Study on the Cell Wall Skeleton Derived from *Mycobacterium bovis* BCG Tokyo 172 (SMP-105): Establishment of Preparation and Analytical Methods

Yuko UENISHI,*[,a] Takashi OKADA,[a] Seiji OKABE,[b] and Makoto SUNAGAWA[c]

[a] *Technology Research & Development Center, Dainippon Sumitomo Pharma Co., Ltd.; 1–3–45 Kurakakiuchi, Ibaraki, Osaka 567–0878, Japan:* [b] *Ehime Plant, Dainippon Sumitomo Pharma Co., Ltd.; 5–1 Sobiraki-cho, Niihama, Ehime 792–0001, Japan: and* [c] *Faculty of Pharmaceutical Sciences, Teikyo Heisei University; 2289–23 Uruido, Ichihara, Chiba 290–0193, Japan.* Received November 13, 2006; accepted March 29, 2007; published online April 9, 2007

Mycobacterial cell walls have diverse adjuvant activities, and in particular, cell wall skeleton (CWS) of *Mycobacterium bovis* BCG has been expected as a drug for tumor immunotherapy. However, its molecular structure–biological activity relationship has not been fully elucidated despite more than 30 years of intensive research. Since it is important to secure purified CWS for such investigation, we established a preparation method of CWS from *M. bovis* BCG Tokyo 172 (SMP-105) and developed accurate, precise, and reliable analytical methods, based on previous reports. Furthermore, we confirmed that SMP-105 is composed of mycolic acids; arabinogalactan consisting of arabinose, galactose, and rhamnose; and peptidoglycan consisting of alanine, glutamic acid, diaminopimeric acid, muramic acid, glucosamine, and galactosamine. We also determined the levels of potential impurities that might be contaminated in the original bacterium or arise during the manufacturing process, such as glucose, mannose, non-constituted amino acids, as well as nucleic acid, trehaolse di-mycolate, and bacterial endotoxins. These results demonstrated that the prepared SMP-105 was of sufficient quality for research into the chemistry, bioactivity, and structure–activity relationship of CWS.

Key words BCG-cell wall skeleton (CWS); preparation; analysis; SMP-105; *Mycobacterium bovis* BCG Tokyo 172

Mycobacterial cell envelopes consist of numerous high molecular weight lipid molecules, most of which induce diverse host immune responses. Among these molecules, cell wall skeleton (CWS) derived from *Mycobacterium bovis* BCG has been reported recently to be potent in anticancer immunotherapeutics *via* host antitumor immunity stimulation.[1—6] Also, the structure of CWS derived from *M. bovis* BCG Pasteur and that of cell walls of *M. tuberculosis* and other mycobacterial species have been studied since the 1970s and speculated to be unique species- and strain-specific complexes consisting of mycolic acid, a very long branched chain-hydroxyl fatty acid, and arabinogalactan, a very large branched chain heteroglycan and extremely stable peptidoglycan part of the cell walls.[7—10] However, their detailed structures have not been clarified, because CWS is a huge polymeric compound that does not dissolve in water or any organic solvent, making it difficult to determine the components of CWS and much more difficult to elucidate its structure–biological activity.

As a tumor immunotherapy agent, the clinical efficacy of CWS from *Mycobacterium bovis* BCG has been recognized in many studies.[11—22] These clinical results have prompted us to research into the chemistry, bioactivity, and structure–activity relationship of CWS derived from *M. bovis* BCG Tokyo 172 in more detail, because no such studies on CWS from *M. bovis* Tokyo 172 have been undertaken to date.

Since the most important first step is to secure purified CWS, we developed preparation methods and analytical methods to produce CWS of sufficient quality for use in these studies. In this paper, we describe an optimized preparation method of CWS from *M. bovis* BCG Tokyo 172 (hereinafter called SMP-105) on the basis of an earlier report,[1] as well as evaluation methods modified with more sensitive and specific technologies. Also, we report analytical results of SMP-105 and show confirmation that the prepared SMP-105 is of sufficient quality for use in research into the chemistry, bioactivity, and structure–activity relationship of CWS.

Experimental

Bacterial Strain and Growth Conditions *M. bovis* BCG Tokyo 172 (ATCC 35737) was grown at 37 °C on Sauton medium as surface pellicles until early stationary phase. Cultivated cells were inactivated at 80 °C for 30 min and harvested by centrifugation.

Preparation of Cell Wall Skeleton (CWS) CWS was prepared according to an optimized and slightly modified method based on an earlier method (Azuma *et al.* in 1974).[1] Briefly, about 600 g wet cell mass was suspended in 2000 ml water, and then disrupted with Mini DeBEE (BEE International) at 35 kpsi. The disrupted cells were centrifuged at 6760×***g***, 25 °C, for 10 min to remove large debris and undisrupted cells. Then supernatant was centrifuged at 18000×***g***, 25 °C, for 1 h to obtain pellet, called whole cell walls (WCW). The yield of WCW from 600 g wet cells was about 190 g. WCW was then incubated with benzonase (Merck Ltd.) at 25 °C for 17 h to digest nucleic acids. WCW was washed 5 times with 1% Triton X-100 by centrifugation, and then treated with pronase (Sigma-Aldrich Co.) at 37 °C for 17 h to digest proteins. The cell walls were collected by centrifugation at 18000×***g***, 25 °C, for 20 min and resuspended in 1% Triton X-100 and gently stirred at 60 °C for 2 h to remove protein digests, and collected and washed with ethanol. Free lipids were removed by extraction with tetrahydrofuran, chloroform–methanol (2 : 1), and methanol consecutively and finally dried to obtain CWS. The yield of dry cell wall skeleton was about 10 g. CWS prepared as such was called SMP-105.

Reagents and Standards All reagents and chemicals used were of analytical or pharmaceutical grade.

Determination Procedure of Mycolic Acids About 30 mg SMP-105 was weighed accurately and 2 ml 0.5 mol/l potassium hydroxide solution in a mixture of ethanol (99.5%), toluene, and water (10 : 10 : 1 v/v/v) was added, and heated at 65 °C for 3 h. After cooling, 1 ml 2 mol/l hydrochloric acid and then 2 ml *n*-hexane were added, and the mixture was shaken vigorously. The upper *n*-hexane layer was taken to another test tube, and this extraction procedure was undertaken 3 times in total. The *n*-hexane layer collected was washed twice with 1 ml water, and the organic solvent was evaporated. The weight of obtained mycolic acids was determined accurately by using a balance.

Separately, for HPLC analysis, about 20 mg SMP-105 was weighed accurately and suspended in a mixture of heptane and ethanol (99.5%) (9 : 1 v/v)

* To whom correspondence should be addressed. e-mail: yuko-uenishi@ds-pharma.co.jp

to make a 5-ml volume of solution as the stock solution. A 300-μl portion of this stock solution was taken and poured into a test tube, an appropriate amount of tricosenoic acid (Tokyo Kasei Kogyo Co., Ltd.) was added as an internal standard, and the solvent was evaporated. To this residue, 1 ml 0.5 mol/l potassium hydroxide solution in a mixture of ethanol (99.5%), toluene, and water (20 : 10 : 1 v/v/v) was added, and heated at 65 °C for 3 h. After cooling, 0.5 ml 2 mol/l hydrochloric acid and then 2 ml *n*-hexane were added, and the mixture was shaken vigorously. The upper *n*-hexane layer was taken to another test tube, and this extraction procedure was undertaken 3 times in total. The *n*-hexane layer collected was washed twice with 1 ml water, and the organic solvent was evaporated.

