INHIBITION OF ALKALINE PHOSPHATASE ISOZYME CONVERSION BY PROTEASE INHIBITORS IN *ESCHERICHIA COLI* K-12

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1. Introduction

A variety of isozyme patterns are observed, on electrophoresis, in alkaline phosphatase of *Escherichia coli* K-12 [1]. The molecular mechanism of isozyme formation has been elucidated as follows: The isozyme 1, with extra arginine residues at the amino-terminal position, is first synthesized and then the residues are removed, resulting in the formation of isozymes 2 and 3 in the bacterial cell [2–4]. A mutant strain, producing only isozyme 1 and a small amount of isozyme 2 [4,5], is supposed to be defective in the proteolytic enzyme which removes the amino-terminal arginine residues from isozymes 1 and 2.

Here we report that the conversion of isozymes was inhibited unexpectedly by endopeptidase inhibitors, antipain and leupeptin, but not by other inhibitors tested, including bestatin, an inhibitor of aminopeptidase B.

2. Materials and methods

Escherichia coli strain K10, a derivative of K-12, was used in this study.

A TG medium [6], a mineral salt solution buffered with Tris–HCl at pH 7.2 and containing 0.2% glucose was supplemented with excess phosphate (6.4×10^{-4} M). Casamino acids (2 mg/ml) and arginine (200 μg/ml) were also added to the medium, if necessary.

Protease inhibitors were obtained from the Institute of Microbial Chemistry, Tokyo. They were inhibitors of serine-thiol protease, antipain [7], leupeptin [8,9], chymostatin [10], elastatinal [11]; inhibitor of carboxypeptidase, pepstatin [12]; inhibitor of aminopeptidase B, bestatin [13]. Oligopeptides were purchased from Sigma Chemical Co.

To prepare alkaline phosphatase sample for electrophoresis, the cells were washed once and resuspended in 0.01 M Tris–HCl (pH 7.2) containing 10^{-3} M $MgSO_4$ (TM buffer) and disrupted with sonication. The cell lysate was then heated in a hot water bath ($\leq 80°C$) for 20 min and the supernatant was applied on electrophoresis.

A 7.5% polyacrylamide gel electrophoresis [14] was performed at a constant 300 V for 3 h at room temperature. After electrophoresis, the gel was stained for alkaline phosphatase activities by soaking in a mixture of naphtol–AS–MX–phosphate and fast blue RR salts in 0.1 M Tris–HCl (pH 8.0) [15].

3. Results and discussion

In *Escherichia coli* alkaline phosphatase, two typical isozyme patterns are observed, on electrophoresis, depending on the nutritional conditions during phosphate starvation (synthesis of the enzyme). All 3 main isozymes are observed when the enzyme sample is prepared from the cells grown in the presence of casamino acids or arginine, but only one (isozyme 3, the most negatively charged band of the 3) is observed in the absence of these agents [5,16,17]. The conversion of isozyme patterns occurs, when the cells are transfered from the former condition to the latter, even when further synthesis of the enzyme is repress-

ed by addition of excess phosphate. Thus, the conversion is apparently inhibited by arginine or casamino acids (see fig.1, lanes 1,10).

Recently, we isolated a mutant strain deficient in the isozyme conversion. The mutation (*iap*) was mapped on 58.5 min of *E. coli* genetic map [18], an opposite of alkaline phosphatase structural gene (*phoA*). The amino-terminal amino acid sequence in isozyme 1′ synthesized in the mutant strain was the same as that in the wild-type strain [2], in spite of the altered mobility caused by the pseudo wild-type structural gene of alkaline phosphatase [3]. These results suggest that the *iap* gene product removes the amino-terminal arginine residues from isozymes 1 and 2.

We examined the effects of various protease inhibitors on isozyme conversion. The exponentially growing bacteria in TG medium supplemented with excess phosphate, casamino acids and arginine were transfered into the same medium without supplemented phosphate. After 9 h incubation at 37°C, the cells were harvested and resuspended into the same medium with excess phosphate (without casamino acids and arginine), to repress further synthesis of the enzyme. To the bacterial suspension, protease inhibitors were added, and then incubated further 14 h. As shown in fig.1, the conversion of isozyme pattern was

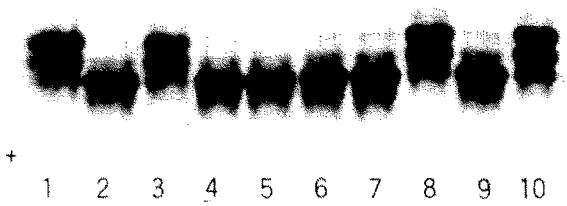

Fig.1. Polyacrylamide gel electrophoresis of *E. coli* alkaline phosphatase. The bacterial cells were grown under phosphate starvation in the presence of casamino acids and arginine, (1); and then transfered into fresh medium supplemented with excess phosphate followed by further incubation, (2); with 400 μg/ml each of antipain, (3); bestatin, (4); chymostatin, (5); elastatinal, (6); leupeptin, (8); pepstatin, (9); casamino acids and arginine, (10).

