Capsaicin-Enhanced Ribosomal Protein P2 Expression in Human Intestinal Caco-2 cells

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Abstract

On the basis of transepithelial electrical resistance (TER) measurements, we found that capsaicin (100 μM)-treated human intestinal Caco-2 cells show a momentary increase in tight-junction (TJ) permeability (decrease in TER) followed by a complete recovery. We used proteome analysis to search for proteins that are associated with the recovery of TJ permeability in capsaicin-treated Caco-2 cells. A protein with a relative molecular mass of 14 kDa was found to be expressed more highly in capsaicin-treated cells than in nontreated cells. Mass spectrometry and sequence analyses revealed that the protein that is expressed significantly upon capsaicin treatment is the ribosomal protein P2; its cDNA sequence was identical to that found in the human genome database. An increase in the amount of cellular filamentous actin (F-actin) was shown after 8 h of incubation with capsaicin. It has been reported that P2 activates elongation factor 2, which stabilizes F-actin filaments, and that the depolymerization of F-actin is associated with the increase in TJ permeability (decrease in TER). Consequently, these results suggest that P2 plays an important role in the recovery of the TJ permeability in capsaicin-treated human intestinal cells.

Keywords: Capsaicin; Tight-junction Permeability; Ribosomal Protein P2; Elongation factor 2; F-actin; Proteome Analysis; Caco-2 Cells

Introduction

Human intestinal Caco-2 cells (Fogh et al. 1977) have been widely used as *in vitro* models to evaluate the transport of absorbed water, ions, and nutrients across the intestinal epithelial barrier (Satsu et al. 2003). The tight-junction (TJ) permeability increase in Caco-2 cells is regulated by various factors, such as food factors (for example, capsaicin and capsianoside.) and chemicals (such as alkylphenolic compounds and EDTA). Such factors can alter the epithelial transport by and barrier function of human intestinal epithelial cells using various mechanisms (cytoskeletal reorganization, redistribution of ZO-1 and occludin, exertion of Rho A, Rac 1, and Cdc 42, *etc.*) (Han et al. 2002; Nusrat et al. 1995; Bruewer et al. 2004; Yap et al. 1998).

In previous studies (Han et al. 2002; Isoda et al. 2001), we showed that the TJ permeability increase (decrease in transepithelial electrical resistance (TER)) in capsaicin-treated (45 min) human intestinal Caco-2 cells is through binding of capsaicin to a capsaicin receptor-like protein. We also suggested that the increase in TJ permeability upon capsaicin treatment occurs due to a cytoskeletal reorganization of actin filaments, particularly due to the decrease in the amount of filamentous actin (F-actin) in Caco-2 cells. In addition, heat shock protein 47 (HSP47), which is activated during capsaicin treatment, plays an important role as a secondary messenger in the increase in TJ permeability by capsaicin treatment.

A preliminary experiment indicated that Caco-2 cells treated with 100 μM capsaicin for a period longer than those of previous studies, showed a decrease followed by a recovery of TER values. The recovery of TER after capsaicin treatment, even without removal of capsaicin, was rapid. This suggests that some proteins, whose expressions are enhanced by capsaicin, facilitate the recovery of TER after the momentary decrease.

Tools such as two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) allow for cellular proteome analysis (Poon et al. 2001; Lim et al. 2002; Fuchigami et al. 2002). Using these techniques, changes in protein expression profiles in capsaicin-treated human intestinal Caco-2 cells, particularly proteins related to the recovery of TJ permeability, can be determined.

In this study, we performed 2-DE and MS to determine the possible mechanism behind the recovery of TER after a momentary decrease in capsaicin-treated Caco-2 cells. We found that the expression of ribosomal protein P2 is enhanced by capsaicin treatment; its role in regulating the recovery of TJ permeability is discussed.

Materials and methods

Cell culture

The Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Sigma), 1% penicillin-streptomycin, and 1% nonessential amino acids (Cosmo Bio). They were incubated in an atmosphere of 5% CO_2 at 37 °C. The cells were passaged at a split ratio of 4 to 8 every 3 or 4 days. For the extraction of proteins, cells were seeded onto Petri plates at a density of 1×10^6 cells per well, followed by the addition of capsaicin (Sigma) (final concentration: 100 μM). After incubating for 45 min, 90 min, 8 h, 16 h, or 24 h, proteins were extracted by cell lysis in 4% (v/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 7 M urea, 2 M thiourea, 25 mM spermine base (Sigma), 1 M ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 5 mM 4-(2-aminoethyl)benzenesulfonylflouride (AEBSF), followed by centrifugation at 100,000 g for 20 min.