Fluorescent Labeling of Mycolic Acids To the resultant residue obtained above, toluene was added to make an estimated 0.3 mg/ml solution. A 200-μl portion of this solution was taken and an appropriate amount of the fluorescent reagent, 9-anthryldiazomethane (ADAM, Funakoshi Co., Ltd.), and toluene were added to make the volume of this solution to 1 ml. The solution was stored at 40 °C for 5 h or more for complete labeling, and this solution was used as the sample solution.

HPLC Conditions for the Determination of Mycolic Acids The HPLC system consisted of binary pumps and a fluorescent detector (Shimadzu LC-10AD$_{VP}$ and RF-10A$_{XL}$, Shimadzu Corporation). Chromatography was conducted using a reversed phase C-30 column (Develosil C30-UG-3, 3 μm, 4.6×150 mm, Nomura Chemical Co., Ltd.). Toluene and methanol were prepared as mobile phases. The initial gradient condition was 0% toluene–100% methanol, with a flow rate of 1.0 ml/min at a column temperature of 50 °C. The toluene concentration was increased linearly from 0 to 10% in the first 15 min, to 50% in the next 15 min, and then to 60% in the following 20 min. The excitation wavelength and measurement wavelength of the fluorescent detector was 365 nm and 412 nm, respectively.

Determination Procedure of Neutral Sugars A 500-μl portion of the stock solution was transferred into a test tube for hydrolysis and evaporated. To this residue, 0.2 ml 0.2 mol/l potassium hydroxide solution in a mixture of ethanol (99.5%), toluene, and water (10 : 10 : 1 v/v/v) was added and heated at 65 °C for 15 min. After neutralizing with 20 μl 2 mol/l trifluoroacetic acid, the organic solvent was removed with a centrifugal evaporator. The residue was hydrolyzed with 50 μl 2 mol/l trifluoroacetic acid by vapor-phase hydrolysis at 100 °C for 2 h. After cooling, an appropriate amount of xylose was added as an internal standard. The solution was treated with anion exchange resins (BioRad AG4-X4 [OH-form], Bio-Rad Laboratories Inc.) and put into another test tube in which 10 μl sodium acetic acid buffer (pH 4.9) had been placed in advance, and then water was added to make the volume of this solution to 5 ml. This solution was used as the sample solution for HPLC analysis.

HPLC Conditions for the Determination of Neutral Sugars The HPLC system consisted of a pump and a pulsed electrochemical detector (PED) (Dionex Corporation). Chromatography was performed with two anion-exchange columns connected in series (CarboPac PA-1, 4.0×250 mm, Dionex Corporation). Water and 10 mmol/l sodium hydroxide solution were prepared as mobile phases. The initial gradient condition was 90% water–10% 10 mmol/l sodium hydroxide solution, with a flow rate of 0.5 ml/min at a column temperature of 15 °C. The concentration of 10 mmol/l sodium hydroxide solution was increased linearly to 100% in 60 min, and equilibrated for 40 min. Before analysis, the columns were washed with 200 mmol/l sodium hydroxide solution for 10 min and stabilized with 90% water–10% 10 mmol/l sodium hydroxide solution for 15 min.

Determination Procedure of Amino Acids and Amino Sugars A 500-μl portion of the stock solution was taken into a test tube for hydrolysis and evaporated. To this residue, an appropriate amount of norleucine as an internal standard and 1 ml 6 mol/l hydrochloric acid were added. The solution was heated at 110 °C for 48 h for the determination of amino acids and at 110 °C for 3 h for amino sugars. After cooling, the contents were transferred to other test tubes and water was added. Hydrochloric acid in the test tubes was then removed with a centrifugal evaporator. A 5-ml quantity of 0.02 mol/l hydrochloric acid was added to these solutions, which were then used as the sample solutions.

HPLC Conditions for the Determination of Amino Acids and Amino Sugars The HPLC system consisted of an amino acid analyzer (Hitachi model L-8800, Hitachi High-Technologies Corporation) equipped with a UV–Vis detector (wavelength at 570 nm for amino acids and amino sugars except for proline, and 440 nm for proline). A column (4.6×80 mm) packed with cation-exchange resin (Hitachi custom ion exchange resin #2620MSC, Hitachi High-Technologies Corporation) was used. Programmed gradient conditions of the mobile phases and column temperature are described in Table 1. The flow rate was 0.19 ml/min and detection was carried out by post-column reaction with ninhydrin.

Table 1. Gradient Program for the Determination of Amino Acids and Amino Sugars

Gradient program (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Solvent D (%)	Column temperature (°C)
0.0	100	0	0	0	50
18.0	100	0	0	0	50
20.0	0	100	0	0	50
32.0	0	100	0	0	60
37.0	0	100	0	0	60
46.0	0	0	100	0	65
55.0	0	0	100	0	50
70.0	0	0	100	0	55
70.1	0	0	0	100	55
81.0	0	0	0	100	55
81.1	100	0	0	0	55
125.0	100	0	0	0	50
140.0	100	0	0	0	50

Solvent A: 7.74 g trisodium citrate dehydrate, 7.08 g sodium chloride, 17.7 g citric acid monohydrate, 120 ml ethanol (99.5%), 10 ml 2-mercaptoethanol, and 8 ml polyoxyethylene (23) lauryl ether were dissolved in 2000 ml water and 0.2 ml *n*-caprylic acid was added.

Solvent B: 10.05 g trisodium citrate dehydrate, 1.87 g sodium chloride, 7.72 g citric acid monohydrate, 3 ml benzyl alcohol, 5 ml 2-mercaptoethanol, and 4 ml polyoxyethylene (23) lauryl ether were dissolved in 1000 ml water and 0.1 ml *n*-caprylic acid was added.

Solvent C: 26.67 g trisodium citrate dehydrate, 2.5 g sodium hydroxide, 54.35 g sodium chrolide, 6.1 g citric acid monohydrate, 5 ml benzyl alcohol, and 4 ml polyoxyethylene (23) lauryl ether were dissolved in 1000 ml water, and 0.1 ml *n*-caprylic acid was added.

Solvent D: 8 g sodium hydroxide, 60 ml ethanol (99.5%), and 4 ml polyoxyethylene (23) lauryl ether were dissolved in 1000 ml water and added with 0.1 ml *n*-caprylic acid was added.

Determination Procedure of Phosphorus About 20 mg SMP-105 was accurately weighed and an appropriate amount of yttrium was added as an internal standard. About 0.5 ml sulfuric acid and then 2 ml nitric acid were added little by little. The solution was heated slowly at first and then strongly until its color became clear to pale yellow. After cooling, 2 ml hydrogen peroxide solution was added until the solution became clear. Then, about 5 ml water and 2 ml hydrochloric acid were added, and dissolved by heating. Water was added to make the volume of this solution to 20 ml, and the resulting solution was used as the sample solution. The sample solution was measured at the wavelength of 177.499 nm by inductively-coupled plasma atomic emission spectrometry (ICPS-8000, Shimadzu Corporation).

Determination Procedure of Water Content About 50 mg SMP-105 was accurately weighed and 5 ml dehydrated toluene was added. To this suspension, 5 ml dehydrated methanol was added. With this solution, water content was determined by Karl-Fisher coulometric titration (Aquacounter AQ-7 Karl Fischer coulometric titrator, Hiranuma Sangyo Co., Ltd.).

Results and Discussion

Establishment of a Preparation Method of CWS Based on the preparation method of CWS described by Azuma *et al.* in 1974,[1] all steps of the preparation procedure, such as choices of cell disruption machine, enzymes, solvents for delipidation, and washing conditions after enzymatic reactions were optimized to yield CWS of consistent and higher quality. Test results of multiple lots (results of one representative lot are summarized in Table 14) demonstrated that SMP-105 prepared by this method were of sufficient quality for use in research into its chemistry, bioactivity, and structure–activity relationship.

