Facile Purification of a C-Terminal Extended His-Tagged *Vibrio mimicus* Arylesterase and Characterization of the Purified Enzyme

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ABSTRACT: *Vibrio mimicus* arylesterase, a 20 kDa protein, is a multifunctional enzyme with thioesterase and chymotrypsin-like activities. Because an affinity His-tag (six consecutive histidine affinity tag) directly to the protein caused the loss of enzyme activity, a hexadecapeptide with His-tag, ADPNSSSVDKLAAALE-HHHHHH encoded from vector pET-20b(+) was constructed to extend from the carboxyl terminus of the arylesterase. This His-tagged protein retained enzyme functions. Thermal unfolding behavior of both proteins was almost identical, and their T_m values were near 54°C as monitored by circular dichroism. Tryptic cleavage of the functional His-tagged enzyme produced two smaller proteins, which still possessed enzyme activity and which suggested that the additional peptide extended on the protein surface. The spacing peptide between His-tag and arylesterase successfully prevented the interference of the His-tag to the enzyme functions. The kinetic studies showed that the esterase and thioesterase activities of the His-tagged enzyme were similar to those of the wild type. On the other hand, the catalytic efficiency of chymotrypsin-like activity of the His-tagged protein was two times higher than that of the wild type.
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KEY WORDS: Arylesterase, enzyme purification, His-tag, recombinant protein.

Esterases (ester hydrolases) (E.C. 3.1.1.), widely distributed in nature, catalyze the hydrolysis of ester bonds (1). Arylesterases (E.C. 3.1.1.2.) show a preferential substrate specificity for aromatic esters (2). The 0.6 kb gene, *etpA*, which encodes an extracellular arylesterase, was isolated and sequenced from the genome of *Vibrio mimicus* (3). The 20 kDa arylesterase is a novel serine arylesterase that also has thioesterase and chymotrypsin-like activities (4,5). It has been proposed to belong to a distinct subfamily (5) within a lipolytic enzyme family (6).

The linkage of the His-tag (six consecutive histidine residues) to an enzyme is useful to facilitate purification by an affinity column (7,8). However, there is no report for the His-tag effects upon enzyme activity and biochemical properties. His-tag added on the amino terminus of the *V. mimicus* arylesterase resulted in a large decrease of esterase activity (Chang, R.-C., unpublished data). In the present study, we found that direct addition of His-tag to the carboxyl terminus of this protein resulted in losing enzyme functions completely. In contrast, a recombinant enzyme with a peptide, ADPNSSSVDKLAAALEHHHHHH encoded from vector pET-20b(+) sequence and spacing the arylesterase and C-terminal His-tag, retained enzyme activity. This recombinant enzyme was easily purified by immobilized Ni-resin column and sequentially characterized. The additional peptide has little effect on the esterase and thioesterase activity but results in doubling the catalytic efficiency of chymotrypsin-like activity.

MATERIALS AND METHODS

Materials. Oligonucleotide primers were made by DNAFax Co. (Taipei, Taiwan), and Taq DNA polymerase and DNA sequencing kit were obtained from HT Biotechnology Ltd. (Cambridge, England) and US Biochemicals (Cleveland, OH), respectively. Isopropyl thio-β-D-galactoside (IPTG) was obtained from B.M. Biochemicals (Mannheim, Germany), while *p*-nitrophenyl butyrate, *p*-nitrophenyl decanoate, α-naphthyl butyrate, decanoyl-CoA, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), *N*-carbobenzoxy-L-phenylalanine *p*-nitrophenyl ester (L-NBPNPE), *N*-carbobenzoxy-D-phenylalanine *p*-nitrophenyl ester (D-NBPNPE), and *N*-carbobenzoxy-D-tyrosine *p*-nitrophenyl ester (D-NBTNPE) were purchased from Sigma Chemical Co. (St. Louis, MO). Ni-NTA resin (6×His-binding resin) was purchased from Novagene Co. (Madison, WI), and DEAE Sepharose CL-6B, Superdex 75 HR10/30 and Q Sepharose Fast Flow gel were the products of Pharmacia Co. (Quarry Bay, Hong Kong). Protein molecular-weight markers were purchased from Novex Co. (San Diego, CA).

