

Reactions of kynurenic acid with hypobromous acid and hypochlorous acid

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Kynurenic acid, a tryptophan metabolite, acts as antagonist or agonist of several receptors. Hypobromous acid (HOBr) and hypochlorous acid (HOCl) are generated by eosinophils and neutrophils. At inflammation sites, kynurenic acid may encounter HOBr and HOCl to generate products. When kynurenic acid was incubated with HOBr under neutral conditions, kynurenic acid generated a single product almost exclusively. This was identified as 3-bromokynurenic acid. Kynurenic acid reacted with HOCl, generating two products. The major product was identified as 3-chlorokynurenic acid with its oxidative decarboxylation product, 3-chloro-4-hydroxy-2(1*H*)-quinolinone as a by-product. Free amino acids suppressed the reactions of kynurenic acid with HOBr and HOCl. Taurine suppressed the HOCl reaction but not the HOBr reaction. An eosinophil peroxidase system containing H₂O₂, NaCl, and NaBr reacted with kynurenic acid, generating 3-bromokynurenic acid under mildly acidic conditions. Although a myeloperoxidase system containing H₂O₂ and NaCl reacted with kynurenic acid to generate 3-chlorokynurenic acid under mildly acidic conditions, the product was altered to 3-bromokynurenic acid by addition of NaBr to the system. These results suggest that 3-bromokynurenic acid and 3-chlorokynurenic acid may be generated from kynurenic acid at inflammation sites in humans, although their formation will be suppressed by coexistent amino acids.

Key Words: kynurenic acid, hypobromous acid, hypochlorous acid, myeloperoxidase, eosinophil peroxidase

Kynurenic acid (KYNA) is a tryptophan metabolite generated by enzymes along the kynurenine pathway.⁽¹⁾ KYNA is an agonist of G-protein coupled GPR35 receptor, which presents predominantly on immune cells and in the gastrointestinal tract.⁽²⁾ The serum level of KYNA was significantly elevated in patients with inflammatory bowel disease, compared to control subjects.⁽³⁾ Several reports suggest that KYNA may have a positive influence on a number of pathologies of the gastrointestinal tract.⁽⁴⁾ KYNA is also an antagonist of both the *N*-methyl-D-aspartate receptor and the α 7 nicotinic acetylcholine receptor.^(5,6) The brain level of KYNA was elevated in patients with schizophrenia and bipolar disorder.^(7,8) KYNA possesses neuroprotective properties, since it blocks some of the neurotoxic effects of excitotoxins.⁽⁹⁾ Recently, the relationship between inflammation and these disorders has received.^(10,11) Eosinophils are a minor component of white blood cells, but are abundant in blood and tissues in various inflammatory disorders.⁽¹²⁾ Eosinophils discharge eosinophil peroxidase (EPO), which catalyzes the oxidation of bromide ion (Br⁻) to hypobromous acid (HOBr) through hydrogen peroxide (H₂O₂) reduction.^(13,14) Although the plasma concentrations are 39–84 μ M for Br⁻ and 100 mM for Cl⁻, EPO uses Br⁻ selectively, resulting in HOBr.⁽¹⁵⁾ The formed HOBr plays an important role in defense mechanisms against parasites. Meanwhile, neutrophils discharge

myeloperoxidase (MPO), which catalyzes the oxidation of chloride ion (Cl⁻) to hypochlorous acid (HOCl) through hydrogen peroxide (H₂O₂) reduction.^(16–18) Neutrophils are a major component of white blood cells. The HOCl formed by MPO plays a central role in host defense mechanisms against infection. Since HOCl can react with Br⁻ to generate HOBr, a portion of the HOCl formed by the MPO system will react with Br⁻ of the plasma concentration converting to HOBr.^(19,20) Under inflammation conditions, KYNA may encounter HOBr and HOCl, and react with them. However, there is little information about the reactions of KYNA with HOBr and HOCl. In the present study, we examined the reactions of KYNA with reagents HOBr and HOCl, and with peroxidases EPO and MPO, using RP-HPLC, and found that both the reagents and the peroxidase systems readily react with KYNA to generate halogenated products.