Development of an Analytical Method for the Determination of Mycolic Acids In earlier reports[1,23—26] on CWS

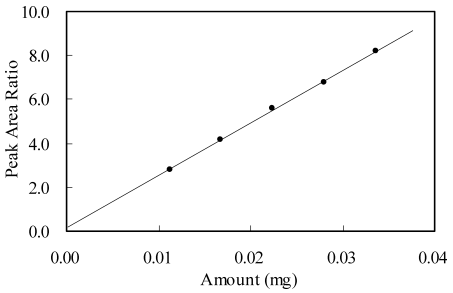

Fig. 1. Linearity for the Determination of Mycolic Acids

Table 2. Linearity for the Determination of Mycolic Acids

Component	Correlation coefficient (r)	y-Intercept	Slope
Mycolic acids	0.9996	0.149	239.1

as well as on cell walls of mycobacterial species, several hydrolytic conditions to extract mycolic acids were described, such as refluxing with 2.5% sodium hydroxide solution in a mixture of methanol and benzene (1 : 1 v/v) for 1 h, and hydrolysis with 10 to 15% potassium hydroxide solution at 90 °C for 3 to 4 h. Based on these conditions, the type and concentration of alkali as well as reaction temperature and period were investigated in order to obtain maximum yield of mycolic acids. As a result, hydrolytic conditions of heating at 65 °C with 0.5 mol/l potassium hydroxide solution in a mixture of ethanol (99.5%), toluene, and water (10 : 10 : 1 v/v/v) for 3 h were adopted. In addition to a conventional method of weighing extracted mycolic acids, HPLC[27—31] was investigated. As mycolic acids do not have an absorption in the ultraviolet region, they were usually labeled with *p*-bromophenacyl bromide (PBPB), 4-bromomethyl-6,7-dimethoxycoumarin, or other fluorescent reagents. As a labeling reagent, 9-anthryldiazomethane (ADAM) was chosen, because ADAM was a popular labeling reagent of fatty acids. Labeling conditions of ADAM were optimized and heating at 40 °C for 5 h or more was adopted. Moreover, a C-30 column rather than a C-18 column, and a gradient program with methanol and toluene were adopted in order to achieve better separation. Also, for the purpose of accurate and precise determination, tricosenic acid was introduced as an internal standard and separately-extracted mycolic acids were used as a reference standard.

This method was validated in terms of specificity, linearity, accuracy, and precision. Mycolic acids were completely separated from other fatty acids, structures of which were not identified. A calibration curve ranging from 50 to 150% (w/w) (corresponding to 0.011 to 0.034 mg of mycolic acids) was linear, with correlation coefficient of 0.9996, passing through the origin (Fig. 1, Table 2). Regarding the accuracy of HPLC, the contents obtained by the weight detremintion method and that by HPLC method were found to be comparable (Table 3). Repeatability of 6 independent analyses was satisfactory, with a relative standard deviation (RSD) of 1.6% (Table 4). Based on the linearity and accuracy data, the range for the determination of mycolic acids by HPLC was 50 to 150%. A representative chromatogram for the determination of mycolic acids is shown in Fig. 2.

Table 3. Comparison of the Content of Mycolic Acids between Weight Determination Method and HPLC Method

Weight (A)	HPLC (B)	A/B
37.9%	37.3%	1.02

Table 4. Repeatablity for the Determination of Mycolic Acids

Preparation time	Content of mycolic acids (%)	Average (%)	S.D. (%)	RSD (%)	90% confidence interval (%)
1	37.64	37.3	0.6	1.6	0.39—1.21
2	37.60				
3	37.64				
4	37.16				
5	36.14				
6	37.35				

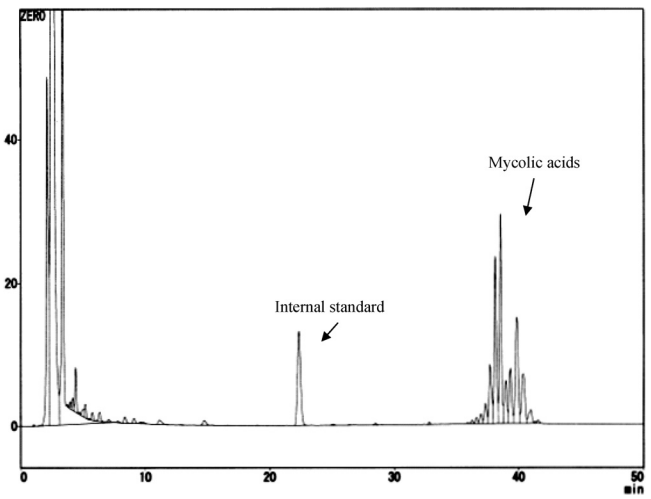

Fig. 2. Representative Chromatogram for the Determination of Mycolic Acids

Development of an Analytical Method for the Determination of Neutral Sugars Although hydrolytic conditions for the determination of neutral sugars described in an earlier report[1] were heating at 100 °C for 8 h with 1 mol/l sulfuric acid, in the current method the type and concentration of acid, reaction temperature and period were optimized, and then hydrolytic conditions of heating with 2 mol/l trifluoroacetic acid at 100 °C for 3 h were adopted. In addition, in order to hydrolyze the ester linkage between mycolic acids and arabinose, SMP-105 was first hydrolyzed with alkali solution, followed by acidic hydrolysis. In doing so, the ratio of arabinose to galactose increased from the range between 1.9 and 2.0 (without alkali hydrolysis) to the range between 2.1 and 2.2 (with alkali hydrolysis), which demonstrated the contents of neutral sugars could not be accurately determined with acidic hydrolysis alone. After hydrolysis with trifluoroacetic acid, the residual acid was removed with a centrifugal evaporator, but it could not be completely done by such evaporation, resulting in different peak profiles on the chromatogram. Therefore, the residual acid was removed with anion-exchange resins before chromatography.

In traditional methods,[7,32—36] neutral sugars are converted to alditol acetates and determined by GC. Amino column chromatography of sugars labeled with 2-aminopyridine is also common. However, anion-exchange chromatography with PED is more favorable because it does not require labeling of sugars. Therefore, this chromatographic method was chosen and a gradient method using water and 10 mmol/l sodium hydroxide solution at a column temperature of 15 °C was adopted for better separation. Under these chromatographic conditions, amino sugars eluted as well, but they were determined not by this method but by amino acid analysis, as described below, because the acid hydrolytic conditions of these amino sugars were not optimized for this chromatography.

