The DmpA aminopeptidase from *Ochrobactrum anthropi* LMG7991 is the prototype of a new terminal nucleophile hydrolase family

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The DmpA (D-aminopeptidase A) protein produced by *Ochrobactrum anthropi* hydrolyses *p*-nitroanilide derivatives of glycine and D-alanine more efficiently than that of L-alanine. When regular peptides are utilized as substrates, the enzyme behaves as an aminopeptidase with a preference for N-terminal residues in an L configuration, thus exemplifying an interesting case of stereospecificity reversal. The best-hydrolysed substrate is L-Ala-Gly-Gly, but tetra- and penta-peptides are also efficiently hydrolysed. The gene encodes a 375-residue precursor, but the active enzyme contains two polypeptides corresponding to residues 2–249 (α-subunit) and 250–375 (β-subunit) of the precursor. Residues 249 and 250 are a Gly and a Ser respectively, and various substitutions performed by site-directed mutagenesis result in the production of an uncleaved and inactive protein. The N-terminal Ser residue of the β-subunit is followed by a hydrophobic peptide, which is predicted to form a β-strand structure. All these properties strongly suggest that DmpA is an N-terminal amidohydrolase. An exploration of the databases highlights the presence of a number of open reading frames encoding related proteins in various bacterial genomes. Thus DmpA is very probably the prototype of an original family of N-terminal hydrolases.

Key words: N-terminal nucleophile amidohydrolase, peptidase, protease precursor, stereospecificity.

INTRODUCTION

Ochrobactrum anthropi LMG7991 contains two distinct intracellular enzymes that hydrolyse D-alanyl-*p*-nitroanilide (D-Ala-*p*-Na) [1]. One of them, DmpB (D-aminopeptidase B), is homologous to the D-alanyl aminopeptidase described by Asano et al. [2,3], and has very similar catalytic properties. The second enzyme, DmpA, is produced in much smaller quantities by the original strain. A DNA fragment was isolated that encoded an original 375-residue open reading frame (ORF) and whose integration in the pUC18 plasmid downstream of the *lacZ* promoter resulted in the production of large amounts of D-Ala-*p*-Na-hydrolysing activity. These experiments suggested strongly that this ORF represented the DmpA structural gene [1]. In this study, we describe the purification of the cloned DmpA protein and demonstrate that the 375-residue precursor is activated by a probably autocatalytic cleavage between residues 249 and 250. A detailed analysis of this cleavage site indicates that its properties are strikingly similar to those of N-terminal nucleophile (N-tn) amidohydrolases. Exploration of the databases (available at the website of the National Center of Biotechnology Information, http://www.ncbi.nlm.nih.gov) shows that DmpA is the prototype of a new family of N-tn hydrolases. The catalytic properties of the enzyme are also analysed.

MATERIALS AND METHODS

Molecular biology, oligonucleotides, kits, enzymes, chemicals and other materials

Components of culture media were from Difco (Detroit, MI, U.S.A.) and Biomérieux (Marcy-l'Etoile, France). Sequencing kits, oligonucleotides, plasmids, purification supports and columns were purchased from Pharmacia Biotech (Uppsala, Sweden). The QuickChange Site-Directed Mutagenesis kit was a Stratagene product (La Jolla, CA, U.S.A.). The *p*-nitroanilide and peptide substrates were from Bachem (Bubendorf, Switzerland) and Sigma (Bornem, Belgium), and protease inhibitors were from Boehringer Mannheim (Mannheim, Germany) or Worthington (Stoke-on-Trent, Staffs, U.K.). The DNA laser sequenators were from Pharmacia Biotech and EMBL (Heidelberg, Germany). The Cybertech CS-1 system (Cybertech, Berlin, Germany) was used to quantify proteins on gels after SDS/PAGE. The Constant Basic System Disintegrator was from Inceltech (Toulouse, France). Protein purification was performed with the help of an Åkta Explorer apparatus (Pharmacia Biotech). The VG Bio-Q Electrospray Triple Quadrupole Mass analyser, upgraded with a platform source, was from Micromass (Altrincham, Cheshire, U.K.), and was connected to a Harvard 11 syringe pump (Harvard Instruments, South Natick, MA, U.S.A.). The TLC plates (Silica gel 60F250) were from Merck (Darmstadt, Germany).

Purification of the DmpA protein produced by *Escherichia coli* DH5α

After transformation with pDML1102 [1], 2–3 colonies producing D-Ala-*p*-Na-hydrolysing activity were inoculated in 250 ml of Luria–Bertani (LB) medium containing ampicillin (50 μg/ml) and cultured during 16 h at 37 °C. Aliquots of this preculture were diluted 500 times in the same medium and the culture incubated for 16 h at 37 °C. The presence of inclusion

Abbreviations used: Dmp, D-aminopeptidase; ORF, open reading frame; D-Ala-*p*-Na, D-alanyl-*p*-nitroanilide; N-tn; N-terminal nucleophile; LB, Luria–Bertani; QSFF, Q–Sepharose Fast-Flow.

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Figure 1 Oligonucleotides designed for mutagenesis of the DmpA cleavage site and plasmids utilized for production of the mutants

WT, wild type.

bodies in the *E. coli* cells was detected by examination of the cells by phase-contrast microscopy.

Extraction of inclusion bodies

Culture (50 ml) was centrifuged and cells were resuspended in 10 ml of 10 mM Tris/HCl buffer, pH 8.0, containing 1 mM EDTA (buffer A). Inclusion bodies were then extracted as described by Goraj et al. [4].

Purification of the soluble active protein

The soluble fraction of DmpA was purified to more than 95 % as follows: 1 litre of culture was centrifuged and cells were resuspended in 50 ml of buffer A. Cells were disrupted with the Constant Basic System Disintegrator and the suspension centrifuged. Activity towards D-Ala-*p*-Na was detected in the supernatant. After dialysis against 50 mM potassium phosphate, pH 6.0, the protein solution was loaded on to a 200-ml Q–Sepharose Fast-Flow (QSFF) column (40 cm × 2.5 cm) equilibrated in the same buffer, and eluted with a 0–0.3 M NaCl linear gradient over a volume of 2 litres. Active fractions were pooled and dialysed against buffer A or diluted twice with 20 mM Tris/HCl, pH 8.0. The sample was then loaded on to the QSFF column equilibrated with buffer A and eluted with a 0.2–0.5 M NaCl linear gradient under the same conditions as above. Active fractions were pooled and the pure protein (60 mg) dialysed against 50 mM potassium phosphate, pH 7.0, concentrated and stored at −20 °C.

Immunodetection of the DmpA

Rabbit antibodies against the purified DmpA enzyme produced in *E. coli* were prepared by Gamma (Liège, Belgium). After SDS/PAGE, electroblotting on to a nitrocellulose membrane and treatment with antibodies, positive bands were revealed with the help of the Bio-Rad ImmunoBlot Alkaline Phosphatase Assay System (Bio-Rad, Nazareth, Belgium). After blotting, standard proteins were stained with 0.1 % (w/v) Ponceau S Red in 5 % (v/v) acetic acid and the membrane was rinsed with water. Prestained standard proteins (low-range, Bio-Rad) were also used.

