JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Construction of a Bifunctional Enzyme Fusion for the Combined Determination of Biogenic Amines in Foods

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Supporting Information

ABSTRACT: Biogenic amines (BAs) are a group of low-molecular-mass organic bases derived from free amino acids. Due to the undesirable effects of BAs on human health, amine oxidase-based detection methods for BAs in foods have been developed. Here, we developed a bifunctional enzyme fusion (MAPO) using a Cu^{2+}-containing monoamine oxidase (AMAO2) and a flavin adenine dinucleotide-containing putrescine oxidase (APUO) from *Arthrobacter aurescens*. It was necessary to activate MAPO with supplementary Cu^{2+} ions, leading to a 6- to 12-fold improvement in catalytic efficiency (k_{cat}/K_M) for monoamines. The optimal temperatures of Cu^{2+}-activated MAPO (cMAPO) for both tyramine and putrescine were 50 °C, and the optimal pH values for tyramine and putrescine were pH 7.0 and pH 8.0, respectively, consistent with those of AMAO2 and APUO, respectively. The cMAPO showed relative specific activities of 100, 99, 32, and 32 for 2-phenylethylamine, tyramine, histamine, and putrescine, respectively. The tyramine-equivalent BA contents of fermented soybean pastes by cMAPO were more than 90% of the total BA determined by HPLC. In conclusion, cMAPO is fully bifunctional toward biogenic monoamines and putrescine, allowing the combined determination of multiple BAs in foods. This colorimetric determination method could be useful for point-of-care testing to screen safety-guaranteed products prior to instrumental analyses.

KEYWORDS: *biogenic amines, monoamine oxidase, putrescine oxidase , bifunctional enzyme fusion*

INTRODUCTION

A biosensor is an analytical device for the detection of an analyte of interest using a biological component that binds or recognizes the analyte. Many biological elements can be used as biosensors, such as antibodies, enzymes, cell receptors, nucleic acids, tissue, microorganisms, and organelles.[1−5] The development of biosensors for rapid, sensitive, and specific methods of detecting undesirable contaminants in foods, such as food-borne pathogens, viruses, and toxins, is of great importance for food safety and security.[4,6,7]

Biogenic amines (BAs) are a group of low-molecular-mass organic bases that contain at least one primary amine group that are produced through naturally occurring decarboxylation of free amino acids by substrate-specific amino acid decarboxylases.[8,9] In the past decade, BAs have been of concern in relation to food hygiene and public health because consumption of foods containing high levels may lead to various types of food-borne diseases, such as "scombroid syndrome" with histamine (HisN)[10] and the "cheese reaction" with tyramine (TyrN).[11] For the rapid and sensitive detection of BAs based on enzyme-linked immunosorbent assay (ELISA) technology, antibodies for individual BAs have been developed.[12−15] Despite remarkable advances in methods of production and use of antibodies, the development of an effective antibody is still challenging due to the complex development process and high production costs. Additionally, if more than one compound is to be detected in the samples, antibodies for each target are required. In contrast, enzymes as the biological element of biosensors have several advantages: (1) well-established recombinant technologies, (2) relatively cost-effective production, and (3) the potential to detect a group of analytes due to the relative promiscuity of enzymes. The ability of an enzyme to detect a group of analytes allows quantification of several compounds of interest simultaneously. Such combined determination of a group of analytes may be useful for point-of-care screening of products containing high levels of contaminants prior to time-consuming instrumental quantification of the individual analytes.

Amine oxidases catalyze oxidative deamination of primary amines to the corresponding aldehydes with ammonia and hydrogen peroxide.[16−18] Recently, we developed an enzymatic method using a monoamine oxidase from *Arthrobacter aurescens*, termed AMAO2, for the combined determination of monoamines including HisN and TyrN.[19] The enzymatic method facilitated quantitation of the monoamine contents of commercial fermented foods, such as cheeses [19] and Korean traditional fermented soybean pastes (*doenjang*).[20] However, due to the specificity of AMAO2 for only biogenic monoamines, the enzymatic assay could not detect diamines, particularly putrescine (Put), a major BA found in many foods.[21,22] Thus, it is important to develop an enzyme assay with broad substrate specificity for detection of multiple BAs in foods. Here, we used a putrescine oxidase from *A. aurescens* (APUO) to develop a bifunctional fusion enzyme of APUO and AMAO2, termed monoamine-putrescine oxidase (MAPO), and investigated its biochemical properties (Figure 1). Additionally, we measured the BA contents of commercial fermented Korean soybean pastes using the fusion enzyme and compared the results with those of a commercially available ELISA-based

