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論 文 内 容 の 要 旨

Chapter 1. Introduction

Subtilisins (E.C. 3.4.21.108) are widely distributed in various organisms including bacteria, archaea and eukaryotes. Among them, bacterial subtilisins, which are represented by subtilisin E from *Bacillus subtilis*, subtilisin BPN' from *B. amyloliquefaciens*, and subtilisin Carlsberg from *B. licheniformis*, have been most extensively studied for structures and functions. Because subtilisins are commercially valuable enzymes, attempts to improve their activity and stability with protein engineering technology have also been made extensively. The subtilisin family includes subtilisins from (hyper) thermophiles and psychrophiles. These thermostable and thermolabile subtilisins have been regarded not only as good models for studying stability-activity-structure relationships of proteins, but also as potential candidates for various biotechnological applications. Bacterial subtilisins are synthesized in a precursor form called pre-pro-subtilisin, in which presequence (signal peptide) and prosequence (propeptide) are attached to the N-terminus of the mature domain. They are secreted in a pro-form (pro-subtilisin) with the assistance of a signal peptide, and activated upon autoprocessing and degradation of the propeptide. Requirement of a propeptide for maturation of its cognate mature domain has also been reported not only for other members of the subtilase family but also for other proteases. However, it remains to be determined whether hyperthemophilic archaeal subtilisins are matured in a similar manner. In this study, the maturation mechanism of Tk-subtilisin from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 was analyzed.

Chapter 2. Analysis of the Maturation Process of Tk-subtilisin

Tk-subtilisin from the hyperthermophilic archaeon *Thermococcus kodakaraensis* is a member of the subtilisin family (Kannan *et al.*, 2001). It consists of a putative signal peptide [Met(-24)-Ala(-1)], propeptide (Gly1-Leu69),

and mature domain (Gly70-Gly398). Tk-subtilisin is synthesized in a prepro-form (Prepro-Tk-subtilisin), secreted in a pro-form (Pro-Tk-subtilisin), and matured to an active form (Tk-subtilisin) upon autoprocessing and degradation of the propeptide (Tk-propeptide). Pro-Tk-subtilisin was inactive in the absence of Ca^{2+}, but was activated upon autoprocessing and degradation of propeptide in the presence of Ca^{2+} at 80℃. This maturation process was completed within 30 min at 80℃, but was bound at an intermediate stage, in which the propeptide is autoprocessed from the mature domain (Tk-subtilisin) but forms an inactive complex with Tk-subtilisin, at lower temperatures. At 80℃, approximately 30% of Pro-Tk-subtilisin was autoprocessed into Tk-propeptide and Tk-subtilisin and the other 70% was completely degraded to small fragments. Likewise, Tk-subtilisin-was inactive in the absence of Ca^{2+}, but was activated upon incubation with Ca^{2+} at 80℃. The kinetic parameters and stability of the resultant activated protein were nearly identical to those of Tk-subtilisin, indicating that Tk-subtilisin^{-} does not require Tk-propeptide for folding. However, only ～5% of Tk-subtilisin^{-} was converted to an active form and the other part was completely degraded to small fragments. Tk-propeptide was shown to be a potent inhibitor of Tk-subtilisin and inhibits Tk-subtilisin in a slow binding mode, similar with its bacterial counterparts. Tk-propeptide may be required to prevent the degradation of the Tk-subtilisin molecules, which are activated later, by those, which are activated earlier.

Chapter 3. Construction of low-temperature adapted mutants of Pro-Tk-subtilisin using directed evolution

Pro-Tk-subtilisin exhibited halo-forming activity only at 80℃ but not at 70℃ and 60℃ because Tk-propeptide is not effectively degraded by Tk-subtilisin and forms an inactive complex with Tk-subtilisin at ＜80℃. Random mutagenesis in the entire Prepro-Tk-subtilisin gene, followed by screening for mutant proteins with halo-forming activity at 70℃ and 60℃, allowed us to identify single Gly56→Ser mutation in the propeptide region responsible for low-temperature adaptation of Pro-Tk-subtilisin. SDS-PAGE analyses and Tk-subtilisin activity assay of Pro-G56S indicated more rapid maturation than Pro-Tk-subtilisin. The resultant active form was indistinguishable from Tk-subtilisin in activity and stability, indicating that Gly56→Ser mutation does not seriously affect the folding of the mature domain. However, this mutation greatly destabilized the propeptide making it unstructured in an isolated form. As a result, G56S-propeptide was more susceptible to proteolytic degradation and less effectively inhibited Tk-subtilisin activity than Tk-propeptide. These results suggest that Pro-G56S is more effectively matured than Pro-Tk-subtilisin at lower temperatures, because autoprocessed G56S-propeptide is unstructured upon dissociation from Tk-subtilisin and is therefore effectively degraded by Tk-subtilisin.

