Biosci. Biotechnol. Biochem., **74** (3), 536–540, 2010

Immunochemical Detection of Food-Derived Isothiocyanate as a Lysine Conjugate

Toshiyuki NAKAMURA,[1] Noritoshi KITAMOTO,[1] Toshihiko OSAWA,[2] and Yoji KATO[1,†]

[1]*Graduate School of Human Science and Environment, University of Hyogo, Himeji 670-0092, Japan*
[2]*Nagoya University Graduate School of Bioagricultural Sciences, Nagoya 464-8601, Japan*

Received October 5, 2009; Accepted December 3, 2009; Online Publication, March 7, 2010
[doi:10.1271/bbb.90728]

In a previous study we prepared monoclonal antibody against allyl isothiocyanate (AITC)-modified lysine (Lys), and found that AITC reacted with Lys under physiological conditions *in vitro* (T. Nakamura *et al.*, *Chem. Res. Toxicol.*, 22, 536–542 (2009)). In the present study, antibodies against benzyl isothiocyanate (ITC), 6-methylsulfinylhexyl ITC and phenethyl ITC modified protein were prepared, and the respective monoclonal antibodies, B6C9, 6MS3D10, and PE3A10 were obtained. These antibodies were applied to ITC detection in food using shredded Wasabia japonica (wasabi) and ground Carica papaya (papaya) seed by trapping ITC with biotin-labeled bovine serum albumin. ITC formation from the wasabi and papaya seed samples was confirmed using the antibodies in a dose-dependent manner. These antibodies might be applicable in identifying food-derived ITC.

Key words: isothiocyanate; lysine adduct; antibody; enzyme-linked immunosorbent assay

Isothiocyanates (ITCs) are present in cruciferous plants as glucosinolates, and are produced by myrosinase when plant cells are injured. Wasabia japonica (wasabi), Carica papaya (papaya), and water cress are sources of allyl ITC (AITC), benzyl ITC (BITC), and phenethyl ITC (PEITC) respectively (Fig. 1). Other known characteristic wasabi compounds include 6-methylsulfinylhexyl ITC (6MSITC) and 6-methylthiohexyl ITC (6MTITC) (Fig. 1).

ITC reacts preferentially with thiols, forming a dithiocarbamate derivative. ITCs and their dithiocarbamates react quantitatively with 1,2-benzenedithiol by a cyclocondensation reaction to give rise to 1,3-benzodithiole-2-thione, which can be quantified by high performance liquid chromatography (HPLC) with UV detection.[1–3] This method alone cannot specify the type of ITC quantified, because 1,3-benzodithiole-2-thione formation is dependent on the N=C=S moiety but not the ITC side chain. In individual cases, sulforaphane (4-methylsulfinylbutyl ITC, SFN) has been detected in broccoli by gas chromatography/mass spectrometry.[4] SFN and its mercapturic acid pathway conjugates have been identified and quantified in human urine by HPLC-tandem mass spectrometry.[5] 6MSITC and its thiol conjugate were detected by HPLC-atmospheric pressure chemical ionization mass spectrometry.[6] Methods of ITC detection in food and in biological samples have been limited to these chemical analyses to date.

Recently, we prepared an antibody to AITC-modified lysine (Lys).[7] In addition to this antibody, in the current study we prepared monoclonal antibodies against food-derived ITC-modified protein using BITC, 6MSITC, and PEITC. Using these antibodies with biotin-conjugated bovine serum albumin (BSA), the formation of ITC from ground food was confirmed.

Materials and Methods

Materials. AITC, BITC, and PEITC were purchased from Tokyo Chemical Industry (Tokyo, Japan). 6MSITC and 6MTITC were kindly donated by Kinjirushi (Nagoya, Japan). *S*-(*N*-Benzylthiocarbamoyl)-L-cysteine (BITC-Cys) was purchased from LKT Laboratories (St. Paul, MN). BSA (A7511, ≥97%), biotinamidocaproyl labeled BSA (biotin-BSA), and streptavidin-peroxidase polymer (ultrasensitive) were from Sigma-Aldrich (St. Louis, MO). Keyhole limpet hemocyanin (KLH) was from Pierce (Rockford, IL). N^{α}-Benzoyl-glycyl-L-lysine (BGK) was from Peptide Institute (Osaka, Japan). N^{α}-Acetyl-L-cysteine (NAC) was from Nacalai Tesque (Kyoto, Japan). All other chemicals were purchased from Wako Chemical (Osaka, Japan).