TER measurement

The TJ permeability of the Caco-2 cell monolayer was evaluated by measuring the TER. TER is considered to be correlated with the change in paracellular permeability of the cell monolayer (Hashimoto et al. 1997). The cell monolayers on Millicell-HA were rinsed with DMEM and then set in a 24-well plate containing DMEM. DMEM was added to the apical

side, and the TER was measured with a Millicell-ERS instrument (Millipore). The TER of the Caco-2 monolayer was measured before and after adding the test sample, and the effect of each test sample is expressed as the TER relative to that at time zero.

Two-dimensional gel electrophoresis

Proteins from capsaicin-treated or nontreated cells were resuspended in 350 μl of 8 M urea, 2% (w/v) CHAPS, 0.5% (v/v) immobilized pH gradient (IPG) buffer (pH 3-10 depending on the pH range of the IPG dry strip to be rehydrated) (Amersham Biosciences), and 10 mM DTT. Solubilized proteins were electrophoresed in the first dimension using a commercial flatbed electrophoresis system (Multiphor II, Amersham Biosciences) and 18-cm IPG dry strips (Amersham Biosciences) with a pH 3-10 linear range. The IPG strip was rehydrated for approximately 14 h at room temperature and the proteins were electrophoresed in gradient mode using an EPS 3501 XL power supply (Amersham Biosciences) under the following conditions: 150 V, 1 mA for 30 min; 300 V, 1 mA for 30 min; 500 V, 1 mA for 1 min; 3500 V, 1 mA for 1.5 h; 3500 V, 1 mA for 7.8 h. After isoelectric focusing, the IPG strips were re-equilibrated for 20 min in 2% (w/v) sodium dodecyl sulfate (SDS), 6 M urea, 30% (v/v) glycerol, 0.05 M Tris–HCl (pH 8.8), and 2% (w/v) DTT and for 20 min in 2% (w/v) SDS, 6 M urea, 30 % (v/v) glycerol, 0.05 M Tris–HCl (pH 8.8), and 5% (w/v) iodoacetamide. The strip was placed on a gradient SDS-PAGE gel (12-14% (w/v)

polyacrylamide) and run at 1000 V, 20 mA for 45 min and at 1000 V, 40 mA for 160 min (Poon et al. 2001). The proteins were visualized by Coomassie Brilliant Blue (CBB) staining (using PhastGel Blue R-350, Amersham Biosciences) for spot analysis. The spot analysis software, ImageMaster 2D Elite (Amersham Biosciences), allows for the magnification of specific fields of view and assists greatly in the visual comparison of proteins between control and experimental gels.

Mass spectrometry

Protein spots of interest were excised from the gel, washed and digested in-gel with trypsin (sequencing grade, Boehringer Mannheim). All matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were acquired using a Voyager-DE STR mass spectrometer (Applied Biosystems). The matrix solution was prepared by dissolving 10 mg of α-cyano-4-hydroxycinnamic acid (Sigma) in 1 ml of 50% acetonitorile and 0.1% trifluoroacetic acid in deionized water. The obtained peptide sequence tags were used to identify proteins by searching databases using BLAST (http://www.ncbi.nlm.nih.gov:80/blast) or the Prospector software MS-Tag (http://prospector.ucsf.edu/).

Preparation of RNA and the cDNA library

Total RNA was extracted from Caco-2 cells incubated with 100 μM capsaicin for 16 h using ISOGEN (Nippon Gene), and poly(A)$^+$RNA was isolated using the Poly(A)$^+$RNA Isolation Kit (Nippon Gene). Double-stranded cDNA was synthesized from Poly(A)$^+$RNA using the Universal RiboClone cDNA Synthesis System (Promega).

