Importance of Interactions of the α -Helices in the Catalytic Domain N- and C-Terminals of the Family 10 Xylanase from *Streptomyces olivaceoviridis* E-86 to the Stability of the Enzyme*

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Abstract: The intact crystal structure of family 10 xylanase (SoXyn10A) from *Streptomyces olivaceoviridis* indicates that the catalytic domain of SoXyn10A consists of nine α -helices ($\alpha 0$ –8) and eight β -sheets ($\beta 1$ –8). Interaction in the α -helices of N-terminal ($\alpha 0$) and C-terminal ($\alpha 8$) of catalytic domain of SoXyn10A by 3 hydrogen bonds and 8 hydrophobic interactions is observed and predicted to playing an important role for the stability of the molecule. Therefore, the importance for the stability and folding of SoXyn10A were examined by using C-terminal truncated mutants of SoXyn10A. The thermostability was gradually decreased when the C-terminal was shortened; however, the enzyme activities were not influenced by the length of the C-terminal. The investigation of the stability using guanidine hydrochloride agreed with the expected results; namely the hydrophobic core was completed at Leu-300 and the resulting stability was not changed if the C-terminal was longer than it. The thermostability slightly decreased when Asn-252 was replaced with Ala, suggesting hydrogen bonding of Gly-303 with Asn-252 is also important for the stability of the molecule. When the effect of the interaction was observed in chimeric xylanases, which have a slight distortion in the structure, the C-terminal 4 amino acids certainly increased the thermostability of the chimeric enzymes. However, the N- and C-terminal of CfXyn10A from *Cellulomonas fimi* displayed by that of SoXyn10A decreased thermostability at the same degree as SoXyn10A, suggesting that the limit temperature of the interaction agrees with that of SoXyn10A and the distortion of both terminals make denaturing of the protein easy.

Key words: endo- β -1,4-xylanase, glycoside hydrolase family 10, thermostability, *Streptomyces olivaceoviridis*

The plant cell wall consists mainly of a complex mixture of polysaccharides such as cellulose, hemicellulose and pectin.¹⁾ Xylan is the major component of the hemicelluloses found in plant cell walls. The backbone of xylan is formed by β -1,4-linked D-xylopyranose units to which several side groups such as α -1,2-linked 4-O-methyl D-glucuronic acid and α -1,3-linked L-arabinofuranose are attached.²⁾ β -Xylanase (EC 3.2.1.8) randomly hydrolyzes β -1,4-glycosidic linkages within the xylan backbone to yield short chain xylooligosaccharides of varying length. Xylanases have many commercial uses, such as in the paper manufacturing, animal feed, bread-making, juice and wine industries, and xylitol and xylooligosaccharide production.³⁾

Thermostability is an important property of industrially significant enzymes. Understanding the structural basis for this attribute will underpin the future biotechnological exploitation of these biocatalysis. However, the term thermostability does not refer to a well-defined physico-

chemical property of a protein, but is generally used to indicate the ability of some proteins to function at elevated temperatures and to withstand them for some time. Such an ability implies that the protein is able to remain folded in its functional native state under such conditions. Because many other factors besides temperature affect protein stability, thermostability at best can only be loosely defined. A number of previous studies have shown that various factors can increase protein thermostability, such as an efficient packing of the hydrophobic core,^{4–6)} electrostatic interactions such as salt bridges and hydrogen bonds,^{5,7,8)} and the stabilization of helix dipoles.⁹⁾

Streptomyces olivaceoviridis E-86 produces both family 10 and 11 xylanases.^{10,11)} The family 10 xylanase (SoXyn10A) has been well characterized.^{10,12–22)} SoXyn10A consists of two domains, a catalytic domain (belonging to GH family 10) and a carbohydrate-binding module (CBM) (belonging to CBM family 13), both of which are connected by a Gly/Pro-rich linker region.¹⁰⁾ The deletion of the CBM of SoXyn10A resulted in about a 20°C decrease in the optimum temperature of SoXyn10A from *Streptomyces olivaceoviridis* E-86 after removal of the C-terminal CBM.¹⁹⁾

* Importance of N- and C-Terminals Interaction in a Xylanase.

