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CARBOHYDRATE RESEARCH

# Characterization of cytosolic sialidase from Chinese hamster ovary cells|qlPart I: Cloning and expression of soluble sialidase in *Escherichia coli*

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**Abstract**

The cDNA of Chinese hamster ovary (CHO) cell cytosolic sialidase was amplified by RT-PCR and cloned into the pGEX-2T plasmid vector encoding for glutathione S-transferase (GST). Screening revealed transformed *Escherichia coli* clones with the constructed plasmid encoding the CHO cell sialidase sequence. After isopropyl-β-D-thiogalactopyranoside (IPTG) induction, SDS-PAGE of the total protein extracts revealed a new protein of about 70 kDa, correlating with the molecular weight of a fusion protein composed of the GST (26 kDa) and the cloned cytosolic CHO cell sialidase (43 kDa). A soluble fusion protein was purified from sonified *E. coli* homogenates by one-step affinity chromatography on Glutathione Sepharose 4B, which showed sialidase activity towards 4-methyl-umbelliferyl-α-D-*N*-acetylneuraminic acid (MUF-Neu5Ac) substrate. Induction of cells with 0.1, 0.5, and 1.0 mM IPTG revealed highest total protein amounts after induction with 1.0 mM IPTG, but highest specific activity for affinity chromatography purified eluates from cultures induced with 0.1 mM IPTG. Therefore, large scale production was performed by inducing cells during exponential growth in a 25 L bioreactor for 3 h with 0.1 mM IPTG after chilling the cell suspension to 25°C. The amount of 26.46 mg of 40-fold purified GST-sialidase with a specific activity of 0.999 U/mg protein was obtained from crude protein extracts by one-step affinity chromatography. 2-Deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en) and Neu5Ac were competitive inhibitors for the sialidase, the former being the more effective one using MUF-Neu5Ac as the substrate. The cytosolic sialidase is capable of desialylating a wide spectrum of different types of gangliosides using a thin-layer chromatography overlay kinetic assay without detergents. This is the subject of the accompanying paper (Müthing, J.; Burg, M. *Carbohydr. Res.* **2001**, *330*, 347–356). © 2001 Elsevier Science Ltd. All rights reserved.

*Keywords:* Affinity chromatography purification; GST-sialidase; Neuraminidase; pGEX-2T plasmid vector

## 1. Introduction

Sialidases (neuraminidases, *N*-acylneuraminosyl glycohydrolases, EC 3.2.1.18) catalyze the hydrolytic removal of sialic acid from sialoglycoconjugates (glycoproteins, polysaccharides, gangliosides). In microorganisms, such as viruses[1–3] and bacteria[4] sialidases are thought to be important in pathogenicity and nutrition.[5] Mammalian sialidase activity is localized in the plasma membrane, lysosomes, Golgi apparatus and the cytosol.[6] Plasma membrane sialidases of different cell types

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play an important role in biochemical processes such as growth control and differentiation,[7,8] developmental modelling of myelin,[9] T cell activation[10] and immune cell interactions.[11,12] A soluble sialidase has been purified from the cytosol of rat liver cells to apparent homogeneity and its properties have been studied in detail.[13] Specific anti-sialidase polyclonal antibodies[14] were then used for immunological discrimination and subcellular localization in rat tissues. Cytosolic sialidase was essentially localized in the cytosolic compartment of various cell types.[15] An extracellular sialidase, which apparently originates from the cytosol of the cells and is released to the cell culture supernatant as a result of damage to the cellular membrane, was identified as responsible for the desialylation of recombinant glycoproteins produced by Chinese hamster ovary (CHO) cells.[16–18] Due to its low abundance in all tissues and cell types, the cDNA encoding for this protein has been isolated and used for the expression of an enzymatically active protein in insect cells.[19] In order to further characterize this enzyme, and because of its low amount in CHO cells, we amplified the cDNA of CHO cell cytosolic sialidase by RT-PCR and cloned it into the pGEX-2T plasmid vector encoding for glutathione S-transferase (GST). The GST-sialidase fusion protein was produced on mg scale and purified from *Escherichia coli* homogenates by one-step affinity chromatography in a soluble and active form. The detailed substrate specificity was elucidated with certain gangliosides by use of a thin-layer chromatography overlay kinetic assay and will be presented in the accompanying paper.[20]