Preparation and characterization of ITC-Lys monoclonal antibody. KLH (5 mg/ml), as a carrier protein, was reacted with four separate aliquots of 10 mM ITC in 65 mM borate buffer (pH 9.0) for 24 h at 37 °C. Each ITC-modified KLH was then dialyzed against phosphate-buffered saline (PBS) for 3 d at 4 °C, with several exchanges of PBS. At the same time, ITC-modified BSA was prepared as a positive control antigen, as described above. The monoclonal antibody was prepared by fusion of myeloma and spleen cells by the polyethylene glycol method, as described previously.[7] Monoclonal antibodies B6C9, 6MS3D10, and PE3A10 were obtained against BITC-KLH, 6MSITC-KLH, and PEITC-KLH, and the immunoglobulin types for these were identified using an Isotyping Kit (Sigma-Aldrich) as IgG_1, IgG_1, and IgG_{2a} respectively. An enzyme-linked immunosorbent assay (ELISA) was done to screen the hybridomas, and competitive ELISA was used for characterization, as described previously.[7]

Preparation of ITC-modified benzoyl-glycyl-lysine. AITC-modified BGK was prepared as described previously.[7] BITC-, 6MSITC-, 6MTITC-, and PEITC-modified BGK were prepared and purified as follows: ITC was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM. BGK (1 mM) was incubated with 10 mM ITC

† To whom correspondence should be addressed. Fax: +81-792-93-5710; E-mail: yojikato@shse.u-hyogo.ac.jp

Abbreviations: ITCs, isothiocyanates; AITC, allyl isothiocyanate; BITC, benzyl isothiocyanate; PEITC, phenethyl isothiocyanate; 6MSITC, 6-methylsulfinylhexyl isothiocyanate; 6MTITC, 6-methylthiohexyl isothiocyanate; SFN, sulforaphane; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; BGK, N^{α}-benzoyl-glycyl-L-lysine; NAC, N^{α}-acetyl-L-cysteine; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; HPLC, high performance liquid chromatography

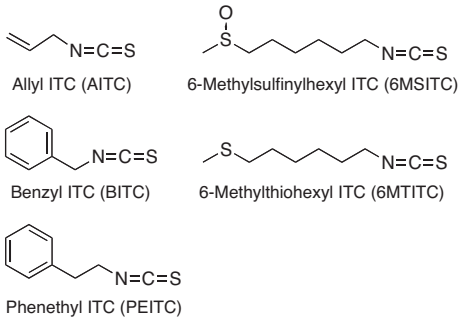

Fig. 1. Chemical Structures of Food-Derived Isothiocyanates (ITCs).

