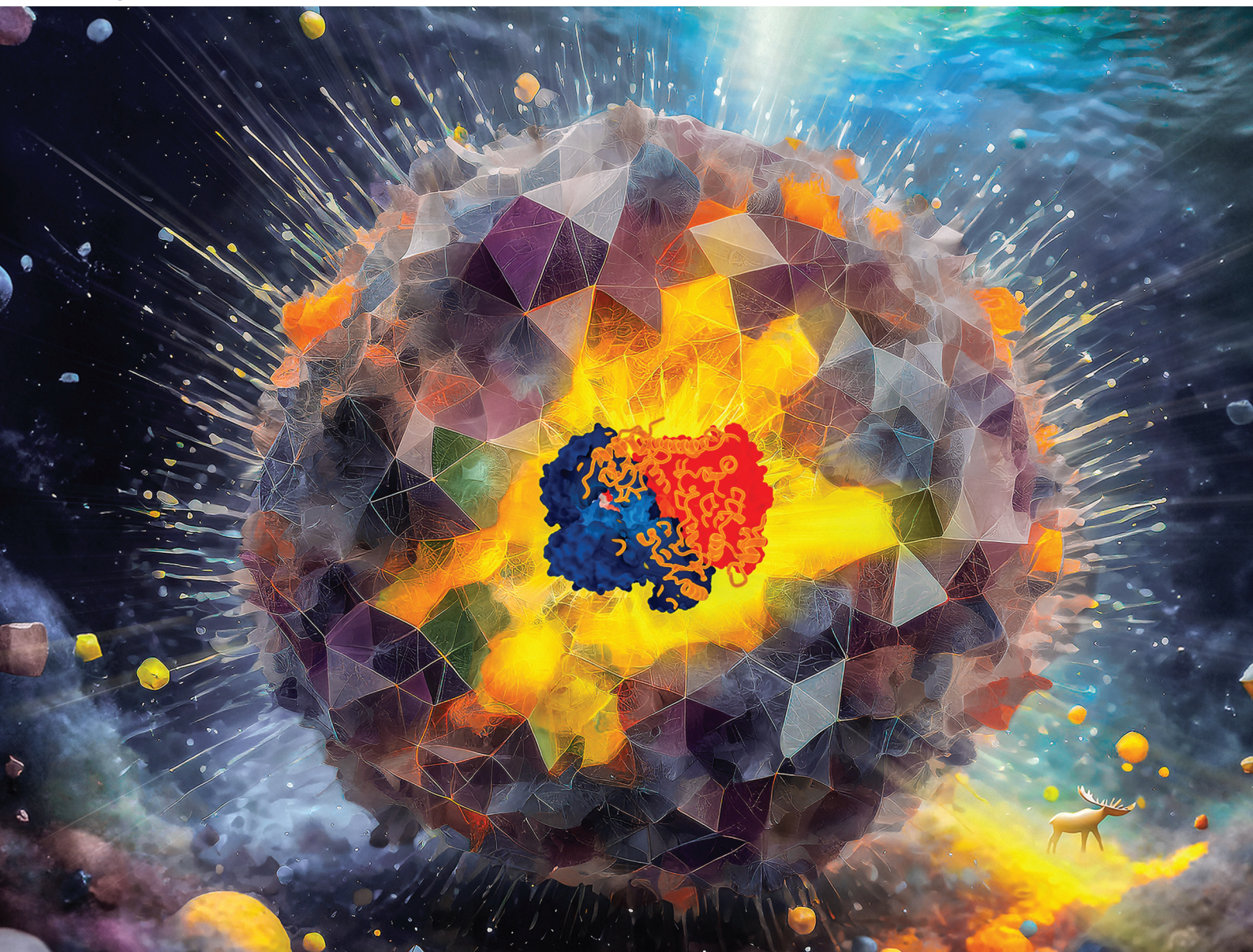


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metaphosphate

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Mild hydrolysis of chemically stable valerolactams by a biocatalytic ATP-dependent system fueled by metaphosphate†