Site-directed mutagenesis of the DmpA cleavage site

To assess the importance of the two residues constituting the cleavage site, the Gly-249 residue was replaced successively by Ala (the least-disturbing possible modification) and Asp. Conversely, the Ser-250 residue was replaced by residues possessing potential nucleophilic groups in their side chains and sometimes encountered in N-tn hydrolases (Thr or Cys) and by a residue devoid of such properties (Ala). The experiments were performed with the help of the QuickChange Site-Directed Mutagenesis kit (Stratagene), according to the supplied protocol. Oligonucleotides used for the mutagenesis reaction are described in Figure 1. The PCR reactions introducing the mutations were performed directly with the pDML1102 plasmid as a template. The reaction mixture contained 10 ng of DNA matrix (40 ng for the S250T mutation), 125 ng of each oligonucleotide (the S250T oligonucleotides were incubated previously for 10 min at 95 °C to denature the secondary structures), all dNTPs at 0.05 mM and 2.5 *Pfu* polymerase units, in a total volume of 50 μl. After heating for 30 s at 95 °C, PCR reactions were performed as follows: 1 min at 55 °C, 8 min at 68 °C, 12 cycles to replace one base, 16 cycles to replace two bases and for the S250T mutation. After completion of the PCR reactions and digestion of the non-mutated DNA matrix with the *Dpn*I enzyme, 1 μl of each PCR mixture was used to directly transform XL1Blue *E. coli* supercompetent cells purchased with the mutagenesis kit. Transformed cells were plated on LB + ampicillin and grown for 16 h at 37 °C. Plasmids extracted from the various clones were analysed by restriction (the G249A mutation created a second *Bss*HII GCGCGC site in the *dmpA* gene) and sequenced on both strands for verification of the presence of the mutation. Plasmids containing the mutated *dmpA* gene were named pDML1103–1107 according to the mutation (see Figure 1) and were then used to transform *E. coli* DH5α cells to produce the corresponding proteins.

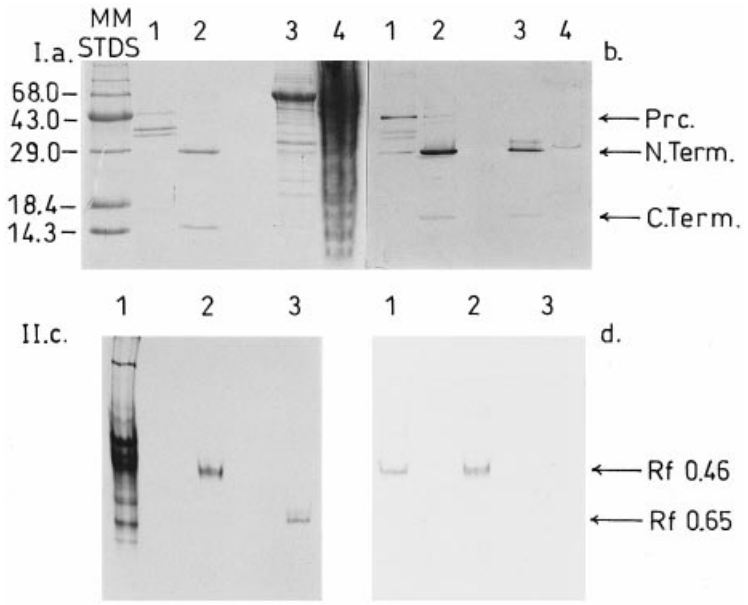

Figure 2 Electrophoretic analysis of DmpA samples

Panel I, Coomassie Brilliant Blue staining of the gel (**a**) and immunoblot revealed with rabbit anti-DmpA antibodies (**b**) after SDS/PAGE (18% polyacrylamide) of the following samples: MM STDS, Gibco-BRL protein molecular-mass standards (kDa); lanes 1, inclusion bodies from *E. coli* containing the uncleaved putative DmpA precursor (Prc.). Note that the intensity of the bands appearing at lower M_r values was found to increase with time upon strorage of the inclusion bodies, a result which suggested that these bands might represent degradation products of the 45000-M_r antibody-recognized protein. Lanes 2, active soluble DmpA purified from *E. coli* (N. Term. and C. Term., N- and C-terminal fragments); lanes 3, active DmpA partially purified from *O. anthropi* LMG7991 (fraction containing the highest activity after chromatography on the Superdex 75 molecular-sieve column [1]); lanes 4, soluble fraction of the total cell extract of *O. anthropi* LMG7991. Panel II, Coomassie Brilliant Blue staining of the gel (**c**) and immunoblot revealed with rabbit anti-DmpA antibodies (**d**) after electrophoresis on non-denaturing 8% polyacrylamide gel of the following samples: lanes 1, partially purified DmpA from *O. anthropi* LMG7991 (fraction containing the highest activity after chromatography on the Superdex 75 molecular-sieve column); lanes 2, active soluble DmpA purified from *E. coli*; lanes 3, active soluble DmpB purified from *O. anthropi* LMG7991. Identical R_F values were obtained with the zymogram method using D-Ala-*p*-Na as a substrate.

Production of the wild-type and modified enzymes

The wild-type protein was purified as described above. For each mutant, 10 ml of LB + ampicillin medium were inoculated with a few colonies and the culture grown for 8 h at 28 °C. These precultures were diluted in 250 ml of the same medium and grown for 16 h at 28 °C. Cells were collected by centrifugation, resuspended in 15 ml of buffer A and disrupted. The DNA was digested with benzonase (16 h at 4 °C) in the presence of 2 mM $MgCl_2$. Cell extracts were then centrifuged, and the supernatants analysed by dot-blot with rabbit anti-DmpA antibodies and by SDS/PAGE (15% polyacrylamide) followed by electroblotting and immunodetection as described above.

Chemical procedures

N-terminal sequences were determined as described previously [5] on a 477A pulsed-liquid sequenator. Approximate M_r values were obtained by SDS/PAGE (15% polyacrylamide), and accurate values by electrospray MS. Isoelectric points were measured by isoelectric focusing on Ampholine PAGplates, pH 3.5–9.5, detection of the active bands with the D-Ala-*p*-Na substrate [1] and measurement of the pH at the position of the active protein. The activity could also be detected directly after electrophoresis on non-denaturing 8% polyacrylamide gels by the same zymogram technique. Protein concentrations were estimated on the basis of the absorbance at 280 nm or with the help of the BCA protein assay kit (Pierce, Rockford, IL, U.S.A.). After SDS/PAGE, the intensities of Coomassie Brilliant Blue-stained bands were compared with those of protein standards. Gel scanning was done using the Cybertech CS-1 System. Substrate acetylation was performed as described previously [1].