for 0–24 h at 37 °C in 90 mM borate buffer (pH 9.0). To isolate the adducts, ITC-modified BGK was isocratically separated by reversed-phase HPLC on a Develosil Combi-RP (20 × 100 mm) column using 0.1% acetic acid/CH_3CN as the eluent at a flow rate of 5.0 ml/min. The major peak was collected, concentrated, and further purified by HPLC. The structures of the purified ITCs-BGK adducts were confirmed by liquid chromatography-mass spectrometry (LC-MS) and ^{1}H-nuclear magnetic resonance (^{1}H-NMR) analysis using the JNM-AL series AL300 (JEOL, Tokyo) in CD_3OD. NMR spectra were kindly provided by Dr. Yoshichika Kawai (The University of Tokushima). The spectral data were as follows: BITC-BGK: ^{1}H-NMR (ppm) 1.44 (m, 2H), 1.58 (m, 2H), 1.74 (m, 1H), 1.92 (m, 1H), 3.47 (m, 2H), 4.07 (m, 2H), 4.45 (m, 1H), 4.68 (s, 2H), 7.27 (m, 1H), 7.27 (m, 2H), 7.29 (m, 2H), 7.44 (t, $J = 7.7$, 2H), 7.53 (t, $J = 7.5$, 1H), 7.84 (d, $J = 7.1$, 2H); LC-MS (ESI+) m/z 457.0 $[M + H]^+$. 6MSITC-BGK: ^{1}H-NMR (ppm) 1.37 (m, 2H), 1.46 (m, 2H), 1.49 (m, 2H), 1.57 (m, 2H), 1.57 (m, 2H), 1.74 (m, 1H), 1.74 (m, 2H), 1.91 (m, 1H), 2.61 (s, 3H), 2.78 (m, 2H), 3.43 (m, 2H), 3.43 (m, 2H), 4.08 (m, 2H), 4.44 (m, 1H), 7.47 (t, $J = 7.5$, 2H), 7.55 (t, $J = 7.3$, 1H), 7.87 (d, $J = 6.8$, 2H); LC-MS (ESI+) m/z 513.0 $[M + H]^+$. 6MTITC-BGK: ^{1}H-NMR (ppm) 1.33 (m, 2H), 1.43 (m, 2H), 1.45 (m, 2H), 1.58 (m, 2H), 1.58 (m, 2H), 1.74 (m, 1H), 1.74 (m, 2H), 1.91 (m, 2H), 2.46 (t, $J = 7.2$, 2H), 2.65 (s, 3H), 3.43 (m, 2H), 3.43 (m, 2H), 4.08 (m, 2H), 4.42 (m, 1H), 7.46 (t, $J = 7.5$, 2H), 7.54 (t, $J = 7.3$, 1H), 7.87 (d, $J = 7.0$, 2H); LC-MS (ESI+) m/z 497.0 $[M + H]^+$. In addition, PEITC-BGK was identified by LC-MS (ESI+) m/z 471.0 $[M + H]^+$.

Preparation of 6MSITC-modified N*-acetyl-cysteine.* 6MSITC-NAC adduct was prepared and purified in a manner similar to that described previously for AITC-NAC, *N*-acetyl-*S*-(*N*-allylthiocarbamoyl)-L-cysteine.[7] A NAC solution (10.9 mg/ml in 50% ethanol) was adjusted to pH 7.8 using 1 M NaOH, and 0.6 ml of 6MSITC solution (13.7 mg/ml in ethanol) was added to 1.2 ml of NAC solution. The mixture was stirred under N_2 for 3 h at room temperature. The sample was dried by evaporation and then purified by HPLC.

The major peak was collected using a Develosil Combi-RP (20 × 100 mm) column with 0.1% acetic acid/CH_3CN (9/1) at a flow rate of 5.0 ml/min. The purified 6MSITC-NAC adduct was identified by ^{1}H-NMR and LC-MS. Spectral data were as follows: ^{1}H-NMR (ppm) 1.37 (m, 2H), 1.46 (m, 2H), 1.61 (m, 2H), 1.7 (m, 2H), 1.89 (s, 3H), 2.56 (s, 3H), 2.75 (m, 2H), 3.45 (d, $J = 13.8$, 1H), 3.61 (t, $J = 6.87$, 2H), 3.87 (d, $J = 13.8$, 1H), 4.57 (m, 1H); LC-MS (ESI+) m/z 369.0 $[M + H]^+$.

Immunochemical identification of ITCs in foods by capture of modified biotin-BSA. Commercial wasabi rhizome was shredded with a grater, and papaya seed was ground with a mortar and pestle. These samples were suspended and diluted in pure water to achieve concentrations of homogenates ranging from 1.56 to 100 mg/ml. As a standard, authentic AITC and BITC were dissolved in DMSO to a concentration of 1 M, and then diluted in 0.1 M phosphate buffer (pH 7.4) to achieve concentrations of AITC and BITC ranging from 0.1 to 10 mM and 0.01 to 1 mM respectively. Biotin-BSA (0.5 mg/ml) was dissolved in 0.1 M phosphate buffer (pH 7.4) and exposed to various concentrations of homogenates or authentic ITCs for 24 h at 37 °C. The reaction mixture was centrifuged (14,000 *g*, 5 min, 4 °C), and the supernatants were collected. The modified proteins in the supernatants were purified with a spin column (Bio-Rad, Micro Bio-Spin 6). The eluate was diluted 20 times with PBS containing 0.05% Tween-20 (TPBS) or immunoreaction enhancer solution (Can Get Signal, Toyobo, Osaka, Japan), and then applied to a microtiter plate. The wells in the microtiter plate had been separately coated with four antibodies (0.01 mg/ml in PBS) overnight at 4 °C. After washing and blocking of the plate using 1% Block Ace aqueous solution (Dainihon Sumitomo Seiyaku, Osaka, Japan) for 1 h at 37 °C, the diluted eluate was added and captured by immobilized antibodies for 1 h at 37 °C. After washing, 100 µl of peroxidase-labeled streptavidin at a 1:5,000 dilution in TPBS was added and this was reacted for 1 h at 37 °C. Color was developed using tetramethylbenzidine (TMB) substrate solution (KPL, Gaithersburg MD), and terminated by the addition of 1 M phosphoric acid. The plate was measured at 450 nm with an xMark microplate spectrophotometer (Bio-Rad).