Materials and Methods

Materials. KYNA was purchased from ChemCruz (Dallas, TX). NaBr (>99.99%), NaCl (>99.99%), KH₂PO₄, K₂HPO₄, MPO from human leukocytes, and EPO human recombinant protein were purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) or TCI (Tokyo). Bromide-free hypobromous acid (HOBr) was prepared by the addition of silver nitrate and subsequent distillation, as previously reported.⁽²¹⁾ The concentration of HOBr was determined spectrophotometrically at 331 nm in 10 mM NaOH using a molar extinction coefficient of 315 M⁻¹ cm⁻¹.⁽²²⁾ Chloride-free sodium hypochlorite (NaOCl) was prepared by the method previously reported.⁽²³⁾ The concentration of NaOCl was determined spectrophotometrically at 290 nm using a molar extinction coefficient of 350 M⁻¹ cm⁻¹.⁽²⁴⁾

HPLC and MS conditions. The HPLC system consisted of LC-10ADvp pumps and an SPD-M10Avp UV-vis photodiode-array detector (Shimadzu, Kyoto, Japan). For the RP-HPLC, an Inertsil ODS-3 octadecylsilane column of 4.6 \times 250 mm and particle size 5 μ m (GL Sciences, Tokyo, Japan) was used. The eluent was 20 mM ammonium acetate (pH 7.0) containing acetonitrile. The acetonitrile concentration was increased from 0 to 14% during 40 min in linear gradient mode. The column temperature was 40°C and the flow rate was 1 ml/min. The electrospray ionization time of flight mass spectrometry (ESI-TOF/MS) measurements were performed on a MicroTOF spectrometer (Bruker, Bremen, Germany) in negative mode.

Spectrometric data. Peak 1: 3-bromokynurenic acid (3-Br-KYNA). ESI-TOF/MS: *m/z* 222 and 224 (1:1), 266 and 268 (1:1). HR-ESI-TOF/MS: 265.946532 obsd (265.945829 calcd for C₁₀H₅BrNO₃⁻). UV: λ_{max} = 248, 320, 330 nm. ¹H NMR (500 MHz,

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DMSO- d_6): δ (ppm/TMS) 8.02 (d, $J = 8.0$ Hz, 1H), 7.61 (d, $J = 8.6$ Hz, 1H), 7.55 (dd, $J = 7.7, 7.7$ Hz, 1H), 7.24 (dd, $J = 7.5, 7.5$ Hz, 1H), ^{13}C NMR (125 MHz, DMSO- d_6): δ (ppm/TMS) 171.7, 161.0, 151.0, 138.2, 131.0, 124.9, 123.0, 122.8, 118.3, 99.3.

Peak 2: 3-chlorokynurenic acid (3-Cl-KYNA). ESI-TOF/MS: m/z 178 and 180 (3:1), 222 and 224 (3:1). HR-ESI-TOF/MS: 221.996068 obsd (221.996344 calcd for $\text{C}_{10}\text{H}_5\text{ClNO}_3^-$). UV: $\lambda_{\text{max}} = 246, 317, 331$ nm. ^1H NMR (500 MHz, DMSO- d_6): δ (ppm/TMS) 8.06 (d, $J = 8.0$ Hz, 1H), 7.67 (d, $J = 8.5$ Hz, 1H), 7.58 (dd, $J = 7.5, 7.5$ Hz, 1H), 7.27 (dd, $J = 7.5, 7.6$ Hz, 1H), ^{13}C NMR (125 MHz, DMSO- d_6): δ (ppm/TMS) 172.0, 162.0, 149.0, 138.2, 131.2, 124.8, 123.0, 122.8, 118.3, 109.0.

Peak 3: 3-chloro-4-hydroxy-2(1H)-quinolinone (3-Cl-HQN). ESI-TOF/MS: m/z 158, 194 and 196 (3:1). HR-ESI-TOF/MS: 193.9996 obsd (194.0007 calcd for $\text{C}_9\text{H}_5\text{ClNO}_2^-$). UV: $\lambda_{\text{max}} = 218, 300$ nm. ^1H NMR (500 MHz, DMSO- d_6): δ (ppm/TMS) 7.81 (d, $J = 8.0$ Hz, 1H), 7.21 (dd, $J = 7.5, 7.6$ Hz, 1H), 7.03 (d, $J = 8.5$ Hz, 1H), 6.89 (dd, $J = 7.5, 7.5$ Hz, 1H), ^{13}C NMR (125 MHz, DMSO- d_6): δ (ppm/TMS) 167.3, 160.6, 137.5, 128.3, 124.3, 121.5, 118.9, 114.2, 99.6.

Quantitative procedures. The concentrations of the products were evaluated according to the integrated peak areas on RP-HPLC chromatograms detected at 240 nm and by the molecular extinction coefficients at 240 nm ($\epsilon_{240\text{ nm}}$). The $\epsilon_{240\text{ nm}}$ value of 25,300 $\text{M}^{-1}\text{cm}^{-1}$ was used for KYNA. The $\epsilon_{240\text{ nm}}$ values of Peaks 1–3 were determined from the integration of proton signals of NMR and the HPLC peak area detected at 240 nm relative to that of KYNA in the mixed solution. The estimated $\epsilon_{240\text{ nm}}$ values were 27,300 $\text{M}^{-1}\text{cm}^{-1}$ for Peak 1, 28,600 $\text{M}^{-1}\text{cm}^{-1}$ for Peak 2, and 22,700 $\text{M}^{-1}\text{cm}^{-1}$ for Peak 3.