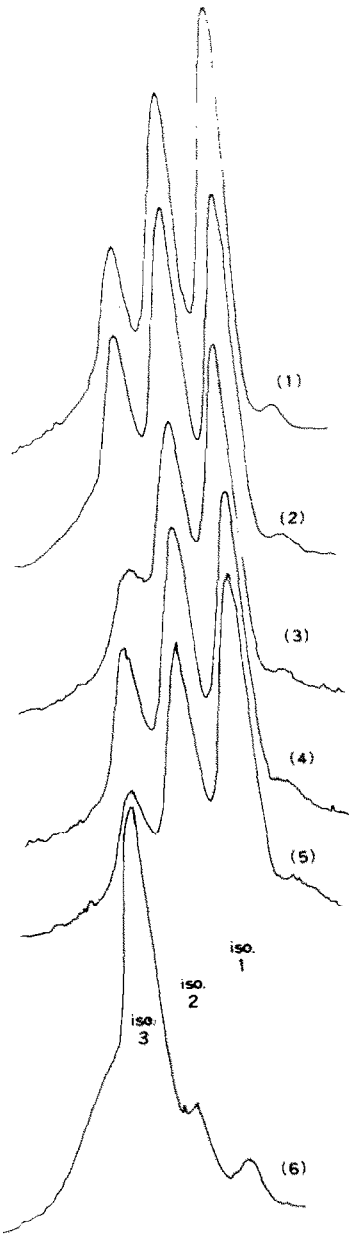

Fig.2. Densitometric scan at 600 nm of electrophoretic gel stained for alkaline phosphatase activities. The experimental procedure was the same as fig.1. (1), after phosphate starvation with casamino acids and arginine (before further incubation); (2) to (6), after further incubation. (2), with casamino acids and arginine; (3), antipain (3.3 mM); (4), leupeptin (3.3 mM); (5), arginine (10 mM); and (6), without inhibitor.

inhibited by antipain (lane 3) and leupeptin (lane 8), as well as casamino acids and arginine (lane 10), but not by other protease inhibitors including bestatin (lane 4), an aminopeptidase B inhibitor.

To test whether the synthesis of alkaline phosphatase was influenced by the protease inhibitors, the inhibitors were added to the bacterial culture at a final concentration of 400 μg/ml, during starvation of phosphate. After overnight incubation, no remarkable difference of the residual growth of the cells during phosphate starvation nor the enzyme activities were observed. The same isozyme patterns as in fig.1 were observed after electrophoresis.

For the comparison of various agents on inhibition of isozyme conversion, densitometric scan was performed on polyacrylamide gel stained for alkaline phosphatase activities and the area of each isozyme peak was measured (fig.2). Since it has been reported that the isozymes 1 and 3 consist of two homologous polypeptides with and without amino-terminal arginine, respectively, and isozyme 2 is composed of one monomer of isozyme 1 and one monomer of isozyme 3 [17,19], the average number of remaining arginine residues in amino-terminal position (N) after incubation with various agents were calculated as follows:

$$N = \frac{\text{area 1} \times 2 + \text{area 2}}{\text{areas 1} + 2 + 3}.$$

As shown in table 1, antipain and leupeptin inhibited conversion at a lower concentration than arginine. A dipeptide, L-arginyl-L-aspartic acid revealed a similar effect as the latter. Less effective inhibition was observed in canavaine, L-glycyl–L-glycyl–L-tyrosyl–L-arginine, *p*-aminobenzamidine (PAB), *N*-α-*p*-tosyl–L-lysine chloromethyl ketone (TLCK) and *p*-tosyl–L-arginine methyl ester (TAME). No inhibition was observed in chymostatin, elastatinal, bestatin, pepstatin, aprotinine (Trasylol), *N*-α-*p*-tosyl–L-lysine methyl ester (TLME), *N*-α-*p*-tosyl–L-arginine amide and dipeptides containing no arginine residue.

It is noteworthy that the inhibitors of endopeptidase, antipain, leupeptin and PAB, inhibited isozyme conversion, whereas, an inhibitor of aminopeptidase B, bestatin did not. This result was unexpected since isozyme conversions were brought about by removing the amino-terminal arginine residues.

It might be possible that the activity of a new type protease, presumably a product of *iap* gene, is inhibited by these protease inhibitors or that the gene product requires proteolysis for its activation and the activation is inhibited by the protease inhibitors.

Table 1
Inhibition of isozyme conversion in vivo[a]

Inhibitor	Concentration (mM)					
	0	0.1	0.33	1.0	3.3	10.0
-(before incubation)	1.17					
-(after incubation)	0.23					
-(standing in cold)	0.39					
Antipain		0.66	1.04	1.21	1.24	
Leupeptin		0.81	1.06	1.18	1.20	
Arginine			0.38	1.06	1.18	1.27
Canavanine			0.36	0.56	0.74	1.15
L-Arginyl–L-aspartic acid			0.33	0.81	1.12	1.17
L-Glycyl–L-glycyl–L-tyrosyl–L-arginine			0.47	0.74	0.87	0.93
TAME						0.63
TLCK						0.56
p-Aminobenzamidine			0.24	0.48	0.56	0.77

[a] The average number of remaining arginine residues in the amino-terminal position (see the text)

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