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学位論文題名

Characterization of PL-14 alginate lyases from some gastropod marine mollusca and their structure-function analyses

(腹足類PL-14型アルギン酸リアーゼの性状と構造・機能連関解析)

学位論文内容の要旨

Alginate lyase (EC 4.2.2.3) is an enzyme that catalyzes the degradation of alginate polymer through a β-elimination reaction. It is found in brown algae, herbivorous marine gastropods, marine and soil bacteria, fungi, and *Chlorella* virus. Recently, enzymatically degraded alginate oligosaccharides with novel physico-chemical and physiological functions have been sought by biopolymer-based industries to expand the application areas of polysaccharides. Although marine gastropods that feed on seaweeds have been considered as major sources for alginate lyases, apart from abalone and turban shell enzymes, general properties and primary structures have not been extensively investigated. The entire amino acid sequences of gastropod alginate lyases have been reported in only three enzymes from abalone and turban shell, and they have been classified as type PL-14 based on hydrophobic cluster analysis. A structure-function study on abalone alginate lyase was performed recently, but the configuration of catalytic residues is not fully understood. To understand the catalytic roles of alginate lyases in alginate degradation and to clarify the range of distribution of PL-14-type alginate lyases in gastropods, it is necessary to study comparatively the enzymatic properties and primary structures of various gastropod alginate lyases from as many species as possible. In the present study, I isolated alginate lyases from the gastropods *Aplysia kurodai* and *Littorina brevicula* and investigated their enzymatic properties and primary structures. *Escherichia coli* expression systems for these cDNAs encoding the gastropod enzymes were also established. Further, catalytically important amino acid residues of these enzymes were assessed by site-directed mutagenesis.

In chapter I, two alginate lyase isozymes, AkAly28 and AkAly33, with approximate molecular masses of 28 kDa and 33 kDa, respectively, were isolated from the digestive fluid of *A. kurodai*. Both AkAly28 and AkAly33 were regarded as endolytic polymannuronate (poly(M)) lyases (EC 4.2.2.3) since they preferably degraded poly(M)-rich substrate producing unsaturated tri- and disaccharides, and rapidly decreased the viscosity of alginate solution in the initial phase of degradation. The optimal pH and temperature of both enzymes were pH 6.7 and 40°C, respectively. The temperature that caused a half inactivation of the two enzymes during 20-min incubation was also similar, i.e., 38°C. However, NaCl requirement and activity toward oligosaccharide substrates of the two enzymes significantly differed. Namely, AkAly28 showed practically no activity in the absence of NaCl and maximal activity at NaCl concentrations higher than 0.2 M, while AkAly33 showed ~20% maximal activity despite the absence of NaCl and maximal activity at around 0.1 M NaCl. The relative amounts of tri- and disaccharide produced from poly(M)-rich substrate by the two enzymes differed in the prolonged reaction time, which may be ascribable to the difference in the oligosaccharide-degrading activity

between AkAly28 and AkAly33. Namely, AkAly28 degraded only a small amount of oligosaccharides smaller than tetrasaccharide, while AkAly33 could degrade oligosaccharides larger than disaccharide, producing disaccharide and α-keto acid (4-deoxy-L-erythro-hexoseulose uronic acid). Analysis of the N-terminal and internal amino acid sequences of AkAly28 and AkAly33 indicated that both enzymes belong to polysaccharide lyase family 14.