Results

Reaction with HOBr. A solution of 100 μM KYNA was incubated with 100 μM HOBr in 100 mM potassium phosphate buffer at pH 7.4 and 37°C for 30 min. When the reaction mixture was analyzed by reversed-phase (RP) HPLC, a product peak, Peak 1, appeared in the chromatogram detected at 240 nm (Fig. 1). The product was isolated by RP-HPLC and identified using MS and NMR (^1H -1d, ^1H - ^1H COSY, ^1H - ^{13}C HMQC, ^1H - ^{13}C HMBC, ^{13}C -1d). Peak 1 showed an ESI-TOF/MS spectrum with $m/z = 222$ and 224 (1:1) and 266 and 268 (1:1) in negative mode. High-resolution (HR) ESI-TOF/MS values of the molecular ion for Peak 1 agreed with the theoretical molecular mass for $\text{C}_{10}\text{H}_5\text{BrNO}_3^-$ attributable to $[\text{KYNA} + \text{Br} - 2\text{H}]^-$ within 3 ppm. ^1H NMR showed four aromatic protons coupled with each other on the ^1H - ^1H COSY spectrum. ^{13}C NMR showed nine aromatic carbons and a carbon attributable to a carboxy group. From these data, Peak 1 was identified as 3-bromokynurenic acid (3-Br-KYNA) (Scheme 1). Figure 2 shows the HOBr dose-dependence of the reaction of KYNA with HOBr when 100 μM KYNA and 0–100 μM HOBr were incubated in 100 mM potassium phosphate buffer at pH 7.4 and 37°C for 30 min. With increasing HOBr concentration, the consumptions of KYNA increased and the yield of 3-Br-KYNA increased. KYNA was converted to 3-Br-KYNA almost exclusively.

Reaction with HOCl. A solution of 100 μM KYNA was incubated with 100 μM HOCl in 100 mM potassium phosphate buffer at pH 7.4 and 37°C for 30 min. When the reaction mixture was analyzed by RP-HPLC, two product peaks, Peaks 2 and 3, appeared in the chromatogram detected at 240 nm (Fig. 3). The products were isolated by RP-HPLC and identified. Peak 2 showed an ESI-TOF/MS spectrum with $m/z = 178$ and 180 (3:1) and 222 and 224 (3:1) in negative mode. HR-ESI-TOF/MS values of the molecular ion for Peak 2 agreed with the theoretical molecular mass for $\text{C}_{10}\text{H}_5\text{ClNO}_3^-$ attributable to $[\text{KYNA} + \text{Cl} - 2\text{H}]^-$ within 3 ppm. ^1H NMR showed four aromatic protons correlated with each other on ^1H - ^1H COSY. ^{13}C NMR showed nine aromatic

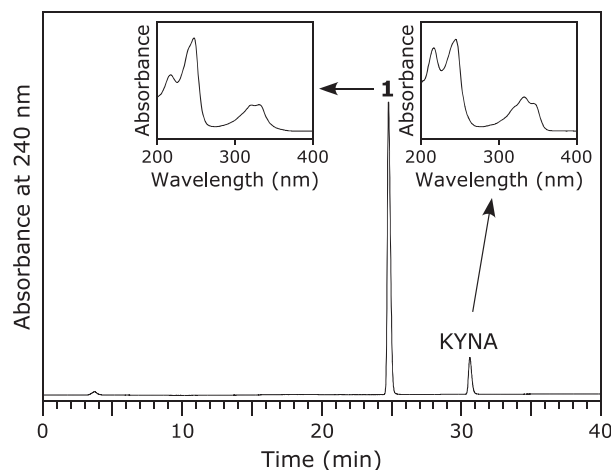
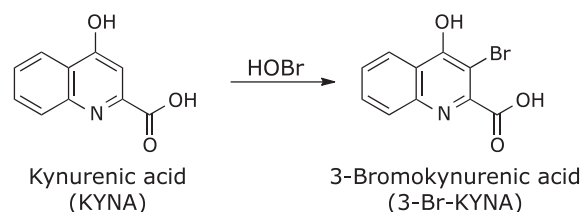


Fig. 1. RP-HPLC chromatogram of a reaction mixture of KYNA with HOBr detected at 240 nm. A solution of 100 μM KYNA was incubated with 100 μM HOBr in 100 mM potassium phosphate buffer at pH 7.4 and 37°C for 30 min. The insets are the UV spectra of Peak 1 and KYNA.



Scheme 1.

