Tea catechins in green tea inhibit the activity of the SARS-CoV-2 main protease via covalent adduction

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Abstract

Here, we examined the inhibitory effects of tea catechins on the SARS-CoV-2 main protease (M^{pro}). Among the eight catechins examined, epigallocatechin 3-(3″-*O*-methyl)gallate (EGCG3″Me), epigallocatechin gallate (EGCG), gallocatechin, gallocatechin gallate, and epigallocatechin inhibited the recombinant M^{pro} enzyme in a dose-dependent manner. Covalent binding of catechins to M^{pro} was confirmed by quinone staining and intact mass spectrometry. Peptide mapping revealed the preferential covalent adduction of catechin to the active site sequence of M^{pro}. Fragmentation analysis revealed 184 liberations from the digested peptides, corresponding to monomethylated gallic acid (D ring) liberation, indicating that the B-ring was bound to the active site. When 10 bottled teas were incubated with M^{pro}, the green teas inhibited the enzyme, whereas the blended and barley tea did not. EGCG covalently reacted with the viral enzyme intracellularly on incubation with cultured cells expressing coronavirus M^{pro}, suggesting that the catechins in green tea can inhibit M^{pro} in infected cells.

Keywords: coronavirus main protease; green tea; catechins; pyrogallol; intracellular reaction

1. Introduction

Green tea is widely consumed in China and Japan as well as in other countries worldwide. Recently, green tea has become increasingly popular, with the expectation of beneficial health-promoting effects. The various biological effects of green tea have been reported (Cabrera, Artacho, & Giménez, 2006). Among these, its antiviral activity has attracted interest toward green tea as a potential alternative medicine. In particular, green tea prevents influenza virus infection (Nakayama, Suzuki, Toda, Okubo, Hara, & Shimamura, 1993).

Recently, COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has spread rapidly globally and remains a threat to human health and life. The occurrence of previous coronavirus outbreaks, such as SARS-CoV-1 and the Middle East respiratory syndrome coronavirus (MERS), suggests that the emergence of another coronavirus in the coming decades is inevitable, indicating that we must continually prepare for future outbreaks.

Food is a critical source of functional chemicals for humans. Tea reportedly prevents coronavirus infection (Joseph, Karthika, Das, & Raj, 2021; J. Liu et al., 2021; Ngwe Tun et al., 2022), and this effect can be explained by the presence of its functional compounds, known as catechins. Tea catechins inhibit the binding of the angiotensin-converting enzyme 2 (ACE2) receptor to the viral spike protein (Ohgitani et al., 2021a, 2021b), which is a critical point in the initial steps of the infection process. Another target molecule for the prevention of virus replication is the viral main protease M^{pro} (3C-like protease) (Hu et al., 2022). This enzyme is critical for the viral replication step of cleaving polypeptides originating from the viral gene. Viral M^{pro} is a thiol enzyme whose active site is well-preserved (Hu et al., 2022). M^{pro} is, therefore, a promising target for antiviral medicine (Owen et al., 2021; Sasaki et al., 2023).

Previous studies have reported the inhibition of recombinant M^{pro} by food- or plant-derived chemicals, such as myricetin (Su et al., 2021; Xiao et al., 2021), quercetin and its relatives (Rizzuti et al., 2021), sulforaphane (Chen et al., 2023), and ellagic acid (Bahun et al., 2022). Previously, we found that several food chemicals have an inhibitory effect on this enzyme (Kato, Higashiyama, Takaoka, Nishikawa, & Ikushiro, 2021). In this previous study, we found that, among the substances examined, epigallocatechin gallate (EGCG), a tea catechin, had a higher inhibitory effect on M^{pro}; however, other

73 catechins have not yet been examined in detail. Another previous study reported that
74 compounds present in green tea beverages and pure tea compounds (e.g., EGCG and
75 gallocatechin gallate) inhibited viral M^{pro} (Ngwe Tun et al., 2022). In addition, catechins,
76 such as gallocatechin gallate (GCG), exhibit an inhibitory effect on intracellularly
77 expressed M^{pro} and pure recombinant M^{pro} (S.-Y. Liu, Wang, Ke, Zhang, Chu, & Bao,
78 2022). However, no direct evidence of the inhibitory effect of tea catechins on M^{pro} has
79 been reported.
80 In this research, therefore, we examined the inhibitory effect of tea catechins,
81 including those found in bottled green tea and sencha (dipped green tea), on viral M^{pro} *in*
82 *vitro*. The covalent adduction of EGCG to intracellularly expressed SARS-CoV-2 M^{pro}
83 was also investigated.
84
85

2. Materials and Methods

2-1. Materials

Maltose-tagged main protease (MBP-M^{pro}) and MBP-cleaved protease (M^{pro}) were prepared as described previously (Kato et al., 2021). Dabcyl-KTSAVLQSGFRKME-Edans was purchased from GL Biochem, Ltd. (Shanghai, China). Epigallocatechin 3-(3″-*O*-methyl)gallate (EGCG3″Me), gallocatechin (GC), GCG, and caffeine were purchased from Fujifilm-Wako Pure Chemical Co. (Osaka, Japan). EGCG, epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and catechin (C) were purchased from Kurita Water Industries Ltd. (Tokyo, Japan). Chymotrypsin (sequencing grade) and ProteaseMAX™ were purchased from Promega (Madison, WI, USA). Amylose resin and Factor Xa were obtained from New England Biolabs Japan Inc. (Tokyo, Japan). Rabbit polyclonal antibodies against M^{pro} (SARS-CoV-2 3CL^{pro}) (GTX135470) were purchased from GeneTex Inc. (CA, USA). The plasmid pLEX307-SARS-CoV-2-3CL WT (#160278) was obtained from Addgene (Resnick et al., 2021). All other chemicals used in this study were of high-quality grade, unless otherwise indicated.

2-2. Sample preparation

Ten bottled teas (eight green and two barley-based teas) were obtained from a retail store (Supplementary Table 1) and were used as bottled tea samples. Commercially available green tea bags (Chiran, Minamikyushu City, Japan) and sencha (green tea leaves) (Shizuoka Prefecture, Japan) were obtained from a retail store. To obtain green tea extracts, boiled water was poured into a tube with a tea bag (green tea), and the tube was gently shaken. For sencha (tea leaves), a small teapot (kyu-su) was used. Tea samples were subsequently aliquoted at 0, 0.5, 1, 2, 3, 4, and 5 min for analysis.

2-3. Measurement of enzyme activity

Enzymatic reactions were conducted as previously described (Kato et al., 2021). In brief, MBP-M^{pro} was incubated with the tested chemical or with diluted tea in 20 mM Tris-HCl buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, and 0.01% Triton X-100 (pH 7.5). Tea catechins (10 mM) were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO (vehicle) was 2% in the reaction mixture. Water was used as the solvent blank for the water-diluted bottled tea (typically

a 50- or 100-fold dilution). MBP-M^{pro} (0.5 μM) was incubated with the test samples (teas) or vehicle at 37°C for 30 min. Then, 10 μM of Dabcyl-KTSAVLQSGFRKME-Edans was added to the solution, which was further incubated for 1 h. The reaction was terminated by adding an equal volume of CH_3CN containing 0.1% formic acid, and 10 μL of the mixture was then injected into a Shimadzu Prominence HPLC liquid chromatograph connected to an RF-10XL fluorescence detector (Shimadzu Co.) using an excitation wavelength of 336 nm and emission wavelength of 490 nm. Separation of the cleaved peptide was performed using a Phenomenex Kinetex XB-C18 column (4.6 × 50 mm, 2.6 μm) with gradient elution using 0.1% trifluoroacetic acid in water (solvent A) and CH_3CN (solvent B) (Kato et al., 2021). The analysis was performed in triplicate. The IC_{50} value was calculated using Prism v.9.2 software (GraphPad Software, LLC., CA, USA).

2-4. Quinone staining

After incubating M^{pro} (0.5 μM) with the test chemical (5 μM) or 50-fold diluted tea, ×4 Laemmli sample loading buffer (Bio-Rad, CA, USA) containing 10 mM dithiothreitol was added to the reaction mixture. The samples (60 ng of protein/lane) were then applied to a sodium dodecyl sulfate-polyacrylamide gel and migrated by electrophoresis (SDS-PAGE; 10% acrylamide). The proteins in the gel were blotted onto a PVDF membrane (Immobilon-P, Merck), and quinones on the membrane were stained with nitroblue tetrazolium (NBT) in 2 M glycine–NaOH (pH 10) as previously described (Paz, Flückiger, Boak, Kagan, & Gallop, 1991). All the experiments were conducted in duplicate.

2-5. Intact mass spectrometry

The M^{pro} enzyme (0.5 μM) was incubated with catechins (0–5 μM, typically 1 μM) or diluted tea (final 1,250-fold dilution) in the Tris-HCl buffer, as shown above, for 30 min at 37°C. After incubation, to avoid adsorption of the protein onto the tube surface, 1/10 volume of CH_3CN was added. The protein (10 μL) was then separated using a Phenomenex bioZen Intact XB-C8 column (2.1 × 100 mm, 3.6 μm), as described previously (Kato et al., 2021). The experiments were performed in duplicate. The deconvolution of the mass spectrum was acquired by UniDec (v.4.4.1) (Marty, Baldwin, Marklund, Hochberg, Benesch, & Robinson, 2015).

2-6. Chymotrypsin digestion

M^{pro} (5 μM) was incubated with EGCG3″Me (10 μM) in Tris-HCl buffer at 37°C for 30 min. Chymotrypsin digestion was performed as previously described (Kato et al., 2021). MS-Digest (https://prospector.ucsf.edu/prospector/mshome.htm) and Sciex OS with a "Bio Tool Kit" plug-in module were used to calculate the theoretical mass numbers of the modified peptides.

2-7. Determination of tea catechins and caffeine by UHPLC

Tea samples were filtered and then diluted with water five times. Subsequently, 2.5 μL of the sample was injected into UHPLC-PDA system (UltiMate3000, Thermo Fisher Scientific), comprising a Luna Omega C18 column (2 × 100 mm, 1.6 μm) with a gradient elution system at a flow rate of 0.4 mL/min. Solvent A was 0.1% formic acid in H_2O, and solvent B was CH_3CN. The linear gradient program was as follows: initial B5%, 14 min B25%, 15 min B5%, and 20 min B5%. The absorbance was monitored at 254 nm. The concentrations of catechins and caffeine in the samples were evaluated by comparison with standard curves of pure catechin and caffeine.

2-8. Transfection of the main protease gene into HEK293 cells

The plasmid (pLEX307) containing the sequence for SARS-CoV-2 M^{pro} was transfected into HEK293 cells cultured on a poly L-lysine-coated dish (10-cm or 6-well dish) using Lipofectamine® 3000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions and previously described methods (Kato et al., 2023; Resnick et al., 2021). In some cases, PEI-MAX (Polysciences Inc., Warrington, PA, USA) was used as the transfection reagent.

2-9. RNA isolation and RT-PCR

Total RNA was isolated from the transfected HEK293 cells using IsoSpin Cell II & Tissue RNA (Nippon Gene) according to the manufacturer's instructions. Single-stranded cDNA was synthesized using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO). PCR was performed with specific primer sets and Quick Taq® HS DyeMix (TOYOBO) according to the manufacturer's instructions. The forward and reverse primer sequences for M^{pro} were 5′-TGACAGGCAAACAGCACAAG-3′ and 5′-CGGCAATTCCAGTTTGAGCAG-3′, respectively. GAPDH was used as the control,

and the forward and reverse primers were 5′-CTGCACCACCAACTGCTTAGC-3′ and 5′-GCCTGCTTCACCACCTTCTTG-3′, respectively. The amplified products were resolved by electrophoresis on 2% agarose gel and stained with GelRed™ (Biotium).