Construction of the recombinant arylesterase gene. Recombinant arylesterase genes were synthesized from plasmid pL662dHE (3) by a two-primer polymerase chain reaction (PCR) method (9). Following are the primers used for the gene whose His-tag directly followed the C-terminal end of

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the wild-type protein: the 5′ end was 5′-TCCGCCGCATCG-CATATGAGCGAAAAGCTTCTTGTT-3′, and the 3′ end was 5′-GGGCTCGAGATGTTTAACCAATTC-3′. These are the primers used for the gene whose His-tag was adjacent to the hexadecapeptide following the C-terminal end of the wild-type: the 5′ end was the same as the former, and the 3′ end was 5′-GAGGATCCG CGAGATGTTTAACCAATTC-3′. The former PCR products were digested with *Nde*I (CA/TATG) and *Xho*I (C/TCGAG) restriction enzymes, and then ligated into a 3.7 kilobase *NdeI*/*Xho*I-restricted pET20b(+) vector (Novagene Co.). The latter PCR products were digested with *Nde*I and *BamH*I (GG/ATCC) restriction enzymes, and then ligated into the *NdeI*/*BamH*I-restricted pET20b(+) vector. The constructed plasmids were cloned from *Escherichia coli* HB101 (Promega Co., Madison, WI) and then were transformed into *E. coli* BL21(DE3) (Novagene Co.) for target protein overexpression. The DNA sequences of the recombinant enzymes were confirmed (10) from plasmids isolated from HB101. Bacterial growth and protein overexpression conditions were as previously described (4,11).

Strategy for the design of functional recombinant protein. The secondary structure of the arylesterase was predicted by the methods of Chou and Fasman (CF) (12) and of Garnier *et al.* (GOR) (13). The C-terminal end of the arylesterase was predicted to fold into an α helix, and the helix to be extended to the last histidine if His-tag was directly attached to the C-terminus of the protein (data not shown). For preventing the effect of His-tag to the enzyme, a recombinant arylesterase was designed with a C-terminal extension of a hexadecapeptide to link His-tag. The extension peptide, ADPNSSSVDKLAAALEHHHHHH, was encoded from vector pET-20b(+), and it increased the protein molecular weight to 24.5 kDa. The third amino acid of the additional peptide, proline, was designed to function as a helix breaker to separate the intact enzyme domain from C-terminal extended peptide. The predicted secondary structure of the additional peptide contains an α helix, which folds into an amphipathic helix from the eighth to the eighteenth amino acid residue (Table 1 and Fig. 1).

Cell growth condition. Bacteria were grown in LB broth, which consisted of 1% Bacto Tryptone, 0.5% Bacto yeast extract, and 1% NaCl (pH value was adjusted to 7.0). For plasmid selection, ampicillin was added at a concentration of 50 μg/mL. Liquid cultures were shaken at 200 rpm at 37°C. The cultures for protein overexpression were induced by adding IPTG to a final concentration of 2.5 mM, when the absorbance value of OD_{600} was approximately 0.6. Cultures were then incubated at 30°C while vigorously shaken until harvesting the cells.

Enzyme extraction and purification. Recombinant arylesterase was purified by affinity chromatography with Ni-

TABLE 1
Secondary Structure Prediction of the Added Peptide, According to the CF and GOR Methods (provided by the Genetics Computer Group, Madison, WI)