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Generally, the removal of CBMs does not affect any of the other properties of the enzymes except that of the hydrolytic capability toward insoluble substrates.¹⁶⁾ However, there are some reports in which the thermostability of family 10 xylanases is reduced after the removal of C-terminal CBMs.^{17–19)} For example, the thermostability displayed by a family 10 xylanase from *Streptomyces halstedii* was reduced when the enzyme lost its C-terminal CBM by proteolytic cleavage.^{17,18)} Therefore, both the N- and C-terminal regions of the CBM seem to play an important role in the stability of the barrel module.

Herein, we report the importance of the interactions between the N- and C-terminal regions of the catalytic domain in determining the stability of the molecule using C-terminal truncated mutants and chimeric xylanases.

MATERIALS AND METHODS

Construction and production of mutant enzymes. To express SoXyn10A and its C-terminal truncated mutants, the appropriate DNA sequences were amplified by the polymerase chain reaction (Fig. 3) and the resulting fragments were cloned into the pQE60 vector (QIAGEN K. K.) as described previously.²³⁾ Each DNA fragment was amplified using a set of primers which included either the *Nco*I or the *Bam*HI restriction sites, enabling the PCR products to be cloned into *Nco*I/*Bam*HI-restricted pQE60. pET28a/CfXyn10A was constructed as described previously.²⁴⁾ The construction and expression of the chimeric xylanases such as FCF-C4 and FCF-C5 are described in another paper.²⁵⁾ CfXyn10A-AESTL and CfXyn10A-AETTL were constructed, as shown in Fig. 4, and, with the aid of the signal sequence from SoXyn10A, the enzymes were produced. The *Stu*I site was introduced by an inverse PCR right after the signal sequence of SoXyn10A and pQE60/SoXyn10A was used as a template, then it was digested with *Stu*I and *Bam*HI. Alternatively, the appropriate CfXyn10A gene was amplified using a primer designed to encode the amino terminal and C-terminal of SoXyn10A with the *Stu*I and *Bam*HI restriction sites. pQE60/CfXyn10A-AESTL and pQE60/CfXyn10A-AETTL were constructed by the adhesion of the appropriate fragments. For the production of the enzymes, a pET28a vector was employed as described in a previous report.²⁴⁾

Circular dichroism spectra and steady state kinetic studies. The circular dichroism (CD) spectra of CfXyn10A, CfXyn10A-AETTL and CfXyn10A-AESTL were measured using conditions reported previously.^{24,26)} Steady-state kinetics were investigated as previously reported;^{24,26)} briefly the reaction mixture containing *p*-nitrophenyl- β -D-xylobioside (PNP-X₂) at various concentrations in 25% (w/v) McIlvaine buffer (a mixture of 0.1 M citric acid and 0.2 M Na₂HPO₄, pH 7.0) containing 0.05% (w/v) bovine serum albumin (BSA) was incubated at 30°C for 5 min and then 50 μ L of enzyme solution was added. The amount of *p*-nitrophenol (PNP) released was determined by monitoring the absorbance at 400 nm with a spectrophotometer (DU-7400; Beckman). PNP-X₂ was synthesized by the method described in a previous paper.²⁷⁾ The xylobiose used in the synthesis was purified

from 'Xylobiose Mixture' (Suntory Holdings Ltd.).

Measurement of enzyme activity. Each assay mixture contained 250 μ L of a 2 mM PNP-X₂ solution, 200 μ L of 0.2 M phosphate buffer, pH 5.7 and 50 μ L of enzyme solution. The reactions were carried out at the specified temperatures for 10 min whereupon they were stopped by the addition of 0.5 mL of a 0.2 M Na₂CO₃ solution, and the amount of PNP released was determined at 408 nm (referred to hereafter as the standard method).

To determine the optimum temperatures of the various enzymes, the enzyme activities were measured at selected temperatures. Each enzyme was adjusted to the same concentration (0.05 U/mL at 30°C) before use in the assay.

The stabilities of the enzymes in guanidine hydrochloride were investigated as follows. The enzyme solution (0.05 U/mL at 30°C) was pre-treated with guanidine hydrochloride at 25°C for 30 min. The residual enzyme activity was then measured at 30°C under the same concentration of guanidine hydrochloride used for the pre-treatment step.

