Inactivation of influenza virus haemagglutinin by chlorine dioxide: oxidation of the conserved tryptophan 153 residue in the receptor-binding site

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Airborne influenza virus infection of mice can be prevented by gaseous chlorine dioxide (ClO_2). This study demonstrated that ClO_2 abolished the function of the haemagglutinin (HA) of influenza A virus (H1N1) in a concentration-, time- and temperature-dependent manner. The IC_{50} during a 2 min reaction with ClO_2 at 25 °C was 13.7 μM, and the half-life time of HA with 100 μM ClO_2 at 25 °C was 19.5 s. Peptides generated from a tryptic digest of ClO_2-treated virus were analysed by mass spectrometry. An HA fragment, ^{150}NLLWLTGK^{157} was identified in which the tryptophan residue (W153) was 32 mass units greater than expected. The W153 residue of this peptide, which is derived from the central region of the receptor-binding site of HA, is highly conserved. It was shown that W153 was oxidized to *N*-formylkynurenine in ClO_2-treated virus. It was concluded that the inactivation of influenza virus by ClO_2 is caused by oxidation of W153 in HA, thereby abolishing its receptor-binding ability.

INTRODUCTION

Influenza virus is an enveloped, negative-sense ssRNA virus with three transmembrane proteins, two of which are known as spike proteins, the haemagglutinin (HA) (Gamblin & Skehel, 2010; Skehel & Wiley, 2000) and neuraminidase (NA) (Xie *et al.*, 2011). The functions of HA are indispensable for the early establishment of infection in a target cell (Skehel & Wiley, 2000). An HA receptor on the target cell binds to a receptor-binding site of HA. The site is a shallow groove made up of three elements, the 190 helix (an α-helix of residues 188–190), the 130 loop (residues 134–138) and the 220 loop (residues 221–228) (Yang *et al.*, 2007). Conserved amino acid residues are present in the receptor-binding site, including Y98, S136, W153, H183, E190 and Y195 (Gamblin & Skehel, 2010; Stevens *et al.*, 2004, 2006). Among these conserved residues, W153 is located at the bottom of the binding site just below the acetamide moiety of the sialic acid (*N*-acetylneuraminic acid) residue of the receptor (Lin *et al.*, 2009).

Chlorine dioxide (ClO_2) is a relatively stable free radical. ClO_2 can inactivate various bacteria (Morino *et al.*, 2011), fungi (Morino *et al.*, 2007) and viruses (Morino *et al.*, 2009; Sanekata *et al.*, 2010). Ogata & Shibata (2008) recently demonstrated that low-concentration (0.03 p.p.m., v/v) ClO_2 gas reduced the mortality of mice exposed to aerosols of influenza A virus (H1N1), whilst Akamatsu *et al.* (2012) recently reported a 6-month continuous inhalation experiment using rats in which they showed that a 0.05 p.p.m. level of ClO_2 had no adverse effect. The effectiveness (Ogata & Shibata, 2008) of low-concentration ClO_2 gas indicates that this might be useful in preventing the transmission of influenza virus (Ogata & Shibata, 2009). Although Ogata & Shibata (2008) showed that ClO_2 inactivated influenza virus, the molecular details of this inactivation mechanism were unclear. The results presented in this paper clearly demonstrate that inactivation of influenza virus by ClO_2 is due to oxidation and elimination of the function of the HA molecule.

Three supplementary figures and a table are available with the online version of this paper.

RESULTS

Inhibition of haemagglutination by ClO_2

The ability of influenza virus particles to agglutinate chicken erythrocytes decreased when virus particles were treated with ClO_2 (Fig. 1a) with an IC_{50} of 13.7 μM at 25 °C for 2 min and a half-life time for HA of 19.5 s at 25 °C with 100 μM ClO_2 (Fig. 1b). This inactivation process was temperature dependent (Fig. 1c), suggesting that the basis of the inhibition involved a chemical reaction. The activation energy of this inactivation process was determined by an Arrhenius plot (Fig. 1d). From the fitted line $y=-1970x+9.31$ ($r^2=0.93$), the activation energy was found to be 16.4 kJ·mol^{-1}·K^{-1}. Erythrocytes pre-treated with bacterial NA did not show agglutination (Fig. 1a), indicating that the agglutination occurred via sialic acid residues in the glycoproteins of the erythrocytes.

