Protein D2 porin of the *Pseudomonas aeruginosa* outer membrane bears the protease activity

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Abstract **We report here our discovery that protein D2 of the outer membrane of *Pseudomonas aeruginosa* is a novel porin bearing protease activity. Homogeneously purified protein D2 hydrolyzed several synthetic peptides according to the Michaelis-Menten kinetics. A specific serine protease inhibitor, diisopropyl fluorophosphate (DFP), inactivated the protease activity and [^{3}H]DFP covalently labeled protein D2. We tested the effect of two monoclonal antibodies raised against protein D2 on the protease activity. One antibody lowered the protease activity to about 20%, while the other enhanced it to about 300% of that without antibody. In addition, the fractions derived from the outer membrane of the protein D2-deficient mutants showed negligible protease activity, whereas similarly fractionated outer membrane proteins of the protein D2-positive parent strain showed strong protease activity.**

Key words: Protein D2; Protease; *Pseudomonas aeruginosa*

1. Introduction

Pseudomonas aeruginosa, which is pathogenic to immunocompromised and cystic fibrosis patients, produces three species of porins (proteins C, D2 and E1) that form channels narrower than the *E. coli* OmpF porin [1]. Among the *P. aeruginosa* porins, protein D2 shows the following unique characteristics: Protein D2 (an M_r of 46 kDa) is composed of the 27-kDa and 19-kDa domain responsible for channel formation and gate formation, respectively [2,3]. This unique structural feature is reflected in its channel function that protein D2 forms a channel of about 400 pico Siemens in 1 M NaCl as reconstituted in lipid bilayers and flickers frequently [4]. The amino acid sequence deduced from the nucleotide sequence of the cloned *opr*D gene showed typical features of the porin proteins [5,6]. Furthermore, clinical and laboratory isolates of *P. aeruginosa* resistant to imipenem, one of the β-lactam antibiotics, were found to lack protein D2 exclusively [7–9]. These results suggest that the protein D2 channel allows preferential permeation of imipenem. In fact, the protein D2 porin was found to have binding affinity towards imipenem and basic amino acids having a similar structure with imipenem, implying that it forms a pore with specificity [10,11]. When the channel conductivity of protein D2 was determined in the presence of lysine, arginine or imipenem, the channel size appeared to be smaller and the opening of the channel became less frequent [12]. These results firmly established in the single molecular level that protein D2 interacts with imipenem and basic amino acids. Close examination of these results raised the question of how the ligand binding site was derived. We hypothesize that the ligand binding activity might be derived from serine protease, since, as we report here, the protein D2 porin shows weak protease activity.

2. Materials and methods

2.1. Bacterial culture and purification of protein D2

P. aeruginosa KG1079 (protein F-deficient strain) was grown in L-broth supplemented with 5 mM $MgCl_2$ for 5 h at 37°C after a 10-fold dilution of fully grown preculture. The bacterial cells were suspended in 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer, pH 7.4 and were passed through a French pressure cell at 1200 kg/cm^2 3 times. Unbroken cells were removed by centrifugation. The membranes were pelleted by centrifugation at 100000×*g* for 45 min at 15°C and suspended in 20 mM HEPES buffer, pH 7.4. The membrane suspension was mixed with 1% (wt/vol) of N-lauroyl sarcosinate and incubated at 30°C for 30 min. The outer membrane proteins were pelleted by centrifugation at 100000×*g* for 45 min at 15°C and washed two times with 200 mM HEPES or sodium phosphate buffer, pH 7.5.

Protein D2 was purified according to the methods described earlier [1]. The outer membrane proteins were mixed with 2% (wt/vol) of β-octylglucoside (β-OG) and the supernatant after centrifugation at 100000×*g* for 45 min was applied onto a DEAE ion-exchange HPLC column (TSK gel DEAE-5PW, 7.5 × 0.75 cm i.d.) equilibrated with 10 mM Tris-HCl buffer, pH 8.0 containing 1% of β-OG or 1% n-octyl-polyoxyethylene (octyl-POE), the column was eluted with a 0 to 0.3 M linear gradient of NaCl in the above buffer. This purification process was repeated until the preparation become homogeneous as checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Fig. 2B, lane 2).