Pos[a]	AA[a]	GlycoS[a]	HyPhil[b]	SurfPr[c]	FlexPr[d]	CF-Pred[e]	GORPred[f]	AI-Ind[g]
1	A	-	1.700	1.571	1.000	—	—	0.900
2	D		1.520	1.647	1.000	—	—	0.900
3	P		1.400	1.727	1.000	T	—	1.300
4	N	G	1.314	2.291	1.000	T	—	1.300
5	S	—	0.971	1.018	1.113	T	—	1.300
6	S	—	0.971	1.100	1.104	T	—	1.300
7	S	—	1.300	1.368	1.085	t		1.100
8	V	—	0.257	0.842	1.061	H	H	0.450
9	D		−0.114	0.634	1.039	H	H	−0.150
10	K		−0.486	0.478	1.010	H	H	−0.450
11	L	—	−0.857	0.651	0.975	H	H	−0.600
12	A	—	−0.800	0.321	0.945	H	H	−0.600
13	A	—	−0.800	0.278	0.924	H	H	−0.600
14	A		−0.900	0.459	0.918	H	H	−0.600
15	L	—	0.100	0.619	0.925	H	H	0.300
16	E	—	0.814	0.833	0.927	H	H	0.600
17	H	—	1.529	1.122	0.924	H	H	0.750
18	H	—	2.243	1.852	0.914	H	H	0.750
19	H		3.243	1.455	1.000	H	H	0.900
20	H		3.200	1.367	1.000	H	H	0.900
21	H		3.200	1.284	1.000	—	H	0.900
22	H	—	3.200	1.206	1.000	—	—	0.900

[a]Abbreviations: Pos, position; AA, amino acid; GlycoS, glycosylation site.
[b]Hydrophilicity (Kyte-Doolittle) (23).
[c]Surface probability according to Emini *et al.* (24).
[d]Chain flexibility according to Karplus-Schulz (25).
[e]Secondary structure according to Chou-Fasman (12).
[f]Secondary structure according to Garnier-Osguthorpe-Robson (13).
[g]Antigenicity index according to Jameson-Wolf (26).

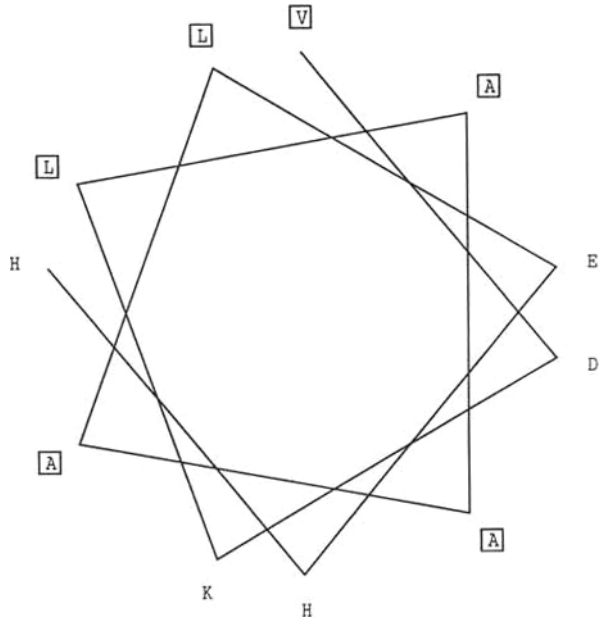

FIG. 1. Secondary structure prediction of the additional peptide. (A) The prediction is according to the CF and GOR methods, which are provided by the Genetics Computer Group (Madison, WI). T means turn, and H means α-helix. From the eighth to the eighteenth amino acid residue, the additional peptide is analyzed with the "Helicalwheel" program provided by the Genetics Computer Group: , hydrophobic.

NTA resin, which is a His-tag binding resin as previously described (9). The protein bound to the column was eluted with 1.0 M imidazole, a histidine analog. For wild-type arylesterase, the harvested cell pellet (from 500 mL-LB broth culture) was suspended in 40 mL of 50 mM Tris buffer (pH 8.0, containing 2 mM EDTA) and then frozen at −70°C for 20 min. After thawing, the suspension was sonicated and then centrifuged. The supernatant of crude extract was separated on a DEAE Sepharose CL-6B column (2.6 cm × 20 cm), equilibrated in 25 mM sodium phosphate buffer, pH 7.0. Elution was carried out with a linear gradient of equilibrating buffer that contained 0.2 to 0.3 M NaCl. The fractions with enzyme activity were pooled and concentrated. Gel filtration was performed on a Superdex 75 column (HR10/30), equilibrated in 25 mM sodium phosphate buffer, pH 7.0, that contained 0.1 M NaCl and 0.02% sodium azide. Elution was carried out with the equilibrating buffer at a flow rate of 0.5 mL/min. The collected enzyme fractions were further purified on a column packed with Q Sepharose Fast Flow gel (1.0 cm × 12 cm), equilibrated under the same conditions as DEAE column chromatography. Protein purity was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) in every step of purification.

