Studies on the Protease of *Pseudomonas*

Part IV. Factors Affecting the Enzyme Production, Especially on the Effect of Calcium

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The extracellular proteinase of *Ps. myxogenes* sp. was found to be produced by cell suspension in a phosphate buffer of pH 7.0 containing a carbon source and calcium ion under aerobic conditions.

Various factors having effect on the proteinase production were studied, and calcium was found to be essential in its production. However, in cells prepared from a synthetic medium without calcium, the enzyme producing ability was possessed similarly as in those from the medium containing calcium, and the production was not altered by amino acid analogues. Thus, it might be assumed that calcium is necessary in the later stages, from the more complex precursor state than the amino acid-stage, in the biosynthetic reaction of proteinase. In the presence of available calcium, the balance between the concentrations of the carbon source and cells was important in production, and by maintaining the balance of both factors the enzymatic activity produced was almost parallel to the cell concentrations. Furthermore, the addition of a nitrogen source promoted production about three-times.

INTRODUCTION

The previous paper¹⁾ has shown that the production of the extracellular proteinase of *Ps. myxogenes* sp. in growing culture was remarkably affected by some components of the synthetic media: 1) Ca was an indispensable agent, 2) a rather high concentration of glucose was required for the promotion of the production, 3) supplementation of growth promoting agents, such as MgSO_4 or corn steep liquor, in an excess amount suppressed production. Moreover, it was pointed out that the proteinase did not appear until glucose had been used up from the media in growing culture.

In order to study whether the above factors are concerned with the net-proteinase production or not, it was desirable to use a cell suspension in production. And, it was soon

shown that proteinase is produced in the absence of growth by shaking the flasks containing a cell suspension in a phosphate buffer of pH 7.0 with a carbon source and calcium ion under aerobic conditions. Further studies were conducted on various factors affecting the enzyme production. From observations on the behavior of calcium and other factors in production by cell suspensions, the phenomenon observed in growing culture was taken into account.

METHODS

The medium used to obtain cells for proteinase production had the following composition: glucose, 4%; $(\text{NH}_4)_2\text{HPO}_4$ and $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1%; KH_2PO_4 , 0.2%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05%; pH 7.0. Resting cell suspensions were obtained by growing *Ps. myxogenes* sp., inoculated from a Bouillon-agar culture, in 100 cc. of this medium in a 500-cc. shaking flask kept on a shaker at 30°C for 16 hrs. Harvesting was accomplished by centrifugation, cells washed thrice with distilled water,

1) K. Morihara, This Bulletin, 20, 243 (1956).

the precipitated material being removed by mild centrifugation and the final suspension adjusted to a concentration of 10 mg. cell material (as dry weight) per one cc. with distilled water. The yield of cells from 100 cc. of this culture was about 300 mg. (dry weight).

To test the proteinase production, one cc. of this cell suspension was added into each solution containing various reagents, adjusting the final volume to 5 cc. and the mixture aerated on a reciprocal shaker of 7 cm amplitude and 130 r.p.m. at 30°C. Proteolytic activity produced in the reaction mixture was measured at various intervals by liquefaction of gelatin using the supernatant, as shown in the previous paper¹⁾. For the examination of the rate of growth, the reaction mixture was diluted to one-tenth with distilled water, and the turbidity was determined at 530 m μ using a Beckmann photometer. The diluted reaction mixture which showed a turbidity equivalent to the optical density of 0.45, contained approximately 0.2 mg. of the dried cells per one cc.

The fructose content was determined by the method of Bertrand, and the amino acid content by the Ninhydrin method²⁾. The oxygen uptake of cells in various carbon sources was measured with the Warburg apparatus, containing 0.2 cc. of 0.05M substrate in the side arm, 1.5 cc. of 0.06M phosphate buffer of pH 7.0 and 5 mg. of cells as dry matter in the main compartment, 0.2 cc. of 20% KOH in the centre well of the vessel, 3 cc. in total volume being shaken at 37°C in air. Oxygen uptake was calculated by subtracting the value of autorespiration, and expressed as Q_{O_2} (μ l. of oxygen uptake/hr./mg. cells).

RESULTS

I. General conditions for the enzyme production in washed cell suspensions.

1) **Effects of calcium and carbon sources.** The cell suspensions were prepared from the cultural medium lacking $CaCO_3$ as described above. Attempts to induce the proteinase biosynthesis were made by shaking the cell suspensions with various reagents under aerobic conditions. The results are shown in Fig. 1. In the absence of $CaCl_2$ little production was observed, while in the reaction mixture containing $10^{-3}M$ $CaCl_2$ without accompanying the growth, the development of activity began within four hours and gradually accelerated to reach a steady vigorous rate within 20 hours. However, even in the presence of $CaCl_2$ ($10^{-3}M$), the addition of

FIG. 1. Effect of Various Reagents in Proteinase Production by Cell Suspensions of *Ps. myxogenus* sp.

