Effects of hydrophobic amino acid substitution in Pleurotus ostreatus proteinase A inhibitor 1 on its structure and functions as protease inhibitor and intramolecular chaperone

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We previously demonstrated that Pleurotus ostreatus proteinase A inhibitor 1 (POIA1) could function as an intramolecular chaperone of subtilisin BPN', as in the case of the propeptide of subtilisin BPN', and that its Phe44→Ala mutant, which lost its tertiary structure, could not assist the refolding of subtilisin BPN'. In this study, we examined the effects of hydrophobic amino acid substitutions at other sites and substitutions of Phe44 with other hydrophobic residues on the structure and functions of POIA1. These mutations were introduced into POIA1cm that had been obtained by the substitution of the C-terminal six residues of POIA1 with those of the propeptide of subtilisin BPN'. When Ile32 or Ile64 was substituted with Ala, the tertiary structure of the resultant mutant was markedly destroyed, and the activities as a protease inhibitor and an intramolecular chaperone were significantly lowered. Among the position 44 mutants, the Phe44→Val mutant was a much less effective intramolecular chaperone with conversion to a digestible inhibitor, possibly owing to destruction of the tertiary structure. On the other hand, the Phe44→Leu or Ile mutant maintained its tertiary structure, and hence could function as a more effective intramolecular chaperone than the Phe44→Val mutant. Furthermore, since the Phe44→Leu mutant was a more susceptible inhibitor than POIA1cm, the halo formed around a colony of Bacillus cells transformed with a plasmid encoding this mutant was larger than others. These results clearly show the close relationship between the tertiary structure and functions of POIA1 as a protease inhibitor and an intramolecular chaperone, and that a combination of such inhibitory properties and intramolecular chaperone activity of POIA1 might affect the diameter of the halo formed around Bacillus colonies in vivo.

Keywords: amino acid substitution/intramolecular chaperone/POIA1/protease inhibitor/subtilisin refolding

Introduction

Pleurotus ostreatus proteinase A inhibitor 1 (POIA1) was isolated as an endogenous inhibitor of proteinase A, a subtilisin-type serine protease, from P. ostreatus (Dohmae et al., 1995). It is composed of 76 amino acids and has no sequence similarity to any other serine protease inhibitors except yeast proteinase B inhibitor 2 (YIB2) (Maier et al., 1979). However, its amino acid sequence is homologous to those of the propeptides of subtilisins, which are intramolecular chaperones of cognate proteases (Shinde and Inouye, 1993), although the similarity between POIA1 and the propeptides is not so high (approximately 20%). Unique features of the propeptides of subtilisins are their additional functions as protease inhibitors as well as intramolecular chaperones of cognate proteases (Ohta et al., 1991; Hu et al., 1996; Kojima et al., 1997). The crystal structure of a complex of the propeptide of subtilisin BPN' or subtilisin E with its cognate protease has revealed that the S1 pocket of the substrate-binding site of subtilisin is occupied by the side chain of the C-terminal residue of the propeptide (Bryan et al., 1995; Gallagher et al., 1995; Janin et al., 1998). Mutational analyses have also shown the importance of the C-terminal region of the propeptide (Wang et al., 1995), and the binding mode of the propeptide and its cognate protease by 'product inhibition' after cleavage between the propeptide and the mature region of subtilisin has been established.

The importance of the C-terminal region of YIB2 and POIA1 has also been shown by results of carboxypeptidase treatment (Maier et al., 1979; Dohmae et al., 1995). On the basis of this, we determined whether YIB2 and POIA1 are unique serine protease inhibitors that utilize their C-terminal region in binding to a protease and demonstrated that their inhibitory activities toward subtilisin BPN' are markedly increased by the substitution of the C-terminal six residues of YIB2 and POIA1 with those of the propeptide of subtilisin BPN' (Kojima et al., 1999, 2001). The subsequent determination of the solution structure of POIA1 by NMR spectroscopy (Sasakawa et al., 2002) showed that the three-dimensional structure of POIA1 is very similar to that of the propeptide of subtilisin BPN' in the complex with a cognate protease, in spite of a sequence similarity of about 20% to the propeptides. These results prompted us to examine the possibility of POIA1 functioning as an intramolecular chaperone of subtilisin. We have recently demonstrated such an additional function of POIA1 by in vivo and in vitro experiments using chimera proteins of POIA1 and subtilisin BPN' (Kojima et al., 2005). Wild-type POIA1 was shown to be an intramolecular chaperone of subtilisin BPN' assisting protease refolding, although its activity is very low compared with that of the propeptide of subtilisin BPN'. When POIA1 was converted to a strong inhibitor of subtilisin BPN' by the substitution of its C-terminal six residues with those of the propeptide, the resultant POIA1 (hereafter named POIA1cm) was shown to function as a more effective intramolecular chaperone of subtilisin BPN' than wild-type POIA1. However, POIA1cm is a resistant inhibitor that is hardly degraded by protease in contrast to the propeptide,
and Bacillus cells transformed with the plasmid encoding POIA1cm exhibit only small halos around their colonies (Kojima et al., 2005).