SDS–PAGE analysis of arylesterase. 15% SDS–PAGE was prepared for analysis. Loading samples were made of equal volumes of protein solution and SDS–PAGE loading dye, which contained 2% SDS and 200 mM dithiothreitol in 100 mM Tris buffer, pH 6.8. Loading samples were boiled for 5 min, then cooled in an ice bath for 10 min. After electrophoresis, separated protein gels were stained by Coomassie brilliant blue and by activity stains. Before activity staining, SDS in the gel was removed by submersion in 25% isopropanol for 30 min twice, then in 0.1 M sodium phosphate buffer, pH 7.0, for 30 min once (14). Esterase and protease activity stains were performed by the methods of Tanksley and Orton (15) and by Dickey and Collin (16), respectively.

Thermal unfolding of wild-type and functional His-tagged arylesterases. Enzyme solutions of 0.15 mg/mL in 25 mM sodium phosphate buffer (pH 7.0) with 0.1 M NaCl were continuously heated from 36 to 72°C (17). The thermal unfolding of the proteins was analyzed by circular dichroism (CD) through monitoring changes in $\Delta\varepsilon$ at 235 and 235.5 nm for the wild-type and the recombinant arylesterase, respectively. The melting temperatures were calculated from the unfolding curves. The CD spectrum was measured on a spectrophotometer (Jasco J-720, Tokyo, Japan), and temperature was adjusted with a coolflow temperature controller (Neslab, Portsmouth, NH) by water circulation within the jacket of the cell.

Thermal inactivation of wild-type and functional His-tagged arylesterases. The experiments were accomplished according to the method described by Shaw *et al.* (18). Enzyme solutions were immersed in a 95°C water bath for various periods, and then cooled by immersion in an ice bath. The remaining enzyme activity was assayed in triplicate of esterase activity with *p*-nitrophenyl butyrate as substrate (19).

Tryptic digestion. The wild-type and the functional His-tagged arylesterases were treated with trypsin. One μL of trypsin (1 mg/mL) was added into a reaction mixture that contained 60 μg arylesterase and 0.3 M NaCl in 25 mM sodium phosphate buffer (pH 7.0). Digestion was carried out at 37°C and sampled periodically. Reaction was stopped by adding an equal volume of SDS–PAGE loading dye and then heated in boiling water for 5 min. Samples with 2 μg of protein were analyzed on 15% SDS–PAGE.

Enzyme assays. Esterase activity for the substrates *p*-nitrophenyl butyrate and *p*-nitrophenyl decanoate was determined at pH 7.0 and 37°C by the increase of absorbance at 346 nm as described (19). Thioesterase activity for decanoyl-CoA and palmitoyl-CoA were determined at pH 7.0 and 37°C by the increase of absorbance at 412 nm with a coupling reaction of DTNB as described (20). Chymotrypsin-like activity with L-NBPNPE, D-NBPNPE, and L-NBTNPE as substrates was determined at pH 7.5 and 37°C by the increase of absorbance at 400 nm as described (21).

RESULTS AND DISCUSSION

Direct addition of His-tag to the C-terminal end of arylesterase. Direct addition of His-tag to the C-terminal of arylesterase resulted in a protein that failed to exhibit esterase and protease activities as shown by the activity stains on SDS–PAGE gel (Fig. 2, lane 2). This recombinant protein could not be purified with an affinity Ni-resin column. Because it has no activity, this protein was not further purified by conventional method.

