-pNA</td>
<td>aminopeptidase</td>
<td>0</td>
</tr>
<tr>
<td>L-gly-pNA</td>
<td>aminopeptidase</td>
<td>0</td>
</tr>
<tr>
<td>L-ala-pNA</td>
<td>aminopeptidase</td>
<td>0</td>
</tr>
<tr>
<td>L-lys-pNA</td>
<td>aminopeptidase</td>
<td>0</td>
</tr>
<tr>
<td>BA-pNA</td>
<td>trypsin</td>
<td>0</td>
</tr>
<tr>
<td>N-CBZ-SAAPF-pNA</td>
<td>chymotrypsin</td>
<td>0</td>
</tr>
</tbody>
</table>

*The units for azocasein hydrolysis are reported as increase at 340 nm of 0.001 A/min and the units for 2% milk hydrolysis are reported as a decrease at 580 nm of 0.001 A/min. Units of activity for hydrolysis of the p-NA substrates are reported as $\mu$mol/min. The values reported are from a single assay for each substrate.*
Table 13. Effect of divalent cations on the purified 34,000 Mr aminopeptidase of *S. lividans*. *

<table>
<thead>
<tr>
<th>Divalent cation</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoCl₂</td>
<td>177</td>
</tr>
<tr>
<td>Ca(CH₃COO)₂</td>
<td>100</td>
</tr>
<tr>
<td>Zn(CH₃COO)₂</td>
<td>100</td>
</tr>
<tr>
<td>SrCl₂</td>
<td>100</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>83</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>77</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>73</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>70</td>
</tr>
<tr>
<td>SrCl₂</td>
<td>39</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>24</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>13</td>
</tr>
<tr>
<td>CrCl₂</td>
<td>7</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>6</td>
</tr>
</tbody>
</table>

*Enzyme was first dialyzed against 10 mM HEPES (pH 8.0) for 24 h at 4°C. Divalent cations were added to a final concentration of 10 mM and incubated at 37°C for 5 min before assaying for activity against L-leu-pNA. The specific activity of the dialyzed enzyme was 3.64 μmole/min/mg and was taken as 100%. The values reported are averages of duplicate assays.
Table 14. Effects of inhibitors on the purified 34,000 M<sub>r</sub> metalloproteinase of *S. lividans*.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,10-phenanthroline</td>
<td>93</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;-EDTA</td>
<td>77</td>
</tr>
<tr>
<td>Bestatin</td>
<td>64</td>
</tr>
<tr>
<td>NTA</td>
<td>60</td>
</tr>
<tr>
<td>EGTA</td>
<td>46</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>0</td>
</tr>
<tr>
<td>Phosphoramidon</td>
<td>0</td>
</tr>
<tr>
<td>Chymostatin</td>
<td>0</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>0</td>
</tr>
<tr>
<td>N-CBZ-PLG-hydroxymate</td>
<td>0</td>
</tr>
<tr>
<td>DIFP</td>
<td>0</td>
</tr>
</tbody>
</table>

a) Activity was measured against L-leu-pNA as the substrate. Activity of the untreated enzyme was 3.64 μmole/min/mg and was taken as 100%. The values reported are averages of duplicate assays.

b) All inhibitors except DIFP were at 10 mM final concentration. DIFP was at 3 mM final concentration.
Table 15. Reactivation of the inactivated 34,000 M₉ metalloproteinase by divalent cations.

<table>
<thead>
<tr>
<th>Divalent cation</th>
<th>% Reactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn(CH₃COO)₂</td>
<td>70</td>
</tr>
<tr>
<td>Ca(CH₃COO)₂</td>
<td>0</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>100</td>
</tr>
</tbody>
</table>