Chapter 4. Mutational and crystallographic analyses of Tk-propeptide

Pro-Tk-subtilisin is characterized by the extremely slow maturation at mild temperatures, but this maturation rate is greatly increased by the single Gly56→Ser mutation in the propeptide region. To analyze the role of Gly56, which assumes a left-handed conformation, the Pro-Tk-subtilisin variants with complete amino acid substitutions at Gly56 were constructed. Comparison of their halo-forming activities suggests that all variants except Pro-G56W mature faster than WT. Pro-G56W and Pro-G56E with the lowest and highest maturation rates, respectively, among 19 variants, as well as WT and Pro-G56S, were overproduced, purified, and characterized. SDS-PAGE analyses and Tk-subtilisin activity assay indicated that their maturation rates increased as WT≤Pro-G56W＜Pro-G56S＜Pro-G56E. The propeptides of these variants were also overproduced, purified, and characterized. The stability and inhibitory potency of these propeptides decreased as WT≥G56W ＞G56S＞G56E, indicating that they are inversely correlated with the maturation rates of Pro-Tk-subtilisin and its derivatives. The crystal structures of these propeptides determined in complex with S324A-subtilisin indicate that the conformation of the propeptide is altered by the mutation, such that the non-glycine residues at

position 56 assume a right-handed conformation and hydrophobic interactions at the core region decrease. These results indicate that Gly56 is required to make the propeptide fold stable. Stabilization of this fold leads to strong binding of Tk-propeptide to Tk-subtilisin, high resistance of Tk-propeptide to proteolytic degradation, and slow maturation of Pro-Tk-subtilisin.

Chapter 5. General conclusion

Tk-subtilisin from the hyperthermophilic archaeon *Thermococcus kodakaraensis* represents a good model in elucidating the mechanism involved in the temperature-dependent maturation of hyperthermophilic proteins. Tk-subtilisin requires Ca^{2+} for its stability as well as for its activity, apart from what has been described for its bacterial counterparts. The maturation of Tk-subtilisin is temperature-dependent and does not require its cognate propeptide for folding since the mature domain, Tk-subtilisin^{-}, was overproduced and activated even in the absence of Tk-propeptide but in the presence of Ca^{2+} and incubation temperature of 80℃. Though the yield was greatly increased when the propeptide is added *in trans*, this finding suggest a unique folding pathway for Tk-subtilisin, different from its bacterial counterparts. Mutational works on the entire Pro-Tk-subtilisin using directed evolution and site-directed mutagenesis revealed the important role of propeptide in the temperature-dependent maturation of Pro-Tk-subtilisin. Structural analyses of the crystal structure of examined mutant propeptides (G56S-, G56W- and G56E-) in complex with the active-site mutant of Tk-subtilisin (S324A-subtilisin) revealed the significant role of Gly56 in keeping the left-handed conformation of Tk-propeptide. The local backbone structure of this residue is greatly changed by the mutation such that the non-glycine residues at position 56 assume a right-handed conformation and hydrophobic interactions at the core region decrease. Rapid maturation of Pro-Tk-subtilisin after secretion into the external medium may induce growth defect of the cells with unknown reasons. With the above results, further study on the effect of other mutations within the entire propeptide region or the hydrophobic core in the propeptide region is noteworthy. The question on whether it is only the Gly56 or possibly other position in the propeptide region that may contribute to rapid maturation of the Pro-Tk-subtilisin at lower temperatures without significantly affecting, its activity and stability at elevated temperatures still arises and awaits to be determined. Whether this hypothesis is possible, this may lead to a hyperthermophilic Tk-subtilisin to have a wide range of temperature which it can be activated. This is not only an interesting contribution on the understanding of structure-function relationship for proteins of hyperthermophilic origin but also of protein with high biotechnological and industrial potential.

論文審査の結果の要旨

本論文は、サチライシンの一種である超好熱古細菌 *Thermococcus kodakaraensis* 由来サチライシン（Tk-サチライシン）の成熟化におけるプロペプチドの役割について研究したものであり、以下に示すように、序論、本論３章、および総括から構成されている。第一章（序論）では、サチライシンは洗剤添加物など産業酵素として広く利用されていること、サチライシンはプロペプチドの自己切断および分解により成熟化すること、プロペプチドは活性阻害剤および分子内シャペロンとして働くことなど、これまで主として中温細菌由来サチライシンを用いて行われてきた研究の背景について触れ、本研究の目的と意義を述べている。第二章では、Tk-サチライシンのプロ体を精製し、その生化学的諸特性を解析することにより、Tk-サチライシンの成熟化は中温細菌由来サチライシン同様、１）成熟体部分のフォールディング、２）プロペプチドと成熟体の間のペプチド結合の自己切断、３）活性部位を塞ぐことにより活性を阻害しているプロペプチドの分解、の３段階のステップで進むことを明らかにしている。また、最初のステップのフォールディングがプロペプチドではなく Ca^{2+} により誘導されるという点で中温細菌由来サチライシンとは大

きく異なることを明らかにしている。さらに、Tk-サチライシンの成熟化は 80℃以上の高温でしか効率良く進まないことを明らかにしている。第三章では、進化工学的手法により 60℃でも効率良く成熟化する Tk-サチライシン変異体をいくつか取得することに成功している。そのうちの一つで、プロペプチド領域の Gly56 が Ser に置換した一アミノ酸変異体 Pro-G56S およびそのプロペプチドをそれぞれ精製し、生化学的に解析することにより、Pro-G56S の成熟化が低温でも進行するのは、変異によりプロペプチドが不安定化するためであることを明らかにしている。第四章では、Gly56 を Ser 以外のアミノ酸に置換した変異体を 18 種類構築し、その成熟化を解析することにより、Gly 以外のアミノ酸に置換した場合はいずれもプロペプチドが不安定化することを明らかにしている。さらに、いくつかの変異体の結晶構造を解析することにより、Gly56 はプロペプチドが安定な構造を形成するのに必要であることを明らかにしている。第五章（総括）では、本研究で得られた結果に基づき Tk-サチライシンの成熟化機構について考察するとともに、産業酵素としての応用の可能性について展望している。

以上のように、本論文は超好熱菌由来サチライシンの成熟化機構に関して新たな知見を見いだした点で意義深い。また、本論文は本酵素の産業的利用を図る上で有益な知見を与えるものである。よって本論文は博士論文として価値あるものと認める。