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Medium-sized 5- and 6-membered ring lactams are molecules with remarkable stability, in contrast to smaller β -lactams. As monomers, they grant access to nylon-4 and nylon-5, which are alternative polyamides to widespread caprolactam-based nylon-6. Chemical hydrolysis of monocyclic γ - and δ -lactams to the corresponding amino acids requires harsh reaction conditions and up to now, no mild (enzymatic) protocol has been reported. Herein, the biocatalytic potential of a pair of heterologously expressed bacterial ATP-dependent oxoprolinases – OplA and OplB – was exploited. Strong activity in the presence of excess of ATP was monitored on δ -valerolactam and derivatives thereof, while trace activity was detected on γ -butyrolactam. An ATP recycling system based on cheap Graham's salt (sodium metaphosphate) and a polyphosphate kinase allowed the use of catalytic amounts of ATP, leading to up to full conversion of 10 mM δ -valerolactam at 30 °C in aqueous medium. Further improvements were obtained by co-expressing OplA and OplB using the pETDuet1 vector, a strategy which enhanced the soluble expression yield and the protein stability. Finally, a range of phosphodonors was investigated in place of ATP. With acetyl phosphate and carbamoyl phosphate, turnover numbers up to 176 were reached, providing hints on a possible mechanism, which was studied by ³¹P-NMR.

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Introduction

Polyamides are synthetic polymers obtained by the repetition of units linked by amide bonds and broadly described as nylon. Based on the repeating units, different types of nylon are obtained, such as the emblematic nylon-6, predominantly used in the textile and plastic industries and produced through the ring-opening polymerization of ϵ -caprolactam.^{1,2} Nylon-5 is obtained in a similar manner from δ -valerolactam and displays ferroelectric properties and high melting point above 250 °C.³ Nylon-5,6 is obtained by the copolymerization of ϵ -caprolactam and δ -valerolactam and exhibits superior properties as fabric in terms of elastic recovery and moisture-absorbance properties.^{4,5} The manufacturing of these polymers implies problematic consequences for the environment

through the release of unreacted monomers§ into the wastewater, which thus needs to be treated before release.⁶ While biological treatments of these wastewaters are still rare, microorganisms able to degrade ϵ -caprolactam have been identified.⁶ Such approach is more challenging in the context of medium-sized lactams, especially γ -butyrolactam and δ -valerolactam, because of their exceptional chemical stability connected to the low ring strain. The increased resonance stabilization of the amide bond and the higher partial C–N double bond character in these lactams are responsible for the resistance of the carbonyl group to hydrolysis, and in general, to nucleophilic attack.^{7,8} The hydrolysis of such molecules requires in fact harsh reaction conditions, such as boiling under reflux in strongly acidic solutions for extended times.

Biotechnological access to δ -valerolactam is currently being investigated as an approach to accessing bio-sourced plastics.⁹ However, the challenging biodegradability of this molecule may hamper its wider acceptance. To the best of our knowledge, no enzymatic approach has been identified for the mild

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‡ Equal contributions.

§ Hazard pictograms are reported for both γ -butyrolactam (GHS07 and GHS08) and δ -valerolactam (GHS07), see ESI† for details.



cleavage of γ -butyrolactam and δ -valerolactam, and surprisingly, a variety of hydrolytic enzymes were shown inactive on these simple molecules.^{7,10} Slight modifications of the ring backbone have important effects on the reactivity, and active enzymes could be identified for bicyclic structures of the type of (\pm)-2-azabicyclo[2.2.1]hept-5-en-3-one (*i.e.* *rac*-Vince lactam, Fig. 1A). These enzymes include members of the amidase signature family and members of the serine protease family, however, all were inactive on monocyclic γ - and δ -lactams.¹⁰ While the absence of proteins active on the amide bond of γ - and δ -lactams from the biocatalytic repertoire of cleaving enzymes is striking, related amide bond-containing monocyclic compounds of similar ring size, or further functionalized, seem less resistant to biohydrolysis.⁷ Other examples include 8-, 9- and 13-membered ring lactams,^{7,11} cyclic imide,¹² 6-aminohexanoate-cyclic dimer,¹³ or dihydrouracil (Fig. 1A).¹⁴ Rich enzymatic diversity promotes hydrolysis of these electronically unrelated structures *via* distinct mechanisms. Active hydrolases relying on a serine-triggered nucleophilic attack of the substrate carbonyl group are frequent and are complemented by mostly zinc-dependent metalloproteins, such as dihydropyrimidinases, hydantoinases, or allantoinases (broadly classified as cyclic amidohydrolases^{15,16}), in which direct attack of the carbonyl group by an activated water molecule is facilitated by substrate coordination to a Lewis acid-acting Zn^{2+} .¹⁷ Finally, the reactivity of β -lactams is due to the strong ring strain of the small lactam ring.⁷