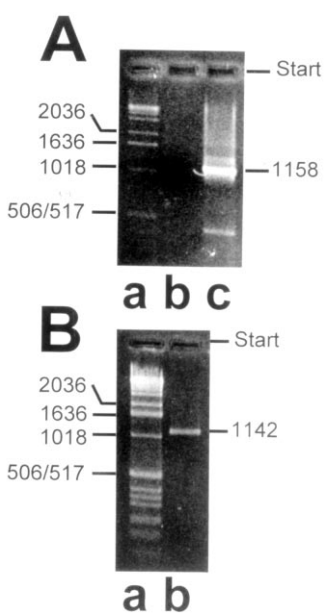


Fig. 1. Agarose gel electrophoresis of RT-PCR products with CHO cell RNA as template (A) and BamHI–EcoRI digest fragment of pMBU6-2 (B). (A) Lane a, 0.25 μg 1 kb-ladder marker X; lane b, control without template; lane c, RT-PCR product of CHO cell RNA. (B) Lane a, 1.0 μg 1 kb-ladder marker X; lane b, purified BamHI–EcoRI sialidase cDNA fragment. DNA was stained with ethidium bromide.

## 2. Results

*Preparation of a cDNA encoding CHO cell cytosolic sialidase by RT-PCR.*—An 1158 bp cDNA fragment of the cytosolic sialidase was obtained by RT-PCR amplification of total RNA from CHO cells using primers complementary to the sialidase sequence [19] (Fig. 1(A), lane c). The 5′ ends of the primers were extended by introducing specific restriction sites. An upstream BamHI site was introduced at the 5′ end and a downstream EcoRI site at the 3′ end of the sialidase cDNA. Taq polymerase activity adds single deoxyadenosines to the 3′ ends of all duplex molecules provided by PCR. The single deoxyadenosine residues were used to achieve direct ligation of the PCR amplification products into the pCR 2.1 vector, which contains single 3′ deoxythymidine residues. The amplified sialidase cDNA was subcloned into the pCR 2.1 vector to eliminate enzymatic modifications. The pMBU6-2 plasmid isolated from the subclone 6-2 harboured the sialidase cDNA within the pCR 2.1 vector. BamHI–EcoRI cleavage and isolation of the cDNA insert from the pMBU6-2 yielded a 1142 bp BamHI–EcoRI fragment encoding for the cytosolic CHO cell sialidase (Fig. 1(B), lane b).

*Cloning of the sialidase cDNA fragment into the pGEX-2T expression vector.*—The isolated BamHI–EcoRI fragment was in-frame ligated into the multiple cloning region of the predigested GST expression vector pGEX-2T (Fig. 2). Twenty clones, isolated from the pool of recombinants, were restriction mapped with BamHI, EcoRI, and PstI. Seven independent clones were found to contain the 1142 bp

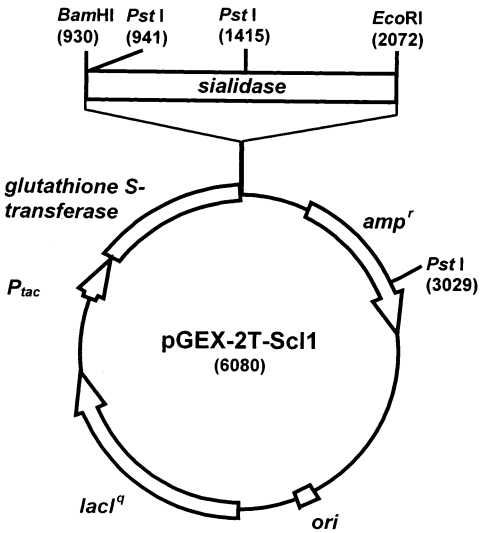


Fig. 2. Map of the glutathione S-transferase fusion vector with the BamHI–EcoRI CHO cell sialidase cDNA fragment insert. The 1142 bp cDNA from pMBU6-2 was cloned between BamHI and EcoRI sites of expression vector pGEX-2T resulting in plasmid pGEX-2T-Scl1 (Scl = sialidase clone).

