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PURIFICATION AND ANALYSIS OF THE STRUCTURE OF α-GALACTOSIDASE FROM Escherichia coli

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SUMMARY: α-Galactosidase, the product of the melA gene, was purified from a strain of Escherichia coli harboring a plasmid carrying melA, which overproduced the α-galactosidase. An apparent molecular weight was determined to be 50 kDa. The amino acid composition of this enzyme was determined. The result indicates that this enzyme is a hydrophilic and acidic protein. We have subjected the purified enzyme to 20 cycles of N-terminal sequence analysis. This verified the translation start site of the melA gene and the predicted N-terminal sequence.

The product of the melA gene, α-galactosidase, has been shown by the genetic analysis to be required for utilization of α-galactoside as a nutrient in Escherichia coli (1). We have cloned the melA gene together with the melB gene, the second structural gene which codes for the melibiose transport carrier, of the melibiose operon (2). We observed 7-fold overproduction of the α-galactosidase in a strain of E. coli harboring a plasmid carrying melA gene under appropriate conditions. We then determined the nucleotide sequence of the promoter region and the N-terminal region of melA. Thus we predicted the amino acid sequence of the N-terminal portion of the α-galactosidase of E. coli (3).

In 1971, Burstein and Kepes reported partial purification of this enzyme (4). The α-galactosidase of E. coli required Mn^{2+} and NAD^{+} for its activity. Unfortunately, purification of this enzyme has never been achieved because of its instability.

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Here we report the purification of this enzyme from the over-producing strain of E. coli. We also determined the amino acid composition of this enzyme and the amino acid sequence of the N-terminal portion, which showed complete agreement with our predicted sequence derived from the DNA sequence (3).

MATERIALS AND METHODS

Bacterium and Plasmid E. coli N1790/pSTY81 (2) was used. Cells were grown aerobically in a minimal medium (5) (Na^+ salts were replaced with K^+ salts), supplemented with 1 % casamino acids, at 37 °C. Melibiose (10 mM) was added to the culture medium to induce the melibiose operon, and cells were harvested 10 hours later at stationary phase.

Enzyme Assay α-Galactosidase was assayed as follows. Enzyme preparation was added to an assay mixture (0.5 ml) containing 10 mM Veronal buffer (pH 8.0), 3 mM $MnSO_4$, 0.15 mM NAD^+, 100 mM 2-mercaptoethanol and 1 mM p-nitrophenyl-α-D-galactopyranoside. After incubation at 37 °C for 10 min, 1 ml of 0.6 M Na_2CO_3 and 1 ml of 0.1 M EDTA were added, and the yellow color of the p-nitrophenol was monitored at 420 nm.

Amino Acid Composition Amino Acid composition of the α-galactosidase was determined by using the Beckman system 6300 as suggested in the manufacturer's manual. Cysteine and tryptophan were not determined.

Amino Acid Sequence Approximately 1 nmol of α-galactosidase was subjected to the sequence analysis in Applied Biosystems model 470A sequencer according to the manufacterer's instructions. The yield and identities of the products were determined on Spectra Physics HPLC system.

Other Methods SDS-polyacrylamide gel electrophoresis (6), silver staining (7), and protein determination (8) were performed as reported.

RESULTS AND DISCUSSION

Purification of α-galactosidase Cells (1.4 g wet weight; obtained from 250 ml culture) were washed and suspended in 7 ml of 10 mM Veronal buffer(pH 7.5). Cells were disrupted by passage through a French press, (1,500 kg/cm^2), and crude cell extract was obtained after centrifugation at 105,000 x g for 90 min. Most of the α-galactosidase activity was recovered in 50 to 80 %-saturated fraction of $(NH_4)_2SO_4$. This fraction was dissolved in a small volume of 10 mM Veronal buffer (pH 7.5), applied to a column of Bio-gel A 1.5m (1 x 120 cm), and eluted with a buffer containing 10 mM Veronal buffer, (pH 7.5), 3 mM $MnSO_4$, 1 mM 2-mercaptoethanol and 1 mM melibiose. Peak fraction of α-galactosidase activity was collected and subjected to DEAE-Sepharose column chromatography. After washing the column with a buffer (same buffer used for gel filtration) containing 180 mM NaCl, linear gradient of 180 mM to 250 mM NaCl was applied, and α-galactosidase was eluted (Fig. 1). We observed a single major peak of protein, which

