

Article

Leucine Aminopeptidase from *Xanthomonas oryzae* pv. *oryzae* with Esterase Activity Toward Heroin: Biochemical and Catalytic Insights

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Abstract

Heroin is a highly addictive drug that exerts its primary effects through activation of μ -opioid receptors. Its principal active metabolite, 6-monoacetylmorphine (6-MAM), significantly contributes to heroin's neurological effects and acute toxicity. Current pharmacotherapies for heroin use disorder, employing opioid receptor agonist or antagonist, are often limited by risks of dependence, tolerance, and/or adverse side effects. In this context, enzyme-based therapy emerges as a promising alternative by rapidly converting drugs into inactive or less harmful metabolites in the blood. As a macromolecule, the enzyme does not cross the blood–brain barrier, thereby avoiding side effects in CNS. Through structure-based computational screening, Xoo-PepA (PDB ID: 3JRU), a leucine aminopeptidase from *Xanthomonas oryzae* pv. *oryzae*, was identified as a potential enzyme capable of hydrolyzing heroin and 6-MAM. Computational and experimental analyses confirm that Xoo-PepA hydrolyzes heroin sequentially to 6-MAM and subsequently to morphine. Enzymatic properties including dependence on metal ions, optimal pH, thermal stability, and substrate specificity were characterized accordingly. Notably, supplementation with Ni^{2+} or Zn^{2+} and TCEP extended Xoo-PepA's half-life at 37 °C from 1 h to over 24 h, highlighting the essential role of metal ions in maintaining structural stability. Moreover, Ni^{2+} enhanced Xoo-PepA's hydrolysis toward peptidase substrate L-leucine-p-nitroaniline by 770-fold, yet conferred no significant activation toward heroin. Mutations in metal ion-coordination residues (e.g., K262A, D267A/E346L) exhibited different activity profiles toward these two types of substrates, suggesting a distinct regulatory mechanism of metal ions may be involved in these activities. This study provides the first demonstration that Xoo-PepA, a non-mammalian, metal-dependent aminopeptidase, can hydrolyze heroin and 6-MAM, shedding light on its functional versatility and biochemical characteristics.

Keywords: heroin abuse; aminopeptidase; catalytic function; biochemical characterization

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1. Introduction

Opioid abuse has emerged as a global public health crisis, affecting an estimated 61 million individuals worldwide. Among these substances, heroin remains one of the most addictive and dangerous illicit opioids, with its widespread use posing serious health and societal challenges [1–3]. In the United States alone, heroin overdose accounted for 174,861 emergency department visits from October 2020 to April 2025, and claimed nearly 162,000 lives between 1999 and 2023, underscoring the magnitude of the epidemic [2,4]. Current treatment options for heroin dependence include methadone maintenance, buprenorphine substitution, and naltrexone-based therapies, where naloxone is used specifically to reverse opioid overdoses [3,5]. While these interventions employing μ -opioid receptor (MOR) agonist or antagonist have shown efficacy, they continue to face significant limitations such as high relapse rates, adverse side effects, and low patient adherence [6,7]. Consequently, there is an urgent need for safer, more effective, and alternative approaches for heroin abuse.

Enzyme therapy has emerged as a promising strategy for treating drug abuse [8–10]. Unlike mechanism-based interventions, enzyme-based approaches directly degrade or eliminate drug molecules in the bloodstream, irrespective of their pharmacological action. By administration of exogenous, a highly efficient enzyme, this approach rapidly converts abused substances into inactive or low-toxicity metabolites, thereby mitigating or abolishing their toxic and physiological effects on target organs [11,12]. Clinical studies have demonstrated its promising effect in cocaine abuse treatment. Teva Pharmaceuticals' TV-1380, a CocH1-HSA fusion protein engineered from human butyrylcholinesterase (BChE), has demonstrated its safety and efficacy in addiction treatment, significantly reducing drug cravings and actual use in trials [13,14]. Meanwhile, the highly active and thermostable cocaine esterase (CocE) mutant RBP-8000 (now designated TNX-1300) rapidly degraded nearly all circulating cocaine into non-toxic metabolites within two minutes in phase 2a clinical trial for cocaine intoxication, and received the "Breakthrough Therapy" designation from the U.S. Food and Drug Administration (FDA) [15,16]. Together, these findings confirm that highly active cocaine-metabolizing enzymes are safe and effective in attenuating its toxicity and pharmacological effects, providing a translational foundation for extending enzyme-based therapies to heroin and other substance use disorders.

Heroin is a semi-synthetic opioid characterized by two acetyl groups that confer high lipophilicity and enable rapid penetration into the brain. Once administered, heroin undergoes rapid hydrolysis by plasma and tissue esterases, initially producing 6-monoacetylmorphine (6-MAM), which is subsequently deacetylated to morphine [17,18]. In the liver, morphine is predominantly metabolized via UDP-glucuronosyltransferase 2B7 (UGT2B7) [19] to form morphine-6-glucuronide (M6G, 9–10%) and morphine-3-glucuronide (M3G, 44–55%), which are the major metabolites excreted in the urine [20]. Following heroin's sequential metabolism, the lipophilicity of its metabolites decreases. 6-MAM is identified as the primary metabolite in the brain [21], exhibits higher binding affinity than heroin and morphine [17], and is essential for heroin-associated rewarding and sensitization properties [22]. Thus, heroin-associated physiological activity and acute toxicity is largely attributable to heroin and its early metabolite 6-MAM [17,22]. These insights suggest that highly efficient enzymes capable of rapidly hydrolyzing heroin and 6-MAM into less toxic, less lipophilic metabolites offer a promising approach to mitigate heroin's pharmacological activity and acute toxicity.

Consequently, the discovery of such highly efficient heroin-metabolizing enzymes is essential for developing alternative therapeutics for heroin use disorder. While sequence-based screening remains the predominant approach for enzyme discovery [23,24], catalytic function can not be reliably inferred from sequence homology alone. In contrast, structure-based methods which predict enzymatic function from three-dimensional

protein structures provide a powerful complementary strategy [25,26]. Fueled by the increasing availability of structural data in public repositories and advances in computational modeling, these approaches now enable accurate functional annotation and mechanistic analysis. For instance, AlphaFold 3 employs a diffusion-based architecture to predict biomolecular complex structures across diverse classes in the Protein Data Bank (PDB), significantly outperforming conventional docking in protein–ligand interaction prediction [27]. By integrating AI-driven structure prediction, potential heroin hydrolases can be systematically identified, establishing a foundation for the development of enzyme-based therapy against heroin abuse.

An integrated screening workflow based on key catalytic residues and enzyme–substrate binding models was developed to identify candidate enzymes capable of hydrolyzing the ester bonds of heroin and 6-MAM. Guided by the heroin metabolism pathway and ester hydrolysis mechanism [17,28], leucine aminopeptidase (LAP) was selected as it possesses a hydrophobic binding pocket compatible with the acetyl group of these substrates [29,30]. Through extensive evaluation, Xoo-PepA (PDB ID: 3JRU), a leucine aminopeptidase from *Xanthomonas oryzae* pv. *oryzae*, was identified as a promising candidate. In this study, recombinant Xoo-PepA was expressed and systematically evaluated in vitro, demonstrating its catalytic activity toward both heroin and 6-MAM for the first time. Key biochemical properties, including metal-dependence, thermal stability, optimal pH, and substrate specificity were thoroughly investigated. Optimal reaction conditions for achieving maximal enzymatic performance were established through comprehensive characterization. These findings provide insights into the functional versatility and biochemical properties of Xoo-PepA and demonstrate its scientific potential as a scaffold for future enzyme engineering toward heroin degradation.

2. Materials and Methods

2.1. Materials

Escherichia coli DH5 α competent cells were procured from Tsingke Biotechnology (Beijing, China). FastPure Plasmid Mini Kit was sourced from Vazyme Biotech (Nanjing, China). HisSep Ni-NTA agarose resin for affinity chromatography was acquired from Yeasen Biotechnology (Shanghai, China). Amicon Ultra-30kD centrifugal filter was purchased from MilliporeSigma (Burlington, MA, USA). BCA Protein Assay Kit was supplied by Beyotime Biotechnology (Shanghai, China). Anti-His mouse monoclonal antibody was purchased from Affinity Biosciences (Cincinnati, OH, USA). Analytical standards of heroin, 6-MAM, and morphine were sourced from Yuansi Standard Science and Technology (Shanghai, China). Aminopeptidase substrates including L-Ala-p-nitroanilide, L-Arg-p-nitroanilide, L-Leu-p-nitroanilide, L-Pro-p-nitroanilide, L-Met-p-nitroanilide, and L-Gly-p-nitroanilide were obtained from Aladdin Biochemical Technology (Shanghai, China).