Received: July 10, 2013
Revised: August 29, 2013
Accepted: September 3, 2013
Published: September 3, 2013

dx.doi.org/10.1021/jf403044m | J. Agric. Food Chem. 2013, 61, 9118−9124

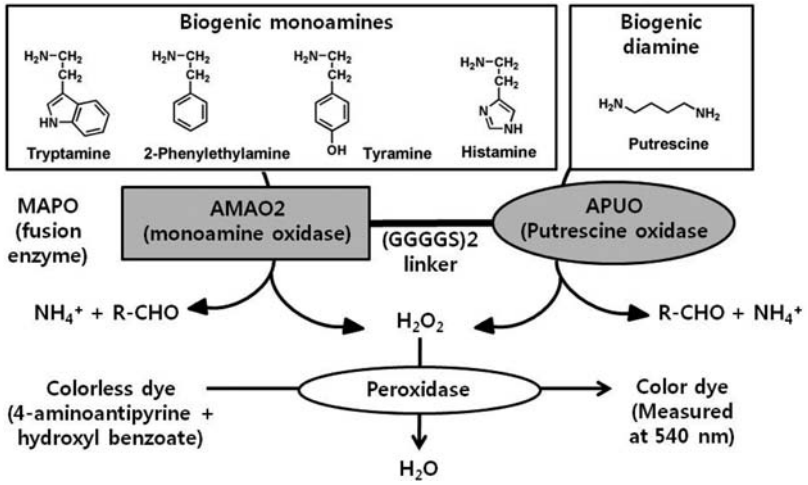

Figure 1. Overview of combined determination of total BAs in foods with a bifunctional fusion enzyme (MAPO) using a monoamine oxidase (AMAO2) and a putrescine oxidase (APUO) from *A. aurescens* TC-1.

Table 1. Sequences of the Oligonucleotides Used in This Study

name	sequence of the oligonucleotide
APUO-Nd-fw	5′-CACGGAGAGGACCATATGCAGAATCTTGAT-3′
APUO-Xh-rev	5′-GTGCTGCTCGAGTCAGGCGACAGGTAC-3′
ATGSEQ_	5′-ATCGACTTTGTAGGGT-3′
AMAO2-rv	5′-ACCACCGCCGAGCCACCGCCACCGTGTCCGCAGGTGCC-3′
APUO-fw	5′-GGTGGCTCGGGCGGTGGTGGGTCGATGCAGAATCTTGATCGCGAC-3′
APUO-Nt-rv	5′-GTGGTGGTGGTGTGCGGCCGCGGCGACAGGTAC-3′

HisN-detection kit and an enzymatic assay using AMAO2 alone.

MATERIALS AND METHODS

Cloning of the Gene Encoding APUO. *A. aurescens* TC1 was cultured and its genomic DNA prepared as described previously.[19] The target gene for a putrescine oxidase (NCBI accession code: YP_945873) was amplified by polymerase chain reaction (PCR) with ExTaq polymerase (Takara Co., Tokyo, Japan), using genomic DNA of *A. aurescens* TC1 as a template and a synthetic primer set (APUO-Nd-fw and APUO-Xh-rev, Table 1). Thirty-five PCR cycles (45 s at 94 °C, 30 s at 55 °C, and 80 s at 72 °C) were performed in a thermal cycler (PTC-200 DNA engine cycler, PerkinElmer Life Sciences, Foster City, U.S.). The resulting PCR product was purified from an agarose gel using a QIAquick Gel Extraction Kit (Qiagen), and digested with *Nde*I and *Xho*I, followed by ligation into the pBL6×HT vector[19] to create pBL6×HAPUO. DNA sequencing analysis was carried out by Bioneer Co. (Daejeon, Korea), and a clone with no PCR error was selected. To produce APUO at a low temperature, the gene for APUO with a $(His)_6$-tag at its N-terminal was obtained via digestion of pBL6×HAPUO with *Nco*I and *Not*I. The gene fragment that had been digested with the corresponding restriction enzymes was extracted and ligated with pET28a (Novagen). The resulting plasmid was designated pET28-6×HAPUO. In the final construct, the recombinant APUO contained eight additional amino acid residues (six His residues, Met, and Glu) at its N-terminus.

Construction of a Bifunctional Fusion Protein of AMAO2 and APUO. To produce an expression construct for MAPO, 3hirty-five PCR cycles (45 s at 94 °C, 30 s at 55 °C, and 80 s at 72 °C) were performed to amplify the AMAO2 gene using pET28-6×HAMAO2[19] as the template with the primers T7 promoter and AMAO2-link-rv (Table 1). The primer AMAO2-rv does not contain the termination codon of the AMAO2 gene, but contains a region for (GGGGS)×2. The gene for APUO was obtained from pET28-6×HAPUO by PCR using the primers APUO-link-fw and APUO-Nt-rv, which contain the nucleotide sequence for (GGGGS)×2 linker peptide in front of the initiation codon for APUO and a *Not*I site for in-frame fusion of APUO with the (His)×6 tag in the pET29a vector. After isolation of the gene fragments, another assembly PCR was conducted. For seven cycles (45 s at 94 °C, 30 s at 55 °C, and 80 s at 72 °C), two gene fragments were assembled by PCR without primers, and then a further 25 cycles (45 s at 94 °C, 30 s at 55 °C, and 2 min at 72 °C) with primers, T7 promoter and APUO-Nt-rv were carried out to amplify the assembled full gene fragment for the fusion protein. The PCR product was digested with *Nde*I and *Not*I, resulting in two fragments (MAPO-NdNt and MAPO-NtNt). First, the MAPO-NdNt fragment was ligated into pET29a (Novagen), to create pET29-MAPO-F. Then, the MAPO-NtNt fragment was ligated into *Not*I-digested pET29-MAPO-F, to create pET29-MAPO6×H. In the final construct, the recombinant MAPO contained a decapeptide linker between the last amino acid of AMAO2 and the first Met of APUO, as well as eleven additional amino acid residues (Ala×3, Leu, Glu, and six His residues) at its C-terminus (Figure S1, Supporting Information, SI).