2-10. EGCG treatment on cultured cells

After transfection, the dish was washed with serum-free Dulbecco's modified Eagle's medium (DMEM), and then EGCG (final concentration of 50 μM in DMEM) was added. After a 60-min incubation at 37°C, the well was washed with ice-cold phosphate-buffered saline (PBS) once and then lysed using 100 mM Tris-HCl (pH 8.6) containing 0.1% Triton X-100, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Protease Inhibitor Cocktail for Use with Mammalian Cell and Tissue Extracts, Nacalai Tesque). The lysate was collected and centrifuged (14,000g, 10 min, 4°C), and the supernatant was concentrated using an Amicon Ultra 10 K ultrafiltration device.

2-11. Isolation of EGCG-modified cellular proteins

The concentrated lysate was mixed with *m*-aminophenyl boronic acid-agarose beads (PBA beads; Sigma A8312) in PBS and incubated at 4°C in a rotator overnight (Tanaka et al., 2011). After centrifugation, the beads were washed three times with 0.1 M phosphate buffer (pH 7.4). Then, aqueous 50 mM glycine-HCl (pH 2.0) containing 1% Triton X-100 was added to elute the EGCG-conjugated proteins. The supernatant was collected, neutralized by adding 1 M Tris, and mixed with 4× loading buffer for SDS-PAGE.

2-12. Gel experiments and blotting

Two gels were prepared, and SDS-PAGE was performed as described above. One gel was stained using Flamingo™ Stain (Bio-Rad) according to the manufacturer's protocol; the other gel was blotted and blocked, and the membrane was then incubated with polyclonal antibodies against M^{pro} (1/4,000–1/10,000) and polyclonal goat anti-rabbit immunoglobulin peroxidase (DAKO). The binding was visualized using Chemi-Lumi One Super chemiluminescent substrate (Nacalai Tesque).

3. Results

3-1. Effect of tea catechins on recombinant viral main protease and successive quinone formation by incubation

First, the inhibitory effects of the eight catechins and caffeine on M^{pro} were investigated. EGCG, GCG, EGC, and GC strongly inhibited M^{pro} (IC_{50} = 1–3 μM; Fig. 1). In contrast, ECG, EC, and C showed lower or no inhibitory activity compared to the other five catechins. In addition, caffeine, which is abundant in green tea, did not affect enzyme activity. M^{pro} enzymes incubated with catechins were subjected to gel electrophoresis and blotted onto PVDF membranes, followed by quinone staining using NBT in alkaline glycinate buffer. As expected, positive staining was observed in the M^{pro} incubated with five catechins, namely, EGCG3″Me, EGCG, GCG, EGC, and GC (Fig. 2). The intensities of the bands correlated with the IC_{50} values (Fig. 1).

3-2. Mass spectrometric analyses of adduction of tea catechins on recombinant viral main protease and its target sequences

Intact MS analysis revealed the binding of one molecule of EGCG3″Me (Δ470), EGCG (Δ456), GCG (Δ456), or EGC (Δ304) to M^{pro} (Fig. 3). The respective chromatograms showed that treatment with ECG, EC, or C did not generate adducted molecules. The MS data showed a similar trend to that obtained for quinone staining after catechin treatment (Fig. 2).

A previous study demonstrated the covalent adduction of EGCG, possibly via the B ring, onto the cysteine residue in the enzyme active site (Kato et al., 2021), because Δ170 liberations (galloyl moiety) were observed. In the current study, when EGCG3″Me was incubated with the enzyme followed by chymotrypsin digestion, six cleaved EGCG3″Me-adducted peptides (X = EGCG3″Me-adducted Cys, DVVRQXSGVTF, NGSXGSVGF, LNGSXGSVGF, SVLAXY, DMXASL, and NIDYDXVSF) were found (Fig. 4A). Among them, the LNGSXGSVGF and NGSXGSVGF signals showed significant intensity. Furthermore, product ion scan of the two peptides revealed adduction of catechin to the cysteine 145 residue and generation of 184 fragments, corresponding to the liberation of the D ring (methylated galloyl moiety) (Fig. 4B). This result suggests that the B ring of EGCG3″Me is conjugated to thiols.

3-3. Effect of bottled tea on recombinant viral main protease

The effects of commercially available bottled teas on M^{pro} activity were also investigated. Eight of the bottled green teas inhibited M^{pro} in a dose-dependent manner, whereas barley tea and blended tea did not inhibit M^{pro} (Supplementary Fig.1). The contents of catechins and caffeine in each bottled tea, estimated by HPLC-PDA (Supplementary Fig. 2), were analyzed (Supplementary Table 1), and the relationship between each concentration of catechin/caffeine and the inhibitory effect was examined. High concentrations of catechins showed inhibitory effects (Supplementary Fig. 3). The sum of the four catechins (EGCG, GCG, EGC, and GC) also correlated with enzyme inhibition, as did the caffeine content. However, caffeine did not inhibit M^{pro} activity (Fig. 1). This may be because green tea, which has higher levels of catechins, contains abundant caffeine. When M^{pro} incubated with tea was applied to the gel, the bottled green teas showed positive quinone staining (Fig. 5); this positive staining correlated with the inhibitory activity of green tea, suggesting that covalent modification of M^{pro} by catechins can cause inhibition.

The chromatograms from intact MS for bottled tea incubated with M^{pro} showed three adducted molecules (Supplementary Fig. 4). Based on the increase in mass, the adduction of EGCG/GCG and EGC/GC in bottled green tea (sample D) was confirmed using intact MS. The actual adduct molecules were not specified because the molecular weights of EGCG and GCG (or EGC and GC) were identical. However, from a content perspective (Supplementary Table 1), EGCG and GC were presumably adducted to M^{pro}.

3-4. Effect of tea extract from dipped tea leaves on recombinant viral enzyme

The effects of manually extracting tea components from the tea leaves by steeping in hot water were also examined. A kyu-su (small teapot) and tea bags were used for the extractions. As shown in Fig. 6, prolonged steeping in hot water increased the catechin concentration and the inhibitory effect on viral M^{pro}.

As described above, EGCG, EGCG3″Me, or other catechins in green tea covalently bound to M^{pro} (Fig. 3). To examine the covalent binding of EGCG to intracellularly expressed M^{pro}, we prepared M^{pro}-overexpressing cells by transfecting a plasmid with the SARS-CoV-2 M^{pro} gene (Resnick et al., 2021). The expression of M^{pro} in the transfected cells was confirmed by RT-PCR and immunoblotting using a polyclonal antibody against M^{pro} (Supplementary Fig. 5).

3-5. Intracellular adduction of EGCG on viral main protease expressed in cultured cells

The cells were incubated with EGCG (50 μM) for 60 min. Next, the EGCG-conjugated proteins were isolated using PBA beads, which bind to catechol moieties (Tanaka et al., 2011). As shown in Fig. 7, protein staining revealed bands in the lane of the EGCG-treated sample, suggesting that EGCG covalently binds to cellular proteins. Among the EGCG–protein complexes, M^{pro} was identified at approximately 30 kDa using an antibody specific to M^{pro}. This result indicated that extracellular EGCG was transferred to biological membranes and bound to intracellular proteins, including the viral M^{pro} expressed in the cell.

4. Discussion

EGCG, EGCG3″Me, and other catechins appear to have bio-functionalities (Kurita, Maeda-Yamamoto, Tachibana, & Kamei, 2010; Umeda, Yano, Yamada, & Tachibana, 2008), including antiviral effects (Song, Lee, & Seong, 2005). In this study, we examined the inhibitory effect of tea catechins on viral M^{pro}, a key element for viral replication. Among the examined compounds, EGCG, EGCG3″Me, GC, GCG, and EGC showed considerable inhibitory effects on viral recombinant M^{pro} at concentrations of just a few micromoles. Quinone staining of the catechin-exposed M^{pro} and intact MS analysis revealed that the reaction was accompanied by the chemical adduction of catechin molecules to the enzyme. Indeed, two EGCG3″Me-adducted peptides [(L)NGSC_{145}GSVG] containing the active site sequence of M^{pro} were predominantly noted, along with other EGCG3″Me-modified peptides, such as SVLAC_{117}Y, NIDYDC_{156}VSF, DMC_{265}ASL, and DVVRQC_{308}SGVTF. In addition, our previous investigation indicated that EGCG, myricetin, and theaflavin have an inhibitory effect on M^{pro} via covalent modification; in the case of EGCG and myricetin, adduction at the cysteine 145 residue of the active site of M^{pro} has been confirmed previously (Kato et al., 2021). In this study, we found that EGCG3″Me has a similar IC_{50} as that of EGCG against viral M^{pro}. Moreover, the adduction occurs at the B ring in EGCG3″Me, as noted by analysis of the mass fragmentation pattern of the methylated galloyl moiety (Fig. 4). These results, along with the results reported in our previous study (Kato et al., 2021), indicate that, from a structural point of view, the B rings in EGCG, EGCG3″Me, GCG, and ECG may contribute to the inhibition of M^{pro} via adduction to the critical thiol.

Tea, including green tea, is often consumed as commercially available bottled tea. Among the bottled teas examined, green tea showed an inhibitory effect at various concentrations. We investigated the relationship between the catechin content and these inhibitory effects and found some correlations. Alternatively, tea is often prepared by steeping tea leaves in hot water. As expected, the longer extraction time of tea when prepared this way using either sencha or tea bags exhibited a higher inhibitory effect on M^{pro}. The amount of tea leaf-derived phytochemicals, such as catechins, can partially explain this inhibitory activity. Tea catechins or black tea theaflavin (and theaflavin-3-gallate) inhibit the M^{pro} of SARS-CoV-2 and reduce cell infection *in vitro* (Chauhan et al., 2022; Du et al., 2021; Ngwe Tun et al., 2022; Ohgitani et al., 2021a, 2021b). Black tea and other herbal teas could also have similar effects.

The M^{pro} enzyme is expressed in cells that are infected with the SARS-CoV-2 virus. Dietary tea catechins are metabolized into methylated or conjugated forms by phase I and II enzymes and are then circulated (Hayashi et al., 2022), indicating that catechins are incorporated into cells because these drug-metabolizing enzymes are expressed intracellularly. Confirmation of the intracellular reactions between tea catechins and M^{pro} remains a challenge (He et al., 2024; Kato et al., 2021). To examine the covalent binding of EGCG to intracellular M^{pro}, we prepared M^{pro} over-expression cells by transfection of a plasmid containing the SARS-CoV-2 M^{pro} gene. This model is safe for handling compared to the virus infection model and is, therefore, easy to apply without extra bio-secure laboratory equipment or experience. In addition, the M^{pro} transfection system is simple and excludes other factors that influence the infection result. To screen for possible inhibitors, cell-based Flip-GFP and cell-based luciferase-Glo assays for the viral M^{pro} enzyme can be applied (Gerber et al., 2022; Ma, Tan, Choza, Wang, & Wang, 2022; Rawson, Duchon, Nikolaitchik, Pathak, & Hu, 2021; Rothan & Teoh, 2021). In the current study, EGCG-conjugated proteins, including M^{pro}, were isolated using PBA beads after 50 μM EGCG was extracellularly supplemented to cultured cells expressing viral M^{pro}. The results indicated that EGCG penetrated the cell and reacted with intracellular proteins. More than ten proteins, including ATP-dependent RNA helicase DDX5 (p68), have previously been identified as EGCG-binding proteins (Tanaka et al., 2011). Furthermore, reduced glutathione cancels the inhibition of M^{pro} activity and adduction by EGCG (He et al., 2024; Kato et al., 2021). These findings indicate that the quinone form of EGCG is an essential transient moiety for covalent adduction. Extracellularly exposed, serotonin-derived quinone reacts with intracellular M^{pro} in cultured cells (Kato et al., 2023). EGCG and other active phytochemicals can become quinone moieties and react with the thiols of M^{pro} and other thiol proteins in cells.