2.2. Molecular Modeling

Xoo-PepA, a peptidase from *Xanthomonas oryzae* pv. *oryzae*, was identified as a promising candidate through our structure-based enzyme screening pipeline. To predict its potential to catalyze heroin or 6-MAM, structural models of Xoo-PepA in complex with heroin or 6-MAM were directly predicted with AlphaFold3 [27]. Under default settings, five structural models were generated, and the top-ranked model exhibiting the highest overall confidence was selected to illustrate the geometric compatibility between the metal coordination site and the substrate acetyl group within the active site. All structural visualizations and measurements were performed using PyMOL 3.0 (The PyMOL Molecular Graphics System, Schrödinger). Subsequent recombinant expression and enzymatic

assays were employed to confirm the hydrolytic activity of Xoo-PepA against heroin and 6-MAM.

2.3. Protein Multiple Sequence Alignment

Xoo-PepA belongs to the M17 leucine aminopeptidase family, whose biochemical properties remain poorly characterized. Given that proteins with homologous sequences often share a common evolutionary origin and exhibit similar structural conformations, biological functions, and physicochemical properties, homologous protein analysis offers a tool to infer Xoo-PepA's structural and functional features. To this end, BLASTp program (available at <https://blast.ncbi.nlm.nih.gov/>, accessed on 10 December 2025) was first employed to identify members of the M17 family of aminopeptidases showing high sequence similarity to Xoo-PepA. Representative members across different species were selected and their protein sequences were retrieved from the Uniprot database. Multiple sequence alignment was conducted using the ClustalW algorithm in MEGA 11, followed by phylogenetic tree construction using the Neighbor-Joining (NJ) method to delineate evolutionary relationships among family members. The alignment results were subsequently imported into the GeneDoc 2.6.02 software for comparative and optimized visualization. The resulting tree was drawn for clarity, with scale adjustments made to branch lengths and node labels. This approach established a theoretical basis for characterizing Xoo-PepA by revealing conserved motifs and evolutionary relationships in the M17 family.

2.4. Plasmid Construction and Enzyme Preparation

- (1) Plasmid construction. The amino acid sequence of Xoo-PepA (Uniprot ID: Q5H4N2) comprises 490 residues. The corresponding cDNA was synthesized by Tsingke Biotechnology (Beijing, China) and cloned into the pET-28a bacterial expression vector with a N-terminal 6×His tag. Mutations at residues involved in metal ion coordination (K262A, D267A/E346L) or oligomer assembly (W410A/Y418A) were selected based on previous studies [31,32], and site-directed mutagenesis was performed to generate the corresponding mutants. The recombinant plasmid was transformed into *E. coli* DH5α competent cells for amplification, and plasmids were extracted using the FastPure Plasmid Mini Kit.
- (2) Enzyme expression and purification. The purified plasmid was introduced into *E. coli* BL21 (DE3) cells for protein expression. Cultures were grown in a Luria–Bertani (LB) broth supplemented with 50 µg/mL kanamycin at 37 °C until OD600 reached 0.6–0.8. Expression was induced with 1 mM IPTG and continued at 17 °C for 16 h. Cells were harvested by centrifugation at 4000 rpm for 20 min at 4 °C, and resuspended and lysed in a lysis buffer (25 mM Tris-HCl, 300 mM NaCl, pH 7.5) using a French Press (Scientz, Ningbo, China). The resulting lysate was clarified by centrifugation at 12,000 rpm for 40 min at 4 °C, and the supernatant was subjected to Ni-NTA affinity chromatography. After removing non-specifically bound proteins with washing buffer (20 mM imidazole in lysis buffer), the target protein was eluted with an elution buffer (200 mM imidazole in lysis buffer). The protein was concentrated using Amicon Ultra-30 kDa filters, and buffer-exchanged into storage buffer (10% (v/v) glycerol in lysis buffer), followed by storage at −80 °C. Protein purity, molecular weight and structural stability in the absence or presence of metal ions were assessed by SDS-PAGE, Native PAGE, and Western blot analysis using anti-His tag primary antibody. Protein concentration was determined using the Enhanced BCA Protein Assay Kit.

2.5. Effect of Metal Ions, Reductant, Temperature and pH

- (1) Metal ions and reductant. The influence of metal ions on enzymatic activity was assessed using 1 mM L-Leu-p-nitroanilide (L-Leu-pNA) as the substrate in the absence or presence of various metal ions. Enzyme reactions were carried out following a 15 min incubation at 37 °C with the indicated metal salts (NiCl₂, CoCl₂, MnCl₂, CuCl₂, ZnCl₂, MgCl₂, FeCl₃, CaCl₂, KCl, or LiCl) at final concentrations of 0.2 mM and 1 mM. Aminopeptidase activity toward L-Leu-pNA was measured at pH 7.4, 37 °C as described in Section 2.6 and in our previous publication [33]. Control groups including no enzyme, heat-inactivated enzyme, EDTA-treated, apo enzyme, and peptidase-inactive mutant (K262A, D267A/E346L, W410A/Y418A) were included for comparison with a Ni²⁺-supplemented wide-type enzyme to assess the contribution of metal ions to L-Leu-pNA hydrolysis. The reaction rate was measured using 1 mM L-Leu-pNA under standardized assay condition. The effect of the reductant was evaluated accordingly by adding 0.1 mM tris-(2-carboxyethyl)phosphine (TCEP) to the above reaction system. The dose-dependent effect of NiCl₂ on Xoo-PepA's aminopeptidase activity was also evaluated. The kinetic parameter of Xoo-PepA toward L-Leu-pNA was determined in the presence of 1 mM NiCl₂, CoCl₂ or ZnCl₂ at pH 7.4 and 37 °C, as described in Section 2.6.
- (2) Temperature. Based on the above analysis, 1 mM Ni²⁺ and 0.1 mM TCEP significantly enhanced the aminopeptidase activity of Xoo-PepA. Therefore, biochemical characterizations were performed with Xoo-PepA in the absence or presence of above supplementation. Temperature dependence was examined by incubating the enzyme at 25–70 °C for 30 min, followed by activity measurement at pH 7.4, 37 °C using 1 mM L-Leu-pNA as the substrate. Thermal stability was determined by incubating Xoo-PepA at 37 °C, sampling at intervals and monitoring its residual aminopeptidase activity over time. Structural stability of Xoo-PepA following 3 h incubation at 37 °C in the absence or presence of Ni²⁺ and TCEP was analyzed by Native PAGE.
- (3) pH. The pH dependence of Xoo-PepA was evaluated by incubating enzymes in buffers ranging from pH 5 to 11, followed by a measurement of its peptidase activity toward L-Leu-pNA accordingly. The buffers used here were 50 mM acetate buffer (pH 5.0–5.5), 50 mM phosphate buffer (pH 6.0–8.0), 50 mM Tris-HCl buffer (pH 8.5–9.0), and 500 mM carbonate buffer (pH 9.5–11.0).

2.6. Aminopeptidase Activity Assay and Substrate Selectivity

- (1) Aminopeptidase activity assay. Aminopeptidase activity of Xoo-PepA toward amino acid p-nitroanilide (pNA) substrates was measured using a microplate reader (Tecan Spark, Männedorf, Switzerland). 1 mM L-Leu-pNA was used as the substrate for evaluating Xoo-PepA's peptidase activity in Section 2.5. Enzyme-catalyzed hydrolysis rates were determined by monitoring the absorbance of products at 405 nm in a 50 mM phosphate buffer (pH 7.4) at 37 °C for 1 h. Initial rates were calculated from the linear portion of reaction curves along with a standard curve of reaction product p-nitroaniline.
- (2) Substrate selectivity. Six aminopeptidase substrates were included: L-Ala-pNA, L-Arg-pNA, L-Leu-pNA, L-Pro-pNA, L-Met-pNA, and L-Gly-pNA. Substrate specificity was initially evaluated at 1 mM substrate concentration in the absence or presence of 1 mM Ni²⁺ and 0.1 mM TCEP. Kinetic profiles were obtained by assaying substrate concentrations ranging from 0.01 mM to 5 mM. All assays were performed in triplicate at pH 7.4, 37 °C. Initial reaction rates were determined as described above. Michaelis–Menten parameters (k_{cat} and K_M) of Xoo-PepA toward these aminopeptidase substrates were estimated using Prism 8.0 (GraphPad Software, Boston, MA, USA).