In chapter II, isolation, cDNA cloning and bacterial expression of another alginate lyase isozyme, AkAly30 of *A. kurodai*, were described. AkAly30 was purified from the digestive fluid of *A. kurodai* by ammonium sulfate fractionation, followed by TOYOPEARL CM-650M column chromatography. The cDNA encoding AkAly30 was amplified by RT-PCR from *A. kurodai* hepatopancreas cDNA. Cloned cDNA that included the entire translational region of AkAly30 consisted of 1,313 bp and encoded the amino acid sequence of 295 residues. The deduced amino acid sequence of AkAly30 comprised an initiation methionine, a putative signal peptide for secretion (18 residues), a putative propeptide (9 residues), and a mature AkAly30 domain (267 residues). AkAly30 shared ~42% identity with molluscan alginate lyases and 21% identity with *Chlorella* virus vAL-1. Such high sequence similarity indicates that AkAly30 is a new member of PL-14-subfamily-3 alginate lyases. An *E. coli* BL21(DE3)-pCold I expression system for recombinant AkAly30 (recAkAly30) was constructed, and recAkAly30 was produced as a fusion protein possessing an N-terminal hexahistidine-tag. The recAkAly30 purified by Ni-NTA affinity chromatography, showed similar biochemical properties to that of native AkAly30 (natAkAly30). The optimal temperature and pH were around 55°C and pH 6.0 in both recAkAly30 and natAkAly30. Temperatures that caused a 50% inactivation during 20-min incubation were 48°C for natAkAly30 and 46°C for recAkAly30. Both enzymes showed considerably high pH stability, i.e., their activities did not significantly decreased at pH 4.5-9.0, e.g., retained more than 80% of the original activity, after incubation at 40°C for 30 min. Both recAkAly30 and natAkAly30 similarly produced tri- and disaccharides as major end products from poly(M)-rich substrate.

In chapter III, the primary structure of a *L. brevicula* alginate lyase isozyme LbAly28 was determined by the cDNA method, and the recombinant LbAly28 (recLbAly28) was produced by an *E. coli* expression system. The cDNA encoding LbAly28 was amplified from *Littorina* hepatopancreas cDNA by the PCR using degenerated primers designed based on the N-terminal and internal amino acid sequences of LbAly28. The deduced amino acid sequence of LbAly28 comprised a putative signal peptide region of 14 residues, a propeptide-like region of 16 residues, and a mature enzyme domain of 260 residues from the N-terminus. The amino acid sequence of the mature LbAly28 domain showed 43-53% identity with other molluscan alginate lyases and 22% identity with the corresponding region of *Chlorella* virus vAL-1. Amino acid residues known to be catalytically important in two PL-14 enzymes, abalone HdAly and *Chlorella* virus vAL-1, were also conserved in LbAly28. This indicates that LbAly28 is also a member of family PL-14. The recLbAly28 was produced with the pCold I-*E. coli* BL21(DE3) system as a fusion protein bearing an N-terminal hexahistidine-tag. General properties of recLbAly28 were similar to those of native enzyme; however, recLbAly28 appeared to be more labile than LbAly28, i.e., the temperature that caused half-inactivation of recLbAly28, 47°C, was approximately 5°C lower than that for LbAly28. This low stability suggests that the

folding structure of recLbAly28 slightly differs from that of native enzyme, since post-translational modification systems, which stimulate maturation of enzyme proteins in eukaryote cells, are absent in prokaryote cells.