Expression of the Genes for APUO and MAPO in *E. coli*. The recombinant *E. coli* BL21(DE3) carrying pET28-6×HAMAO2,[19] pET28-6×HAPUO or pET29-MAPO6×H was cultured in Luria–Bertani medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl) supplemented with kanamycin (20 μg/mL) at 37 °C until the optical density at 600 nm reached 0.5, and then 0.2 mM isopropyl β-D-thiogalactoside (IPTG) was added to the culture broth to induce gene expression. After induction for 20 h at 20 °C with shaking, the cells were harvested, and then resuspended in 50 mM Tris-HCl (pH 7.5), followed by sonication using an ultrasonic sonicator (model: VC750, Sonic and Materials Inc., Newtown, U.S.). After clarification of the cell extract by centrifugation (10 000*g*, 4 °C, 20 min), recombinant APUO was purified using an AKTAprime plus (GE Healthcare, Uppsala, Sweden) equipped with a His-Trap column (GE Healthcare) as described previously.[19]

Activity Assay of Amine Oxidases. All BAs used in this study were purchased from Sigma (St. Louis, U.S.). The activities of amine oxidases were measured according to the horseradish peroxidase-coupled reaction method.[19] The absorbance of the assay mixture was measured using a microplate reader (VERSAmax, Molecular Devices,

dx.doi.org/10.1021/jf403044m | J. Agric. Food Chem. 2013, 61, 9118–9124

U.S.) at a wavelength of 540 nm. To determine the optimal conditions for APUO and the fusion protein, the reaction mixtures were incubated at a temperatures of 10 to 70 °C at pH 7 or a pH of 5.0–11.0 at the optimum temperature for each enzyme. To investigate the effects of supplemental Cu^{2+} ions on the activity of MAPO, purified MAPO was incubated in 100 mM potassium–phosphate buffer (pH 7.0) containing 0.1 mM $CuSO_4$ at 4 °C overnight, followed by dialysis to remove excess Cu^{2+} ions.

Kinetic Analysis of Amine Oxidases. A reaction mixture was prepared by mixing 40 μL substrate solution in 50 mM phosphate buffer (pH 7.0) and 60 μL chromogen solution, consisting of 1 U horseradish peroxidase, 1 mM 4-aminoantipyrine, and 10 mM hydroxyl benzoate in one well of a 96-well plate. After being prewarmed for 5 min at 35 °C in the microplate reader, 20 μL of the amine oxdiases were added to the solution, followed by incubation at 35 °C. To determine the initial rate of the reaction, the amount of H_2O_2 released in the reaction was measured in the microplate reader. The absorbance was converted into BA levels using a standard curve prepared using TyrN as the standard. The kinetic parameters were calculated by fitting the data to the Michaelis–Menten equation. When substrate inhibition was observed, the kinetic parameters were calculated using the data in the following equation, using SigmaPlot software (ver. 11; Systa Software, Inc., U.S.):

$$\frac{v}{[E]_o} = \frac{k_{cat}}{1 + \frac{K_M}{[S]} + \frac{[S]}{K_i}}$$

where k_{cat}, K_M, and K_i are the catalytic constant, the Michaelis constant of the substrate, and the inhibition constant of the corresponding substrate, respectively. $[E]_o$ and $[S]$ denote the concentrations of the enzyme and substrate, respectively.

Analysis of BAs in foods using HPLC and ELISA Kit. Three commercial fermented soybean pastes were purchased from grocery stores in Sejong, Korea. Samples (5 g) were extracted with 25 mL of hot water (70 °C) twice. The extracts were combined and filtered through a filter paper (Advantech, Tokyo, Japan). To detect the BAs in the samples using HPLC, the amine group of BAs was modified by dansyl chloride, and then the dansylated samples were analyzed by HPLC as described previously.[19] To identify BAs, the extract plus an internal standard mixture (10 mg/kg of each) was analyzed. Each BA was quantified using the corresponding standard curve. HisN in the samples was quantified using an HisN-specific ELISA kit, Veratox for histamine kit (Neogen Corporation, Barcelona, Spain) was conducted as described previously.[23]

Enzymatic Quantification of Biogenic Amines. To quantify BAs, a reaction mixture was prepared by mixing 40 μL of the diluted extracted samples and 60 μL chromogen solution used for the kinetic analysis in one well of a 96-well plate. After being prewarmed for 5 min at 35 °C in the microplate reader, 20 μL of enzyme mixture (AMAO2 or fusion enzyme; 3.36 U/mL for TyrN as the substrate) was added to the solution, followed by incubation at 35 °C. The reactions were monitored in the microplate reader continuously until no further change in absorbance at 540 nm occurred (normally, 30 min). TyrN-equivalent BA contents were calculated using the TyrN standard curve.

