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Large-scale production and homogenous purification of long chain polysialic acids from *E. coli* K1

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ABSTRACT

The study of new biomaterials is the objective of many current research projects in biotechnological medicine. A promising scaffold material for the application in tissue engineering or other biomedical applications is polysialic acid (polySia), a homopolymer of α2,8-linked sialic acid residues, which represents a posttranslational modification of the neural cell adhesion molecule and occurs in all vertebrate species. Some neuroinvasive bacteria like, e.g. *Escherichia coli* K1 (*E. coli* K1) use polySia as capsular polysaccharide. In this latter case long polySia chains with a degree of polymerization of >200 are linked to lipid anchors. Since in vertebrates no polySia degrading enzymes exist, the molecule has a long half-life in the organism, but degradation can be induced by the use of endosialidases, bacteriophage-derived enzymes with pronounced specificity for polySia.

In this work a biotechnological process for the production of bacterial polysialic acid is presented. The process includes the development of a multiple fed-batch cultivation of the *E. coli* K1 strain and a complete downstream strategy of polySia. A controlled feed of substrate at low concentrations resulted in an increase of the carbon yield ($C_{product}/C_{substrate}$) from 2.2 to 6.6%. The downstream process was optimized towards purification of long polySia chains. Using a series of adjusted precipitation steps an almost complete depletion of contaminating proteins was achieved. The whole process yielded 1–2 g polySia from a 10-l bacterial culture with a purity of 95–99%. Further product analysis demonstrated maximum chain length of >130 for the final product.

1. Introduction

5-*N*-Acetylneuraminic acid (Neu5Ac) is 1 out of >50 naturally occurring derivatives in the family of sialic acids (Sia). These 9-carbon α-keto acid sugars form the non-reducing end in a wide variety of oligosaccharide chains linked to proteins and lipids. Sialic acids are abundant in the animal kingdom. They are not found in plants and arthropods, but in certain types of pathogenic bacteria (Angata and Varki, 2002). Due to their "outermost" location sialic acids are implicated in a variety of vital biological processes such as embryogenesis, neural cell growth and differentiation. In higher vertebrates, loss of sialic acid from the cell surface indicates biological aging. While Neu5Ac is the major Sia derivative found in mammals and pathogenic bacteria, other derivatives can be predominant in other species. The structures of four major Sia derivatives found in nature are shown in Fig. 1a.

In some neuroinvasive bacteria and in higher vertebrates an unusual polymerization product of Neu5Ac, the so-called polysialic acid (polySia), is produced. This homopolymer of α2,8-glycosidically linked Neu5Ac residues (see Fig. 1b) provides in higher vertebrates (including mammals) a posttranslational modification of the neural cell adhesion molecule. PolySia is expressed during all steps of embryonic development with maximal levels in the perinatal phase. The polymer is concentrated to the nervous

Abbreviations: a.c., acetate; b.l.d., below limit of detection; BPB, bromphenol blue; BCP, bromcresol purple; CA, colominic acid; cetavlon, cetyltrimethyl ammonium bromide; DMB, 1,2-diamino-4,5-methylenedioxybenzene; DO, dissolved oxygen; DP, degree of polymerization; *E. coli*, *Escherichia coli*; K1-polySia, polysialic acid isolated from E.coli K1; Kdn, 5-deamino-3,5-dideoxyneuraminic acid; K_M, Monod factor; μ, growth rate factor; MWCO, molecular weight cut-off; n.c., not calculated; Neu5Ac, 5-*N*-acetylneuraminic acid; Neu5Gc, 5-*N*-glycolylneuraminic acid; OD, optical density; P, product; PEG, polyethyleneglycol; PhR, phenol red; polySia, polysialic acid; q_P, production rate factor; S, substrate; Sia, sialic acid; SEC, size exclusion chromatography; TB, tryptane blue; TBA, thiobarbituric acid; $\dot{V}_{feed}$, feed rate; V_R, medium volume; X, biomass; XC, xylene cyanol; $Y_{a/b}$, yield factor.

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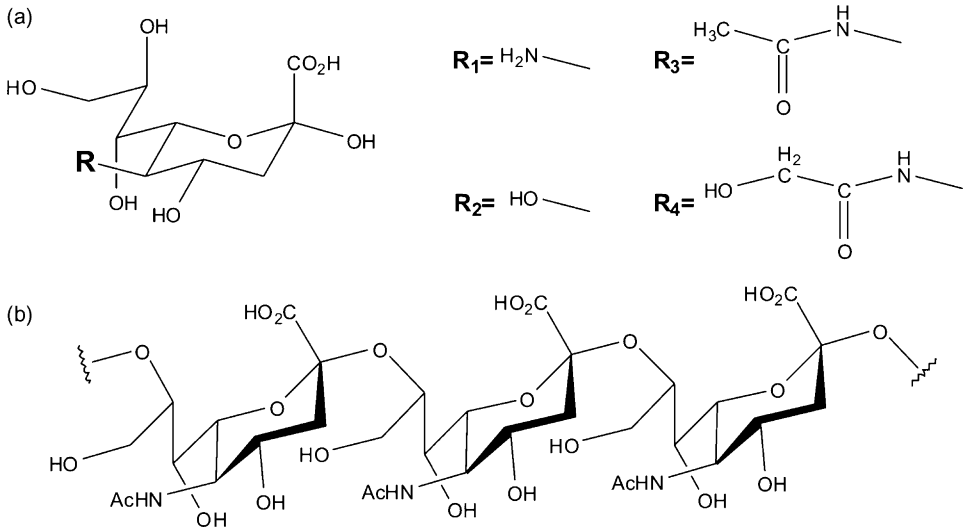