This method was validated in terms of specificity, linearity, accuracy, and precision, and additionally in terms of the quantitation limits for rhamnose, glucose and mannose. Arabinose, galactose, rhamnose, glucose, mannose, glucosamine, galactosamine, and the internal standard (xylose) were clearly separated (Table 5). Calibration curves ranging from 7.5 to 30% (w/w) arabinose (corresponding to 0.3 to 1.2 mg), from 5 to 20% (w/w) galactose (0.2 to 0.8 mg), and from 0.2 to 1.2% (w/w) rhamnose, glucose, and mannose (0.008 to 0.048 mg) were linear, with correlation coefficient of 0.99 or more. However, the curves of arabinose, galactose, rhamnose, and mannose did not pass through the origin, while that of glucose did (Fig. 3, Table 6). Therefore, we decided that calibration curves were to be produced for every analytical run. Recovery rates calculated by determining samples spiked with arabinose at three different concentrations ranging from 7.5 to 22.5% (equivalent to 0.3 to 0.9 mg) and galactose from 5 to 15% (0.2 to 0.6 mg) were 91.3 to 117.3% and 88.9 to 112.2%, respectively. Meanwhile, those

Table 5. Specificity for the Determination of Neutral Sugars

Component	Retention time (min)	Relative retention time
Arabinose (Ara)	47.10	0.59
Rhamnose (Rham)	49.55	0.62
Galactosamine (GalN)	53.53	0.67
Galactose (Gal)	60.02	0.75
Glucosamine (GlcN)	66.37	0.83
Glucose (Glc)	70.33	0.88
Xylose (Xyl) (Internal standard)	79.93	1.00
Mannose (Man)	82.92	1.04

Table 6. Linearity for the Determination of Neutral Sugars

Component	Correlation coefficient (r)	y-Intercept	Slope
Arabinose	0.9999	1.0344	7.6555
Rhamnose	0.9992	0.0271	10.6314
Galactose	0.9986	1.1254	10.9151
Glucose	0.9995	0.0136	17.8417
Mannose	0.9992	−0.0172	15.9351

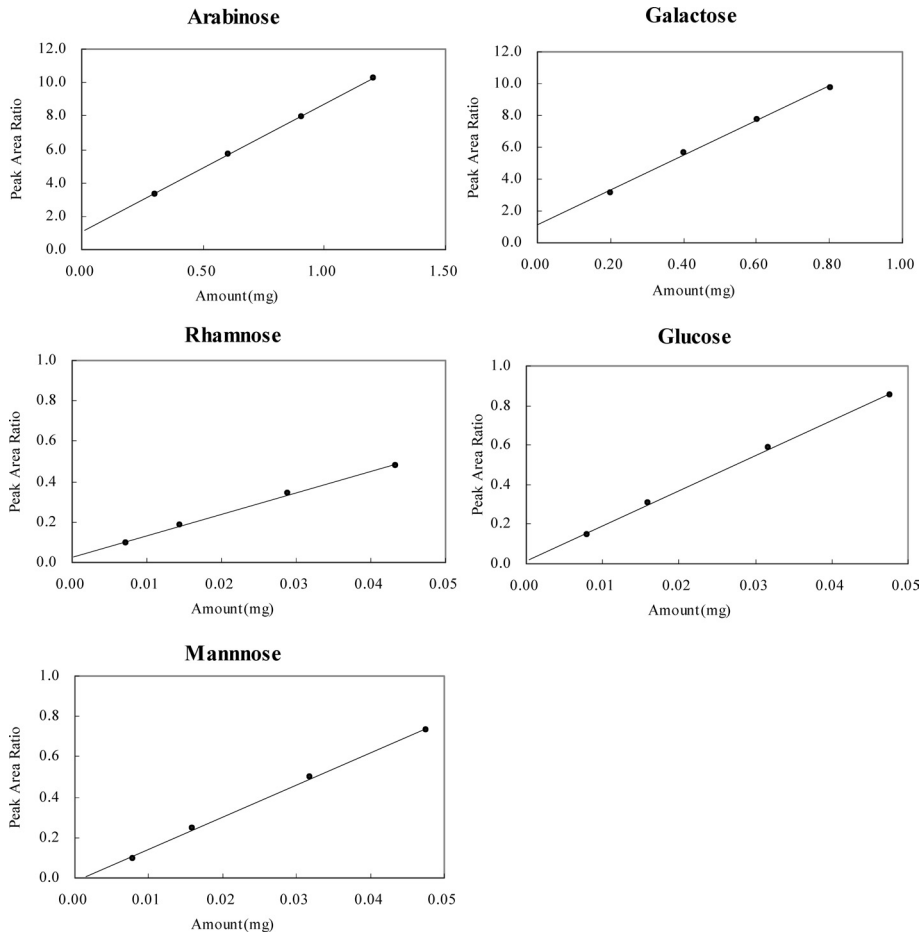

Fig. 3. Linearity for the Determination of Neutral Sugars

Table 7. Accuracy for the Determination of Neutral Sugars (%)

Component	7.5% spiked (%)			15% spiked (%)			22.5% spiked (%)			Average (%)	95% confidence interval (%)
Arabinose	113.3	117.3	116.0	91.3	102.7	107.3	98.7	98.2	104.9	105.5	84.0—127.0

Component	5% spiked (%)			10% spiked (%)			15% spiked (%)			Average (%)	95% confidence interval (%)
Galactose	112.2	110.2	102.2	90.2	93.2	95.2	90.2	88.9	92.2	97.2	73.3—121.1

Component	0.2% spiked (%)			0.4% spiked (%)			0.8% spiked (%)			Average	95% confidence interval (%)
Rhamnose	92.9	115.2	126.3	91.0	110.5	116.1	91.5	95.7	104.0	104.8	86.8—122.8
Glucose	109.8	104.7	74.3	105.6	113.2	120.8	97.1	93.3	102.2	102.3	78.9—125.7
Mannose	143.5	133.4	77.7	114.8	114.8	84.4	101.7	99.2	95.4	107.2	82.5—131.9

Table 8. Repeatability for the Determination of Neutral Sugars

Sample	Arabinose (%)	Galactose (%)	Ara/Gal molar ratio	Rhamnose (%)	Glucose (%)	Mannose (%)
First lot	20.8	12.3	2.0	0.1	0.5	0.2
	17.8	10.8	2.0	0.2	0.5	0.2
	18.3	11.5	1.9	0.3	0.5	0.2
Average	19.0	11.5	2.0	0.2	0.5	0.2
S.D.	1.6	0.8	0.1	0.1	0.0	0.0
RSD	8.4	7.0	—	50.0	0.0	0.0
Second lot	19.4	11.5	2.0	0.2	0.9	0.3
	18.7	11.7	1.9	0.3	0.9	0.3
	21.4	13.1	2.0	0.3	0.9	0.4
Average	19.8	12.1	2.0	0.3	0.9	0.3
S.D.	1.4	0.9	0.1	0.1	0.0	0.1
RSD	7.1	7.4	—	33.3	0.0	33.3

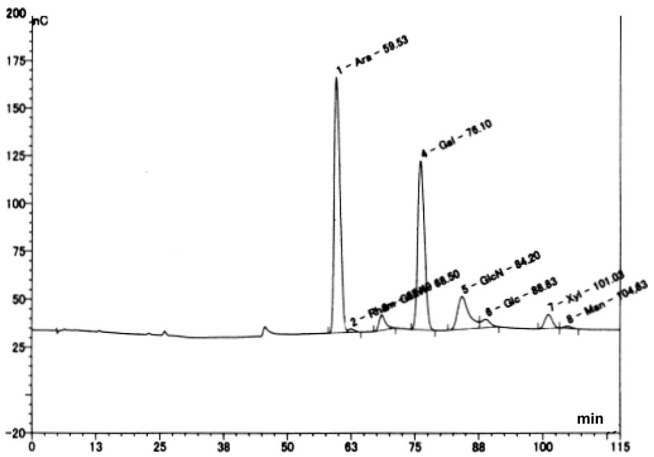

Fig. 4. Representative Chromatogram for the Determination of Neutral Sugars

of rhamnose, glucose, and mannose produced by determining samples spiked with from 0.2 to 0.8% (0.008 to 0.032 mg) levels were 91.0 to 126.3%, 74.3 to 120.8%, and 77.7 to 143.5%, respectively (Table 7). This data indicated that the recovery rates varied from sugar to sugar and also the degradation rates due to hydrolysis could not be neglected (data not shown); thus, we concluded that standard solutions should be hydrolyzed along with sample solutions for each analytical run and the contents of neutral sugars should be corrected for the degradation rates of each sugar. Regarding the repeatability obtained from 3 different preparations of 2 samples, the standard deviation (S.D.) of arabinose was 1.4 to 1.6% and that of galactose was 0.8 to 0.9%, while those of rhamnose, glucose, and mamnose were 0.0 to 0.1% (Table 8). Quantitation limits of rhamnose, glucose, and mannose, which were considered from the calibration curve at lower concentrations, were 0.2%. As a result of the linearity, accuracy, and quantitation limits, the ranges for the determination of arabinose and galactose were 7.5 to 30% and 5 to 20%, respectively, and that of rhamnose, glucose and mannose was 0.2 to 1.2%. A representative chromatogram for the determination of neutral sugars is presented in Fig. 4.