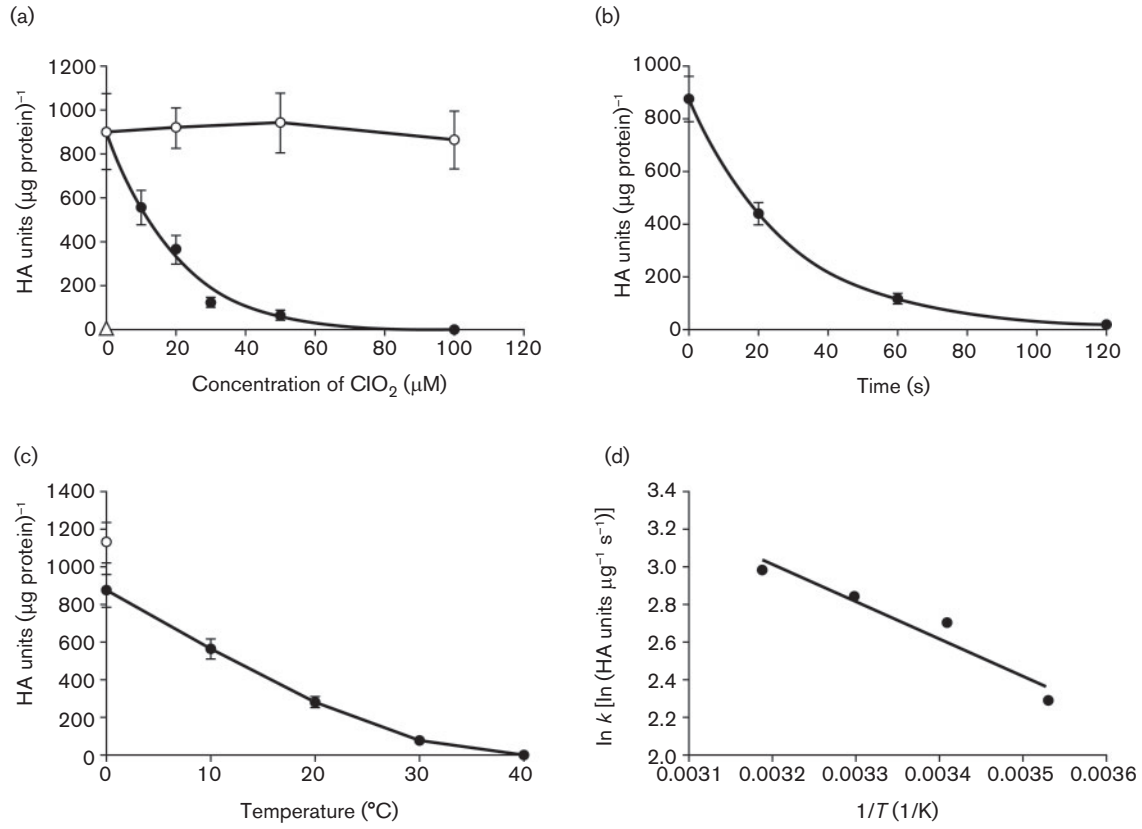

Fig. 1. Inactivation of influenza virus HA by exposure to ClO_2. (a) Influenza virus particles were treated with various concentrations of ClO_2 at 25 °C for 2 min and then were subjected to a haemagglutination assay using untreated chicken erythrocytes (●) or bacterial NA-treated chicken erythrocytes (△). As a control, ClO_2 pre-treated with an excess amount of $Na_2S_2O_3$ was added to the HA assay (○). (b) Time course of the inactivation of haemagglutination by exposure to 100 μM ClO_2 at 25 °C. (c) Temperature dependence of the inactivation of haemagglutination by exposure to 100 μM ClO_2 for 2 min. (d) An Arrhenius plot of the temperature dependence of the inactivation of haemagglutination by exposure to 100 μM ClO_2 for 2 min. k, Rate constant; T, absolute temperature (K). Data are shown as the means ± SD of four independent experiments. Error bars are omitted in (d).

Inhibition of receptor binding of HA by ClO_2

The ClO_2-mediated inactivation of the ability of HA to bind its specific receptor was examined by direct binding of sialyl-α(2,6)-lactose-*N*-acetylated *p*-phenylenediamine-derivatized human serum albumin conjugate (hereafter referred to as sialyl–albumin conjugate) to a microtitre plate pre-coated with HA. The ability of the conjugate to bind HA on the plate decreased markedly when HA was treated with ClO_2 (Fig. S1, available in JGV Online). Taken together, these results suggested that ClO_2 abolishes the ability of HA to bind to its specific receptor.