The thermostabilities of the enzymes were determined as follows. The enzyme solution (0.05 U/mL at 30°C) was pre-incubated at various temperatures for 2 h. The residual enzyme activity was then measured at 45°C.

RESULTS

Interaction of the N- and C-terminals in the catalytic domain of SoXyn10A.

SoXyn10A consists of 436 amino acids.²³⁾ SoXyn10A consists of a catalytic domain and a CBM, and a Gly-rich linker which couples the two modules.²³⁾ The crystal structure of intact SoXyn10A shows that the catalytic domain consists of nine α -helices (α 0–8) and eight β -sheets (β 1–8) and that the domain is completed at the end of α 8, at residue Asn-301.²⁰⁾ The linker, starting at Gly-302 and finishing at Ser-316, is followed by the family 13 CBM running from Gly-317 through to Thr-436.²⁰⁾ As shown in Fig. 1, interactions between the α -helices of the N-terminal (α 0) and C-terminal (α 8) of the catalytic domain of SoXyn10A are observed. Hydrophobic interactions are formed by Leu-5, Ala-8 and Ala-9 in α 0, Phe-16 in β 1, and Leu-282, Phe-283, Tyr-293, Val-296, Leu-297 and Leu-300 in α 8. Three hydrogen bonds, namely N δ 2 and O δ 1 of Asn-301 in α 8 hydrogen bonding with O of Glu-2 and Ne2 of Gln-11 in α 0, respectively, and O η of Tyr-293 hydrogen bonding with O ϵ 1 of Glu-37, cover the hydrophobic core so that it does not appear on the outer surface of the molecule. These interactions are predicted to be important for the stability of the molecule. The catalytic domain alone (residues 1–303, abbreviated as Cat (303)) expressed in *Escherichia coli* shows the enzymatic activity;¹⁹⁾ therefore, we constructed C-terminal truncated mutants (Cat (299), residues 1–299; Cat (300), residues 1–300; and Cat (301), residues 1–301) (Fig. 2) to investigate the importance of these regions for the thermostability of the enzyme.

Enzyme activity and stability of the C-terminal truncated mutants of SoXyn10A.

The kinetic parameters of the mutant enzymes for PNP-

Fig. 1. Interaction of the N- and C-terminals of the catalytic domain of SoXyn10A.

(A) Whole view of the crystal structure of SoXyn10A complexed with arabinofuranosylxylotriase (pdb code 1v6v).²¹⁾ (B) Close view of the interface between the N- and C-terminals of the catalytic domain. Hydrogen bonds are shown as dashed lines colored in light blue and the side chains of the amino acids forming the hydrophobic core are shown in orange. The N-terminal region including α -helices, α_0 and α_1 , is shown in pink and the C-terminal region from α -helix α_8 is shown in green. Bound arabinofuranosylxylotriase was colored in blue.

Fig. 2. Construction of C-terminal truncated mutants of SoXyn10A.

SoXyn10A consists of 436 amino acids comprising a catalytic domain, a CBM and a Gly-rich linker.²⁰⁾ The intact crystal structure of SoXyn10A suggests the catalytic domain consists of 301 amino acids (1–301) and that the linker starts at Gly-302 and ends at Ser-316 followed by the CBM which runs from Gly-317 to Thr-436.²⁰⁾ The C-terminal truncated mutants were constructed by PCR according to the method described in a previous report.²⁰⁾

X_2 are shown in Table 1. There are no significant differences in either the K_m or k_{cat} values of any of the enzymes, indicating that the deletion of the C-terminal of the enzyme did not affect enzyme activity. In the crystal structure of SoXyn10A, the hydrophobic core of the catalytic domain is completed at Leu-300 and several hydrogen bonds covering the core prevent the hydrophobic amino acids appearing the outer surface of the molecule. To confirm the hypothesis that the hydrogen bonds help to protect the hydrophobic core, the stabilities of the mu-

Table 1. Activity of native and mutants of SoXyn10A and CfXyn10A against PNP- X_2 .