*Enzyme was dialyzed against 1000 vol of 20 mM Tris·HCl (pH 8.0). The enzyme was first inactivated by incubating with 1,10-phenanthroline for 30 min at ambient temperature. Divalent cations were added to a final concentration of 10 mM and incubated for 15 min at ambient temperature before assaying for activity against L-leu-pNA. The specific activity of the untreated enzyme was 3.64 μmole/min/mg and was taken as 100%. The values reported are averages of duplicate assays.
Figure 27. Temperature optimum for activity of the 34,000 M₇ leucine aminopeptidase. A graph showing the effect of temperature on the leucine aminopeptidase activity. Enzyme activity is reported here as Δ O. D.₄₁₀/5 min. Assay buffer (100 mM HEPES, pH 8.0) was pre-warmed to each assay temperature for 5 min prior to addition of the enzyme and the substrate. The assay was monitored in a quartz cuvette placed in a temperature controlled cuvette holder. The temperature inside the cuvette holder had been calibrated earlier with a temperature microprobe. The values reported are averages of duplicate assays.
Figure 27
Figure 28. pH optimum for activity of the 34,000 $M_r$ leucine aminopeptidase. A graph showing the effect of pH on the leucine aminopeptidase activity. The enzyme activity is reported here as $A O. D_{410/5}$ min. Enzyme was incubated in MES (pH 6.4 - 7.0) ○; HEPES (7.0 - 8.2) ▲; and Tris (pH 8.0 - 8.8) ■, before assaying for proteinolytic activity against L-leu-pNA. Values reported are averages of duplicate assays. The buffer pH was adjusted either with 2 N HCl or 2N NaOH as required.
Figure 29. Lineweaver-Burk plot of the initial rate of hydrolysis of L-leu-pNA by 550 ng of the purified leucine aminopeptidase against various concentrations of L-leu-pNA (0.1 mM to 3.0 mM). The velocity, $V$, is in $\mu$mol/min/mg and the substrate concentration, $[S]$, is in mM. Values reported are averages of duplicate assays.
LINEWEAVER-BURK PLOT OF S. LIVIDANS AMINOPEPTIDASE ACTIVITY

Km = 300 μM
Vmax = 4.2 μmol/min/mg
Kcat = 2.3/sec

Figure 29
Figure 30. Eadie-Hofstee plot of the initial rate of hydrolysis of L-leu-pNA by 550 ng of the purified leucine aminopeptidase against various concentrations of L-leu-pNA (0.1 mM to 3.0 mM). The velocity, $V$, is in $\mu$mol/min/mg and the substrate concentration, $[S]$, is in mM. Values reported are averages of duplicate assays.
EADIE-HOFSTEE PLOT
OF S. LIVIDANS AMINOPEPTIDASE ACTIVITY