Kinetic measurements

The enzyme activity was measured in 50 mM potassium phosphate, pH 8.0, or 100 mM Tris, pH 8.0, at 30 °C. For substrates containing a *p*-nitroaniline leaving group, variations of absorbance were monitored with the help of HP8452A or Uvikon spectrophotometers, at 405 nm for v_0 measurements ($\Delta\epsilon = 11\,500\ M^{-1}\cdot cm^{-1}$) and at 440 nm ($\Delta\epsilon = 2250\ M^{-1}\cdot cm^{-1}$) for complete time-course analysis. With substrates containing an N-terminal D-alanine, the released D-alanine was quantified by the D-amino-acid oxidase method [6]. Liberation of L-alanine from the peptide N-terminus was measured by oxidation with L-alanine dehydrogenase [7] or by quantification on TLC (see below). The k_{cat} and K_m parameters were obtained by non-linear regressions of the v_0 values using the ENZFITTER software package (Elsevier Biosoft, Cambridge, U.K.) or by analysis of the complete time-courses with the help of the integrated Henri–Michaelis equation [8]. Estimated errors on v_0 values were $< 10\%$. The degradation of non-chromogenic substrates was monitored by withdrawing samples at various times, separating the substrates and products by TLC at 20 °C and detecting their amino groups using ninhydrin [9]. Degradation of unstable substrates in the absence of enzyme was also monitored to account for their spontaneous degradation. The TLC solvent was *n*-butanol/acetic acid/5% NH_4OH in water (5.5:3:1.5, v/v/v). After ninhydrin revelation, the resulting picture was digitalized and analysed by densitometry with the Cybertech CS-1 system. Quantification was done by comparison with known

quantities of standard peptides and amino acids corresponding to the products expected in the reaction mixture. The estimated errors on product quantification were $\pm 20\%$.

RESULTS

Production and purification of DmpA

E. coli DH5α cells harbouring the pDML1102 plasmid contained inclusion bodies but also a high amount of soluble D-Ala-*p*-Na-hydrolysing activity. After purification to more than 95% homogeneity, only one protein band was detected upon non-denaturing PAGE (Figure 2, panel II) and electrofocusing (results not shown, pI = 5.0), but gel electrophoresis in the presence of SDS revealed two polypeptides with respective M_r values of about 30000 and 15000 (Figure 2, panel I). These were electro-transferred on to a PVDF membrane and submitted to N-terminal amino acid sequencing, which yielded the TSQTPTR-KPR and SIIVVLATDL sequences for the large and small peptides, respectively. These corresponded to residues 2–11 and 250–259 of the protein deduced from the sequence of the cloned gene. Antibodies were raised against the purified protein.

Presence of a potential precursor in the inclusion bodies

Inclusion bodies were solubilized and submitted to SDS/PAGE. The highest-M_r protein revealed by Coomassie Brilliant Blue staining reacted with the antibodies raised against the soluble and active DmpA (Figure 2, panel I). Its migration rate corresponded to an M_r value of about 45000, indicating that it might represent a precursor of the active enzyme. This hypothesis was corroborated by the identification of the first 10 N-terminal residues of the solubilized protein. These corresponded exactly with the N-terminal sequence of the large polypeptide, which was preceded by the Met residue corresponding to the first ATG codon of the gene. Attempts to obtain active enzyme by successive denaturation of the inclusion bodies and renaturation remained unsuccessful. The active protein was submitted to electrospray MS and two peptides exhibiting M_r values of 26564.3 ± 2.6 and 13736.8 ± 0.6 were found. These values corresponded well with the M_r values calculated on the basis of the sequences for residues 2–249 (M_r 26565) and 250–375 (M_r 13737). These results suggested that the active protein was derived from the precursor by elimination of the N-terminal Met residue and cleavage of the Gly-249–Ser-250 peptide bond without additional loss of residues (Figure 3).

Absence of detectable precursor in *O. anthropi*

A sample of partially purified enzyme from an *O. anthropi* culture [1] was submitted to non-denaturing PAGE and to polyacrylamide-gel isoelectric focusing. In both cases, a zymogram revealed the presence of D-Ala-*p*-Na-hydrolysing activities

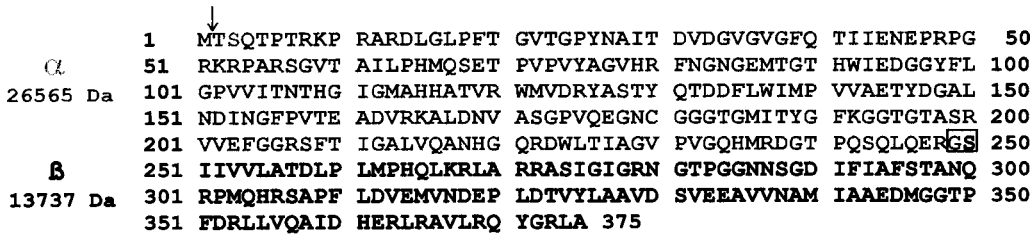

Figure 3 Sequence of the DmpA amidohydrolase

Hydrolysis of the Gly-249–Ser-250 peptide bond (boxed) cleaves the polypeptide precursor into two subunits: an α N-terminal and a β C-terminal peptides (by analogy with penicillin acylase). Note that the N-terminal M residue is also absent in the mature active protein (arrow). M_r values were determined by electrospray MS.

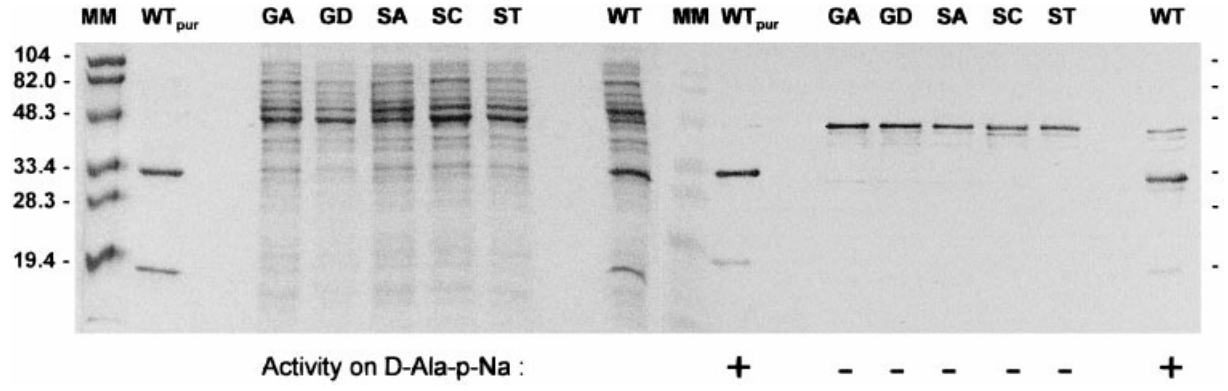

Figure 4 Electrophoretic analysis of DmpA mutants and correlation with activity using D-Ala-*p*-Na as a substrate

Coomassie Brilliant Blue-stained gel (left) and immunoblots revealed with anti-DmpA antibodies (right) after SDS/PAGE (15% polyacrylamide) of the following samples: MM, pre-stained molecular-mass standards (Bio-Rad); WT_{pur}, wild-type purified DmpA protein (1.5 μg) produced by *E. coli* DH5α; GA, GD, SA, SC, ST and WT, 15 μl of 10-fold diluted cell supernatants (equivalent to 25 μl of culture) containing the various mutants (G249A, G249D, S250A, S250C and S250T) and the non-mutated (WT) DmpA enzyme. +, activity found; −, no activity found.