In a parallel study to investigate the inhibitory properties of POIA1, we have found that a Phe → Ala substitution at different sites (positions 44 and 56) of POIA1cm has different effects on the POIA1 structure, although both substitutions convert POIA1cm to digestible inhibitors (Kojima and Hisano, 2002). The tertiary structure of POIA1cm with a Phe44 → Ala substitution is destroyed, whereas that of POIA1cm with a Phe56 → Ala substitution is maintained. These Phe → Ala substitutions at different sites of POIA1cm also showed different effects on the intramolecular chaperone activity of POIA1cm; POIA1cm with a Phe56 → Ala substitution not only functions as an intramolecular chaperone with essentially the same activity as POIA1cm, but also produces a large halo around a Bacillus colony, because it is a digestible inhibitor, whereas POIA1cm with a Phe44 → Ala substitution exhibits no such activities. These results strongly indicate the importance of the tertiary structure of POIA1 functioning as an intramolecular chaperone of subtilisin BPN'.

In this study, we therefore planned substitutions of hydrophobic residues at other sites on the basis of results of a sequence comparison of POIA1 and the propeptides of subtilisins. As shown in Fig. 1, Ile32 and Ile64 of POIA1 are more hydrophobic and larger than Val at the corresponding sites of the propeptides. Furthermore, Val65 of the propeptide of subtilisin BPN' was identified as a residue whose substitution with Ile resulted in the partial formation of the tertiary structure in the propeptide of subtilisin BPN' that do not possess a tertiary structure in the isolated state (Kojima et al., 1998). Therefore, we considered that the corresponding residue Ile64 of POIA1 is important for the POIA1 structure. Similarly, Ile32 was considered to be involved in the structural formation and maintenance of POIA1, although the substitution of the corresponding residue Val37 in the propeptide of subtilisin BPN' with Ile did not induce positive effects on the acquisition of the tertiary structure in the propeptide (Kojima et al., 1998). Because a Phe → Ala substitution is a drastic substitution with large decreases in side chain volume and hydrophobicity, we also examined the effects of the substitution of Phe44 with other hydrophobic amino acids, namely, Val, Leu, and Ile. Through such substitutions, we intend to clarify the relationships between the tertiary structure, inhibitory properties and function of POIA1cm as an intramolecular chaperone in more detail.

These substitutions were introduced into POIA1cm whose C-terminal six residues had been substituted with those of the propeptide of subtilisin BPN', because this mutant is a stronger and more resistant inhibitor of subtilisin BPN' than wild-type POIA1 (Kojima et al., 2001), thus making the effects of substitutions of hydrophobic residues on the inhibitory properties clearer.

**Materials and methods**

Site-directed mutagenesis and construction of expression plasmids

The pUC18-derived POIA1cm-encoding plasmid constructed in a previous study (Kojima et al., 2001) was named pUCPOcm. Amino acid substitutions in the POIA1cm or POIA1cm region in the chimera protein with subtilisin BPN' were carried out with a Quik Change Mutagenesis kit using mutation primers and pUCPOcm or pPOcmSubN (Kojima et al., 2005) as a template, respectively. Newly designed mutations in this study are substitutions of Ile32 or Ile64 with Ala and of Phe44 with Ile, Leu or Val.

The subsequent construction of pET11d-derived expression plasmids of mutated POIA1cm or its chimera proteins with subtilisin BPN' with a Ser221 → Cys substitution in Escherichia coli from mutated pUCPOcm or pPOcmSubN was carried out by essentially the same procedures described previously (Kojima et al., 2001, 2005). An expression plasmid of a chimera protein of mutated POIA1cm and subtilisin BPN' in Bacillus subtilis from mutated pPOcmSubN was also constructed as described previously (Kojima et al., 2005).