Identification of ITCs in shredded wasabi and ground papaya seed by LC-MS/MS. Suspensions of shredded wasabi and of ground papaya seed (1.56 to 100 mg/ml) were prepared as described above and incubated with BGK (1 mM) in water for 24 h at 37 °C. The reaction mixture was centrifuged (14,000 *g*, 5 min, 4 °C), and the supernatants were collected. The sample was analyzed by LC-MS using a quadruple tandem mass spectrometer (API-3000, Applied Biosystems) connected to an Agilent 1100 HPLC system. HPLC separation utilized a gradient system of solvent A (0.1% acetic acid) and solvent B (CH_3CN) on a Develosil ODS-HG-3 (2.0 × 50 mm) column at a flow rate of 0.2 ml/min. The gradient program was as follows: 0 min (A 100%), 7 min (A 30%), 7.5 min (A 30%), 7.6 min (A 100%), and 15 min (A 100%). Quantification of AITC-BGK and BITC-BGK was performed by multiple reaction monitoring (MRM) of 407.0/308.0 $[M + H]^+$ and 457.0/308.0 $[M + H]^+$ respectively. A parameter for the detection of PEITC-BGK (471.0/308.0 $[M + H]^+$) was also included in the scanning program.

Results

Specificity of monoclonal antibodies to ITC-modified protein

BITC, 6MSITC, and PEITC (10 mM) were separately reacted with KLH in borate buffer (pH 9.0) for 24 h, and the modified KLHs obtained were used as immunogens. Spleen cells from an immunized mouse and myeloma cells were fused by the polyethylene glycol method. After repeated screening and cloning of hybridomas, three novel monoclonal antibodies, B6C9 (BITC), 6MS3D10 (6MSITC), and PE3A10 (PEITC), were obtained. The immunoglobulin types of the antibodies were identified as IgG. None of the antibodies recognized native BSA on noncompetitive indirect ELISA (data not shown).

Competitive ELISA was used to characterize the cross-reactivity of the antibodies with the ITC-related compounds. We have reported that the A4C7 antibody specifically recognized AITC-modified BGK but not native BGK (a Lys derivative), allylamine (an analog of AITC), or AITC-modified NAC.[7] In this study, the antibody also did not react with BITC-BGK or 6MSITC-BGK (Fig. 2Aa). The B6C9 antibody, which was prepared by immunization of BITC-modified KLH, recognized BITC-BGK but not BGK, AITC-BGK, or 6MSITC-BGK (Fig. 2Ab). The 6MS3D10 antibody recognized 6MSITC-BGK but not BGK, AITC-BGK, or BITC-BGK (Fig. 2Ac). Although the B6C9 antibody did not react with BITC-Cys or benzoic acid, it recognized PEITC-BGK at approximately 10-times lower levels than BITC-BGK (Fig. 2Ba). The 6MS3D10 antibody recognized 6MTITC-BGK and 6MSITC-NAC at approximately 100-times and 20-times lower levels than that against 6MSITC-BGK, respec-