In chapter IV, structure-function analyses were conducted for AkAly30 and LbAly28 enzymes using theoretical modeling methods. Site-directed mutagenesis for AkAly30 and LbAly28 were performed to assess catalytically important amino acid residues that had been found in PL-14 abalone enzyme HdAly and *Chlorella* virus enzyme vAL-1. Amino acid residues that extrude toward the active cleft and play key roles in the catalytic reaction and/or substrate binding of HdAly and vAL-1 were conserved in both AkAly30 and LbAly28. Replacements of Lys99, Arg128, Tyr140 and Tyr142 of recAkAly30 and Lys97, Arg123, Tyr135 and Tyr137 of recLbAly28 by Ala and/or Phe greatly decreased their activities as in the case of HdAly and/or vAL-1 enzymes. These results also indicate that both AkAly30 and LbAly28 are PL-14 alginate lyases. His213, an essential residue for *Chlorella* virus enzyme to exhibit activity at pH 10.0, was originally replaced by Asn120 in AkAly30 and by Arg115 in LbAly28. The reverse replacement of Asn120 by His in recAkAly30 increased its activity at pH 10.0 from 8 to 93 U/mg; however, the activity level at pH 7.0, i.e., 774.8 U/mg, was still much higher than that at pH 10.0. This indicates that Asn120 is not directly related to the pH dependence of AkAly30, unlike His213 of vAL-1. S219, which was critically important for vAL-1 and is a highly conservative residue in PL-14 enzymes, was replaced by T121 in LbAly28. Replacement of such a residue i.e., S126 in AkAly30 and T121 in LbAly28, by Ala also decreased their specific activities to 15~39%, which suggests that these residues are important for the catalytic actions of both AkAly30 and LbAly28. Interestingly, the mutation of T121 to Ala also decreased the specific activities toward sodium alginate and random(MG)-rich substrate less than it decreased the activity toward poly(M)-rich substrate for LbAly28. Thus T121 is considered to be an important residue for substrate-recognition of LbAly28. Another basic amino acid mutant K94A of LbALy28 and its corresponding mutant R92A of HdAly, whose mutation sites were located in the entrance of the active cleft of LbALy28 and HdAly, showed a decrease in activities of up to ~25% of their wild-type enzymes. This suggested that the residues surrounding the active cleft also participated in the enzyme-substrate binding. Almost complete inhibition of recombinant *Haliotis*, *Aplysia*, *Littorina* and *Chlorella* virus lyases by site-directed mutants of lysine and arginine indicated that they were the key residues which more directly and commonly involved in the catalytic mechanism of PL-14 enzymes.

In conclusion, both AkAly30 and LbAly28 were considered to be the superior materials for protein-engineering studies on gastropod alginate lyases since these enzymes could be expressed in *E. coli* with a sufficient amount for the determination of enzymatic properties. The higher thermal stabilities of AkAly30 and LbAly28 may be advantageous for the production of recombinants in *E. coli* cells. Further, it was confirmed that the catalytically important amino acid residues are highly conserved among bacteria, *Chlorella* virus, and gastropod PL-14 enzymes. The structural similarities among all sources of enzymes suggest the common origin of PL-14 alginate lyase genes. Studies on the distribution of PL-14-type alginate lyase genes in connection with the host phylogeny studies are important to elucidate the molecular evolution process of PL-14 enzymes.

学位論文審査の要旨

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学位論文題名

Characterization of PL-14 alginate lyases from some gastropod marine mollusca and their structure-function analyses

(腹足類PL-14型アルギン酸リアーゼの性状と構造・機能連関解析)

海洋無脊椎動物による海藻多糖の分解・消化機構と，それに関連する酵素に対するバイオテクノロジー分野での関心が高まっている。アルギン酸リアーゼは，褐藻の主要構造多糖であるアルギン酸をβ-脱離機構により切断する酵素であり，アルギン酸のポリマンヌロン酸領域に作用するポリMリアーゼ（EC 4.2.2.3）とポリグルロン酸領域に作用するポリGリアーゼ（EC 4.2.2.11）に大別される。また一次構造の疎水クラスター解析に基づき，多糖リアーゼファミリー（PL）-5, -6, -7, -14, -15, -17 および-18 に分類される。本酵素は，海洋微生物や土壌細菌，植食性腹足類などに分布するが，腹足類の酵素に関する研究は微生物の酵素のそれに比べて知見が少ないのが現状である。本研究は，腹足類として無楯目（Anaspidae）および中腹足目（Mesogastropoda）に属するアメフラシおよびタマキビガイに着目し，これらが産生するアルギン酸リアーゼの酵素特性や一次構造を，既報のアワビなどの原始腹足目（Archeogastroposa）が有する酵素と比較解析するとともに，cDNA を利用した大腸菌発現系の構築，さらに部位特異変異法による触媒活性に関与するアミノ酸残基の解析を行ったものである。これらの研究成果に基づき，海産腹足類のアルギン酸リアーゼに関する生化学的および分子生物学的知見が深められた。本研究成果の概要は以下の通りである。