Development of an Analytical Method for the Determination of Amino Acids and Amino Sugars Based on hydrolysis conditions of amino acids and amino sugars of CWS described in earlier reports,[1,37—42] such as heating with 6 mol/l hydrochloric acid at 105 to 110 °C for 15 to 16 h, in the current method hydrolytic conditions with 6 mol/l hydrochloric acid at 110 °C for 48 h for amino acids and for 3 h for amino sugars were developed. Mobile phases and other conditions were established based on the conditions of amino acid analysis in which amino sugars including glucosamine, muramic acid, and galactosamine could also be separated from each other.

Validation studies for this method were conducted in terms of specificity, linearity, accuracy, precision, and quantitation limits. All amino acids and amino sugars were separated from each other and the specificity was satisfactory (Table 9). Calibration curves of amino acids and amino sugars ranging from 0.1 to 10% (w/w) (corresponding to 0.002 to 0.2 mg) were linear, with correlation coefficient of 0.999 or more, and passed through the origin (Fig. 5, Table 10). Recovery rates of alanine, glutamic acid, diaminopimeric acid, glucosamine, muramic acid, and galactosamine, calculated by determining samples spiked with 3 different concentrations (1% [equivalent to 0.02 mg], 3% [0.06 mg], and 5% [0.1 mg]) of each amino acid and amino sugar were 86.0 to 98.3%, 87.0 to 103.9%, 87.9 to 101.8%, 69.3 to 98.6%, 81.4 to 141.6%, and 81.6 to 91.5%, respectively (Table 11). With variable recovery rates such as these, and since degradation

Table 9. Specificity for the Determination of Amino Acids and Amino Sugars

Component	Retention time (min)	Relative retention time	Degree of separation
Asparagines (Asp)	14.343	0.29	—
Threonine (Thr)	17.529	0.35	2.88
Serine (Ser)	19.021	0.38	1.25
Muramic acid (Mur)	20.420	0.41	0.86
Glutamine (Glu)	23.240	0.46	1.64
Glycine (Gly)	33.883	0.68	6.80
Alanine (Ala)	36.428	0.73	1.48
Cystine (Cys)	39.562	0.79	2.72
Valine (Val)	40.503	0.81	1.43
Diaminopimeric acid (DAP)	42.104	0.84	2.23
Methionine (Met)	43.094	0.86	1.25
Isoleucine (Ile)	46.351	0.93	3.44
Leucine (Leu)	48.111	0.96	1.65
Norleucine (N-Leu) (Internal standard)	50.072	1.00	1.76
Tyrosine (Tyr)	52.046	1.04	1.65
Phenylalanine (Phe)	54.248	1.08	2.04
Glucosamine ($GlcNH_2$)	56.284	1.12	1.92
Galactosamine ($GalNH_2$)	57.720	1.15	1.01
Lysine (Lys)	64.440	1.29	5.61
Histidine (His)	67.258	1.34	3.11
Arginine (Arg)	79.669	1.59	10.34
Glutamine (Glu)	23.220	0.46	—
Proline (Pro)	25.283	0.50	1.49

rates due to hydrolysis could not be neglected (data not shown), we concluded that standard solutions should be hydrolyzed along with sample solutions for each run and the contents of amino acids and amino sugars should be corrected for the degradation rate of each compound. Regarding repeatability of 6 independent analyses, RSDs of alanine, glutamine, diaminopimeric acids, glucosamine, muramic acid, and galactosamine were 3.8, 2.8, 2.6, 6.7, 3.9, and 0.0%, respectively (Table 12). Quantitation limits determined 6 times with a standard solution at a concentration of 0.1% were 0.1% for amino acids, except for muramic acid (0.2%) and proline (0.3%). From the results of linearity, accuracy, and quantitation limits, the range of amino acids and amino sugars except for muramic acid and proline was 0.1 to 10%, while those of muramic acid and proline were 0.2 to 10%

Table 10. Linearity for the Determination of Amino Acids and Amino Sugars

Component	Correlation coefficient (r)	y-Intercept	Slope
Alanine	1.0000	-2.486×10^4	2.706×10^7
Glutamine	1.0000	-5.006×10^3	1.642×10^7
Diaminopimeric acid	1.0000	-1.594×10^3	1.606×10^7
Glucosamine	0.9998	-1.291×10^4	1.062×10^7
Muramic acid	1.0000	-1.190×10^3	2.819×10^6
Galactosamine	0.9999	-1.623×10^3	1.023×10^7

