A novel aminopeptidase with unique substrate specificity was purified from a culture broth of *Sphingomonas capsulata*. This is the first reported aminopeptidase to demonstrate broad substrate specificity and yet release glycine and alanine with the highest efficacy. On a series of pentapeptide amides with different N-terminal amino acids, this enzyme efficiently releases glycine, alanine, leucine, proline, and glutamate with the lowest turnover value of 370 min\(^{-1}\) for glutamate. At pH 7.5 (pH optimum) and 25 °C, the kinetic parameters for alanine para-nitroanilide were found to be \(k_{\text{cat}} = 7600\) min\(^{-1}\) and \(K_m = 14\) mM. For alanine \(\beta\)-naphthylamide, they were \(k_{\text{cat}} = 860\) min\(^{-1}\) and \(K_m = 6.7\) mM. Polymerase chain reaction primers were designed based upon obtained internal sequences of the wild type enzyme. The subsequent product was then used to acquire the full-length gene from an *S. capsulata* genomic library. An open reading frame encoding a protein of 670 amino acids was obtained. The translated protein has a putative signal peptide that directs the enzyme into the supernatant. A search of the amino acid sequence revealed no significant homology to any known aminopeptidases in the available data bases.

The rising interest, both basic and applied, in aminopeptidases (EC 3.4.11) from different sources has led to the discovery of a number of enzymes that differ from each other in cellular location, catalytic mechanism, and substrate specificity (1–3). The majority of bacterial monoaminopeptidases are intracellular or membrane-bound metalloenzymes (1). Based on substrate specificity, bacterial monoaminopeptidases can be divided into two basic categories, specific aminopeptidases, and exopeptidases.

Proline and glycine are among the most difficult residues for aminopeptidases to hydrolyze because of their unique structures. Proline is unusual because of its cyclic structure, and glycine is identified by the lack of a side chain. Nature has developed a family of enzymes that recognize proline exclusively (4). A monoaminopeptidase that preferentially releases glycine with high efficiency has not yet been described and thus would be of high interest.

In this report we describe a novel extracellular monoaminopeptidase from *Sphingomonas capsulata*. This enzyme has a clear preference for N-terminal glycine and alanine. Because of this characteristic, this monoaminopeptidase has the potential to significantly enhance the degree of protein hydrolysis (5) when used as a supplement to endoproteases and other exopeptidases.

**EXPERIMENTAL PROCEDURES**

**Materials**

Chemicals used as buffers and reagents were commercial products of at least reagent grade. para-Nitroanilides of L-amino acids and peptide substrates were from Sigma or Bachem. Pentapeptide amides were synthesized at the Core Laboratories (Louisiana State University). *S. capsulata* strain IFO 12533 was purchased from the Institute for Fermentation (Osaka, Japan). A Whatman glass microfiber 2.7-µm filter and Nalgene Filterware equipped with a 0.45-µm filter were used for filtering buffers and supernatants. Protein purification was performed on an Amersham Pharmacia Biotech fast performance liquid chromatography device with column supports and resins from the same. Ultrafiltration units (10-, 180-, and 350-ml) and membranes were from Amicon. The Tricine\(^1\) gels and polyvinylidene difluoride membranes used in the peptide separation and sequencing process were from Novex. The molecular weight of proteins was estimated using Novex Multi-Mark pre-stained and Mark 12 SDS-PAGE markers. Endopeptidase Glu-C (V8 protease) was obtained from Roche Molecular Biochemicals. Assays were performed on a THERMOmax microplate reader, Shimadzu spectrophotometer UV160U, or Hewlett Packard Series 1050 HPLC system with column supports from Vydac, Inc. The protein sequencer was used from Applied Biosystems (model 476A). The sequencing reagents were purchased from PerkinElmer Life Sciences.

**Purification of 66-kDa Aminopeptidase**

Cultivation of *S. capsulata* strain IFO 12533 was performed for 15 h at 31 °C, 250 rpm, and initial pH value of 7.45. In 1.5 liters of medium composed per liter of 10 g of bactopeptone, 5 g of yeast extract, 3 g of NaCl, 2 g of K\(_2\)HPO\(_4\), 0.1 g of MgSO\(_4\)•7H\(_2\)O, and 5 g of glucose (autoclaved separately).