Recently, an ATP-dependent enzymatic system involved in the degradation pathway of ϵ -caprolactam was identified in *Pseudomonas jessenii* by proteomics analysis.^{18,19} The two subunits PjCapA and PjCapB function in pair and were shown to catalyze the hydrolysis of ϵ -caprolactam (Fig. 1B). On a

sequence level, the two enzymes are closely related to oxoprolinases OplA and OplB, which are responsible for the endergonic ring opening of L-pyrroglutamic acid (5-oxo-L-proline) to form L-glutamic acid with concomitant hydrolysis of ATP (Fig. 1C).²⁰ While bacterial homologues are made of two separate fragments encoded by two distinct genes,²⁰ eukaryotic oxoprolinases are large one-fragment proteins (*e.g.*, rat Oplah MW: ~137 kDa).¹⁸ These enzymes are also related to ATP-dependent carboxylases, such as acetone and acetophenone carboxylases.¹⁹ Hydrolysis of ϵ -caprolactam to the corresponding ω -amino acid 6-aminocaproic acid by PjCapAB was demonstrated on 2 mM substrate in the presence of 1 equivalent of ATP, leading to the formation of 1.4 mM of product. Since the presence of bicarbonate was found necessary for the formation of the product, and highest velocity was obtained in the presence of a large excess of bicarbonate (50 mM), bicarbonate was postulated to be involved in the reaction mechanism through the formation of a carboxyphosphate intermediate from ATP.¹⁹ This hypothesis was derived from analogy with related enzymes acetone and acetophenone carboxylases. ATP-dependent phosphorylation of bicarbonate is indeed a vital process in a number of biological processes, through which carboxyphosphate can transfer the carboxy group to a variety of acceptors, including acetone and acetophenone,^{21,22} or ammonia.²³

OplAB from *Pseudomonas putida* KT2440 was recently shown to be involved in δ -valerolactam catabolism in the context of whole cells utilizing this molecule as sole carbon source, however no activity in the hydrolysis of δ -valerolactam toward the formation of 5-aminovaleric acid could be detected with the purified proteins *in vitro*. Involvement of these enzymes in the degradation of ϵ -caprolactam was also demonstrated in the whole cells.²⁴ With the goal to establish a generally applicable biocatalytic platform for the ring opening of γ - and δ -lactams under mild reaction conditions, and given the sequence identity between PpOplA and PjCapA (80%) and between PpOplB and PjCapB (86%), we decided to explore the catalytic activity of the OplAB system from *Pseudomonas putida* KT2440 in the ring cleavage of a range of chemically stable lactams toward the corresponding ω -amino acids (Fig. 2). Our strategy relied on the heterologous expression of both enzymes in *E. coli* and their investigation in hydrolytic reactions *in vitro*.

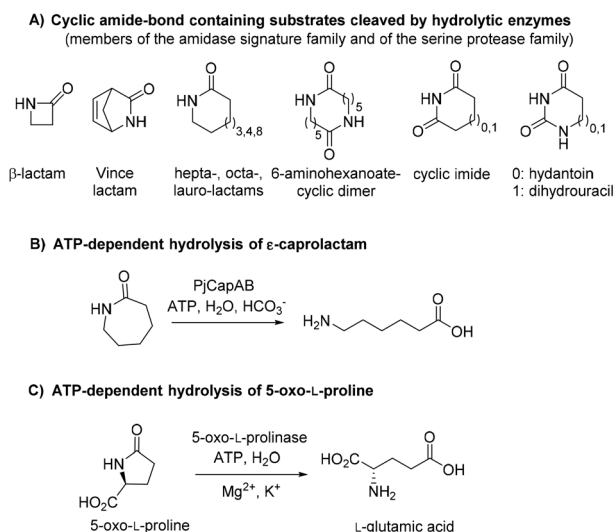


Fig. 1 State-of-the art in the biohydrolysis of lactams and lactam-like compounds: (A) cyclic amide-bond containing substrates cleaved by hydrolytic enzymes;^{7,15,16} (B) ATP-dependent hydrolysis of ϵ -caprolactam by PjCapAB;^{18,19} (C) hydrolysis of oxoprolinase by ATP-dependent oxoprolinase.²⁰

Results and discussion

Individual access to PpOplA and PpOplB and activity assays

The synthetic genes coding for PpOplA and PpOplB, both flanked with the restriction sites *Nde*I at the N-terminus and *Hind*III at the C-terminus and codon optimized for expression in *E. coli*, were ordered and cloned in the vector pET28a(+). Given our interest to study the effect of both proteins separately in view of getting insights on the role of each in the mechanism, we decided to attempt their over-expression individually. The heterologous over-expression of both proteins carrying a N-terminal His-tag in *E. coli* BL21 (DE3) in lysogeny