Table 1 Activity of DmpA on Xaa-*p*-Na substrates

Values have S.D.s of ±10%. The following substrates were also hydrolysed by DmpA: L-Leu-*p*-Na (0.5% of Gly-*p*-Na), L-Met-*p*-Na (0.2%) and L-Val-*p*-Na (0.02%). Acetyl-D-Ala-*p*-Na, D-Leu-*p*-Na and D-Phe-*p*-Na were not hydrolysed significantly (< 0.005%). ND, not determined.

Substrate	DmpA		
	k_{cat} (s^{-1})	K_m (*mM*)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Gly-*p*-Na*	70	3	23000
Gly-*p*-Na†	70	3	23000
L-Ala-*p*-Na*	0.5	0.6	800
L-Ala-*p*-Na†	0.56	0.36	1550
D-Ala-*p*-Na*	4.0	0.54	7500
D-Ala-*p*-Na†	3.3	0.52	6300
L-Lys-*p*-Na†	0.11	0.4	275
L-Arg-*p*-Na†	0.14	0.4	350
L-Phe-*p*-Na†	ND	ND	17

* In 100 mM Tris/HCl, pH 8.0, at 30 °C.
† In 50 mM potassium phosphate, pH 8.0, at 30 °C.

Table 2 Hydrolysis of dipeptides by DmpA

The substrate concentrations were 10 mM unless otherwise stated. The rate values have S.D.s of ±20%. The following compounds were not hydrolysed significantly (< 0.005 μmol/min per mg of enzyme): acetyl-Gly-Gly, D-Ala-Gly, L-His-Gly, L-Asp-Gly, benzoyl-Gly-L-Ala, phenylacetyl-Gly-L-Ala, Gly-D-Phe, Gly-L-Asp, Gly-L-Gln and L-Trp-L-Phe.

Dipeptide	Rate of hydrolysis (μmol/min per mg of enzyme)
Gly-Gly	0.09
Gly-Gly-NH_2	0.25
Gly-Gly-OC_2H_5	0.5
L-Ala-Gly	0.07
L-Ser-Gly	0.005
L-Phe-Gly	0.11
L-Leu-Gly	0.04
L-Arg-Gly	0.13
L-Lys-Gly	0.13
Gly-L-Ala	0.09
Gly-D-Ala	0.02
Gly-L-Ser	0.02
Gly-L-Phe	0.4
L-Arg-L-Phe	0.44
L-Lys-L-Phe	0.35
L-Leu-L-Leu	0.06
L-Leu-L-Arg	0.09
L-Phe-L-Phe (3 mM)	0.07

in positions identical to those observed with the fully purified DmpA produced by *E. coli*. In the first case, a positive response to the antibodies after electroblotting was also observed (Figure 2, panel IId). After SDS/PAGE, a similar analysis of the same partially purified fractions highlighted two polypeptides in the same positions as those observed with the purified *E. coli* protein (Figure 2, panel Ib). The active enzymes produced by *E. coli* and *O. anthropi* also behaved similarly upon molecular-sieve filtration on Superdex 75 and chromatography on QSFF. By contrast, the 45000-M_r inactive precursor was never observed in *O. anthropi* cell extracts.

Table 3 Hydrolysis of tripeptides by DmpA

The substrate concentrations were 10 mM unless otherwise stated. The rate values have S.D.s of ±20%. The following compounds were not hydrolysed significantly: acetyl-Gly-Gly-Gly, acetyl-L-Ala-Gly-Gly and D-Leu-Gly-Gly.

Tripeptide	Initial rate of hydrolysis (μmol/min per mg of enzyme)
L-Ala-Gly-Gly	1.25
D-Ala-Gly-Gly	0.04
Gly-Gly-L-Ala	0.70
L-Phe-Gly-Gly	0.50
L-Leu-Gly-Gly	0.60
L-Ser-L-Ser-L-Ser	0.65
L-Leu-L-Leu-L-Leu	0.13
L-Phe-L-Phe-L-Phe (1 mM)	0.18

Table 4 Influence of the length of the peptide chain on the activity of DmpA

The substrate concentration was 2 mM in all cases. Values have S.D.s of ±20%.

Substrate	Initial rate of hydrolysis (μmol/min per mg of enzyme)
Gly_2	0.02
Gly_3	0.11
Gly_4	0.22
Gly_5	0.14

DmpA mutant analysis

At the stationary phase of growth, all *E. coli* cells producing the wild-type enzyme and the five mutants contained inclusion bodies, which were visualized by phase-contrast microscopy. Moreover, SDS/PAGE analysis of cell supernatants followed by Western-blot detection with anti-DmpA antibodies showed that all the mutant-producing cells contained a soluble protein whose electrophoretic mobility corresponded to that of the wild-type DmpA precursor, a 45 kDa protein, and which was recognized by the antibodies (Figure 4). By contrast, under the same conditions, the supernatant from cells producing the wild-type DmpA contained both uncleaved soluble precursor and cleaved protein. Incubating all cell extracts for several hours at 30 °C alone or in the presence of purified active wild-type DmpA failed to further cleave the soluble precursors. Of all samples, only the cell extract containing the wild-type enzyme was active on the D-Ala-*p*-Na substrate. This showed that cleavage and activity were related phenomena and that the intact Gly-249–Ser-250 site was essential for processing of the precursor into an active enzyme. Note that host cells that did not contain the plasmid or which harboured a plasmid devoid of the *dmpA* insert did not produce inclusion bodies and that the corresponding supernatants were devoid of proteins yielding positive Western-blot responses.