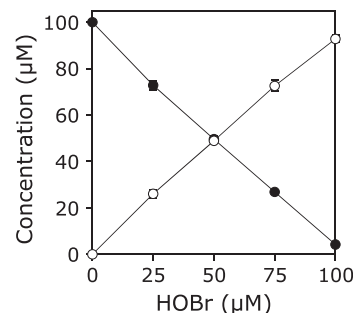
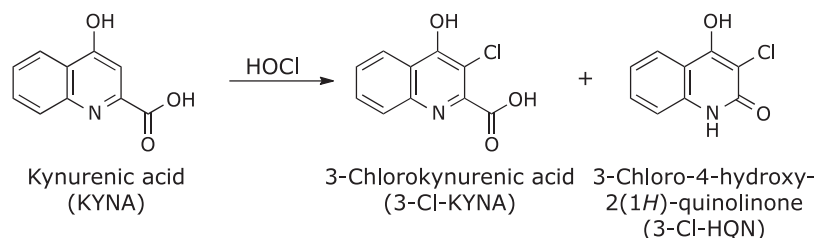


Fig. 2. HOBr dose-dependence of the concentration changes in KYNA and 3-Br-KYNA when a solution of 100 μM KYNA was incubated with 0–100 μM HOBr in 100 mM potassium phosphate buffer at pH 7.4 and 37°C for 30 min. KYNA (closed circle), 3-Br-KYNA (open circle). All the reaction mixtures were analyzed by RP-HPLC. Means \pm SD ($n = 3$) are presented.

carbons and a carbon attributable to a carboxy group. Peak 2 was identified as 3-chlorokynurenic acid (3-Cl-KYNA) (Scheme 2). Peak 3 showed an ESI-TOF/MS spectrum with $m/z = 194$ and 196 (3:1) in negative mode. HR-ESI-TOF/MS values of the molecular ion for the Peak 3 agreed with the theoretical molecular mass for $\text{C}_9\text{H}_5\text{ClNO}_2^-$ attributable to $[\text{KYNA} + \text{Cl} - \text{CO} - 2\text{H}]^-$ within 3 ppm. ^1H NMR showed four aromatic protons correlated with each other on ^1H - ^1H COSY. ^{13}C NMR showed nine aromatic carbons but no carbon of a carboxy group. Peak 3 was identified as 3-chloro-4-hydroxy-2(1H)-quinolinone (3-Cl-HQN) (Scheme 2). Figure 4 shows the HOCl dose-dependence of the reaction of



Scheme 2.

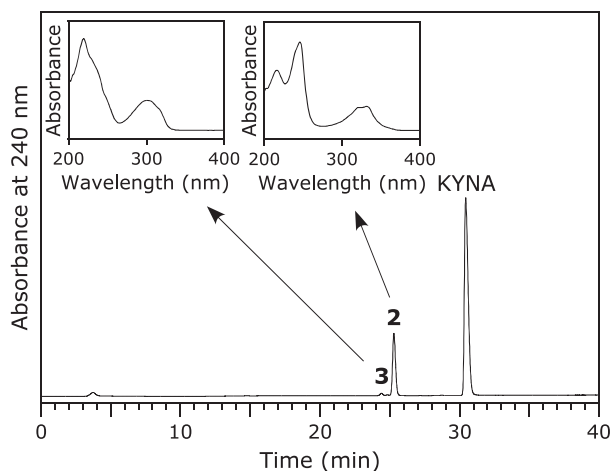


Fig. 3. RP-HPLC chromatogram of a reaction mixture of KYNA with HOCl detected at 240 nm. A solution of 100 μM KYNA was incubated with 100 μM HOCl in 100 mM potassium phosphate buffer at pH 7.4 and 37°C for 30 min. The insets are the UV spectra of Peak 2 and Peak 3.

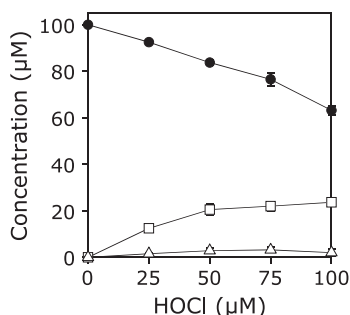


Fig. 4. HOCl dose-dependence of the concentration changes in KYNA and products, 3-Cl-HQN and 3-Cl-KYNA, when a solution of 100 μM KYNA was incubated with 0–100 μM HOCl in 100 mM potassium phosphate buffer at pH 7.4 and 37°C for 30 min. KYNA (closed circle), 3-Cl-KYNA (open square), and 3-Cl-HQN (open triangle). All the reaction mixtures were analyzed by RP-HPLC. Means \pm SD ($n = 3$) are presented.

KYNA with HOCl. With increasing HOCl concentration, the consumptions of KYNA and both the yields of 3-Cl-KYNA and 3-Cl-HQN increased. The reactivity of HOCl to KYNA was lower than that of HOBr. The yield of 3-Cl-KYNA was higher than that of 3-Cl-HQN.