EGCG (50 μM) was found to bind to cellular proteins, including viral M^{pro} (Fig. 7). However, ingested EGCG is found at approximately several nM to a few μM in plasma (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). The bioavailability of EGCG3″Me could be higher than that of EGCG (Oritani, Setoguchi, Ito, Maruki-Uchida, Ichiyanagi, & Ito, 2013), but still, it may not be enough to prevent viral replication in infected cells. It is worth mentioning that, unlike Western medicine, food contains various chemicals, and the sum of their functions is the key to food functionality. Therefore, if people drink green tea or consume fruits and vegetables, various potentially

active phytochemicals are incorporated simultaneously. Alternatively, the combination of EGCG or other phytochemicals within a nano-delivery system (Dai et al., 2020) could overcome this bioavailability issue.

5. Conclusions

The occurrence of the COVID-19 pandemic and previous coronavirus outbreaks suggests that the emergence of another coronavirus in the coming decades is highly likely. There are many ways to fight SARS-CoV-2 infection. Among these, viral M^{pro} is a promising target molecule. We hypothesized that some food-derived chemicals could have medicinal functions that inhibit M^{pro} through daily food intake and focused on the ability of tea catechins to combat viral replication. We confirmed the inhibitory effects of catechins on recombinant viral M^{pro} accompanied by covalent adduct formation. The active site C145 of the enzyme was predominantly adducted by the B ring (a pyrogallol moiety) of methyl-EGCG.

Previous studies have shown that catechins can prevent viral infection in virus-infected cell models, and some catechins can inhibit the pure recombinant M^{pro} enzyme. However, to the best of our knowledge, there is no direct evidence that extracellular EGCG enters cultured cells and binds to the viral M^{pro} enzyme. In this study, when cultured cells expressing the viral enzyme were exposed to EGCG, EGCG-modified M^{pro} was detected in the cell lysate. This suggests that even extracellularly supplemented EGCG can react with viral M^{pro} expressed in infected cells. Thus, catechins may act as inhibitors of M^{pro} *in vivo*. Although there are still some unsolved issues, such as low bioavailability and *in vivo* occurrence, our findings indicate that the catechins present in green teas may provide a possible defense against coronavirus infection and replication.

CRediT authorship contribution statement

Yoji Kato: Conceptualization, Methodology, Investigation, Writing-Original Draft & Editing, Supervision, Project Administration, Funding Acquisition. **Sakiko Suzuki, Akari Higashiyama**: Investigation. **Mitsugu Akagawa, Ichiro Kaneko, Miyu Nishikawa, Shinichi Ikushiro**: Investigation, Methodology, Writing-Review & Editing, Supervision.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

This work was partially supported by grants from the Iijima Foundation for the Promotion of Food Science and Technology.

Data availability

Data will be made available on request.

Acknowledgments

We thank Dr. Toshio Niwa (Shubun University) for the helpful advice on experimental design. The authors would like to thank Enago (www.enago.jp) for the English language review.

411 **Figure legends**

412

413 **Figure 1.** Dose-dependent inhibition of viral recombinant M^{pro} exposed to catechins.
414 Recombinant maltose-tagged main protease (MBP-M^{pro}) was incubated with various
415 concentrations of catechins or caffeine for 30 min. The enzyme activity was evaluated by
416 measuring the cleaved peptide fragment, as described in the Materials and Methods
417 section. IC_{50} values are shown in each panel.

418

419 **Figure 2.** Covalent adduction and quinone formation on viral M^{pro} following exposure to
420 catechins. M^{pro} (0.5 μM) was incubated with catechins or caffeine (5 μM). Two gels were
421 used to separate proteins by electrophoresis. One gel was used for protein staining and
422 the other was blotted onto a PVDF membrane for quinone staining. (Top panel) Quinone
423 staining of the membrane was performed as described in the Materials and Methods.
424 (Bottom panel) Protein staining of the gel was performed using Flamingo gel stain.
425 EGCG3″Me, epigallocatechin 3-(3″-*O*-methyl)gallate; EGCG, epigallocatechin gallate;
426 GCG, gallocatechin gallate; EGC, epigallocatechin; GC, gallocatechin; ECG, epicatechin
427 gallate; EC, epicatechin; C, catechin.

428

429 **Figure 3.** Covalent adduction of reactive catechins on the viral recombinant enzyme. M^{pro}
430 (0.5 μM) was incubated with tea catechins (1 μM) for 30 min and subjected to intact MS
431 analysis. Mass spectrum data obtained were deconvoluted using UniDec (v.4.4.1), as
432 described in Materials and Methods. The abbreviations used are mentioned in the legend
433 for Figure 2.

434

435 **Figure 4.** Chymotrypsin digestion of EGCG3″Me-modified M^{pro}. X indicates
436 EGCG3″Me-adducted Cys. (A) Extracted ion chromatograms of EGCG3″Me-modified
437 peptides. (B) MS/MS analysis of covalently EGCG3″Me-modified peptides at the active
438 site, NGSCGSVGF/LNGSCGSVGF sequence. NGSXGSVGF ($[M+H]^+$ 1,297.4) and
439 LNGSXGSVGF ($[M+H]^+$ 1,410.5) were located at the active site. Liberation of the
440 methyl galloyl moiety (Δ184), the D ring, was observed.

441

442 **Figure 5.** Quinone formation and enzyme inactivation by incubation with bottled tea. The
443 recombinant viral M^{pro} (0.5 μM) was incubated with 50-fold diluted bottled teas (A to J)

and subjected to SDS-PAGE, followed by blotting onto a PVDF membrane for quinone staining. One gel was used to stain recombinant M^{pro}. To estimate M^{pro} enzyme activity after exposure to tea, 135-fold diluted bottled tea was reacted with MBP-M^{pro} (0.5 μM) for 30 min. The enzyme activity was estimated as described in the legend for Figure 1.

Figure 6. Time-dependent loss of enzymatic activity after incubation with tea. Tea from a kyu-su (small teapot) was diluted by 1/600, and the tea bags were diluted by 1/700 with water and then reacted with MBP-M^{pro}. Tea catechins were quantified by UHPLC-PDA, as described in the Materials and Methods section. The abbreviations used are mentioned in the legend for Figure 2.

Figure 7. Covalent modification of intracellularly expressed viral M^{pro} by extracellularly supplemented EGCG. HEK293 cells were transfected into the plasmid with the viral M^{pro} sequence and then exposed to 50 μM EGCG for 60 min in culture medium. After incubation, cells were collected and lysed. The lysate was applied to PBA beads to isolate EGCG-conjugated proteins. Bound proteins were subjected to SDS-PAGE on two gels. One gel was stained for protein, and the other gel was used for blotting and stained with specific polyclonal antibodies against M^{pro} (1/4,000 dilution). Arrowhead indicates the theoretical position of M^{pro}.

Supplementary Materials

Supplementary Table 1. Concentrations of catechins and caffeine in ten bottled teas. EGCG3″Me, epigallocatechin 3-(3″-*O*-methyl)gallate; EGCG, epigallocatechin gallate; GCG, gallocatechin gallate; EGC, epigallocatechin; GC, gallocatechin; ECG, epicatechin gallate; EC, epicatechin; C, catechin. * Sum of four catechins (EGCG, GCG, EGC, and GC).

Supplementary Figure 1. Dose-dependent inhibition of MBP-M^{pro} in bottled tea, including green and barley tea. Letters (A–J) indicate the bottled teas described in the remarks in Supplementary Table 1. Samples A to H were green teas, tea "I" was a cocktail of several teas (except green tea), and tea "J" was barley tea.

Supplementary Figure 2. Chromatograms of tea catechins. (Top) Standard of eight catechins and caffeine. (Bottom) Typical chromatogram of bottled green tea.

Supplementary Figure 3. Relationship between catechin concentration and inhibition. Catechins and caffeine in ten bottled teas were analyzed. The correlation between % inhibition and the concentration of each chemical was plotted. "4-catechins sum" means the total concentration of EGCG, GCG, EGC, and GC, which showed a strong inhibitory effect on M^{pro} ($<IC_{50}$ = 3 μM). EGCG3″Me was excluded from the sum because EGCG3″Me was not found in the commercial bottled teas examined. The abbreviations used are listed in Supplementary Table 1.

Supplementary Figure 4. Adduction of tea catechins from bottled green tea to recombinant viral enzymes. M^{pro} (0.5 μM) was incubated with bottled tea (1,250-fold dilution) for 30 min and subjected to intact MS analysis. Mass spectrum data obtained were deconvoluted using UniDec (v.4.4.1), as described in Materials and Methods. The abbreviations used are listed in Supplementary Table 1.

Supplementary Figure 5. Validation of SARS-CoV-2 M^{pro} expression by HEK293 cell transfection. (A) Total mRNA was collected from the transfected cells, and the sequences of M^{pro} and GAPDH (control) were amplified and applied to an agarose gel along with

the DNA ladder marker (Gene ladder 100, Nippon gene). The agarose gel electrophoresed was stained with GelRed™. The theoretical length of the PCR product for the M^{pro} was 224 bp. (B) Cells were lysed 24 and 48 h after transfection, and the cellular lysate was collected. The lysate was separated on a gel and used for blotting. Immunostaining was performed using an antibody against M^{pro} (1/10,000 dilution). The MagicMark™ XP Western Protein Standard was used as the marker. Arrowhead indicates the theoretical position of the main protease. Protein staining using Flamingo™ Fluorescent Protein Gel Stain was also performed to determine the quantity of protein applied.