Five cc of the reaction mixture contained 10 mg of cells (as dry weight), M/50 phosphate buffer of pH 7.0, and the others as follows:

- A, 0.2% fructose, $10^{-3}M$ $CaCl_2$ (○—○)
- B, 0.2% glucose, $10^{-3}M$ $CaCl_2$, 5 mg $CaCO_3$ (●—●)
- C, 0.2% Na-pyruvate, $10^{-3}M$ $CaCl_2$ (●—●)
- D, 0.2% fructose (Δ — Δ)
- E, $10^{-6}M$ $CaCl_2$ (×—×)

calcium-removing agents such as EDTA* ($10^{-3}M$), Na-oxalate ($5 \times 10^{-3}M$), Na-citrate ($5 \times 10^{-2}M$) and NaF ($5 \times 10^{-2}M$) inhibited production. It might be presumed that these agents combined with calcium, making calcium unavailable to the cells. Interestingly, as seen in Fig. 2, the inhibitory effect is seen at every stage of production, that is, the development of the production is immediately stopped by the addition of a calcium-removing agent (EDTA, $10^{-3}M$) which was shown not to affect the stability of the proteinase which had already been produced. The inhibition, however, was recovered by increasing the concentration of $CaCl_2$, i.e. $5 \times 10^{-3}M$ $CaCl_2$ to $10^{-3}M$ EDTA. On the contrary, after shaking the reaction mixture without $CaCl_2$, $CaCl_2$ was added at various intervals and its effect was studied (Fig. 3). In any case, production by the addition of $CaCl_2$ was not observed immediately but after a lag period. By shaking the mixture without $CaCl_2$ for four hours, and then adding $CaCl_2$, the maximum activity attained was the same as that when $CaCl_2$ was added initially, whilst when $CaCl_2$ was added after more than an 8-hour shaking period the maximum activity attained was lowered in spite of

2) S. Moore, W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

* ethylenediamine tetraacetate-Na.

FIG. 2. Effect of EDTA in Proteinase Production.

Five cc. of the reaction mixture contained 10 mg. of cells (as dry weight), M/50 phosphate buffer of pH 7.0, 0.5% fructose and 10^{-3} M CaCl_2 , and was shaken (O—O). At the various steps of the reaction, shown by the arrows in the figure, 0.05 cc. of 10^{-1} M EDTA was added into the reaction mixture and then the reaction was continued (Δ --- Δ).

shortening the lag period to some extent. Perhaps the material which may be transformed into the enzyme in cells might be utilized in spite of the non-production of the enzyme. By further study, it was shown that the rate of utilization of fructose was unchanged with or without CaCl_2 . Accordingly, it is difficult to consider that CaCl_2 is concerned in the metabolism of carbon source.

The indispensable effect of CaCl_2 was substituted with SrCl_2 but not by other ions such as BaCl_2 , MgCl_2 , CoCl_2 , MnCl_2 , NiCl_2 , ZnCl_2 and CdCl_2 (Table I). From the table, it is apparent that the effect of CaCl_2 or SrCl_2 is developed by the cation.

The production, similarly, does not occur in the absence of a carbon source, and also, the absence of phosphate. As a carbon source (activity^{*1}, expressed as $[\mu]/\text{cc.}$, shown in parenthesis), fructose (5.5) could be replaced with other substances such as glucose (4.2), ethanol (3.2), gluconate (4.2), pyruvate (4.0), succinate (4.0), fumarate (4.2), lactate (4.2) etc., and various

*1 The activity measured after shaking for 16 hrs. in the reaction mixture containing 10 mg. of cells (as dry weight), M/50 phosphate buffer of pH 7.0, 10^{-3} M CaCl_2 and various carbon sources at their optimal concentrations. In the reaction mixture containing glucose or ethanol, 5 mg. of CaCO_3 was added for neutralization.

FIG. 3. Effect of Calcium in Proteinase Production.

