Acta Chemica Scandinavica B 33 (1979) 632—636

Comparison of Membrane Proteins from Lactic Streptococci by Gel Electrophoresis

M. PÄKKILÄ [a] and RAILI FORSÉN [b]

[a] Department of Biochemistry, University of Oulu, SF-90100 Oulu 10, Finland and [b] Public Health Laboratory, Kajaanintie 46 D, SF-90100 Oulu 10, Finland

Slime forming Group N *Streptococcus* strains isolated from the Finnish fermented milk "viili" and non-slime forming control strains were characterized by comparing electrophoretic patterns of the membrane proteins. Cells of the non-slime forming *S. cremoris* Hki I and II, *S. lactis* Hki I and II and *S. diacetilactis* Hki, the slime forming *S. cremoris* Biotypes A (T_5) and B (T_1), and reference organism *S. faecalis* KTL were harvested in the late logarithmic phase of culture growth. Membranes were isolated by sonic treatment, lysozyme digestion and osmotic shock followed by digestion with DNAse and RNAse and incubation in sodium dodecyl sulfate – phosphate solution. The membrane proteins were separated in 5 % polyacrylamide gels containing 0.1 % of sodium dodecyl sulfate (SDS).

The membrane proteins from non-slime forming and slime forming strains differ from each other in regard to the number of the polypeptides, the amount of corresponding proteins and the distribution of molecular weight. The number of bands in the electrophoretic patterns of slime forming strains was smaller (10) than in those of non-slime forming strains (13 – 14). The slowly moving band 21 was the first major band in slime forming strains. Three or four of the fastest bands, MW 15 000 – 41 200, were major bands in the non-slime forming strains. All but two or three bands in the slime forming strains were major bands. Proteins Nos. 17 – 21, MW 80 000 – 100 000, were common in non-slime forming strains and extremely rare in slime forming strains. *S. faecalis*, the reference organism from Group D, showed a group specific pattern where especially proteins with high molecular weight were missing.

Bacterial membranes provide a useful experimental system for investigating membrane functions. Biosynthesis of wall components together with other surface components of the procaryotic cell are important subjects in the investigation of membranes. Lactic streptococci – Group N streptococci – include strains with or without slime forming capacity, whose capsular and slime substances have a characteristic carbohydrate and protein composition.[1,2] Characterization of lactic streptococcus "species" and biotypes by comparing their membrane proteins makes our studies on differences in polypeptides in the biosynthesis of extracellular components more complete.[3] According to Machtiger and Fox [4] bacterial mutants can be divided into two classes according to the alteration in membrane proteins: (1) those with deficiencies in the activities of membrane associated enzymes and (2) those with increased or decreased amounts of protein as determined by SDS polyacrylamide gel electrophoresis (PAGE). In the present study a comparison between lactic streptococcus species and slime forming biotypes was made on this principle.

The aim of this study was (a) to develop an isolation method for membrane proteins from slime forming or non-slime forming lactic streptococci suitable for SDS-PAGE in tubes and (b) to compare the protein patterns and the amounts of different proteins found in slime forming and non-slime forming strains.

METHODS

Bacteria strains and growth conditions. Non-slime forming control lactic streptococci *Streptococcus cremoris* Hki I and II, *S. lactis* Hki I and II and *S. diacetilactis* Hki were obtained from the Laboratory of the Foundation for

0302-4369/79/090632-05$02.50

Chemical Research, Biochemical Institute, Helsinki. The slime forming strains were isolated from the formented milk product "viili". Their isolation and characteristics have been described earlier.[5] The strains chosen for the present work were: *S. cremoris* T_1 (Biotype B) and *S. cremoris* T_5 (Biotype A). A reference strain of Group D streptococci, *i.e. S. faecalis* KTL III, was obtained from a clinical specimen. Stock cultures were subcultured using Dextrose Broth (Difco Laboratories) as described earlier.[5]

Isolation of membranes. Cultures of bacteria were grown for 20 h in Dextrose Broth, and they were harvested in the late logarithmic phase.[5] Cell membranes were prepared by modifying the procedures described by Baird-Parker and Woodroffe,[6] Vorbeck and Marinetti [7] and Emdur *et al.*[8] followed by treatment with DNAase and RNAase as described by Salton and Freer.[9] A preceding ultrasonic treatment was calibrated for achievement of the most effective detaching of the extracellular slime and capsule material. Cells (1 g of wet weight) from 0.8 liter of culture were washed thrice with 0.05 M Na-K-phosphate buffer (pH 7.0), suspended in 5 ml of the same buffer and subjected to sonic treatment in an MSE (Measuring & Scientific Equipment, Ltd., England) 100-W sonic disintegrator (25 Kc/s) for 20 min, the temperature being kept below 10 °C. The mixture obtained was centrifuged at 6000 *g* for 15 min at 4 °C in an IEC Model B-20 centrifuge (International Equipment Co., Needham Hts., Mass., U.S.A.).