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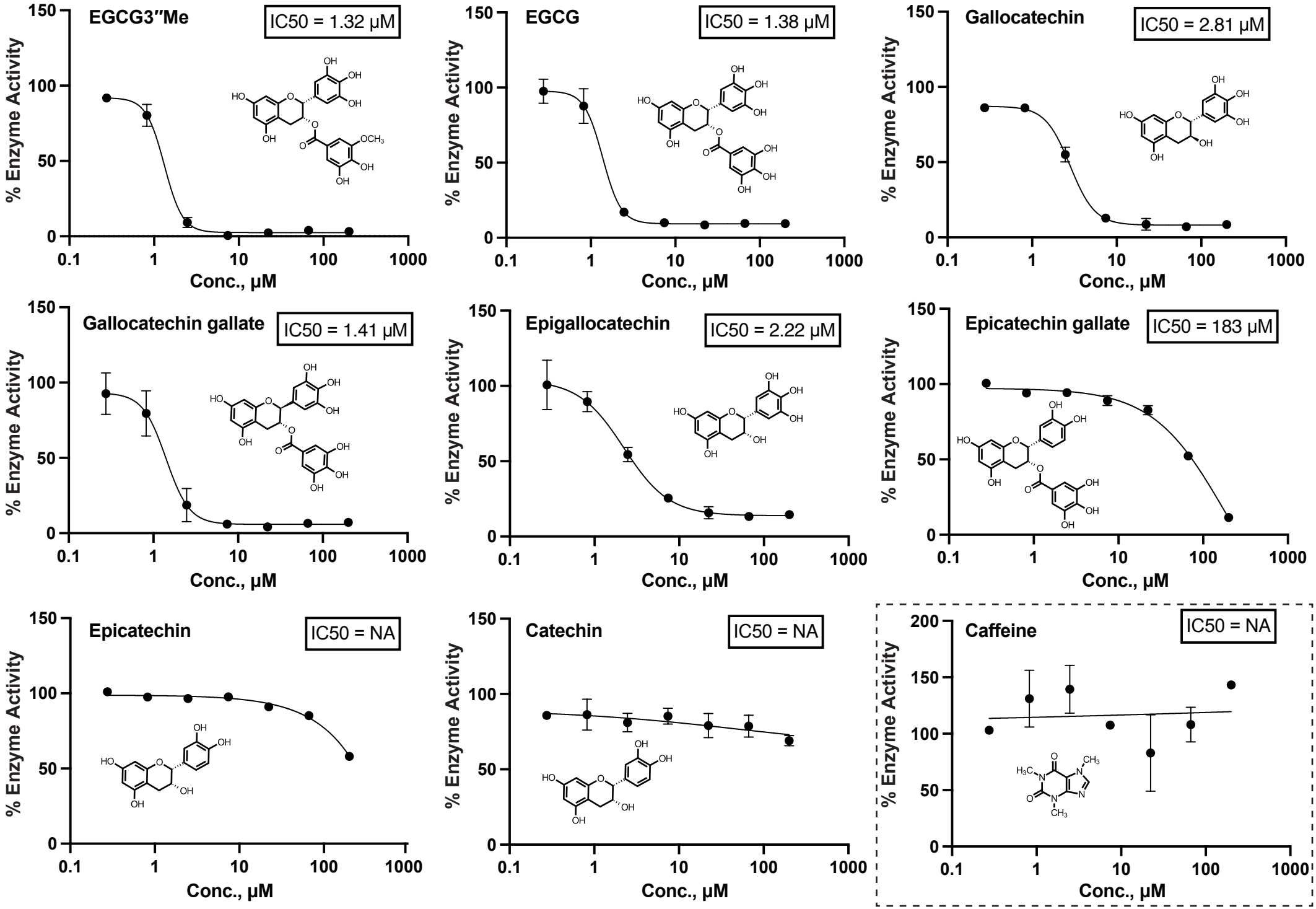

EGCG3″Me
IC50 = 1.32 μM
EGCG
IC50 = 1.38 μM
Gallocatechin
IC50 = 2.81 μM
Gallocatechin gallate
IC50 = 1.41 μM
Epigallocatechin
IC50 = 2.22 μM
Epicatechin gallate
IC50 = 183 μM
Epicatechin
IC50 = NA
Catechin
IC50 = NA
Caffeine
IC50 = NA
% Enzyme Activity
Conc., μM
0.1
1
10
100
1000
0
50
100
150
200

Figure 2

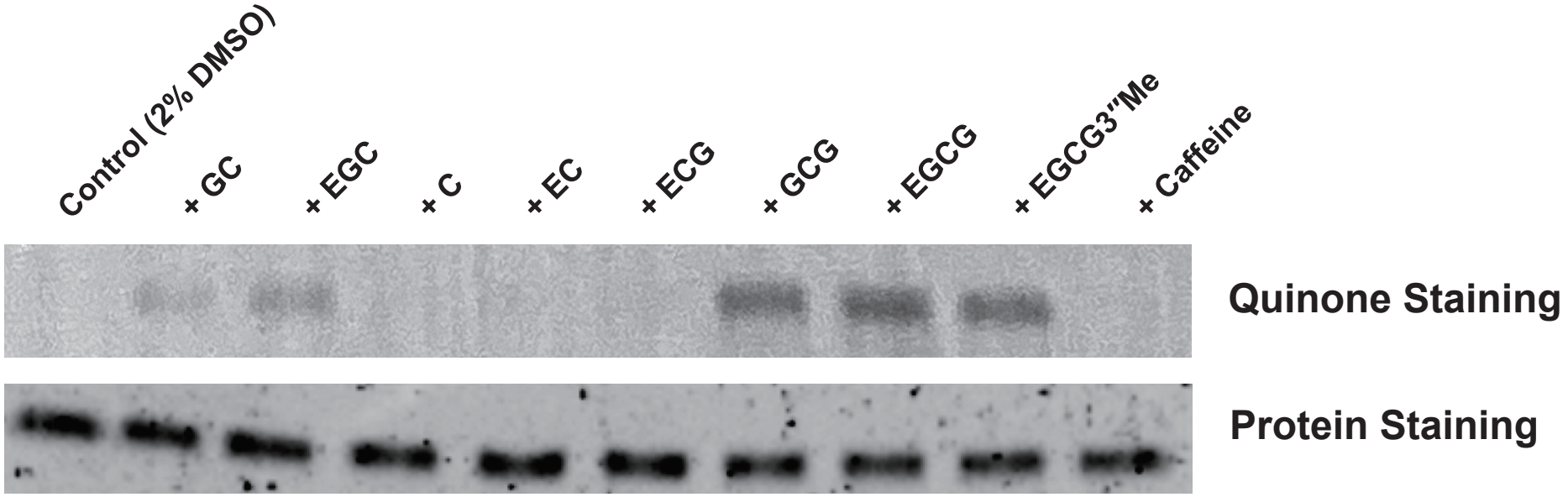

Control (2% DMSO)
+ GC
+ EGC
+ C
+ EC
+ ECG
+ GCG
+ EGCG
+ EGCG3''Me
+ Caffeine
Quinone Staining
Protein Staining

Figure 3

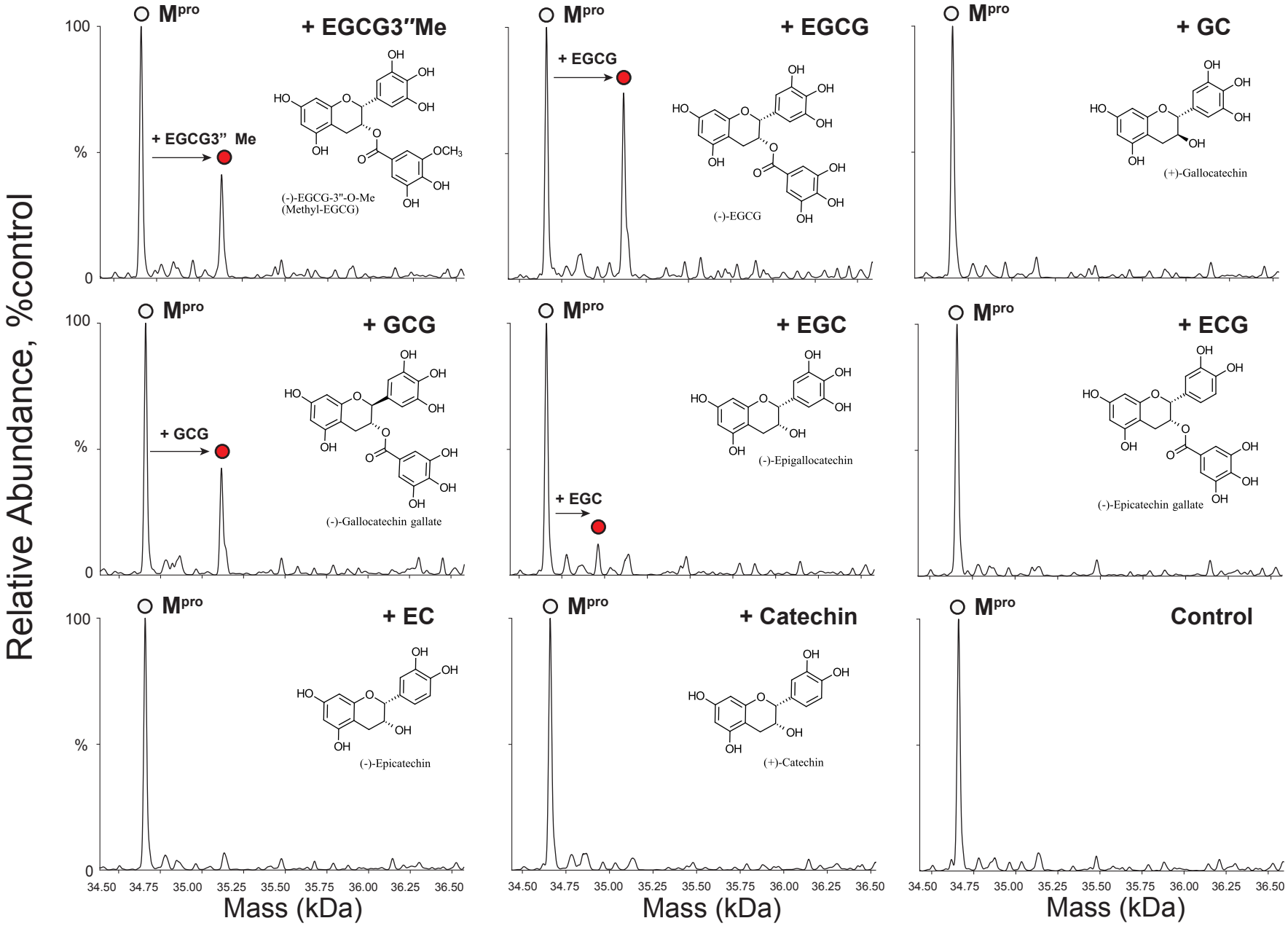
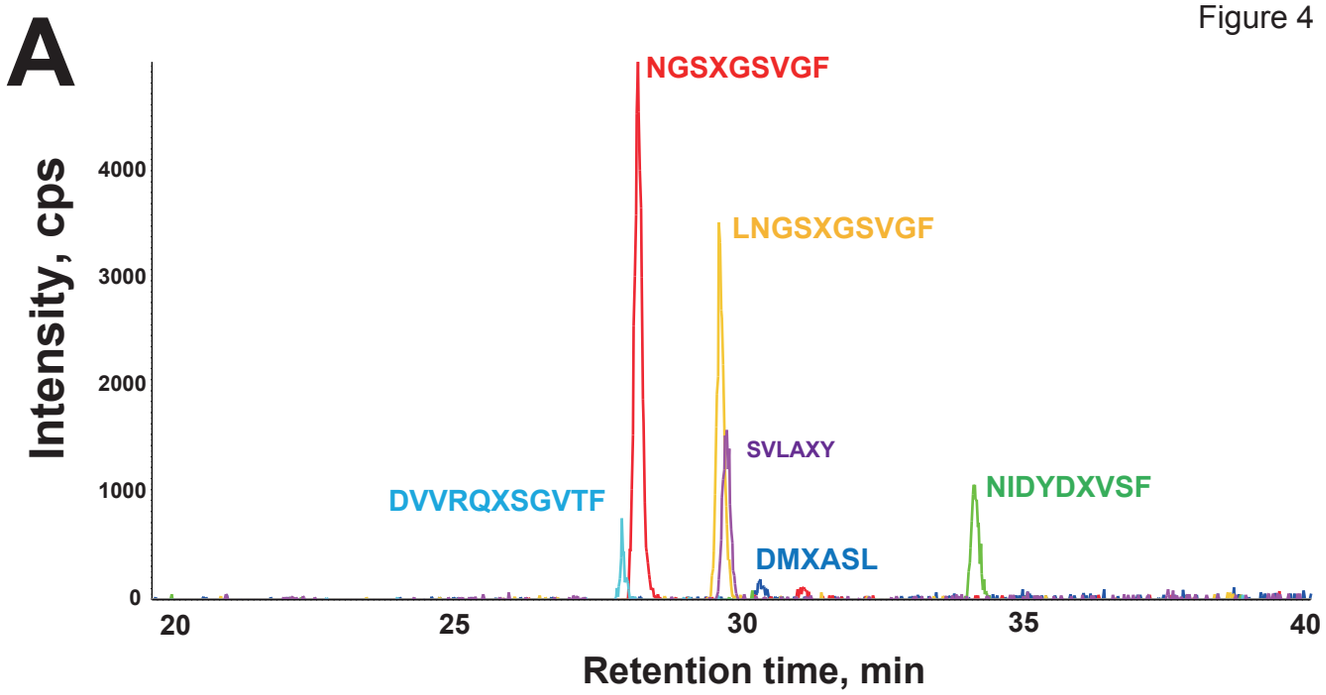
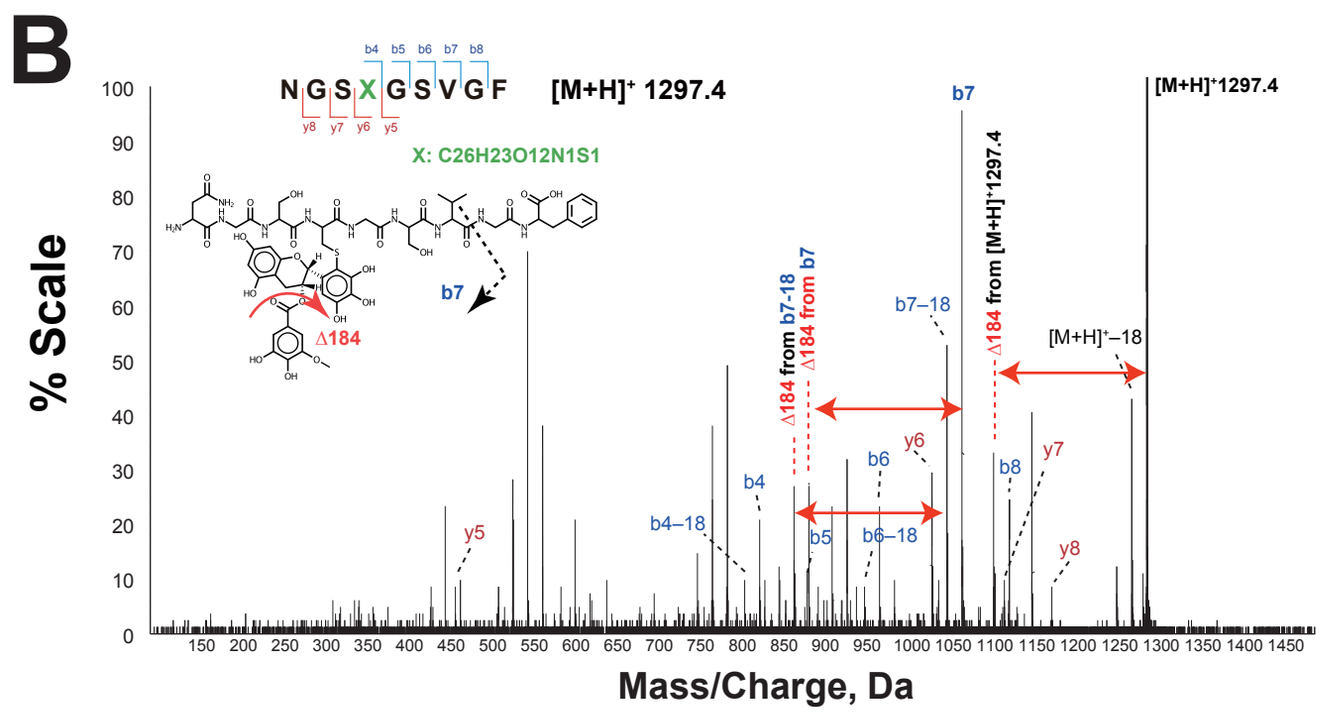