2.2. Determination of protease activity

The enzyme activity was assayed according to the method described by Kawabata et al. [13]. Typically, 2 μl of tert-butoxycarbonyl (Boc)-Gln-Ala-Arg-methylcoumarin (MCA) in dimethyl sulfoxide (DMSO) was added to a 0.2 ml solution containing 10 mM HEPES buffer, pH 7.5, 5 mM octaethyleneglycol dodecylether ($C_{12}E_8$) (Buffer A) and appropriate amount of protein D2, then the mixture was incubated at 37°C. Fluorescence of 7-amino-4-methylcoumarin (AMC) liberated by the hydrolysis of MCA-peptides was measured at 440 nm with an excitation wavelength of 380 nm using a Hitachi fluorescence spectrophotometer 650-10S.

2.3. Monoclonal antibody against protein D2

Monoclonal antibody (MAb) against protein D2 was raised according to the procedure described earlier [14].

2.4. Isolation of the protein D2-deficient mutants

The KG1079 cell suspension (100 μl) grown in 10 ml of L-broth containing 5 mM $MgCl_2$ at 37°C for 6 h was plated on agar containing 0.18 or 0.27 μg per ml of DX8739, one of the carbapenem antibiotics, and the plates were incubated at 37°C. Colonies grown on these DX8739-impregnated plates were selected. More than 90% of the DX8739-resistant mutants were devoid of protein D2.

3. Results and discussion

3.1. Protease activity of the homogeneously purified protein D2

Homogeneously purified protein D2 dissolved in 10 mM

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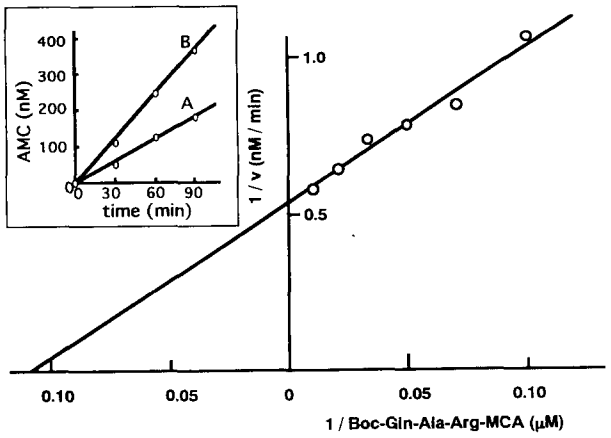

Fig. 1. Hydrolysis of Boc-Gln-Ala-Arg-MCA. Protein D2 (3.35 μM) purified to homogeneity was mixed with Boc-Gln-Ala-Arg-MCA in 150 μl of Buffer A. An aliquot (60 μl) of the reaction mixture was withdrawn at time indicated and diluted with 1 ml of 100 mM Tris-HCl, pH 8.0. The amount of AMC produced was determined. The amount of AMC in the mixture without protein D2 was subtracted from the value obtained. Inverse values of the reaction rate were plotted against the reciprocal of the substrate concentration. Inset: Time course of Boc-Gln-Ala-Arg-MCA hydrolysis. Protein D2 (3.73 (A) or 7.45 μM (B)) was mixed with 200 μM of Boc-Gln-Ala-Arg-MCA in 200 μl of Buffer A and incubated at 37°C.

HEPES buffer, pH 7.5, in the presence of 5 mM $C_{12}E_8$, was mixed with Boc-Gln-Ala-Arg-MCA (200 μM) and incubated at 37°C. Fig. 1 inset shows that the hydrolysis of synthetic substrate proceeded in time after protein D2 was added to the reaction mixture and that the rate of substrate hydrolysis was linearly proportional to the amount of protein D2 added to the mixture. The results depicted in Fig. 1 show that the hydrolytic reaction proceeds according to the Michaelis-Menten kinetics and the values of K_m and k_{cat} were calculated to be 9.0 μM and 8.6×10^{-6} s^{-1}, respectively. Although the K_m value was reasonably low, the k_{cat} value was 6 orders of magnitude lower than that of trypsin against this peptide.