RT-PCR and 3'- and 5'-RACE amplification

RT-PCR was performed using the degenerate primers S1 and A1 and the template of cDNA library using the Expand High-Fidelity PCR System (Roche). S1 and A1 were designed on the basis of the partial amino acid sequence determined by MALDI-TOF MS (Table 1). The PCR cycle consisted of 1 min denaturation at 94 °C, 1 min annealing at 52 °C, and 1 min extension at 72 °C for 35 cycles. Expected PCR fragments were cloned into pGEM-T Easy vector (Promega) and sequenced. To obtain the full-length cDNA, 3'- and 5'-RACE amplifications were carried out using the poly(A)$^+$RNA from Caco-2 cells as the template. The primers used in these experiments are shown in Table 1. For 3'- RACE, the 3' -Full RACE Core Set (Takara) was used. The first-strand cDNA was synthesized with the oligo (dT) adaptor primer, which was provided with the set. The specific primers for the nested 3'-RACE reactions used in the first and second PCRs were S2 and S3, respectively. For 5'-RACE, the 5' -Full RACE Core Set (Takara) was used. For the nested 5'-RACE reactions,

A2 was used as the anchor primer, while S2 and A3 were used in the first PCR and S3 and A4 were used in the second PCR. The resulting cDNA clones were subcloned into the pGEM-T Easy vector for further sequencing.

Measurement of cellular F-actin

The relative content of F-actin was determined by a fluorescent phalloidin binding assay. Caco-2 cells that had been incubated on a slide chamber for 3 days were rinsed with phosphate-buffered saline (PBS) and then incubated with capsaicin (100 μM) for 0, 8, 16, or 24 h. After incubation, the cell monolayer was fixed with acetone/methanol (1/1 = v/v), and then the actin was stained for 20 min with rhodamine-phalloidin (Wako) diluted tenfold with PBS. The stained cells were extracted with 2 ml of methanol, and the fluorescence intensity of the extract was determined using a Fluoroscan Ascent FL fluorescence spectrophotometer (Labsystems) with excitation-emission wavelengths of 545-578 nm.

Statistical analysis

Statistical analysis was carried out using Student's t-test, and relationships were considered statistically significant at $P < 0.05$.

Results and discussion

TER measurement shows that the TJ permeability of the Caco-2 monolayer increased significantly ($p < 0.05$ vs control) upon treatment with capsaicin (100 μM and 1 mM) (Fig. 1). When treated with 100 μM capsaicin, the TJ permeability recovered after 60 min, followed by a complete recovery after 90 min. However, upon treatment with 1 mM capsaicin, the TJ permeability increased irreversibly (Fig. 1). These results suggest that the barrier function was disrupted by the high concentration of capsaicin (1 mM). However, treatment at low concentration (100 μM) resulted in the recovery of the barrier function. This indicates that the permeability of the intestinal TJ can be regulated by the low concentration of capsaicin (100 μM).

To elucidate the mechanism behind the recovery of the barrier function, we used 2-DE and MS. The extracted proteins were first subjected to 2-DE (Fig. 2 A). In particular, the protein spot indicated by arrows in Fig. 2 B shows the highest expression in capsaicin-treated cells. The isoelectric point of this protein is in the range of 3.8-4.3, and it has a relative molecular mass of 14 kDa. To identify this protein, the spot was subjected to tryptic digestion and MALDI-TOF MS analysis. Fig. 3 shows the MALDI-TOF peptide map trace of the protein spot. Results of protein identification by database search are shown in Table 2. The protein spot has a sequence closest to that of ribosomal protein P2;

the matched peptides cover 72% (83/115 of amino acids) of P2 (shown as lines in Fig. 4).

By MS analysis, we were unable to obtain the complete amino acid sequence of the protein expressed upon capsaicin treatment. Therefore, we carried out the isolation and analysis of cDNA of P2 from Caco-2 cells to examine whether there might be some discrepancy or diversity from the known nucleotide or amino acid sequence. The nucleotide and deduced amino acid sequences of the full-length cDNA clone of the protein P2 from Caco-2 cells are shown in Fig. 4. These sequences, as well as the 5'-upstream nucleotide sequence, are identical to those of the human ribosomal protein P2 found in the databases.