Relative Abundance, %control
Mpro
+ EGCG3″Me
+ EGCG3″ Me
(-)-EGCG-3"-O-Me
(Methyl-EGCG)
+ EGCG
(-)-EGCG
+ GC
(+)-Gallocatechin
+ GCG
(-)-Gallocatechin gallate
+ EGC
(-)-Epigallocatechin
+ ECG
(-)-Epicatechin gallate
+ EC
(-)-Epicatechin
+ Catechin
(+)-Catechin
Control
100
%
0
34.50 34.75 35.00 35.25 35.50 35.75 36.00 36.25 36.50
Mass (kDa)

Figure 4


A
Intensity, cps
4000
3000
2000
1000
0
NGSXGSVGF
LNGSXGSVGF
SVLAXY
NIDYDXVSF
DVVRQXSGVTF
DMXASL
20
25
30
35
40
Retention time, min


B
% Scale
b4 b5 b6 b7 b8
N G S X G S V G F
y8 y7 y6 y5
[M+H]+ 1297.4
X: C26H23O12N1S1
b7
Δ184
[M+H]+1297.4
b7–18
Δ184 from b7-18
Δ184 from b7
Δ184 from [M+H]+1297.4
[M+H]+–18
b4
b6
y6
b8
y7
b4–18
b5
b6–18
y5
y8
100
90
80
70
60
50
40
30
20
10
0
150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000 1050 1100 1150 1200 1250 1300 1350 1400 1450
Mass/Charge, Da

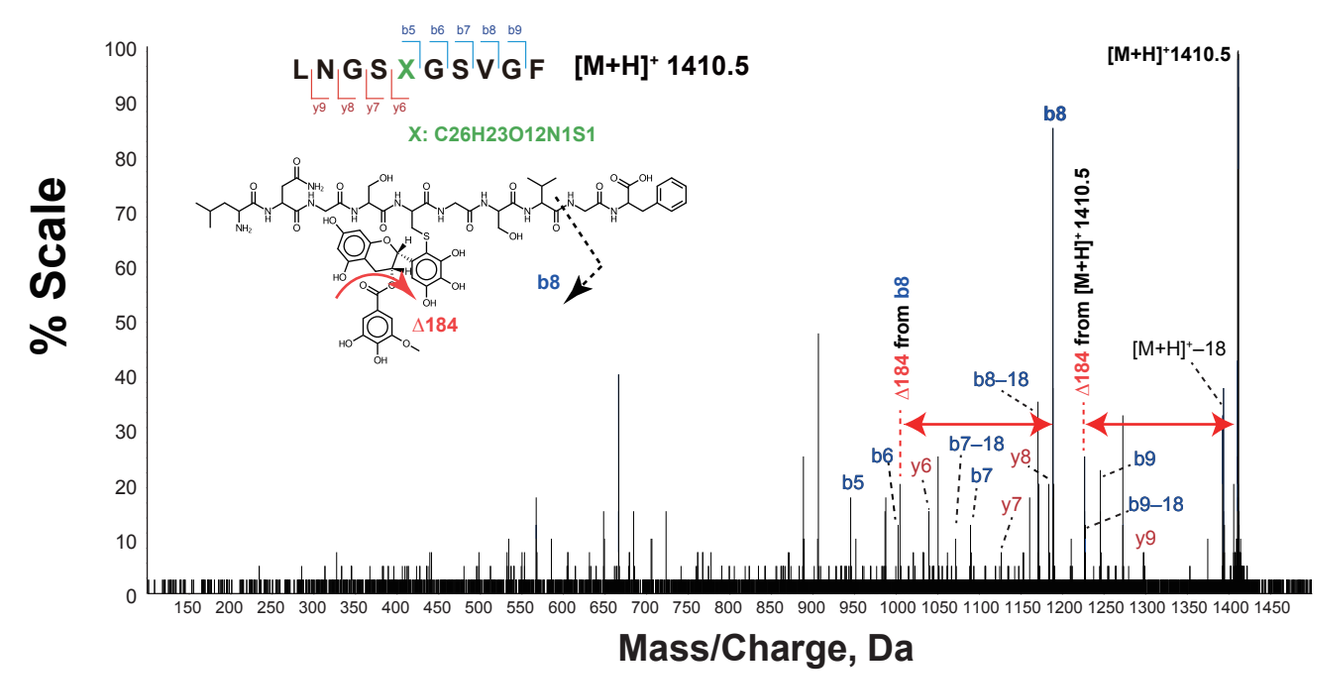
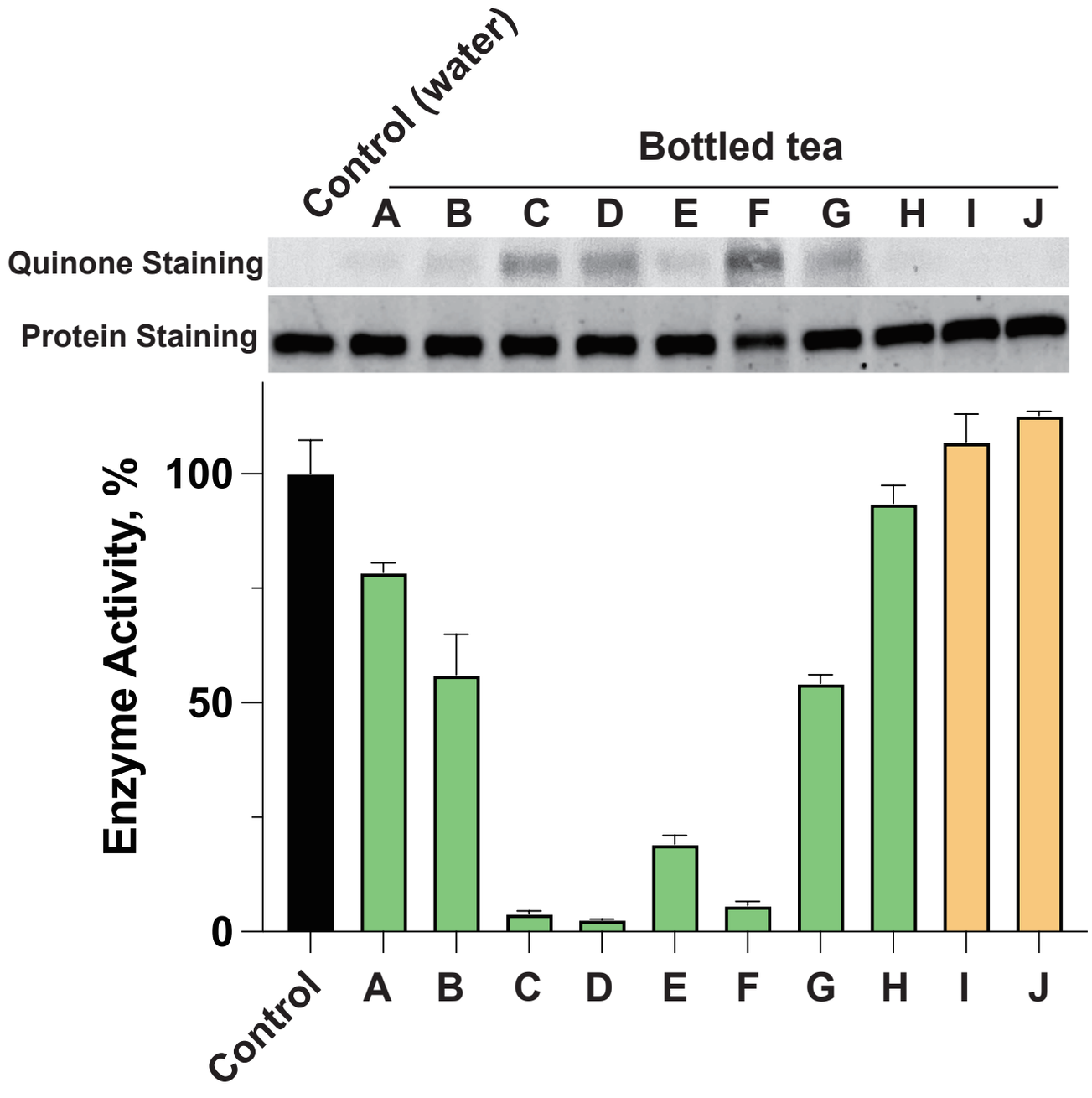