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/mM)
SoXyn10A	2.1	40	19
Cat (303)	2.1	42	20
Cat (299)	1.9	41	22
CfXyn10A	0.012	7.0	583
CfXyn10A-AETTL	0.012	8.2	683
CfXyn10A-AESTL	0.015	10.8	720

CfXyn10A	1	A-TTLKEAADQAGR	14	- - -	-305	YAAVMEAF	312
SoXyn10A	1	AESTLGAAAQSGR	14	- - -	-293	YTAVLNALNGG	303
CfXyn10A-AETTL	1	AETTLKEAADQAGR	14	- - -	-305	YAAVMEAFNGG	315
CfXyn10A-AESTL	1	AESTLGAAAQSGR	14	- - -	-305	YAAVLNALNGG	315

Fig. 3. Construction of CfXyn10A-AESTL and CfXyn10A-AETTL.

A pQE60 vector with the SoXyn10A signal sequence was constructed using the inverse PCR method. The CfXyn10A gene was amplified by PCR, and it was introduced into the vector at the *StuI/BamHI* restriction site.

tants in guanidine hydrochloride were measured. The results of the stability measurements in guanidine hydrochloride are shown in Fig. 4 (A). The stabilities of all of the enzymes tested, except that of Cat (299) which displayed a reduction in its stability relative to the other enzymes, were the almost the same. In the crystal structure of SoXyn10A, the hydrophobic core of the catalytic domain is completed at Leu-300 and several hydrogen bonds covering the core prevent the hydrophobic amino acids appearing the outer surface of the molecule. The guanidine hydrochloride weakens both the hydrophobic and electrostatic interactions. The difference in the stability seems to be observed when the key interaction stabilizing the SoXyn10A molecule is broken. That is to say, Leu-300 which completes the hydrophobic core of N- and C-terminals, greatly contributes to the stability of the molecule. This result suggests that the hydrophobic core in the N-terminal-C-terminal interaction is complete at residue Leu-300. In contrast, the thermostability gradually decreased in relation to the decreasing length of the C-terminal (Fig. 4 (B)). These denaturations of the enzymes were non-reversible. The optimum temperatures of each of the mutants were 60, 55, 50 and 45°C for Cat (303),

Fig. 4. The stability in guanidine hydrochloride (A), thermostability (B) of the C-terminal truncated mutants of SoXyn10A and thermostability of the Cat (303)-N252A mutant (C).

●, Cat(303); ○, Cat(303)-N252A; ▼, SoXyn10A; ■, Cat(299); ▲, Cat(300); ◆, Cat(301).

Fig. 5. Interaction between Gly-303 and Asn-252 in the N- and C-terminal interaction of Cat (303).

Fig. 6. The thermostability of chimeric xylanases and their C-terminal truncated mutants.

○, FCF-C4 (299); ●, FCF-C4 (303); □, FCF-C5 (299); ■, FCF-C5 (303).

Fig. 7. The stability in guanidine hydrochloride (A), CD spectroscopy (B) and thermostability (C) of CfXyn10A, CfXyn10A-AESTL and CfXyn10A-AETTL.

×, CfXyn10A-AETTL; ★, CfXyn10A; ●, Cat (303); +, CfXyn10A-AESTL.

Cat (301), Cat (300) and Cat (299) respectively. The vertical axis of Fig. 4 (B) shows that the activity of each of the enzymes is referenced to 1.0 at 30°C, reflecting the specific activity of each of the enzymes at the various temperatures. The activities of all of the enzymes up to 45°C are identical, indicating that the loss of enzyme activity directly reflects the stability of the N- and C-terminal interactions. To our surprise, the stability of Cat (303) and Cat (301) were not the same, but that was explained by the crystal structure of Cat (303). An additional hydrogen bond between Asn-252 and Gly-303 was observed (Fig. 5) and it seemed to influence the stability of the molecule. To test the hypothesis, the N252A mutant was constructed. The stability of the N252A mutant, as shown in Fig. 4 (C), supports the concept that the hydrogen bond between Asn-252 and Gly-303 relates directly to the stability of the catalytic domain. The number of hydrogen bonds and hydrophobic interactions are directly related to the thermostability of the enzyme.

Effect of the interaction of the N- and C-terminal regions of the catalytic domains of the chimeric enzymes.