What is the relationship between the recovery of the barrier function and the significant expression of ribosomal protein P2? P2 is a member of the well-conserved acidic P (phosphor) protein family in the eukaryotic ribosome (Gonzalo et al. 2002; Lavergne et al. 1987). It plays an important role in the elongation step of protein synthesis and in the activation of elongation factor 2 (EF-2) (Vard et al. 1997). It has been reported that EF-2 is activated by the homologous carboxy-terminal region of P2 (Furukawa et al. 1992). EF-2 interacts with globular actin (G-actin), stabilizes filament structure, and causes lateral association of F-actin (Bektas et al. 1994; Bektas et al. 1998). F-actin is a ubiquitous intracellular protein, which in filamentous form constitutes a major component of the cytoskeleton, and plays an essential role in cell motility and mechanics (Holmes et al. 1990;

Dadabay et al. 1991). Moreover, it has been suggested that the cytoskeletal reorganization of actin filaments mediates the increase in TJ permeability (decrease in TER) (Lim et al. 2002). F-actin is a major component of the adherens junction between intestinal cells, and the assembly of tight junctions between epithelial cells seems to require prior formation of adherens junctions (Alberts et al. 2002). Therefore, it is highly possible that P2, whose expression is enhanced by the capsaicin treatment, activates EF-2 to restore the F-actin in human intestinal Caco-2 cells followed by the decrease in TJ permeability (increase in TER). These results clearly show that the P2-mediated recovery of TJ permeability is related to the capsaicin-enhanced expression of P2. Furthermore, one recent study has shown that PaCa-2 and BxPC-3 cells transfected with ribosomal protein P2-antisense oligonucleotide showed a change in F-actin expression, as determined by proteome analysis (Gardner-Thorpe et al. 2003).

In the previous study (Isoda et al. 2001), we examined whether the capsaicin-induced increase in TJ permeability (decrease in TER) is associated with the cytoskeletal reorganization of the actin filaments by determining the cellular F-actin amount in Caco-2 cells after 45 min of treatment with capsaicin (0, 200, and 300 μM). The amount of cellular F-actin was found to decrease significantly in the capsaicin-treated Caco-2 cells. This reduction is probably due to the depolymerization of F-actin into G-actin as a form of cytoskeletal reorganization (Rayment et al. 1993; Savala et al. 1998). To confirm the

hypothesis, we determined the amount of cellular F-actin in Caco-2 cells treated with 100 μM capsaicin using a longer time scale (0, 8, 16, and 24 h), which is comparable to that used for the proteome analysis (Fig. 2 B). An increase in the amount of F-actin was shown in 8 h and this continued until 24h of incubation (Fig. 5). These and the previous results indicate that there is a close correlation between the amounts of P2 and F-actin.

In summary, we have investigated the mechanism of the recovery of TJ permeability in capsaicin-treated human intestinal Caco-2 cells. The expression of ribosomal protein P2 was enhanced by the capsaicin-treatment, which is followed by the increase in the amount of F-actin and the recovery of TJ permeability. This result suggests that P2 stabilizes F-actin and recovers the TJ permeability through the activation of EF-2. This is the first report showing that the expression of P2 is related to the recovery of TJ permeability in human intestinal Caco-2 cells.

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Table 1

Primers used for PCR and RACE

Primer	Sequence
S 1 (262－281 sense)	5’- GTIGGIAT(ACT)GAIGCIGAIGA-3’
A1 (370－389 antisense)	5’-GC(GCAT)ACIGCICCICCIGCIGG-3’
S2 (324－341 sense)	5’-CATTGAAGACGTCATTGC-3’
S3 (335－352 sense)	5’-TCATTGCCCAGGGTATTG-3’
A2 (357－369 antisense)	5’-TACACTGGCAAGC-3’
A3 (303－320 antisense)	5’-TTTCCATTCAGCTCACTG-3’
A4 (297－314 antisense)	5’-TTCAGCTCACTGATAACC-3’

The degenerate primers S1 and A1 were derived from the amino acid sequence determined by the MS analysis. “I” indicates inosine. The primers S2, S3, A2, A3, and A4 were derived from the sequence of the ribosomal protein P2 cDNA fragment.

Table 2

Comparison of observed and theoretical masses derived from the tryptic digests of the spot of interest (indicated by the arrow in Fig. 2 B).