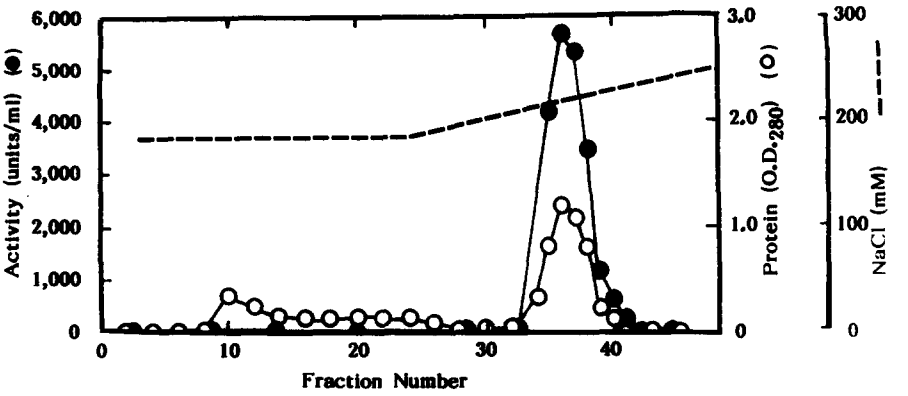

Fig. 1. Elution profile of the α-galactosidase from the DEAE-Sepharose column. Six mg of protein was applied to a column, eluted, and fractions were assayed as described in the text. One unit of activity is defined as the release of 1 nmol p-nitrophenol per min.

corresponds to the α-galactosidase activity. The peak fraction was collected, and stored frozen until use. Purity of the enzyme was checked on SDS-polyacrylamide gel electrophoresis. After silver staining we observed only one band corresponding to Mr=50 kDa (Fig. 2). This apparent molecular weight corresponds well to that we reported previously (2) and to that calculated from the deduced amino acid sequence (51 kDa)(9). We detected no minor band on SDS-polyacrylamide gel electrophoresis. We obtained 3 mg of purified enzyme starting from 1.4 g of wet cells. Since total protein in the crude extract was about 80 mg, the α-galactosidase consists of at least 4 % soluble cellular proteins. The increase in specific activity of the purified enzyme was around 20-fold compared with the crude cell extract.

The activity of the purified α-galactosidase required both Mn^{2+} and NAD^{+} (data not shown), as reported with a partially purified enzyme (4). Judging from the elution volume of α-galactosidase in Bio-gel A 1.5m, molecular

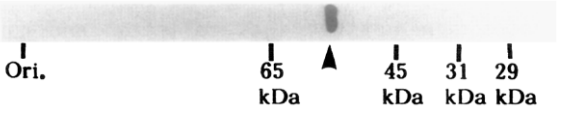

Fig. 2. SDS-polyacrylamide gel electrophoresis of the purified α-galactosidase. Purification of the α-galactosidase, gel electrophoresis, and staining were carried out as described in the text. Molecular weight markers used were bovine serum albumin (65 kDa), ovalbumin (45 kDa), DNase I (31 kDa), and carbonic anhydrase (29 kDa).