Purification of mutated POIA1cm and their chimera proteins with subtilisin BPN'

The expression plasmid of mutated POIA1cm or its chimera protein with subtilisin BPN' constructed in pET11d was transferred into E. coli BL21(DE3). The subsequent cultivation of E. coli and the purification of the proteins were carried out essentially as described previously (Kojima et al., 2001, 2005).

Elucidation of inhibitory properties and intramolecular chaperone activities of mutated POIA1cm

The measurement of the inhibitory activities of mutated POIA1cm toward subtilisin BPN' using the synthetic substrate N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide and the subsequent determination of inhibitor constants (K_i) were carried out by the same procedures as described previously (Kojima et al., 2001). The in vitro intramolecular chaperone activity of mutated POIA1cm was investigated by dialyzing chimera proteins of mutated POIA1cm and subtilisin BPN' with a Ser221 → Ala substitution at 10 μM against 50 mM Tris–HCl (pH 7.0) containing 0.5 M (NH_4)_2SO_4, 1 mM CaCl_2 and 5 mM 2-mercaptoethanol at 4°C for 2 days, followed by an SDS-polyacrylamide gel electrophoresis analysis of the precipitated proteins, as described previously (Kojima et al., 2005). The in vivo intramolecular chaperone activity of mutated POIA1cm was examined by halo formation around a colony of Bacillus cells transformed with an expression plasmid encoding the chimera protein of mutated POIA1cm.

### Fig. 1. Amino acid sequences of POIA1, POIA1cm and three subtilisins (BPN', E. and Carlsberg). The residues of POIA1 examined in the previous and this study are underlined.
Circular dichroism spectral measurement

The circular dichroism (CD) spectra of mutated POIA1cm at 50 µM in 50 mM sodium phosphate buffer (pH 7.0) were measured with a JASCO-J720 CD spectrophotometer. The path length of the cell was 1 mm. Temperature was regulated by circulating electrostatically controlled water through a jacket surrounding the cell.

Results

Inhibitory properties of mutated POIA1cm

The inhibitory activities of five mutants of POIA1cm toward subtilisin BPN' were measured using a synthetic substrate. Their incubation time dependence until inhibitory activity measurements after mixing the mutant and protease was also investigated to determine whether the mutant was changed into a digestible inhibitor. The results for an incubation time of zero are shown in Figs 2A and 3A, along with those of the F44A and/or F56A mutant, and the results showing the incubation time dependence are shown in Figs 2B–C and 3B–D. The inhibitor constants ($K_i$) of all the mutants were obtained from the inhibitory profile at an incubation time of zero and are summarized in Table I, along with those of the F44A and F56A mutants.

These values show that the abilities of the I32A and I64A mutants of POIA1cm to inhibit subtilisin BPN' are weaker than that of the F56A mutant of POIA1cm, and that the ability of the position 44 mutants of POIA1cm to inhibit subtilisin BPN' increases as the hydrophobicity of the amino acid incorporated into this site increases. All the mutants examined in this study were considered to be digestible temporary inhibitors, since their inhibitory activities decrease when the incubation time after mixing the mutant and protease is increased, as shown in Figs 2 and 3. The rates of decreases in the inhibitory activities of I32A and I64A mutants of POIA1cm are basically similar, and the order of the rates of the position 44 mutants of POIA1cm is F44A (fastest) > F44V > F44L > F44I (slowest), which is consistent with the order of the inhibitor constants of the mutants.

Then, an electrophoretic analysis of the proteins in the mixture of mutated POIA1cm and subtilisin BPN' was carried out. It was found that the band of mutated POIA1cm gradually becomes thinner as incubation time increases (data not shown), in a similar manner to the case of the F44A or F56A mutant of POIA1cm (Kojima and Hisano, 2002). These results clearly show that the time-dependent decrease in the inhibitory activity of mutated POIA1cm is due to the degradation of mutated POIA1cm by subtilisin BPN', as demonstrated for the other mutants of POIA1cm and protease inhibitors or their mutants that exhibit temporary inhibition (Kojima et al., 1993, 1999, 2001; Kojima and Hisano, 2002).

CD spectra of mutated POIA1cm

Because previous studies showed that the F44A mutant of POIA1cm lost its tertiary structure, whereas the F56A mutant maintained its tertiary structure (Kojima and Hisano, 2002), the CD spectra of the mutants generated in this study were measured. The results are shown in Fig. 4A and B, along with those of POIA1cm and its F44A mutant for comparison.