The culture broth supernatant (~1 liter) was obtained by initial centrifugation followed by filtration using a Whatman glass microfilter and Nalgene Filterware 0.22-µm filters consecutively. The filtrate was concentrated using an Amicon spiral ultrafiltration system equipped with a PM-10 ultrafiltration membrane. The sample was equilibrated with 10 mM phosphate buffer, pH 6.0, until the conductivity and pH value were equal to the loading buffer, 50 mM MES buffer, pH 6.0. The filtered solution was loaded onto a 240-ml column containing ~180 ml of SP-Sepharose fast flow, pre-equilibrated with 50 mM MES buffer, pH 6.0. Protein with aminopeptidase activity was eluted with a 240-mI gradient from 0 to 0.2 M NaCl in 50 mM MES buffer, pH 6.0. Fractions with enzymatic activity toward Ala-pNA were pooled, desalted using a PM-10 membrane, and equilibrated with 20 mM phosphate buffer, pH 7.0.

The pooled solution was then loaded onto a 20-ml Amersham Pharmacia Biotech Mono Q Bead column equilibrated with 20 mM phosphate buffer, pH 7.0. Protein with aminopeptidase activity did not bind to the column and was collected in the flow-through. The flow-through

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\(\text{1 The abbreviations used are: Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; pNA, para-nitroanilide; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinopropanesulfonic acid.}\)
Aminopeptidase from Sphingomonas sphaeroides

Aminopeptidase activity was monitored using Ala-pNA as the substrate. A stock solution of 100 mg/ml Ala-pNA in dimethyl sulfoxide was diluted with 50 mM phosphate buffer, pH 7.5, to a concentration of 2 mi/ml. The reaction mixture consisted of 2.5 ml of the enzyme solution and 0.5 ml of Ala-pNA. The reaction was initiated by addition of the enzyme and was followed for 5 min at 25 °C. The reaction was terminated by adding 5 ml of 0.1 M HCl. The resulting samples were measured at 280 nm for the tyrosine residue.

Enzymatic Hydrolysis of Pentapeptides (see structures in Table II) was performed at pH 7.5 in 50 mM MOPS buffer at 21 °C. Concentrations of the peptides in the incubation mixtures were between 1.10 and 1.14 mi. To stop the reactions, 50-μl aliquots of the incubation mixture were added to 190 μl of 100 mg/ml Ala-pNA in dimethyl sulfoxide (100 mg/ml) was diluted with 980-μl aliquots of sodium acetate-Tris-HCl buffer (0.125 mi) that had different pH values between 5.0 and 8.5. The resulting pH value of the substrate solutions was measured. A stock solution (0.05 mg/ml) of the aminopeptidase in 50 mM phosphate buffer was diluted 5-fold by 10 mM Tris-HCl buffer, pH 7.5. The reaction mixture contained 200 μl of the substrate solution and 10 μl of an enzyme solution at room temperature.

Temperature Optimum—An aliquot (970-μl) of 50 mM MOPS buffer, pH 7.5, was incubated for 15 min at the chosen temperature, which was maintained using a thermostated (Shimadzu cell positioner CPS-240A) microplate photometer. The 30-μl volume of the reaction mixture was added. The reaction was initiated by adding 7 μl of enzyme solution. Initial velocities were monitored over a 2-min period at 405 nm.

Thermal Stability—An enzyme aliquot (10-μl) was added to 190 μl of 50 mM phosphate buffer, pH 7.5, which had been preincubated for 30 min at the chosen temperature. The sample was placed on ice after a 20-min incubation. The samples were then assayed at room temperature following the protocol shown above.

Sequential Release of N-terminal Amino Acid Residues from a Natural Peptide—Leucine enkephalin was dissolved in 1 ml of 50 mM MOPS buffer, pH 7.5, to a final concentration of 1 mg/ml. Enzymatic hydrolysis was initiated by 8.3 μg of S. capsulata aminopeptidase. After incubation at room temperature (21 °C), aliquots of the incubation mixture were added to 0.1 n HCl to terminate the reaction. The free amino acids of the sample were analyzed by reverse-phase HPLC (8).

Cloning

Construction of a Genomic DNA Library—Genomic DNA was isolated from S. capsulata IFO 12533 using a QiaGen Tip-500 column as per the manufacturer's instructions. The library was constructed by ligating Sau3A partially digested (5–7-kilobase) S. capsulata IFO 12533 chromosomal DNA into the BamHI sites of the vector pJ1678 (8) (Fig. 1) and transformed into Escherichia coli XL1 Blue MR supercompetent cells (Stratagene, Inc.).