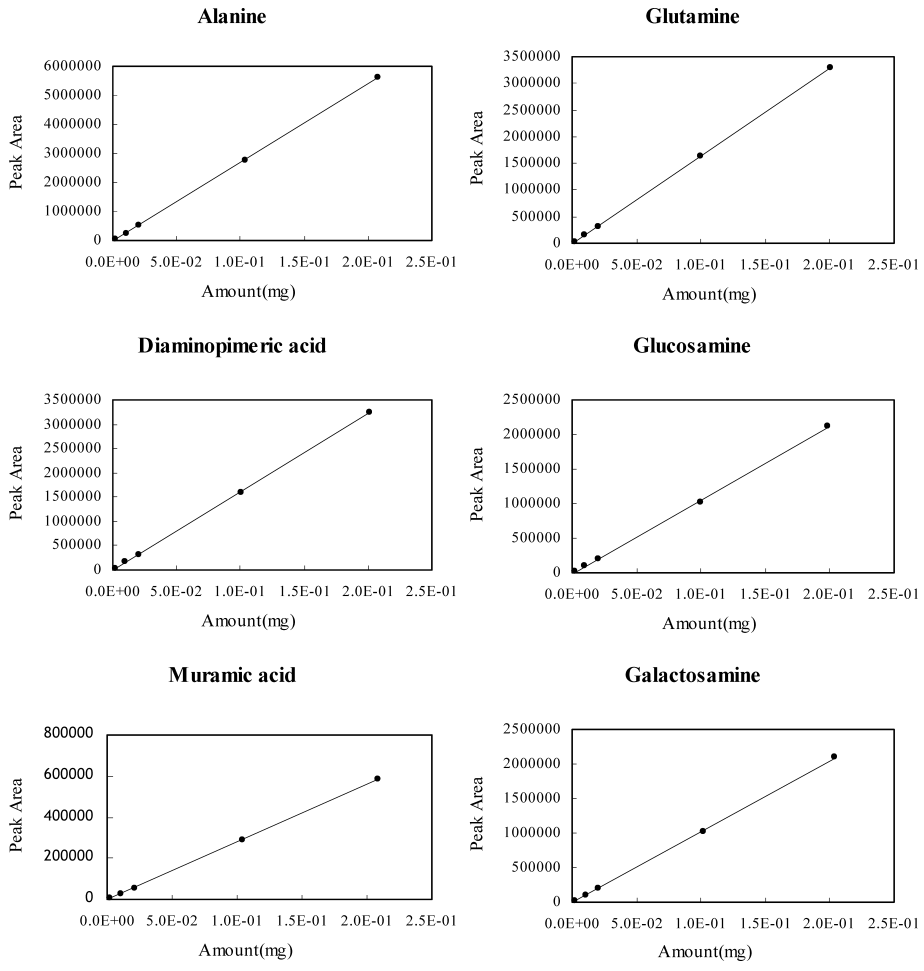

Fig. 5. Linearity for the Determination of Amino Acids and Amino Sugars

Table 11. Accuracy for the Determination of Amino Acids and Amino Sugars

Component	Recovery rate (%)										95% confidence interval (%)
	Spiked with 1%			Spiked with 3%			Spiked with 5%			Average	
Alanine	92.4	89.8	86.0	92.8	96.2	98.3	96.4	92.4	94.9	93.2	84.7—101.7
Glutamine	100.0	90.3	87.0	98.2	103.2	103.9	103.0	98.4	101.0	98.3	85.5—111.1
Diaminopimeric acid	100.0	93.4	87.9	96.7	100.3	101.8	100.0	94.7	98.4	97.0	89.7—104.3
Glucosamine	74.6	98.6	69.3	94.7	75.7	85.9	85.1	83.8	77.5	82.8	76.9—88.7
Muramic acid	114.5	141.6	105.2	114.5	89.9	102.0	97.0	93.3	81.4	104.4	67.1—141.7
Galactosamine	89.1	89.1	89.1	91.5	81.6	87.5	86.3	85.0	82.8	86.9	81.4—92.4

Table 12. Repeatability for the Determination of Amino Acids and Amino Sugars

Component	Content (%)							S.D. (%)	RSD (%)
	n=1	n=2	n=3	n=4	n=5	n=6	Average		
Alanine	2.5	2.6	2.6	2.6	2.5	2.5	2.6	0.1	3.8
Glutamine	3.5	3.6	3.6	3.6	3.5	3.5	3.6	0.1	2.8
Diaminopimeric acid	3.7	3.8	3.8	3.9	3.7	3.8	3.8	0.1	2.6
Glucosamine	2.7	3.1	3.0	3.0	3.1	3.2	3.0	0.2	6.7
Muramic acid	4.7	5.2	5.1	5.0	5.1	5.3	5.1	0.2	3.9
Galactosamine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.0	0.0
Others	2.3	2.5	2.7	2.5	2.5	2.5	2.5	0.1	4.0
Total	19.9	21.3	21.3	21.1	20.9	21.3	21.0	0.5	2.4

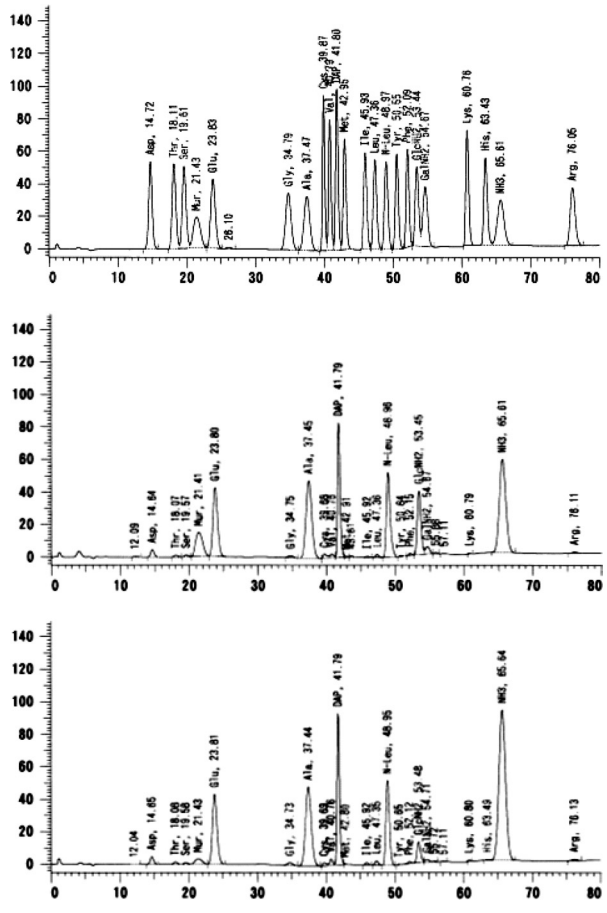

Fig. 6. Representative Chromatograms of the Standard Solution, and the Sample Solutions (Hydrolysis for 3 and 48 h) for the Determination of Amino Acids and Amino Sugars

Table 13. Accuracy for the Determination of Phosphorous

Sample	Concentration (%)	Recovery rate (%)	95% confidence interval (%)
No spiked	0.092	—	78.2—104.8
Spiked with 0.05%	0.138	92.0	
Spiked with 0.1%	0.183	91.0	
Spiked with 0.2%	0.285	96.5	

and 0.3 to 10%, respectively. A representative chromatogram for the determination of amino acids and amino sugars is presented in Fig. 6.

A point to be noted for this method is that glucosamine and muramic acid were reported[43] to be present as *N*-acetyl glucosamine and *N*-glycolyl muramic acid in SMP-105; however, these were not identified in this study, because they were hydrolyzed and only glucosamine and muramic acid were detected.

This method was also applicable to non-constituent amino acids, judging from their validation results.

Development of an Analytical Method for the Determination of Phosphorous (P) The content of phosphorous was determined with a colorimetric method in an earlier report,[1] but ICP analysis was adopted here because of increased sensitivity and convenience. Samples were incinerated with sulfuric acid and nitric acid, followed by ICP analysis. Samples spiked with phosphorus at the levels equivalent to 0.05, 0.1 and 0.2% were determined, and recovery rates calculated were satisfactory, at 91.0 to 96.5%, as shown in Table 13.

Development of a Method for the Determination of Water Content As SMP-105 is hygroscopic, water content was determined by the Karl Fisher titration method. Samples were suspended in dehydrated toluene, dehydrated methanol was added, and the obtained suspension was shaken vigor-

Table 14. Summary Results of the Determination of SMP-105 (lot no. S005)

Test item	Result		
	Content		Molar ratio
Fatty acids (dried basis)			
Mycolic acids	38.8%	—	—
Other fatty acids	0.3%	—	—
Neutral sugars (dried basis)			
Arabinose	23.0%	1.741 μmol/mg	2.1 (against Gal)
Galactose (Gal)	13.6%	0.837 μmol/mg	1.0 (against Gal)
Rhamnose	0.4%	0.030 μmol/mg	0.12 (against DAP)
Glucose	0.6%	0.037 μmol/mg	—
Mannose	0.6%	0.035 μmol/mg	—
Subtotal	38.2%	—	—
Amino acids and amino sugars (dried basis)			
Alanine	2.8%	0.389 μmol/mg	1.6 (against DAP)
Glutamic acid	3.6%	0.277 μmol/mg	1.1 (against DAP)
Diaminopimelic acid (DAP)	4.2%	0.242 μmol/mg	1.0 (against DAP)
N-Acethyl glucosamine	4.9%	0.242 μmol/mg	1.0 (against DAP)
N-Glycolyl muramic acid	6.9%	0.236 μmol/mg	1.0 (against DAP)
Galactosamine	0.5%	0.033 μmol/mg	0.14 (against DAP)
Total non-constituent amino acids	1.3%	—	—
Subtotal	24.2%	—	—
Phosphorus	0.1%	0.029 μmol/mg	0.12 (against DAP)
Water	2.3%	—	—
Total (excluding water content)	101.7%	—	—
Description	White mass and powder		

ously to extract water. The water content of a representative lot was 2.3% as shown in Table 14 and those of the other multiple lots were 1.0 to 3.3%.