The supernatant containing capsule and slime substances was discarded. Cells were washed with 0.05 M Na-K-phosphate buffer (pH 7.0) and 20 mg was suspended in one ml of a solution consisting of 0.01 M Na-K-phosphate buffer (pH 7.0) and 0.6 M sucrose with 0.005 M $MgCl_2$ and 0.01 M NaCl. Lysozyme (0.1 mg/ml, E. Merck AG, Darmstadt, Germany) was added and the suspension was incubated at 37 °C for 30 min. The protoplasts were collected by centrifuging at 10 000 *g* for 15 min, suspended in distilled water at 4 °C and exposed to osmotic shock for 25 min. Crude membranes were obtained by centrifuging at 35 000 *g* for 20 min. 20 mg of membranes was suspended in 1 ml of a solution consisting of 0.01 M Na-K-phosphate buffer (pH 7.0), 0.005 M $MgCl_2$, and 0.01 M NaCl, and then subjected to enzymatic treatment with DNAase I (20 μg/ml, Sigma Chemical Company, St. Louis, Mo., U.S.A.) and RNAase (20 μg/ml, Calbiochem, Los Angeles, U.S.A.) at 37 °C for 15 min. The membrane fraction was collected by centrifuging at 35 000 *g* for 20 min and washed three times with 0.1 M NaCl. No intact cells were present in the membrane preparations when examined by gram stained smears. Slime substance contamination was scanty or negligible as determined by the double immunodiffusion technique [10] using antiserum against the slime substances.[3]

The protein content of membranes was examined using human serum albumin (Behringerwerke AG, Marburg-Lahn, Germany) as the standard.[11] An average yield of 415 mg of membrane material was obtained per isolation. Isolated membranes were used for experiments immediately or stored at −20 °C until required.

Polyacrylamide gel electrophoresis (PAGE). Preparation of protein solutions was performed as described by Weber and Osborn.[12] Samples were prepared by solubilizing the proteins from 2.5 mg of membrane substance in 1 ml of 0.01 M sodium phosphate (pH 7.0) containing 1 % SDS and 1 % 2-mercaptoethanol, followed by incubation at 37 °C for 3 h. After incubation the protein solution was dialyzed overnight against 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 % SDS and 0.1 % 2-mercaptoethanol. The reference protein, human serum albumin, was prepared in the same manner. From 100 to 150 μg of protein was applied per gel.

SDS-PAGE was performed in 5 % gels by modifying the procedure of Weber and Osborn [13] as described in our previous work.[3] Molecular weight groups of the electrophoretical protein bands were calculated as in an earlier work.[3] The migration of human serum albumin was used as reference, and three molecular weight groups were established: 15 000 to 38 000, 41 200 to 79 000 and 80 000 to 100 000.

Densitometric scanning and quantitative correlation of the amounts of different proteins in the protein patterns were made using a densitometer, Quick Scan Model 1020 (Helena Laboratories, Beamont, Texas, U.S.A.), and evaluating the peaks as follows: the highest absorbance level obtained at 550 nm is assigned to be 100 %; bands with absorbance from 50 to 100 % are called major bands; bands with absorbance up to 25 % are called minor bands; and bands between major and minor bands are called intermediate bands.

RESULTS

Electropherograms of membrane proteins from eight bacteria, *i.e.* three lactic streptococci species including five strains, two slime forming biotypes and one reference organism from Group D, are shown in Fig. 1. Protein patterns of *S. cremoris* Hki I and II are identical to each other (Fig. 1a) as are protein patterns of *S. lactis* Hki I and II (Fig. 1b). These protein patterns collected for comparison in Table 1 show many similarities. Bands Nos. 5 and 6 are alternative bands. In other respects differences are quantitative. The third control species, *S. diacetilactis*, is reminiscent of the above two species, but has additional species