RESULTS

Expression and Characterization of APUO. The gene for APUO was successfully amplified using a synthetic primer set (APUO-fw-Nd and APUO-rv-Xh; Table 1) and cloned into pBL6×HT vector with a constitutive promoter. However, the expression level was extremely low (data not shown). To improve the expression level, the vector system was changed to a pET vector. After cloning the APUO gene into the pET28a vector, the recombinant APUO was successfully overproduced in *E. coli* BL21(DE3) through IPTG-induced fermentation at 20 °C and homogeneously purified using Ni-NTA chromatography (Figure 2, lane 2). Generally, approximately 150 mg of pure APUO was obtained per liter of cell culture.

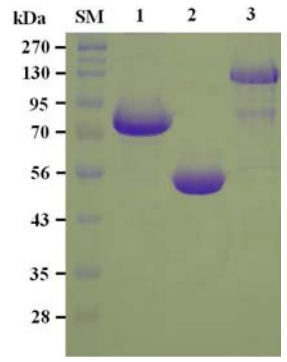

Figure 2. SDS-PAGE of the purified proteins AMAO2, APUO, and MAPO. The recombinant enzymes were purified according to the steps described in the Materials and Methods. The purified proteins were analyzed by 10% acrylamide gel electrophoresis and stained with Coomassie brilliant blue. Lane M, protein size markers; lanes 1–3, purified AMAO2, APUO, and MAPO, respectively. The numbers on the left indicate the sizes of the markers (in kDa).

Next, for the construction of a bifunctional fusion enzyme (MAPO), the C-terminus of AMAO2 was fused with the N-terminus of APUO in-frame through a decapeptide linker, the amino acid sequence of which was (GGGGS)×2. The DNA fragment containing the gene for MAPO was ligated into the pET29a vector. For expression of the MAPO gene, the recombinant plasmid, named pET29-MAPO6×H, was transformed into *E. coli* BL21(DE3), and IPTG induction at 20 °C was conducted. SDS-PAGE revealed that the recombinant fusion enzyme MAPO, the molecular weight of which was approximately 120 kDa (the molecular weight of the deduced amino acid sequence of MAPO was 124 169 Da) was successfully expressed and purified by Ni-NTA chromatography (Figure 2, lane 3). Generally, 45 mg of pure MAPO were obtained per liter of cell culture.

Enzymatic Properties of APUO. Using Put as the substrate, APUO showed the maximum activity at 55 °C and pH 8.0 (Figure 3A,B). Interestingly, APUO exhibited a broad pH profile, exhibiting more than 90% of the maximum activity at pH 7 to 10. To investigate the substrate specificity of APUO, the specific activities for six BAs (HisN, TyrN, and 2-phenylethylamine (PheN) as monoamines; Put and cadevarine (Cad) as diamines; spermidine (Spd) as a polyamine) were measured. The specific activity of APUO for Put was 154.3 U/mg and the relative activity of APUO was 100:8.8:2.5 for Put:Cad:Spd, respectively (Figure 3C). APUO showed the highest activity toward Put, and longer aliphatic di- and polyamines, such as Cad and Spd, were poor substrates, an order of magnitude lower than Put. APUO did not show activity toward monoamines.

Enzymatic Properties of MAPO. To confirm the bifunctional activity of MAPO toward monoamines and Put, its activity was determined at pH 7.0 and 50 °C using TyrN and Put as substrates. The specific activities of MAPO for TyrN and Put were 56 and 116 U/mg, respectively. Given the almost 2.5-fold increase in the molecular weight of the fusion protein compared with that of APUO, the specific activity of MAPO for Put (75% of that of APUO for Put) was compatible with that of APUO. In contrast, the activity of MAPO for TyrN was lower compared with that of AMAO2 (312 U/mg for TyrN). AMAO2 is a copper-containing amine oxidase that requires a Cu^{2+} ion as a cofactor for the posttranslational modification of a tyrosine residue to TPQ and catalytic turnover.[16,24] After

