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Immunological Identification of *Thiobacillus ferro-oxidans* and *Thiobacillus thiooxidans*

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*Thiobacillus ferrooxidans*와 *Thiobacillus thiooxidans*의 면역학적 동정

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ABSTRACT

Detergent soluble fractions were obtained from *T. ferrooxidans* ATCC 13598 and the *T. thiooxidans* ATCC 8085 which were treated with 3% of Tween 20. The detergent soluble antigen(crude antigen) fractions of the *T. ferrooxidans* and the *T. thiooxidans* were sujected to hydroxyapatite. In the case of *T. thiooxidans*, further purification was carried out on the DEAE-cellulose column chromatography.

The antigen fractions, such as the hydroxyapatite peak-1(Tf, HA-1) and peak-2 from *T. ferrooxidans*(Tf, HA-2), and hydroxyapatite peak-1(Tt, HA-1), DEAE-cellulose peak-1(Tt, DP-1) and peak-2(Tt, DP-2) from *T. thiooxidans* were compared each other with the homologous and the heterologous antisera against to the *Thiobacillus* species.

The hydroxyapatite peak-2 fraction from the *T. ferrooxidans*(Tf, HA-2) and DEAE-cellulose peak-2 fraction from the *T. thiooxidans*(Tt, DP-2) were represented the type-specific immuno-reactivities between the *T. ferrooxidans* and the *T. thiooxidans* on the several sets of double gel diffusion analysis.

The type-specific antigen fractions from both of the bacteria were mainly composed of protein with entierly different electrophoretic mobility on the SDS-polyacrylamide gel electrophoresis. However, the PAS positive banding patterns on the electrophorogram showed wide range of common antigenic properties in the *T. ferrooxidans* and the *T. thiooxidans*, respectively.

INTRODUCTION

Generally, the genus *Thiobacillus* is a gram-negative, motile rod, short and obligatory acidophilic. This chemolithoautotrophic organism is capable of using the reduced iron and sulfur as energy source for the autotrophic growth. Because of the growing ability in the acid-mine wastes and the production of sulfuric acid which is able to leach the low grade uranium ore, the *T. ferrooxidans* are economically very important.

It is strongly believed that the *Thiobacillus* species are closely related genetically,

since they have a common function in oxidizing certain compounds containing the inorganic sulfur and utilizing carbon dioxide as a sole carbon source. The bacteria has an outer cell envelope which is typical structure of gram-negative bacteria, however, the envelope is known to have a number of unusual properties(Remsen and Lungren, 1966; Wang *et al.*, 1970) The cell envelope has been investigated on the viewpoint that this structure coming into direct contact with the acidic environment might exhibit peculiarities or adaptation, which enable the organism to use a soluble and insoluble inorganic substances(Wang *et al.*, 1970; Wang and Lundgren, 1968). On the other hand, the taxonomy of the Thiobacilli has recently been introduced by Hutchinson, Johnstone and White(1965, 1966, 1967). This method, however, has a disadvantage to analyse directly the relationships among a group of the microorganisms to compare the over-all base composition of their DNA complement. (Hill, 1966; Jackson *et al.*, 1968)

Although the methods described above are good process for identification of related bacteria, it required so many kinds of the tedious work and detailed procedure for the purpose. These facts led to the conclusion that a new taxonomic survey of the Thiobacilli were required and as many strains as possible should be also investigated using a large number of differential tests.

In the case of Thiobacilli species, however, there are no previous reports concerning the taxonomic aspect with certain kinds of antigen fractions which were derived from the cell envelope, purifed LPS or polysaccharide except Veital *et al*(1973).

In this regard, the immunological approaches on the identification of Thiobacilli species are introduced in this laboratory with various kinds of theantigens, but the results obtained were not showed clearct type-specificity with these antigens in the Thiobacilli species,(Rhee, 1976; Rhee *et al.*, 1975).

For the purpose of purifying the antigens from the *Thiobacillus* species, the authors selected the bacterial strains of the *T. ferrooxidans* ATCC 13598 and the *T. thiooxidans* ATCC 8085 and the coulmn chromatographic procedures, such as, hydroxyapatite ard DEAE-cellulose for the purification process were introduced.