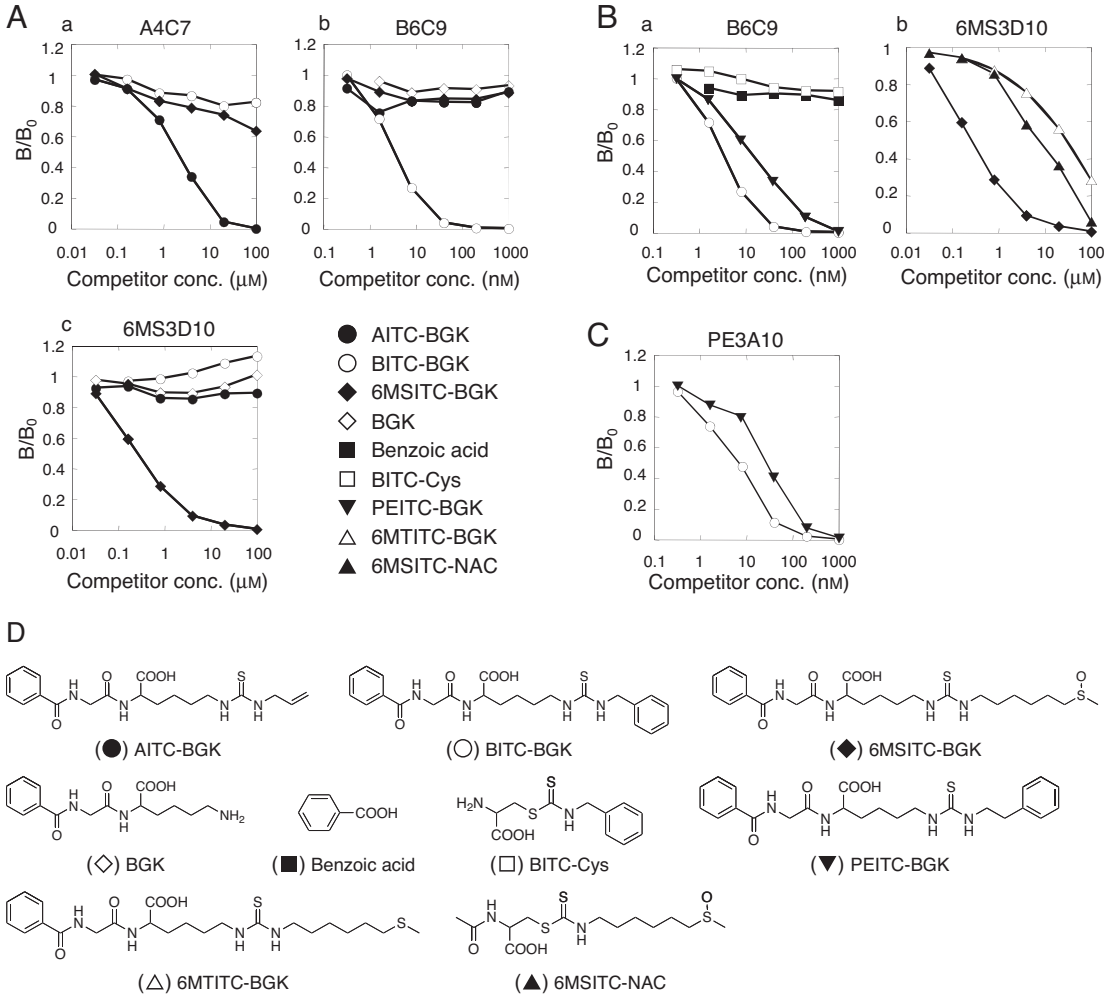

Fig. 2. Characterization of Monoclonal Antibodies by Competitive ELISA.

A, Cross-reactivity of the A4C7 (a), B6C9 (b), and 6MS3D10 (c) antibodies with ITC-BGK (ITC-benzoyl-glycyl-lysine) and BGK as competitors as determined by competitive ELISA. B, Competitive ELISA with the B6C9 (a) and 6MS3D10 (b) antibodies. C, Competitive ELISA of the PE3A10 antibody. The cross-reactivity of the antibody with the competitors was expressed as B/B_0, in which B is the amount of the antibody bound to the coating antigen in the presence of the competitor and B_0 is the amount in the absence of the competitor. D, Chemical structures of the competitors used. The symbols used are (●) AITC-BGK, (○) BITC-BGK, (◆) 6MSITC-BGK, (◇) BGK, (■) benzoic acid (an analog of BITC), (□) BITC-NAC, (▼) PEITC-BGK (an analog of BITC-BGK), (△) 6MTITC-BGK (an analog of 6MSITC-BGK), and (▲) 6MSITC-NAC.

tively (Fig. 2Bb). The PE3A10 antibody to PEITC-KLH reacted with BITC-BGK as well as PEITC-BGK (Fig. 2C), indicating that the PE3A10 and B6C9 antibodies have similar reactivity. The detection limits of A4C7, B6C9, 6MS3D10, and PE3A10 were approximately 1 μM, 1 nM, 0.1 μM, and 10 nM respectively. The chemical structures of the competitors used are shown in Fig. 2D.

