Terminal Amino Acid Sequences of Alkaline Amylase from Alkalophilic *Bacillus* sp. MB 809 and Their Homology

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Alkaline α-amylase expressed in the transformant, *Bacillus subtilis* MB809, containing alkaline amylase gene cloned from alkalophilic *Bacillus* sp. AL-8, was purified through four step separation processes. The purified alkaline α-amylase had molecular weight of approximately 59,000 daltons on SDS-PAGE and Sephadex G-100 gel filtration. Amino acid sequence of terminal portion of the enzyme was analyzed with pure amylase eluted from the SDS-PAGE gel. N-terminal amino acid sequence of α-amylase was determined by the Edman degradation method and resulted in NH_2-ser-thr-ala-pro-ser-(ile)-lys-ala-gly-thr-(ile)-leu-. For C-terminal amino acid sequencing, purified α-amylase was digested with carboxypeptidase A and B, and reverse-phase HPLC gradient elution system resulted in -thr-trp-pro-lys-COOH.

KEY WORDS ☐ **Alkalophilic *Bacillus* sp., alkaline amylase, amino acid sequence**

The mechanism in molecular levels that allow the organism to adapt to extreme environment is one of the most interesting subjects to be solved in microbiological ecology.

Except for a few cases, little attempt to study the mechanisms of alkalophilicity of alkaline amylases in biochemical characters has been made yet. In the previous papers (2, 3), it has been reported that one of the alkalophilic bacteria isolated from soil produced alkaline amylase and that the alkaline amylase gene was cloned and expressed in *E. coli* and *Bacillus subtilis*.

Amino acid sequences of amyalses from several strains of *Bacillus* species (5, 14) and the differences of N-termini between two types of liquefying and saccharifying amylase of the genus *Bacillus* (13) have been recently studied and characterized.

In this paper, an attempt has been made to characterize the differences of amino acid sequences of N- and C-termini between several α-amylases from neutrophilic *Bacillus* and alkalophilic *Bacillus* isolated in this work.

MATERIALS AND METHODS

Bacterial strains

Alkalophilic *Bacillus* sp. AL-8 isolated from soil was used as a donor strain of alkaline amylase gene. And a transformant harboring the gene, *B. subtilis* MB 809 (3), which produce alkaline amylase 1.8 fold to the original strain. *Bacillus* sp. AL-8, was used as a producer of the alkaline amylase.

Preparation and assay of α-amylase

Cells of the culture were eliminated by centrifugation and the resulting culture broth was added to four fold cold acetone. After keeping overnight, the precipitate was loaded to DEAE-cellulose column, and eluted out with a linear gradient of 0.2 M NaCl in the 0.1 M Na_2CO_3 solution.

The fractions of amylase were pooled, further purified through Sephadex G-100 and G-75, and eluted out with 50 mM glycine buffer (pH 10).

Pure amylase was finally prepared by SDS-polyacrylamide gel electrophoresis, eluted out with 50 mM glycine buffer (pH 10), and precipitated by 9 fold cold acetone (6). Amylase activity was measured based on the procedure of Somogyi-Nelson method (4). Protein quantity was determined at 280 nm by the method of Lowry *et al.* (7) with bovine serum albumin as a standard. A unit of amylase activity was defined as 1.0 g of reducing sugar per mg of protein.

Chemicals for terminal sequencing of amylase

Phenylisothiocyanate (PITC), N,N-dimethyl-N-allyamine buffer, and trifluoroacetic acid were purchased from Sigma Chemical Co. Solvents for high performance liquid chromatography (HPLC)

were obtained from Merck Korea Ltd. Carboxypeptidase A and B were donated from Institute of Genetic Engineering, KAIST.

Determination of terminal amino acid sequence of alkaline amylase

N-terminal sequencing of the alkaline amylase was carried out by the Edman degradation methods described in the book of protein sequencing (12).

Standard phenylthiohydantoin (PTH)-amino acids and amino acid derivertives obtained for the N-terminal sequences analysis were separated and identified by a reversed phase HPLC (Waters Co.) with isocratic elution system (1) with Bondapak C_{18} column as in Table 1. For the C-terminal sequencing of the alkaline amylase, carboxypeptidase method (12) was adopted. For the analysis of amino acids released from C-terminus of the enzyme, the derivatives of amino acids with phenylisothiocyanate (PITC) to yield the phenylthiocarbamyl (PTC)-amino acids were determined by reversed phase HPLC system (PICO-TAG method) (12).