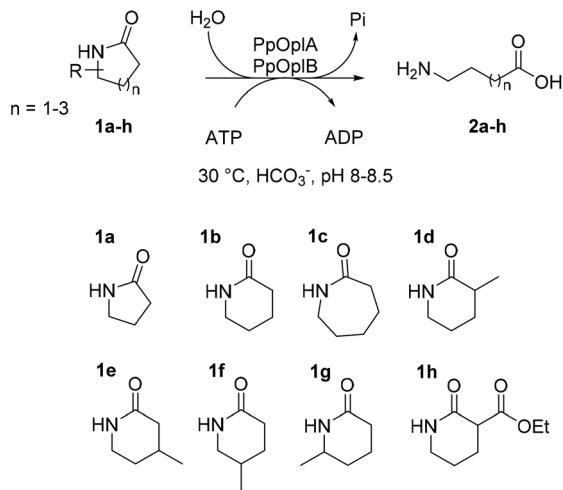


Fig. 2 Enzymatic hydrolysis of medium-sized lactams to the corresponding ω -amino acids by the pair of ATP-dependent oxoprolinases OplA and OplB from *Pseudomonas putida* KT2440 and overview of investigated lactams **1a–h**.

broth under isopropyl β -D-1-thiogalactopyranoside (IPTG) induction at 30 °C resulted in poor soluble expression, as was the case in trials at lower temperatures (20 °C, data not shown), despite good overall over-expression (see ESI, Fig. S1†). The expression was thus performed under autoinduction conditions. The presence of ammonium bicarbonate in the lysis buffer during cell lysis by sonication was crucial for the release of soluble protein (see ESI, Fig. S2 and S3†), especially in the case of PpOplA, while in general PpOplB showed low soluble expression or low release as soluble protein. Due to the very low amounts of PpOplB obtained after purification by immobilized metal affinity chromatography (IMAC, see ESI, Fig. S4†), biotransformations were first performed by combining both cell-free extracts (CFEs) obtained by collecting both supernatants upon cell lysis by sonication.

The first tests were run on 2 mM δ -valerolactam (**1b**) in aqueous ammonium bicarbonate buffer (50 mM, pH 8.5) at 30 °C for 16 h in the presence of 1 eq. ATP and equal amounts of total protein in CFEs of PpOplA and PpOplB. The formation of 5-aminovaleric acid (**2b**) was monitored by gas chromatography after derivatization with ethyl chloroformate (see ESI, Fig. S8–S16†), and reached 1.4 mM (70% conversion), indicating strong activity of the pair PpOplA/PpOplB in the hydrolysis of **1b** (Table 1, entry 2). Full conversion could be obtained in the presence of excess of ATP (2.5 eq., Table 1, entry 3). Under these conditions, no product formation could be detected with γ -butyrolactam (**1a**). The use of a large excess of ATP was found detrimental to the reaction with **1b** as conversion dropped to 35% with 12.5 mM of ATP. Up to 80% conversion could be reached on 5 mM **1b** with 7.5 mM ATP (Table 1, entry 6). Under the tested conditions, no conversion was observed on 10 mM **1b**. Conversions on ϵ -caprolactam (**1c**) remained low at both 2 mM and 5 mM substrate concentration (max. 25%, Table 1, entries 9 and 10), highlighting the preference of the pair PpOplA/PpOplB for the 6-membered ring lactam.

Table 1 Hydrolysis of **1a–c** to the corresponding amino acids **2a–c** by CFEs of PpOplA and PpOplB expressed individually in *E. coli*^a

Entry	Substrate	[1a–c] (mM)	[ATP] (mM) (eq.)	[2a–c] formed (mM)	Conv. to 2a–c (%)
1	1a	2	5 (2.5)	n.d.	n.a.
2	1b	2	2 (1)	1.4	70
3	1b	2	5 (2.5)	~2	>99
4	1b	2	12.5 (6.25)	0.7	35
5	1b	5	5 (1)	2.1	42
6	1b	5	7.5 (1.5)	4.0	80
7	1b	5	10 (2)	3.1	62
8	1b	10	25 (2.5)	n.d.	n.a.
9	1c	2	5 (2.5)	0.5	25
10	1c	5	10 (2)	0.8	16

n.d.: not detected, n.a.: not applicable. ^a Reaction conditions: ammonium bicarbonate buffer (50 mM, pH 8.5) containing MgCl₂ (4 mM), CFEs of PpOplA and PpOplB (11.4 mg mL⁻¹ each, total protein content), [ATP] as indicated, [**1a–c**] as indicated, incubation at 30 °C for 16 h and 140 rpm. Reactions performed in duplicates and analyzed by gas chromatography after derivatization of the amino acid and use of a calibration curve with reference materials.