2.7. Enzymatic Assay of Heroin and 6-MAM

Catalytic activity of Xoo-PepA against heroin and 6-MAM was determined and confirmed by HPLC analysis. Enzymatic reactions were initiated by mixing 50 μ L of heroin solution (50–5000 μ M for kinetics study) with 50 μ L of enzyme solution (20 μ M). After incubation at 37 °C for 2 h, reactions were quenched with 50 μ L of 0.5 M HCl, followed by addition of 150 μ L of 20% acetonitrile. Samples were vortexed, centrifuged at 12,000 rpm for 5 min, filtered through a 0.22 μ m syringe filter (Tianjin Navigator Lab Instrument, Tianjin, China), and transferred to HPLC vials for analysis. For 6-MAM, identical conditions were applied except that incubation was extended to 24 h. Control groups including no-enzyme, heat-inactivated enzyme, EDTA-treated, apo enzyme, and peptidase-inactive mutant (K262A, D267A/E346L, W410A/Y418A) groups were included in parallel to determine the level of non-enzymatic hydrolysis. The product formation observed in the no-enzyme group and heat-inactivated enzyme group was subtracted from that of the enzymatic reaction group to determine the real enzymatic reaction rate.

HPLC analysis of heroin, 6-MAM and morphine was performed using the Thermo Ultimate 3000 HPLC-UV detection system (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was achieved on a Welch Xtimate C18 column (4.6 $\times$ 250 mm, 5 μ m) using a gradient elution program at a flow rate of 1 mL/min. The mobile phase consisted of (A) 0.1% trifluoroacetic acid (TFA) in water and (B) acetonitrile. The gradient elution program was as follows: 10% B for 2 min, linear increase to 25% B from 2 to 20 min, held at 25% B for 4 min, returned to 10% in 2 min, and equilibrated for another 2 min, for a total runtime of 28 min. 50 μ L samples were injected, and absorbance of analytes was monitored at 210 nm. Heroin, 6-MAM and morphine in the reaction mixtures were quantified using calibration curves generated with authentic standards. Initial reaction rates obtained at varying substrate concentrations were used for kinetics analysis. Michaelis–Menten parameters for heroin and 6-MAM hydrolysis were determined by nonlinear regression using Prism 8.0.

3. Results

3.1. Predicted Structural Compatibility of Xoo-PepA with Heroin or 6-MAM

Among the screened enzymes, Xoo-PepA emerged as a promising candidate. This metal-dependent hydrolase belongs to the leucine aminopeptidase (LAP) family, which canonically catalyzes the cleavage of N-terminal amide bonds in peptides (Figure 1B). Given that both heroin and 6-MAM contain hydrolysable acetyl ester bonds, and that this reaction pathway is well characterized (Figure 1A) [17], it is hypothesized that Xoo-PepA might also exhibit hydrolytic activity toward these small-molecule esters.

Following this hypothesis, AlphaFold3 were employed to predict the complex structure of Xoo-PepA with heroin or 6-MAM. Figures 1C,D show both heroin and 6-MAM bind within the catalytic pocket of Xoo-PepA, adjacent to the binuclear Zn^{2+} cluster and the key residue Lys274. The carbonyl oxygen of the 3'-acetyl group in heroin is positioned 2.9–4.8 Å from Zn^{2+} and Lys274, within the typical distance range for a nucleophilic attack, and forms stable interactions with Lys274. In contrast, the carbonyl oxygen group of 6-MAM is positioned further away (4.8–10.1 Å), resulting in weaker interaction with Zn^{2+} and Lys274. The structural superimposition of two complexes reveals high overlapping binding poses (Figure 1E). Notably, the 3'-acetyl carbonyl group of heroin is positioned closer to the catalytic Zn^{2+} and Lys274 than that of 6-MAM, suggesting a higher catalytic activity toward heroin. This structural feature also implies that Xoo-PepA may preferentially hydrolyze the 3'-acetyl group of heroin. Overall, structural analyses suggest Xoo-PepA may be capable of hydrolyzing heroin and 6-MAM, though this remains to be confirmed experimentally.

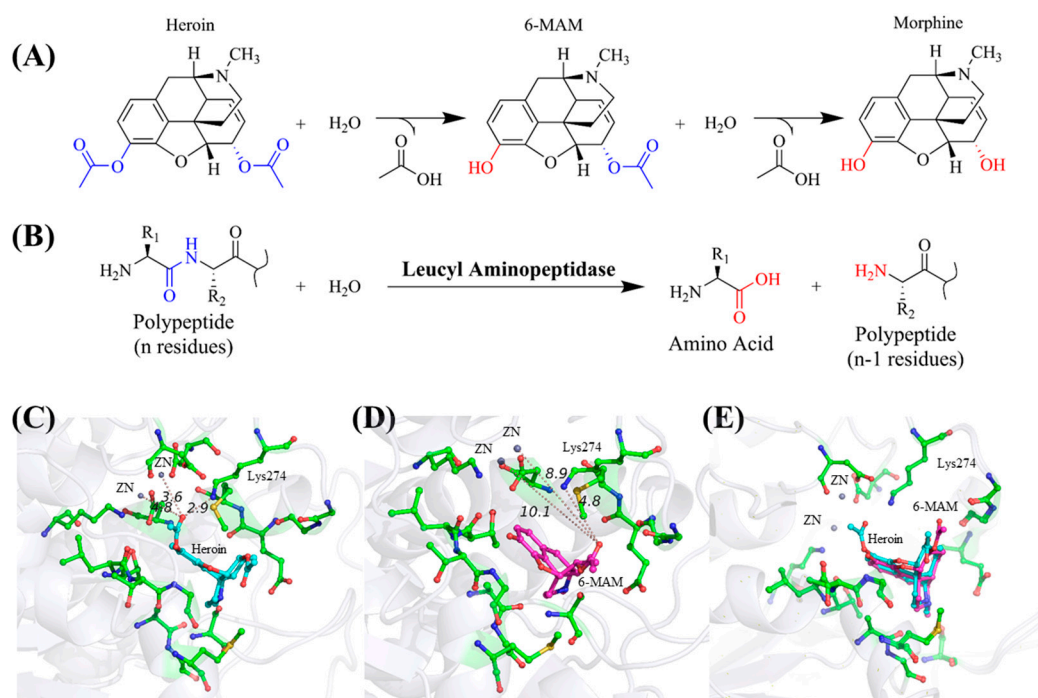


Figure 1. Structural insights support that Xoo-PepA potentially catalyzes the hydrolysis of ester bonds in heroin and 6-MAM. (A) Hydrolysis pathway of heroin and 6-MAM; (B) Canonical hydrolytic function of Leucyl Aminopeptidase toward peptide; (C,D) AlphaFold3-predicted structure of Xoo-PepA in complex with heroin (C) and 6-MAM (D); (E) structural superposition of these two complexes.

3.2. Sequence Analysis of Xoo-PepA with Its Homologs

Xoo-PepA is an M17 leucine aminopeptidase identified in *Xanthomonas oryzae* pv. *oryzae*, with its sequence available in the UniProt database (Uniprot ID: Q5H4N2). To assess the sequence conservation within the M17 family, 13 representative homologs were selected for analysis: *Stenotrophomonas maltophilia* (Uniprot ID: B2FMS4), *Xylella fastidiosa* (Uniprot ID: Q9PH08), *Methylobacillus flagellatus* (Uniprot ID: Q1H4U4), *Neisseria meningitidis* (Uniprot ID: Q9JTI8), *Escherichia coli* (Uniprot ID: A1AJG3), *Sus scrofa* (Uniprot ID: P28839), *Homo sapiens* (Uniprot ID: P28838), *Solanum lycopersicum* (Uniprot ID: Q42876), *Arabidopsis thaliana* (Uniprot ID: P30184), *Bos taurus* (Uniprot ID: P00727), *Mus musculus* (Uniprot ID: Q9CPY7), *Plasmodium vivax* (Uniprot ID: A5K3U9), and *Plasmodium falciparum* (Uniprot ID: Q8IL11). Blast analysis suggests protein sequence similarity of homologs with Xoo-PepA ranges from 35% to 85%, and the sequence similarities are as follows: *S. maltophilia* (85%), *X. fastidiosa* (79%), *M. flagellatus* (53%), *N. meningitidis* (53%), *E. coli* (48%), *S. scrofa* (42%), *H. sapiens* (41%), *P. falciparum* (40%), *S. lycopersicum* (39%), *P. vivax* (39%), *A. thaliana* (37%), *B. taurus* (35%), *M. musculus* (35%). Multiple sequence alignment analyses revealed that Xoo-PepA contains conserved motifs characteristic of M17 leucine aminopeptidases, including catalytically essential residues shared across the family (Figure 2A). The strong conservation of active-site residues provides a molecular basis for Xoo-pepA's substrate recognition and metal-dependent catalytic activity, thereby facilitating mechanistic insights into its function.