The purpose of this study is to compare the antigen fractions obtaine from the *T. ferrooxidans* and the *T. thiooxidans* with their homologous and heterologous antisera, and to characterize their properties by SDS-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

1. Bacterial strains and growth

The chemosynthetic autotophic bacteria, *i. e.*, *T. ferrooxidans* ATCC 13598, *F. ferrooxidans* ATCC 13661 (designated *T. ferrooxidans* ATCC 13661 or Tf-1), *F. sulfooxidans* ATCC 14119(designated *T. ferrooxidans* ATCC 14119 or Tf-2), *T. ferrooxidans* Japan(designated TfOJ), *T. intermedius* ATCC 15466(Ti), *T. novellus* ATCC 8093(Tn), *T. thioparus* ATCC 8158 (Tth), and *T. thiooxidans* ATCC 8085 (Tt) were obtained from the American Type Culture Collection and were propagated in the media as recommened from the ATCC. All bacterial strains were cultured in 20 L carboy under the forced aeration at 28°C for 7 days.

2. Preparation of antisera against *Thiobacillus* species

Antisera to the whole organisms described above were raised in 3 rabbits weighing 2.0 to to 2.5kg, each organism(antigen). Injection of the antigen with Freund's complete adjuvant were given intradermally, subcutaneously and intramuscuarly at 10 day intervals for 5 times using 5 to 10 mg proteins of sonicated bacterial suspension in each injection.

The blood were obtained by cardiac puncture at 10 days after the last immunization: the antisera were collected, millipore filtered, and kept at -50°C until use. The antibody titrations were carried out by the procedures of paper electrophoresis, agglutination and hemagglutination tests described in elsewhere(Kwapinski, 1972).

3. Fractionation of antigen fractions

The bacterial strains selected for the fractionation of antigen were the *T. thiooxidans* ATCC 8085 and the *T. ferrooxidans* ATCC 13598. The organisms cultured were harvested by centrifugation(8,000×g, 15min) and then the packed cells were washed with ice cold physiological saline for 3 times and suspended in 1mM phosphate buffer, *p*H8.0.

The suspended cells were then sonicated for 1 hour at 4°C and treated with Tween 20 at final concentratration of 2~3%. The lysed cells were then centrifuged at 13,000 ×g for 30 min and the supernatant fractions were dialysed to 1mM phosphate buffer, *p*H8.0 until the detergents were from the samples.

The dialysed crude antigen fractions were subjecterd to a column of hydroxyapatite using 1mM phosphate buffer, *p*H8.C as an eluent with peristaltic pump at 4°C. After the elution of peak-1fraction, a linear salt gradient was applied with 1mM phosphate buffer, *p*H8.0 containing 1 to 400mM KCl. The elution was then monitored by UV absorption at 280nm.

The *T. ferrooxidans* antigen fractions obtained form the hydroxyapatite column chromatography were used as a test antigen, however, the *T. thiooxidans* antigen fraction from the hydroxyapatite was further purified using a DEAE-cellulose chromatography equilibrated with 1mM phosphate buffer, *p*H8.0. The chromatography was carried out by an initial elution with equilibration buffer, then followed by a linear salt gradient elution with 1mM phosphate buffer, *p*H8.0, containing KCl from 1 to 400mM at 4°C. The elution was monitored by UV absorption at 280nm.

It was found that the hydroxyapatite peak-2 fraction from the *T. ferrooxidans* was eluted at the KCl concentrations of 400 mM and the DEAE-cellulose peak-2 fraction from the *T. thiooxidans* was eluted at the KCl concentrations of 200mM in elution buffer, and then these procedues were applied for further experiment hereafter.

4. Double gel diffusion

1.5gm of purified agar(Difco, USA) was dissolved in 100ml of 0.14M NaCl solution containing merthiolate (1 : 10,000). The melted and deaerated agar solution was dispensed in the petri dish.

The antigen fractions used in the double gel diffusion analysis were crude antigen, hydroxyapatite peak-1 and 2 from the *T. ferrooxidans* and the crude antigen, hydroxyapatite peak-1, DEAE cellulose peak-1 and 2 fractions from the *T. thiooxidans*, respectively.

Antigen fractions mentioned above were reacted with undiluted antisera against to the *Thiobacillus* species. The double gel diffusion tests were carried out in moisture chamber at 37°C for 24 hrs and consecutively for 2 days at 4°C. After the completion

of the immune-reaction, the agar petri dish was dialysed with saline at 4°C.

5. Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electropheresis was performed on 7.5% polyacrylamide gel without SDS as described by Stoklosa *et al*(1974). Acrylamide, bisacrylamide, 2-mercaptoethanol, and SDS were purchased from Eastman Organic Chemical Co., Rochester, N.Y. The samples were suspended in a solution containing 1% SDS and 1% 2-mercaptoethanol 10mM sodium phosphate buffer, *p*H 7.2 and then incubated at 37°C for 16 hrs. Since the addition of urea into the solubilizing solution was found to have no significant effect on the pattern of bands, the urea was not routinely used.

Prior to application to the gel, the sample was mixed with sucrose solution and bromophenol blue was used as a tracking dye. The sample mixture(200μg of protein per gel) was applied to a 5×100mm gel, and electrophoresis was carried out at 6 mA/gel for 6 hrs at room temperature.

The protein bands were detected by staining with the amidoblack 10B, and the gels were destained by washing several times with 7% acetic acid. For carbohydrate or glycoprotein the gels were stained with the periodic acid-Schiff reagent according to the method of Fairbanks *et al* (1971).

The estimation of molecular weight of each antigen fraction banding patterns obtained in the SDS-polyacrylamide gel electrophoresis were determined from a standard curve of log molecular weight versus relative electrophoretic mobility to BPB by the method described elsewere (Shapiro *et al.*, 1969; Weber and Osborn, 1969).

RESULTS

To obtain the type specific antigen fractions from sonicated and detergent treated (Tween 20) crude antigen fractions of the *T. ferroxidans* and *T. thiooxidans*, the hydroxapatite and the DEAE-cellulose column chromatography were applied.

The crude antigen fraction from the *T. ferrooxidans* showed two kinds of peaks on the hyroxyapatite column chromatography as shown in Fig. 1. The peak-1 fraction was eluted with 1mM phosphate buffer, pH 8.0, however, the peak-2 fraction was eluted at the KCl concentrations of 400mM in the same buffer.

The chromatographic elution profile from the hydroxyapatite are showed relatively higher optical density at 280nm comparing to that of peak-2 fraction.

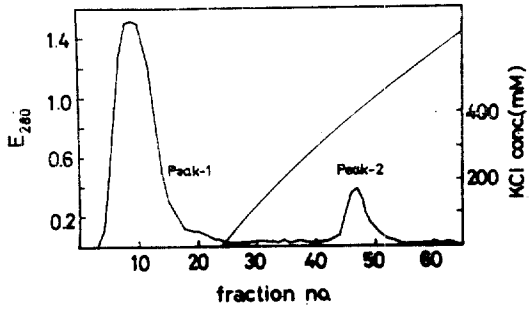

Fig. 1. Hydroxyapatite column chromatography of Tween 20 soluble fraction from *T. ferrooxidans*, ATCC 13598. Peak-1 fraction was eluted with 1mm phosphate buffer, *p*H 8.0 and a linear gradient was done from 1 to 400mM KCl in the same buffer.

On the other hand, the crude antigen fraction from the *T. thiooxidans* showed only one of the peak on the hydroxyapatite chromatography, and furthermore there were observed any detectable fraccions when it was eluted with high salt containing elution buffer(Fig. 2).

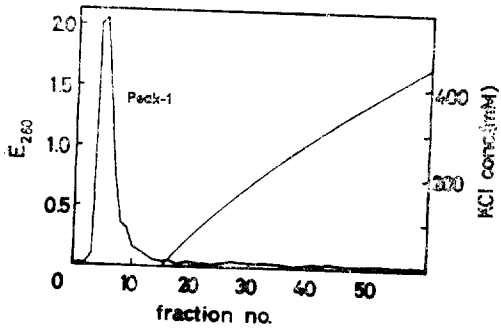

Fig. 2. Hydroxyatite column chromatography of Tween 20 soluble fraction from *T. thiooxidans*, ATCC 8085. Peak-1 fraction was eluted with 1mM phosphate buffer, *p*H 8.0 and a linear gradient was done at KCl concentrations from 1 to 400mM in the same buffer.

As shown in Fig. 3, two kinds of the antigen fractions were detected on the DEAE-cellulose column chromatography with different salt containing elution buffer. The DEAE-cellulose peak-1 fraction from the *T. thiooxidans* was eluted with 1mM phosphate buffer, *p*H 8.0, whereas, the peak-2 fraction was eluted at KCl concentrations of 200mM in the same elution buffer.

