Studies on the Protease of *Pseudomonas*

Part VI. On the Role of Ca in the Enzyme Production (supplemented)

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A discussion was made on the step of the incorporation of Ca in the synthesizing reaction of the proteinase of *Ps. myxogenes* sp.

When the cell suspensions containing a carbon source without addition of Ca were shaken aerobically, the secreted protein content was found to be remarkably lower than that of the suspensions containing Ca, while total amounts of secreted organic nitrogen materials were almost the same despite of the presence or absence of Ca. From the experimental result of an auto-splitting phenomenon of the proteinase by Ca-removal treatment, it was assumed that the above result occurs by auto-digestion of the proteinase secreted in the medium where Ca is absent. Accordingly, the step of incorporation of Ca would be considered to occur in the synthesized Ca-free proteinase.

INTRODUCTION

In a previous paper¹⁾ of this series, it has been shown that Ca, which is required as an essential factor²⁾ for the proteinase production of *Ps. myxogenes* sp., is a constituent of the produced enzyme itself, while the removal of Ca from the enzyme molecule causes inactivation. Further, it has also been indicated³⁾ that the resting cells of the bacteria could produce the enzyme by shaking them with the addition of only a carbon source and Ca ion without accompanying growth. That is, nitrogen material within the cells contributes to the enzyme synthesis. Further, from the experiment on amino acid analogues³⁾ in the enzyme production, it was assumed that in any step of the enzyme-synthesizing reaction Ca might be required from the precursor substance which is a more complicated substance than amino acids.

Therefore, the following two pathways are to

be considered for the problem concerning the step of incorporation of Ca in the enzyme-synthesizing reaction;

1. Precursor + Ca → Precursor-Ca → Proteinase
2. Precursor → Ca-free

Proteinase + Ca → Proteinase

In order to clarify this problem, the following experiment was performed.

EXPERIMENTAL AND RESULTS

I. Researches for other substances constituted with Ca

As will be seen in the following paper⁴⁾, a precursor material immunologically related to the proteinase cannot be found within the cells. However, whenever a protein or high molecular peptide which incorporates with Ca within the cells at the time when the cells are shaken for enzyme formation, is formed this protein or peptide might be assumed to be the precursor of the enzyme. This also may clarify that the incorporation of Ca occurs at a precursor state of enzyme synthesis.

The following experiment was undertaken to clarify this question. The washed cells of the bacteria was prepared as already shown in the previous paper³⁾. Each

1) K. Morihara, This Bulletin, **23**, 60 (1959).

2) K. Morihara, *ibid.*, **20**, 243 (1956).

3) K. Morihara, *ibid.*, **23**, 49 (1959).

4) K. Morihara, This Bulletin, **24**, 467 (1960).

100 ml contained 200 mg of the cells as dry weight in M/50 phosphate buffer of pH 7.0, with 1% fructose, and 10^{-3} M CaCl_2 composed of $20 \mu\text{C}$ $\text{Ca}^{45}\text{Cl}_2$ of final concentration, and was shaken aerobically in 500-ml shaking flasks at 30°C for 5, 10 and 16 hrs., respectively. Cells at various ages were collected by centrifugation, washed with distilled water several times, and crushed with alumina in the cold. After centrifugation at 10,000 r. p. m. for 30 minutes in the cold, the proteolytic inactive clear supernatant was subjected to paper-electrophoresis using M/20 Tris-buffer (pH 8.5) containing M/100 Ca-acetate as a buffer solution by 0.5 mA/cm at about 5°C . After a 16-hours run, the paper was dried in the atmosphere. The detection of protein or peptide was performed by Amidoschwarz, brom phenol blue or ninhydrin on a part of the paper, and contact printing to X-ray film (radio-autography) on the remaining strip was made for about two months. Exposure of X-ray film was not observed at all in the fraction corresponding to the colour reaction of protein or peptide.

From the result obtained, it can be said that the protein or peptide constituted with Ca is not formed within the cells at the time when enzyme production occurs.

II. Differences in characters of the organic nitrogen materials secreted from the cells in the presence or absence of Ca ion

In the previous paper³⁾, it has been shown that the maximum activity attained by cell suspensions with a carbon source is remarkably lowered when Ca ion is added after more than an 8-hours shaking period. The same phenomenon was also observed in the washed cells which were previously shaken with a carbon source even in the presence of Ca ion for more than 8-hours. On the other hand, decrease of the enzyme producing ability was not observed in those cells which were shaken for 24 hrs. without a carbon source or even in the presence of not only carbon but also nitrogen sources.