Five cc. of the reaction mixture contained 10 mg. of cells (as dry weight), M/50 phosphate buffer of pH 7.0 and 0.5% fructose, and was shaken (E, Δ --- Δ). At various intervals of shaking, i.e. 0 (A), 4 (B), 8 (C) and 16 (D) hours as shown by the arrows in the figure, 0.05 cc. of 10^{-1} M CaCl_2 was added into the reaction mixture, and further the reaction was continued (O—O).

amino acids such as histidine (8.1), glutamate (5.3), aspartate (5.6), leucine (3.5), and casein hydrolysate^{*2} (4.0) etc., but not by starch, dextrin, glycogen, sucrose, lactose, cellobiose, maltose, galactose, mannose, ribose, arabinose, xylose etc. and various proteins which were not metabolized by the cells. While, citrate, acetate, various sugar alcohols such as glycerol, mannitol and various peptides are slightly effective in enzyme production. The pH of the reaction mixture, however, could not be kept constant at pH 7.0 during the reaction with some useful carbon sources: By using glucose (or ethanol), the pH of the reaction mixture was lowered to a value below 5.0 within four hours' reaction, thereby inhibiting the production completely (so that the addition of CaCO_3 was required to keep the pH at neutrality in the reaction mixture), while with pyruvate the pH rose above 8.0, at which the production was not optimum as will be shown later. The difference in activity by different carbon sources may be reasoned partly by the change of pH during reaction. It is of interest to note that glucose which showed a high value of oxygen consumption ($Q_{O_2}=126$) was not so

*2 "KAZAMINOSAN" by Nissan Co.

TABLE I. EFFECT OF VARIOUS METAL IONS ON ENZYME PRODUCTION

Five cc. of the reaction mixture contained 10mg. of cells (as dry weight), M/50 phosphate buffer of pH 7.0, 0.2% of fructose and various metal ions as shown in the table, and shaken for 16 hrs.

Metal	(M)	Enz. activity ([u]/cc.)	Metal	(M)	Enz. activity ([u]/cc.)	Metal	(M)	Enz. activity ([u]/cc.)
None		0.2	BaCl ₂	10 ⁻⁵	0.3	NiCl ₂	10 ⁻³	0.0
CaCl ₂	10 ⁻³	5.5	MgCl ₂	10 ⁻³	0.5		10 ⁻⁵	0.5
SrCl ₂	10 ⁻²	6.2		10 ⁻⁵	0.4	ZnCl ₂	10 ⁻³	0.2
	10 ⁻³	//	CoCl ₂	10 ⁻³	0.0		10 ⁻⁵	//
	10 ⁻⁴	0.7		10 ⁻⁵	0.6	CdCl ₂	10 ⁻³	//
	10 ⁻⁵	0.3	MnCl ₂	10 ⁻³	0.0		10 ⁻⁵	//
BaCl ₂	10 ⁻³	0.5		10 ⁻⁵	0.5			

effective as fructose which had a low value ($Q_{O_2}=40$).

On the other hand, even when the suitable reagents mentioned above were contained production was not observed under anaerobic conditions. Further study proved the reaction to be inhibited almost completely by various metabolic inhibitors, such as KCN, NaN₃, methylene blue, monojodacetate, arsenite, dinitrophenol, α , α' -dipyridyl ($10^{-3}M$, respectively) and streptomycin and chloramphenicol ($10 \gamma/cc.$ respectively), but not by penicillin G ($100 \gamma/cc.$). Fukumoto et al.³⁾ have shown that the inhibitory effect of chloramphenicol on amylase production of *B. amyloliquefaciens* is diminished by addition in a later stage of reaction, but in this case such a phenomenon was not observed.

2) Effects of pH and phosphate. At various pH values, adjusted with phosphate and Tris-buffer* solutions, the production in cell suspensions was studied as shown in Fig. 4. Using these buffer solutions in the reaction mixture containing fructose, the change of pH throughout the reaction was inconsiderable except with M/50 phosphate buffer at the alkali-side. The figure shows that the optimum pH is approx. pH 7. High concentration of buffer solution revealed to suppress the production; in M/5 phosphate buffer a suppression in production was observed and also, the optimum pH shifted to 6.0.

It is of interest to note that a difference in production is observed with or without phosphate, especially at pH 6.0. Fukumoto et al.⁴⁾ have found that amylase production by cell suspensions of *B. amyloliquefaciens* could hardly be observed without phosphate when sugars were used as the carbon source, while a slight production was seen in the case of organic acid salts. In

FIG. 4. Effect of pH in Proteinase Production.

Five cc. of the reaction mixture contained 10mg. of cells (as dry weight), 0.2% fructose, $10^{-3}M$ CaCl₂ and various buffer solutions varying the pH values:

- I. M/5 phosphate buffer (●—●)
- II. M/50 phosphate buffer (○—○)
- III. M/50 Tris-buffer (adjusted by HCl, △—△)

The reaction was done for 16 hrs.

order to clarify this problem, the effects of various concentrations of phosphate in proteinase production were studied at various pH values, adjusted with M/50 Tris-buffer (Table II).