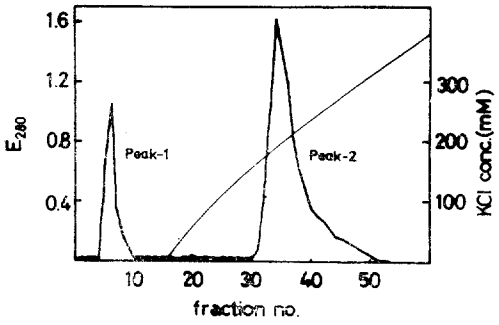

Fig. 3. DEAE-cellulose column chromatogaphy of hydroxyapatite peak-1 fraction from *T. thiooxidans* ATCC 8085. DEAE-cellulose peak-1 fraction was eluted with 1mM phosphate buffer, *p*H 8.0 and a linear gradient was applied at KCl concentrations from 1 to 400mM in the same buffer.

According to the chromatographic results from the *T. ferrooxidans* and the *T. thiooxidans* they represented very different antigenic moiety each other.

For the detailed survey on the relationship among the antigen fractions such as the crude antigens, the hydroxyapatite peak-1 and 2 fractions from the *T. ferrooxidans* ATCC 13598 and the *T. thiooxidans* ATCC 8085, several sets of double gel diffusion analysis were conducted with various antisera to the *Thiobacillus* specie.

As shown in Fig. 4, the antigen fractions from the *T. thiooxidans* and the *T. ferrooxidans* were strongly reacted to their homologous antibody, however, the number of precipitation line was decreased slightly according to their purification process.

The fact the decreased number of immunoprecipitation line in double gel diffusion test with hydroxyapatite peak-1 antigen fraction of the *T. thiooxidas* to homolous antibody suggests that the hydroxyapatite absorbed some kinds of antigen components from the crude antigen fraction although the hydroxyapatitie chromatographic behavior was shown only one peak.

The DEAE peak-1 fraction of the *T. thiooxidans* showed 3 or 4 kinds of immunoprecipitation lines with homologous antiserum, however, the DEAE peak-2 showed 1 or 2 kinds of immunoprecipitation lines including major precipitation line with homologous antiserum.

The *T. thiooxidans* DEAE-cellulose peak-1 antigen fraction was reacted faintly with heterologous antiserum(*T. ferrooxidans* antiserum), however, the antigen fraction, DEAE-cellulose peak-2 was not reached at all with the *T. ferrooxidans* antiserum. These results suggest that the DEAE-cellulose peak-2 fraction is the type specific antigen fraction isolated from the *T. thiooxidans*.

As shown in Fig. 4, the antigen fract-

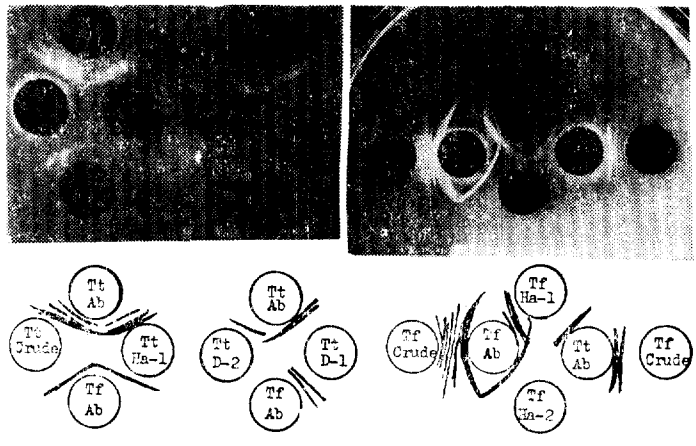

Fig. 4. Double gel diffusion pattern of antigen fractions from *T. thiooxidans*, ATCC 8085 with antisera to *T. thiooxidans*, and *T. ferrooxidans* ATCC 13598.

ions such as the crude antigen and the hydroxyapatite peak-1 from the *T. ferrooxidans* showed a wide ranges of cross-reactivity between homologous and heterologous(*T. thiooxidans* antiserum) antisera, whereas the hydroxyapatite peak-2 fraction from the *T. ferrooxidans* was illustrated the type specific immunoprecipitation with the antiserum against to *T. ferrooxidans*,

As a further index to the relationship between the antigen fractions from the *T. ferrooxidans* and the antisera to the *Thioqacillus* species, another severalsets of immunodiffusion tests were performed(Fig. 5). The crude antigen fraction of the *T. ferrooxidans* showed a strong immunoprecipitaion lines among the antisera to the *T. ferroxidans* species. This is suggested that they possessed a considerable amounts of common antigens in each other.