To further elucidate the evolutionary relationship of Xoo-PepA, a phylogenetic tree was constructed using M17 leucine aminopeptidases from these 14 species (Figure 2B). The analyzed LAPs from these organisms are resolved into two major clades with high bootstrap values. One clade included bacterial species such as *X. oryzae* pv. *oryzae*, *S. maltophilia*, *X. fastidiosa*, *M. flagellatus*, and *E. coli*, with *X. oryzae* pv. *oryzae* and *S. maltophilia* forming a close subclade and *E. coli* grouping separately. *N. meningitidis* branches off

earlier, indicating it is more distantly related to the other bacteria. The second clade comprises eukaryotic organisms, including mammals (*B. taurus*, *Sus scrofa*, *M. musculus*, *H. sapiens*), plants (*S. lycopersicum*, *A. thaliana*), and protozoans (*P. vivax*, *P. falciparum*). The resulting phylogenetic tree reveals a clear evolutionary divergence between prokaryotic and eukaryotic lineages. Xoo-PepA and LAP from *S. maltophilia* and *X. fastidiosa* have the highest sequence similarity and probable functional conservation.

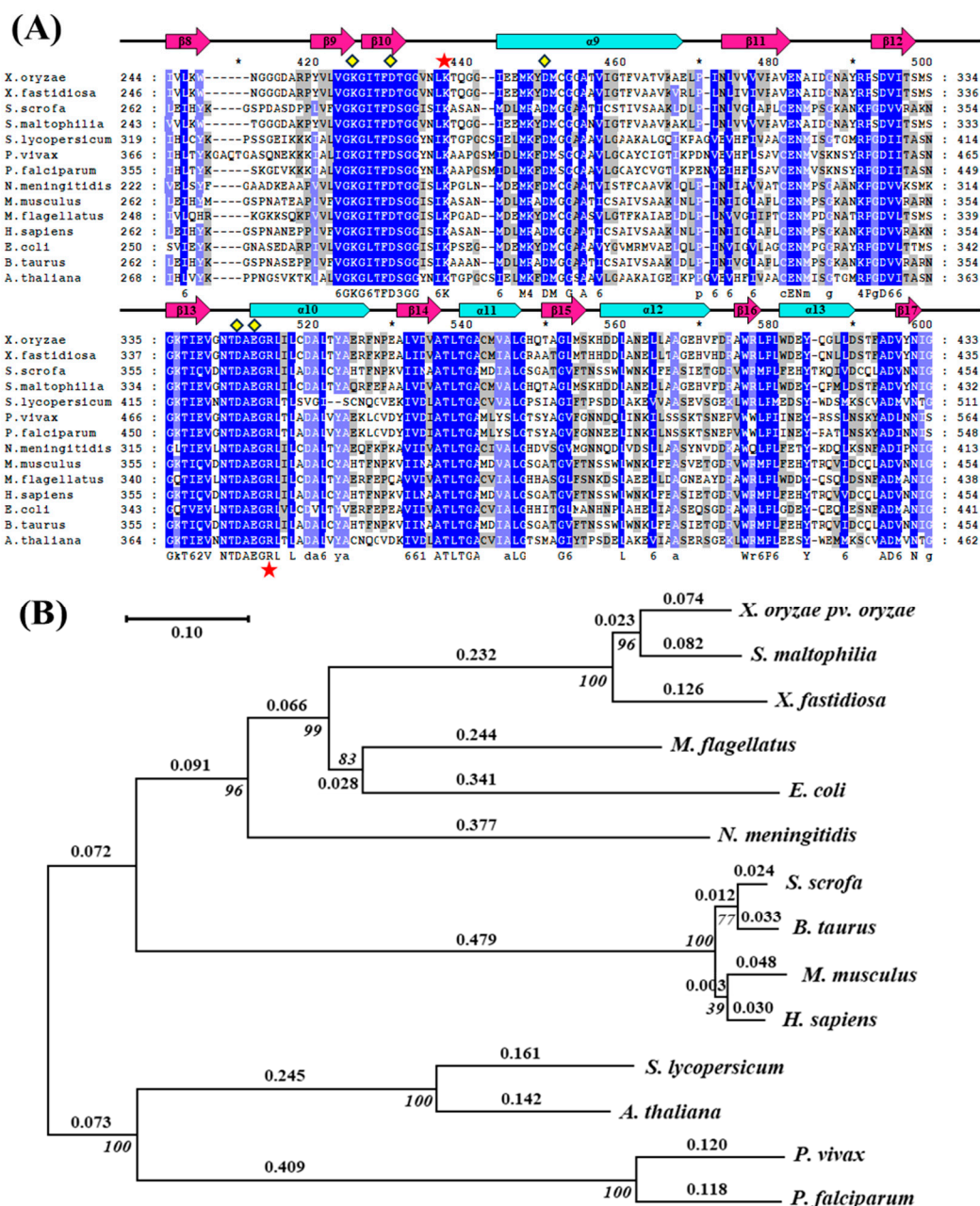


Figure 2. Sequence alignment and phylogenetic analysis of Xoo-PepA with other members of LAP family. **(A)** Multiple sequence alignment of the catalytic domains of M17 LAPs. Conserved metal-binding residues (yellow squares) and active site residues (red asterisks) are highlighted. **(B)** Phylogenetic tree illustrating evolutionary relationships among Xoo-PepA and homologous LAP sequence. The tree is drawn to scale, with branch lengths representing evolutionary distances in units of amino acid substitutions per site and bootstrap support values shown at the nodes.

3.3. Recombinant Xoo-PepA Expression and Purification

To prepare recombinant Xoo-PepA for biochemical and functional analyses, its cDNA was cloned into a pET28a vector and expressed in *E. coli* BL21(DE3). The recombinant protein was purified by nickel affinity chromatography. Xoo-PepA was successfully expressed, yielding approximately 5 mg enzyme per liter of culture. The purity and size of the obtained enzyme were confirmed by SDS-PAGE and Western blot analysis, revealing a purity exceeding 95% and an apparent monomeric molecular mass of ~53 kDa (Figures 3A,B). Figure 3C suggests the apo form is unstable even at low temperature, i.e., without 37 °C incubation, and that metal ions such as Ni²⁺ are essential for its structural integrity. In addition, SDS-PAGE analysis in Figure 3D suggests that the observed bands in native gels consist of intact Xoo-PepA subunits without degradation, arguing against nonspecific proteolysis.

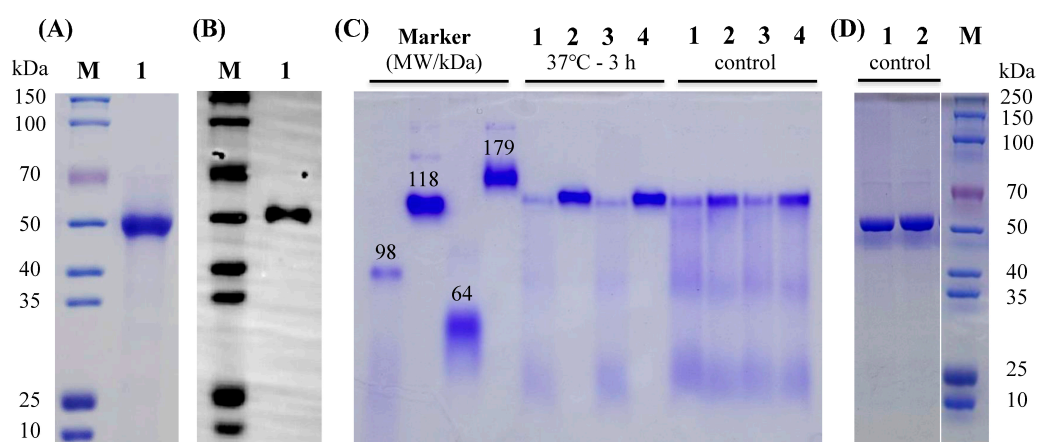


Figure 3. Characterization of purity, molecular weight and structural stability of recombinant Xoo-PepA. (A,B) SDS-PAGE and Western blot analysis of recombinant Xoo-PepA. Line M, molecular weight marker; Line 1, Xoo-PepA purified from cell lysates. (C) Native PAGE of Xoo-PepA under various conditions. Sample 1–4: 1, Xoo-PepA; 2, Xoo-PepA + Ni²⁺; 3, Xoo-PepA + TCEP; 4, Xoo-PepA + Ni²⁺ + TCEP. Equal amounts of enzymes were incubated at 37 °C for 3 h in the absence or presence of supplements, samples without incubation served as controls, and equal volumes of samples collected before and after incubation were loaded for analysis. In-house proteins were used as markers, whose theoretical MW are 64 kDa, 98 kDa, 118 kDa and 179 kDa, respectively. (D) SDS-PAGE analysis of Xoo-PepA in the absence or presence of Ni²⁺ in no-incubation control group. Original images can be found in Supplementary Materials.