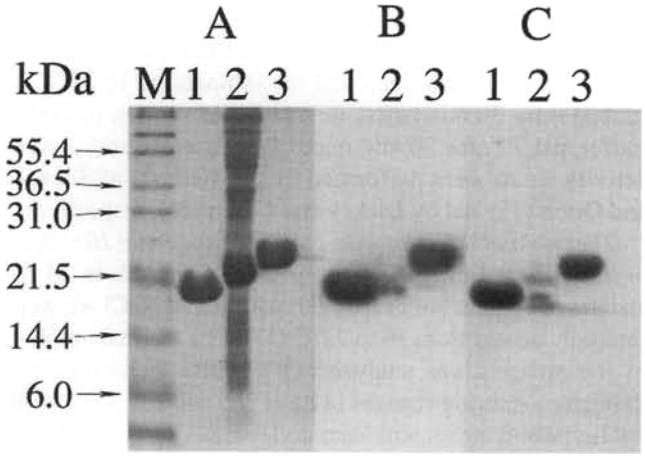

FIG. 2. Protein, esterase, and protease activity analysis for wild-type and His-tagged arylesterases on SDS–PAGE. Part A, Coomassie brilliant blue stains. Part B, esterase activity stains. Part C, protease activity stains. Lane M, protein molecular weight markers; lane 1, purified wild-type arylesterase, 2 μg; lane 2, crude extract of the overexpressed recombinant protein whose His tag was directly added on the C-terminus end of the wild type; lane 3, purified recombinant protein, 2 μg, whose His tag was spaced with a hexadecapeptide.

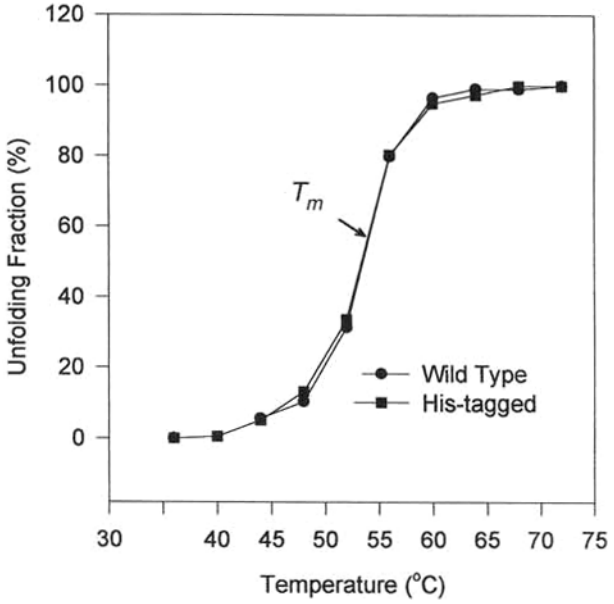

FIG. 3. Thermal unfolding profiles of wild-type and functional His-tagged arylesterases. T_m (54°C) values were calculated for both enzymes from the curves of unfolding fractions vs. temperature.

Protein purification. The wild-type enzyme was sequentially purified by ion exchange (DEAE Sepharose CL-6B), gel filtration (Superdex 75) and a second ion exchange (Q Sepharose Fast Flow) chromatography (Fig. 2, lane 1). Ten mg of purified wild-type protein was obtained from 1 L of LB broth culture. The His-tagged protein with a spacing hexadecapeptide was purified on an affinity Ni-resin column (Fig. 2, lane 3), and 12 mg of purified protein was obtained from 1 L of LB broth culture. Both purified proteins showed a single band on SDS–PAGE stained by Coomassie brilliant blue (A), as well as esterase activity (B) and protease activity (C) in Figure 2. It revealed that the recombinant protein retained enzyme activity.

That this functional His-tagged protein could be successfully purified on an affinity Ni-resin column implied that the spaced His-tag was not buried in the interior of the protein and made the purification by affinity column possible. This result supports the prediction of protein secondary structure.

Thermal unfolding and thermal inactivation of wild-type and functional His-tagged arylesterases. The unfolding fraction vs. temperature profiles revealed that thermal denaturation of these two proteins is almost identical (Fig. 3). T_m, melting point, is the temperature at which half of the protein molecules have unfolded. The calculated T_m values were 53.5 and 53.7°C for the wild-type and His-tagged arylesterases, respectively. In the experiment of protein thermal inactivation, the residual esterase activity after heat treatment at 95°C is shown in Figure 4. Although the protein was completely unfolded and presumably had no enzyme activity at this high temperature, cooling on ice immediately after heat treatment could recover enzyme activity. The heated times for wild-type and His-tagged proteins to lose half of the enzyme activity at 95°C were 25 min and 18.5 min, respectively. The difference could be due to the refolding ability between the two proteins after heat treatment and suggests that the additional peptide did not significantly alter the intact protein structure, but somehow it did affect protein refolding after heating at high temperature.