RESULTS AND DISCUSSION

Purification of alkaline amylase and its molecular weight

The culture broth of the transformant, *B. subtilis* MB 809, grown for 16 hours was added to cold acetone and the precipitate containing alkaline amylase was further purified by means of DEAE-cellulose, Sephadex G-100, and Sephadex G-75 column chromatography. After Sephadex G-75 column chromatography, the specific activity of the purified alkaline amylase was 467.8 unit per mg protein as shown in Table 2. The yield of the purified alkaline amylase was about 15% on Sephadex G-75 chromatography.

Freeze-dried alkaline amylase eluted from the band on SDS-polyacrylamide gel electrophoresis (PAGE) was furnished for the terminal sequencing of the enzyme.

From SDS-PAGE, the molecular weight of the enzyme was estimated to be about 59,000 daltons (Fig. 1) and confirmed by the gel filtration on Sephadex G-100.

Amino acid sequence of terminal portion and homology study

Table 1. *HPLC condition for PTH-amino acid analysis.*

Column	: μ-Bondapak C_{18} (7.9 mm×30)
Column temperature	: 30°C
Detector	: 254 nm spectrophotometer
Elution solvent	: 38% CH_3CN-0.013 M Na-acetate (pH 5)
Total retention time	: 18 min
Sample size	: 10 μl

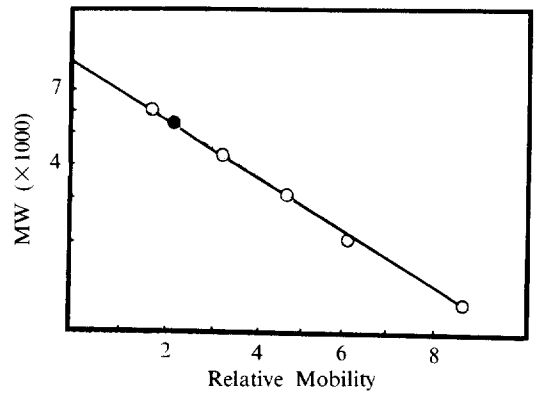

Fig. 1. *SDS-PAGE and determination of the molecular weight of the purified α-amylase by SDS-PAGE.* The molecular weight (59,000 daltons) of the purified α-amylase was interpolated (●). The standard markers (○) were bovine albumine (MW 66,000), egg albumine (MW 45,000), glyceraldehyde 3- phosphate dehydrogenase (MW 36,000), trypsin inhibitor (MW 20,100), lacto-albumin (MW 14,000).

Table 2. *The purification of alkaline α-amylase from Bacillus sp. MB809.*

Purification step	Total activity(U)	Total protein(mg)	Specific activity(U/mg)	Yield (%)	Purification (fold)
1. Culture extract	118,750	7,000	16.96	100	1
2. Acetone fraction	63,250	2,308	27.4	53.3	1.6
3. DEAE-cellulose column	49,045	1,443	34	41.3	2
4. Sephadex G-100 column	30,714	295	104	25.9	6.1
5. Sephadex G-75 column	17,310	37	467.8	15	27.6
6. SDS-PAGE elution	13,041	2	6520.5	10	384.5

The N-terminal amino acid sequence of the alkaline α-amylase of the transformant was analyzed by HPLC. The sequence was determined to be NH_2-ser-thr-ala-pro-ser-ile-lys-(ala)-gly-thr-ile-leu-, and was generally different from the sequence of α-amylase derived from another species of genus *Bacillus* which have been reported earlier by others (9, 10, 11, 14).

However, it was found that the N-terminal sequence of this alkaline amylase was quite similar to those of neutral amylase of *Bacillus subtilis*, which formed saccharifying type α-amylase of molecular weight 42,000 (5).

N-terminal sequence of saccharifying type α-amylase from the neutrophilic *Bacillus* showed a homology in N-terminal portion with -thr-ala-pro-ser-ile-lys-, as shown in Table 3.

On the other hand, N-terminal sequence even originated from alkalophilic *Bacillus* sp. #707 (10), which produced liquefying type α-amylase, showed almost no homology with that of the saccharifying type α-amylase of transformant alkalophilic *Bacillus* sp. MB 809.

To determine C-terminal sequence of the pure enzyme, the alkaline amylase was reacted in the solution containing carboxypeptidase A and B. Free amino acids released were treated by PICO-TAG method (12), and determined by HPLC.