Accordingly, it could be considered that the nitrogen substance which should be transformed to the enzyme would be consumed by shaking cells with a carbon source despite of the presence or absence of Ca ion, that is, in both cases of production or no-production of the enzyme. Hence, the following question arises: what substance is secreted in the medium without Ca by consumption of nitrogen substance of cells, while the enzyme is seen at the extracellular site in the presence of Ca.

To clarify this question, the nature of the organic nitrogen materials secreted from the cells in either the presence or absence of Ca was examined.

Two hundred mg of the washed cells (as dry weight) which were prepared as shown in the previous paper³⁾ was added to the reaction mixture containing M/50 phosphate buffer of pH 7.0 in the presence or absence of 0.5% fructose and also 10^{-3} M CaCl_2 , respectively, to make the total mixture up to 100 ml, in 500-ml shaking flasks, and incubated on a shaker at 30°C for 16 hours. Following this procedure, a clear supernatant was obtained from the reaction mixture by centrifugation at 5000 r. p. m. for 30 minutes.

The fructose content was measured by the method of Bertrand, and the proteolytic activity was determined by the usual method²⁾. The contents of organic nitrogen materials were examined on 0.5 ml of the supernatant (made up to 6.5 ml, in final) at 500 m μ by the method of Lowry et al.⁵⁾. The determination of the secreted protein content was made on one ml of the solution of the isolated materials from the supernatant, by the method of Lowry et al.⁵⁾ (final to 6.5 ml) at 500 m μ . Isolation was made from 50 ml of the supernatant by such treatments as rivanol precipitation (0.1%), elution with 0.5 M CaCl_2 , fractional precipitation of acetone (40~60%) and the final volume was made up to 10 ml with water. The results are given in Table I.

From the table, it is clear that organic nitrogen substances are secreted in the medium by shaking the cells with fructose in almost the same amount regardless of the presence or absence of Ca, while only a little secretion is seen in the absence of carbon source. On the other hand, the content of protein secreted in the medium in the absence of Ca is found to be almost one-fifth of that observed in the presence of Ca. The above results show that a low molecular organic nitrogen material is secreted when the cells are shaken with a carbon source in the absence of Ca.

III. Effect of Ca-removal treatment on the nature of enzyme protein

The low molecular organic nitrogen material seen in the medium in the absence of Ca could be caused by autodigestion of the proteinase secreted in the medium. To clarify this point, the following experiment was performed.

A portion of 0.75 ml of the reaction mixture containing the crystalline proteinase (670 [u]/ml) and 0.25 M Tris-buffer of pH 8.0 was kept at 50°C . for two hours in the presence or absence of 0.125 M EDTA. When treated in the absence of EDTA was proteolytic activity found to be unchanged (670 [u]/ml), while activity was remarkably diminished (19 [u]/ml) in the presence of

5) O.H. Lowry et al., *J. Biol. Chem.*, **193**, 265 (1951).

TABLE I. DIFFERENCES IN THE NATURE OF THE ORGANIC NITROGEN MATERIALS SECRETED BY THE CELLS IN THE PRESENCE OR ABSENCE OF Ca.

Composition of the reaction mixture		Fructose utilized mg/100ml	Proteolytic activity [u]/ml	Organic nitrogen contents secreted (OPT.D.)	Protein contents secreted (OPT.D.)
fructose	CaCl ₂				
+	+	275	5.5	0.25	0.21
+	-	265	0.2	0.23	0.04
-	+	—	0.0	0.045	0.0

FIG. 1. Paper-electrophoresis of the Proteinase Treated in the Presence or Absence of EDTA at 50°C.

0.1 ml of the reaction mixture treated in the presence (▨) or absence (□) of 0.125 M EDTA at 50°C, was subjected to paper-electrophoresis. The phoresis was done by 0.5 mA/cm (450 V) at about 5°C for 16 hrs., using M/20 Tris buffer (pH 8.5) containing M/100 calcium-acetate as buffer solution.

EDTA.

