# CHARACTERIZATION AND BIOTECHNOLOGICAL APPLICATION OF EUBACTERIAL GLYCOPROTEINS

P. Messner, S. Küpcü, M. Sára, D. Pum and U.B. Sleytr

Zentrum für Ultrastrukturforschung and Ludwig Boltzmann-Institut für Ultrastrukturforschung, Universität für Bodenkultur, A-1180 Wien, Austria.

## SUMMARY

This contribution summarizes the structural and chemical characterization of crystalline bacterial cell surface layers (S-layers) and gives an overview on its biotechnological application potential. These supramolecular structures represent ideal model systems for learning how nature has accomplished production and maintainance of structures at the nanometer level. Crystalline S-layers consist of regularly arranged protein or glycoprotein molecules and exhibit pores of identical shape and size in the two-dimensional lattice. This makes them particularly useful for the production of a completely new type of ultrafiltration membrane. Chemical modification allows for specific adaptations of the S-layer, either for ultrafiltration purposes or for coating with monolayers of specific molecules such as enzymes, antigens or haptens.

These studies have clearly shown that the protein or glycoprotein molecules are of great interest as patterning elements for nanometer technologies and in the development of biosensors. S-layers can also be used as supports for Langmuir-Blodgett films or as carriers for immunomodulating agents in the development of a new generation of vaccines.

## 1. INTRODUCTION

Crystalline bacterial surface layers (S-layers) are the outermost cell envelope component of almost every taxonomic group of walled eubacteria and archaebacteria (1,2). While the existence of archaebacterial S-layer glycoproteins was known since the discovery of archaebacteria (3,4), the occurrence of covalently linked carbohydrates on eubacteria has been questioned for a long time. In the course of the structural characterization of the S-layers of taxonomically closely related thermophilic eubacteria, chemical analyses have provided the first evidence for the existence of glycosylated surface layer arrays in these strains (5). Proteolytic degradation and chemical characterization of purified S-layers of several thermophilic and mesophilic *Bacillaceae* have indicated that frequently glycosylated S-layer proteins can be found in this family (see Table 1).

At the present time there is a growing interest in the miniaturization of optical, chemical and electronic devices (6,7). Because of their crystalline arrangement, and thus highly ordered localization of functional groups, S-layers have been found particularly useful for specific aspects of biotechnological applications.

## 2. ULTRASTRUCTURE OF S-LAYERS

S-layers can be considered the simplest biological membranes developed during evolution. Freeze-etched preparations of whole bacterial cells have shown that S-layer lattices can exhibit oblique (p2), square (p4) or hexagonal (p6) symmetry. The centre-to-centre spacing of the morphological units, which is a strain-specific feature, can range from 5 to 35 nm (2).

## 3. ISOLATION AND CHEMICAL CHARACTERIZATION

In eubacterial S-layers the individual subunits are linked to each other and to the underlying cell envelope layer by non-covalent bonds (8). Usually, they can be isolated by extraction with chaotropic agents (9). During removal of the disrupting agent the S-layer subunits frequently reassemble in a self assembly process into lattices identical to those observed on the intact cells. Thus, the information for the formation of two-dimensional protein or glycoprotein crystals resides in the amino acid sequence of the S-layer protein itself.

S-layers are composed of a single protein or glycoprotein species with molecular weights ranging from 40,000 to 200,000 (2). The S-layer protein contains a high amount of acidic and hydrophobic amino acids (1), among the basic amino acids lysine predominates. With S-layers it was shown for the first time that frequently eubacteria and archaebacteria are capable of glycosylating proteins (3,4,11). Degradation of

TABLE 1. GLYCAN STRUCTURES OF EUBACTERIAL S-LAYER GLYCOPROTEINS[a]

*Bacillus stearothermophilus* NRS 2004/3a

(-2αLRhap1-2αLRhap1-3βLRhap1-)
and
(-4βManpA2,3diNAc1-3αGlcpNAc1-4βManpA2,3diNAc1-6αGlcp1-)

*Clostridium thermohydrosulfuricum* L111-69

(-4αDManp1-3αLRhap1-)

*Clostridium thermosaccharolyticum* D120-70

(-3[βDGlcp1-6]βDManp1-4αLRhap1-3αDGlcp1-4[αDGalp1-2]αLRhap1-)
and
(-4βDGlcpNAc1-3[(αDGalp)$_{0.5}$1-4]βDManpNAc1-)

*Bacillus alvei* CCM 2051

(-3βDGalp1-4[αDGlcp1-6]βDManpNAc1-)

*Clostridium symbiosum* HB25

(-6αDManpNAc1-4βDGalpNAc1-3αDBacpNAc1-4αDGalpNAc1-$PO_3H$-)

[a]For review see reference 12

glycopeptide I of *Bacillus stearothermophilus* has shown that the glycan chain is linked by an N-glycosidic linkage via rhamnose to the amido nitrogen of an asparagine residue (11). On a *Clostridium* strain we have observed O-glycosidically linked carbohydrate chains (unpublished results). In eubacteria the type of linkage between the polypeptide chain and the carbohydrate residue is frequently different from those detected in eukaryotic glycoproteins. So far, the structures of the carbohydrate chains of the S-layer glycoproteins of several thermophilic and mesophilic *Bacillaceae* have been elucidated by a combination of degradation experiments and $^1$H- and $^{13}$C nuclear magnetic resonance measurements (12 and Table 1).