3.4. Biochemical Characterization of Xoo-PepA

Given that Xoo-PepA belongs to the M17 family of metal-dependent aminopeptidases and exhibits high sensitivity to environmental conditions [32,34,35], its biochemical properties, including the influence of environmental factors (pH and temperature) and chemical agents (metal ions and reducing agents) on enzymatic activity were assessed.

(1) Effect of metal ions and thiol reducers. Xoo-PepA is typically classified as a Zn-dependent peptidase, with two catalytic zinc ions bound in each monomer. To elucidate the regulatory role of metal ions in the catalytic activity of Xoo-PepA, the effects of various metal ions on the hydrolysis of L-leucine-pNA were systematically evaluated. Several divalent metal ions such as Ni²⁺, Co²⁺, Zn²⁺ and Mn²⁺ at concentrations of 200 μM and 1 mM enhanced the catalytic activity of Xoo-PepA, with Ni²⁺ exhibiting the most pronounced effect (Figure 4A). Notably, the addition of 1 mM Ni²⁺ increased the reaction rate of Xoo-PepA by approximately 770-fold. The peptidase activity of Xoo-PepA showed a dose-dependent increase with rising Ni²⁺ concentration (Figure 4B). Although the highest activity

of Xoo-PepA was observed at 3 mM Ni^{2+} , precipitate was observed in the reaction mixture at this concentration due to the solubility limitation in the buffering solution. Therefore, 1 mM Ni^{2+} was used in the subsequent study. Moreover, mutations of residues involving metal ion coordination in the active site (K262A, D267A/E346L) and mutations of residues critical for oligomer assembly (W410A/Y418A) resulted in almost undetectable activity for L-leucine-pNA, as demonstrated in Figure 4C. This further confirms the essential role of metal binding in conical peptidase activity of Xoo-PepA. To compare the metal dependence of these divalent metal ions, kinetic parameters of Xoo-PepA toward L-leucine-pNA were determined in the presence of 1 mM Ni^{2+} , Co^{2+} or Zn^{2+} . As shown in Figure 4D, Xoo-PepA exhibited the strongest substrate binding affinity and highest catalytic efficiency (k_{cat}/K_M) in the presence of Ni^{2+} . The kinetic parameters follow the order: K_M , $\text{Ni}^{2+} < \text{Zn}^{2+} < \text{Co}^{2+}$; k_{cat} , $\text{Ni}^{2+} \sim \text{Co}^{2+} >> \text{Zn}^{2+}$. Consequently, the catalytic efficiency with Ni^{2+} was 12-fold and 44-fold higher than with Co^{2+} and Zn^{2+} , respectively. In addition, Ni^{2+} binding markedly stabilizes the structure of Xoo-PepA (Figure 3C), suggesting that the metal ion is critical for maintaining the protein's proper conformation, oligomeric state and catalytic activity. These stimulatory effects are consistent with previous reports on M17 aminopeptidases [32,34,36], which demonstrate that specific metal ion bindings are required not only for catalytic activity, but also for inducing conformational changes into a catalytically competent state. Accordingly, Ni^{2+} was included as a cofactor in the subsequent investigations to stabilize the enzyme conformation and preserve high enzymatic activity.

Xoo-PepA functions as a multimer and each monomer contains six cysteine residues highly susceptible to oxidation, which may disrupt protein structure and impair function. To explore this vulnerability, the effect of the reducing agent TCEP on Xoo-PepA's enzymatic activity was further investigated. Experimental results revealed that the addition of TCEP to maintain the cysteine residues in a reduced state did not adversely affect the enzyme, but instead slightly enhanced Xoo-PepA's catalytic activity in the presence of stimulatory metal ions such as Ni^{2+} and Co^{2+} (Figure 4A). Therefore, TCEP was included in the enzyme preparation along with Ni^{2+} to maintain the native conformation and optimal activity of Xoo-PepA.

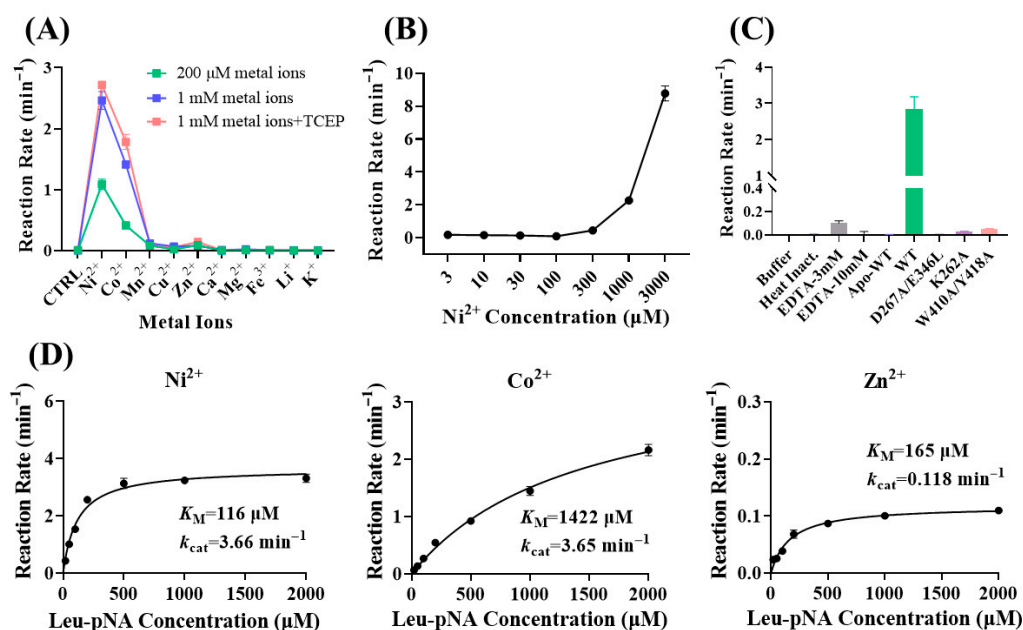


Figure 4. Effect of metal ions and thiol reducing agent on Xoo-PepA activity. (A) Reaction rate of Xoo-PepA in the presence of various metal ions at concentrations of 200 μM and 1 mM, or in the presence of 0.1 mM TCEP with 1 mM of the indicated metal ions. (B) Reaction rate of Xoo-PepA

with different concentrations of Ni^{2+} toward L-Leu-pNA. (C) Reaction rate of 1 mM L-Leu-pNA under various control settings: Buffer refers no-enzyme control, Heat Inact. refers heat-inactivated enzyme, EDTA-3 mM & 10 mM, Apo-WT refers wide-type enzyme without Ni^{2+} , WT and peptidase-inactive mutants (K262A, D267A/E346L, W410A/Y418A). (D) Kinetic data of Xoo-PepA against L-Leu-pNA in the presence of 1 mM Ni^{2+} , Co^{2+} , and Zn^{2+} . Reaction rates are expressed as mean $\pm$ standard deviation in $\mu\text{M}/\text{min}$ per μM enzyme.

(2) Effect of pH. To investigate the influence of pH on Xoo-PepA's catalytic activity, enzymatic assay was performed across a range of pH conditions using 1 mM L-leucine-pNA as the substrate. In the absence of any cofactors, Xoo-PepA exhibited maximal catalytic activity in pH 5.5 buffer system (Figure 5A). However, when both 1 mM Ni^{2+} and 0.1 mM TCEP were included in the reaction system, the optimal pH shifted to pH 7.4 (Figure 5B). The pH activity profile displayed a characteristic bell-shaped curve, with enzymatic activity increasing from pH 5.0 to 7.4 and declining when pH exceeded 7.4. Notably, enzyme activity was severely compromised under extreme pH conditions—approximately 80% loss at both pH 6.5 and pH 10.0, and a complete loss of activity at pH 6.0. In contrast, ~70% of its maximal activity was retained within the physiological pH range of 7.0–8.0, indicating Xoo-PepA functions across a narrow pH range. Subsequent assays were all carried out at pH 7.4.

Aminopeptidase inactivity below pH 6.5 and narrow working range were also reported in several homologous M17 LAPs [37]. While Xoo-PepA exhibits the maximal activity at pH 5.5 without any cofactors, it shows no activity at this pH when Ni^{2+} and TCEP are added. This suggests an acidic environment not only alters the ionization state of active-site residues, but also disrupts the metal coordination environment due to proton-induced depletion of Ni^{2+} , thereby cooperatively regulating enzyme activity. Moreover, metal precipitation in the reaction mixture may also play a role in the decline in activity occurred in basic environment [32,37,38].