Immunochemical determination of ITCs in foods

We developed a biotin tagged ITC-detection method by capture ELISA (Fig. 3A). This method specifically detects ITC-modification of a target compound (BSA). To verify the method, commercial AITC and BITC were incubated with biotin-BSA, and the conjugate formed was analyzed (Fig. 3B). Reactivity increased with increasing AITC or BITC concentration.

Using this method, we attempted to identify food-derived ITCs. Suspensions of shredded wasabi and of ground papaya seed were incubated with biotin-labeled BSA, and then capture ELISA carried out. The wasabi sample was specifically recognized by the A4C7 antibody but not by the other monoclonal antibodies (Fig. 3C). Although 6MSITC is one of the characteristic compounds in wasabi, the 6MS3D10 antibody did not react with the sample, probably because of the smaller amount of 6MSITC than AITC in wasabi.[8] To improve sensitivity, binding between the immobilized antibody and ITC-modified BSA was enhanced by replacing conventional TPBS with an immunoreaction enhancer solution. As shown in Fig. 3C (inset), 6MSITC modification was also confirmed in the wasabi sample. The product of ground papaya seed incubated with biotin-BSA reacted with both B6C9 and PE3A10 antibodies, which have overlapping epitope specificity (Fig. 2). These results suggest that food-derived ITC can be identified and semi-quantified by novel antibodies by the biotin tagged method. Indeed, by replacing biotin-BSA with a lysine peptide, BGK, the specific formation of AITC-Lys (from wasabi) and BITC-Lys (from papaya seed) could be confirmed by LC-MS/MS (Fig. 3D). In addition, PEITC-BGK was not detected in the reaction mixture of papaya seed with BGK.