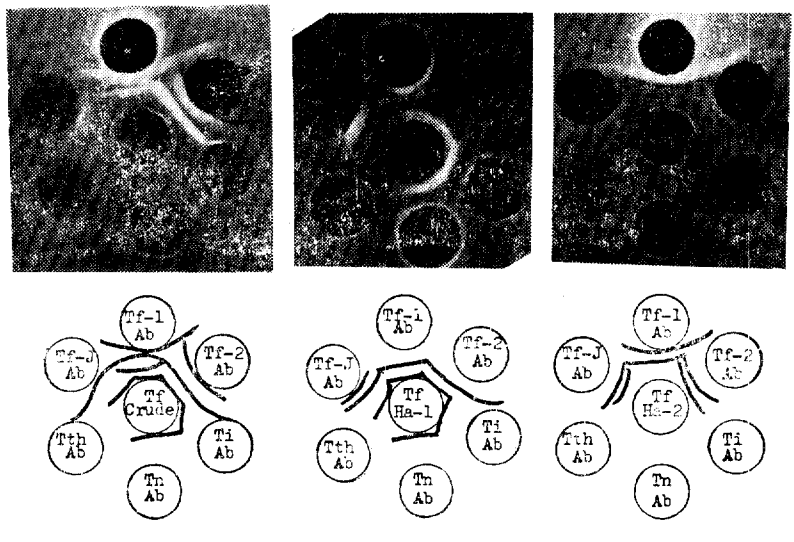

Fig. 5. Double gel diffusion pattern of antigen fractions from *T. ferrooxidans* ATCC 13598 with antisera to *Thibacillus*. species.

On the other hand, a weak precipitation lines were observed between the antisera to the *T. intermedius* and the *T. novellus*, whereas the antiserum to the *T. thioparus*

was not reacted at all with the crude antigen fraction of the *T. ferrooxidans*. Another kind of antigen fraction, the hydroxyapatite peak-1 fraction of the *T. ferrooxidans*, was strongly reacted with antisera to other *Thiobacillus* species and the overall immunoreactivities with antisera to another *Thiobacillus* species were the same as described in the crude antigen fraction.

The hydroxyapatite peak-2 fraction of the *T. ferrooxidans* which already displayed an unique immuno-specificity between the *T. ferrooxidans* and the *T. thiooxidans*, revealed strong immunoreativity in the homologous antiserum and two of the *T. ferrooxidans* strain(Tf-1, Tf-J), but failed to react with antisera to *T. novellus*, *T. intermedius* and *T. thioparus*, respectively.

Similar sets of double gel diffusion analyses were adopted with antigen fractions of the *T. thiooxidans* ATCC 8085. As illustrated in Figs 6 and 7, the overall creatitvities of the *T. thiooxidans* antigen fractions with the antisera against to the *T. ferroxidans* species were very much similar to those of the *T. ferrooxidans* antigen fractions. The crude antigen fraction of the *T. thiooxidans* showed wide cross-reactions among the *T. ferrooxidans* antisera, however, the reaction was failed to observe with antiserum to the *T. thioparus*.

The other kind of antigen, the hydroxyapatite peak-1 fraction of the *T. thiooxidans*, has a distinctly clear immunoprecipitation with antisera to the *T. ferrooxidans* species, however, some other kinds of precipitation lines were disappeared that the hydroxyapatite absorbed some portion of the antigenic moiety from the crude antigen fraction.

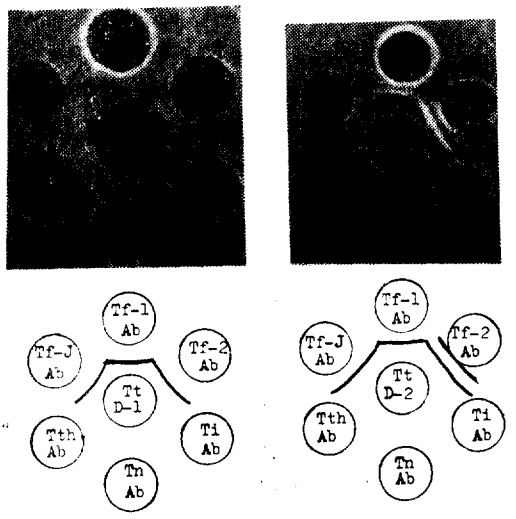

Fig. 6. Double gel diffusion pattern of antigen fractions from *T. thiooxidans* ATCC 8085 with antisera to *thiobacillus* species.