It was evident from the investigations described above that the 4 amino acids of the C-terminal of the catalytic domain (*i.e.* those involved in the interaction between the C- and N-terminals) were greatly involved in the stability of the enzyme. In order to examine whether the interaction of the C- and N-terminal regions was also effective in the chimeric xylanases, in which modules 4 or 5 were replaced by the CfXyn10A from *C. fimi* producing a slight distortion on the inside of their structure and additional strain in the folding of molecules,²⁵⁾ the thermostabilities of the chimeric xylanases were investigated. The results are depicted in Fig. 6 and they indicate that it is possible to make thermostable proteins even if the inside of the protein is distorted if both ends of the protein molecule are still able to firmly interact with each other.

Introduction of the interactions of the N- and C-terminals in CfXyn10A.

Because the interaction of the C- and N-terminals of SoXyn10A was effective for the stability of the molecule, the interactions of SoXyn10A were introduced in the other family 10 xylanase. CfXyn10A from *C. fimi* was selected for the target enzyme because the crystal structure of CfXyn10A has been reported²⁸⁾ and the amino acid sequence of N- and C-terminals of CfXyn10A is different from those of SoXyn10A (Fig. 3).

The introduction of the N- and C-terminal interactions to CfXyn10A did not alter the observed kinetic parameters of the enzyme relative to the native CfXyn10A (Table 1). The stability of the chimeric CfXyn10A enzymes along with the native CfXyn10A are shown in Fig. 7. The stabilities of both chimeric CfXyn10A enzymes in guanidine hydrochloride were almost the same as for the native CfXyn10A (Fig. 7 (A)), indicating that the replacement of both terminals by those of SoXyn10A did not affect the overall structure of the enzyme. This is also supported by the CD spectra results (Fig. 7 (B)) and the kinetic studies (Table 1). In contrast, the thermostabil-

ity of the CfXyn10A enzyme decreased to the same level as that of Cat (303) (Fig. 7 (C)) in spite of the stabilities of CfXyn10A, CfXyn10A-AETTL and CfXyn10A-AESTL in guanidine hydrochloride being the same. The observation of a decrease in the thermostability of CfXyn10A mutants would indicate that the temperature limit for the interaction is 60°C (Fig. 7 (C)). The distortion of both terminals makes it easier to denature the protein. This corresponds to the observation with the *S. halstedii* xylanase in which XysVW, having an elongated linker region was less stable than Xys1S.^{17,18)}

DISCUSSION

Thermostability is one of the most important factors of enzymes that catalyze industrially relevant reactions. Therefore, understanding of the structural basis for thermostability has attracted considerable interest. In several reports, the structure of the thermostable family 10 xylanase was solved and the implications for the evolution of thermostability in family 10 xylanases and other (α/β)₈-barrel enzymes were discussed.^{29,30)} The structural analysis of family 10 xylanases outlined in the papers suggests that thermostability is acquired mainly by improved hydrophobic packing,^{29,30)} favorable interactions of charged sidechains with helix dipoles,^{29,30)} and the introduction of proline residues at the N-terminus of helices.²⁹⁾ However, the authors do not refer to the most terminal helices of the enzyme (*e.g.* $\alpha 0$) or the interaction of the N- and C-terminals which we were able to examine in this study and we have demonstrated that the interactions of the N- and C-terminal regions of the catalytic domain play a critical role in the stability of family 10 enzymes.

The hypothesis that the N- and C-terminal regions of family 10 xylanases are implicated in the thermostability of the enzyme has been suggested in several reports.^{10-19,31)} A family 10 xylanase from *S. halstedii* JM8 has been reported as consisting of an N-terminal family 10 catalytic domain, a C-terminal cellulose-binding domain and a Gly-rich linker connecting the two domains.³²⁾ The recombinant enzyme of this xylanase was proteolytically cleaved in the middle of the Gly-rich linker when the gene was expressed in *S. lividans* and the degraded enzyme (Xys1S) was found to be more stable than the wild type enzyme (Xys1L).¹⁷⁾ In addition, the length of the C-terminal was increased and decreased relative to Xys1S and the stability of these enzymes were less than that of Xys1S.¹⁸⁾ The other evidence to support the above hypothesis can be found in the thermostabilizing domain. This domain was first located in *T. maritima* XynA.³¹⁾ Removal of this domain significantly reduced the observed thermostability of the enzyme.³¹⁾ The homologue of this domain has been found in several xylanases and finally it was demonstrated that the thermostabilizing domain is actually a xylan-binding domain;^{14,15)} therefore it is apparent that the terminal of the catalytic domain is quite important to the stability of the molecule. The idea is supported by the several results. An additional hydrogen bond between Asn-252 and Gly-303 in SoXyn10A increased the thermostability of the enzyme (Fig. 4 (C)). The other evidence to support the above idea was found in the other family 10