From the table, it can be seen that the effect of phosphate is observed remarkably at pH 6, slightly at 7 and not at 8, with the concentration of the phosphate at over $10^{-3}M$. And also, the utilization of fructose is shown to be almost parallel with production in various pH values with or without phosphate.

It is a well-known fact that phosphorus plays an important role in metabolism, that is, the oxidative phosphorylation in living organisms. Then, the ques-

3) J. Fukumoto, T. Yamamoto, D. Tsuru, *J. Agr. Chem. Soc. Japan*, **31**, 545 (1957).

* Tris (hydroxymethyl) amino methane.

4) J. Fukumoto et al., *J. Agr. Chem. Soc. Japan*, **31**, 425 (1957).

TABLE II. EFFECT OF PHOSPHATE AT VARIOUS pH RANGES

Five cc. of the reaction mixture containing 10 mg. of cells (as dry weight), 0.5% fructose (25 mg.), 10^{-3} M CaCl_2 , M/50 Tris-buffer adjusted at pH 6, 7 and 8, and various concentrations of phosphate adjusted in each pH value as shown in the table was shaken for 16 hrs.

Phos- phate concn. (M)	10 ⁻² Enz. activity ([u]/cc.)	10 ⁻³		10 ⁻⁴ Enz. activity ([u]/cc.)	10 ⁻⁵ Enz. activity ([u]/cc.)	0	
		Enz. activity ([u]/cc.)	Fructose utilized (mg.)			Enz. activity ([u]/cc.)	Fructose utilized (mg.)
pH 6	4	4	11.2	3.5	0.5	0.5	2.6
7	5.5	5.5	12.7	4.6	4.5	4.5	10.1
8	3	3	11.7	3	3	3	11.2

tion may arise as to whether coupled phosphorylation does not occur in production at pH 8. To clarify this question the effect of dinitrophenol (DNP), which is known as an inhibitor of oxidative phosphorylation, was studied at various pH values. The results showed that the production was inhibited almost completely by the addition of 10^{-3} M DNP in the reaction mixture containing 10^{-3} M phosphate at all pH ranges.

These results suggest that production could occur only when coupled with the oxidative phosphorylation at all pH ranges; in other words, phosphorus is necessitated even at pH 8. It has already been pointed out⁵⁾ that organic phosphorus kept within the cell might be used for the production of amylase in cell suspensions of mould. By adopting this view, it might be said that the difference in the requirement of phosphate is exclusively attributed to the difficulty or ease in the utilization of phosphorus kept in the cell-body by varying the pH-values.

3) **Effect of nature of cells.** It was determined whether the enzyme producing ability of the cells is affected by differences in nutrition or age. Even by varying the concentration of glucose, with or without

TABLE III. ENZYME PRODUCING ABILITIES OF CELLS CULTIVATED IN DIFFERENTIATED MEDIUM

The basic medium contained 1% $(\text{NH}_4)_2\text{HPO}_4$ and $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.2% KH_2PO_4 , and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, with other factors as shown in the table. After shaking culture (100 cc of these media in 500 cc. shaking flasks) for 16 hrs. at 30°C, the cell suspensions were prepared as described in the paragraph: "Method".

The production was assayed as usual, using cell suspensions of 10 mg. (as dried cell) in M/50 phosphate buffer of pH 7.0 containing 0.2% fructose and 10^{-3} M CaCl_2 , and shaken for 16 hrs. Cells yielded was shown as dry weight.

"MEDIUM" for obtaining cells					Enz. activity ([u]/cc.)
Glucose %	CaCO_3 %	C.S.L. %	MgSO_4 %	Cells yielded (mg/100 cc.)	
1	0.05			280	4.4
"				200	5.4
2	0.2			250	5.5
4	0.5	0.05		380	"
"				300	"
7	2.0	0.2		654	5.2
"			0.45	810	6.0

CaCO_3 , or adding an excess amount of growth promoting agent such as MgSO_4 or corn steep liquor in

TABLE IV. EFFECT OF AGE ON CELLS

The medium used was 4% glucose, 1% $(\text{NH}_4)_2\text{HPO}_4$ and $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.2% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ with or without 0.5% CaCO_3 . The shaking culture and production in cell suspensions was performed by the same method as shown in Table III. Cells yielded was shown as dry weight.