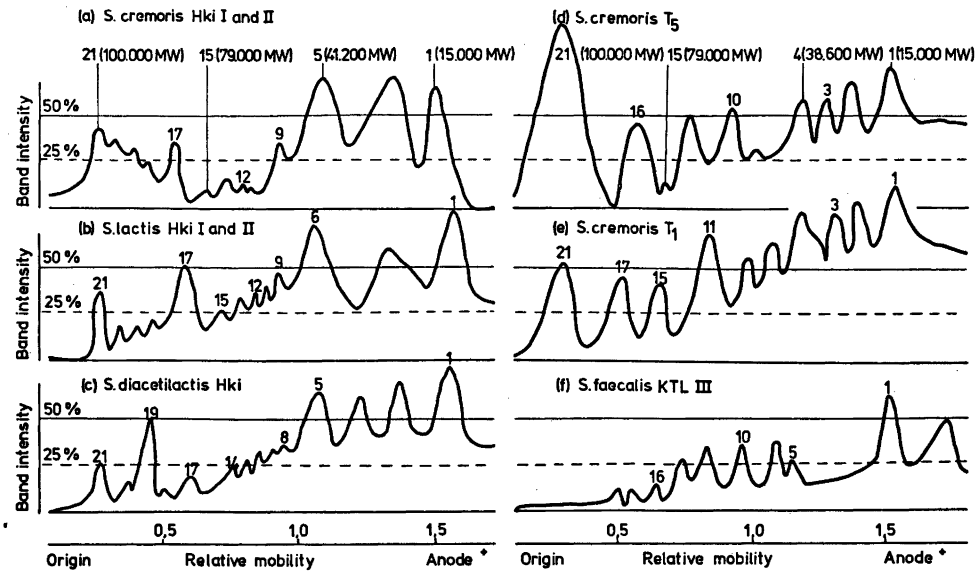

Fig. 1. Densitometric scans of polyacrylamide gels obtained after electrophoresis in the presence of sodium dodecyl sulfate, and Coomassie blue staining of the membrane proteins from streptococci strains. (a) *S. cremoris* Hki I and II; (b) *S. lactis* Hki I and II; (c) *S. diacetilactis* Hki; (d) *S. cremoris* T_5 (Biotype A); (e) *S. cremoris* T_1 (Biotype B) and (f) *S. faecalis* KTL III. Band intensity levels at 550 nm – 50 and 25 % – are marked. Some bands are numbered for guidance. Apparent MW ranges 15 000, 38 600, 41 200, 79 000 and 100 000 are marked. Relative mobility, human serum albumin as reference (marked 1.0).

specific proteins, *i.e.* bands Nos. 8 and 14 (Fig. 1c). Moreover, band No. 15 is missing only in this organism.

Slime forming biotypes A (*S. cremoris* T_5) and B (*S. cremoris* T_1) show characteristic protein bands in all three molecular weight groups (Fig. 1d and e). *S. cremoris* T_1 (Biotype B) has similar characteristics with *S. lactis* in the differentiation tests, *i.e.* growth at pH 9.2.[5] These organisms have protein band No. 6 in common, and it is a major band in both cases. The reference organism, *S. faecalis*, Group D, shows an additional band with the smallest molecular weight, and especially the proteins with MW from 80 000 to 100 000 differ from those of Group N organisms (Fig. 1f).

The occurrence and amounts of proteins are compared in Table 1. The total number of protein bands, 10, is smaller in slime forming strains, but the proportion of major bands is distinctly higher, *i.e.* 70 – 80 % of all protein bands. Non-slime forming strains show from 13 to 14 protein bands of which only 23 to 35.7 % are major bands. Intermediate bands dominate (38.5 – 46 %) and the occurrence of minor bands (21.5 – 38.5 % is noticeable.

DISCUSSION

The results obtained from lactic streptococci membrane proteins are compared in the same manner as by Machtiger and Fox,[4] noting that some proteins are missing and the amounts of some proteins are increased or decreased. It was observed that: (i) bands Nos. 3, 4, 7, 10 and 16 were missing in all non-slime forming strains but band No. 4 was present in *S. diacetilactis* Hki, (ii) bands Nos. 5, 8, 12, 14, 18, 19 and 20 were not present in slime forming strains, (iii) band No. 14 was absent in all but the *S. diacetilactis* control strain, (iv) band No. 17 was a major band only in *S. lactis* non-slime forming strains and (v) bands Nos. 9, 11, 13 and 21 of slime forming strains were major bands while corresponding bands in non-slime

Table 1. Comparison of the SDS-PAGE protein bands of non-slime forming strains *S. cremoris*, *S. lactis* and *S. diacetilactis* and slime forming strains *S. cremoris* T_5 (Biotype A) and T_1 (Biotype B) regarding the major (+ + +), intermediate (+ +) and minor (+) bands and molecular weights as described in the "Polyacrylamide gel electrophoresis (PAGE)" section.