Tryptic peptide number*	Observed mass (m/z)	Theoretical mass (m/z)	Corresponding sequence
T1	2156.1174	2156.1201	MRYVASYLLAALGGNSSPSAK
T2	1868.9705	1868.9758	YVASYLLAALGGNSSPSAK
T3	1772.8919	1772.9030	ILDSVGIEADDDRLNK
T4	1256.6825	1256.6850	NIEDVIAQGIGK
T5	2774.3965	2774.4325	LASVPAGGAVAVSAAPGSAPAAGSAPAAAEEK
T6	2902.4946	2902.5274	LASVPAGGAVAVSAAPGSAPAAGSAPAAAEEKK

*Corresponds to those in the MALDI-TOF spectrum (Fig. 3).

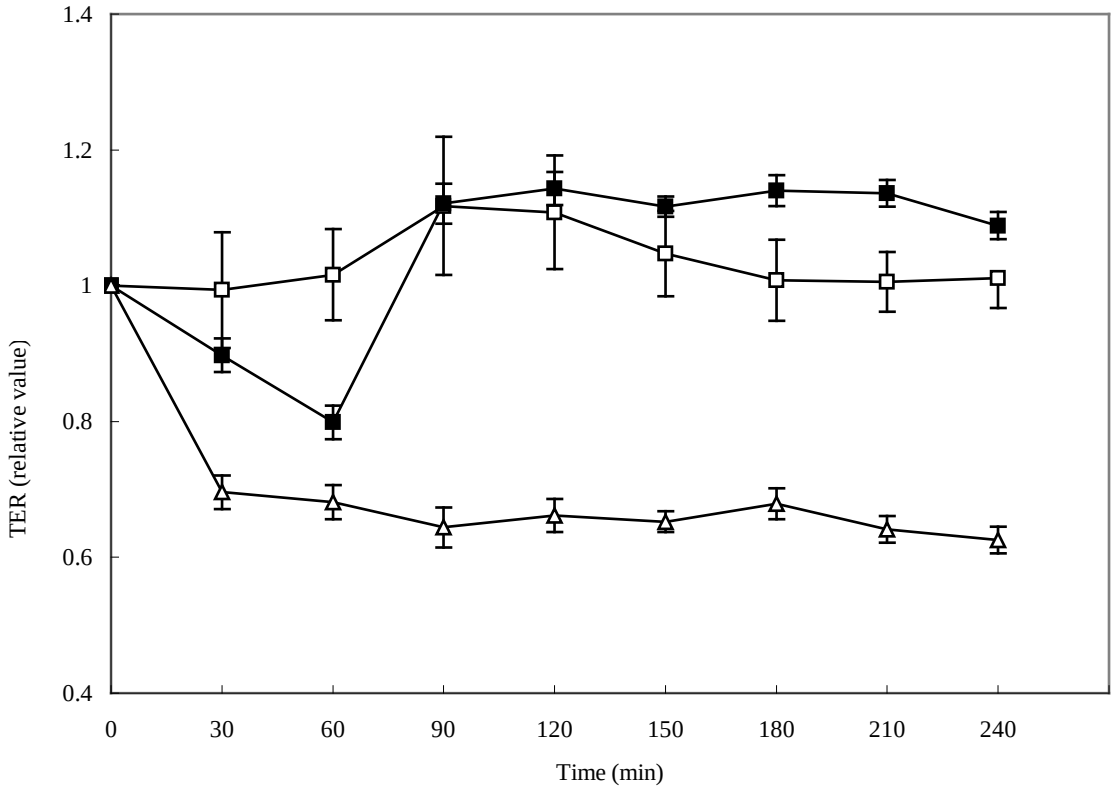

Fig. 1. Effect of capsaicin on TER. TER values are presented as relative to those at time zero. Capsaicin concentrations are 0 μM (open boxes), 100 μM (filled boxes), and 1 mM (open triangles).