Cells obtained from the medium with CaCO_3				Cells obtained from the medium without CaCO_3			
Age (hrs.)	pH	Cells yielded (mg/100 cc.)	Enz. activity ([u]/cc.)	Age (hrs.)	pH	Cells yielded (mg/100 cc.)	Enz. activity ([u]/cc.)
20	6.0	334	5.5	16	6.0	300	5.9
44	6.5	374	"	20	5.5	310	5.5
68	7.0	478	"	44	4.0	555	1.3
92	"	425	0.7	96	3.0	715	0.5
100	"	353	0.5				

5) O. Tanabe and K. Tonomura, *Bull. Ferm. Res. Inst. Japan*, 9, 29 (1953).

the synthetic medium, in which proteinase production was profoundly affected in growing culture as shown in the previous paper¹³, the proteinase producing abilities of cells prepared from these media were shown to undergo only a slight change (Table III). However cells harvested from natural medium such as bouillon or peptone possessed little producing ability even when not only fructose, but also pyruvate was used as a carbon source.

The effect of age is shown in Table IV. Cells obtained from a medium containing 4% glucose without CaCO_3 , cultured for more than 44 hrs. showed a remarkable decrease in enzyme producing ability owing to the acidification of the medium, while, in the presence of CaCO_3 in the medium producing ability was almost unchanged for 68 hrs. and then decreased remarkably along with the lysis of cells.

II. Mutual relation between the enzymatic activity produced and the concentration of each component necessitated in production by cell suspensions.

1) Effects of concentrations of carbon sources and calcium ion. Fig. 5 shows the effects of concentrations of various carbon sources in proteinase production of resting cells. From the figure, it may be seen that a stronger inhibition occurs along with the increase

FIG. 5. Effects of Concentrations of Various Carbon Sources.

Five cc. of the reaction mixture contained 10 mg. of cells (as dry weight), M/50 phosphate buffer of pH 7.0, 10^{-3} M CaCl_2 and in varying the concentrations of the various carbon sources: A, glucose (5–10 mg. BaCO_3 was added in accompanying with the increase of the concentrations from 0.2% to 1.0%, (●—●) B, fructose (○—○) C, glycerol (●—●) D, ethanol (BaCO_3 was added as same as in A, ▲—▲) E, Na-gluconate, Na-lactate, Na-pyruvate, Na-fumarate or Na-succinate (△—△) F, Na-citrate (×—×) The reaction was done for 16 hrs.

FIG. 6. Effect of Concentrations of CaCl_2 in Various Amounts of Carbon Sources.

The reaction mixture contained 10 mg. of cells (as dry weight), M/50 phosphate buffer of pH 7.0, and the others as shown in the figure, and was shaken for 16 hrs.

of the concentration of various carbon sources, such as glucose, ethanol, gluconate, pyruvate, lactate, succinate, fumarate and citrate, but it is not so strong in the case of fructose and glycerol. This result might lead to the consideration that inhibition occurred because of the lack of utilizable calcium by combination with organic acids, i.e., 1) in glucose or ethanol, where a large amount of acids is formed (thus making the addition of CaCO_3 or BaCO_3 necessary in the reaction mixture, for neutralization) the inhibition is more remarkable in comparison with that of fructose or glycerol, where much less acids are formed. 2) All of the various organic acids inhibit production at high concentrations. Thus, the following experiment on the effect of the concentration of CaCl_2 in varying concentrations of organic acids and fructose was carried out for comparison. The results are shown in Fig. 6. As expected, the inhibition occurring in one percent of citrate or pyruvate is shown to be recovered by increasing the concentration of calcium, that is, in the former $5 \times 10^{-2}\text{M}$, and in the latter 10^{-2}M is necessitated as optimal concentration. Namely, it might be said that some carbon sources, especially various organic acids, are not only utilized as carbon sources in production at low concentrations, but also as calcium-removing agents at their high concentrations. Therefore, the optimum concentration of calcium is differ-

TABLE V. EFFECT OF CELL CONCENTRATIONS IN PROTEINASE PRODUCTION

The reaction mixture consisted of M/50 phosphate buffer of pH 7.0, 10^{-3}M CaCl_2 (in 40 mg. of cells, 10^{-2}M CaCl_2 is used), and various concentrations of cells and fructose as shown in the table, making a total volume of 5 cc., and was shaken for 16 hrs.

Fructose concentration % (mg.)		Cell concentration (mg. as dry weight)				
		5	10	20	30	40
0.05 (2.5)	Enz. activity ([u]/cc.)	1.0	0.5	0		
	Fructose utilized (mg.)	2.5	2.5	2.5		
0.2 (10)	Enz. activity ([u]/cc.)	2.7	5.5	3.0	0.5	0
	Fructose utilized (mg.)	5.2	10	10	10	10
0.5 (25)	Enz. activity ([u]/cc.)	2.5	5.5	10.5	6.8	4.5
	Fructose utilized (mg.)	5.0	12.7	25	25	25
1.0 (50)	Enz. activity ([u]/cc.)	2.0	5.0	11.0	17.0	23
	Fructose utilized (mg.)	4.6	12.3	25	38.3	50

entiated by the kinds of carbon sources and their concentrations.