Different control reactions in the absence of ATP, PpOplA, PpOplB, or both PpOplA and PpOplB, were performed in parallel; no amino acid could be detected in any of these cases. No product could be detected when the reaction was run in potassium phosphate buffer (50 mM, pH 7.5) in absence of ammonium bicarbonate, confirming past observations with homologous PjCapAB that bicarbonate is crucial for activity.¹⁹ Noteworthy is that activity was strongly diminished upon freezing and thawing of the enzyme preparations, therefore CFEs were prepared fresh before each round of biotransformation. A mixture of lyophilized whole cells separately expressing PpOplA and PpOplB was found active in the hydrolysis of 2 mM **1b** with 5 mM ATP, however lower conversions were achieved (max. 50% conversion, data not shown). The limitation in using a mixture of whole cells is likely due to the necessary transfer from the first intermediate obtained with PpOplA to the second biocatalyst preparation, which requires exchange from one cell to another. The use of purified proteins was impaired due to the low amounts of PpOplB retrieved (see ESI, Fig. S4†) and first attempts did not lead to any conversion (data not shown). Early studies already indicated the tendency of the purified fragment B from *Pseudomonas putida* to aggregate,²⁵ affecting activity. Given the negative impact of high concentrations of ATP on conversion (see Table 1), a stepwise addition of ATP was implemented. Addition of three batches of 5 mM ATP over 2 h led to a great increase in the formation of **2b** (up to >99% conversion from 6 mM **1b**, and 6.9 mM **2b** obtained from 8 mM **1b**, Table 2, entries 1 and 2). On **1c**, the effects remained modest (up to 1.4 mM **2c**, Table 2, entries 4–6), confirming the preference of the PpOplAB system for the valerolactam ring under these reaction conditions.

Co-expression of PpOplA and PpOplB in the pETDuet1 vector

Given the poor stability of the enzyme preparations upon storage and the low soluble expression of PpOplB (*vide supra*),



Table 2 Stepwise addition of ATP in the hydrolysis of **1b–c** by CFEs of PpOplA and PpOplB expressed individually^a

Entry	Substrate	[1a–c] (mM)	[2a–b] formed (mM)	Conv. to 2a–b (%)
1	1b	6	~6	>99
2	1b	8	6.9	86
3	1b	10	6.7	67
4	1c	6	1.4	24
5	1c	8	1.2	15
6	1c	10	1.2	12

^a Reaction conditions: ammonium bicarbonate buffer (50 mM, pH 8.5) containing MgCl₂ (4 mM), CFEs of PpOplA and PpOplB (11.4 mg mL⁻¹ each, total protein content), ATP (15 mM final concentration, 5 mM added at 0 h, 1 h and 2 h), [**1b–c**] as indicated, incubation at 30 °C for 16 h at 140 rpm. Reactions performed in duplicates and analyzed by gas chromatography after derivatization of the amino acid and use of a calibration curve with reference materials.

improvements in the protein expression were necessary for further investigations of the catalytic activity. We decided to co-express PpOplA and PpOplB using a co-expression vector, taking into account that the crystal structure of the homologous caprolactamase system from *Pseudomonas jessenii* revealed a heterotetrameric assembly, in which each CapB subunit interacts with a CapA subunit. We anticipated that co-expression of both subunits of the OplAB system within the same cell would favor the formation of the tetrameric assembly upon protein expression, likely impacting positively the stability of the whole assembly. In addition, such protocol grandly simplifies the preparation of the biocatalyst. The use of the pETDuet1 vector with two multiple cloning sites allowed successful co-expression of both enzymes in *E. coli* BL21 (DE3) under autoinduction in terrific broth at 24 °C. Noteworthy was the significant increase in the soluble expression of PpOplB and the comparable yields of soluble protein between PpOplA and PpOplB (Fig. 3). This method allowed the generation of a

stable assembly in solution, as demonstrated by the successful co-purification of both proteins by IMAC. Since only PpOplA bears a His-tag at the N-terminus, this points out strong interactions between the two subunits and the formation of a protein complex.²⁶ A 1 : 1 molar yield of the soluble assembly was confirmed by densitometric analysis of SDS-PAGE (see Fig. 3 and ESI, Table S1†). The presence of bicarbonate in the lysis buffer contributed to higher protein yields, but was found in this case not to be mandatory for good release of soluble proteins, which was also obtained in Tris and HEPES buffers for both proteins (see ESI, Fig. S5†).