Modification of HA by ClO_2 as revealed by mass spectrometry (MS)

The precise nature of the ClO_2-induced modification of HA was investigated next. A tryptic digest of ClO_2-treated virus particles was performed, and the resulting peptides were separated by HPLC and the fractions subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. A fraction with a retention time of 25.0−25.5 min (Fig. S2, fraction 45) showed a mass-to-charge ratio (m/z) value of 976.55 (Fig. 2a). The tryptic fragment with m/z=976.55 corresponded to a peptide comprising ^{150}NLLWLTGK^{157} of HA (H3 numbering), containing a single tryptophan, W153. However, the m/z value of 976.55 was 32 mass units greater than that anticipated based on the amino acid sequence. Moreover, this peak was absent from virus particles that were not treated with ClO_2 (data not shown). The amino acid sequence of the peptide deduced from tandem MS (MS/MS) was ^{150}NLLWLTGK^{157}, except that W153 was 32 mass units larger than expected (Fig. 2b). This residue was tentatively assigned as *N*-formylkynurenine (FK) (Fig. 2a, inset) based on previous reports (Ogata, 2007; Stewart *et al.*, 2008). The other MS peaks of the HPLC fractions are shown in Table S1.

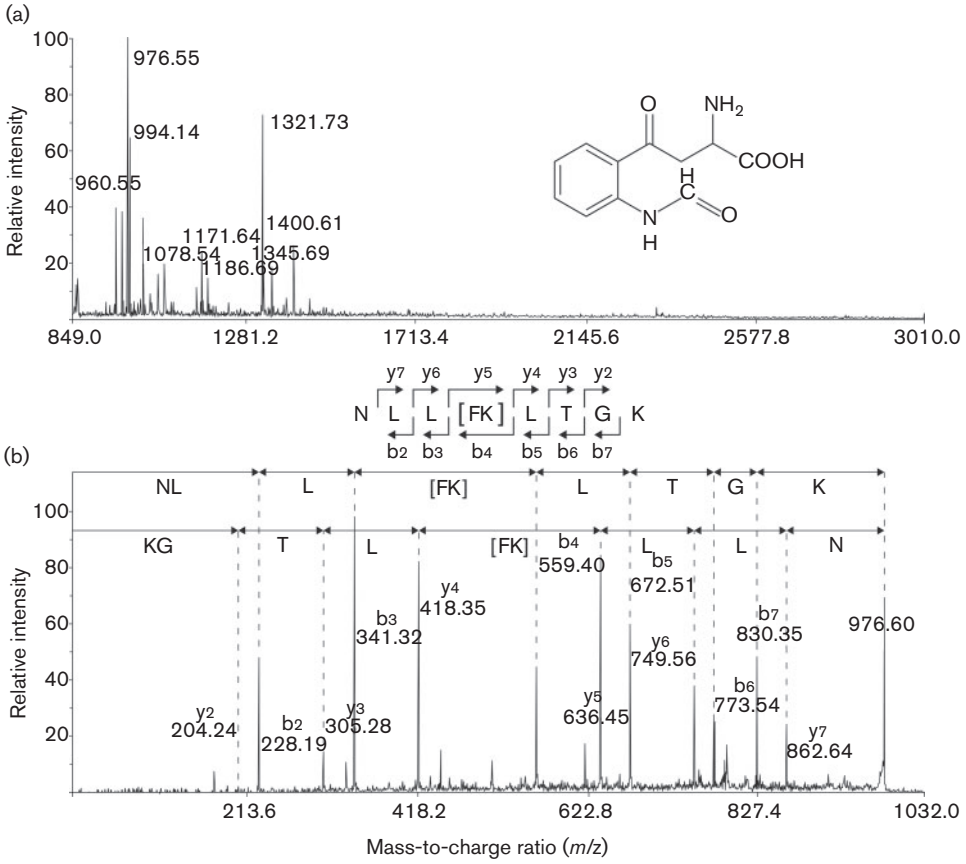

Fig. 2. MALDI-TOF MS analysis of HPLC fractions. (a) Peptides released from the ClO_2-treated virus particles after proteolysis with trypsin were subjected to HPLC. Fraction 45 (Fig. S2) was subjected to MALDI-TOF MS. The inset shows the structure of *N*-formylkynurenine. (b) The peak with $m/z=976.55$ in (a) was subjected to MS/MS analysis. The amino acid sequence deduced from the MS/MS spectrum is shown above (b). Numbering of the b and y fragment ions is based on the nomenclature of Biemann (1988).