Reaction with HOCl in the presence of NaBr. A solution of 100 μM KYNA was incubated with 100 μM HOCl in the presence of 0–100 μM NaBr in 100 mM potassium phosphate buffer at pH 7.4 and 37°C for 30 min (Fig. 5). Consumption of

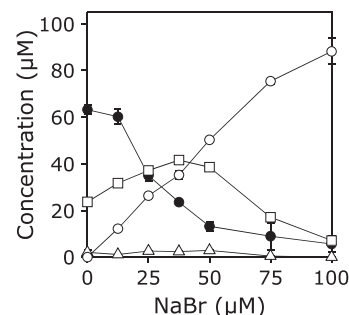


Fig. 5. NaBr dose-dependence of the concentration changes in KYNA and products in the reaction with HOCl. A solution of 100 μM KYNA was incubated with 100 μM HOCl in the presence of 0–100 μM NaBr in 100 mM potassium phosphate buffer at pH 7.4 and 37°C for 30 min. KYNA (closed circle), 3-Br-KYNA (open circle), 3-Cl-KYNA (open square), and 3-Cl-HQN (open triangle). All the reaction mixtures were analyzed by RP-HPLC. Means \pm SD ($n = 3$) are presented.

Table 1. Effects of additives on the reactions of KYNA with HOBr^{a)}

Additives	KYNA (μM)	3-Br-KYNA (μM)
None	4.0 \pm 0.2	94.1 \pm 1.2
NH ₄ Cl	80.8 \pm 1.6	22.4 \pm 1.4
CH ₃ NH ₂ /HCl	1.2 \pm 1.7	98.6 \pm 0.8
(CH ₃) ₂ NH/HCl	0.3 \pm 0.4	97.9 \pm 1.0
(CH ₃) ₃ N/HCl	0.0 \pm 0.0	99.7 \pm 1.5
(CH ₃) ₄ NCl	0.0 \pm 0.0	97.8 \pm 1.7
Gly	65.4 \pm 1.3	32.1 \pm 1.0
Lys	88.0 \pm 0.2	9.2 \pm 0.1
Met	93.4 \pm 0.6	3.7 \pm 0.2
Cys	93.1 \pm 1.1	1.7 \pm 0.1
Taurine	10.7 \pm 1.6	86.8 \pm 0.6
Ascorbic acid	72.7 \pm 0.1	3.5 \pm 0.2
Urea	9.6 \pm 2.5	92.1 \pm 2.6
Glucose	10.8 \pm 0.4	90.2 \pm 2.0
CH ₃ COONa	1.9 \pm 2.3	98.9 \pm 2.0

^{a)}Concentrations of KYNA and 3-Br-KYNA when a solution of 100 μM KYNA was incubated with 100 μM HOBr in 100 mM potassium phosphate buffer (pH 7.4) at 37°C for 24 h in the presence of the 1 mM additives. All the reaction mixtures were analyzed by RP-HPLC. Means \pm SD ($n = 3$) are presented.

KYNA and the yield of 3-Br-KYNA increased with increasing NaBr dose. At 100 μM NaBr, the major product was 3-Br-KYNA.

Effects of additives on the reaction with HOBr. To obtain information about the effects of coexistent compounds on the reactions of KYNA with HOBr, experiments were carried out in the presence of various additives. Table 1 shows the concentrations of KYNA and 3-Br-KYNA when a solution of 100 μM KYNA and 1 mM additives was incubated with 100 μM HOBr in 100 mM potassium phosphate buffer (pH 7.4) at 37°C for 24 h.

Although ammonium chloride suppressed the reaction, methylamine, dimethylamine, trimethylamine, and tetramethylammonium chloride had no effect. Amino acids suppressed the reaction. Taurine had no effect. Ascorbic acid suppressed the reaction. Urea, glucose, and sodium acetate had no effect.

Effects of additives on the reaction with HOCl. To obtain information about the effects of coexistent compounds on the reactions of KYNA with HOCl, experiments were carried out in the presence of various additives (Table 2). Ammonium chloride, methylamine, and dimethylamine strongly suppressed the reaction, while trimethylamine and tetramethylammonium chloride showed little effect. In the case of tetramethylammonium chloride, the yield of 3-Cl-HQN was comparable to that of 3-Cl-KYNA, probably due to facilitation of the decarboxylation of 3-Cl-KYNA by tetramethylammonium ion, generating 3-Cl-HQN. Amino acids and taurine suppressed the reaction almost completely. Ascorbic acid suppressed the formations of 3-Cl-KYNA and 3-Cl-HQN almost completely, although a certain amount of KYNA was consumed. Urea, glucose, and sodium acetate showed little effect.