Tryptic digestion of wild-type and functional His-tagged arylesterases. The trypsin-digested proteins were analyzed on SDS–PAGE (Fig. 5). The wild type (lanes 1–6) was rather intact after protease treatment; however, the His-tagged pro-

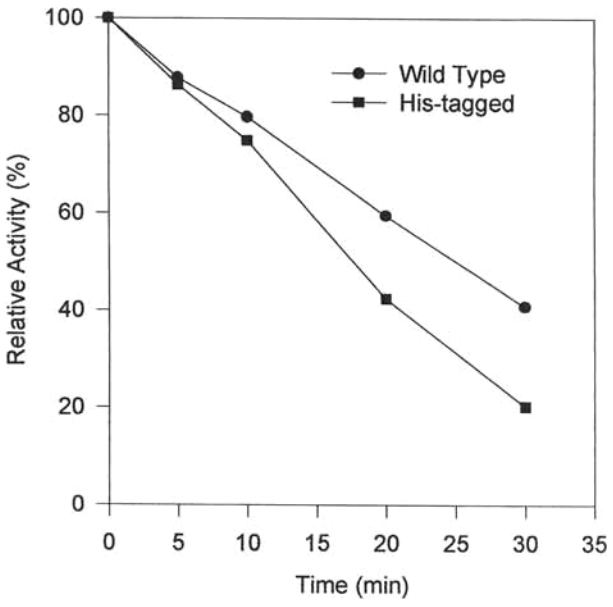

FIG. 4. Thermal inactivation of the wild-type and functional His-tagged arylesterases at 95°C. The heated wild type and His-tagged proteins retained half of the enzyme activity after heating at 95°C for 25 min and 18.5 min, respectively.

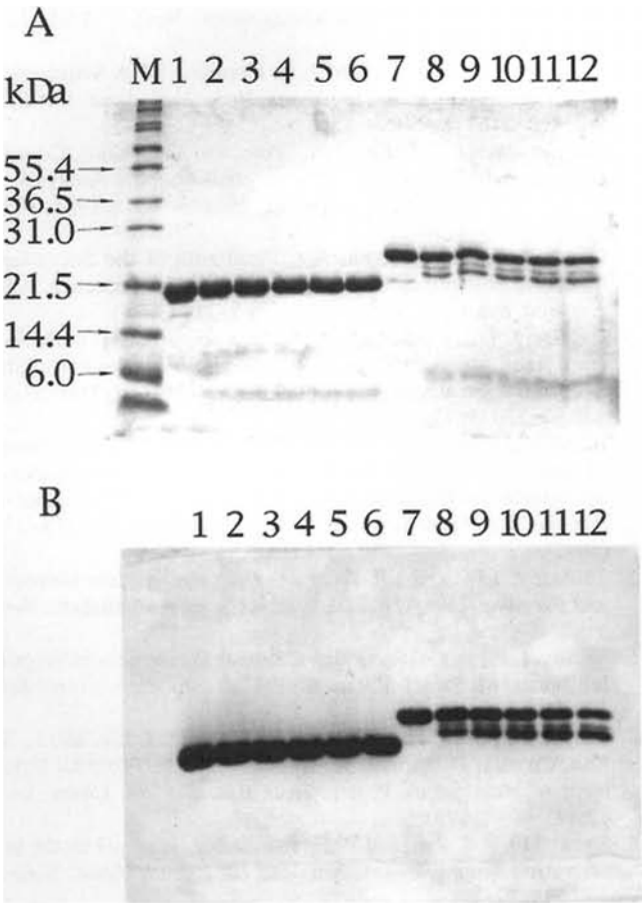

FIG. 5. Tryptic digestion of wild-type and recombinant arylesterase. The serial-digested proteins were separated on 15% SDS–PAGE. Part A, protein Coomassie brilliant blue stains. Part B, esterase activity stains. Lane M, protein molecular weight markers. Lanes 1–6, the wild type was digested for 0, 5, 10, 20, 30, 40 min, respectively. Lanes 7–12, the recombinant was digested for 0, 5, 10, 20, 30, 40 min, respectively.

tein (lanes 7–12) was digested into two slightly smaller proteins, which still possessed enzyme function as monitored by activity stain (B).