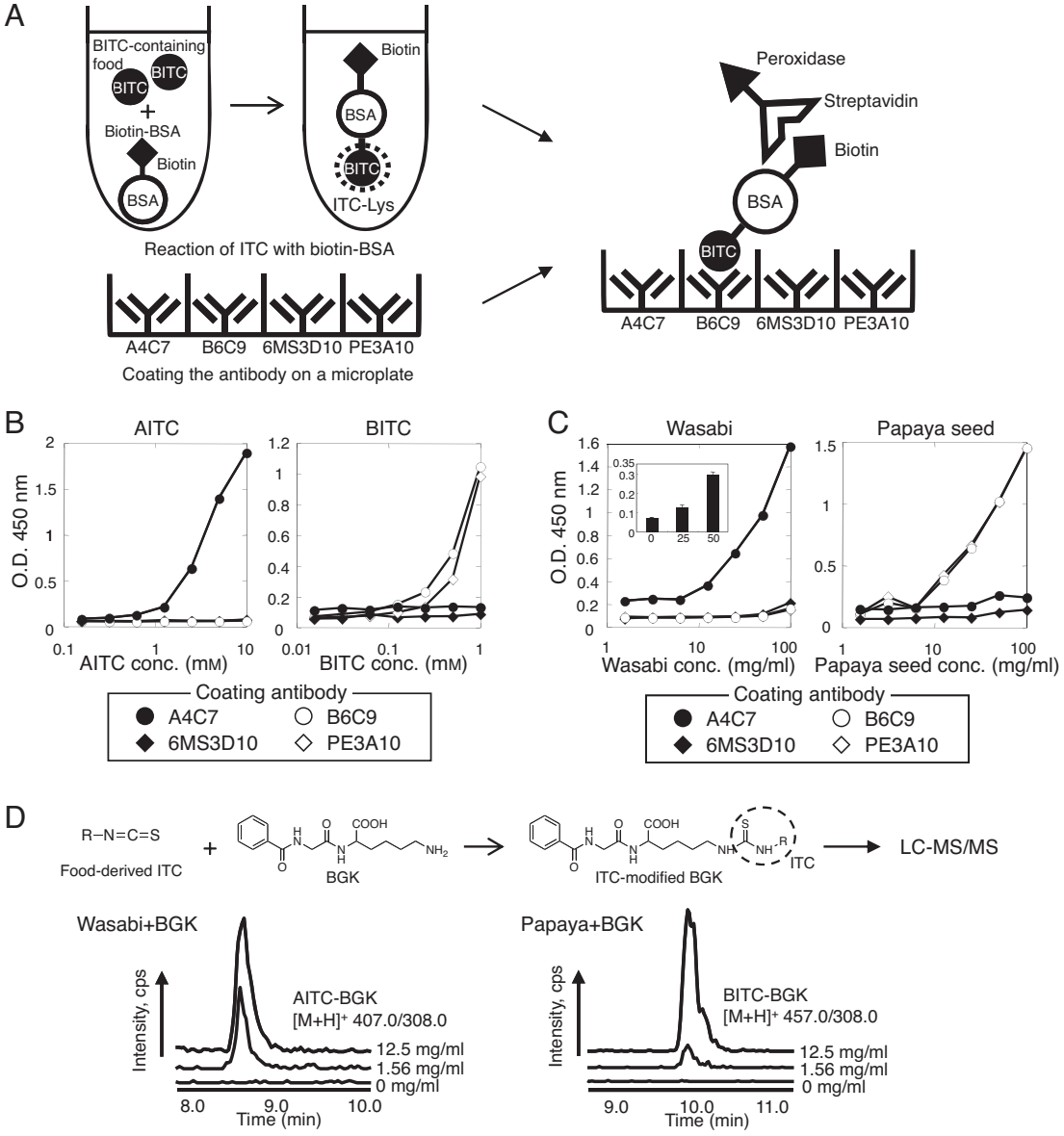

Fig. 3. Capture Assay of Food-Derived ITC Using Biotin-BSA as Carrier Protein.

A, Scheme for the reaction of immobilized antibody on a microplate with ITC-modified biotin-BSA as an illustration using a BITC-containing food. ITC in food reacts with biotin-BSA at neutral pH. The modified BSA was captured by immobilized antibody. The binding was evaluated by reaction with peroxidase-labeled streptavidin. B, Confirmation of the assay using pure ITCs. Biotin-BSA (0.5 mg/ml) was exposed to authentic AITC (0.1 to 10 mM) or BITC (0.01 to 1 mM) for 24 h at 37 °C. Symbols: (●) the A4C7 antibody, (○) the B6C9 antibody, (◆) the 6MS3D10 antibody, (◇) the PE3A10 antibody. C, The reactivity of the antibodies with food-derived ITC-modified biotin-BSA was measured. Shredded wasabi and ground papaya seed (1.56 to 100 mg/ml) were suspended individually in water and then incubated with biotin-BSA (0.5 mg/ml) for 24 h at 37 °C. The symbols are the same as in B. Control and wasabi samples (0, 25, 50 mg/ml) were also reacted with the 6MS3D10 antibody in an immunoreaction enhancer solution instead of TPBS. Then the binding of biotin-BSA to the antibody was evaluated by reaction with peroxidase-labeled streptavidin (C, inset in wasabi). D, Shredded wasabi and ground papaya seed (1.56–100 mg/ml) were reacted with BGK (1 mM), as a model Lys residue, for 24 h at 37 °C. The MRM 407.0/308.0 $[M + H]^+$ for AITC-BGK and 457.0/308.0 $[M + H]^+$ for BITC-BGK were scanned by LC-MS/MS.

Discussion

In the present study, we developed three novel monoclonal antibodies against BITC, 6MSITC, and PEITC-modified protein. All these antibodies recognized the corresponding ITC-modified BSA (data not shown), and peptides that contained ITC-modified Lys. Although we also obtained a monoclonal antibody reactive to SFN-modified protein, the antibody did not react with free SFN-Lys derivative (data not shown).