Kinetic characterization of DmpA

Activity was first measured towards Xaa-*p*-Na chromogenic substrates (Table 1). For these artificial peptide analogues, the highest activity was recorded with Gly and D-Ala derivatives. An activity profile was also established with a set of peptide substrates (Tables 2–4). Hydrolysis of tripeptides exhibiting different residues at their N- and C-termini was monitored to determine the

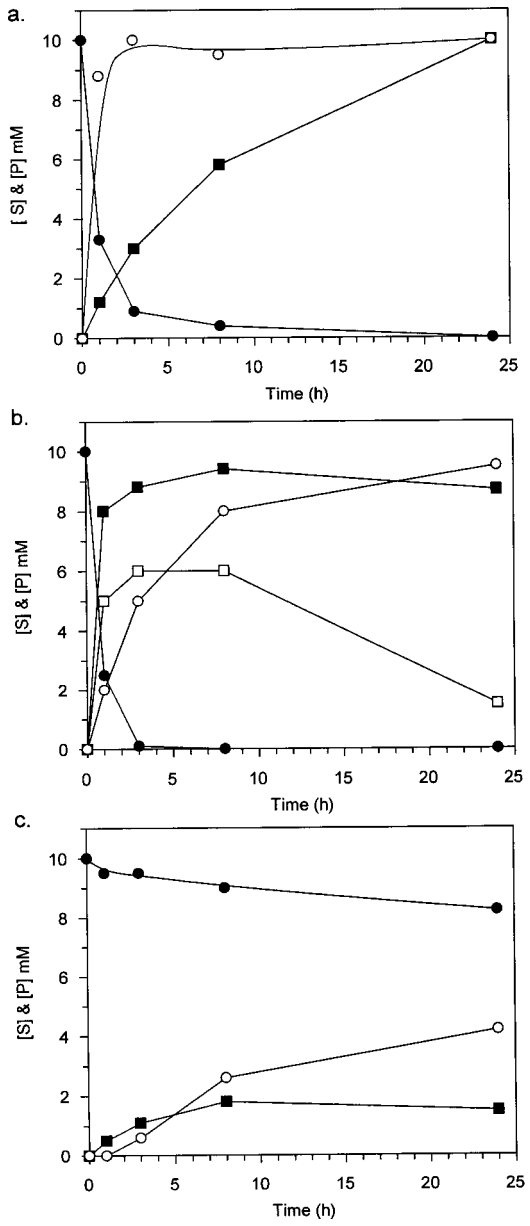

Figure 5 Hydrolysis of 10 mM tripeptides: Gly-Gly-L-Ala (a), L-Ala-Gly-Gly (b) and D-Ala-Gly-Gly (c) by 9.2 μM DmpA at 30 °C in 50 mM potassium phosphate, pH 8.0

Products were quantified after TLC and treatment of the plates with ninhydrin, as explained in the text. Gly and Gly-L-Ala (**a**) could not be well separated, and were quantified by comparison with the Gly standard. (**a**) ○, Gly + Gly-L-Ala; ●, Gly-Gly-L-Ala; ■, L-Ala. (**b**) ○, Gly; □, Gly-Gly; ●, L-Ala-Gly-Gly; ■, L-Ala. (**c**) ○, Gly; ●, D-Ala-Gly-Gly; ■, D-Ala.

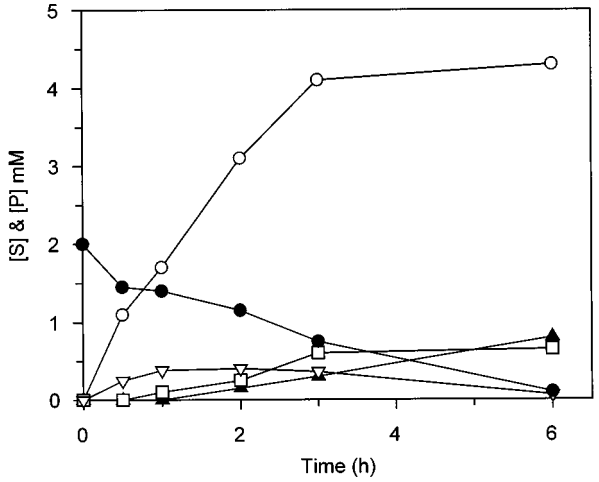

Figure 6 Hydrolysis of 2 mM Gly_5 by 2.1 μM DmpA in 50 mM potassium phosphate, pH 8.0, at 30 °C

The accumulation of the various intermediates is also shown: ○, Gly; ▲, Gly_2; □, Gly_3; ▽, Gly_4; ●, Gly_5.

Table 5 Activity of DmpA on simple ester and amide substrates

The substrate concentration was 10 mM in all cases. The values have S.D.s of ±20%.

Substrate	Initial rate of hydrolysis (μmol/min per mg of enzyme)
Gly-NH_2	1.4
L-Ala-NH_2	0.09
D-Ala-NH_2	0.23
Gly-OCH_3	1.2
L-Ala-OCH_3	0.09
D-Ala-OCH_3	1.2

preferential site of DmpA action. Figure 5 shows that the first products of tripeptide hydrolysis were always the N-terminal residues, and that the release of C-terminal residues was significantly delayed when compared with the release of N-terminal residues. These results suggest that C-terminal residues were only liberated when the dipeptide formed during the first reaction step was hydrolysed by DmpA. Note that tripeptides were better substrates than dipeptides (Tables 2 and 3), with the exception of D-Ala-Gly-Gly, which was a very poor substrate: in this case, the Gly-Gly dipeptide was not an observable product and was thus hydrolysed as soon as it was formed. Peptide degradation studies in the Gly_n series showed that the products appeared as the result of the release of one Gly from one extremity (exoprotease) and that the better substrates were tetra-, penta- and tripeptides (in that order, see Table 4 and Figure 6), whereas the dipeptide accumulated. An activity profile was also determined for residues in the N1 (the first N-terminal residue) and N2 (the following residue) positions, their configuration and their number (Tables 2–4). Acetylation of the substrate N-terminus suppresses the activity, a result showing that DmpA only recognizes substrates with a free N-terminal amino group. DmpA thus belongs to the aminopeptidase group of enzymes (EC 3.4.11). However, DmpA was also active towards D-Ala-NH_2, D-Ala-OCH_3 (Table 5) and D-Ala-*p*-Na (Table 1). Hence, DmpA is also a D-Ala-amidase-esterase. Note that the specific activity of DmpA towards D-Ala-*p*-Na was much weaker than that of the Dap enzyme, a strict D-aminopeptidase [2].

DmpA inhibition

The following protease inhibitors were tested unsuccessfully (30 min pre-incubation at 30 °C, at the concentration advised by

the supplier but also with 10-fold higher concentrations): antipain, aprotinin, bestatin, chymostatin, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), EDTA, leupeptin, pefabloc SC and 1,10-phenanthroline.

DISCUSSION

DmpA as a new N-tn amidohydrolase

The sequence of the purified DmpA protein confirmed that its structural gene was the ORF identified downstream of the *lacZ* promoter on the pDML1102 plasmid that encoded a 375-residue polypeptide. However, the soluble active protein produced in *E. coli* consisted of two distinct polypeptides corresponding to residues 2–249 (α-subunit) and 250–375 (β-subunit), as shown by N-terminal sequencing and electrospray MS (Figure 3). The absence of signal peptide was in agreement with the intracellular location of the protein. The enzyme was not purified from the original *O. anthropi* strain, where the quantities produced were too small, but the analysis of a crude preparation by immunological methods indicated the presence of two polypeptides of sizes similar to those of the α- and β-subunits. Upon overproduction in *E. coli*, large quantities of inclusion bodies that contained the complete and uncleaved polypeptide accumulated, but failed to yield active enzyme after solubilization. In some cases, small quantities of the large polypeptide were obtained in a soluble form (Figure 4), but it was not further processed upon incubation of the crude extract. In the mature protein, the two subunits remain associated by non-covalent bonds, since the sequence highlights only one Cys residue at position 180 of the precursor.