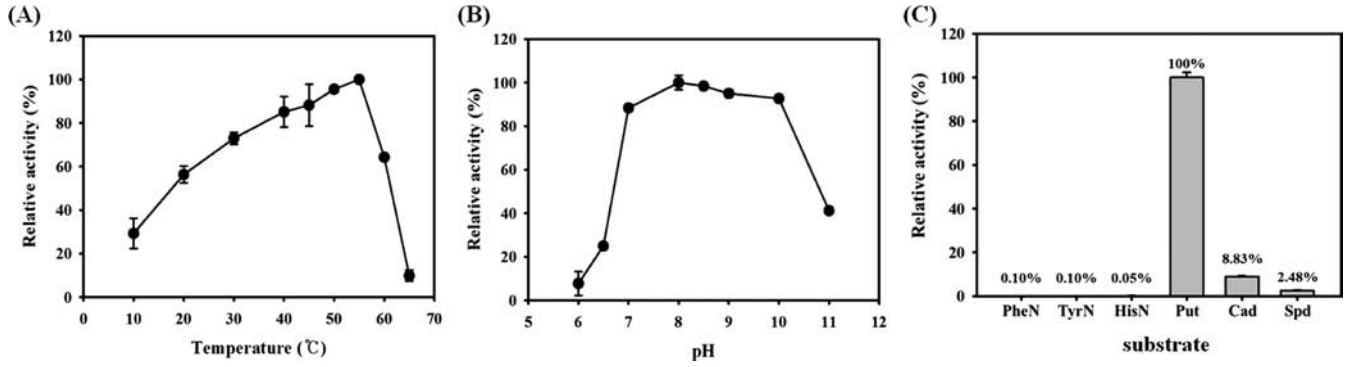

Figure 3. Biochemical properties of APUO. (A) Effect of temperature on activity. (B) Effect of pH on activity. (C) Substrate specificity under optimum conditions (pH 7.0 and 50 °C).

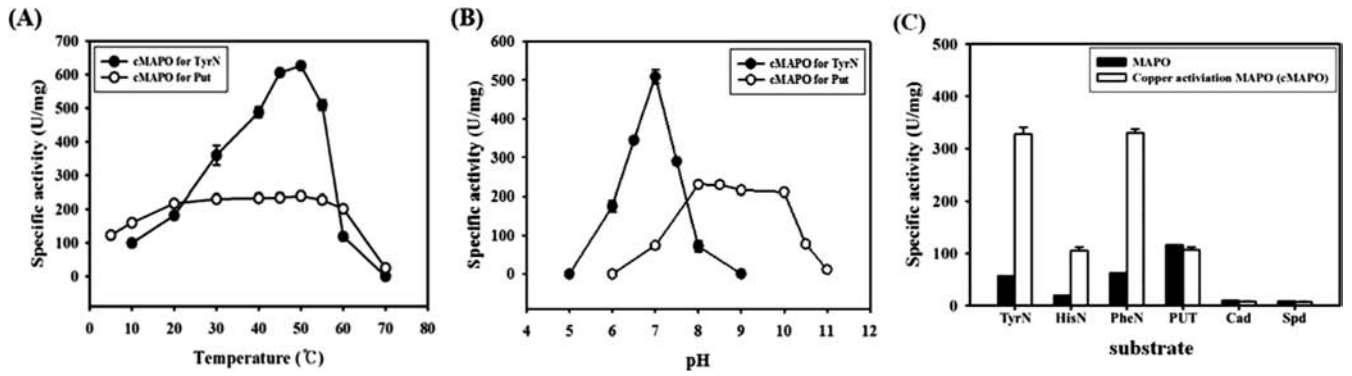

Figure 4. Biochemical properties of cMAPO. (A) Effect of temperature on activity. (B) Effect of pH on activity. (C) Substrate specificity under optimum conditions (pH 7.0 and 50 °C for TyrN, PheN, and HisN; pH 8.0 and 50 °C for Put, Cad, and Spd).

Table 2. Kinetic Parameters of AMAO2, APUO, MAPO, and cMAPO for Various Substrates