1. アメフラシの消化液から分子量 28,000 と 33,000 のエンド型のアルギン酸リアーゼ（AkAly28 および AkAly33）を単離した。それらは，pH 6.7 および 40°C において最大活性を示し，38°C までは安定であるという類似した性質を示した。その一方で，両者は NaCl 要求性と反応機作で相違が認められた。AkAly28 は最大活性を示すために 0.2 M 以上の濃度の NaCl を必要としたが AkAly33 は必要とせず，また，AkAly28 は 4 糖以上の大きさのオリゴ糖や多糖を分解するが AkAly28 は 3 糖以上を分解する点で異なっていた。さらに，これらに加えて，分子量 30,000 のアルギン酸リアーゼ AkAly30 の単離にも成功し，これは AkAly28 と類似の特性を示した。これらの結果より，アメフラシには特性の異なる複数のアルギン酸リアーゼアイソフォームが存在することを明らかにした。

2. 次に，PCR 法により AkAly30 の cDNA を増幅し，その塩基配列を解析することにより全アミノ酸配列を推定した。その結果，AkAly30 は既報のアワビやサザエのアルギン酸リアーゼのアミノ酸配列と約 40%のアミノ酸が一致し，PL-14 型に分類された。さらに，クローン化した cDNA を用いて大腸菌発現系の構築に成功し，250 ml 培養規模の組換え大腸菌体から約 0.6 mg の高純度かつ高活性の組換え AkAly30 を精製した。この系を用いて 7 種類の AkAly30 点変異体を作出し，触媒活性に関与するアミノ酸の同定を試み，Lys99, S126, R128, Y140 および Y142 は本酵素の触媒活性に重要なアミノ酸であり，特に Lys99 と R128 はその酵素活性に必須の残基であることを明らかにした。
3. タマキビガイの肝膵臓からも 3 種類のアルギン酸リアーゼを単離し，この中で分子量 28,000 の LbAly28 の酵素特性を解析した。その結果，これらタマキビガイの酵素は極めて高い温度安定性および pH 安定性を示すことを明らかにした。すなわち，酵素活性の至適温度は約 50°C であり，45°C で 30 分間の処理では失活せず，また 30°C の処理においては pH 5〜10 の広い pH 範囲で安定であった。これら 3 種のタマキビガイ酵素のうち，最も分子量の小さい LbAly28 について PCR により cDNA を増幅した。その演繹アミノ酸配列は 260 残基から成り，既報のアワビやサザエおよび本研究で明らかにしたアメフラシのアルギン酸リアーゼのアミノ酸配列と約 40%のアミノ酸同一率を示した。このことから，タマキビガイのアルギン酸リアーゼも PL-14 型の酵素であることを明らかにした。さらに，この cDNA を利用して大腸菌発現系を構築するとともに，この系を利用した 7 種類の点変異体を作出し触媒活性に関与するアミノ酸を解析した。その結果，タマキビガイの酵素の触媒残基と推定されるアミノ酸は，アメフラシや他の PL-14 型アルギン酸リアーゼと同一の位置に存在し，種類もほぼ同一であることを明らかにした。以上の結果を総合し，腹足類の PL-14 型アルギン酸リアーゼにおいては，触媒残基は極めて良く保存されていると結論づけた。

以上，本研究は海産腹足類のアメフラシおよびタマキビガイからそれぞれ 3 種のアルギン酸リアーゼを単離し，それらのうちの主要アイソザイムについて cDNA クローンニングと大腸菌発現系を構築し，さらに合計 14 種類の部位特異変異体を作出することによって海産腹足類の PL-14 型アルギン酸リアーゼの触媒活性に関与するアミノ酸残基を特定し，本酵素のタンパク質工学的基盤を整備したものとして高く評価できる。よって審査員一同は申請者が博士（水産科学）の学位を授与される資格のあるものと判定した。