The cleavage of the 249–250 peptide bond could result from either the action of a cytoplasmic protease or self-processing. The facts that this cleavage, although very specific (see below), occurred in both *E. coli* and *O. anthropi*, and that the unprocessed protein was not observed in the latter strain, argued in favour of the second hypothesis. The cleaved Gly–Ser peptide bond is identical to the autocatalytic processing sites of the AcyII cephalosporin acylases produced by various *Pseudomonas* strains [10,11], and is generally similar to that found in N-tn amidohydrolases (Table 6).

Numerous site-directed mutagenesis results have been obtained with various N-tn hydrolases: 20S proteasomes [12,13], penicillin acylases [14,15], *Bacillus subtilis* PRPP amidotransferase [16,17], *Flavobacterium meningosepticum* aspartyl glucosaminidase [18] and the MIP (intein-extein) *in vitro* splicing system [19,20]. By analogy, five mutants were produced at the DmpA cleavage site. All the modified proteins were produced with similar good yields but remained both uncleaved and inactive, even when Ser-250 (position n) was replaced by other potential nucleophilic residues such as Thr or Cys. With the other N-tn hydrolases, mutations performed either at the $n-1$ or n positions, and which prevent cleavage, always result in a loss of activity [12,14,15,17,18,30]. Conversely, in position n, substitution by a residue with a nucleophilic side-chain sometimes allows processing but only yields an active enzyme in the case of the 20 S proteasome Thr → Ser mutant [12]. This suggests that the geometries necessary for cleavage and activity are different, and this is not surprising, since the free α-amino group of residue n that acts as a general base in the enzymic activity cannot play the same role in the cleavage reaction.

An analysis of the DmpA sequence with the help of the Chou and Fasman algorithm [31] shows that the N-terminal Ser of the β-subunit is located at the N-terminus of a β-sheet structure, in exactly the same way as the active nucleophile residue in N-tn hydrolases. The N-terminal Ser-1–Lys-18 peptide of the small β-subunit is highly hydrophobic (results not shown) and the fact that this subunit is not well recognized by antibodies raised against the native protein (Figure 2) suggests that most of it is buried inside the protein core. All these results indicate that DmpA might be the first representative of a new subfamily of N-tn hydrolases. The crystal structure of DmpA, which has now been solved at a resolution of 1.82 Å [C. Bompard-Gilles, V. Villeret, L. Fanuel, J.-M. Frère and J. Van Beeumen, unpublished work], confirms this assumption and shows a clear similarity between the fold of DmpA and that of the N-tn amidohydrolases of known structure [32].

A search of the databases (see the Introduction) revealed that the genomes of *Pyrococcus horikoshii*, *Pseudomonas aeruginosa*, *Ps. fluorescens*, *Bordetella pertussis* and *Neisseria meningitidis* contained ORFs encoding putative proteins very similar to DmpA (Table 6). When these sequences are compared with that of DmpA, Gly and Ser residues align with the position-249–250 Gly/Ser dyad of DmpA. Moreover, the sequences downstream of this dyad are nearly identical (Table 6) but, as in other N-tn hydrolases, the sequences upstream of the cleavage site are much less conserved. Lower degrees of similarity were found with putative proteins encoded by ORFs in the genomes of *Mycobacterium leprae* and *M. tuberculosis*. In these cases, alignments highlight striking similarities downstream of a potential cleavage site formed by an Asn/Thr dyad, which is reminiscent of the Gln/Thr or Asp/Thr cleavage sites in other N-tn hydrolases (Table 6). Surprisingly, these two putative mycobacterial proteins exhibit a significant similarity with the well-characterized amidase NylC, a nylon hydrolase produced by *Flavobacterium* sp. K172 [29]. It is thus very tempting to assume that all these proteins are cleavage-activated N-tn hydrolases and that the DmpA family can be divided into two subfamilies, containing either Gly/Ser- or Asn/Thr-cleavage sites. It can also be expected that all these proteins, when isolated, will exhibit amidohydrolase activities, as already demonstrated for the NylC enzyme, and that the members of the 'Gly/Ser' subfamily will behave as aminopeptidases.

It should be noted finally that the consequences of the elimination of the N-terminal Met of DmpA on the enzyme's activity remain undetermined.

Enzymic characterization of DmpA

DmpA liberates the N-terminal residues from peptide substrates with an efficiency depending on the peptide length: $Xaa_5 \cong Xaa_4 \cong Xaa_3 \gg Xaa_2$. Tripeptides of the form Xaa-Gly-Gly are hydrolysed with a consistently higher efficiency than the corresponding Xaa-Gly dipeptides; in the Gly_n series, the tetrapeptide is the best substrate. To allow recognition, the substrate α-amino group must be free (peptides whose N-terminus has been modified are not degraded) and optimal activity requires an N-terminal residue in an L-configuration. Thus DmpA may possess a negative charge in the substrate-binding pocket that could stabilize the N-terminal positive charge of the peptide substrate and position it in a catalytically productive geometry, as observed in the PepC aminopeptidase [33]. Its catalytic profile for residues at the first N-terminal position in dipeptides is: basic amino acid (Arg, Lys) > Phe > aliphatic amino acid (Leu, Gly, Ala) > hydroxylated amino acid (Ser). An acidic residue in the first or second N-terminal position precludes hydrolysis. This underlines the importance of the N-terminal charge of the substrate on its hydrolysis: a double positive charge (basic amino acid) favours substrate binding and hydrolysis, whereas an ionized acidic group is detrimental to the activity. Dipeptides exhibiting a His or a Trp residue at the N-terminus are not hydrolysed. A Phe at the second position from the N-terminus increases the DmpA activity

Table 6 Alignment of the cleavage sites of N-tn and potential N-tn amidohydrolases

The N- and C-terminal residues of the cleaved peptide bond are shown in bold. The cleavage either eliminates an N-terminal propeptide of variable length or yields two subunits with or without the concomitant elimination of a propeptide or an internal peptide (both processes occur in penicillin acylase [25]). Some other enzymes might also be related to N-tn hydrolases [26]: *Pseudomonas* 7A glutaminase-asparaginase [27] and *Lactobacillus* 30a histidine decarboxylase [28]. aa, amino acids. Addresses in data banks: a, DDBJ|AB009466; b, gnl|PAGP|*P. aeruginosa*_contig 163; c, gb|AF004848; d, gnl|Sanger|*B. pertussis*_contig 578; e, gnl|TIGR|GNMCQ43R; f , gb|U00014|*M. leprae* cosmid B1549_C2_208; g, EMBL|Z73902|*M. tuberculosis* H37Rv.