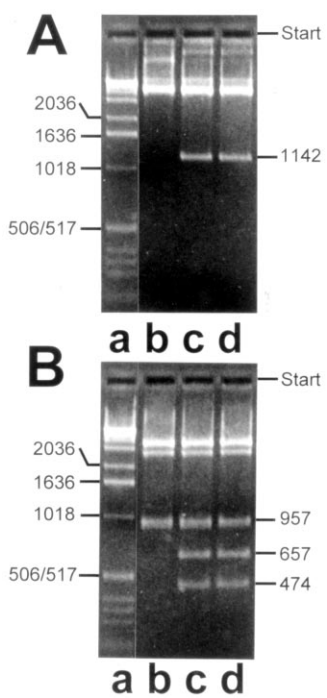


Fig. 3. Agarose gel electrophoresis of endonuclease digest of pGEX-2T-Scl1 and pGEX-2T-Scl2 replicated in *E. coli* M15 pREP4 transformands. (A) BamHI–EcoRI and (B) BamHI–EcoRI-PstI digest. Lanes a, 0.5 μg 1 kb-ladder marker X; lanes b, control without insert; lanes c, pGEX-2T-Scl1; lanes d, pGEX-2T-Scl2. DNA was stained with ethidium bromide. DNA fragments of digestion are listed in Table 1.

sialidase cDNA fragment. The BamHI–EcoRI digest of two representatives, pGEX-2T-Scl1 and pGEX-2T-Scl2, is shown in Fig. 3(A) (lanes c and d, respectively); fragments of BamHI–EcoRI-PstI digest are depicted in Fig. 3(B) (lanes c and d) and listed in Table 1. The deduced sialidase amino acid sequence encodes a total number of 376 amino acids. Compared to the native CHO cell cytosolic sialidase the pentapeptide Met–Ala–Thr–Cys–Pro was replaced by the Gly-Ser-dipeptide.

*Expression and purification of the GST-sialidase fusion protein on an analytical scale.*— Treatment of clones with IPTG at growth temperature of 37°C gave negative results. The expression of soluble GST-sialidase was achieved by lowering the temperature to 25°C, indicating the entrapment of GST-sialidase in inclusion bodies after induction at 37°C. The expression and recovery of the GST-sialidase fusion protein in *E. coli* M15 pGEX-2T-Scl1 and pGEX-2T-Scl2 total protein extracts after induction with 2 mM IPTG is demonstrated in Fig. 4. Coomassie Blue stained protein extracts exhibited in both clones a protein band of approximately 70 kDa, corresponding to the additive molecular weight of a fusion protein composed of GST (26 kDa) and sialidase (43 kDa) (Fig. 4(A), lanes a and b), whereas GST but not the fusion protein was detected in the clone with pGEX-2T (religated) vector (Fig. 4(A), lane d). The affinity chromatography purified soluble GST-sialidase fusion proteins of pGEX-2T-Scl1 and pGEX-2T-Scl2 are shown in Fig. 4(B) (lanes a and b, respectively). No glutathione-binding host proteins were isolated from the bacterial lysates without IPTG induction (Fig. 4(B),

Table 1
Endonuclease digest fragments of pGEX-2T-Scl1 and pGEX-2T-Scl2

| Fragment origin | Number (bp) | Restriction sites [a] | | |
|---|---|---|---|---|
| | | BamHI | PstI | EcoRI |
| pGEX-2T | 3981 | 930 | 3029 | |
| pGEX-2T | 957 | | 3029 | 2072 |
| Insert [b] | 657 | | 1415 | 2072 |
| Insert [b] | 474 | | 941/1415 | |
| Insert [b] | 11 | 930 | 941 | |

[a] See Fig. 2.
[b] BamHI–EcoRI sialidase cDNA fragment.