enzyme	substrate[a]	k_{cat} (sec^{-1})	K_M (mM)	k_{cat}/K_M (sec^{-1}/mM^{-1})	K_i[b] (mM)	kinetics mode
AMAO2[c]	PheN	5.12	0.012	429	0.84 ± 0.08	substrate inhibition
	TyrN	3.95	0.030	133	1.08 ± 0.09	substrate inhibition
	HisN	5.91	0.41	14.6	3.51 ± 0.34	substrate inhibition
	Put	ND[d]	ND[d]	0.15	ND[d]	linear
	Cad	6.19×10^{-2}	1.27×10	4.88×10^{-3}	ND[d]	saturation
	Spd	4.29×10^{-2}	7.04	6.14×10^{-3}	ND[d]	saturation
APUO	Put	31.8 ± 0.94	0.30 ± 0.02	106 ± 6.7	19.1 ± 3.2	substrate inhibition
	Cad	1.72 ± 0.03	0.36 ± 0.02	4.78 ± 0.24	ND[d]	saturation
	Spd	0.29 ± 0.01	0.35 ± 0.04	0.83 ± 0.09	ND[d]	saturation
MAPO	PheN	2.09 ± 0.12	0.057 ± 0.01	37.0 ± 4.4	0.90 ± 0.12	substrate inhibition
	TyrN	2.07 ± 0.13	0.061 ± 0.01	34.2 ± 3.9	0.96 ± 0.13	substrate inhibition
	HisN	3.17 ± 0.16	0.45 ± 0.03	7.07 ± 0.51	3.30 ± 0.31	substrate inhibition
	Put	16.2 ± 0.4	0.68 ± 0.03	23.8 ± 1.0	12.3 ± 1.0	substrate inhibition
	Cad	0.74 ± 0.01	0.78 ± 0.04	0.95 ± 0.04	ND[d]	saturation
	Spd	0.19 ± 0.01	1.33 ± 0.18	0.14 ± 0.02	ND[d]	saturation
cMAPO	PheN	14.5 ± 1.0	0.03 ± 0.01	453 ± 68	0.78 ± 0.12	substrate inhibition
	TyrN	15.3 ± 0.6	0.07 ± 0.01	222 ± 18	0.76 ± 0.05	substrate inhibition
	HisN	15.2 ± 0.5	0.28 ± 0.02	53.2 ± 1.6	6.73 ± 0.72	substrate inhibition
	Put	21.4 ± 0.6	0.51 ± 0.01	42.0 ± 1.1	22.3 ± 3.6	substrate inhibition
	Cad	1.03 ± 0.03	0.78 ± 0.06	1.33 ± 0.01	ND[d]	saturation
	Spd	0.12 ± 0.01	1.68 ± 0.21	0.07 ± 0.01	ND[d]	saturation

[a]PheN, phenylethylamine; TyrN, tyramine; HisN, histamine; Put, putresine; Cad, cadevarine; Spd, spermidine. [b]Inhibition constant at high substrate concentrations. [c]Kinetic parameters for AMAO2 were referred from Lee et al.[19] [d]Not determined.

incubation of MAPO in the presence of 0.1 mM Cu^{2+} ions, followed by dialysis to remove excess Cu^{2+} ions, the monoamine oxidase activity of MAPO for TyrN increased, 6-fold, compared with that prior to activation (327 U/mg for TyrN after Cu^{2+} activation). In contrast, the activity for Put was not affected by Cu^{2+} ion activation (107 U/mg for Put after activation) because bacterial Put oxidases, including APUO, use flavin adenine dinucleotide (FAD) as a cofactor, not the Cu^{2+}

dx.doi.org/10.1021/jf403044m | J. Agric. Food Chem. 2013, 61, 9118−9124

Table 3. Analysis of Biogenic Amine Contents in the Doenjang Samples

sample	HPLC (mg/kg) monoamines[a]	HPLC (mg/kg) total BAs[b]	ELISA[c] (mg/kg)	AMAO2[d] (mg/kg)	cMAPO[e] (mg/kg)
S1	60.8 ± 1.2	68.4 ± 1.4	42.2 ± 3.2	57.1 ± 2.2	63.4 ± 3.9
S2	228.8 ± 1.4	249.3 ± 1.7	86.5 ± 10.4	236.3 ± 1.6	226.4 ± 2.6
S3	113.2 ± 0.6	191.0 ± 1.7	35.1 ± 2.1	127.8 ± 1.8	182.4 ± 6.1

[a]Sum of the contents of TrpN, PheN, TyrN, and HisN by HPLC. [b]Sum of the contents of TrpN, PheN, TyrN, HisN, Put, Cad, and Spd by HPLC. [c]HisN contents measured by Veratox histamine kit. [d]TyrN-equivalent BA contents by AMAO2. [e]TyrN-equivalent BA contents by cMAPO.

ion.[17] The fusion protein activated with Cu^{2+} ions is referred to as "cMAPO." The optimal temperatures of cMAPO for both TyrN and Put were 50 °C, and the optimal pH values for TyrN and Put were pH 7.0 and 8.0, respectively (Figure 4A,B), consistent with those of AMAO2[19] and APUO (Figure 3A,B), respectively. The relative specific activities of cMAPO were 100, 99, 32, 32, 2.3, and 2.1 for PheN, TyrN, HisN, Put, Cad, and Spd, respectively (Figure 4C).

Kinetic Analysis of APUO and MAPO. The kinetic parameters of the two individual enzymes (AMAO2 and APUO) and the fusion enzymes before and after Cu^{2+} activation (MAPO and cMAPO, respectively) were determined using various BAs as substrates (Figures S2−S4 in the SI); the kinetic parameters are summarized in Table 2. The kinetic analysis revealed that Put was the optimum substrate for APUO. The relative catalytic efficiencies (k_{cat}/K_M) for Put, Cad, and Spd of APUO were 100:4.5:0.8, respectively. The K_M values of APUO for Put, Cad and Spd were almost equal (~0.3 mM), but the k_{cat} values contributed most to the determination of the catalytic efficiencies of APUO. Substrate inhibition was observed only for Put with a 19.1 mM K_i, an order of magnitude higher than the K_i values of AMAO2 for monoamines, such as TyrN and HisN.[19]