Enzyme		Cleavage site and position in the precursor		Precursor length	Result of cleavage	Reference
Proteasome 20 S (β-subunit)						
*Thermoplasma acidophilum**	1	MNQTLET**GT**TTVGITLKDAV	20	211 aa	Propeptide	[12]
Human (Z)†	36	LPKVRKT**GT**TIAG	48	Propeptide	[12,13]	
Glutamine PRPP amidotransferase						
*Bacillus subtilis**	4	EIKGLNE**EC**GVFG	16	465 aa	Propeptide	[16]
Saccharomyces cerevisiae†	1	**MC**GILG	6		Propeptide	[16]
Chicken†	4	EELGIRE**EC**GVFG	16		Propeptide	[16]
Aspartylglucosaminidase or glycosylasparaginase						
Human*	198	TEDDRGH**DT**IGMVVIHKTGH	217	346 aa	2 Subunits	[21]
Flavobacterium meningosepticum†	189	IVNIENH**DT**IGMIALDAQGN	208	340 aa	2 Subunits	[18]
γ-Glutamyltranspeptidase‡						
E. coli	383	LAPYESN**QT**THYSVVDKDGN	402	580 aa	2 Subunits	[22]
Human	374	YTP-DDG**GT**AHLSVVAEDGS	392	580 aa	2 Subunits	[22]
Penicillin acylase						
*E. coli**	282	GLAGYPT**TS**NMWVIGKSKAQ	301	846 aa	2 Subunits	[14,15,23]
Kluyvera citrophila†	282	GLAGYPT**TS**NMWVIGKNKAQ	301	844 aa	2 Subunits	[24]
Cephalosporine acylase‡						
AcyI *Pseudomonas* sp. SE83	360	LSGGESA**DT**THVTVADAMGN	379	558 aa	2 Subunits	[10]
AcyII *Pseudomonas* sp. SE83	232	ASDAAGG**GS**NNWAVAPGRTA	251	774 aa	2 Subunits	[10]
Pseudomonas sp. SY-77-1	191	PPDLADQ**GS**NSWAVAPGKTA	210	311 aa	2 Subunits	[11]
DmpA or putative proteins potentially related to DmpA						
O. anthropi	242	QSQLQER**GS**IIVVLATDLPL	261	375 aa	2 Subunits	This study
Pyococcus horikoshii	231	GRGGEGK**GS**IIMIIATDAPL	250	361 aa	?	(a)
Ps. aeruginosa	233	EEGTPGM**GS**IVVILATDAPL	252	370 aa	?	(b)
Ps. fluorescens§		ERGTPGM**GS**IVVIIATDAPL		?	(c)	
Bordetella pertussis¶		PAGQPEK**GS**IILLLATDAPL		?	(d)	
Neisseria meningitidis¶		TMMQEN**GS**LIVWHGQDKPL		?	(e)	
Mycobacterium leprae	236	KSPLSAL**NT**TIGVVATDATL	255	362 aa	?	(f)
Mycobact. tuberculosis H37rv	225	GAFNTPF**NT**TIGVIACDAAL	244	344 aa	?	(g)
6-Aminohexanoate oligomer hydrolase (nylon hydrolase)						
Flavobacterium	259	PPVTEAG**NT**TISAIVTNVRM	278	355 aa	?	[29]

* Enzymes whose three-dimensional structure have been determined and for which the involvement of the β-subunit N-terminal residue in the catalytic process has been demonstrated.
† Enzymes exhibiting catalytic properties and sequence analogies with the first group.
‡ Potential N-tn hydrolases [32].
§ Incomplete ORF.
¶ Complete ORF not available.

towards dipeptide substrates and the best-hydrolysed dipeptide substrate was L-Arg-L-Phe. It is also interesting to note that the stereospecificity requirements do not appear to be absolute, since the Gly-D-Ala dipeptide and the D-Ala-Gly-Gly tripeptide were hydrolysed significantly, although 4- and 30-fold less efficiently than the L-isomers, respectively. Among all the peptides tested, L-Ala-Gly-Gly was the best substrate.

Simple amino acid amides and ester derivatives were also hydrolysed by DmpA, with Gly derivatives as the best substrates. However, in this case, marked differences were observed when compared with the 'normal' peptides. First, the *p*-nitroanilide derivatives of L-Arg, L-Lys, L-Leu and L-Phe were much poorer substrates than Gly-*p*-Na, in sharp contrast to the results obtained with the dipeptides. Secondly, and more strikingly, the D-Ala derivatives were hydrolysed 3–10-fold faster than their L-Ala counterparts. However, this does not hold for larger amino acids, since D-Leu-*p*-Na and D-Phe-*p*-Na were not hydrolysed at all. The recognition of D-Xaa derivatives thus seems to be limited to D-Ala: DmpA is thus also a D-Ala-amidase-esterase. We verified that two well-characterized L-aminopeptidases, microsomal leucine aminopeptidase from pig kidney and aminopeptidase I from *Streptomyces griseus*, were nearly inactive on the D-Ala-*p*-Na stereoisomer (D-Ala-*p*-Na/L-Ala-*p*-Na = 0.005 and 0.01, respectively). Moreover, no mention of this type of D-stereospecific activity for L-aminopeptidases is found in the literature [33]. This double L-aminopeptidase/D-amidase-esterase activity seems to be an original property of the DmpA enzyme, although it has been shown that the exact structure of the substrate might influence the stereospecificity of lipases [34,35] and DD-peptidases [36], a phenomenon that is generally attributed to steric hindrance or to the quality of the leaving group.

We demonstrate here that the specificity profile for chromogenic *p*-nitroanilide substrates reflects the activity of the enzyme on simple amide and ester derivatives rather than on 'normal'

peptides. The former compounds probably bind into the enzyme active site in a somewhat different way, resulting in a modified position of their scissile bonds relative to the enzyme catalytic groups. More detailed hypotheses attempting to explain such a 'changing' stereospecificity will have to rely on the detailed analysis of the DmpA protein's three-dimensional structure. Nevertheless, the kinetic results show that attempts to characterize a new peptidase with chromogenic substrates can only be hazardous, as also noted by Niven [37,38].

Possible physiological roles of DmpA and DmpB

Thus *O. anthropi* produces both DmpA, an L-aminopeptidase, and DmpB [1] or Dap [2], two very similar D-aminopeptidases. To date, this latter activity has only been found in this species, which has been recently separated from the *Achromobacter* genus [39] and is still poorly characterized. These D- and L-aminopeptidases are probably parts of the general peptidase pool of *O. anthropi*, enzymes used by these highly proteolytic bacteria to extract amino acids from peptides present in the medium. In this respect, it is interesting to note that DmpA appears to exhibit a rather wide specificity profile and might thus contribute to the hydrolysis of a vast number of small peptides transported into the cell. Similarly, *Lactococcus lactis* possesses a set of proteases, among which are 8–10 intracellular or membrane-bound aminopeptidases, that produce the essential amino acids from milk proteins (for instance, see [40]). The physiological role of the DmpA-like proteins whose encoding gene was found in other species remains mysterious.