Reaction of KYNA with EPO systems. Figure 6A and B show the pH dependence of concentrations of KYNA and the products in the reaction of KYNA with an EPO/H₂O₂/Cl⁻ system without or with Br⁻, respectively. A 100 μM KYNA solution with 100 μM H₂O₂, 100 mM NaCl, and 0.2 unit/ml EPO in 100 mM

potassium phosphate buffer (pH 2.8–9.6) in the absence or presence of 100 μM NaBr was incubated at 37°C for 30 min, and the reaction was terminated by addition of 1 mM *N*-acetylcysteine (AcCys). In the absence of NaBr, the reactions were slow even under mildly acidic conditions. Small but comparable amounts of 3-Br-KYNA and 3-Cl-KYNA were generated. No reaction was observed above pH 7. In the presence of 100 μM NaBr, KYNA was consumed and 3-Br-KYNA formed under mildly acidic conditions. The maximum consumption of KYNA was observed at pH 4–5. At around pH 7, the reaction was still observed. 3-Cl-KYNA and 3-Cl-HQN were not generated over the pH range examined.

Reaction of KYNA with MPO systems. Figure 7A and B show the pH dependence of concentrations of KYNA and the products in the reaction of KYNA with an MPO/H₂O₂/Cl⁻ system with or without Br⁻, respectively. In the absence of NaBr, 3-Cl-KYNA was formed under mildly acidic conditions. Small amounts of 3-Br-KYNA were also generated, although 3-Cl-HQN was not detected. The maximum consumption of KYNA was observed at around pH 5. No reaction was observed above pH 7. In the presence of 100 μM NaBr, 3-Br-KYNA was formed under mildly acidic conditions. 3-Cl-KYNA and 3-Cl-HQN were not generated over the pH range examined. No reaction was observed above pH 7.

Table 2. Effects of additives on the reactions of KYNA with HOCl^{a)}

Additives	KYNA (μM)	3-Cl-KYNA (μM)	3-Cl-HQN (μM)
None	66.3 ± 3.8	19.5 ± 0.5	3.1 ± 1.4
NH ₄ Cl	94.4 ± 0.8	0.5 ± 0.1	0.0 ± 0.0
CH ₃ NH ₂ /HCl	97.0 ± 1.0	0.6 ± 0.2	0.0 ± 0.0
(CH ₃) ₂ NH/HCl	92.4 ± 0.6	2.4 ± 0.2	0.0 ± 0.0
(CH ₃) ₃ NH/HCl	48.2 ± 1.5	21.7 ± 0.4	5.7 ± 0.1
(CH ₃) ₄ NCl	59.5 ± 0.7	12.1 ± 0.1	10.9 ± 0.3
Gly	98.1 ± 0.4	0.2 ± 0.1	0.0 ± 0.0
Lys	97.7 ± 0.1	0.2 ± 0.2	0.0 ± 0.0
Met	97.3 ± 0.1	0.5 ± 0.1	0.0 ± 0.0
Cys	98.6 ± 1.2	0.2 ± 0.2	0.0 ± 0.0
Taurine	96.0 ± 3.0	0.7 ± 0.1	0.0 ± 0.0
Ascorbic acid	74.7 ± 0.1	0.3 ± 0.2	0.0 ± 0.0
Urea	58.6 ± 0.8	26.6 ± 0.7	2.6 ± 0.2
Glucose	59.5 ± 1.5	21.6 ± 0.4	3.3 ± 0.2
CH ₃ COONa	59.0 ± 2.7	20.9 ± 0.6	3.8 ± 0.5

^{a)}Concentrations of KYNA, 3Cl-KYNA, and 3-Cl-HQN when a solution of 100 μM KYNA was incubated with 100 μM HOCl in 100 mM potassium phosphate buffer (pH 7.4) at 37°C for 24 h in the presence of the 1 mM additives. All the reaction mixtures were analyzed by RP-HPLC. Means ± SD (n = 3) are presented.