Band No.	*S. cremoris* Hki I and II	*S. lactis* Hki I and II	*S. diaceti-lactis* Hki	*S. cremoris* T_5 (A)	*S. cremoris* T_1 (B)	MW $\times 10^3$
1	+ + +	+ + +	+ + +	+ + +	+ + +	15.0
2	+ + +	+ + +	+ + +	+ + +	+ + +	
3				+ + +	+ + +	
4			+ + +	+ + +	+ + +	38.6
5	+ + +		+ + +			41.2
6		+ + +			+ + +	
7				+ +		
8			+ +			
9	+ +	+ +	+ +		+ + +	
10				+ + +		
11	+	+ +	+ +		+ + +	
12	+	+ +	+ +			
13	+	+ +		+ + +		
14			+ +			
15	+	+ +		+	+ +	79.0
16				+ +		80.0
17	+ +	+ + +	+		+ +	
18	+	+	+			
19	+ +	+	+ + +			
20	+ +	+	+			
21	+ +	+ +	+ +	+ + +	+ + +	100.0
Total number	13	13	14	10	10	
Major	3 (23 %)	4 (31 %)	5 (35.7 %)	7 (70 %)	8 (80 %)	
Interm.	5 (38.5 %)	6 (46 %)	6 (42.8 %)	2 (20 %)	2 (20 %)	
Minor	5 (38.5 %)	3 (23 %)	3 (21.5 %)	1 (10 %)	–	

forming strains were minor or intermediate bands.

The absence of seven definite protein bands in slime forming strains is probably an important finding. The earlier studies [3] have also shown that slime forming parent strains exhibited smaller number of protein bands and had quantitative differences as compared with their altered non-slime forming variants. This observation is in agreement with the hypothesis that the biosynthesis of slime substance is due to the incomplete synthesis of bacterial wall polymers.[13] In addition, the predominance of major bands in connection with slime forming capacity further resolves the differences. However, two major protein bands of the particulate fraction [3] – major band I, approximate MW 60 000 and major band II, approximate MW from 45 000 to 50 000 – were not present in plasma membrane preparations. Earlier studies [14] showed that the amino acid and vitamin requirements are more complicated in slime forming biotypes than in non-slime forming strains. This can result from deficiences in transport systems. Further characterization of differences regarding peripheral and integral plasma membrane proteins from lactic streptococci is going on with the intention to elucidate the function of membrane proteins. According to Machtiger and Fox [4] membrane protein mutants with deficiences in activities of membrane bound enzymes have been useful in biochemical studies of transport systems.

By comparing the MW distribution of electrophoretical protein bands, it can be observed that in slime forming strains only 20 %

of bands have MW ≥ 80 000, while non-slime forming strains have 38 % of their protein bands in the highest MW group (80 000 – 100 000). It can be concluded that the MW of membrane proteins is relatively smaller in slime forming than in non-slime forming strains. Earlier studies [3] concerning cell proteins of the particulate fraction indicated higher total number of polypeptides (26), from which only bands Nos. 23 to 26 were in the highest MW group of 80 000 – 100 000. Two of the bands appeared in most lactic streptococci strains. They represented on an average 15 % of all protein bands in the pattern. Differences in membrane proteins between slime forming and non-slime forming lactic streptococci regarding the proteins with MW ≥ 80 000 are evident also in comparing the MW distribution in particular preparations.

The results show that the membranes from non-slime forming and slime forming strains differ from each other in regard to the number of polypeptides, the amounts of corresponding proteins and the distribution of molecular weight. Markovitz [15] assumed that M antigen (colanic acid) polymerase(s) is located on the inner or outer membrane of *E. coli*, and a mutation that alters the structure of the membranes could activate or inactivate the function of polymerases by disturbing the organization of the membrane. Differences in the membrane proteins of lactic streptococci can analogously be explained by alteration of the structure of the membranes and activation or inactivation of enzymes participating in slime antigen biosynthesis. Further results concerning characterization of membrane proteins from non-slime forming and slime forming lactic streptococci obtained by isoelectric focusing (IEF) will be published later.

Acknowledgements. We are indebted to VALIO Finnish Co-operative Dairies' Association for financial support of this research project.

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Received May 28, 1979.