Comparison of the kinetic parameters of MAPO and cMAPO showed that Cu^{2+} activation increased k_{cat}/K_M for monoamines by 6.5- to 12-fold. The relative k_{cat}/K_M values for PheN, TyrN, and HisN for cMAPO were 100:49:11.7, respectively. Given the relative k_{cat}/K_M for PheN, TyrN and HisN of cMAPO (100:31:3.4),[19] the values of k_{cat}/K_M for TyrN and HisN of cMAPO were improved (1.7- and 3.6-fold for TyrN and HisN, respectively; Table 2). Of the diamines (Put and Cad) and Spd, Put was the optimum substrate of cMAPO, consistent with the substrate specificity of APUO. After Cu^{2+} activation, the k_{cat}/K_M for Put of the fusion enzyme increased by 1.7-fold, due to cooperative effects—an increase in k_{cat} and a decrease in K_M for Put. No such activity improvement due to Cu^{2+} activation was observed for Cad or Spd. MAPO and cMAPO were also inhibited by the substrate at high concentrations. The tendency for substrate inhibition toward MAPO and cMAPO was similar to those for AMAO2 and APUO, except that of HisN against cMAPO. The K_i values for HisN of cMAPO (6.73 mM) increased, about 2-fold, compared to that of AMAO2 (3.51 mM).

Determination of BA Contents in Fermented Soybean Pastes. Previously, we attempted enzymatic determination of monoamines, including TyrN and HisN, using AMAO2 for fermented soybean pastes, that are traditional Korean seasonings.[20] Of the 16 samples, we selected three as model foods with differing ratios of BA contents; high HisN (S1, 56% of total BAs), high TyrN (S2, 55%), and high Put (S3, 28%; the Put ratios of the others were 7.3 and 3.8%; Table 3). The contents of BAs were determined by HPLC, a HisN-specific ELISA kit, AMAO2, and cMAPO.

The total BA contents of S1, S2, and S3, by HPLC, were 68.4, 249.3, and 191.0 mg/kg, and the monoamine and putrescine levels accounted for 81−99% of total BAs in the samples (Table S1 in the SI). On the basis of the BA contents determined by HPLC analysis, the samples were diluted to adjust the concentration to within the detection ranges of the ELISA and enzymatic assay. The HisN-specific ELISA kit detected more than 90% of HisN determined by HPLC, but the amount of HisN determined using the ELISA kit showed no correlation with the total BA contents (61%, 35%, and 18% of the total BA contents for S1, S2, and S3, respectively; Table 3 and Table S1 in the SI). The TyrN-equivalent BA contents determined by AMAO2 alone were 94−113% of the total monoamines and 83% and 95% of the total BAs for S1 and S2, respectively, with Put contents less than 10% of the total BAs. In contrast, on using the S3 sample, which contains a high Put concentration, only 67% of the total BAs were detected by AMAO2. In the case of cMAPO, the TyrN-equivalent BA contents of samples S1, S2, and S3 were 63.4, 226.4, and 182.4 mg/kg, respectively, translating into 93, 91, and 96% of the total BA in these samples, respectively. These results suggest that enzymatic quantification of BA content using cMAPO resulted in detection of more than 90% of the total BA contents of the food samples tested.

DISCUSSION

Due to the undesirable effects of BAs on human health, various methods for their quantitation in foods have been developed. Despite the high resolution and sensitivity of chromatography-based methods,[22,25,26] such instrumental analyses require expensive equipment and are time-consuming. To conduct rapid and easy tests of BAs in foods, colorimetric assays and amperometric biosensors using amine oxidases have been developed.[20,27−29] Because biosensors are often used as a reference method for preliminary screening of safety-guaranteed products prior to instrumental analyses, amine oxidases with broad substrate specificity are required for the determination of total BAs in foods. However, the substrate specificity of the amine oxidase is a drawback. To address the specificity problem, enzyme sensor arrays using amine oxidases with differing specificities[30] and the use of a diamine oxidase from pea seedlings[27] have been investigated. In this study, we developed a bifunctional fusion protein with broad substrate specificity for the major BAs, such as HisN, TyrN, and Put.

BLAST searches revealed that *A. aurescens* TC1 contains one gene for putrescine oxidase (NCBI accession code: YP_945873.1) of which predicted amino acid sequence shares 87.8% and 74.4% identities with those from *A. globiformis* (ZP_09279529) and *Rhodococcus erythropolis* (ABY74497). These bacterial putrescine oxidases are FAD-containing enzymes. An FAD molecule noncovalently binds to these bacterial putrescine oxidases,[17] whereas an FAD molecule is covalently attached to another FAD-containing amine oxidase,

a monoamine oxidase B from human (MAO-B) through a covalent bond between Cys397 and the C8α position of flavin.[31] In APUO, the amino acid residue corresponding to Cys397 for MAO-B is an alanine residue, consistent with the putrescine oxidase from *R. erthropolis* (RPUO).[17] The relative catalytic efficiencies (k_{cat}/K_M) of APUO were similar to that of RPUO.[17] The specific activity and catalytic efficiency of APUO allowed its use as a fusion partner for AMAO2.