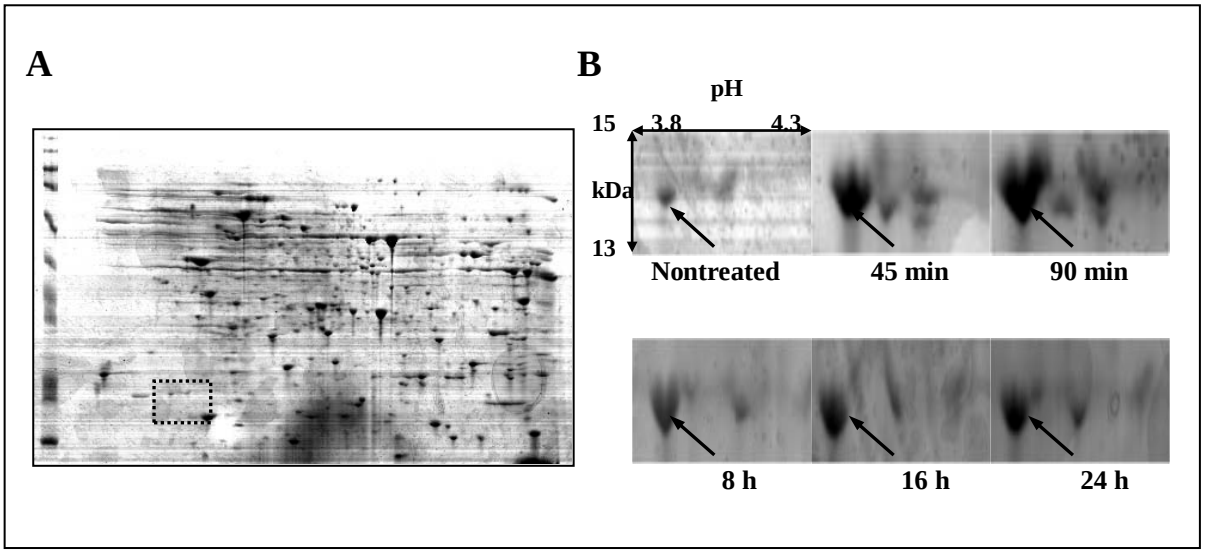

Fig. 2. Two-dimensional gel electrophoresis of proteins from nontreated human intestinal Caco-2 cells (A) and time-dependent expression of a protein induced by capsaicin treatment (B). In panel (B), the boxed region in panel (A) and the corresponding regions of the capsaicin-treated samples are magnified. The arrowed spot has an isoelectric point in the range of 3.8-4.3, and its molecular mass is approximately 14 kDa. 2-DE was performed by the isoelectric focusing of proteins using immobilized pH 3-10 strips, followed by second-dimension separation on 12-14% polyacrylamide gels. The separated proteins were stained with CBB.

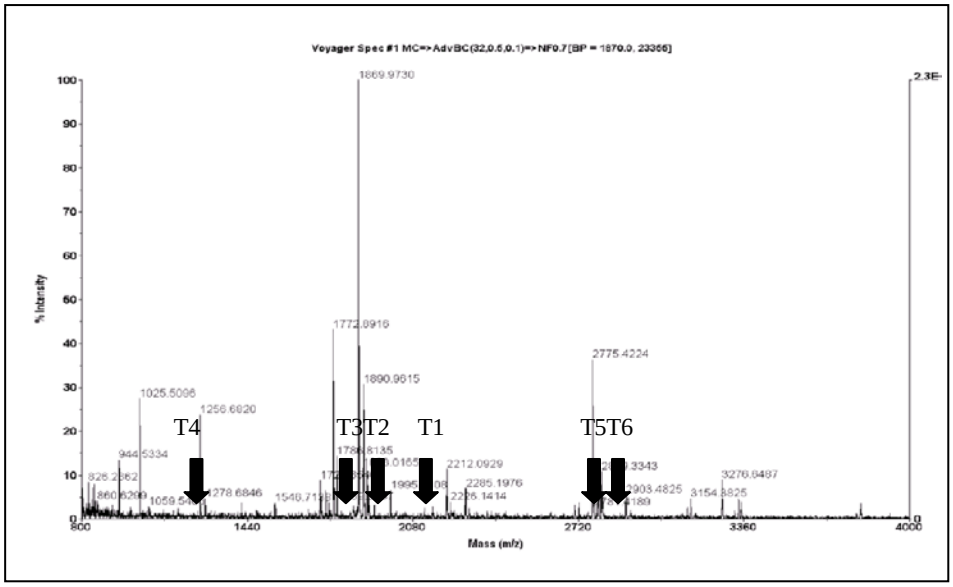

Fig. 3. MALDI-TOF reflector mass spectrum of tryptic digests of the spot of interest (indicated by arrow in Fig. 2 B).