Modification of synthetic peptide with ClO_2

A synthetic peptide of sequence NLLWLTGK treated with ClO_2 showed a novel peak with a retention time of 26.3 min, hereafter referred to as the 'ClO_2-modified peptide' (data not shown). As a control, another peptide, NLL**G**LTGK, with a substitution of G for W (bold), was also treated with ClO_2. This G-substituted peptide did not show any detectable change in mass after ClO_2 treatment (data not shown). These results suggested that the ClO_2-induced modification was caused by the presence of a tryptophan residue in peptide NLLWLTGK. To further verify this interpretation, the absorbance and fluorescence emission spectra of the ClO_2-modified peptide were compared with those of the original NLLWLTGK peptide. The absorbance and fluorescence spectral changes of the ClO_2-modified peptide indicated that its tryptophan residue was covalently modified (data not shown).

MS analysis of the ClO_2-modified peptide

The MALDI-TOF MS peak of the ClO_2-modified peptide ($m/z=976.79$) (Fig. S3a) was almost identical to the peak liberated after tryptic digestion from the ClO_2-treated virus particles (Fig. 2a). The spectrum obtained by MS/MS using the $m/z=976.79$ peak as a precursor ion also gave almost identical peaks (Fig. S3b) to those generated from the HA fragment released from the ClO_2-treated virus particles after trypsin digestion (Fig. 2b). Moreover, the amino acid composition of the ClO_2-modified peptide showed no tryptophan, although kynurenine was detected (data not shown). (Note that the formyl moiety of FK is removed under the acid hydrolysis conditions used for the amino acid analysis.) These results indicated that the synthetic peptide NLLWLTGK was also oxidized to NLL[FK]LTGK by ClO_2.

DISCUSSION

ClO_2 reacts non-specifically with tryptophan and tyrosine residues in proteins (Napolitano *et al.*, 2005; Ogata, 2007; Stewart *et al.*, 2008). However, it should be noted that ClO_2 does not necessarily react with all of the tryptophan and tyrosine residues in a specific protein (Ogata, 2007). The different reactivity of ClO_2 against amino acid residues in

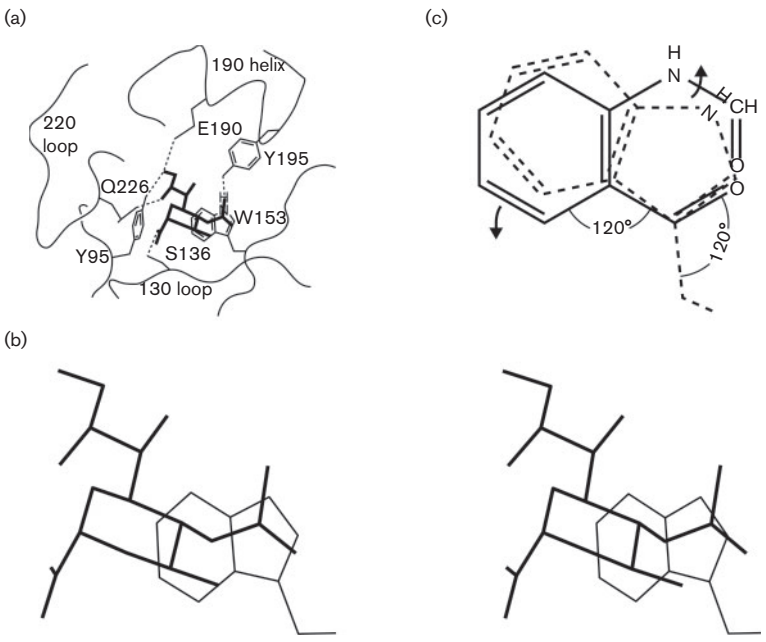

Fig. 3. The receptor-binding site of HA and possible movement of the conserved W153 residue after oxidation to FK by ClO_2. (a) The receptor-binding site of HA co-crystallized with a truncated receptor (Lin *et al.*, 2009) is shown. Only a terminal sialic acid residue of the receptor is shown (bold lines). Highly conserved amino acid residues in the receptor-binding site in HA are also shown. Putative hydrogen bonds that fix the sialic acid residue of the receptor to HA are represented by dotted lines. This view shows the receptor-binding site of HA looking from the receptor down to the tip of HA. (b) An enlarged view of the centre of the receptor-binding site of HA (stereogram). (c) Possible movement of the W153 residue (dotted lines, facing view of the indole ring) after oxidation to FK (solid lines) by ClO_2. Arrows indicate the possible movement of W153 when it is oxidized to FK. The double bond of the formyl group is not exactly to scale because this bond may be tilted upwards.