Development of Methods for the Determination of Other Potential Impurities As SMP-105 was produced by disrupting *M. bovis* BCG Tokyo 172, methods for the determination of nucleic acid, trehalose di-mycolate, and bacterial endotoxins derived from the original bacterium and the preparation process were developed and their levels in SMP-105 were preliminarily investigated.

To determine the level of nucleic acid, SMP-105 was disrupted with a bead mill (0.5 mm i.d. zirconia beads) and nucleic acid was extracted with a TE buffer (10 mM Tris–HCl, 1 mmol/l EDTA, pH 7.5, PicoGreen dsDNA Quantitation Kit, Molecular Probe Inc.). The level of nucleic acid in SMP-105 was determined using a PicoGreen assay (PicoGreen dsDNA Quantitation Kit, Molecular Probe Inc.). The result was less than 0.05% when comparing to λ-Hind III digest (Takara Bio Inc.) as a reference standard.

There was also a possibility of contamination of trehalose di-mycolate, which causes toxicity and granulomatogenic activity in mice. Trehalose di-mycolate was extracted from SMP-105 by shaking in a mixture of chloroform and methanol (4 : 1 v/v) and was analyzed by thin-layer chromatography with a silica-gel plate (Merck Ltd.) and a developing solvent of chloroform, methanol, acetone, and acetic acid (100%) (90 : 10 : 6 : 1 v/v/v/v).[43] The level of trehalose di-mycolate in SMP-105 was less than 0.05% by comparing the color strength of spots of trehalose di-mycolate with that of a known concentration.

A test method for bacterial endotoxins, which might be contaminated in SMP-105 during the preparation process, was also investigated. As a result of determination by gel-clot technique, the level of bacterial endotoxins in SMP-105 was quite low, not more than 0.0015 EU/mg.

Summary of Analysis Results More than 10 lots of SMP-105 derived from *M. bovis* BCG Tokyo 172 were prepared and analyzed with the above-mentioned methods. The test results of a representative lot (lot no. S005) of SMP-105 are shown in Table 14. The content of mycolic acids in SMP-105 was found to be 38.8%, and those of arabinose and galactose were 23.0% and 13.6%, respectively, with a molar ratio of 2.1 : 1. Meanwhile, the contents of alanine, glutamic acid, diaminopimeric acid, *N*-acethyl glucosamine, and *N*-glycolyl muramic acid were 2.8, 3.6, 4.2, 4.9, and 6.9%, respectively. Also, phosphorus was contained at 0.1%.

The composition analysis data of this lot and the other lots (data not shown) suggested that CWS of consistent quality can be prepared with this method.

The total content of this lot was 101.7% and those of the other lots were from 97.0 to 101.7%. This good material balance indicated that the analytical methods were relatively accurate, precise, and reliable when taking into consideration that the methods were adopted with specific techniques chosen according to the subjects, and that some parts of the analytical procedures, particularly the hydrolytic steps, were not always simple or easy to perform.

In an earlier report,[1] CWS derived from *M. bovis* BCG Pasteur consisted of 34.2% mycolic acids, 38.6% arabinogalactan with arabinose and galactose in a molar ratio of 2.8 : 1, and 20.1% amino acids. The results of SMP-105, which was derived from *M. bovis* BCG Tokyo 172, and those of CWS from BCG Pasteur were comparable except for several respects:

The first was that the molar ratio of arabinose against galactiose of SMP-105 was 2.1, compared with 2.8 of CWS from BCG Pasteur and 2.4 of cell walls of *Mycobacterium tuberculosis*.[35]

The second difference was that the molar ratios of alanine and glutamic acid against diaminopimeric acid of SMP-105

were 1.6 and 1.1, compared with 1.08 and 1.0 of CWS from BCG Pasteur, while those of glucosamine and muramic acid were similar. Such differences between SMP-105 from BCG Tokyo 172 and CWS from BCG Pasteur might be attributable to the variety of the composition of mycobacterial species as well as the difference and variability of analytical procedures.

Furthermore, it was reported[1] that rhamnose was not a constituent of CWS from BCG Pasteur, but in later papers,[7,35] it was found to be incorporated into the structure of cell walls of *M. tuberculosis*, *M. bovis*, and other mycobacterial species as well as *Nocardia*. In the current study, as rhamnose was detected at the constant level of 0.3 to 0.4% in multiple lots of SMP-105, which was approximately equivalent to the molar ratio 0.1 against diaminopimeric acid, it was considered to be one of the constituents. Meanwhile, glucose and mannose were detected in the relatively broad range from 0.4 to 0.7 and from 0.5 to 1.0, respectively, and they have never been reported to be constituents of cell walls of any mycobacterium. Therefore, it was difficult to consider that these two sugars should be incorporated into the structure of SMP-105. Regarding galactosamine, as it was assumed by Draper *et al.*[44] to be a minor component of cell walls of *M. tuberculosis*, *M. bovis* BCG (Danish strain), *M. leprae*, and other slow-growing mycobacteria, and it was actually detected in SMP-105 at the constant level of 0.5% or molar ratio of 0.1 against diaminopimeric acid, it might be one of the constituents. Regarding these sugars, further studies are needed to clarify the structure of SMP-105.

From amino acid analysis, non-constituent amino acids, including asparagine, valine, arginine, cysteine, glycine, threonine, isoleucine, leucine, phenylalanine and serine, were detected in lot no. S005 at the level of 0.2% for the first two and 0.1% for the others. Also, total contents of these non-constituent amino acids in this lot amounted to about 1.3%, and those in the other lots were 1.0 to 1.3%, suggesting that some proteins from the original bacterium might have resided in SMP-105, but they have not yet been identified.

In addition, methods for the determination of nucleic acid, trehaolse di-mycolate, and bacterial endotoxins, which might be contaminated in the original bacterium or arise during the preparation process, have preliminarily been investigated and these levels were determined to be very low, demonstrating that the prepared SMP-105 was of sufficient quality for use in research into its chemistry, bioactivity, and structure–activity relationship.

Conclusion

We established a preparation method of SMP-105 from *M. bovis* BCG Tokyo 172, and also developed accurate, precise, and reliable evaluation methods, based on earlier reports. Furthermore, we confirmed that SMP-105 is composed of a mycolic acid–arabinogalactan–peptidoglycan complex and that the levels of possible impurities were quite low. These results demonstrated that the SMP-105 as prepared was of sufficient quality for research into the chemistry, bioactivity, and structure–activity relationship of this CWS. These studies are now in progress and some interesting results, such as unique compositions of mycolic acids of SMP-105, are being obtained. These data and other results will be reported in our future papers.

Acknowledgements We would like to express our deep appreciation to Dr. Ichiro Azuma of Hokkaido Pharmaceutical University School of Pharmacy and Dr. Ikuya Yano of Japan BCG Laboratory for their kind and valuable discussion and advice. We would also thank Dr. Jiro Akimaru and Mr. Toyohiro Nishikawa of Dainippon Sumitomo Pharma Co., Ltd. for their technical efforts in the establishment of this preparation method, and also Ms. Tomoko Sugiyama for her technical assistance in method development.