xylanases. Introducing a disulphide bond between the disordered N and C termini of CmXyn10B from *Cellvibrio mixtus* conferred an increase in thermostability.^{33,34)} When the C-terminal of the catalytic domain of *S. lividans* XynA was elongated by four amino acids, X-ray crystallography data was successfully obtained at 1.2 Å resolution compared to the parental resolution of 2.6 Å.³⁵⁾

The investigations using chimeric enzymes suggested that the interaction is applicable in protein engineering because it is still possible to use the protein if it has some degree of strain inside the structure. Attempts to increase the thermostability of CfXyn10A were unsuccessful; however we have demonstrated the possibility of using these interactions to change the stability of the enzyme without changing other properties such as substrate specificity. The interaction of the N- and C-terminals is observed in all barrel type enzymes. Although a thermostabilizing domain does not exist,^{14,15)} this interaction is still expected to be able to be exploited in proteins with a thermostabilizing domain to change the stability of TIM-barrel proteins. In some proteins, the technique of circular permutation in which the conventional N-terminal is connected with the C-terminal to generate new N- and C-terminals has been studied.³⁵⁾ Therefore, the interaction of the N- and C-terminals observed in this study may be applicable to other proteins even when the protein does not have a (β/α)₈-barrel structure.

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**放線菌 *Streptomyces olivaceoviridis* E-86 由来
ファミリー 10 キシラナーゼの触媒ドメイン中
に存在する N 末端および C 末端 α ヘリックスの
酵素安定性における重要性**

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放線菌 *Streptomyces olivaceoviridis* E-86 由来ファミリー 10 キシラナーゼ (SoXyn10A) は触媒モジュール, 糖結合モジュールおよび両モジュールを繋ぐリンカーからなる。われわれは, これらすべてを含む結晶構造解析に成功した。その結果, 触媒ドメインは九つの α ヘリックス ($\alpha 0$ –8) と八つの β シート ($\beta 1$ –8) からなり, 触媒ドメイン中の N 末端 α ヘリックス ($\alpha 0$) と C 末端 α ヘリックス ($\alpha 8$) とが相互作用していることが明らかになった。結晶構造から, 両 α ヘリックス間には SoXyn10A の安定化に重要な結合があると予想された。そこで, SoXyn10A の安定化に寄与している $\alpha 0$ – $\alpha 8$ 間の結合を明らかにするために, SoXyn10A の C 末端欠損変異体を作成し, 欠損が酵素の安定化に及ぼす影響について調べた。触媒ドメインの C 末端のアミノ酸を一つずつ削り込んだ変異体では, 欠損の程度に準じて SoXyn10A の熱安定性が段階的に減少したが, 酵素活性には変化がみられなかった。塩酸グアニジンを用いて各変異体の安定性を比較した結果, 結晶構造から予測したとおり, $\alpha 8$ 中の Leu-300 が $\alpha 0$ と相互作用し疎水性コアを形成していることを明らかにした。さらに, リンカー中の Asn-252 を Ala に置換すると熱安定性がやや減少し, $\alpha 8$ 中の Gly-303 と Asn-252 間の水素結合もまた, SoXyn10A の安定性に重要であることを明らかにした。次に, 同じファミリー 10 キシラナーゼである *Cellulomonas fimi* の CfXyn10A と SoXyn10A とのキメラ酵素を作成し, これらの相互作用について検証した結果, C 末端の四つのアミノ残基の付加により, 熱安定性が上昇した。しかしながら, CfXyn10A に SoXyn10A の N 末端, C 末端を導入した場合には, SoXyn10A と同程度の熱安定性の減少が確認できた。N 末端と C 末端の相互作用の温度限界は SoXyn10A と一致しており, 両末端のゆがみによりタンパク変性しやすくなることを明らかにした。