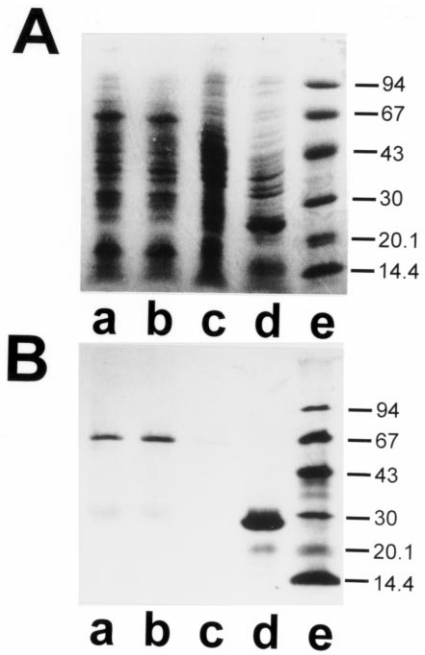


Fig. 4. SDS-PAGE of GST-sialidase fusion protein in total protein extracts (A) and purified by glutathione affinity chromatography (B) of *E. coli* M15 pGEX-2T-Scl1 and pGEX-2T-Scl2 cells after induction with 2 mM IPTG. Lanes a, *E. coli* M15 pGEX-2T-Scl1; lanes b, *E. coli* M15 pGEX-2T-Scl2; lanes c, *E. coli* M15 pGEX-2T-Scl1 without IPTG; lanes d, *E. coli* M15 pGEX-2T (religated vector). Proteins were stained with Coomassie Blue (A, lane e: 3 μg LMW calibration kit proteins) and silver stain reagent (B, lane e: 0.8 μg LMW calibration kit proteins).

lane c). Only GST but not the GST-sialidase fusion protein was detected in the religated vector (Fig. 4(B), lane d). Neither GST nor fusion protein was found in lysates of controls without IPTG induction as shown for pGEX-2T-Scl1 (Fig. 4(B), lane c). Both, GST and GST-sialidase fusion protein were immunologically identified with anti-GST antibody as evidenced by Western blot analysis (not shown).

*IPTG-induction and specific activity of GST-sialidase.*—Sialidase activity towards MUF-Neu5Ac was detected in supernatants of small scale lysates of *E. coli* M15 clones pGEX-2T-Scl1 and pGEX-2T-Scl2, but not in untransformed *E. coli* M15 host cells or cells with pGEX-2T, the latter without the insert. After induction with 1.0 mM IPTG (3 h, 25°C, optical density 1.0), the supernatant of lysates of pGEX-2T-Scl1 revealed higher volumetric activities compared to clone pGEX-2T-Scl2. Using 0.5, 1.0, and 2.0 mM IPTG concentrations, volumetric activities observed were reverse to the IPTG concentrations, i.e., highest activity was obtained with 0.5 mM and lowest activity with 2 mM IPTG. To further optimize the conditions for large scale GST-sialidase production (see below), *E. coli* pGEX-2T-Scl1 cultures (250 mL volumes, flask scale) were induced with 0.5 mM IPTG at three different optical densities. The highest specific enzyme activity was obtained in affinity chromatography purified eluates during logarithmic growth. Induction of cells with 0.1, 0.5, and 1.0 mM IPTG revealed highest total protein amounts after induction with 1.0 mM IPTG, but highest specific activity for affinity chromatography purified eluates from cultures induced with 0.1 mM IPTG (data summarized