To determine the protein nature of the enzyme inactivated by the treatment of EDTA, 0.1 ml of the treated reaction mixture was subjected to paper-electrophoresis (width of the paper 2 cm, length 15 cm). The buffer solution used was the same as that mentioned in section I of this paper, and phoresis was also done on the same condition. After drying in the atmosphere, the paper was immersed in 0.2% brom phenol blue solution saturated with HgCl₂, and then washed repeatedly by distilled water until the colour disappeared in the washed solution. The paper was cut into pieces, each 0.5 cm in length, eluted with 6.0 ml of 0.01 N NaOH, and the optical density read at 575 mμ within 30 minutes with a Beckmann photometer. The results are shown in

Fig. 1.

The weak colour reaction of the inactivated enzyme solution treated with EDTA compared with that of its absence might be considered to be caused by splitting the enzyme protein into small fragments. Further, it was found that little precipitation is seen even if a two times volume of acetone is added to the inactivated enzyme solution.

Accordingly, it could be said that Ca-free proteinase cannot maintain its original protein structure owing to instability occurring by auto-digestion or for other reasons.

SUMMARY AND DISCUSSION

The extracellular proteinase of *Ps. myxogenes* sp. is constituted with Ca as an essential component as shown in the previous paper¹⁾. This paper deals with a study concerning the step of incorporation of Ca in the synthesizing reaction of the proteinase of the bacteria.

A protein or peptide substance constituted from Ca is not found within the cells of the bacteria which have been shaken in the presence of a carbon source and Ca ion, while the proteinase is accumulated at the extracellular site. As it is sure that nitrogen material within the cells (which has been assumed to be a more complicated substance than amino acid, which is a precursor substance) contributes to the enzyme synthesis, the above result might suggest that Ca is not incorporated at the precursor state in the enzyme synthesis, or even if the incorporation really does occur at that state, the precursor substance is immediately secreted in the medium. However, we have not found the precursor material which is secreted in the medium where the material is activated autocatalytically as seen in *Streptococcal* proteinase⁶⁾.

6) S.D. Elliott, *J. Exptl. Med.*, **92**, 201 (1950).

Accordingly, it is difficult to consider the latter case to occur.

The total amount of organic nitrogen material secreted in the medium containing a carbon source by shaking the bacterial cells aerobically, is found to be identical in both the presence or absence of Ca ion, but the secreted protein content is remarkably diminished (to almost one-fifth) in the absence of Ca ion. Further, it is clear that the removal of Ca from the enzyme molecule, by treatment with EDTA at 50°C, results not only in inactivation of the enzymatic action but also exhibits the auto-splitting phenomenon of the enzyme protein.

These results may lead to the assumption that Ca-free proteinase would be secreted in the

medium regardless of the existence of Ca ion, and the enzyme is accumulated when it is stabilized by the constitution of Ca ion in the medium, whereas the auto-splitting phenomenon of the enzyme would take place in absence of the Ca ion.

Therefore, the incorporation of Ca in the proteinase synthesis by the bacteria is considered to occur in the completely synthesized Ca-free proteinase secreted in the medium.

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Studies on the Protease of *Pseudomonas*

Part VII. An Immunological Study of the Crystalline Proteinase

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An immunological study was made on the crystalline proteinase of *Ps. myxogenes* sp.

By application of this method, it was shown that not only the determination of the purity of the enzyme which is used as an antigen, but a comparison of protein nature of the enzyme with proteinases of other origins is also attainable.

INTRODUCTION

Recently, in the study of enzymes, immunological methods have been applied to examine the protein nature¹⁻³⁾ of enzyme and to

analyze⁴⁻⁷⁾ the mechanism of enzyme formation.

Since the author has succeeded in the prepa-

1) S.D. Elliott, *J. Exptl. Med.*, **92**, 201 (1950).

2) T. Amano et al., *Med. J. Osaka Univ.*, **7**, 593 (1956).

3) T. Wada, *J. Biochem.*, **46**, 329 (1959).

4) M. Cohn and A.M. Torriani, *J. Immunol.*, **69**, 471 (1952).

5) M. Cohn and A.M. Torriani, *Biochim. Biophys. Acta*, **10**, 280 (1953).

6) M.R. Pollock, *J. Gen. Microbiol.*, **14**, 90 (1956); **15**, 154 (1956).

7) M. Nomura and T. Wada, *J. Biochem.*, **45**, 629 (1958).