The activity of co-expressed PpOplA and PpOplB (now described as PpOplAB) was investigated on a range of lactams (Fig. 2). To simplify the analysis and avoid time consuming derivatization for GC analysis, a method was developed that allowed for co-analysis of substrate and product by LC-MS, directly from the aqueous reaction mixture. Calibration curves were generated for all reference amino acids (see ESI, Fig. S17–S22†). PpOplAB was used as CFEs on 10 mM **1a–h** in the presence of 12.5 mM ATP (total protein content of 8 mg mL⁻¹, corresponding to ~7 μM of PpOplAB based on densitometric analysis, see Table S1†). Strongest activity could be observed on **1b** (full consumption) and 5-methylpiperidin-2-one (**1f**, leading to 7.9 mM product, Table 3), while conversion on **1c** remained modest (2.3 mM **2c** formed, Table 3, entry 3). For the first time, some product could be observed in the conversion of γ -butyrolactam (**1a**), corresponding to ~4% substrate consumption (see ESI, Fig. S26†). With other methyl derivatives of δ -valerolactam, conversions were moderate (max. 2.4 mM product, Table 3) and substitution closest to the amide bond (especially 3- and 6-position) impacted the conversion negatively the most. In contrast, substitution furthest to the carbonyl had almost no impact on activity, pointing at possible steric hindrance near the reactive moiety with **1d**, **1e** and **1g**. Finally, 3-ethoxycarbonyl-2-piperidone (**1h**) with a large and electron-withdrawing substituent in α -position to the carbonyl was not accepted (data not shown). All substituted

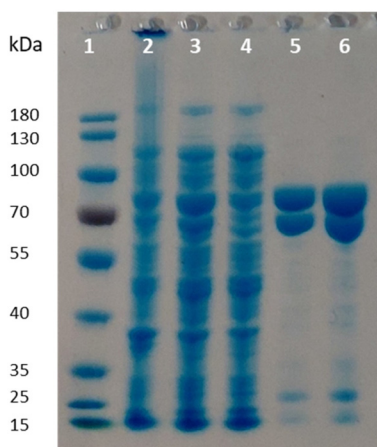


Fig. 3 SDS-PAGE following co-expression of PpOplA and PpOplB using the pETDuet-1 vector (MW PpOplA including His-tag: ~76 kDa, MW PpOplB: ~63 kDa). Lane 1: PageRuler™ Prestained Protein Ladder; lane 2: pellet; lane 3: supernatant (soluble fraction); lane 4: flow through from IMAC; lanes 5 and 6: desalted purified enzyme fraction.

Table 3 Hydrolysis of lactams **1a–h** with co-expressed PpOplAB^a

Entry	Substrate	[2a–h] (mM)	Conv. to 2a–h (%)
1	1a	n.d. ^b	~4
2	1b	7.7 (±0.8)	77 ^c
3	1c	2.3 (±0.1)	23
4	1d	1.3 (±0.1)	13
5	1e^d	2.4 (±0.1)	24
6	1f	7.9 (±0.3)	79
7	1g	1.4 (±0.0)	14
8	1h	n.f.	n.a.

^a Reaction conditions: 8 mg mL⁻¹ PpOplAB CFE (total protein content), 10 mM **1a–h**, 12.5 mM ATP, ammonium bicarbonate buffer (39 mM, pH 8.5), 8 mM MgCl₂, 30 °C, 900 rpm, 24 h. Reactions performed in triplicates and analyzed by LC-MS (standard deviation indicated). ^b n.d.: not determined, product formation not quantified. ^c 4% substrate consumption (see ESI, Fig. S26†). ^d n.f.: not formed, n.a.: not applicable. ^e Substrate fully converted. See ESI, Fig. S26–S33.† ^f *E*-Value of 6, see text and ESI.†



phate provides hints that the role of bicarbonate in the reaction is to generate a carboxyphosphate intermediate for substrate activation. PpOplAB is not effective in the hydrolysis of γ -butyrolactam. However, the trace activity observed is promising and suggests that the chemical stability of this molecule may be tackled in the future using biological tools.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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