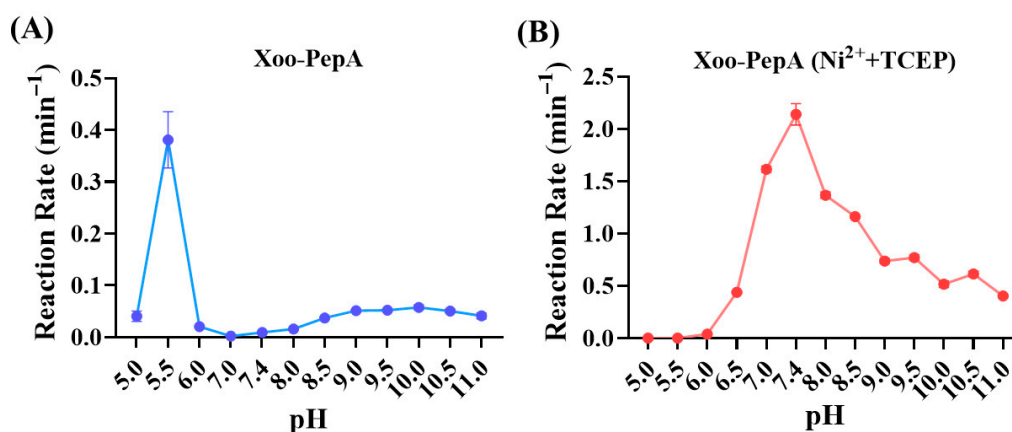


Figure 5. Effect of pH on Xoo-PepA activity. (A,B) Reaction rate of Xoo-PepA-catalyzed hydrolysis of L-leucine-pNA across a range of pH conditions in the absence (A) or presence (B) of 1 mM Ni^{2+} and 0.1 mM TCEP. Data are presented as mean $\pm$ standard deviation in $\mu\text{M}/\text{min}$ per μM enzyme.

(3) Thermostability. To assess the temperature dependence of Xoo-PepA activity, enzymatic assays were performed across a range of temperatures using 1 mM L-leucine-pNA as the substrate. In the absence of cofactors, Xoo-PepA exhibited maximal activity at 42 °C and sharply declined above this temperature (Figure 6A). However, supplementation with Ni^{2+} and TCEP increased the optimal temperature to 55 °C, with comparable enzymatic activity observed from 37 °C to 55 °C (Figure 6B). Thermal stability of Xoo-PepA was further evaluated by monitoring residual activity over time. Activity–time

profiles reveal that the apo form of Xoo-PepA lost nearly all activity within 2 h at 37 °C (Figure 6C). In contrast, enzymes supplemented with Ni^{2+} and TCEP retained approximately 60% of initial activity after 24 h incubation at the same temperature (Figure 6D). In addition, Xoo-PepA in the presence of Zn^{2+} also remained stable over a 24 h incubation period (Figure 6D), suggesting divalent metal ions such as Ni^{2+} and Zn^{2+} play a critical role in maintaining the enzyme structural integrity and enhancing its thermostability. To access the structural stability of Xoo-PepA in the presence of Ni^{2+} and TCEP, native PAGE was performed using samples collected before and after 3 h incubation at 37 °C. Results in Figure 3C align with data in Figures 6C,D, suggesting an essential role of Ni^{2+} in maintaining Xoo-PepA's structural integrity and thermostability.

All these data demonstrate that Ni^{2+} and TCEP substantially broaden Xoo-PepA's functional temperature range (25–55 °C) and dramatically enhance its thermal stability, extending its half-life at 37 °C from 1 h to more than 24 h. Together, TCEP and Ni^{2+} supplementation not only significantly boost the catalytic activity of Xoo-PepA but also markedly improve its thermal stability, indicating a metal-ion-dependent mechanism underlying its functional and structural dynamics, a feature observed or proposed in members of M17 aminopeptidases [32,37,38].

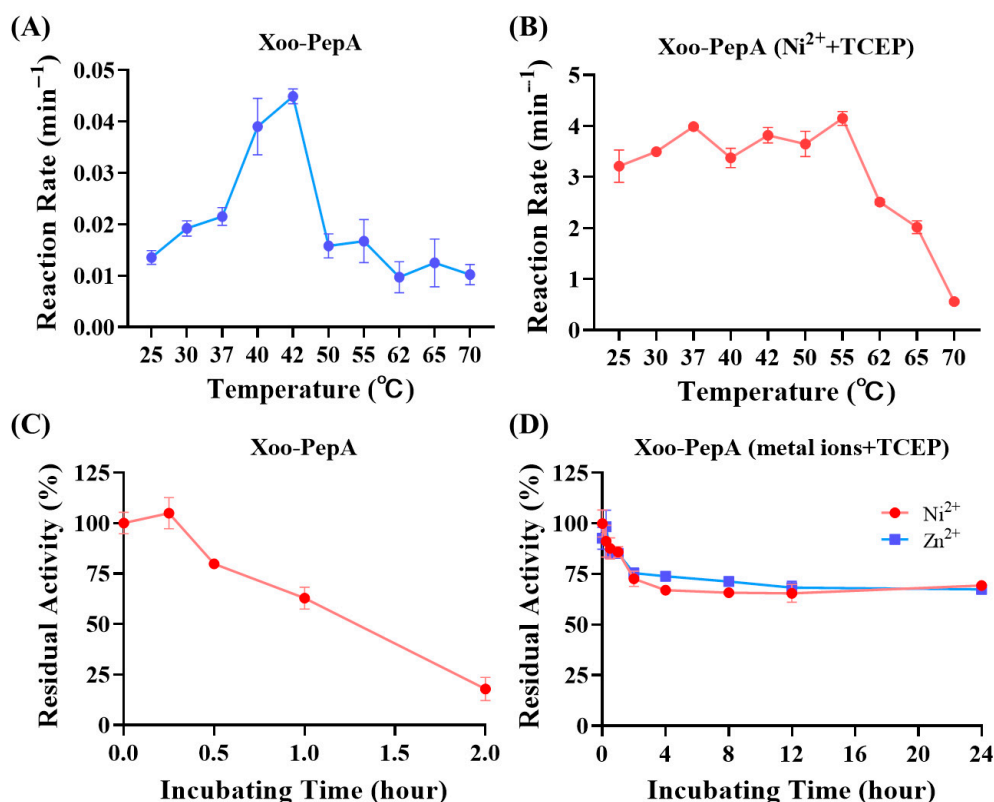


Figure 6. Thermostability of Xoo-PepA. (A,B) Effect of temperature on Xoo-PepA's activity toward L-leucine-pNA in the absence (A) or presence (B) of 1 mM Ni^{2+} and 0.1 mM TCEP. (C,D) Residual activity of Xoo-PepA supplemented without (C) or with (D) Ni^{2+} or Zn^{2+} and TCEP after incubation for various times at 37 °C. The initial reaction rate is represented in $\mu\text{M}/\text{min}$ per μM of enzyme, with the error bar indicating the standard deviation of three replicates.

3.5. Substrate Profiling of Xoo-PepA Toward Peptidase Substrates

The M17 family aminopeptidases share a highly conserved hydrophobic pocket in its structure, which largely determines the substrate recognition specificity. Although its members are often annotated as leucine aminopeptidases (LAP), which typically cleave leucine residues from N-terminal of peptides and proteins, many often exhibit a broader

substrate specificity. Although Xoo-PepA is taxonomically classified as a LAP, its true substrate specificity remains unclear. Therefore, experimental characterization of Xoo-PepA's substrate profile is crucial to clarify its biological function and potential role in pathogenesis.

Substrate specificity profiling using amino acid p-nitroanilide derivatives reveals that, in the absence of Ni^{2+} and TCEP, Xoo-PepA exhibited dramatically diminished activity across all tested substrates, with only L-leucine-pNA, L-arginine-pNA and L-alanine-pNA showing detectable hydrolysis (Figure 7A). In contrast, in the presence of Ni^{2+} and TCEP, Xoo-PepA can effectively hydrolyze a range of these substrates, displaying classical Michaelis–Menten kinetics. Under these optimized conditions, the highest catalytic efficiency was observed for L-leucine-pNA ($4.11 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$), followed by L-arginine-pNA ($3.21 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$) and L-methionine-pNA ($2.61 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$), while much lower catalytic efficiency was obtained for alanine-, glycine-, and proline-p-nitroanilide (Figures 7B,C). All these results demonstrate that activated Xoo-PepA exhibits a defined yet broad substrate specificity for cleavage of N-terminal leucine, arginine, and methionine residues in peptides and proteins, thereby expanding its functional substrate profile beyond an exclusive preference for N-terminal leucine.