any given protein might depend on whether these residues are easily accessible to ClO_2 or not. This study demonstrated that ClO_2 reacts with W153 preferentially, if not specifically, in HA (Fig. 2). W153 is located on the surface of HA (Fig. 3a) and is presumably easily accessible to ClO_2. It is also possible that the geometry of W153 in HA facilitates the reaction with ClO_2. The binding ability of HA to its specific receptor on a target cell is crucial for establishing infection (Skehel & Wiley, 2000). Because W153 in HA is highly conserved in almost all subtypes of influenza virus (Knossow & Skehel, 2006; Martín *et al.*, 1998), it is believed to play a crucially important role in the function of HA (Meisner *et al.*, 2008). As shown in Fig. 3(a, b), W153 is located at the bottom of the receptor-binding site just below the sialic acid residue of the receptor (Lin *et al.*, 2009). In fact, the acetamide moiety (Fig. 3b) of the sialic acid is in van der Waals contact with the indole ring of W153 (Lin *et al.*, 2009; Martín *et al.*, 1998). It is speculated that W153 together with Y195, which makes a hydrogen bond with the pyrrole nitrogen of W153, provides an optimal geometric structure that allows stable binding of the sialic acid residue of the receptor to HA. This proposed interaction explains why oxidation of W153 by ClO_2 is likely to abolish the function of HA (Fig. 1).

It was shown previously by isotopic labelling experiments that ClO_2 oxidizes tryptophan to FK by transferring two atoms of oxygen from ClO_2 (Ogata, 2007). In this reaction, the pyrrole ring of tryptophan is split, and the benzene ring of W153 will shift as shown in Fig. 3(c, lower arrow). In addition, because the *N*-formyl bond in FK is a freely rotatable σ bond, the oxygen atom of the formyl group may rotate upwards (in the direction of the sialic acid residue; Fig. 3c, upper arrow) to accommodate the bulky CO moiety. These possible conformational changes in the binding site of HA are likely to perturb its interaction with the sialic acid residue. Additionally, such a conformational change in the receptor-binding site of HA would no longer enable formation of the necessary hydrogen bonds involving Y95, Y195, E190 and Q226 (Fig. 3a, dotted lines) to hold the sialic acid residue in place (Sauter *et al.*, 1992). The structural alterations to the binding site caused by the oxidized W153 would therefore hinder the function of the HA molecule.

METHODS

Virus and reagents. Influenza A virus [A/New Caledonia/20/99 (H1N1)] was obtained from Dr Y. Okuno of the Department of Infectious Diseases, Osaka Prefectural Institute of Public Health, Japan. Antibodies were purchased from MyBiosource. Peptides (>98 % pure) were purchased from Pi Proteomics. Recombinant HA protein of A/New Caledonia/20/99 (H1N1) was purchased from Protein Sciences. ClO_2 was prepared using $NaClO_2$ and HCl as described previously (Ogata, 2007). The sialyl–albumin conjugate was obtained from IsoSep AB (Kallin *et al.*, 1986). This conjugate has 15 moles of the oligosaccharide moiety per mole of the protein. Trypsin was obtained from Promega. All other reagents were analytical grade and were obtained from Nacalai Tesque. Influenza virus particles were prepared in embryonated chicken eggs based on the method described by Herrmann (1978). Approximately 3.5 mg virus protein in 1 ml was obtained from 100 eggs using this method.

Treatment of virus particles and HA with ClO_2. Unless otherwise specified, the standard reaction for the treatment of virus particles or HA protein with ClO_2 was carried out in a 1 ml reaction mixture containing 10 mM sodium phosphate buffer (pH 7.0), 130 mM NaCl, 300 μM ClO_2 and virus particles (100 μg protein ml^{-1}) or HA protein (100 μg ml^{-1}). The reaction was started by the addition of ClO_2 and was continued at 25 °C for 2 min. The reaction was terminated by the addition of a twofold molar excess of $Na_2S_2O_3$ (Ogata, 2007). For a

control experiment without ClO_2 treatment, ClO_2 that had been premixed with a twofold molar excess of $Na_2S_2O_3$ was added to the reaction mixture. For the reaction with virus particles, the reaction mixture was next heated at 100 °C for 3 min, followed by the addition of 38 µl trypsin solution (1 mg ml^{-1}). The mixture was incubated at 37 °C for 5 h and then centrifuged at 20 000 ***g*** at 4 °C for 1 h. The supernatant was recovered and a 100 µl aliquot was subjected to HPLC.