On 15 min after the enzyme reaction, lysine was in the first place eluted out, followed by

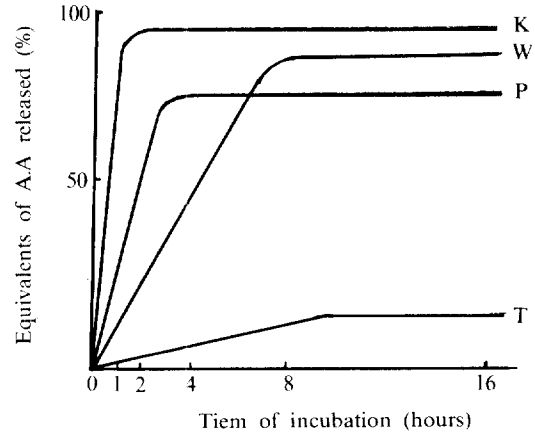

Fig. 2. *The release of amino acid from α-amylase during digestion with carboxypeptidase A and B.*
Peak K, lysine; W, tryptophane; P, proline; T, threonine.

Table 3. *N-terminal sequences of α-amylase from Bacillus strains.*

α-amylase	Mol. wt	Sequence	Ref.
B. licheniformis ERY R9 (liquefying, thermostable)	62,000	N-Ala-Asn-Leu-Asn-Gly-Thr-Leu-Met-Glu -Tyr-Phe-Glu-X-Tyr-Met-Pro-Asn-Asp-	14
B. amyloliquefacience (liquefying, thermostable)	50,000	N-Val-Asn-Gly-Thr-Leu-Met-Glu-Tyr-Phe -Glu-Trp-Tyr-Pro-Asn-Asp-	9
B. subtilis Marburg (saccharifying)	67,000	N-Leu-Thr-Ala-Pro-Ser-Ile-Lys-(Ser) -Gly-(Thr)-Ile-Leu-	11
B. subtilis (saccharifying, neutrophilic)	42,000	N-Thr-Ala-Pro-Ser-Ile-Lys-Ala-Gly-Thr -Ile-Leu-	5
Bacillus sp. #707 (liquefying, alkalophilic)	55,370	N-His-His-Asn-Gly-Thr-Asn-Gly-Thr-Met-Gln-Thr-	10
Bacillus sp. MB 809 (saccharifying, alkalophilic)	59,000	N-Ser-Thr-Ala-Pro-Ser-Ile-Lys-(Ala) -Gly-(Thr)-Ile-Leu-	this work

Table 4. *C-terminal sequences of α-amylase from Bacillus strains.*

α-amylase	Sequence	Ref.
B. licheniformis	COOH - Thr-Arg-Val-Thr-Gly-Lys-Gln-His-Phe-	8
B. amyloliquefaciens	Thr-Arg-Val-Thr-Gly-Lys-Gln-Gln-Phe-	8
B. subtilis T2N26	Gln-Ile-Val-Phe-Phe-Leu-Tyr-Leu-Phe-Phe-	11
B. licheniformis 584 (heat pH stable)	Arg-Gln-Val-Tyr-Ile-Ser-Val-Ser-Gly-Gly-	14
B. stearothermophilus	Pro-Trp-Ala-Val-Leu-Arg-Pro-Glu-His-Trp-	14
Bacillus sp. #707 (alkaline, liquefying)	Lys-Asn-Val-Trp-Ile-Ser-Val-Ser-Gly-Gly-	10
Bacillus sp. MB 809 (alkaline, saccharifying)	Lys-Pro-Trp-Thr-	This work

proline and tryptophan, shown as Fig. 2. Thus, the C-terminal sequence of the alkaline amylase was defined to be -thr-trp-pro-lys-COOH.

As shown in Table 4, almost no homology could be observed in C-terminal sequence of α-amylase from *Bacillus* strains, except between *B. licheniformis* and *B. amyloliquefaciens*.

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초 록: 호알카리성 *Bacillus* sp. MB 809의 알카리성 아밀라제의 말단 아미노산 서열과 그 상동성

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호알칼리성 *Bacillus* sp. AL-8로부터 알칼리성 amylase gene이 클론된 형질 전환주 *Bacillus subtilis* MB 809의 알칼리성 α-amylase를 4단계 정제과정을 통해 순수 정제하였다. 정제된 알칼리성 α-amylase는 SDS-PAGE 와 Sephadex G-100 젤 여과 크로마토그래피를 통해 59,000 dalton의 분자량을 갖는 것으로 나타났다. SDS-PAGE 젤로부터 용출한 amylase로 효소 단백질 말단의 아미노산 서열을 분석하였다. α-amylase의 NH_2- 말단 부위의 아미노산 서열은 Edman 분해 방법에 의해 결정되었는데 그 결과는 NH_2-ser-thr-ala-pro-ser-(ile)-lys-ala-gly-thr-(ile)-leu 으로 밝혀졌다. COOH- 말단 부위의 아미노산 서열은 정제된 α-amylase를 carboxypeptidase A와 B로 분해시킨 후 역상 HPLC gradient elution system을 수행한 결과 thr-trp-pro-lys-COOH로 밝혀졌다. 이들 서열의 상동성에 관해 토의하였다.