The B6C9 and PE3A10 antibodies exhibited cross-reactivity towards BITC and PEITC (Fig. 2), probably due to the similar structures of these Lys adducts (Fig. 1). Similarly, the 6MS3D10 antibody recognized the 6MTITC-Lys adduct to some extent. This antibody reacted with Cys conjugate, a predominant ITC conjugate.[9] Although Cys conjugate is known to be unstable,[10,11] covalent modification of ITC with Cys from α-tubulin in cells has been identified by mass spectrometry.[12] The 6MS3D10 antibody might be useful for detection of ITC-Cys adducts in cell and tissue samples if the adducts are stably isolated.

When testing our antibodies with food-derived ITC-biotin-BSA conjugates, we saw that A4C7 and 6MS3D10 reacted with the reaction mixture of shredded wasabi and biotin-BSA (Fig. 3C). Even though 6MSITC

is present in amounts approximately 6.5-times less than AITC in wasabi,[8] we were able to detect 6MSITC. We established a brief and convenient assay for the detection of ITCs in food homogenates. BITC-Lys was detected by the B6C9 antibody in the mixture of ground papaya seed and biotin-BSA (Fig. 3C). While the PE3A10 antibody, prepared from PEITC-KLH as the immunogen, reacted with this mixture, PEITC was not present in the papaya seed, and this was due to cross-reaction between antibody B6C9 and PE3A10.

With regard to sensitivity and selectivity, chemical analysis by LC-MS/MS is generally superior to immunochemical methods. However, as compared with LC-MS/MS, our immunochemical assay can handle many samples at once. The sensitivity of the immunochemical assay was increased by the use of immunoreaction enhancer solution. It has been reported that accumulation of quercetin metabolite in human atherosclerotic lesions was immunohistochemically proven by a monoclonal antibody specific to the quercetin metabolite.[13] In this way, an immunochemical technique is a powerful tool not only for identifying molecules in foods, but also for evaluating the localization of target molecules in tissues and in cellular components. The antibodies obtained might be useful tools for the identification of ITC in these applications.

Acknowledgments

We thank Kinjirushi Co., Ltd. (Nagoya, Japan) for kindly providing 6MSITC and 6MTITC. We thank Dr. Yoshichika Kawai (The University of Tokushima) for helpful technical assistance, and Dr. Yoshimasa Nakamura (Okayama University) for helpful advice.

References

1) Zhang Y, Cho CG, Posner GH, and Talalay P, *Anal. Biochem.*, **205**, 100–107 (1992).
2) Zhang Y, Wade KL, Prestera T, and Talalay P, *Anal. Biochem.*, **239**, 160–167 (1996).
3) Zhang Y and Talalay P, *Cancer Res.*, **58**, 4632–4639 (1998).
4) Chiang WCK, Pusateri DJ, and Leitz REA, *J. Agric. Food Chem.*, **46**, 1018–1021 (1998).
5) Egner PA, Kensler TW, Chen JG, Gange SJ, Groopman JD, and Friesen MD, *Chem. Res. Toxicol.*, **21**, 1991–1996 (2008).
6) Yamaguchi H, Noshita T, Kidachi Y, Umetsu H, Fuke Y, and Ryoyama K, *Chem. Pharm. Bull.*, **56**, 715–719 (2008).
7) Nakamura T, Kawai Y, Kitamoto N, Osawa T, and Kato Y, *Chem. Res. Toxicol.*, **22**, 536–542 (2009).
8) Fukuchi Y, Kato Y, Okunishi I, Matsutani Y, Osawa T, and Naito M, *Food Sci. Technol. Res.*, **10**, 290–295 (2004).
9) Conaway CC, Krzeminski J, Amin S, and Chung FL, *Chem. Res. Toxical.*, **14**, 1170–1176 (2001).
10) Hong F, Freeman ML, and Liebler DC, *Chem. Res. Toxicol.*, **18**, 1917–1926 (2005).
11) Kassahun K, Davis M, Hu P, Martin B, and Baillie T, *Chem. Res. Toxicol.*, **10**, 1228–1233 (1997).
12) Mi L, Xiao Z, Hood BL, Dakshanamurthy S, Wang X, Govind S, Conrads TP, Veenstra TD, and Chung FL, *J. Biol. Chem.*, **283**, 22136–22146 (2008).
13) Kawai Y, Nishikawa T, Shiba Y, Saito S, Murota K, Shibata N, Kobayashi M, Kanayama M, Uchida K, and Terao J, *J. Biol. Chem.*, **283**, 9424–9434 (2008).