Haemagglutination assay. A round-bottomed, 96-well polystyrene microtitre plate (Biotec) was used to measure the haemagglutination ability of virus particles. Virus particles that had been treated or not with ClO_2 were suspended in PBS. The virus particles were then diluted by twofold serial dilutions and placed in each well of the plate (50 µl per well). Fresh chicken erythrocytes, treated or not with *Clostridium perfringens* NA (0.39 U ml^{-1}, 37 °C for 10 min in PBS), were suspended in PBS at a concentration of 4×10^7 cells ml^{-1} and then added to each well (50 µl per well). The plate was kept at 4 °C for 1 h. Agglutination of erythrocytes was observed by eye: a sheet of erythrocytes was formed on the bottom of the well if agglutination occurred; otherwise the erythrocytes formed a red 'spot' on the bottom in the centre of the well. One haemagglutinin unit was defined as the minimum amount of virus that agglutinated erythrocytes under the assay conditions described above.

Receptor-binding assay of HA. Recombinant HA, treated or not with ClO_2 as described above, was diluted with PBS to a concentration of 20 µg ml^{-1}. One hundred microlitres of this solution was then placed in each well of a 96-well, flat-bottomed polystyrene microtitre plate (MaxiSorp; Nunc) at 25 °C for 30 min. Each well of the plate was washed three times with 100 µl PBS containing 0.01 % (v/v) Tween 20 and once with 100 µl distilled water. Equine skeletal muscle myoglobin (Sigma-Aldrich) solution in PBS (1 mg ml^{-1}) was placed in each well (100 µl per well) at 25 °C for 5 min to block the protein-uncoated area of the well. The wells were washed as described above, air dried and stored at room temperature, protected from light, until use. For the receptor-binding assay, 100 µl sialyl–albumin (1.5 µg ml^{-1}), as a model of truncated HA receptor, was placed in each well at 25 °C for 5 min. The wells were then washed as described above, and 100 µl HRP-labelled anti-human serum albumin antibody (diluted to 0.8 µg ml^{-1}) was placed in each well at 25 °C for 5 min. The wells were then washed as described above before finally adding peroxidase substrate solution (Bio-Rad). The reaction was allowed to continue at 37 °C for 10 min before quenching by the addition of 20 mM HCl (100 µl per well). The plate was scanned using a microplate reader (model MPR-A4; Toyosoda) to measure the absorbance at 415 nm. The ability of the 96-well plate to bind HA was measured in a separate experiment. Here, after the binding, washing and drying steps as described above, 100 µl HRP-labelled anti-influenza A virus (H1N1) HA antibody (diluted to 0.5 µg ml^{-1}) was placed in each well. The colour-developing reaction was performed as described above to quantify the amount of HA bound to each well of the plate.

HPLC of peptides. Peptides (5–20 nmol in 100 µl) were resolved on a Cosmosil 5C18-AR-300 column (4.6 × 250 mm; Nacalai Tesque) mounted on an HPLC system. After injection of a peptide sample, the column was eluted with 0.1 % (v/v) trifluoroacetic acid containing two consecutive linear gradients of acetonitrile (0–12 %, v/v, from 0 to 1 min, and then 12–60 % , v/v, from 1 to 60 min) using a flow rate of 1 ml min^{-1}. An elution profile of the peptides was obtained by monitoring the absorbance of the eluate at 230 or 280 nm. Fractions of the eluate were lyophilized and dissolved in distilled water for further analysis.

MS of peptides. Peptide samples (10–500 pmol) were subjected to either MALDI-TOF MS to measure their intact mass, which is actually the *m*/*z* ratio (Biemann, 1988), or MS/MS, after electron spray ionization of precursor ions, to analyse the amino acid sequences of the peptides, as described previously (Ogata, 2007).

Amino acid analysis of peptides. The amino acid composition of the peptides (~200 pmol) was analysed using an amino acid analyser (model L-8500; Hitachi) after acid hydrolysis (in 6 M HCl at 110 °C for 22 h) under reduced pressure and in the presence of nitrogen. Thioglycolic acid (4 %, v/v) was added to the HCl to prevent decomposition of tryptophan during hydrolysis.

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