Polymerase Chain Reaction Amplification of Aminopeptidase Coding Sequences—The following primers were synthesized based on amino acid sequence data obtained from peptide fragments obtained following cyanogen bromide and V8 protease digestion of the purified aminopeptidase. Forward primer, 5′-GCTCTAGATCCCTCCC-3′; reverse primer, 5′-ACYYTMYGYACCC-3′ (R = A or G, Y = C or T, N = A or G or C or T). Amplification reactions were prepared in a 25-μl volume containing 100 ng forward and reverse primers, 1 μg of S. capsulata IFO 12533 chromosomal DNA as template, 1× polymerase chain reaction buffer (PerkinElmer Life Sciences), 200 μM each of dATP, dCTP, dGTP, and dTTP, and 0.5 units of AmpliTaq Gold (PerkinElmer Life Sciences). Reactions were incubated in a Stratagene Robocycler 40 (Stratagene) programmed for 1 cycle at 95 °C for 10 min, 35 cycles each at 95 °C for 1 min, 44 °C for 1 min, and 72 °C for 1 min, and 1 cycle at 72 °C for 7 min. The resulting product of ~190 base pairs was cloned into vector pCR1.1TOPO as per the manufacturer's instructions (Invitrogen, Inc.).

Identification of Aminopeptidase Clones—The genomic S. capsulata IFO 12533 library was screened by colony hybridization using a polymerase chain reaction-generated probe with the Genius chemiluminescent system (Roche Molecular Biochemicals) as per the manufacturer's instructions.

DNA Sequence Analysis of S. capsulata IFO 12533 Aminopeptidase Gene—DNA sequencing of two aminopeptidase-containing clones, pMR004.1–7 and pMR004.1–14, was performed with an Applied Biosystems model 373A automated DNA sequencer (Applied Biosystems, Inc.) on both strands using (a) the Primer Island Transposition method (Applied Biosystems, Inc.) as per the manufacturer's instructions and (b) the primer walking technique using dye-terminator chem-
Oligonucleotide sequencing primers were synthesized by Operon Technologies, Inc.

RESULTS

Localization and Purification of the Native Enzyme—The aminopeptidase was obtained from the supernatant of *S. capsulata* IFO 12533. Periplasmic extraction of the whole cells was also performed, but the enzyme was not present in the extract. It is evident from this result that the *S. capsulata* aminopeptidase is a secreted enzyme. The purification led to a protein that migrated as a single band of 66 kDa on SDS-PAGE (Fig. 2).

Physicochemical Properties of the Enzyme—A specific activity of 105 units/mg was determined for Ala-pNA under the condition described above, assuming that the \(A_{280}\) of a 1 mg/ml solution of the aminopeptidase is 1.89. The theoretical extinction coefficient of the enzyme was calculated based on the deduced protein sequence (10).

We were able to determine the kinetic parameters for Ala-pNA (\(k_{\text{cat}} = \frac{7600 \pm 850}{\text{min}^{-1}}\), and \(K_m = 14 \pm 2\) mM) and alanine \(\beta\)-naphthylamide (\(k_{\text{cat}} = 860 \pm 90\) min \(^{-1}\), and \(K_m = 6.7 \pm 1.1\) mM). However, the kinetic parameters for the other amino acid \(\alpha\)-nitroanilides and \(\beta\)-naphthylamides could not be accurately measured. This can be attributed to a combination of large \(K_m\) values, as well as poor solubility of the synthetic substrates. In terms of relative activity, the *S. capsulata* aminopeptidase preferably hydrolyzes alanine \(\alpha\)-nitroanilide. It also demonstrates high efficacy on \(\alpha\)-nitroanilides of leucine, methionine, glycine, and aspartic and glutamic acids (Table I).

The lower estimation of turnover numbers of the *S. capsulata* aminopeptidase on a series of pentapeptide amides with different N-terminal amino acids are shown in Table II. The enzyme exhibited the highest “turnover” on the pentapeptide amide with N-terminal glycine, followed by alanine, leucine, glutamate, and proline.

A study of the hydrolysis of several natural peptides, catalyzed by the *S. capsulata* aminopeptidase, revealed that the enzyme is capable of hydrolyzing a variety of peptide bonds. Among the bonds most readily hydrolyzed was a Gly-Gly bond (Table III). This is very unusual. The Gly-Gly bond is extremely resistant to enzymatic hydrolysis, probably because of a lack of side chain groups on both the N-terminal and penultimate amino acids. The *S. capsulata* aminopeptidase also hydrolyzed the peptides YAGFL and EALELARGAIFQA-amide with obvious “bottlenecks” at phenylalanine (data not shown). It is important to stress that it also releases N-terminal proline (Table II). This aminopeptidase, meanwhile, does not split off N-terminal amino acids with a penultimate proline and thus possesses no proline aminopeptidase activity.