Conversely, bacteria producing enzymes that act on molecules containing amino acids in the D-configuration, such as DmpB, could hydrolyse bacterial cell-wall-degradation products present in the medium. Some strains of *Bacillus*, a highly proteolytic bacterial genus, also produce enzymes acting on D-amino acid-containing peptides [41,42]. However, it seems that these enzymes do not take part in intracellular peptidoglycan recycling. So far, no enzymic activity has been discovered that liberates a peptidoglycan-degradation product exhibiting an N-terminal D-amino acid residue in the cell cytoplasm. A D-Ala-D-Ala dipeptidase (VanX) involved in vancomycin resistance has been described and well studied [43], but its sequence does not exhibit any similarity with those of Dap and DmpB.

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REFERENCES

1 Fanuel, L., Thamm, I., Kostanjevecki, V., Samyn, B., Joris, B., Goffin, C., Brannigan, J., Van Beeumen, J. and Frère, J.-M. (1999) Cell. Mol. Life Sci. **55**, 812–818
2 Asano, Y., Nakazawa, A., Kato, Y. and Kondo, K. (1989) J. Biol. Chem. **264**, 14233–14239
3 Asano, Y., Kato, Y., Yamada, A. and Kondo, K. (1992) Biochemistry **31**, 2316–2328
4 Goraj, K., Renard, A. and Martial, J. A. (1990) Protein Eng. **3**, 259–266
5 Ledent, P., Duez, C., Vanhove, M., Lejeune, A., Fonzé, E., Charlier, P., Rhazi-Filali, F., Thamm, I., Guillaume, G., Samyn, B. et al. (1997) FEBS Lett. **413**, 194–196
6 Frère, J.-M., Leyh-Bouille, M., Ghuysen, J. M., Nieto, M. and Perkins, H. R. (1976) Methods Enzymol. **45**, 610–636
7 Williamson, D. H. (1996) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), Verlag Chemie, Weinheim
8 De Meester, F., Joris, B., Reckinger, G., Bellefroid-Bourguignon, C., Frère, J.-M. and Waley, S. G. (1987) Biochem. Pharmacol. **36**, 2393–2403
9 Randerath, K. (1966) Thin Layer Chromatography, 2nd edn., Verlag Chemie and Academic Press, New York and London
10 Matsuda, A., Toma, K. and Komatsu, K.-I. (1987) J. Bacteriol. **169**, 5821–5826
11 Matsuda, A. and Komatsu, K.-I. (1985) J. Bacteriol. **163**, 1222–1228
12 Seemüller, E., Lupas, A. and Baumeister, W. (1996) Nature (London) **382**, 468–470
13 Zwickl, P., Grziwa, A., Pühler, G., Dahlmann, B., Lottspeich, F. and Baumeister, W. (1992) Biochemistry **31**, 964–972
14 Slade, A., Horrocks, A. J., Lindsay, C. D., Dunbar, B. and Virden, R. (1991) Eur. J. Biochem. **197**, 75–80
15 Choi, K. S., Kim, J. A. and Kang, H. S. (1992) J. Bacteriol. **174**, 6270–6276
16 Zhou, G., Broyles, S. S., Dixon, J. E. and Zalkin, H. (1992) J. Biol. Chem **267**, 7936–7942
17 Mäntsälä, P. and Zalkin, H. (1984) J. Biol. Chem. **259**, 14230–14236
18 Guan, C., Cui, T., Rao, V., Liao, W., Benner, J., Lin, C.-L. and Comb, D. (1996) J. Biol. Chem. **271**, 1732–1737
19 Shao, Y., Xu, M.-Q. and Paulus, H. (1996) Biochemistry **35**, 3810–3815
20 Xu, M.-Q. and Perler, F. B. (1996) EMBO J. **15**, 5146–5153
21 Oinonen, C., Tikkanen, R., Rouvinen, J. and Peltonen, L. (1995) Nat. Struct. Biol. **2**, 1102–1108
22 Suzuki, H., Kumagai, H., Echigo, T. and Tochikura, T. (1989) J. Bacteriol. **171**, 5169–5172
23 Schumacher, G., Sizmann, D., Haug, H., Buckel, P. and Böck, A. (1986) Nucleic Acids Res. **14**, 5713–5727
24 Barbero, J. L., Buesa, J. M., de Buitrago, G. G., Mendez, E., Perez-Aranda, A. and Garcia, J. L. (1986) Gene **49**, 69–80
25 Sizmann, D., Keilmann, C. and Bock, A. (1990) Eur. J. Biochem. **192**, 143–151
26 Pietrokovski, S. (1994) Protein Sci. **3**, 2340–2350
27 Lubkowski, J., Wlodawer, A., Ammon, H. L., Copeland, T. D. and Swain, A. L. (1994) Biochemistry **33**, 10257–10265
28 Gallagher, T., Rozwarski, D. A., Ernst, S. R. and Hackert, M. L. (1993) J. Mol. Biol. **230**, 516–528
29 Negoro, S., Kakudo, S., Urabe, I. and Okada, H. (1992) J. Bacteriol. **174**, 7948–7953
30 Souciet, J.-L., Hermodson, M. A. and Zalkin, H. (1988) J. Biol. Chem. **263**, 3323–3327
31 Chou, P. Y. and Fasman, G. D. (1978) Adv. Enzymol. Relat. Areas Mol. Biol. **47**, 45–148
32 Brannigan, J. A., Dodson, G., Duggleby, H. J., Moody, P. C. E., Smith, J. L., Tomchick, D. R. and Murzin, A. G. (1995) Nature (London) **378**, 416–419
33 Barrett, A. J. and Rawlings, N. D. (1998) Handbook of Proteolytic Enzymes, Academic Press, London
34 Holmquist, M., Martinelle, M., Berglund, P., Groth Clausen, I., Patkar, S., Svendsen, A. and Hult, K. (1993) J. Prot. Chem. **12**, 749–757
35 Kazlauskas, R. J. (1994) Trends Biotechnol. **12**, 464–472
36 Damblon, C., Ledent, P., Zhao, G.-H., Jamin, M., Dubus, A., Vanhove, M., Raquet, X., Christiaens, L. and Frère, J.-M. (1995) Lett. Pep. Sci. **2**, 212–216
37 Niven, G. W. (1995) Biochem. Biophys. Acta **1253**, 193–198
38 Niven, G. W. (1991) J. Gen. Microbiol. **137**, 1207–1212
39 Holmes, B., Popoff, M., Kiredjian, M. and Kersters, K. (1988) Int. J. Syst. Bacteriol. **38**, 406–416
40 Niven, G. W., Holder, S. A. and Stroman, P. (1995) Appl. Microbiol. Biotechnol. **44**, 100–105
41 Tanizawa, K., Asano, S., Masu, Y., Kuramitsu, S., Kagamiyama, H., Tanaka, H. and Soda, K. (1989) J. Biol. Chem. **264**, 2450–2454
42 Asano, Y., Ito, H., Dairi, T. and Kato, Y. (1996) J. Biol. Chem. **271**, 30256–30262
43 Reynolds, P. E., Depardieu, F., Dutka-Malen, S., Arthur, M. and Courvalin, P. (1994) Mol. Microbiol. **13**, 1065–1070

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