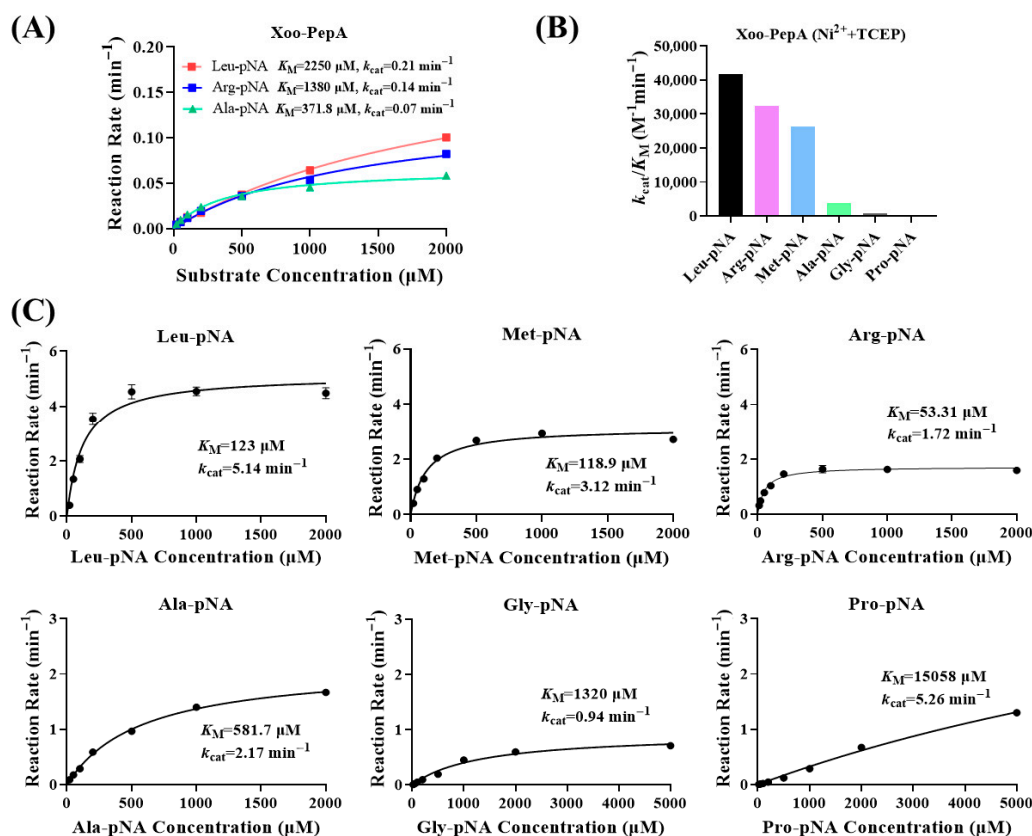


Figure 7. Substrate specificity of Xoo-PepA as an aminopeptidase. (A) Kinetic data for amino acid p-nitroanilide substrate hydrolysis catalyzed by Xoo-PepA in the absence of Ni^{2+} and TCEP. Only three substrates exhibited detectable activity. (B) Relative catalytic efficiency (k_{cat}/K_M) of Xoo-PepA toward six amino acid p-nitroanilide substrates in the presence of Ni^{2+} and TCEP. (C) Kinetic data for six amino acid p-nitroanilide substrate hydrolyses catalyzed by Xoo-PepA in the presence of Ni^{2+} and TCEP. Reaction rates are presented as mean $\pm$ standard deviation in $\mu\text{M}/\text{min}$ per μM enzyme.

3.6. Catalytic Activity of Xoo-PepA Toward Heroin and 6-MAM

Through computational screening and molecular simulation, Xoo-PepA was identified as a potential enzyme capable of hydrolyzing heroin and 6-MAM. To verify this

prediction, the catalytic activity of Xoo-PepA toward these substrates was assessed through HPLC-based quantification. Compared to the enzyme-free control or the heat-inactivated enzyme control, incubation of a 20 μM enzyme with 200 μM heroin or 6-MAM at 37 $^{\circ}\text{C}$ led to the formation of more 6-MAM or morphine, confirming Xoo-PepA can hydrolyze both compounds (Figure 8A–C).

The effects of Ni^{2+} and TCEP on Xoo-PepA's activity toward heroin and 6-MAM were also evaluated. Surprisingly, the addition of these supplements did not significantly enhance the activity toward heroin (Figure 8C), despite the significant role in activating Xoo-PepA for canonical peptidase substrates. To further investigate the role of metal ions in heroin hydrolysis, EDTA chelation and mutations of residues involved in metal ion coordination (K262A, D267A/E346L) or oligomer assembly (W410A/Y418A) were employed for this test. Product formation in the EDTA-treated group was significantly reduced, i.e., comparable to heat-activated enzyme controls. While K262A and W410A/Y418A retained similar activity to wide-type enzymes, D267A/E346L showed reduced product formation compared to WT. These results suggest that metal ions are also involved in heroin hydrolysis, albeit to a lesser extent than in canonical peptidase activity. Given the markedly improved thermal stability conferred by Ni^{2+} and TCEP addition, the supplemented enzyme was used in subsequent kinetic characterizations of heroin and 6-MAM hydrolysis.

Kinetic analysis revealed that heroin and 6-MAM hydrolysis catalyzed by Xoo-PepA follows the Michaelis–Menton kinetics, with the following parameters: $K_M = 1376 \mu\text{M}$, $k_{\text{cat}} = 0.09 \text{ min}^{-1}$ (Figure 8D, for heroin), and $K_M = 838.6 \mu\text{M}$, $k_{\text{cat}} = 0.0028 \text{ min}^{-1}$ (Figure 8E, for 6-MAM). Despite its relatively low catalytic activity, Xoo-PepA, an aminopeptidase, demonstrates the ability to catalyze the hydrolysis of esters such as heroin and 6-MAM. This highlights Xoo-PepA's catalytic versatility toward non-native substrates, and its potential as an ester-hydrolyzing biocatalyst and as a scaffold for re-engineering for heroin degradation applications.

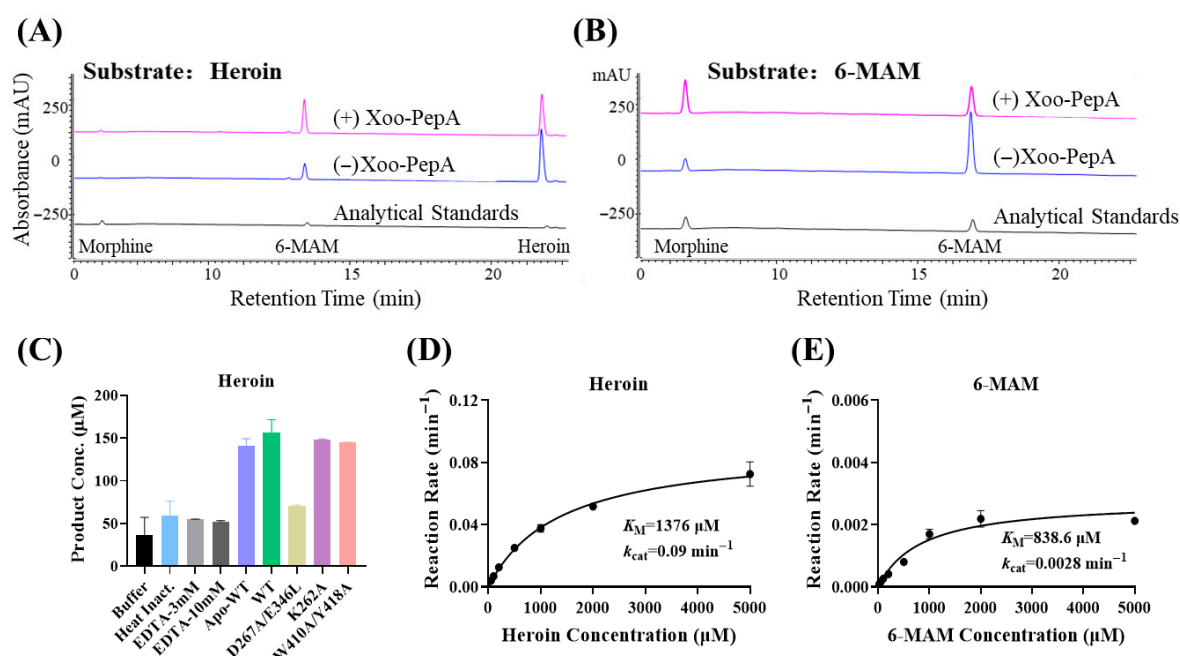


Figure 8. Experimental data confirm that Xoo-PepA catalyzes the hydrolysis of heroin and 6-MAM. (A,B) HPLC–UV chromatograms at 210 nm of heroin (A) or 6-MAM (B) reactions with or without Xoo-PepA. Chromatograms are vertically offset for clarity in this stacked plot, and negative mAU values result from baseline shifting to facilitate visual comparison and do not represent actual negative absorbance. (C) Product formation from the hydrolysis of 1 mM heroin for 2 h under various