% Scale
b5 b6 b7 b8 b9
L N G S X G S V G F
y9 y8 y7 y6
[M+H]+ 1410.5
X: C26H23O12N1S1
b8
Δ184
[M+H]+1410.5
Δ184 from b8
Δ184 from [M+H]+1410.5
[M+H]+–18
b8–18
b7–18
b6
y6
b7
y8
b5
b9
y7
b9–18
y9
100
90
80
70
60
50
40
30
20
10
0
150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000 1050 1100 1150 1200 1250 1300 1350 1400 1450
Mass/Charge, Da

Figure 5


Control (water)
Bottled tea
A B C D E F G H I J
Quinone Staining
Protein Staining
Enzyme Activity, %
100
50
0
Control
A B C D E F G H I J

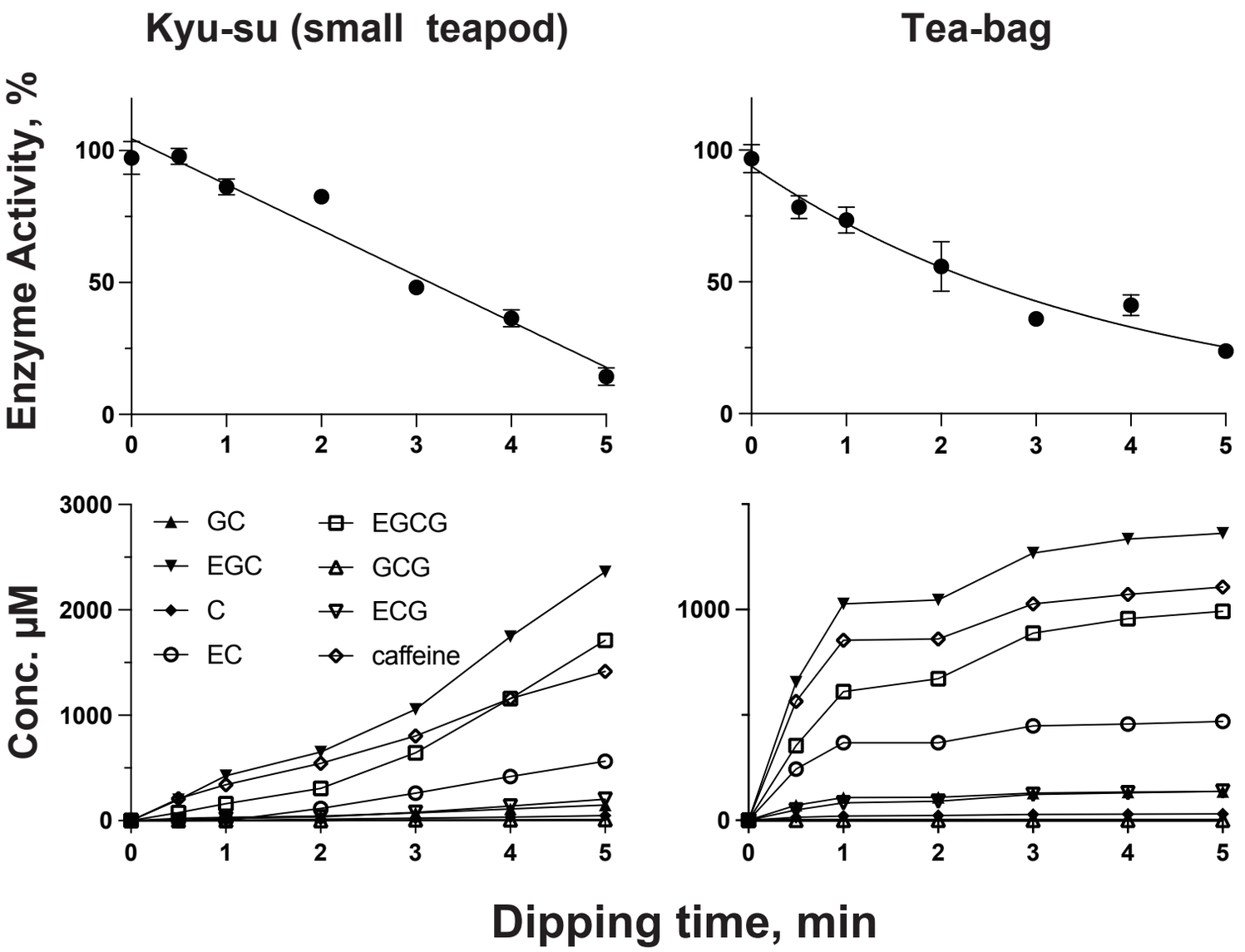
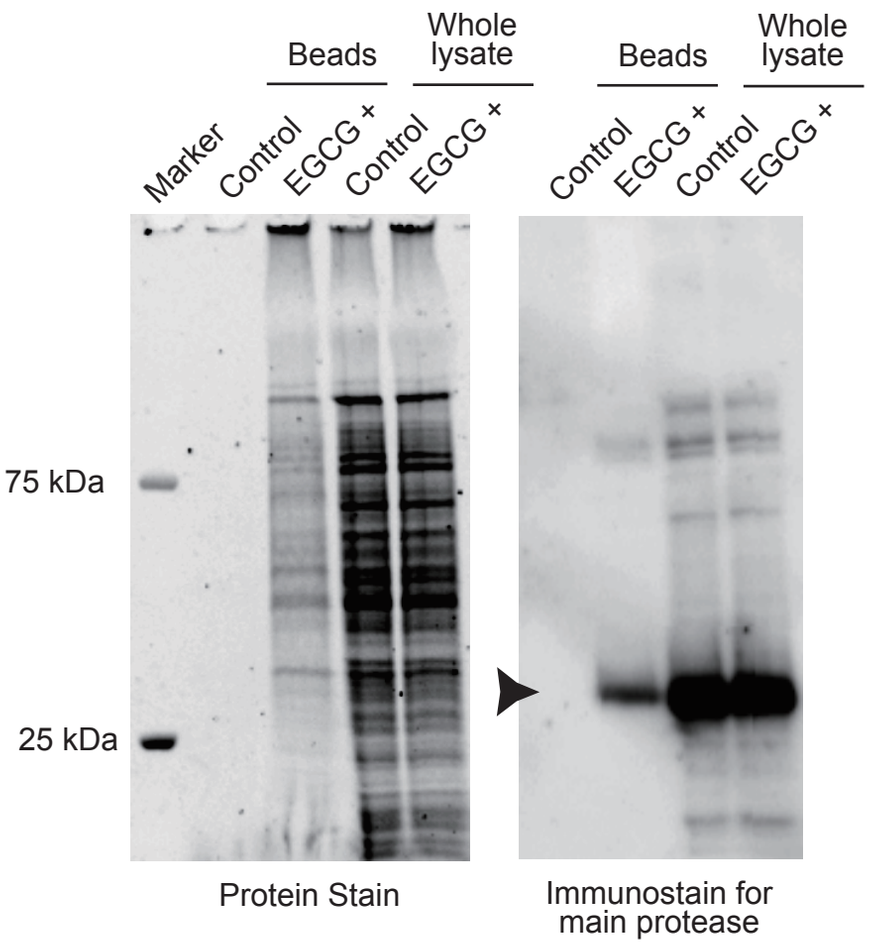

Kyu-su (small teapod)
Tea-bag
Enzyme Activity, %
100
50
0
0
1
2
3
4
5
Conc. μM
3000
2000
1000
0
GC
EGC
C
EC
EGCG
GCG
ECG
caffeine
Dipping time, min

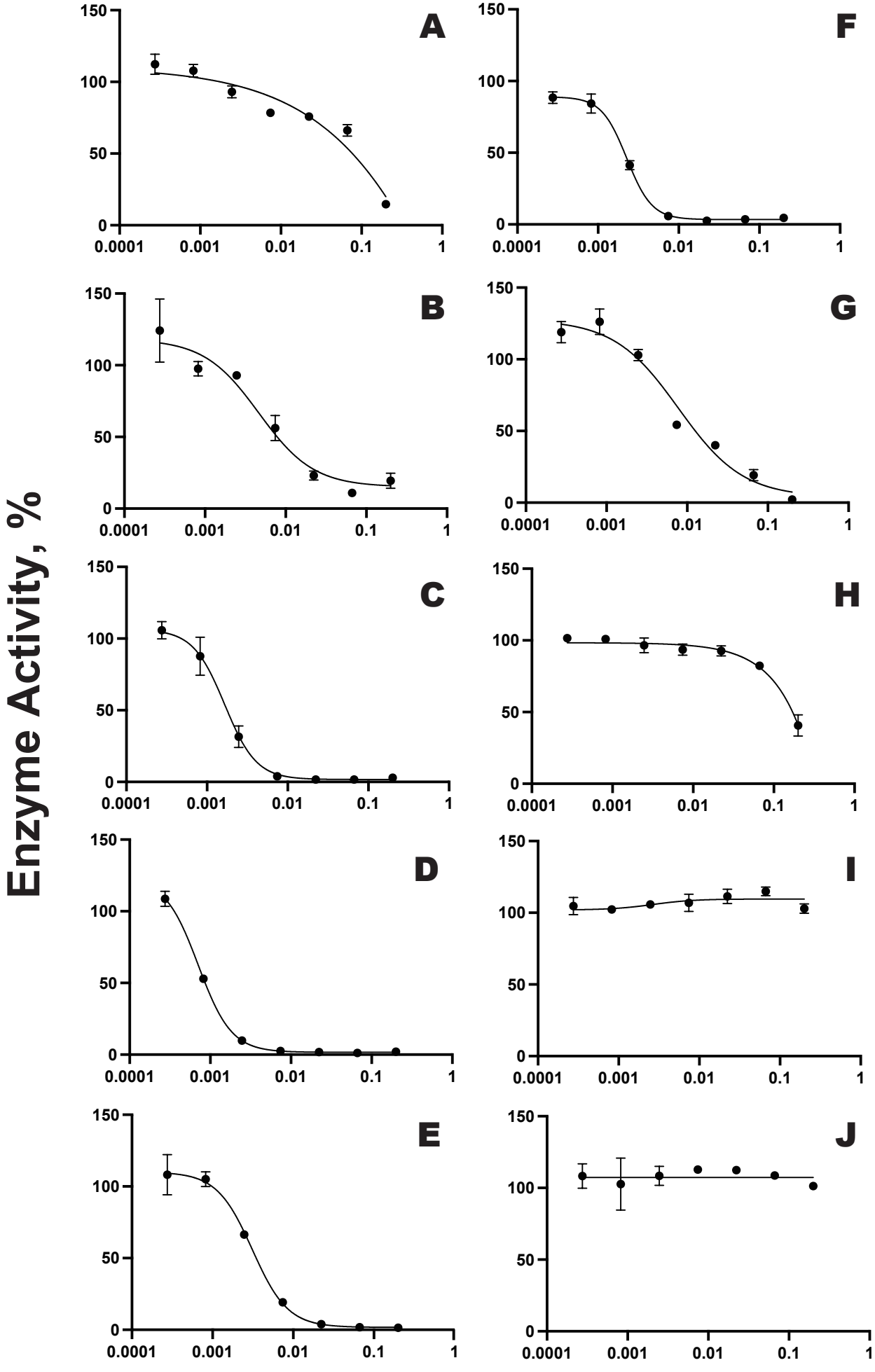
Figure 7


Beads
Whole lysate
Beads
Whole lysate
Marker
Control
EGCG +
Control
EGCG +
Control
EGCG +
Control
EGCG +
75 kDa
25 kDa
Protein Stain
Immunostain for main protease