Fig. 1. (a) Neuraminic acid (R_1) is a 9-carbon sugar with a carboxylate function at position one. Major derivatives found in nature are 5-deamino-3,5-dideoxyneuraminic acid (Kdn, R_2), 5-*N*-acetylneuraminic acid (Neu5Ac, R_3) and 5-*N*-glycolylneuraminic acid (Neu5Gc, R_4); (b) Structure of the homopolymer of α2,8-linked 5-*N*-acetylneuraminic acid (colominic acid). The figure has been adapted from Muhlenhoff et al. (1998).

system and known to be involved in a multitude of plastic processes such as neuronal cell migration, neurite outgrowth, axonal pathfinding and synapse formation (Kleene and Schachner, 2004). In bacterial pathogens polySia is an important virulence factor because the highly hydrated polysaccharide covers bacterial specific antigens. By this molecular mimicry bacteria are protected against the host immune system. *Escherichia coli* serotype K1, a major cause of bacterial meningitides in neonatants (Bliss and Silver, 1996) uses this patho-mechanism. In the classification of bacterial capsules the K1-antigen belongs to the group 2 capsules (Whitfield, 2006). Investigations in mice have shown that polySia can be regarded as a kind of growth support for nerve cells in the developing brain. Consequently, genetic interference with polySia synthesis in mice caused a lethal phenotype, characterized by dramatic aberrations in the central nervous system (Weinhold et al., 2005; Angata et al., 2006). In contrast, most recent studies demonstrate that reactivation of polySia biosynthesis in the adult brain induces and supports neuronal regeneration (El Maarouf et al., 2005, 2006). The beneficial biological functions together with the fact that this bio-identical material provides interesting chemical features for modifications, functional decorations, and, based on the observed tendency to form self-aggregates, for the formation of solids, led us to assume, that polySia should be a suitable scaffold material in biomedical applications (Bruns et al., 2007; Stark et al., 2008).

To enable comprehensive testing of this hypothesis the provision of large amounts of homogenous and highly purified material is prerequisite. Protocols exist for the isolation of polySia from encapsulated bacteria, mainly *E. coli* K1. However, previously described methods were just working in a small scale and did not focus on the production of long and lipid-anchor-free product, but concentrated on the isolation of colominic acid (Uchida et al., 1973; Rodriguez-Aparicio et al., 1988), a hydrolysate of capsular polySia with a medial polymer length between 30 and 60 units (Mcguire and Binkley, 1964). Moreover, considerable investments have been made in optimizing culture conditions towards increased capsule synthesis (Rodriguez-Aparicio et al., 1988). In these studies, it has been shown that production of polySia in bacteria is thermo-regulated (Orskov et al., 1984; Puente-Polledo et al., 1998; Whitfield, 2006) and depended on pH-value and aeration of the growth media (Rodriguez-Aparicio et al., 1988).

The production process described in this study was optimized with regard to productivity in polySia biosynthesis by controlled process conditions (Surribas et al., 2006) and purity of the final product. For this purpose a reproducible and controllable fed-batch cultivation of *E. coli* K1 bacteria with a high product-yield was developed and coupled with an effective process monitoring and downstream process especially for highly polymerized polySia (Scheper et al., 1984, 1987; Marose et al., 1998, 1999).

2. Materials and methods

2.1. Material

2.1.1. Microorganism and chemicals

The wild type strain *E. coli* B2032/82 serotype K1 is an original clinical isolate. The bacteria were grown in a complex medium at 37 °C for 10 h. Stock cultures were prepared with 50% glycerol and stored at −80 °C.

2.1.2. Chemicals and growth media

All bulk chemicals were purchased from Sigma–Aldrich, Taufkirchen, Germany. Deionized water was prepared with ARIUM from Sartorius Stedim Biotech, Göttingen, Germany.

During the experiments two different kinds of media were applied: a complex medium consisting of yeast extract ($10\,g\,l^{-1}$) and tryptone ($10\,g\,l^{-1}$) and a basic defined medium containing NaCl ($1.2\,g\,l^{-1}$), K_2SO_4 ($1.1\,g\,l^{-1}$), $CaCl_2$ ($13\,mg\,l^{-1}$), $MgSO_4{\cdot}7H_2O$ ($0.15\,g\,l^{-1}$), $FeSO_4{\cdot}7H_2O$ ($1\,mg\,l^{-1}$), $CuSO_4{\cdot}5H_2O$ ($1\,mg\,l^{-1}$), K_2HPO_4 ($6.67\,g\,l^{-1}$), KH_2PO_4 ($0.25\,g\,l^{-1}$) (Rodriguez-Aparicio et al., 1988). In addition to the defined medium, different substrates (glucose, xylose, sorbitol, mannose) and nitrogen (N)-sources ($(NH_4)_2SO_4$, L-proline, glutamate) were added in varying concentrations.

2.2. Cultivation of bacteria

2.2.1. Shaking flask cultivations and preculture

All cultivations were performed in 500 ml shaking flasks with 100 ml medium. The bacteria were incubated at 37 °C and $120\,min^{-1}$. The cell-suspension used as inoculum for all bioreactor cultivations was obtained by growing the bacteria in