To examine the substrate specificity of the protein D2 protease, we compared the rates of hydrolysis of various MCA-peptides with that of Boc-Gln-Ala-Arg-MCA (Table 1). The results show that the protein D2 efficiently hydrolyzed the peptide bond at the carboxyl side of the arginine and lysine residues, but hydrolysis of the peptide bond adjacent to Phe, Ala or Val was negligible.

Next, we investigated the effect of the specific serine protease inhibitor, diisopropyl fluorophosphate (DFP), on the activity of protein D2 protease. We treated protein D2 with 1 mM DFP for 60 min at 37°C, then determined the protease activity in the presence of 200 μM of Boc-Gln-Ala-Arg-MCA at 37°C. As shown in Fig. 2A, the activity was mostly abolished by the DFP treatment, indicating that protein D2 is likely a member of a serine protease family. To assure that this protease activity is attributed to protein D2, we analyzed protein D2 treated with [^{3}H]DFP on SDS-PAGE and fluorography. Fig. 2B shows only a single radioactive band throughout the lane and the location of this band corresponded to that of the protein D2 stained with Coomassie brilliant blue. However, radioactive DFP was inefficiently incorporated into protein D2 that may be due to partial inactivation of protein D2 during the course of purification as discussed below. This protein band was confirmed to be protein D2 as tested by the Western blotting method using MAbs specific to protein D2 (data not shown). We concluded from these results that protein D2 possesses protease activity in addition to the channel function.

3.2. *The protease activity of the fractions from protein D2-deficient strain*

Although the results shown above established that protein D2 bears a protease activity, the catalytic activity (k_{cat}) of protein D2 appeared to be extremely low, leaving some question on the physiological significance of the protease activity. We considered the possibility that protein D2 might be inactivated. Then, the outer membrane proteins were solubilized with 1.5% of octyl-POE and subjected to the DEAE chromatography in the buffer containing 1% of octyl-POE. The fractions eluted at about 0.15 M NaCl contained protein D2 and other proteins and hydrolyzed Boc-Gln-Ala-Arg-MCA at the rate of 5.7×10^{-11} mol·s^{-1}·mg protein^{-1}. The K_m value to Boc-Gln-Ala-Arg-MCA was nearly identical to that (8.3×10^{-6} M) of the highly purified protein D2. The calculated k_{cat} value appeared to be 0.013 s^{-1}, as the protein D2 content (20% which was determined by the densitometer tracing of the gel) in this fraction was normalized. To establish that this proteolytic reaction is carried out by protein D2 itself, but not by other proteins contained in this fraction, the protein D2-deficient strains were isolated by the method described above and confirmed to be lacking protein D2 in the outer membrane by SDS-PAGE (data not shown). The outer membrane proteins from the protein D2-deficient mutant were fractionated as above in the buffer containing 1% of octyl-POE. We found that the fractions where protein D2 was expected to be eluted, i.e. about 0.15 M NaCl, showed an undetectable protease activity. When the plasmid carrying the *opr* D gene was introduced into the protein D2-deficient strain by transformation, the outer membrane fraction fully recovered protein D2 and the protease activity was concomitantly recovered (to be published).

In order to make sure that the protease activity in this fraction is attributed to protein D2, we tested the effect of MAbs specific to protein D2 on the protease activity of this fraction. YD15, one of the MAbs as shown in Fig. 3 inset, was added to the above partially purified protein D2 and mixed with 10 μM Boc-Gln-Ala-Arg-MCA, then incubated at 37°C. YD15 inhibited the protease activity in a concentration-dependent manner (Fig. 3). Another MAb, YD6, in-

Table 1
Proteolytic activity of protein D2 towards various MCA substrates

Substrates	Relative rate
Bz-Arg-MCA	57
Bz-Arg-Arg-MCA	7
Boc-Gln-Ala-Arg-MCA	100
Boc-Gln-Arg-Arg-MCA	11
Boc-Glu(OBzl)-Ala-Arg-MCA	60
Boc-Gln-Gly-Arg-MCA	97
Z-Phe-Arg-MCA	42
Boc-Val-Leu-Lys-MCA	24
Suc-Ala-Ala-Pro-Phe-MCA	0
Suc-Ala-Pro-Ala-MCA	3
Suc-Ala-Ala-Pro-Val-MCA	0

Protein D2 (6.9 μM) was mixed with 200 μM of MCA substrates and incubated at 37°C. The rate of increase in fluorescence due to hydrolysis was expressed as a percentage of that of Boc-Gln-Ala-Arg-MCA.