The gene fusion technique is useful in the development of bifunctional biocatalysts.[32,33] Generally, fusion proteins contain a linker peptide between the fusion partners, the nature of which is important for retention of the functions of both enzymes. Poly glycine and glycine-rich flexible sequences $(GGGGS)_n$ (usually $n \leq 6$) are frequently used linkers that are thought to be resistant to proteolysis, and presumably confer conformational flexibility. Thus, these linkers are often used to construct bi- or multifunctional fusion proteins.[32,34,35] Here, a linker peptide with a duplicate GGGGS sequence was used, resulting in the independent functioning of the two moieties of the fusion enzyme with activities fully compatible with those of the individual enzymes.

The monoamine oxidase used in this study (AMAO2) belongs to the copper-containing amine oxidases, and requires a copper ion as a cofactor for the conversion of a tyrosine residue to a redox cofactor, 2,4,5-trihydroxyphenylalanine quinone (TPQ), which is essential for catalytic function.[36] The catalytic activity of recombinant AMAO2 produced in *E. coli* was not affected by further incubation with Cu^{2+} after purification, suggesting that the Tyr393 of AMAO2 was completely modified to TPQ during purification.[19] However, the purified fusion protein (MAPO) showed relatively low activity for monoamines compared with AMAO2, and the incubation of MAPO with Cu^{2+} ion markedly increased the activity for TyrN without changing the activity for Put (Figure 4C). This suggests that the Tyr residue of the fusion enzyme was *not* completely modified to TPQ. The reason for this difference in the rate of TPQ modification between AMAO2 and MAPO is unknown.

The fusion of AMAO2 and APUO not only yielded the bifunctional fusion enzyme protein, but also improved the activity toward two important monoamines, TyrN and HisN (Table 2). In a previous study, low activity of AMAO2 for HisN resulted in a reduced HisN detection range, up to 120 μM, during a 30-min assay. Thus, these results suggest that the fusion enzyme could expand the detection range. In terms of specific activity for Put (0.1 mM), the k_{cat}/K_M value of cMAPO for Put was only 39.6% of that of APUO, which translates to 19% of that for TyrN (Table 2). In our previous study, however, the k_{cat}/K_M value for HisN of AMAO2, which corresponded to 11% of that for TyrN, did not restrict its application to quantification of monoamines, including HisN.[19,20] Thus, despite the reduction in cMAPO activity toward Put, the fusion enzyme can be used for the simultaneous determination of multiple BAs. The results of the analysis of BAs in food samples support our conclusion (Table 3).

Because of its importance, commercially available HisN-specific ELISA kits are used to determine HisN contents in various foods. Additionally, the HisN contents determined by ELISA show good agreement with the total BA contents by HPLC in the foods tested.[23,37,38] However, as shown in Table 3, in foods with a low or a high ratio of HisN to the other BAs, estimation of total BA content based solely on HisN content would likely be inaccurate. The results in Table 3 clearly show that the fusion enzyme yielded better performance than either the HisN-specific ELISA kit or AMAO2 alone. The combination of AMAO2 and APUO will likely exhibit a performance similar to that of cMAPO. However, the activity toward HisN of AMAO2 was improved by the fusion with APUO of cMAPO, as discussed above. Additionally, upon immobilization of the individual enzymes on the electrodes of BA biosensors, AMAO2 and APUO could be present in uneven proportions. However, when the fusion enzyme is used, AMAO2 and APUO are immobilized in identical proportions.

■ ASSOCIATED CONTENT

Supporting Information

Contents of BAs in the *doenjang* samples using HPLC (Table S1); contruction strategy of pET29-MAPO6×H for the production of MAPO enzyme (Figure S1); kinetics analysis of APUO for putrescine, cadaverine, and spermidine (Figure S1); kinetics analysis of MAPO for 2-phenylethylamine, tyramine, histamine, putrescine, cadaverine, and spermidine (Figure S3); and kinetics analysis of cMAPO for 2-phenylethylamine, tyramine, histamine, putrescine, cadaverine, and spermidine (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported by the Converging Research Center Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (no. 20090083290).

■ ABBREVIATIONS USED

AMAO2,*Arthrobacter aurescens* monoamine oxidase 2; APUO,*Arthrobacter aurescens* putrescine oxidase; MAPO,enzyme fusion with AMAO2 and APUO; cMAPO,Cu^{2+}-activated MAPO; Cad,cadevarine; HisN,histamine; PheN,2-phenylethylamine; Put,putrescine; TrpN,tryptamine; TyrN,tyramine; Spd,spermidine; ELISA,enzyme-linked immunosorbent assay; IPTG,isopropyl-D-thiogalactoside; TPQ,2,4,5-trihydroxyphenylalanine quinone

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