Leu-pNA: $K_m = 304 \mu M$
$V_{max} = 4.2 \mu mol/min/mg$

Figure 30
D) Discussion

Determination that *S. lividans* possesses only one major extracellular leucine aminopeptidase activity. Aretz *et al.* [1989] have indicated that *S. lividans* TK24 contained both aminopeptidases and chymotrypsin-like serine proteinases. Their data suggest in general terms that the serine proteinase activity was strong rather than weak [Aretz *et al.*, 1989]. Recently, others [Daza *et al.*, 1990] have used *S. lividans* 66 to clone a putative activator gene, *safA*, from *S. griseus* that enhances proteinase production in *S. lividans*. They chose to work with *S. lividans* due to absence of significant levels of host proteinolytic activity. Chang *et al.* [1990] have also found that *S. lividans* TK64 exhibited little or no proteinolytic activity when tested against casein or skinned milk. The authors utilized this property to clone the neutral proteinase gene (*npr*) from *S. cacaoi* into *S. lividans* TK64. Thus it seems that there is some disagreement in the literature about the presence or absence and the relative activity of various proteinases in *S. lividans*. To address this issue, I carried out two experiments to determine further the ensemble of proteinolytic activities of *S. lividans* 1326. First, I tested the activities of *S. lividans* 1326 culture supernatant on general proteinase substrates such as milk and azocasein (Table 9). While the concentrated cell free broth indicated some azocasein hydrolyzing activity, the purified leucine aminopeptidase showed no ability to hydrolyze azocasein. Gibb [1987] has also
observed that *S. lividans* 1326 contained proteinolytic activity only against azocasein. Thus it is possible that the minor azocasein hydrolyzing activity that I noticed in the concentrated cell free broth may be contributed by an unknown proteinase. Another extremely weak proteinase activity that could hydrolyze the synthetic substrate pro-pNA was also noted in the concentrated cell free broth of *S. lividans*. This pro-pNA hydrolyzing activity has been demonstrated to be chiefly intracellular (Dr. M. Butler, personal communication), which would suggest that the activity that I observed in the concentrated cell free broth against pro-pNA may be due to release of this enzyme from mycelia that had lysed during the course of the fermentation. Several 10-liter fermentation cultures of *S. lividans* were then analyzed for the activities of various proteinases on synthetic substrates as a function of time. I have repeatedly observed that culture broths of *S. lividans* 1326 demonstrated proteinolytic activity only against L-leucine pNA, and significant levels of this activity appeared only after the cessation of exponential growth. In several different experiments, only background levels of proteinase activity on the synthetic substrates succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroanilide (SAAPF-pNA; chymotrypsin-like proteinase substrate), or benzoyl-arginine-p-nitroanilide (BAPNA; trypsin-like proteinase substrate) were found in the culture supernatants of *S. lividans* 1326. DIFP, which is a potent, irreversible inhibitor of serine proteinases, was tested for its ability to inhibit the extracellular L-Leu-pNA hydrolytic activity of *S. lividans* 1326. No inhibition of this hydrolysis activity was observed, indicating that if any enzymes possessing serine proteinase activity are being produced by *S. lividans*, then they must be a negligible
portion of the total proteinase activity. After SDS-PAGE, only a single separated from the concentrated culture broth from *S. lividans* 1326 bound extremely weakly to $^3$H-DIFP, whereas several proteins from the culture broth of *Streptomyces* sp. C5-A13 bound the $^3$H-DIFP strongly (Fig. 21), as expected from previous results [Gibb *et al.* 1987, 1989]. Additionally, the DNA of several streptomycetes has previously been probed with a mixed, consensus oligonucleotide probe deduced from the active site of serine proteinases [Vinci, 1988]. Whereas the DNAs of *S. griseus*, *S. coelicolor*, *S. longisporus*, and *Streptomyces* sp. C5 contained several DNA fragments which hybridized intensely with the "serine active site" probe, only one DNA fragment of *S. lividans* hybridized very weakly with the serine proteinase active site probe [Vinci, 1988]. This extremely weak band could easily be the result of non-specific hybridization, and cannot be construed as proof for or against the presence of a serine proteinase gene in *S. lividans*. These data suggest that *S. lividans* 1326 contains at most one, minor serine proteinase with, at best, very weak proteinase activity. On the other hand, most of the other streptomycetes that have been tested in this laboratory contain strong serine proteinase activities, as demonstrated by, DIFP-sensitive cleavage of general substrates like casein and azocasein [Gibb, 1987, Vinci, 1988]. Thus, it appears that *S. lividans* 1326 is unusual with respect to most streptomycetes in its complement of extracellular proteinases.