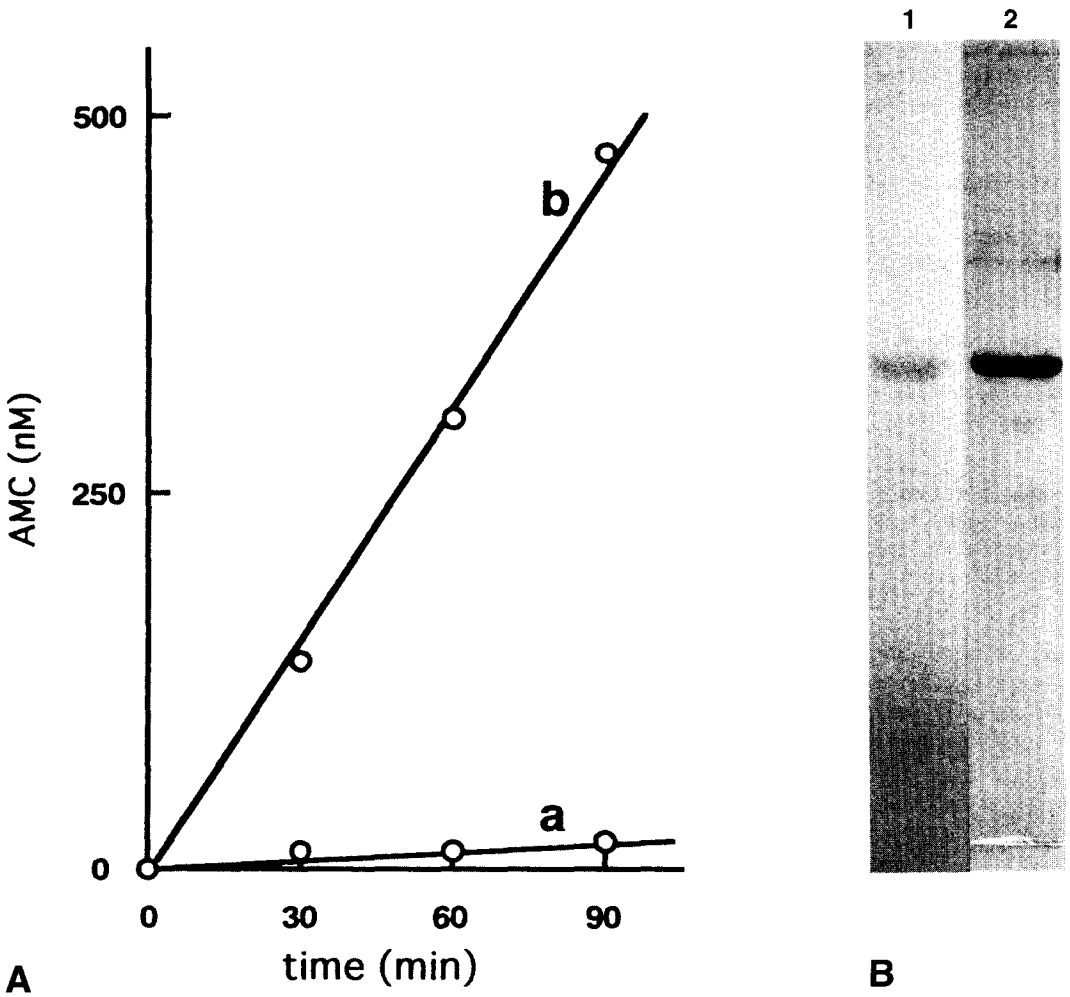

Fig. 2. Effect of DFP on the enzyme activity and specific labeling of protein D2. A: Protein D2 (3.55 μM) in 200 μl of Buffer A was mixed with 1 mM DFP dissolved in DMSO or an equivalent amount of DMSO without DFP and the mixture was preincubated at 37°C for 60 min. The enzymatic reaction was initiated by addition of 100 μM of Boc-Gln-Ala-Arg-MCA and incubated at 37°C for the desired period of time. (a): with 1 mM DFP; (b): without DFP. B: Protein D2 (8.7 μM) was mixed with 40 μM of [^{3}H]DFP (New England Nuclear) and incubated at 37°C for 12 h. The reaction mixture was diluted with a 20-fold volume of cold acetone, centrifuged at 16000×g for 10 min and subjected to SDS-PAGE. The gel was treated with PPO, dried and subjected to fluorography with Kodak X-ray film at −70°C for 2 months (lane 1). The gel was stained with Coomassie brilliant blue (lane 2). An arrow indicates the location of protein D2.