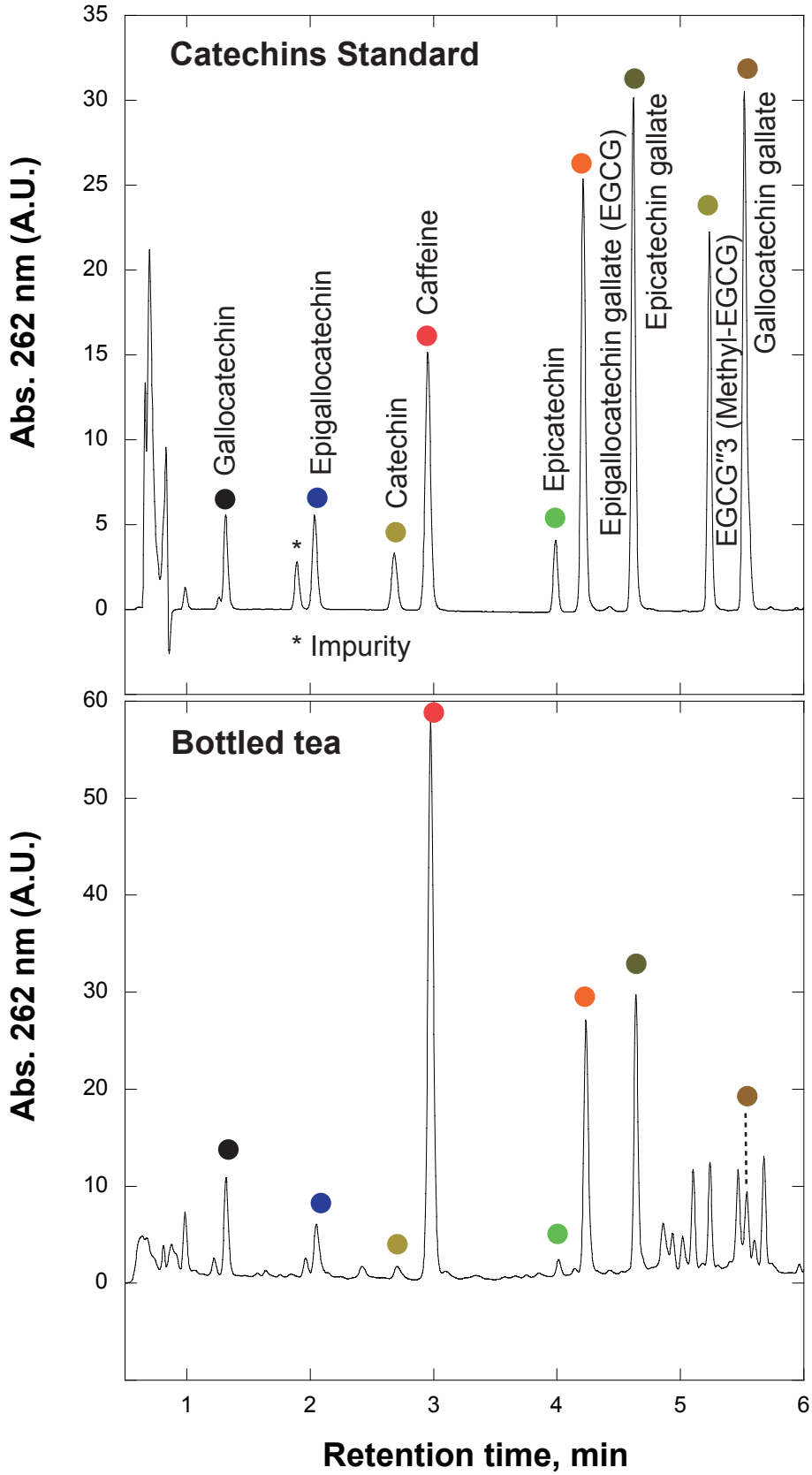
Supplementary Table 1

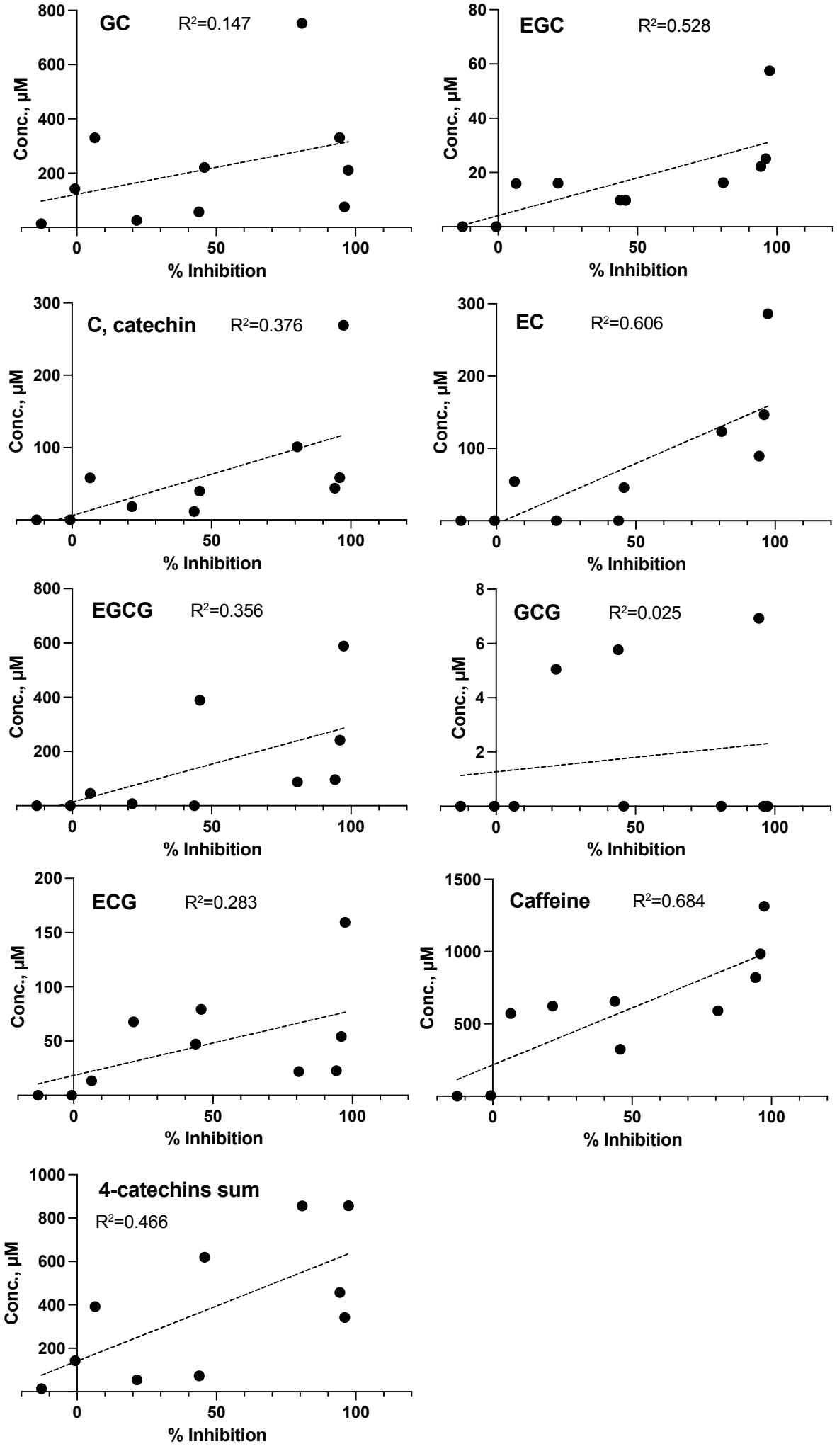
Concentrations of catechins and caffeine in ten bottled teas