2) **Effect of concentrations of cells.** Although it has been shown that a rather high concentration of glucose is required for the promotion of enzyme production in growing culture, such a phenomenon was not recognized in production experiments by cell suspensions. Moreover, we have not found such specific promoting substance among various carbon sources that could be designated as an "inducer" for enzyme production in cell suspensions. Thus, the effects of cell concentrations in production were studied, as seen in Table V, when the concentration of cells was in a range of 5 to 40 mg., activity produced was almost parallel to the cell concentrations in the reaction mixture containing 1.0% fructose; this was also observed in the range of 5 to 20 mg. in a mixture containing

FIG. 7. Effect of Concentrations of CaCl_2 in the Reaction Mixture Containing Various Contents of Cells.

The reaction mixture contained M/50 phosphate buffer of pH 7.0, various concentrations of CaCl_2 as shown in the figure, and the others as follows:

- A, 40 mg. of cells (as dry weight), 1.0% fructose ($\times$ — $\times$)
- B, 20 mg. of cells (as dry weight), 0.5% fructose ($\triangle$ — $\triangle$)
- C, 10 mg. of cells (as dry weight), 0.5% fructose ($\circ$ — $\circ$)
- D, 5 mg. of cells (as dry weight), 0.5% fructose ($\bullet$ — $\bullet$)
- E, D+0.1% $(\text{NH}_4)_2 \text{HPO}_4$ ($\bullet$ — $\bullet$)

The reaction was done for 16 hrs.

0.5% fructose and in the range of 5 to 10 mg. in a mixture containing 0.2% fructose. Further, the utilization of fructose is also parallel to the cell concentrations. However, the higher the cell concentrations in comparison to the concentration of fructose, i.e., when the cell concentration is 30 mg. or above in 0.5% fructose and above 20 mg. in 0.2% fructose, the higher inhibition is observed.

On the other hand, the required concentration of calcium was not changed by increasing the cell concentration in a range of 5 to 20 mg. (in five cc. of the reaction mixture) notwithstanding the addition of a nitrogen source, but when the cell concentration was 40 mg. a higher concentration ($10^{-2}M$) was required to obtain a maximum activity (Fig. 7).

From the above results, it may be concluded that the enzymatic activity produced in cell suspensions, is

controlled by the ratio of the concentration of the carbon source and the cells, when available calcium is present. By keeping both of them in balance, activity produced is proportional to the cell concentrations in the reaction mixture.

III. Effects of nitrogen sources and amino acid analogues.

The following experiment was conducted on the effects of various nitrogen sources in production, by cell suspensions containing other reagents which were essential for production as mentioned above (Fig. 8). It may be seen that the addition of a nitrogen source such as $(NH_4)_2HPO_4$ or leucine promotes the production to about three times of that without the supplement of a nitrogen source, with a slight increase of growth and utilization of fructose (65 and 75% utiliza-

FIG. 8. Effects of Nitrogen Sources and Amino Acid Analogues on Proteinase Production.

Five cc. of the reaction mixture contained 10 mg. of cells (as dry weight), $M/50$ phosphate buffer of pH 7.0, 0.5% fructose, $10^{-3}M$ $CaCl_2$ and various nitrogen sources as follows:

A, no addition (○—○)	E, 0.1% $(NH_4)_2HPO_4$, 0.1% DL-norleucine (○—○)
B, 0.1% $(NH_4)_2HPO_4$ (●—●)	F, 0.1% L-leucine, 0.1% DL-norleucine (×—×)
C, 0.1% L-leucine (×—×)	G, omitted $10^{-3}M$ $CaCl_2$ in each flask, respectively (△—△)
D, 0.1% DL-norleucine (○—○)	

tion, in the experiment shown in the figure in the presence of $(\text{NH}_4)_2\text{HPO}_4$ and leucine respectively in comparison with 50% utilization without a nitrogen source). This promoting effect was also observed with other nitrogen sources, such as NH_4Cl , NaNO_3 as inorganic nitrogen sources, various amino acids and yeast extract etc. Nevertheless $(\text{NH}_4)_2\text{SO}_4$, NaNO_2 , cysteine, cystine and methionine had rather inhibitive effects on production and various protein nitrogen sources did not affect production.