**Physiological regulation of LapA.** Preliminary studies done at the shake flask level indicate maximal production of the extracellular leucine aminopeptidase activity
(amongst the media tested, single set of experiments) when *S. lividans* was cultivated in the J medium. Analysis of the constituents of the J medium reveal autoclaved milk (a proteinase substrate) as the major medium component. Even though this scenario conforms, at first glance, to the previously postulated hypothesis of induction of proteinases by the substrates that they hydrolyze [Gibb, 1987], it is interesting to note that *S. lividans* has no detectable milk hydrolyzing activity on its own [this work; Daza *et al.*, 1990; Chang *et al.*, 1990]. The volumetric production of LapA decreased by 11-fold and the specific production of LapA decreased by 2.3-fold when milk was omitted from the J medium, to give the K medium (Table 10). On further analyses, it was deduced that autoclaved milk may not be the only medium component that is affecting LapA production from *S. lividans*. This is supported by the fact that the M medium which supports only 15% of the volumetric production of LapA when compared to the Lap production in the J medium, also contains autoclaved milk as the major protein constituent. Yeast extract is an additional medium component that seems to be contributing to increased production of the LapA. This observation can be fortified by the data presented in Table 10. The G medium, while showing a 5-fold decrease in volumetric production of LapA as compared to the J medium, seems to sustain a slightly better production of LapA as compared to the M medium. Even though the G medium does not contain autoclaved milk, it does contain pancreatic digest of casein. Furthermore the G medium also contains yeast extract which is missing from the M medium. That the volumetric production of LapA from the G medium is much less than what could be obtained from the J medium may be
explained by the fact that the G medium contains a pancreatic digest of casein instead of autoclaved milk as in the J medium. The T medium, which contains a pancreatic digest of casein (but not autoclaved milk), but does not contain yeast extract, yielded the least amount of LapA, suggesting further the need of having autoclaved milk and yeast extract together in the medium to enhance production of this leucine aminopeptidase.

**Purification and characterization of LapA.** While FS-IEF initially seemed to be an attractive procedure to sequester active aminopeptidase into workable volumes in a single step, it also seemed to concentrate a proteinase inhibitor along with an unknown brown pigment. Alternatively, it is possible that the leucine aminopeptidase was inactivated by this procedure. I observed a 34-fold final purification of the aminopeptidase activity when FS-IEF was included in the initial purification protocol (data not shown), but since the unknown pigment could never be fully removed from the active enzyme, I decided to abandon this strategy and seek out a better method to purify the leucine aminopeptidase.

A successful purification protocol was developed that resulted in a 33-fold purification of the extracellular leucine aminopeptidase activity from *S. lividans*. Since the enzymatic activity responsible for hydrolysis of L-leu-pNA did not adhere to the DEAE anion exchange matrix at pH 6.0, 7.0 or 8.0, nor did it adhere to CM cation exchange matrix at all three of these pH values, a stronger ion exchanger in the form of the Mono-Q matrix was utilized. Even with the Mono-Q matrix, a "wash-
through" peak of leucine aminopeptidase activity was always observed during the initial stages of the purification protocol. Fractionation of the protein responsible for this wash-through activity by Superose-6 gel filtration reproducibly resulted in a protein of the same molecular mass as that which eluted from the Mono-Q column at ca. 250 mM NaCl concentration. Furthermore, this wash-through activity eluted at ca. 250 mM NaCl concentration when reloaded on the Mono-Q resin. Thus, this wash-through activity was likely to be due to elution of the enzyme from the column after overloading of the Mono-Q resin beyond its capacity to hold this particular enzymatic activity. The two-fold loss in activity observed during the initial ion exchange chromatography step involving the Mono-Q HR 10/10 matrix was presumably due to inactivation of the enzyme. The G-75 gel filtration step was omitted from the final purification protocol because it did not afford any significant advantage during the purification procedure.

The purified enzyme is not inhibited by DIFP, aprotinin, or chymostatin, indicating it to be other than a typical serine proteinase. This enzyme is also not inhibited by N-CBZ-PLG-hydroxymate indicating that it is not a collagenase, an assumption that is further supported by the lack of ability of this enzyme to hydrolyze the collagenase substrate FALGPA. That this enzyme does not belong to the thermolysin superfamily is indicated by the inability of this enzyme to hydrolyze FAGLA and the inability of phosphoramidon to inhibit enzymatic activity against L-leu-pNA. The purified enzyme was almost totally inactivated by 1,10-phenanthroline, and significantly inactivated by Na₂-EDTA, NTA, and EGTA, suggesting it to be a
metalloproteinase. Enzymatic activity against L-leu-pNA diminished significantly if the enzyme was stored at 4°C in the absence of Ca++ for more than one week. Enzyme preparations in a buffer containing Ca++ did not show any significant decrease in the L-leu-pNA hydrolyzing activity even after storage for up to a month at 4°C. This would suggest that Ca++ is potentially required for the stability of the enzyme but is not necessary for its activity against L-leu-pNA. The only synthetic substrate, amongst those tested, that could be hydrolyzed by this enzyme was L-leu-pNA. These data, combined with the fact that bestatin could inhibit activity of this enzyme suggests that this is a zinc metalloproteinase (aminopeptidase), perhaps with a preference for cleavage on the carboxyl side of leucine. Co++ ions seem to completely reactivate this enzyme which had previously been inactivated by 1,10-phenanthroline. The positive influence of Co++ has been reported for other leucine aminopeptidases from Bacillus spp. [Wagner et al., 1979], Saccharomyces spp. [Trumbly and Bradley, 1983], and Actinomycyes viscosus [Nakamura et al., 1984].