creased the protease activity a three-times higher level than that without YD6 (data not shown). On the contrary, murine immunoglobulin from normal serum exerted an undetectable effect on the protease activity (Fig. 3).

We are interested in whether the amino acid sequence of protein D2 shares similarity with other proteases. We conducted a computer-aided homology search of the amino acid sequence between protein D2 and other serine proteases (Fig. 4). Alignment of the amino acid sequence between Trp^{115} and Gly^{346} of protein D2 and those of trypsin and chymotrypsin showed low overall sequence similarity. However, His^{156}, Asp^{208} and Ser^{296} sequence of protein D2 seems to have a sequence similarity with the catalytic triad, His^{57}, Asp^{102} and Ser^{195} of chymotrypsin and that of His^{55}, Asp^{99}

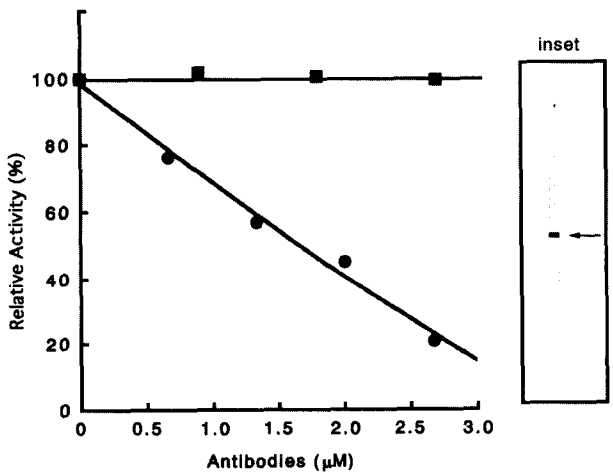

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Fig. 3. Effect of monoclonal antibody on the protease activity of the partially purified protein D2. The various amounts of YD15 or normal serum immunoglobulin were added to the partially purified protein D2 and incubated at 37°C for 30 min. Enzyme reaction was started by tipping Boc-Gln-Ala-Arg-MCA (10 μM) in the above mixture. Protease activity in the presence of antibodies was expressed as percent of that in the absence of antibody. Symbols, ●, YD15; ■, Normal serum immunoglobulin. Inset: Specificity of the MAbs, YD 15 was tested by immunoblotting of the whole outer membrane proteins. An arrow indicates the location of protein D2.