control settings: Buffer refers no-enzyme control, Heat Inact. refers heat-inactivated enzyme, EDTA-3 mM & 10 mM, Apo-WT refers wide-type enzyme without Ni^{2+} , WT and peptidase-inactive mutants (K262A, D267A/E346L, W410A/Y418A). (D,E) Michaelis–Menten kinetic profiles for Xoo-PepA-catalyzed hydrolysis of heroin (D) and 6-MAM (E) at 37 °C. Reaction rates are reported as mean $\pm$ standard deviation in $\mu\text{M}/\text{min}$ per μM enzyme.

4. Discussion

Current treatments for heroin use disorders using μ -opioid receptor agonist or antagonist such as methadone, buprenorphine, and naltrexone show efficacy but are limited by high relapse rates, adverse effects, and poor adherence [6,7,39]. Clinically, acute detoxification is a prerequisite to stabilize patients before long-term abstinence and rehabilitation. Enzymatic therapy holds promise for mitigating acute toxicity and supporting addiction treatment, particularly in drug overdose [14,16,40]. In an effort to search for an efficient heroin hydrolase capable of hydrolyzing heroin and its primary active metabolite 6-MAM, Xoo-PepA, an aminopeptidase from *Xanthomonas oryzae* pv. *Oryzae*, was identified through computational screen and experimental validation. This study demonstrates for the first time that Xoo-PepA catalyzes heroin and 6-MAM hydrolysis, sequentially converting heroin to 6-MAM and then to morphine. This unexpected esterase-like activity in an aminopeptidase reveals a new capability for this enzyme class, shedding light on its intrinsic catalytic versatility and biocatalyst potential.

Xoo-PepA is a leucine aminopeptidase from *Xanthomonas oryzae* pv. *oryzae*, the bacterial pathogen responsible for rice bacterial white leaf blight [41]. As a member of the metal-dependent M17 aminopeptidase family, it has been proposed as a potential target for novel antibacterial agents [42,43]. M17 LAPs are evolutionarily conserved, cytoplasmic enzymes found across bacteria, plants, and mammals, typically requiring divalent metal cations, most commonly Zn^{2+} for activity [29,35,44]. They catalyze the removal of N-terminal amino acids from polypeptides and play key roles in protein turnover and processing [29,30,44]. Xoo-PepA features two distinct metal-binding sites that are critical for both substrate recognition and catalysis, endowing it with functional versatility that extends beyond canonical aminopeptidase activity [35,45]. Despite its annotation as a leucine-specific aminopeptidase, M17 family members often exhibit broader substrate specificity than annotated [30,35]. Moreover, as a metalloenzyme, Xoo-PepA's activity is highly sensitive to environmental conditions such as pH, temperature and metal ion availability. Consequently, the biochemical and functional properties of Xoo-PepA were extensively characterized to enable a more comprehensive understanding of its catalytic mechanism and its potential as a scaffold for further re-engineering toward a detoxifying agent against heroin.

Metal-dependence studies revealed that Ni^{2+} supplementation enhanced Xoo-PepA's catalytic activity toward the peptidase substrate L-leucine-pNA by approximately 770-fold, yet it showed no significant activation effect on heroin hydrolysis. However, EDTA addition significantly decreased its activity toward heroin, suggesting metal ions may still play a regulatory role in this reaction. Structural analysis indicates K262, D267, D285, D344, E346 coordinate metal ions within the active site, with D267 and E346 each interacting with two metal ions [31]. W410/Y418 are conserved residues involved in oligomer assembly through Pi-Pi stacking interactions between chains [32]. Therefore, mutations of these residues are hypothesized to compromise active site metal binding or disrupt the oligomer state of Xoo-PepA. Activity assays of WT, K262A and D267A/E346L toward L-Leu-pNA and heroin revealed that these mutants exhibited almost no activity for L-Leu-pNA. In contrast, while D267A/E346L showed significantly decreased hydrolytic activity toward heroin, K262A and W410A/Y418A show comparable activity as wide-type enzymes. These findings suggest the canonical metal-dependent active site of Xoo-PepA is likely

involved in heroin hydrolysis; however, this activity appears to be less dependent on metal ions than canonical peptidase activity. Distinct catalytic mechanisms with differing dependencies on metal ion identity and coordination geometry may be engaged in peptide and ester hydrolysis.

Although its hydrolytic activity toward heroin appears less sensitive to metal ion availability, results indicate a divalent metal cofactor such as Zn^{2+} or Ni^{2+} is essential for Xoo-PepA's structural integrity and thermostability. Xoo-PepA functions as a multimer, with each subunit containing six cysteine residues that are highly prone to oxidation. Oxidation of these residues can compromise structural integrity and impair enzymatic function. To mitigate this risk, the reducing agent TCEP was included in assays to preserve Xoo-PepA in its native conformation. In addition, metal ion addition adjusts the optimal pH of Xoo-PepA to pH 7.4, thereby positioning its peak activity within the physiological pH range of 7.0–8.0. Together, these findings reveal a metal-ion-dependent mechanism that regulates both the catalytic function and structural conformational dynamics of Xoo-PepA, a feature previously reported for other members of the M17 aminopeptidase family [32,37,38]. Although Xoo-PepA is taxonomically annotated as a leucine aminopeptidase, it effectively hydrolyzes L-leucine-, L-arginine-, and L-methionine-pNA. Substrate profiling data demonstrate Xoo-PepA exhibits a broadened yet selective substrate preference for cleaving N-terminal Leu, Arg, and Met residues in peptides and proteins, thereby expanding its functional scope beyond a strict preference for Leu.

The observed promiscuous esterolytic activity of Xoo-PepA extends beyond its canonical role in peptide bond cleavage. Structural and evolutionary analysis suggests that the bimetallic active site and surrounding stabilization residues are evolutionarily configured for peptide substrates, resulting in low catalytic efficiency due to suboptimal geometry and hydrophobic complementarity for ester substrates such as heroin. These structural insights may provide directions for enzyme rational engineering to enhance Xoo-PepA's esterolytic activity, including reshaping the substrate-binding pocket, expanding the hydrophobic access tunnel, tuning metal coordination geometry, and introducing strategic mutations to improve transition-state stabilization during ester hydrolysis [46,47].

5. Conclusions

In summary, this study identifies Xoo-PepA, an aminopeptidase from *Xanthomonas oryzae* pv. *oryzae*, as a hydrolase capable of catalyzing the sequential conversion of heroin to 6-MAM and then to morphine. Substrate preference profiling demonstrates that its peptidase catalytic scope extends beyond a strict Leu specificity to include N-terminal Leu, Arg, and Met residues. The expansion in catalytic scope and hydrolytic activity observed in an aminopeptidase reveals a previously unappreciated functional plasticity within the family of Xoo-PepA. As a metalloenzyme, divalent metal ions are essential for Xoo-PepA's structural stability and biochemical properties. Peptidase activity, optimal pH, and thermostability are regulated by a metal-dependent mechanism. While its canonical peptidase activity is significantly enhanced by metal ions, its ability to hydrolyze heroin appears considerably less dependent on metal ion availability. Structural analysis reveals that this promiscuous esterolytic activity of Xoo-PepA toward heroin or 6-MAM arises from a bimetallic active site evolutionarily configured for peptide substrates, thereby providing a direction for rational engineering toward enhanced heroin degradation.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biom16020298/s1>. Original images of Figure 3 can be found in supplementary materials

Author Contributions: H.L.: investigation, methodology, formal analysis, visualization, writing—original draft. Q.H.: investigation, formal analysis, visualization, writing—original draft. N.X.: investigation, methodology. X.S.: methodology, validation. Y.L.: validation, resources. Y.H.: resources. B.W.: resources. J.W.: resources, funding acquisition. J.Y.: software, investigation, project administration, funding acquisition. S.H.: conceptualization, formal analysis, project administration, writing—review and editing. X.C.: conceptualization, project administration, writing—review and editing, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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