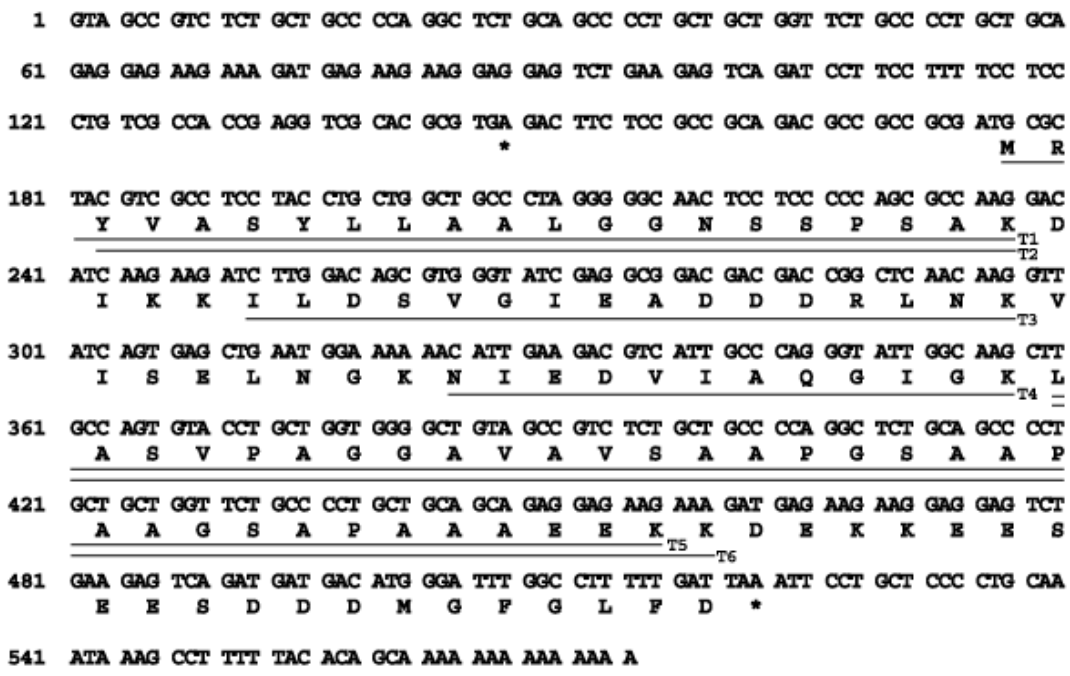

Fig. 4. Nucleotide and deduced amino acid sequences of ribosomal protein P2. An asterisk indicates a stop codon. The peptide fragments determined by MS analysis (Table 2) are indicated by lines under the sequences.

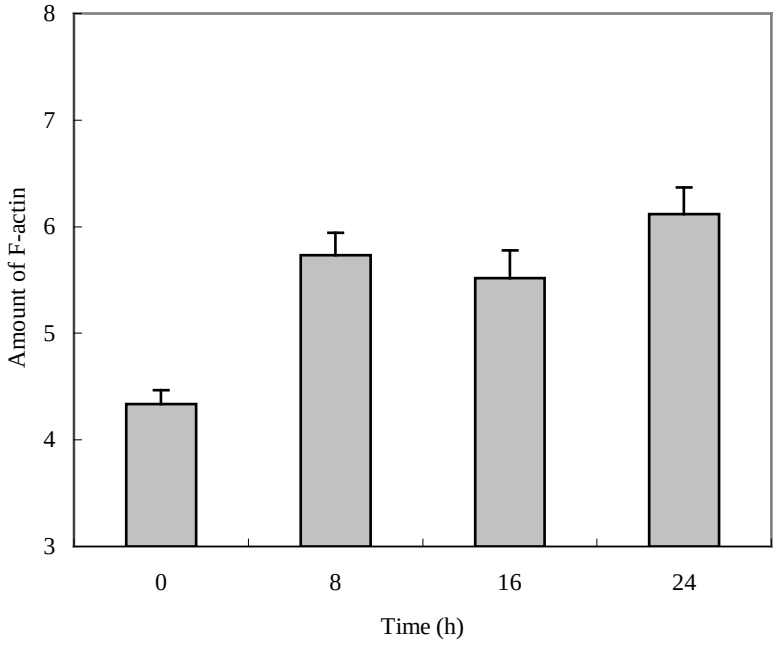

Fig. 5. Effect of 100 μM capsaicin on F-actin. Standard deviations were less than 10 %.