Although POIA1cm exhibits a spectrum typical for a protein composed of both α-helices and β-sheets, as revealed by tertiary structure determination by NMR spectrometry, the
CD spectra of I32A and I64A mutants of POIA1cm clearly show the marked destruction of such a tertiary structure of POIA1. Because an intensity minimum is observed between 200 and 210 nm for the CD spectra of both mutants, in contrast to that of the F44A mutant typical for a protein in a random coil state, the tertiary structure of POIA1 composed of two \( \alpha \)-helices and four \( \beta \)-strands is considered to be slightly maintained by the mutation of Ile32 \( \rightarrow \) Ala or Ile64 \( \rightarrow \) Ala. The fact that the CD intensity of the I32A mutant of POIA1cm between 200 and 210 nm is larger than that of the I64A mutant of POIA1cm suggests that the residual tertiary structure of POIA1 is larger for the I32A mutant than for the I64A mutant.

As for the position 44 mutants of POIA1cm, the F44V mutant exhibits a spectrum that resembles that of a protein in a random coil state, as in the case of the F44A mutant (Kojima and Hisano, 2002). However, the larger CD intensity of the F44V mutant of POIA1cm between 210 and 230 nm than that of the F44A mutant of POIA1cm suggests the presence of the partial tertiary structure of POIA1 in the F44V mutant. In contrast, from the CD spectra of the F44L and F44I mutants of POIA1cm, it is considered that most of the tertiary structure of both mutants is retained, although it may be slightly destructed on the basis of the difference in spectra between both mutants and POIA1cm.

Then, the temperature dependences of the CD intensities at 220 nm of F44L and F44I mutants of POIA1cm were investigated to compare their thermal stabilities with that of POIA1cm. The intensities are represented as values relative to that of each mutant at 25°C, and are shown in Fig. 4C along with that of POIA1cm. The ratios of the reduced intensities of each mutant caused by denaturation are smaller than that of POIA1cm, perhaps, due to the partial denaturation of each mutant at 25°C. \( T_{1/2} \), which is defined as the temperature at the midpoint of decreasing intensity, is estimated to be 45°C for the F44L mutant and 46°C for the F44I mutant, whereas that of POIA1cm is 59°C.

In vitro intramolecular chaperone activities of mutated POIA1

Because POIA1cm was shown to function as an intramolecular chaperone of subtilisin BPN’ by in vivo and in vitro experiments in addition to the original protease inhibitor (Kojima et al., 2005), such functions of the mutated POIA1cm constructed in this study were also examined and their relationships with the function of POIA1cm as a
protease inhibitor and with the tertiary structures of the mutants were investigated.

Figure 5 shows the electrophoretic patterns of proteins produced by refolding and the subsequent processing of chimera proteins in which mutated POIA1cm was attached to the N-terminus of subtilisin BPN’ with a Ser221 → Cys substitution. Because the different amounts of processed subtilisin BPN’ molecules, at the same amount of proteins subjected to refolding, are considered to be due to the production of precipitates as a result of misfolding, the amount of processed subtilisin BPN’ molecules is closely related to the intramolecular chaperone activity of the mutated POIA1cm.

As shown in Fig. 5, the amounts of subtilisin BPN’ molecules from the chimera protein with the I32A and I64A mutants of POIA1cm were markedly reduced as well as the total amounts of proteins in the gel, indicating that both mutants of POIA1cm are much less effective intramolecular chaperones of subtilisin BPN’ than POIA1cm. Similarly, the intramolecular activities of the F44V and F44I mutants of POIA1cm are also shown to be markedly lower than that of POIA1cm. In contrast, the F44L mutant of POIA1cm was demonstrated to be a more effective intramolecular chaperone than these mutants, although still less effective than POIA1cm.

In vivo intramolecular chaperone activities of mutated POIA1cm

Then, the propeptide-encoding region of the entire subtilisin BPN’ gene was substituted with the mutated POIA1cm gene newly constructed in this study, and their in vivo intramolecular chaperone activities were investigated by measuring the diameters of the halos around the Bacillus colonies transformed with the plasmid encoding the mutated POIA1cm.

As shown in Fig. 6A, the Bacillus colony transformed with the plasmid encoding POIA1cm with an Ile32 → Ala or Ile64 → Ala substitution exhibited only a small halo, which is considerably smaller than that in the case of the F56A mutant of POIA1cm but is slightly larger than that in the case of the F44A mutant of POIA1cm.

