Germination Promotant for Plants Seed, produced by Microorganisms

Part IV. The Germination Promotant for Rice Plant Seeds, produced by Streptomyces sp. S-580. Isolation and Structure of Another Active Crystal (Factor-D).*

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Received March 25, 1958

A crystalline substance (Factor-D), by which the activity of Factor-E in promoting the germination of rice plant seeds could be increased synergistically, was isolated from the active crude syrup from which Factor-E was also isolated. This Factor-D has been proved by synthesis to be 1-methyl-2,4-imidazoledione (1-methyl hydantoin). This substance has not been isolated from culture filtrates of any microorganisms.

INTRODUCTION

It has been already reported1) that a neutral crystalline substance (Factor-E), which is effective for the germination of rice plant seeds at the dilution range of $10^{-5}$–$10^{-7}$, was isolated from the active crude syrup obtained from the culture filtrate of Streptomyces sp. S-580. This substance was further proved by hydrolysis and synthesis to be L-prolyl-L-valine anhydride (diketopiperazine), a new diketopiperazine. In marginalia of that report the author has made reference that the activity of Factor-E could be increased synergistically by another crystalline substance isolated from the same active crude syrup. This substance, Factor-D, has been isolated by a silica-gel chromatography and proved by synthesis to be 1-methyl-2,4-imidazoledione (1-methyl hydantoin). It may also be stated that Factor-
D is solitary effective for the promotion of germination of rice plant seeds at the concentration range of $10^{-8}$ to $10^{-4}$M. Whereas, when it is used with Factor-E (in the concentration range of $10^{-4}$ to $10^{-8}$M), the synergistically effective concentration range of Factor-D was $10^{-5}$ to $10^{-6}$M. In 1913 1-methyl hydantoin had been isolated by D. Ackermann from the culture filtrate of certain putrifying bacteria cultured in medium added with creatinine. However this substance has not been isolated from culture filtrates of any microorganisms grown on a conventional medium in the absence of creatinine.

EXPERIMENTAL AND RESULTS

1) Isolation of Factor-D from the active crude syrup (A.C.S.).

Factor-D was isolated by a silica-gel chromatography from A.C.S. in the following manner. (a) 30 grm of dried silica (Mallinckrodt, 100 mesh) was thoroughly mixed with 22 ml of distilled water in a mortar and then mixed with 180 ml of pure chloroform to be gelatinized. (b) The silica-gel obtained was introduced into a glass tube with a diameter of 2.2 cm and 24 cm in height, (c) then, 1 grm. of A.C.S. dissolved in 1 ml of pure chloroform was charged into the silica-gel column and was eluted at a flow rate of 1.5-2.0 ml/min. with each 100 ml of chloroform-n-butanol mixture saturated with distilled water (volume-percentage of n-butanol in the mixture were as follows: 0, 5, 10, 15, 20, 30, 40 and 50, respectively). (d) Each 10 ml of the eluant was collected into a test tube and titrated with N/20 NaOH; the chromatograph obtained is shown in Fig. 1, and fractions of tubes No. 33-35 were combined and evaporated in vacuo. (e) Presently, a crystalline substance appeared in the pale-brown syrup, and then repeated crystallizations from ethanol gave colorless prisms (Factor-D) as shown in Fig. 2.

2) Properties of Factors-D.

Factor-D is a colorless prism (derived from ethanol) having a weak acidic character, m.p. 157°C; easily soluble in acetone, alcohols and water, soluble in chloroform and ethyl acetate, very slightly soluble in benzene and ethyl ether, but insoluble in n-hexane and petroleum ether. Analytical data of this substance was as follows: Anal. Calcd. for $\text{C}_4\text{H}_6\text{O}_2\text{N}_2$: C, 42.1; H, 5.3; N, 24.6; mol. wt., 114.1; Found: C, 42.5; H, 5.5; N, 24.1; mol. wt. (performed

![Fig. 2. Crystalline Form of Factor-D (from ethanol) (magnification x 80)](image-url)
by the micro-Rast method, dissolving in camphor), 109. Therefore, this substance was proved to have the empirical formula: C₄H₆O₂N₂. The UV-spectrum of this substance in methyl alcohol showed no significant absorption in the wave-length range of 220-400 m\(\mu\), but showed a strong end-absorption. The infra-red spectrum of this substance in Nujol mull is shown in Fig. 3. The ninhydrin reaction test to this substance was negative but the test with sodium nitroprusside was positive. Furthermore, by a paper chromatography (filter paper, Toyoroshi No. 50; solvent, n-butanol: acetic acid : water = 4:1:5; ascending for twenty or twenty-four hours at room temperature; development of spots, a chlorine-starch method employed), this substance could be detected to have \(R_F = 0.62 \pm 0.06\).

3) Synthesis of 1-methyl-2,4-imidazoledione (1-methyl hydantoin).

According to the method described by L. Baumann, 1-methyl hydantoin was formed from urea and sarcosine which had been formed from methylamine hydrochloride and potassium cyanide in a formaldehyde solution.