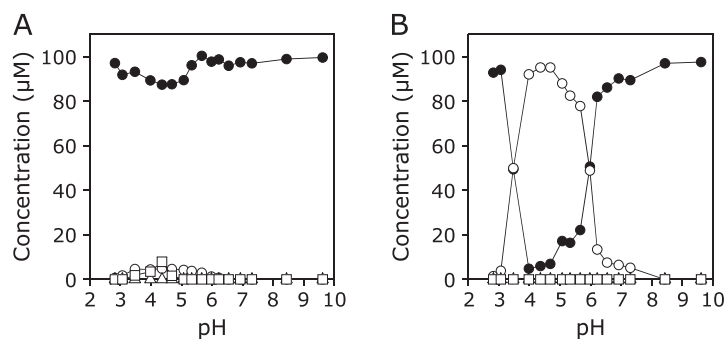


Fig. 6. (A) pH dependence of concentrations of KYNA and products in the reaction of KYNA with an EPO/H₂O₂/Cl⁻ system. A 100 μM KYNA solution with 100 μM H₂O₂ and 100 mM NaCl in the presence of 0.2 unit/ml EPO in 100 mM potassium phosphate buffer (pH 2.8–9.6) was incubated at 37°C for 30 min and the reaction was terminated by addition of 1 mM AcCys. (B) pH dependence of concentration of products in the reaction of KYNA with an EPO/H₂O₂/Cl⁻ system. A 100 μM KYNA solution with 100 μM H₂O₂, 100 mM NaCl, and 100 μM NaBr in the presence of 0.2 unit/ml EPO in 100 mM potassium phosphate buffer (pH 2.8–9.6) was incubated at 37°C for 30 min and the reaction was terminated by addition of 1 mM AcCys. KYNA (closed circle), 3-Br-KYNA (open circle), 3-Cl-KYNA (open square), and 3-Cl-HQN (open triangle). All the reaction mixtures were analyzed by RP-HPLC.

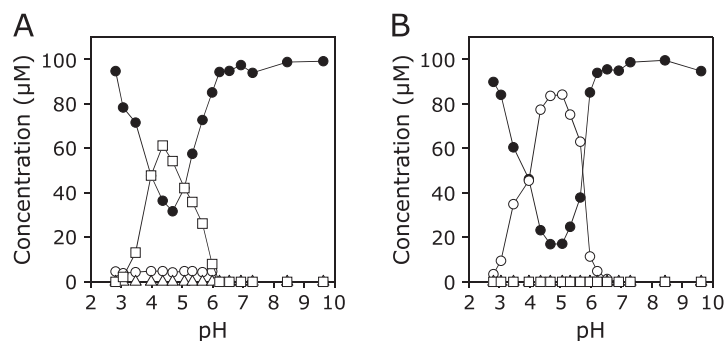


Fig. 7. (A) pH dependence of concentrations of KYNA and products in the reaction of KYNA with an MPO/H₂O₂/Cl⁻ system. A 100 µM KYNA solution with 100 µM H₂O₂ and 100 mM NaCl in the presence of 0.2 unit/ml MPO in 100 mM potassium phosphate buffer (pH 2.8–9.6) was incubated at 37°C for 30 min and the reaction was terminated by addition of 1 mM AcCys. (B) pH dependence of concentration of products in the reaction of KYNA with an MPO/H₂O₂/Cl⁻/Br⁻ system. A 100 µM KYNA solution with 100 µM H₂O₂, 100 mM NaCl, and 100 µM NaBr in the presence of 0.2 unit/ml MPO in 100 mM potassium phosphate buffer (pH 2.8–9.6) was incubated at 37°C for 30 min and the reaction was terminated by addition of 1 mM AcCys. KYNA (closed circle), 3-Br-KYNA (open circle), 3-Cl-KYNA (open square), and 3-Cl-HQN (open triangle). All the reaction mixtures were analyzed by RP-HPLC.

Discussion

3-Br-KYNA and 3-Cl-KYNA were reportedly synthesized from an ethyl ester derivative of KYNA by treatment with Br₂ and SO₂Cl₂, respectively, and subsequent hydrolysis.⁽²⁵⁾ The present study showed that 3-Br-KYNA and 3-Cl-KYNA were generated directly as the major products of the reaction of KYNA with HOBr and HOCl, respectively, under neutral conditions. We recently showed that rebamipide and methotrexate reacted with HOBr, resulting in corresponding brominated products as major products.^(26,27) Methotrexate reacted with HOCl generating a corresponding chlorinated methotrexate, whereas rebamipide did not react with HOCl. Like methotrexate, KYNA reacted with both HOBr and HOCl. The reactivity of HOBr to KYNA was higher than that of HOCl (Fig. 2 and 4), probably due to the higher electrophilicity of HOBr compared to HOCl.⁽²⁸⁾ In the presence of NaBr, HOCl generated 3-Br-KYNA in addition to 3-Cl-KYNA, probably due to the formation of HOBr.⁽²⁹⁾ At serum concentration of Br⁻ (about 60 µM), the yield of 3-Br-KYNA was higher than that of 3-Cl-KYNA (Fig. 5). Amines had enhanced on the reactions of KYNA with HOBr, whereas they suppressed the reactions with HOCl almost completely (Table 1 and 2). It has been reported that CH₃NH₂ and (CH₃)₂NH react with HOBr and HOCl to generated corresponding bromamines and chloramines, respectively, and that second-order rate constants for the reactions of phenol with bromamines of CH₃NH₂ and (CH₃)₂NH are several orders higher than those with chloramines.⁽³⁰⁾ Similarly, KYNA would react strongly with bromamines of them to generate 3-Br-KYNA, whereas KYNA would not react with the chloramines. Free amino acids of 1 mM suppressed the reactions of KYNA with both HOBr and HOCl. For HOCl, even Gly suppressed the reaction almost completely. Taurine suppressed the HOCl reaction but not the HOBr reaction. The reaction of KYNA *in vivo* with HOBr and especially HOCl should be suppressed by free amino acids and taurine, since the total concentration of free amino acids in human plasma is several mM and the concentration of taurine is high in neutrophils (19 mM) and eosinophils (15 mM).^(31,32) An EPO/H₂O₂/Cl⁻/Br⁻ system reacted with KYNA, generating 3-Br-KYNA exclusively under mildly acidic conditions (Fig. 6B). Although an MPO/H₂O₂/Cl⁻ system reacted with KYNA to generate 3-Cl-KYNA under mildly acidic conditions with small amounts of 3-Br-KYNA (Fig. 7A), this was probably due to Br⁻ as contaminant in NaCl or phosphate buffer. An MPO/H₂O₂/Cl⁻/Br⁻ system of 100 µM NaBr generated 3-Br-KYNA almost exclusively (Fig. 7B).