Norleucine, however, when added to the reaction mixture containing $(\text{NH}_4)_2\text{HPO}_4$, was shown to diminish activity, to a point corresponding to the activity in the absence of $(\text{NH}_4)_2\text{HPO}_4$, that is, it retracted the promoting effect of the nitrogen source, while it did not diminish the promoting effect of leucine, which is homologue. However, norleucine itself does not affect the production at all in the reaction mixture not containing other nitrogen sources. It was ascertained by further study, that norleucine is utilized by the cells in spite of the presence or absence of other nitrogen sources, by determination of the amino acid content by the Ninhydrin method and by paper-partition chromatography.

The same phenomenon as seen in the case of norleucine, was also observed with other amino acid analogues such as norvaline, 5-methyl-tryptophane and β -hydroxy glutamic acid. The latter two, however, diminished the promoting effect to a certain extent in the case their homologue amino acids are used as the nitrogen source.

IV. Others.

The following study was attempted to investigate the effects of various anions and cations in production. It was found that production is inhibited by the addition of Cu, Co, Fe, Sn and Ni but not affected by Zn, Mg, Cd, Ba, Mn and Sr at concentrations of 10^{-3}M , respectively. Among the substances used as anions, remarkable inhibition was observed with those

TABLE VI. EFFECTS OF VARIOUS SUBSTANCES CONTAINING SULFUR ON PROTEINASE PRODUCTION

Five cc. of the reaction mixture contained 10 mg. of cells (as dry weight), $\text{M}/50$ phosphate buffer of pH 7.0, 0.2% fructose, 10^{-3}M CaCl_2 and various substances shown in the table, and it was shaken for 16 hrs. as usual.

Substances	(M)	Inhibition (Percentage)
Na_2SO_4	10^{-3}	82
	10^{-4}	//
	10^{-5}	0
NaHSO_3	10^{-3}	82
	10^{-4}	//
$\text{Na}_2\text{S}_2\text{O}_3$	//	84
$(\text{NH}_4)_2\text{SO}_4$	//	71
MgSO_4	//	82

containing sulfur in concentrations of 10^{-4}M , as shown in Table VI. Further study, revealed that the SO_4 ion does not only affect the activity of the proteinase but is also an indispensable agent for the growth of bacteria. It is however obscure, how this inhibition occurred. Accordingly, the inhibition by $(\text{NH}_4)_2\text{SO}_4$, cystine, cysteine or methionine mentioned above might be due to the sulfur component. Anions such as Cl, Br, I, F, BO_3 , CO_3 in concentrations of 10^{-3}M did not affect production.

Further experiments were carried out to investigate the effect of base analogues. 8-Azaguanine ($5 \times 10^{-3}\text{M}$) did not inhibit the production but rather accelerated it, similarly to the addition of a nitrogen source, while it suppressed growth completely in growing culture. However, the presence of a nitrogen source seemed to diminish the effect partly as seen in Table VII. Though thiouracil ($5 \times 10^{-3}\text{M}$) was shown to inhibit production remarkably, the reason for inhibition i.e., whether it is caused by the sulfur component or not is obscure.

DISCUSSION

The above data shows that the proteinase

TABLE VII. EFFECT OF BASE ANALOGUES ON PROTEINASE PRODUCTION

The conditions are the same as in Table VI.

Base analogues	(M)	Others	Final pH	Enz. activity ([μ]/cc.)
No addition			6.8	5.5
8-Azaguanine	5×10^{-3}		7.1	13.0
"		$(\text{NH}_4)_2\text{HPO}_4$ 0.1%	"	9.5
Thiouracil	5×10^{-3}		6.3	1.0
		Uracil $5 \times 10^{-3}\text{M}$	6.7	11.0
Thiouracil	5×10^{-3}	"	6.4	6.0

production in cell suspensions of *Ps. myxogenes* sp. occurs in the presence of a carbon source, phosphate (differentiated by pH values), and calcium ion under aerobic conditions.

However, this reaction is not affected by amino acid analogues, which are generally known^{6,7,8)} to inhibit the incorporation of corresponding amino acids into the cellular protein. Therefore, it may be assumed that the production of resting cells in the absence of nitrogen source does not involve free amino acid utilization, but rather utilizes the pre-formed complex precursor substance, which is formed within the cells grown in the synthetic medium without the addition of calcium. Thus, calcium might be necessary in any step of the biosynthetic reaction from the precursor state, together with metabolism of a carbon source which perhaps might be the energy supplier in proteinase production. The following scheme may be advanced to explain:

On the role of calcium in production, it is difficult to assume that calcium is involved in the metabolism of the cells, from experimental results showing that no difference is observed in the utilized ratio of carbon source either in its presence or absence.