As shown in Fig. 3, the infra-red spectrum of Factor-E is identical with that of synthesized 1-methyl hydantoin, furthermore on the paper chromatography, the \(R_F\) of the former is identical with that of the latter and a mixed melting point of the both gave no depression. Therefore, from these evidences, Factor-D has been proved to be 1-methyl-2,4-imidazoledione (1-methyl hydantoin).

4) Effect of Factor-D on the germination of rice plant seeds.

Fujisaka-No. 5 harvested in 1956 was used in this experiment. Conditions on the germination of rice plant seeds, determination of the activity in promoting the germination and preparation of the test solution were the same as described in the previous papers. However, the test solutions used in this experiment were diluted with 10⁻⁴M ammonium sulfate solution. As indicated in Table I, Factor-D, alone, showed a promoting effect on the germination of rice plant seeds in the concentration range of 10⁻³-10⁻⁴ M. Furthermore

### TABLE I. EFFECT OF FACTOR-D ON THE GERMINATION OF RICE PLANT SEEDS

<table>
<thead>
<tr>
<th>Concentration (M)</th>
<th>Stem Elongation (%)</th>
<th>Root Elongation (%)</th>
<th>Prophyll and Crown Root Formation Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.56 (21)</td>
<td>2.66 (32)</td>
<td>40</td>
</tr>
<tr>
<td>10⁻³ M</td>
<td>1.89 (21)</td>
<td>2.71 (32)</td>
<td>40</td>
</tr>
<tr>
<td>10⁻⁴ M</td>
<td>2.10 (32)</td>
<td>3.10 (44)</td>
<td>40</td>
</tr>
<tr>
<td>10⁻⁴ mol</td>
<td>2.30 (32)</td>
<td>3.30 (44)</td>
<td>40</td>
</tr>
<tr>
<td>10⁻⁵ mol</td>
<td>2.50 (32)</td>
<td>3.50 (44)</td>
<td>40</td>
</tr>
<tr>
<td>10⁻⁶ mol</td>
<td>2.70 (32)</td>
<td>3.70 (44)</td>
<td>40</td>
</tr>
</tbody>
</table>

NOTES
1) Treated with 10⁻⁴mol ammonium sulfate solution without Factor-D.
2) Figures in the brackets show the promoting ratio against control.
5) L. Baumann, J. B. C., 21, 565 (1915).
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(I) Effects on Promoting Ratio of Stem and Root Elongation Against Control.

Nine days after start of experiment incubated in dark condition at 18°C, stem and root elongation were determined. Factor-D and E were diluted with 10^{-4} mol ammonium sulfate solution as test solutions.

(refer to the previous papers1, 6, 7)

FIG. 5. Synergistic Action of Factor-E and Factor-D in Promoting the Germination of Rice Plant Seeds.


Nine days after start of experiment incubated in dark condition at 18°C, prophyll and crown root formation ratio were determined. Also six days after, interim-germination ratio was determined. And Factor-D and Factor-E were diluted with 10^{-4} mol ammonium sulfate solution as test solutions. (refer to the previous paper1, 6, 7)
Factor-D showed, synergistically, a promoting effect on the germination in the concentration range of $10^{-5}-10^{-6}$ M with $10^{-4}-10^{-6}$ M of Factor-E. For example as shown in Figs. 4 and 5, mixtures of $10^{-4}$ M Factor-D and $10^{-4}-10^{-6}$ M of Factor-E were synergistically effective for promoting germination, however, activities of mixtures of $10^{-4}$ M Factor-D and the same concentrations of Factor-E were found to be similar to those in case of $10^{-4}$ M Factor-D alone. (These activities were less than that of the culture filtrate of St. sp. S-580. Consequently, studies concerning this fact are now in progress.)

Acknowledgements The author wishes to express his sincere thanks to Prof. K. Sakaguchi, Univ. of Tokyo for his guidance throughout this work. Thanks are also due to Prof. Y. Sumiki and Prof. T. Asai, Univ. of Tokyo for their kind advice and helpful suggestions. Micro-analysis and the infra-red spectrum were carried out in this department.


Studies on the Opalescence in Sodium-caseinate Solution Developed by the Milk Coagulating Enzymes

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Received February 7, 1958

Both rennet and pepsin are used as a coagulant in cheese-making process. The proteolytic action of these two milk-coagulating enzymes was studied. In this report, results of the experiments on the development of the opalescence in sodium-caseinate solution by two enzymes are reported. Some properties of the opalescent materials and the change in the distribution of nitrogen and phosphorus are also included.

These experiments show the difference of the mode of proteolytic action on casein by rennet and by pepsin, which may be concerned with the ripening of cheese.

INTRODUCTION

Rennet used for coagulating milk in cheese making, also hydrolyses casein and its proteolytic action may be significant for the ripening of cheese. Pepsin instead of rennet was also used as a coagulant in cheddar cheese making.13 Experiments were conducted on the proteolytic action of milk-coagulating enzymes in order to clarify the change of casein during cheese-ripening.

This report presents the results of the experiments on the development of the opalescence in sodium-caseinate solution by the action of rennet and pepsin.

EXPERIMENTAL

Materials and methods

Casein was prepared from unpasteurized cow's skim milk by the method of Hipp.3 Casein was prepared by the method of Warner2 and β-casein by the method