Only \(o\)-phenanthroline demonstrated an inhibitory effect among the class-specific inhibitors tested. In this case, the residual activity was found to be 4% of the initial. Neither EDTA nor phenylmethylsulfonyl fluoride influenced the performance of the enzyme.

Certain inorganic anions that form salts with zinc of low solubility were found to have inhibitory properties. Among the

| Amino acid \(\alpha\)-nitroanilide | Relative activity |
|---------------------------------|------------------|
| Ala                             | 100              |
| Leu                             | 27               |
| Met                             | 24               |
| Gly                             | 14               |
| Asp                             | 14               |
| Glu                             | 12               |
| Lys                             | 3                |
| Pro                             | 0                |
| Ile                             | 0                |
| Val                             | 0                |
| Phe\(^a\)                       | 0                |

\(^a\) Solubility of \(\alpha\)-nitroanilides of glutamate and phenylalanine is lower than 7.7 mM. Their suspensions were used.
Amino acid sequences of the other bands were inconclusive. The digestion with cyanogen bromide resulted in fragments with molecular masses of 40, 25, 22, 20, 17, 10, 6, 5, and 4 kDa. The sequence FKDEPNPYDKARMADAKV was obtained from the 22- and 17-kDa fragments. The N-terminal sequence of the 10-kDa fragment was determined to be AVNG....

**Table III**

| Substrate | Released amino acid | Amount of released amino acid (nmol) after incubation |
|-----------|---------------------|-------------------------------------------------------|
| Leucine enkephalin | Tyr | 0.24 | 2.46 | 2.46 | 2.46 |
| | Gly | 0.40 | 5.10 | 4.70 |
| | Phe | 0.08 | 0.10 | 0.47 |
| | Leu | 0.11 | 0.09 | 0.43 |

The sequence of leucine enkephalin is Tyr-Gly-Gly-Phe-Leu.

**Discussion**

Bacteria hydrolyze different proteins to acquire essential amino acids from the pool of free amino acids and peptides. Certain organisms, such as the nutritionally fastidious Lactococcus lactis, apply a non-direct mechanism and employ a cascade of endo- and exopeptidases to release N-terminal glycine (1, 13). Others, such as Xanthomonas citri, have developed a more rational method, producing aminopeptidases with broad substrate specificity (14). In this report, we show that *S. capsulata* secretes a unique enzyme that preferably liberates N-terminal glycine. Because of a combination of large *Km* values for both para-nitroanilides and β-naphthylamides coinciding with low solubilities for these compounds, it is impossible to carry out a comprehensive study of the substrate specificity of the *S. capsulata* aminopeptidase utilizing kinetic data for these artificial substrates. Nonetheless, a few remarks can be made. This enzyme apparently discriminates similar amino acids effectively releasing leucine but not isoleucine or valine (Table I); results for alanine and β-naphthylamide unambiguously show that the structure of the leaving group for a substrate affects both *Kcat* and *Km*. Taking this into account and expecting that the substrate preference of an aminopeptidase toward derivatives of amino acids, such as para-nitroanilides, and natural peptides can be substantially different (15), the study of the hydrolysis of non-protected peptides catalyzed by the *S. capsulata* aminopeptidase was warranted. Comparative analysis of the Pro-pNA and Pro-Ala-Pro-Tyr-Lys-amide highlights the misleading role of the para-nitroanilide group. Apparently, it hinders the binding of at least some amino acid residues, for example proline, by the enzyme. More importantly, the *S. capsulata* aminopeptidase demonstrates an unusual substrate pattern with the order of preference Gly > Ala > Leu in terms of relative activity (Table II). A plausible explanation for these features could be a catalytic pocket that is not deep and exhibits very limited flexibility.

Leucine aminopeptidases are widely distributed in bacteria (1). Normally they are completely passive toward glycine (16). There are a few bacterial aminopeptidases described in the literature that demonstrate a reasonable ability to release alanine. The alanine-specific aminopeptidase N from *E. coli* (17) was not shown to release N-terminal glycine. The bimolecular constant for the thiol aminopeptidase from *X. citri* was almost 40-fold greater for alanine β-naphthylamide in comparison to glycine β-naphthylamide (14). It is highly unlikely that this enzyme is capable of cleaving a Gly-Gly bond.