Table 1. Amino acid composition of the purified α-galactosidase

Amino acid	Number of residues	
	Determined[a]	DNA sequence[b]
Asx	43	44
Thr	28	29
Ser	14	14
Glx	48	47
Pro	23	23
Gly	28	28
Ala	43	43
Val	28	28
Met	14	16
Ile	31	32
Leu	40	39
Tyr	19	19
Phe	11	11
His	16	17
Lys	19	20
Arg	22	22
Cys	N.D.[c]	12
Trp	N.D.[c]	7
	Total	451

[a]Values given were corrected for degradation during hydrolysis. Total number of amino acid residues was taken as 451 (9).
[b]Amino acid composition predicted by the DNA sequence (9).
[c]Not determined.

mass of an active form of the enzyme was about 100,000 (data not shown). Therefore, this α-galactosidase seems to function as a dimer. Schmid and Schmitt (10) purified α-galactosidase from E. coli, which is specified by the Raf-plasmid. Judging from the molecular weight, oligomeric structure, amino acid composition and properties of the enzyme, the enzyme encoded by the plasmid is a different one from the enzyme encoded by chromosomal melA. Staining of the polyacrylamide gel for glycoprotein gave no positive reaction (data not shown), suggesting the lack of a detectable carbohydrate moiety in the enzyme. The purified α-galactosidase was not stable. Thus it was not suitable for further characterization of enzyme activity. However, it is suitable for structural analysis because it is a highly purified enzyme.

Amino Acid Composition The amino acid composition of the purified α-galactosidase was determined and is shown in Table 1. The amino acid composition of the purified protein closely matches the composition predicted from the DNA sequence of the melA gene reported by Liljestrom and Liljestrom (9). This

Table 2. N-Terminal sequence analysis of the α-galactosidase

Amino acid	Cycle	1	2	3	4	5	6	7	8	9	10
Major		Ser	Ala	Pro	Lys	Ile	Thr	Phe	Ile	Gly	Ala
Minor		Met	Met	Ser	Ala	Pro	Lys	Ile	Thr	Phe	Ile
Deduced[a]		Met	Met	Ser	Ala	Pro	Lys	Ile	Thr	Phe	Ile
Amino acid	Cycle	11	12	13.	14	15	16	17	18	19	20
Major		Gly	Ser	Thr	Ile	Phe	Val	Lys	Asn	Ile	Leu
Minor		Gly	Ala	Gly	Ser	Thr	Ile	Phe	Val	Lys	Asn
Deduced		Gly	Ala	Gly	Ser	Thr	Ile	Phe	Val	Lys	Asn

Approximately 1 nmol of the α-galactosidase was subjected to the sequence analysis. The ratio of major amino acid:minor amino acid was about 4:1.
[a]Sequence deduced from the DNA sequence analysis (3, 9).

verifies that the protein we purified is really the α-galactosidase, product of melA, and that the DNA sequence of melA is most likely to be correct. The amino acid composition agrees well with the fact that the α-galactosidase is a soluble protein. Also, it indicates that this enzyme is an acidic protein similar to other α-galactosidases (10).

Amino Acid Sequence of N-Terminal Portion We have subjected the purified α-galactosidase to 20 cycles of N-terminal sequence analysis (Table 2). We detected two amino acid residues (serine as major one and methionine as minor one) at N-terminus, and two residues at any cycle of sequence analysis. The ratio of the two residues was about 4:1. It seemed that our preparation of α-galactosidase contained two proteins. Fortunately, however, our sequence analysis revealed that the sequence of the first 18 residues of the major one was exactly same as that of the 18 residues starting from the third residue of the minor one (Table 2). This means that the major one lacks the first two methionine residues of the minor one. It is likely that the removal of N-terminal two methionine residues has been taken place during the purification step. Alternatively, the removal of the two methionine residues might occur in the cells. At present we do not know whether the removal of the two methionine residues affects the activity of the α-galactosidase or not. We, however, were able to determine the amino acid sequence of the 22 residues of N-terminal region of the α-galacto-

sidase, which showed perfect agreement with the sequence deduced from the DNA sequence (3, 9). Thus, the results verified the translation starting point of the melA gene and the predicted N-terminal sequence (3, 9).

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