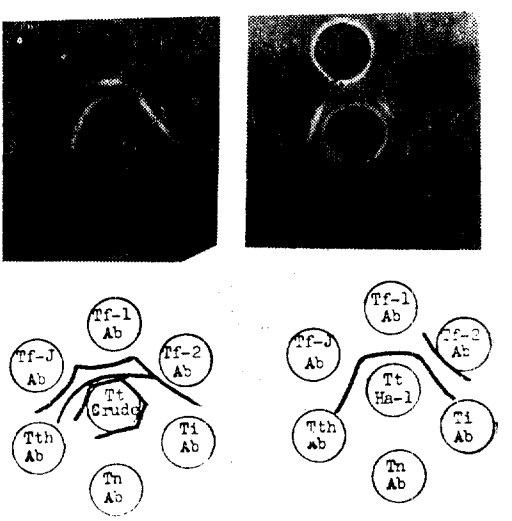

Fig. 7. Double gel diffusion pattern of antigen fractions from *T. thiooxidans* ATCC 8885 with antisera to *thiobacillus* species.

The comparisons were made with DEAE-cellulose peak 1 and 2 antigen fractions from the *T. thiooxidans*. More clear precitiation line was observed on the DEAE-cellulose peak-2 fraction of the *T. thiooxidans* than that of peak-1 fraction(Fig. 7)

The antigen fraction, DEAE-cellulose peak-2 fraction of the *T. thiooxidans* were

Fig. 8. SDS-Polyacrylamide gel electrophoresis pattern of antigen fractions from *T. ferrooxidans* ATCC 13598 and *T. thiooxidans* ATCC 8085.
A; *T. ferrooxidans* hydroxyapatite peak-1(PAS staining)
B; *T. ferrooxidans* hydroxyapatite peak-2(protein staining)
C; *T. thiooxidans* DEAE-cellulose peak-1(PAS staining)
D; *T. thiooxidans* DEAE-cellulose peak-2(protein staining)

shown the type specificity on immunodiffusion analysis with antiserum to the *T. ferrooxidans* 13598, however, in this analysis it showed a wide cross-reactivites among the *T. ferrooxidans* 13598, however, in this analysis it showed a wide cross-reactivities among the *T. ferrooxidans* species which formerly isolated as a *F. ferrooxidans* or a *F. sulfooxidans*, respectively. Especilally, a strong precipitation line was observed between the antiserum to the *T. ferrooxidans* strain 2 which was originally isolated as a *F. sulfooxidans* ATCC 14119, indicating the possibility of the cultural conditions and choice of nutrients influence the composition of bacterial antigens(Vestal *et al.*, 1973).

The SDS-polyacrylamide gel electophoretic patterns of various antigen fractions from the *T. ferrooxidans* and the *T. thiooxidans* were illustrated in Fig. 8. The antigen fractions of the *T. ferrooxidans* obtained from the hydroxyapatite column chromatography represented entirely different characters each other. The hydroxyapatite peak-1 fraction revealed 2 or 3 kinds of banding patterns which stained strongly with PAS, whereas, the hydroxyapatite peak-2 fraction was a protein with a trace of carbohydraee detected on the electrophoregram.

On the other hand, the DEAE-cellulose peak-1 fraction from the *T. thiooxidans* showed 3 kinds of PAS positive banding patterns on electrophoregram which were similar pattern with the *T. ferrooxidans*, however, the DEAE-cellulose peak-2 fraction was staired positively with Amidoblack 10B, indicating the antigen fraction was mainly composed of proteins.

Comparing the results obtained from double gel niffusion analyses and polyacrylamide gel electrophoresis, the antigenic moieties which represent the type-specificities were the proteins with different electrophoretic mobility, however, the polysaccharide glycoprotein was the common antigenic group between the *T. ferrooxidans* and the *T. thiooxxidans*, respectively.

The estimated molecular weights of the type specific antigenic moiety from the *T. ferrooxidans* and the *T. thiooxidans* were approximately 23,000 and 84,000 daltons, respectively, which contain protein and trace amount of carbohydrate.