Aminopeptidase from *S. capsulata* occupies a unique niche among proteases. This is the first reported enzyme...
that hydrolyzes natural peptides with bimolecular constant values that are similar for glycine and alanine or probably even higher for glycine. This extraordinary substrate preference is undoubtedly exhibited in the hydrolysis of leucine enkephalin catalyzed by the *S. capsulata* aminopeptidase (Table III). A quick and complete release of tyrosine and both glycine residues from this peptide was observed after a 1-h reaction, yet the Phe-Leu bond was nearly untouched. This clearly demonstrates the substantially higher catalytic efficacy of this enzyme for amino acids with small, rather than large, side chains.

A high $k_{cat}$ value, at least 5400 min$^{-1}$, for releasing glycine is also uncommon. Another interesting feature of the *S. capsulata* aminopeptidase is that, for an unknown reason, it is able to distinguish between similar amino acid residues, like tyrosine and phenylalanine, in the case of non-protected peptides (Table III).

Metalloaminopeptidases are predominant in bacteria (1). There are several indirect indications that this is a zinc metalloenzyme. First, effective inhibition of the *S. capsulata* aminopeptidase by o-phenanthroline, but not by phenylmethylsulfonyl fluoride or p-chloromercuribenzoic acid, was observed. The enzyme is also inhibited by certain anions, whose zinc salts have low solubility product value. We assume that EDTA, another strong chelator of transition metals, shows practically no inhibitory effect because of its voluminous structure and polyanionic nature resulting in its inability to penetrate close enough to the zinc of the catalytic site. A neutral pH optimum (18) for the *S. capsulata* aminopeptidase and a putative zinc binding domain HExXH (15) in its amino acid sequence are both indications of a zinc metalloenzyme.

Atomic absorption spectroscopy confirms the presence of one atom of zinc per molecule of enzyme. In addition, four atoms of iron were also detected. A plausible explanation might be non-specific binding of this metal to the protein molecule. The results that have been presented prove the scientific novelty of the *S. capsulata* aminopeptidase. We believe that this enzyme will receive significant attention from the food industry, as well. It has an "industrial" pH optimum, high specific activity, and great performance in releasing alanine and glycine, two amino acids that give considerably strong sweetness (19), in natural peptides.

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FIG. 3. The gene and the protein sequence.
REFERENCES

1. Gonzales, T., and Robert-Baudouy, J. (1996) *FEMS Microbiol. Rev.* **18**, 319–344
2. Watson, R. R. (1976) *Methods Microbiol.* **9**, 1–14
3. Schomburg, D., and Salzmann, M. (eds) (1991) *Enzyme Handbook. Class 3: Hydrodases*, Vol. 5, Springer-Verlag, Berlin
4. Cunningham, D. P., and O’Connor, B. (1997) *Biochim. Biophys. Acta* **1343**, 160–186
5. Adler-Nissen, J. (1985) *Enzymic hydrolysis of food proteins*, Elsevier Science Publishing Co., Inc., New York
6. Heinrikson, R. L., and Meredith, S. C. (1984) *Anal. Biochem.* **136**, 65–74
7. Sloma, A. P., Outrup, H., Dambmann, C., and Aaslyng, D. (April 22, 1997) U. S. Patent 5622850
8. Sloma, A. P., Sternberg, D. C., Adams, L. F., and Brown, S. H. (September 28, 1999) U. S. Patent 5958728
9. Sanger, F., Nicklen, S., and Coulson A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
10. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) *Protein Sci.* **4**, 2411–2423
11. von Heijne, G. (1984) *J. Mol. Biol.* **173**, 243–251
12. Rawlings, N. D., and Barrett, A. J. (1995) *Methods Enzymol.* **248**, 183–228
13. Tan, P. S. T., Poolman, B., and Konings, W. N. (1993) *J. Dairy Res.* **60**, 269–286
14. Osada, H., and Isono, K. (1986) *Biochem. J.* **233**, 459–463
15. Blinkovsky, A. M., Byun, T., Brown, K. M., Golightly, E. J., and Klotz, A. V. (2000) *Biochim. Biophys. Acta* **1480**, 171–181
16. Gonzales, T., and Robert-Baudouy, J. (1996) *FEMS Microbiol. Rev.* **18**, 319–344
17. Chapelet-Tordo, D., Lazdunski, C., Murgier, M., and Lazdunski, A. (1977) *Eur. J. Biochem.* **81**, 299–305
18. Fersht, A. (1985) *Enzyme Structure and Mechanism*, pp. 405, W. H. Freeman and Company, New York
19. Nishimura, T., and Kato, H. (1988) *Food Rev. Int.* **4**, 175–194

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