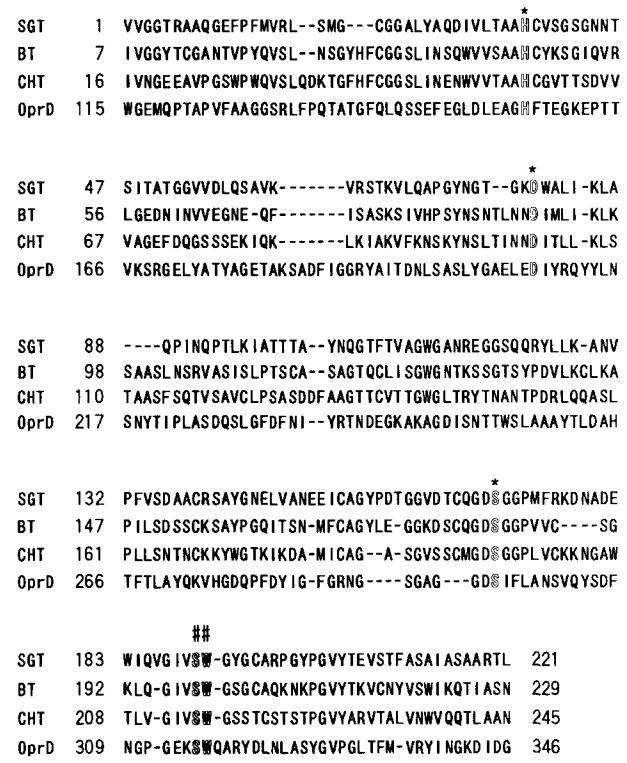

Fig. 4. Alignment and comparison of the amino acid sequence of protein D2 with that of serine proteases. The amino acid sequence from Trp^{115} to Gly^{346} of protein D2 (OprD) was compared with that of serine proteases, including *Streptomyces griseus* trypsin (SGT), bovine trypsin (BT) and bovine chymotrypsin (CHT). The residues of the catalytic triad forming the substrate binding sites are indicated by * and #, respectively.

and Ser^{192} of trypsin. Moreover, Ser^{315} and Trp^{316} of protein D2 coincide with Ser^{214} and Trp^{215} of trypsin in forming the substrate binding S_1 and S_2 subsites, respectively, indicating that the subsite structure in the protein D2 protease is similar to that of serine proteases. As Huang et al. recently proposed the topology of protein D2 [15], we superimposed His^{156}, Asp^{208} and Ser^{296} of protein D2 on this model. His^{156} and Asp^{208} were localized on the extracellular loop L3 and L4, respectively, and Ser^{296} was at the second residue from the extracellular surface of membrane spanning β-strand 12. Therefore it is likely that the proteolytic reaction is carried out at the extracellular mouth or near it of the protein D2 channel.

Function of this protease remains to be clarified. However, we propose on the basis of these results and those of others [10,11] that the protein D2 porin binds peptides, hydrolyses them and facilitates the permeation of peptides and amino acids small enough to pass through the protein D2 pore. Alternatively, it is more likely that the main function of the active site is to bind ligands and facilitate the diffusion through the pore. It is possible therefore that the binding site was derived originally from the serine protease. Among known channel proteins, this is the first report, to the best of our knowledge, demonstrating that a channel protein bears a protease activity.

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References

[1] Yoshihara, E. and Nakae, T. (1989) J. Biol. Chem. 264, 6297–6301.
[2] Yoshihara, E. and Nakae, T. (1992) FEBS Lett. 306, 5–8.
[3] Yoshihara, E. and Nakae, T. (1993) Biochem. Biophys. Res. Commun. 194, 1460–1465.
[4] Ishii, J. and Nakae, T. (1993) FEBS Lett. 320, 251–255.
[5] Yoneyama, H., Yoshihara, E. and Nakae, T. (1992) Antimicrob. Agents Chemother. 36, 1791–1793.
[6] Huang, H., Siehnel, R.J., Bellido, F., Rawling, E. and Hancock, R.E.W. (1992) FEMS Microbiol. Lett. 97, 267–274.
[7] Quinn, J.P., Dudek, E.J., DiVincenzo, C.A., Lucks, D.A. and Lerner, S.A. (1986) J. Infect. Dis. 154, 289–294.
[8] Büscher, K.H., Cullman, W., Dick, W., Wendt, S. and Opferkuch, W. (1987) J. Infect. Dis. 156, 681–684.
[9] Lynch, M.J., Drusano, G.L. and Mobley, H.L.T. (1987) Antimicrob. Agents Chemother. 31, 1892–1896.
[10] Trias, J. and Nikaido, H. (1990) Antimicrob. Agents Chemother. 34, 52–57.
[11] Trias, J. and Nikaido, H. (1990) J. Biol. Chem. 265, 15680–15684.
[12] Ishii, J. and Nakae, T. (1996) FEMS Microbiol. Lett. 136, 85–90.
[13] Kawabata, S., Miura, T., Morita, T., Kato, H., Fujikawa, K., Iwanaga, S., Takada, K., Kimura. T. and Sakakibara, S. (1988) Eur. J. Biochem. 172, 17–25.
[14] Hosaka, M., Gotoh, N. and Nishino, T. (1995) Antimicrob. Agents Chemother. 39, 1731–1735.
[15] Huang, H., Jeanteur, D., Pattus, F. and Hancock, R.E.W. (1995) Mol. Microbiol. 16, 931–941.