Several studies have reported the concentrations of halogenated

derivatives of tyrosine (Tyr), 3-bromotyrosine (3-Br-Tyr) and 3-chlorotyrosine (3-Cl-Tyr) in animals and humans, which were probably generated from Tyr by EPO and MPO. In wild-type mice, the concentration of free 3-Br-Tyr in the peritoneal fluid was comparable to that of free 3-Cl-Tyr. Treatment by cecal ligation and puncture greatly increased the concentrations of both 3-Br-Tyr and 3-Cl-Tyr, with the average concentration of 3-Cl-Tyr three times higher than that of 3-Br-Tyr.⁽³³⁾ In healthy volunteers, the concentration of 3-Cl-Tyr in proteins recovered from airways was five times higher than that of 3-Br-Tyr, while in severe asthmatics the concentrations of both 3-Br-Tyr and 3-Cl-Tyr were higher than in healthy volunteers; the average concentration of 3-Br-Tyr was seven times higher than that of 3-Cl-Tyr.⁽³⁴⁾ Another study showed that the concentration of free 3-Br-Tyr in human plasma of healthy male volunteers was three times higher than that of free 3-Cl-Tyr.⁽³⁵⁾ In contrast, only one study reported the concentrations of halogenated nucleosides in humans. The concentration of 8-bromo-2'-deoxyguanosine (8-Br-dGuo) in urine from healthy volunteers was similar to that of 8-chloro-2'-deoxyguanosine (8-Cl-dGuo), while urinary 8-Br-dGuo and 8-Cl-dGuo levels from diabetic patients were 8-fold the levels in healthy volunteers.⁽³⁶⁾ These reported results imply that both bromination and chlorination of living substances occur in healthy humans, and that inflammatory diseases greatly increase those levels. In humans, the concentration of KYNA is 0.003 µM in saliva, 0.004–0.060 µM in plasma, 0.14–1.58 µM in brain, and 4–40 µM in urine, whereas the concentration of Tyr ranges from 8 to 82 µM (median 30 µM) in plasma and 7 to 366 µM (median 64 µM) in urine.^(4,37) The halogenated derivatives of Tyr are detected in human plasma.⁽³⁵⁾ The present study showed that *in vitro* EPO and MPO systems readily converted KYNA to its halogenated derivatives under mildly acidic conditions. There is a possibility that the halogenated derivatives of KYNA form *in vivo*, especially in urine, although the reaction rate constants of KYNA with HOBr and HOCl are not determined.

KYNA is an agonist of G-protein coupled GPR35 receptor, which presents predominantly on immune cells and in the gastrointestinal tract.⁽²⁾ KYNA may have a positive influence on a number of pathologies of the gastrointestinal tract.⁽⁴⁾ KYNA is also an antagonist of both the *N*-methyl-D-aspartate receptor and the α7 nicotinic acetylcholine receptor.^(5,6) KYNA possesses neuroprotective properties, since it blocks some of the neurotoxic effects of excitotoxins.⁽⁹⁾ It has been reported that halogenated analogues of KYNA, such as 7-chlorokynurenic acid, 5,7-dichlorokynurenic acid, and 5,7-dibromokynurenic acid, are selective antagonists at the glycine modulatory site of the *N*-

methyl-D-aspartate receptor complex.^(38,39) However, the biological activities of 3-Br-KYNA and 3-Cl-KYNA have not been clarified. And also, the formations of 3-Br-KYNA and 3-Cl-KYNA are unclear in humans. If 3-Br-KYNA and 3-Cl-KYNA formed in body fluids and show biological activities, they may affect the pathophysiology of various diseases with inflammation.

The present study showed that KYNA readily reacts with reagents of HOBr and HOCl, and enzyme systems of EPO and

MPO, resulting in halogenated products *in vitro*. These results suggest that 3-Br-KYNA and 3-Cl-KYNA may be generated from KYNA by EPO and MPO in inflammation sites in humans.

Conflict of Interest

No potential conflicts of interest were disclosed.

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