DISCUSSIONS

Little is known with certainty about the antigenic structure of presumably related strains of the *Thiobacillus* species. The antigens such as endotoxins and LPS of gram-negative bacteria are generally composed of polysaccharide, lipid, and a small amonts of protein. (Halegua et al., 1974; Hirt and Vestal, 1975; Schnaitman, 1970) Especially, the polysaccharide moiety of gram-negative bacteria endows the antigen with serological specificity which plays an important role on the immunological identification of related species. (De Araujo *et al.*. 1963; Benett and Tomabene, 1974; Hollingdale, 1975; Bernstein *et al.*, 1975)

As reported previously, the authors have tried to isolate the type specific component from the *T. thiooxidans* or the *T. ferrooxidans* with an expectation of showing critical immunologic specificity concerning to immunological classification, however, the isolated anutigen fractions such as purified LPS, soluble fraction from the cell wall or the cytoplasmic component and the detergent soluble fractions were not showed any critical role on the determination of type specificity in Thiobacillus species. (Rhee, 1976)

Previous results obtained in the laboratory by the radioimmunometric/polyacylamide gel electrophoresis technique were showed a typical differences on the electrophoregrams of the *T. ferrooxidans* or the *T. thiooxidans* which were solubilized with phenol/actic acid mixture; this implies that the type specific antigen moieties were failed to be extracted with conventional methods which has been employed in this laboratory. (Rhee *et al.*, 1975)

The fractionation procedures presented here suggest that the presence of one or a few such type speeific major protein antigen fractions from both of the *T. ferrooxidans* and the *T. thiooxidans* seems to be originated from the cytoplasm of the bacteria, since it is believed that the glycoprotein fractions which was derived from the cell wall are the common antigenic fraction between the *T. ferrooxidans* and the *T. thiooxidans*, respectively.

In this regard, it is of great interest that the *T. ferrooxidans* or the *T. thiooxdans* appears not to have the type-specific antigens on their LPS or polysaccharide moiety in contrast to the other gram-negative bacteria. Thus, it is strongly believed that the envelopes of these bacteria contain both glycoproteins bearing common antigenicity, since the *T. ferrooxidans* and the *T. thiooxidans* have a structually different type-specific antigen moiety according to the results polyacrylamide gel elctrophoresis.

However, further investigation should be continued to get highly purified antigen fraction, and more detailed physicochemical properties of the purified antigen fractions from both of the *T. ferrooxidans* and *T. thiooxidans* are needed to elucidate the defined structural moiety of the type specific antigen.

The fractionation procedures presented here suggest that the presence of one or a few such major protein antigen fractions in the *T. ferrooxidans* or the *T. thiooxidans* will be a type-specific feather. The fractionation procedures presented here suggest that the presence of such type-specific major protein antigen fraction in nach of the *T. ferrooxidans* or the *T. thiooxidans* are originated from cytoplasm, whereas, it is supposed that the glycoprotein fractions which showed wide cross-re-

activties between the *T. ferroxidans* and the *T. thiooxidans* are originated from the cell wall.

摘 要

ATCC 균주인 *T. ferrooxidans* ATCC 13598 및 *T. thiooxidans* ATCC 8085 2균주에 3%의 Tween 20을 처리한 후 detergent soluble 분획으로 각 균체의 특이항원을 분리하기 위하여 hydroxyapatite 및 DEAE-cellulose column chromatography를 행하였다. *T. ferrooxidans*로부터 분리된 항원인 hydroxyapatite peak-1 및 peak-2항원(Tf, HA-1)(Tf, HA-2)과 *T. thiooxidans*로부터 분리된 DEAE-cellulose peak-1(Tt, DP-1) 및 peak-2(Tt, DP-2) 항원분획을 *Thiobacillus* species에 대한 각각의 항혈청과 반응시킨 결과 *T. ferrooxidans*의 hydroxyapatite peak-2 및 *T. thiooxidans*의 DEAE-cellulose peak-2 분획이 double gel diffusion에서 특이항원 성분으로 나타났으며 상기 type-specific한 분획의 성상을 SDS-polyacrylamide gel 전기영동 한 후, 단백질 및 당단백질에 대한 염색을 실시한 결과 *T. ferrooxidans* 특이 항원인 hydroxyapatite peak-2 분획과 *T. thiooxidans* 특이항원분획(DEAE peak-2)은 모두 순수 단백질로서 상당한 분자량의 차이를 보였고 당단백질인 PAS positive banding pattern은 모두 공통항원성을 갖고 있었다.

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