Similarly, the in vivo intramolecular chaperone activities of position 44 mutants of POIA1cm can be compared by measuring the diameters of the halos around the Bacillus colonies. Figure 6B shows that the Bacillus cells transformed with the plasmid encoding the F44L mutant of POIA1cm showed the largest halo in this group including POIA1cm, whereas the Bacillus cells transformed with the plasmid encoding the F44A mutant of POIA1cm exhibited the smallest halo in this group.

These results of the in vitro intramolecular chaperone activity of mutated POIA1cm using the Bacillus cells are considered to be essentially consistent with the results of in vitro experiments and the inhibitory properties of the POIA1cm mutants (see Discussion for detail).

Discussion

In this paper, we found that Ile32 and Ile64 are also important for the maintenance of the tertiary structure and the functions
and the mutants of POIA1cm possessing a substitution of either of respectively. Such locations of these residues might have expression plasmids in which the propeptide region of the subtilisin BPN et al. spectrometry (Sasakawa B. subtilis gene was substituted with the mutated POIA1cm were transferred into B. subtilis UOT0999 cells, and the transformants inoculated onto an LB plate containing 1% skim milk were incubated at 37°C for 39 h.

The tertiary structure of POIA1 determined by NMR spectroscopy (Sasakawa et al., 2002) indicates that Ile32 and Ile64 are located in the loop regions between the α3-helix and the β2-strand, and the α2-helix and the β1-strand, respectively. Such locations of these residues might have resulted in the partial retention of the tertiary structure in the mutants of POIA1cm possessing a substitution of either of these residues with Ala. In contrast, Phe44 is positioned at the center of the hydrophobic core of POIA1 and, therefore, substitution with Ala would produce a larger effect on the tertiary structure, thus leading to a significant suppression of the functions of POIA1cm as a protease inhibitor and an intramolecular chaperone. Although Phe56 is also located near the molecular interior of POIA1, its substitution with Ala in POIA1cm was found to induce only a small effect on the tertiary structure and functions. These results suggest that the effects of the substitution of a hydrophobic residue on the structure and functions of POIA1cm depend on the location of the residue substituted rather than on the change in side-chain volume or hydrophobicity by mutation, as demonstrated for many other proteins, and that the same Phe → Ala substitution produces different effects.

Because Phe44 → Ala substitution destroyed the tertiary structure of POIA1cm, Phe44 of POIA1cm was substituted with an amino acid more hydrophobic than Ala, and it was found that even the F44I mutant of POIA1cm became more susceptible to proteolytic attack by subtilisin BPN’ and less effective as an intramolecular chaperone in vitro than POIA1cm, as a result of a decrease in thermal stability (13°C of T½). The order of the ability of the position 44 mutants of POIA1cm to function as a protease inhibitor (inhibitor constant and resistance to proteolysis) and an intramolecular chaperone in vitro (efficiency of processing after refolding) was found to be the same as that of the structural stability, except the case that the F44I mutant is a less effective intramolecular chaperone than the F44L mutant. These results also clearly show the close relationship between the structure and functions of POIA1.

The diameter of halos around the colonies of the Bacillus cells transformed with the chimera gene of mutated POIA1cm and subtilisin BPN’ is related to the in vivo intramolecular chaperone activity and inhibitory properties of the respective mutants, because mutated POIA1cm binds to a refolded subtilisin BPN’ for a while after assisting the refolding of subtilisin BPN’ and autocatalytic processing follows. Small halos observed in the cases of the I32A and I64A mutants of POIA1cm are considered to be due to a marked reduction in their intramolecular chaperone activity as shown by in vitro experiments, although these mutants were converted into digestible inhibitors. In the case of the F44V mutant of POIA1cm, a larger halo was observed than in the case of the I32A or I64A mutant of POIA1cm, which may be a result of its susceptibility to proteolytic attack and an intramolecular chaperone activity higher than that of the above mutants. In contrast, a similar halo diameter for the F44I mutant of POIA1cm is considered to be due to its resistance to proteolytic attack, although its intramolecular chaperone activity is higher than that of the F44V mutant of POIA1cm. In the case of the F44L mutant of POIA1cm, an intramolecular chaperone activity higher than the F44V or F44I mutant and a moderate susceptibility to proteolysis might result in the formation of a halo larger than that of the F44V or F44I mutant of POIA1cm.

In conclusion, the results of this study strongly show that the stability of the tertiary structure of POIA1 is closely related to the POIA1 functions as a protease inhibitor and an intramolecular chaperone, because the tertiary structure of POIA1 is required for the protection of POIA1 from proteolytic attack and for the 'template' effect of POIA1 to assist protease refolding.
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References


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