From observations on the behavior of calcium and other factors in production by cell suspensions, it might be possible to explain the various phenomena seen in the proteinase production by growing culture. When glucose is used as carbon source, it is metabolized very rapidly so that its intermediary products (various organic acids) are fastly formed before they can be used up. Accordingly, it

might be considered that intermediary products of glucose may combine with calcium, as observed in the experiments using various organic acids such as gluconate, pyruvate, succinate, fumarate, citrate etc., thus making calcium unavailable to the bacteria, as has been suggested by Mandels and Reese⁹⁾ on the cellulase production by mould. Upon consumption of these intermediary products after the disappearance of glucose, calcium would be released, this being essential for proteinase production. This might be an answer for the question why proteinase does not appear until glucose is used up in the medium in growing culture.

Accordingly, the production in growing culture would be affected by the conditions, at the time when calcium is released, and not by the natures of cells as seen in this paper. This corresponds to the time when normal cellular multiplication no longer takes place as shown by Nomura et al.⁷⁾ in amylase formation of *B. subtilis*. Thus, the production in growing culture may be regarded to be similar to that of cell suspensions described above.

The enzymatic activity produced in cell suspensions is controlled by the ratio of the concentrations of the carbon source and the cells. When the balance of both factors is kept in suitably, the enzymatic activity produced is almost parallel to the cell concentrations, while in the unbalanced condition, a lower production of enzyme is obtained. Therefore, the remarkable increase in production along with the increase in the concentrations of glucose suitably supplemented with other factors in growing culture might be occurred by the increase in concentrations of cells simultaneously maintaining the balance optimally with the concentrations of carbon sources, which must be intermediary products of glucose. On the other hand, a remarkable decrease in production by the addition of a growth promoting agent, even at a high con-

6) H. O. Halvorson and S. Spiegelman, *J. Bact.*, **64**, 207 (1952), **65**, 496, 601 (1953).

7) M. Nomura et al., *J. Biochem.* **43**, 841 (1956).

8) J. Fukumoto et al., *J. Agr. Chem. Soc. Japan*, **31**, 545 (1957).

9) M. Mandels and E. T. Reese, *J. Bact.*, **73**, 269 (1957).

centration of glucose in the growing culture, might be caused by an unbalance of both factors, i. e., the lack of carbon sources for production.

This assumption leads to the next consideration that factors affecting the growth and proteinase production are not always the same. The following facts give support to a difference between these factors: 1) Calcium is essential for production but is not necessarily essential for growth, 2) SO_4 ion inhibits production remarkably, while it is required as an indispensable agent for growth, 3) 8-Azaguanine does not only inhibit but rather accelerates production, while growth is inhibited completely.

SUMMARY

Factors affecting the proteinase production in cell suspensions of *Ps. myxogenes* sp. were studied and the following facts are shown:

1) The production was observed in cell suspensions in a phosphate buffer of pH 7.0, containing a carbon source and calcium ion under aerobic conditions, without accompanying growth. The calcium ion could be replaced with strontium but not by others, and inhibition was observed in the presence of calcium-removing agents such as EDTA, Oxalate, Citrate and NaF. The carbon sources which were to be utilized by the cells were all found to be effective, and production was inhibited by various metabolic inhibitors.

2) The optimum pH was about 7.0, and the promoting effect of phosphate was observed remarkably at pH 6 and slightly at 7 but not at 8.

3) The enzyme producing abilities by nutritionally differentiated types of cells prepared from synthetic media (of which the components were differentiated) in various concentrations of glucose (1-7%), in the pre-

sence or absence of CaCO_3 or excess addition of a growth promoting agent, were almost identical with one other. Furthermore ability was not altered throughout the stationary phase when the pH of the cultural medium was kept at neutrality.

4) The production was more inhibited along with the increasing of the concentrations of carbon sources such as organic acids and glucose or ethanol in which were observed high acid formation, but not in fructose or glycerol in which low acid formation was seen. The inhibition, however, was recovered to some extent by the further addition of calcium. In other words, it could be said that the optimum concentration of calcium was differentiated by the sorts of carbon sources or by their concentrations.

5) The balance between the concentrations of carbon source and cells was important for the production, and when kept the balance of both factors suitably the enzymatic activity produced was parallel to the concentrations of cells.

6) The addition of a nitrogen source in the reaction mixture promoted production about three times. However, in the presence of amino acid analogues, which did not affect the production in the reaction mixture without a nitrogen source, the promoting effect of the nitrogen source was diminished except when their homologues were used.

7) The production was inhibited remarkably by the addition of various substances containing sulfur, while 8-Azaguanine, base analogue, did not affect the production.

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