Comparison of S. lividans LapA with other streptomycete leucine aminopeptidases.
Table 16 shows the variety of leucine aminopeptidases that are produced by different streptomycetes. Morihara [1967] demonstrated the presence of a leucine aminopeptidase from S. fradiae. While the author did not mention the molecular weight of this proteinase, he showed its requirement for Ca++ for its activity. Uwajima et al. [1973] purified a 19,000 M, leucine aminopeptidase with a pI value of 7.4 from culture supernatents of S. peptidofaciens. This enzyme was inhibited by
EDTA and 1,10-phenanthroline. Enzyme inactivated by EDTA was successfully reactivated by addition of Ca++, indicating requirement of Ca++ for enzymatic activity. Two aminopeptidases of Mr 25,000 and Mr 23,000 have been purified from *S. griseus* [Vosbeck *et al.*, 1973]. Both enzymes were shown to require Ca++ for their enzymatic activity. Another Ca++-requiring leucine aminopeptidase has been purified from *Streptomyces* spp. 771 by Krest’yanova [1983]. More recently, Vitale *et al.* [1986] have purified an extracellular leucine aminopeptidase from *S. rimosus* as a byproduct of an industrial oxytetracycline fermentation. This enzyme is stable over a broad pH range, has a Mr of 27,000, a pI of 7.3 and requires Ca++ for its leucine aminopeptidase activity. My data indicate that the 34,000 Mr proteinase from *S. lividans* is a metalloproteinase (leucine aminopeptidase) which falls within the general category of other reported streptomycete leucine aminopeptidases, but it is different from other reported streptomycete leucine aminopeptidases in that it apparently utilizes Zn++ as its cation instead of Ca++.
Table 16. Leucine aminopeptidases from *Streptomyces* spp.

<table>
<thead>
<tr>
<th>Producer microorganism</th>
<th>Mr</th>
<th>Cation requirement</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. lividans</em></td>
<td>34,000</td>
<td>Zn**</td>
<td>This work</td>
</tr>
<tr>
<td><em>S. fradiae</em></td>
<td>nkt*</td>
<td>Ca**</td>
<td>Morihara <em>et al.</em>, 1967</td>
</tr>
<tr>
<td><em>S. rimosus</em></td>
<td>27,500</td>
<td>Ca**</td>
<td>Vitale <em>et al.</em>, 1986</td>
</tr>
<tr>
<td><em>S. griseus</em></td>
<td>25,000</td>
<td>Ca**</td>
<td>Vosbeck <em>et al.</em>, 1973</td>
</tr>
<tr>
<td><em>S. griseus</em></td>
<td>23,000</td>
<td>Ca**</td>
<td>Vosbeck <em>et al.</em>, 1973</td>
</tr>
<tr>
<td><em>S. peptidofaciens</em></td>
<td>19,000</td>
<td>Ca**</td>
<td>Uwajima <em>et al.</em>, 1973</td>
</tr>
<tr>
<td><em>Streptomyces</em> 771</td>
<td>nkt*</td>
<td>Ca**</td>
<td>Krest’yanova <em>et al.</em>, 1983</td>
</tr>
</tbody>
</table>