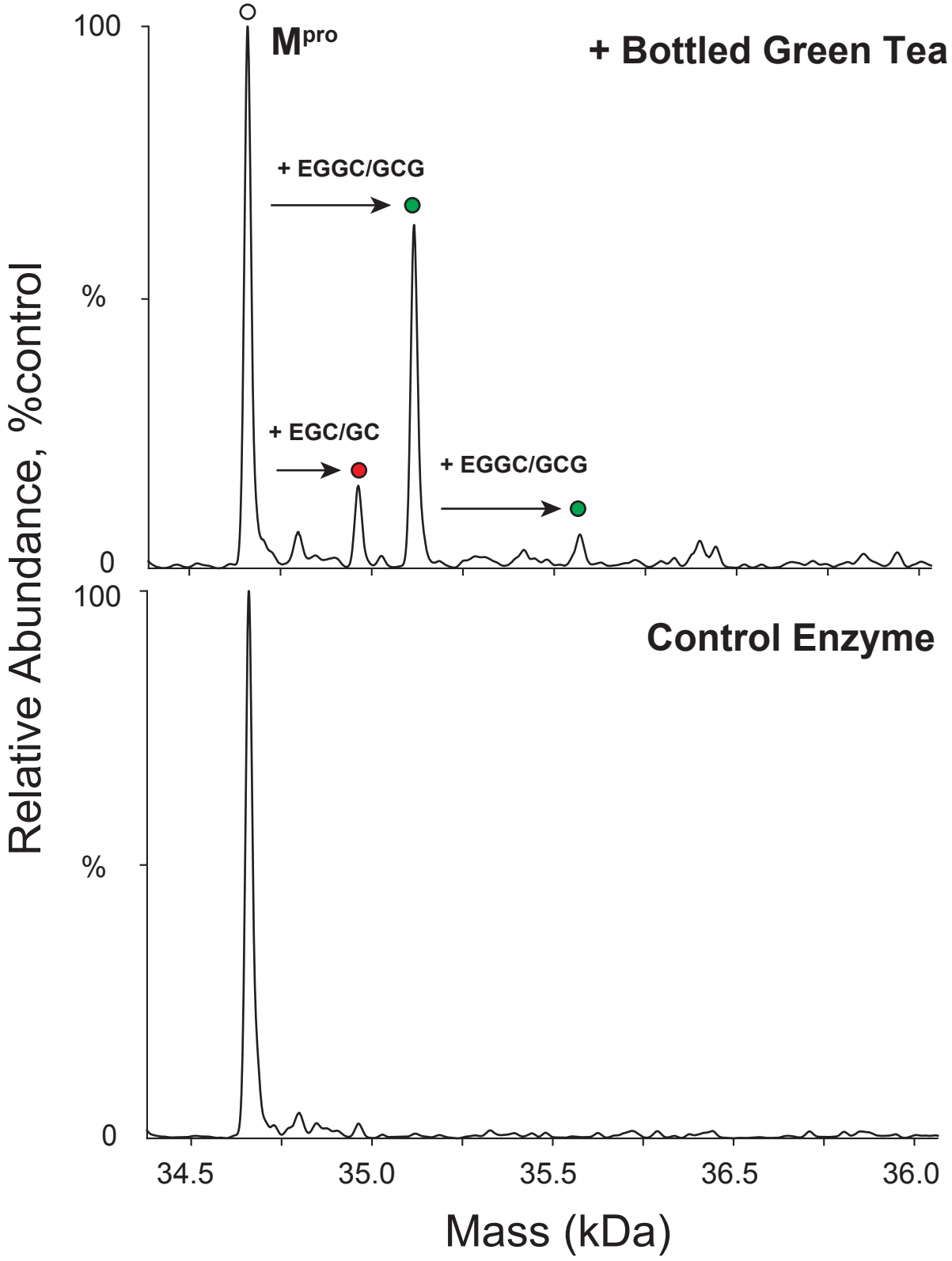
Bottled tea	GC (μM)	EGC (μM)	C (μM)	Caffeine (μM)	EC (μM)	EGCG (μM)	GCG (μM)	ECG (μM)	4 catechins* (μM)	Remarks
A	25.7	16.0	18.5	623.5	0.0	7.7	5.1	67.7	54.6	Green tea
B	57.2	9.8	11.6	655.3	0.0	0.0	5.8	47.3	72.8	Green tea
C	75.6	25.1	58.4	984.4	146.6	241.3	0.0	54.3	342.0	Green tea, with Health Claim
D	210.9	57.5	269.1	1312.7	286.2	588.9	0.0	159.4	857.2	Green tea, with Health Claim
E	752.4	16.3	101.0	591.1	123.3	87.9	0.0	21.9	856.5	Green tea, Low temp. extraction
F	331.3	22.2	43.8	820.5	89.3	96.7	6.9	22.7	457.1	Green tea, with Health Claim
G	220.8	9.7	39.8	324.5	46.0	388.9	0.0	79.3	619.4	Green tea, with Health Claim
H	330.3	15.9	58.1	572.2	54.4	45.9	0.0	13.4	392.1	Green tea, with Maccha
I	142.7	0.0	0.0	4.6	0.0	0.0	0.0	0.0	142.7	Blend tea
J	13.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	13.6	Barley tea

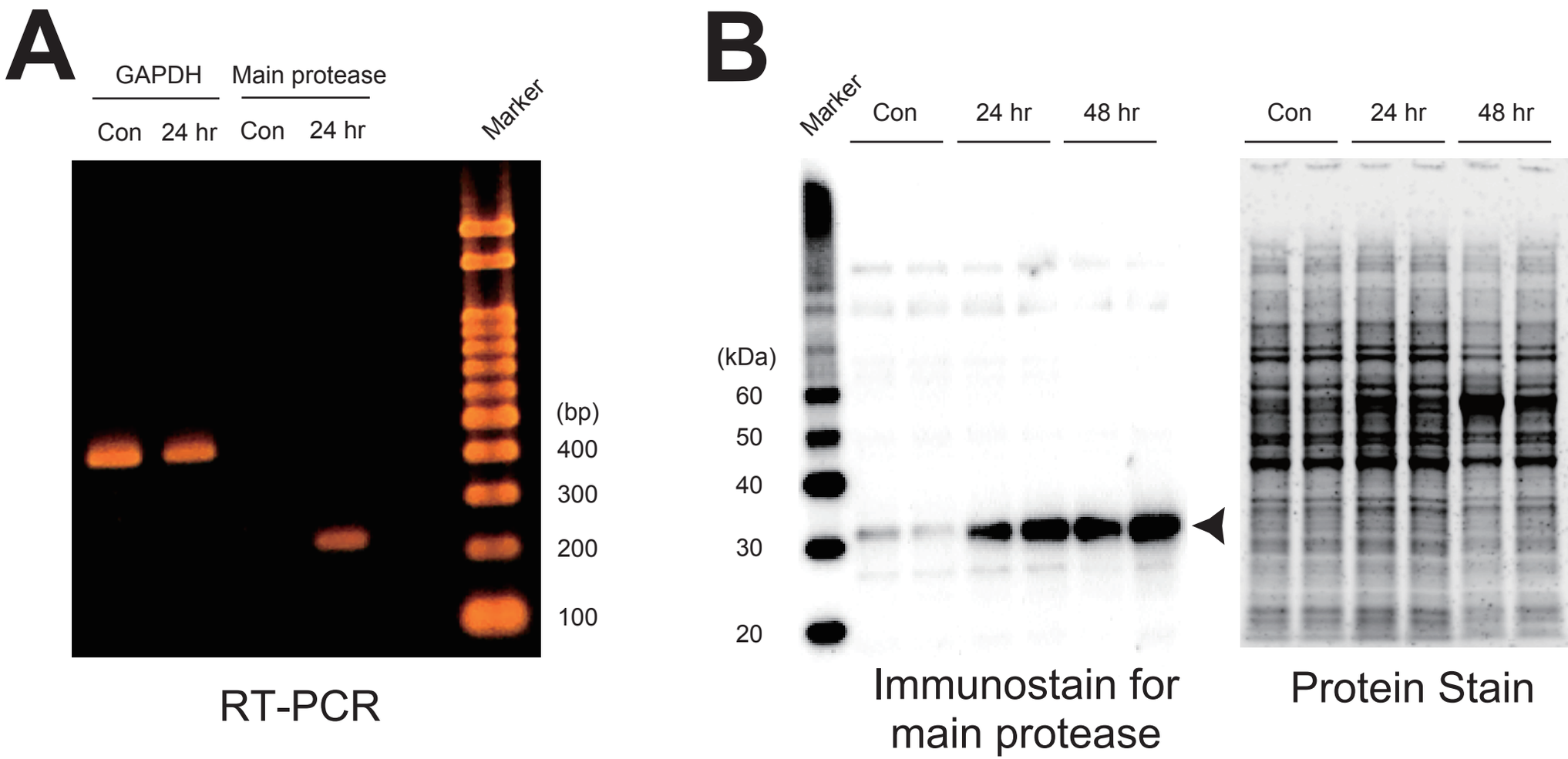

A
B
C
D
E
F
G
H
I
J
150
100
50
0
0.0001
0.001
0.01
0.1
1
Enzyme Activity, %
Dilution Factor

Supplementary Figure 2




GC
R²=0.147
EGC
R²=0.528
C, catechin
R²=0.376
EC
R²=0.606
EGCG
R²=0.356
GCG
R²=0.025
ECG
R²=0.283
Caffeine
R²=0.684
4-catechins sum
R²=0.466
Conc., µM
% Inhibition


100
Mpro
+ Bottled Green Tea
+ EGGC/GCG
%
+ EGC/GC
+ EGGC/GCG
0
100
Control Enzyme
%
0
34.5
35.0
35.5
36.5
36.0
Mass (kDa)
Relative Abundance, %control


A
GAPDH
Main protease
Con
24 hr
Con
24 hr
Marker
(bp)
400
300
200
100
RT-PCR
B
Marker
Con
24 hr
48 hr
Con
24 hr
48 hr
(kDa)
60
50
40
30
20
Immunostain for main protease
Protein Stain

Extracellular Inhibition of M^{pro} by catechins

Intracellular Adduction of EGCG to M^{pro}

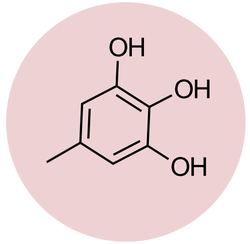
Tea catechins

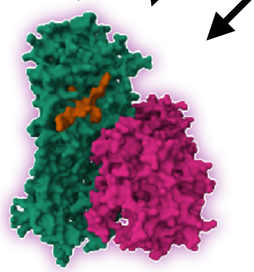
C | EC | ECG

EGC | GC | GCG

EGCG Me | EG CG

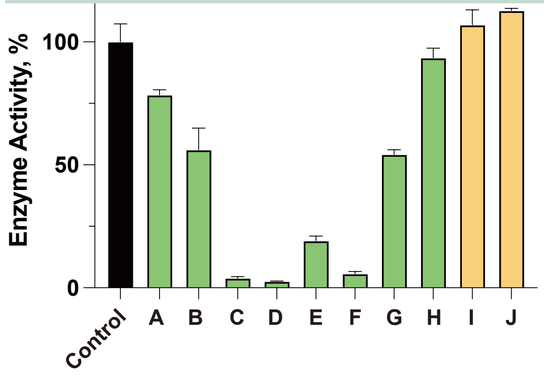
Key structure



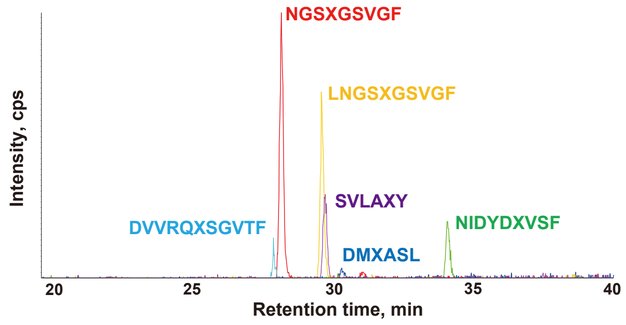


SARS-CoV-2 M^{pro} enzyme

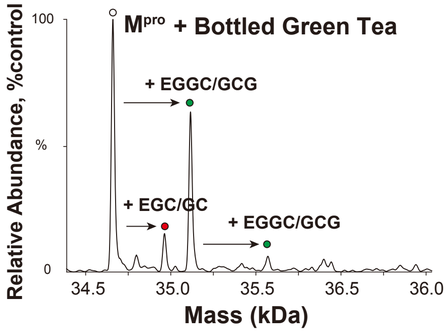
Inhibitory effect by bottled tea



Targetted sequences



Adducted catechins



Plasmid

Transfection

Viral M^{pro} gene

Plasmid

Translation

EG CG

Intracellular Adduction

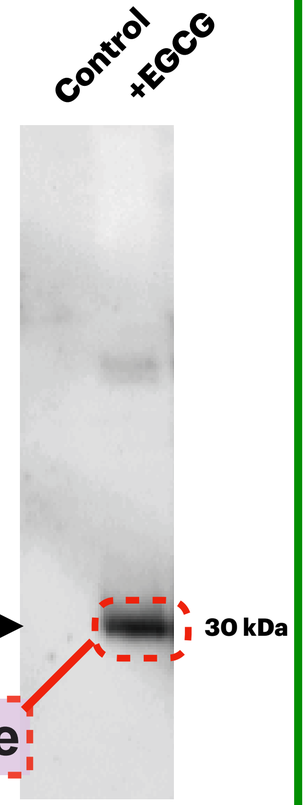
M^{pro} enzyme

PBA beads

EGCG-modified M^{pro} enzyme

Viral enzyme-expressing HEK cell

Precipitated by PBA beads



Immunostain Anti-M^{pro} poAb

Graphical Abstract