The only difference between both enzymes was the C-terminal extension of the His-tagged protein. There are two lysine residues located in the C-terminus of this protein, one in the extension peptide, and another one located next to the C-terminal amino acid of wild-type arylesterase sequence. These two could be the sites for protease digestion that cleaved the His-tagged protein into two smaller proteins. It also served as evidence that the additional peptide exposed on the protein surface thus readily hydrolyzed and consequently did not interfere with the enzyme intact structure.

Enzyme kinetic studies. The His-tagged arylesterase had the same functions as the wild type to hydrolyze esters, thioesters, and amino acid derivatives. The results of enzyme kinetic studies are shown in Table 2. In general, both wild-type and His-tagged enzymes exhibited similar activities when hydrolyzing ester and thioester substrates. The shorter acyl chainlength of *p*-nitrophenyl ester (*p*-nitrophenyl butyrate) was preferred to the longer one for both enzymes. As for thioester substrates, the His-tagged enzyme had similar k_{cat} values (18.5, 18.6 s^{-1}) and the same K_m values (31.1 μM) for palmitoyl-CoA and decanoyl-CoA. However, the wild type preferred to hydrolyze the longer acyl chainlength of acyl-CoA (palmitoyl-CoA).

The chymotrypsin-like activity of the His-tagged enzyme showed twice the catalytic efficiency value of that of the wild type. The increased activity was largely contributed by the decreased K_m values. It suggests that the additional peptide of the recombinant enzyme might slightly change the enzyme conformation and increase the enzyme affinity toward amino acid derivatives.

Conclusion. In general, His-tagged protein purification is

TABLE 2
Kinetic Parameters for Wild-Type and Recombinant Arylesterase[a]

	Wild-type arylesterase			Recombinant arylesterase		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM)
Esters						
p-Nitrophenyl butyrate	331.1	122.1	0.369	476.7	161.1	0.344
p-Nitrophenyl decanoate	741.5	210.7	0.284	766.1	214.9	0.280
Acyl-CoA						
Decanoyl-CoA	33.6	17.9	0.512	31.1	18.5	0.597
Palmitoyl-CoA	22.7	13.0	0.569	31.1	18.6	0.597
Amino acid derivative						
L-NBPNPL	22.2	7.4	0.332	10.6	7.2	0.680
L-NBTNPE	35.7	73.1	2.044	21.9	88.3	4.030

[a]The activities in solution were determined as described in the the Materials and Methods section. Kinetic data were calculated from the Lineweaver-Burk plots (22) and linear regression plots. Each data point is the average value of three independent measurements. K_m, k_{cat}, and k_{cat}/K_m have the units of μM, s^{-1}, and s^{-1} $μM^{-1}$, respectively; L-NBPNPE, *N*-carbobenzoxy-L-phenylalanine-*p*-nitrophenyl ester; L-NBTNPE, *N*-carbobenzoxy-L-tyrosine-*p* nitrophenyl ester.

easy and rapid, but a different arrangement of His-tag in the recombinant protein may result in different effects on protein structure and functions. The resulting alterations may involve changes in many biochemical characteristics, including kinetic behavior, substrate specificity, and thermostability.

His-tag can cause loss of enzyme activity, such as by direct addition to the C-terminus of the *V. mimicus* arylesterase. Our experimental results revealed that a spacing of hexadecapeptide between C-terminus and His-tag can both facilitate enzyme purification and retain enzyme functions. The functional His-tagged protein has been crystallized (11), and the X-ray crystallographic study for this protein is underway. The kinetic study showed that chymotrypsin-like activity of the enzyme was even increased by this modification. It implied that the C-terminus of the enzyme can be engineered to alter or improve enzyme activity and biochemical properties. For both industrial applications and basic research, we have introduced a new method to rearrange the His-tag for functional protein processing.

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