*n*not known.
CHAPTER IV

Summary and future experiments

I have been successful in purifying the neutral proteinase encoded by the \textit{snpA} gene of \textit{Streptomyces} sp. C5 from cultures of \textit{S. lividans}(pANT42) and \textit{S. galilaeus}(pANT42). This proteinase was significantly different from other previously reported neutral proteinases. Based on its small size, inhibition pattern, and substrate specificity, and in conjunction with other recent reports, I believe that I have isolated a novel proteinase which might represent a new sub-class of metalloproteinases. The gene responsible for this proteinolytic activity has been localized by my efforts onto a 1.1 kb DNA fragment. During subcloning experiments, \"\textit{snpR}\\text{"}, a putative potential "activator" gene that enhanced proteinase production in \textit{S. lividans} has been identified and separated (as a 1.35 kb fragment) from the \textit{snpA} gene by subcloning separately into pIJ702. The putative \textit{snpR} gene, which is transcribed divergently, lies upstream to the \textit{snpA} gene on plasmid pANT42. The deduced gene product (analyzed by collaborators of this laboratory), \"\textit{SnR}\\text{"},
indicates a DNA binding motif and has considerable resemblance to the LysR family of regulatory proteins.

I have also been successful in proving that *S. lividans* 1326, which was used as the host microorganism for cloning purposes, has a very small ensemble of proteinolytic activities. The major extracellular proteinase activity from *S. lividans* 1326, under growth conditions tested, appears to be an aminopeptidase active against L-leu-pNA which is represented by a single enzyme of 34,000 M,. Serine proteinase activity was found to be at best in trace amounts by undertaking experiments using synthetic substrates typically preferred by serine-like proteinases. This result was also strengthened by performing labelling experiments with the cell-free broth of *S. lividans* using ³H-DIFP, which binds irreversibly to the active site of serine proteinases. Only a single, weak band was observed on the subsequent autoradiogram. The positive control, C5-A13, which is an overproducer of serine proteinases, indicated strong binding with the ³H-DIFP as evident by an intense band on the X-ray film.

I have set the stage for more experiments to be performed to keep the proteinase project productive. Even though I have proven through genetic analysis that the *snpA* gene is indeed from *Streptomyces* sp. C5, the SnpA enzyme needs to be characterized from its producer. The major problem in purifying proteins from *Streptomyces* sp. C5 is the presence of copious amounts of a dark brown pigment which is difficult to remove and which interferes with standard protein isolation procedures [Gibb, 1987]. Now that pure SnpA is available, antibodies can be raised
in rabbits against this proteinase. Such antibodies can be conjugated to agarose beads by standard protocols to construct an affinity matrix for a single step purification of the SnpA from Streptomyces sp. C5. Thus this part of the research work is poised for a strong continuation. The antibodies, in conjunction with genetic analyses, would also provide a powerful tool in analyzing the regulation of the snpA gene, both in the native and in the cloned state. The snpR gene can also be studied further to understand its mechanism of regulating the proteinase activities in S. lividans. Whether or not this gene acts as a global regulator for other extracellular hydrolytic activities could prove to be a significant finding.

That the snpA gene is active early in the exponential phase of cell growth is an interesting result. The promoter for this gene can be further characterized and could potentially be used to construct a "secretion cassette". Such a cassette could conceivably be used to clone foreign genes, the advantage being that the cloned gene will then be expressed and secreted early in the fermentation. This would primarily economize time and would make this streptomycete system more competitive with other fast growing organisms like Escherichia and Bacillus species. During early stages of growth, only a few proteins are secreted out in the medium. Thus, downstream processing would be much easier as the correctly folded, predominant protein in the extracellular broth at this time would be the cloned gene product.

The major leucine aminopeptidase, of M, 34,000, from S. lividans has been purified and characterized biochemically. Antibodies can be raised in rabbits against the purified Lap in order to study its regulation in S. lividans.
BIBLIOGRAPHY


191


characterization of a gene encoding extracellular metalloprotease from *Streptomyces lividans*. Gene **111**:125-130.