## 4. S-LAYERS AS NATURE-TAILORED ULTRAFILTRATION MEMBRANES WITH WELL DEFINED PROPERTIES

Since S-layers represent the outermost cell envelope component, they must possess pores large enough for enabling the passage of nutrients and the exit of exoproteins and metabolic degradation products. Due to the regular arrangement of subunits within this two-dimensional protein crystal, the pores can be expected to show a well defined size and morphology (13).

From permeability and electron microscopy studies it has been demonstrated that the "functional pore sizes" of S-layers range from ca. 4 to 5 nm (14,15). The observation that S-layers function as isoporous molecular sieves led to the conclusion that they may be suitable for the development of a completely new type of ultrafiltration membrane (Sleytr and Sára, patents pending and granted). Moreover, since S-layers are two-dimensional arrays of identical protein or glycoprotein subunits, it is evident that functional groups on the polypeptide chain and/or on the carbohydrate moiety such as carboxyl, amino or hydroxyl groups, must be presented on each protomer in an identical position and orientation.

For rejection curve determination of S-layer ultrafiltration membranes (SUM) globular test proteins with Mw. ranging from 17,000 to 67,000 were applied. These studies revealed that SUM exhibit steep rejection curves in general between Mw. 30,000 and 45,000. Stability tests of SUM indicated that the membranes are resistant to a broad spectrum of organic solvents and diluted acids and bases (13,16,17).

## 5. SURFACE PROPERTIES

The cell surface of S-layer carrying bacteria does not exhibit a net negative charge, which on the other hand is typical for S-layer deficient organisms (1,18,19). This was confirmed by labeling native cells with polycationized ferritin. Chemical modification studies of S-layers have indicated an intramolecular neutralization of carboxyl groups by surface-located free amino groups (20,21). Since cross-linking of the S-layer protein with glutaraldehyde involves free amino groups, free carboxyl groups remaining on the S-layer surface are responsible for a net negative surface charge in cross-linked S-layers. With some glycosylated S-layers it was found that carboxyl groups can also originate from covalently attached carbohydrate chains (22).

All interactions between the S-layer surface and materials in the ambient environment are strongly determined by the physicochemical properties of the carbohydrate residues, which represent the first contact region (12,22). Since the surface-located carbohydrate chains possess closely arranged hydroxyl groups, which can be chemically modified, labeling with polycationized ferritin is possible. The PCF molecules form a densly packed "superlattice" on the surface of the S-layer lattice.

## 6. S-LAYERS AS SUPPORT FOR COVALENT ATTACHMENT OF MACROMOLECULES

As already described in section 5 both carboxyl and hydroxyl groups can be activated (Fig. 1). Depending on the size of the macromolecules (e.g. enzymes like glucose oxidase, Mw. 150,000) dense packing of foreign protein can be obtained at each morphological unit of the S-layer (23). Such a high binding capacity has never been observed with synthetic polymers. The immobilized enzymes also retain considerable portions of their original activity (23).

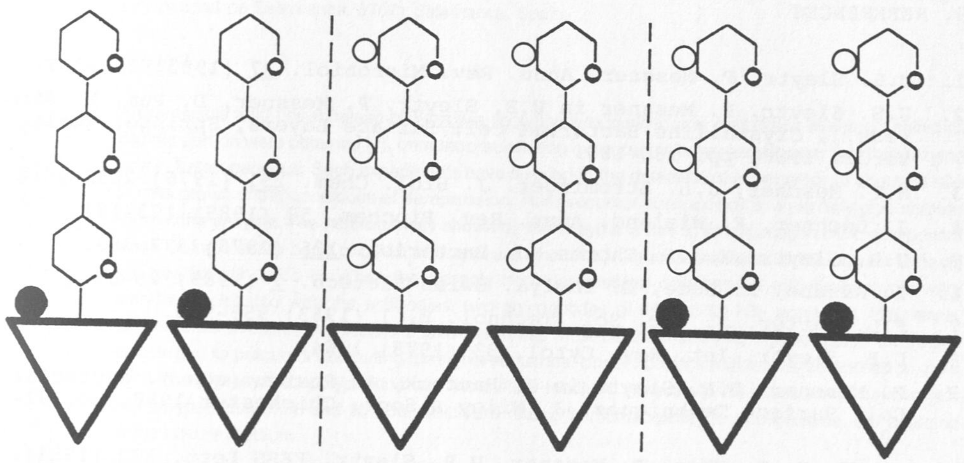

Figure 1. Schematic drawing illustrating the immobilization potential of S-layer glycoproteins. The ligands (○,●) can be linked either to the protein moiety (▽), or to the glycan chains (-⬡-), or to both of them.

## 7. S-LAYERS AS CARRIER FOR IMMUNOLOGICALLY ACTIVE MOLECULES

The functional groups of the S-layers can also be used for the immobilization of antigens or haptens, such as carbohydrate capsules of pathogenic bacteria or tumor-associated carbohydrates. First analyses of the immunological response show an increased carbohydrate specific T-cell response, which could not be obtained with common carrier proteins (e.g. bovine serum albumin or keyhole limpet hemocyanin) (submitted for publication).

## 8. CONCLUDING REMARKS

The unique feature of S-layers is their crystalline structure and consequently their isoporosity. This represents the basis for all considerations concerning technical applications. From the limited data available it is now quite obvious that two-dimensional S-layer arrays have a great biotechnological application potential.

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