References

1) Azuma I., Ribi E. E., Meyer T. J., Zbar B., *J. Natl. Cancer Inst.*, **52**, 95—101 (1974).
2) Meyer T. J., Ribi E. E., Azuma I., Zbar B., *J. Natl. Cancer Inst.*, **52**, 103—111 (1974).
3) Nakajima H., Kawasaki K., Oka Y., Tsuboi A., Kawakami M., Ikegami K., Hoshida Y., Fujiki F., Nakano A., Masuda T., Wu F., Taniguchi Y., Yoshihara S., Elisseeva O. A., Oji Y., Ogawa H., Azuma I., Kawase I., Aozasa K., Sugiyama H., *Cancer Immunol. Immunother.*, **53**, 617—624 (2004).
4) Akazawa T., Masuda H., Saeki Y., Matsumoto M., Takeda K., Tsujimura K., Kuzushima K., Takahashi T., Azuma I., Akira S., Toyoshima K., Seya T., *Cancer Res.*, **64**, 757—64 (2004).
5) Yoo Y. C., Hata K., Lee K. B., Azuma I., *Arch. Pharm. Res.*, **25**, 522—527 (2002).
6) Seya T., Matsumoto M., Tsuji S., Begum N. A., Nomura M., Azuma I., Hayashi A., Toyoshima K., *Arch. Immunol. Ther. Exp.*, **49** (Suppl. 1), S13—21 (2001).
7) McNeil M. R., Daffe M., Brennan P. J., *J. Biol. Chem.*, **266**, 13217—13223 (1991).
8) Brennan P. J., Nikaido H., *Annu. Rev. Biochem.*, **64**, 29—63 (1995).
9) McNeil M. R., "Genetics of Bacterial Polysaccharides", ed. by Goldberg J. B., CRC Press, Washington, D.C., 1999, pp. 207—223.
10) Brennan P. J., *Tuberculosis*, **83**, 91—97 (2003).
11) Yamamura Y., Azuma I., Taniyama T., Sugimura K., Hirao F., Tokuzen R., Okabe M., Nakahara W., Yasumoto K., Ohta M., *Ann. N.Y. Acad. Sci.*, **277**, 209—227 (1976).
12) Yamamura Y., Ogura T., Yoshimoto T., Nishikawa H., Sakatani M., *Gann*, **67**, 669—677 (1976).
13) Yasumoto K., Manabe H., Ueno M., Ohta M., Ueda H., *Gann*, **67**, 787—795 (1976).
14) Nishikawa H., Yasaki S., Yoshimoto T., Sakatani M., Itoh M., Masuno T., Namba M., Ogura T., Hirao F., Azuma I., Yamamura Y., *Gann*, **69**, 819—824 (1978).
15) Yamamura Y., Sakatani M., Ogura T., Azuma I., *Cancer*, **43**, 1314—1319 (1979).
16) Yasumoto K., Manabe H., Yanagawa E., Nagano N., Ueda H., Hirota N., Ohta M., Nomoto K., Azuma I., Yamamura Y., *Cancer Res.*, **39**, 3262—3267 (1979).
17) Hayashi A., Doi O., Azuma I., Toyoshima K., *Proc. Jpn. Acad.*, **74** (B), 50—55 (1998).
18) Tsuji S., Matsumoto M., Takeuchi O., Akira S., Azuma I., Hayashi A., Toyoshima K., Seya T., *Infect. Immun.*, **68**, 6883—6890 (2000).
19) Azuma I., Seya T., *Int. Immunopharmacol.*, **1**, 1249—1259 (2001).
20) Matsumoto M., Seya T., Kikkawa S., Tsuji S., Shida K., Nomura M., Kurita-Taniguchi M., Ohigashi H., Yokouchi H., Takami K., Hayashi A., Azuma I., Masaoka T., Kodama K., Toyoshima K., Higashiyama M., *Int. Immunopharmacol.*, **1**, 1559—1569 (2001).
21) Begum N. A., Ishii K., Kurita-Taniguchi M., Tanabe M., Kobayashi M., Moriwaki Y., Matsumoto M., Fukumori Y., Azuma I., Toyoshima K., Seya T., *Infect. Immun.*, **72**, 937—948 (2004).
22) Ishii K., Kurita-Taniguchi M., Aoki M., Kimura T., Kahiwazaki Y., Matsumoto M., Seya T., *Immunol. Lett.*, **98**, 280—290 (2005).
23) Minnikin D. E., Parlett J. H., Magnusson M., Ridell M., Lind A., *J. Gen Microbiol.*, **130**, 2733—2736 (1984).
24) Kaneda K., Naito S., Imaizumi S., Yano I., Mizuno S., Tomiyasu I., Baba T., Kusunose E., Kusunose M., *J. Clin. Microbiol.*, **24**, 1060—1070 (1986).
25) Kaneda K., Imaizumi S., Mizuno S., Baba T., Tsukamura M., Yano I., *J. Gen. Microbiol.*, **134**, 2213—2229 (1988).
26) Kaneda K., Imaizumi S., Yano I., *Microbiol. Immunol.*, **39**, 563—570 (1995).
27) Butler W. R., Jost K. C., Kilburn J. O., *J. Clin. Microbiol.*, **29**, 2468—2472 (1991).
28) Floyd M. M., Silcox V. A., Jones W. D., Butler W. R., Kilburn J. O., *J. Clin. Microbiol.*, **30**, 1327—1330 (1992).

29) Guthertz L. S., Lim S. D., Jang Y., Duffey P. S., *J. Clin. Microbiol.*, **31**, 1876—1881 (1993).
30) Hagen S. R., Thompson J. D., *J. Chromatogr. A*, **692**, 167—172 (1995).
31) Butler W. R., Guthertz L. S., *Clin. Microbiol. Rev.*, **14**, 704—726 (2001).
32) Misaki A., Seto N., Azuma I., *J. Biochem.* (Tokyo), **76**, 15—27 (1974).
33) Daffe M., Brennan P. J., McNeil M., *J. Biol. Chem.*, **265**, 6734—6743 (1990).
34) McNeil M., Daffe M., Brennan P. J., *J. Biol. Chem.*, **265**, 18200—18206 (1990).
35) Daffe M., McNeil M., Brennan P. J., *Carbohydr. Res.*, **249**, 383—398 (1993).
36) Besra G. S., Khoo K.-H., McNeil M. R., Dell A., Morris H. R., Brennan P. J., *Biochemistry*, **34**, 4257—4266 (1995).
37) Misaki A., Yukawa S., Tsuchiya K., Yamasaki T., *J. Biochem.* (Tokyo), **59**, 388—396 (1966).
38) Wietzerbin-Falszpan J., Das B. C., Azuma I., Adam A., Petit J. F., Lederer E., *Biochem. Biophys. Res. Commun.*, **40**, 57—63 (1970).
39) Wietzerbin-Falszpan J., Das B. C., Gros C., Petit J. F., *Eur. J. Biochem.*, **32**, 525—532 (1973).
40) Wietzerbin J., Das B. C., Petit J. F., Lederer E., Leyh-Bouille M., Ghuysen J. M., *Biochemistry*, **13**, 3471—3476 (1974).
41) Phiet P. H., Wietzerbin J., Zissman E., Petit J. F., Lederer E., *Infec. Immun.*, **13**, 677—681 (1976).
42) Lee R. E., Brennan P. J., Besra G. S., *Curr. Top. Microbiol. Immunol.*, **215**, 1—27 (1996).
43) Baba T., Natsuhara Y., Kaneda K., Yano I., *Cell. Mol. Life Sci.*, **53**, 227—232 (1997).
44) Draper P., Khoo K.-H., Chatterjee D., Dell A., Morris H. R., *Biochem. J.*, **327**, 519—525 (1997).