Table 2
GST-sialidase activities and yields of affinity chromatography purified enzyme of *E. coli* pGEX-2T-Scl1 obtained with three different IPTG concentrations

| IPTG [a] (mM) | Purification step [b] | Total protein [c] (mg) | Total activity (U) | Specific activity (U/mg) | Purification factor [d] | Yield [e] (%) |
|---|---|---|---|---|---|---|
| 0.1 | Supernatant | 34.14 | 0.275 | 0.008 | 1.0 | 100 |
| | Eluate | 0.157 | 0.100 | 0.636 | 79.2 | 36 |
| 0.5 | Supernatant | 29.77 | 0.320 | 0.011 | 1.0 | 100 |
| | Eluate | 0.206 | 0.111 | 0.541 | 49.2 | 35 |
| 1.0 | Supernatant | 29.06 | 0.392 | 0.013 | 1.0 | 100 |
| | Eluate | 0.267 | 0.155 | 0.581 | 44.7 | 40 |

[a] Induction was performed in 250 ml cell suspensions (2 h, 25°C) at an optical density of 1.
[b] Supernatant from centrifuged cell lysates obtained by sonication; pooled fractions after Glutathione Sepharose affinity chromatography ( = eluate).
[c] Determined according to Bradford.[35]
[d] Calculated for specific activities.
[e] Yields of flow-through fractions ranged within 3–8% (not listed).

Table 3
GST-sialidase activities and yields of affinity chromatography purified enzyme of *E. coli* pGEX-2T-Scl1 produced on bioreactor scale [a]

| Purification step [b] | Total protein [c] (mg) | Total activity (U) | Specific activity (U/mg) | Purification factor [d] | Yield (%) |
|---|---|---|---|---|---|
| Supernatant | 4503.21 | 113.35 | 0.025 | 1 | 100 |
| Concentrate | 4163.08 | 94.47 | 0.023 | 1 | 83.3 |
| Eluate | 26.46 | 26.44 | 0.999 | 40 | 23.3 |

[a] Induction with 0.1 mM IPTG was performed in 25 L cell suspension (3 h, 25°C) at an optical density of 1.5.
[b] Supernatant from centrifuged cell lysates obtained with a high pressure homogenizer; ultrafiltrate after ultra- and diafiltration (= concentrate); pooled fractions after Glutathione Sepharose affinity chromatography (= eluate).
[c] Determined according to Bradford.[35]
[d] Calculated for specific activities.

in Table 2). According to this data, the clone pGEX-2T-Scl1 was chosen for large-scale bioreactor production using a concentration of 0.1 mM IPTG for induction during exponential growth after chilling the cell suspension to 25°C.

*Production and purification of the GST-sialidase fusion protein on preparative scale.*—Cells of the clone pGEX-2T-Scl1 were induced during exponential growth at an optical density of 1.5 with 0.1 mM IPTG for 3 h at 25°C. In total, 26.46 mg of 40-fold purified GST-sialidase was obtained from a 25 L batch with a specific activity of 0.999 U/mg protein (see Table 3). The inhibitory effect on the activity of the sialidase was investigated with 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en) and Neu5Ac. Both are competitive inhibitors for the sialidase, the former being higher effective as depicted in Table 4. The SDS-PAGE separated GST-sialidase and its corresponding immunodetection with polyclonal anti-GST antibody after Western blot membrane transfer is shown in Fig. 5(A and B), respectively. This enzyme preparation was used for the following characterization of its ganglioside hydrolyzing specificity. The cytosolic sialidase is capable to desialylate a wide spectrum of different types of gangliosides using a thin-layer chromatography overlay kinetic assay without detergents.[20]

## 3. Discussion

A soluble CHO cell sialidase[21] which can degrade recombinant glycoproteins expressed

Table 4
Inhibitory effects of Neu5Ac and Neu5Ac2en on GST-sialidase activity

| Inhibitors | Sialidase residual activity | |
|---|---|---|
| | (mU) [a] | (%) |
| Without | 0.203 | 100 |
| Neu5Ac (2 mM) | 0.168 | 83 |
| Neu5Ac (5 mM) | 0.163 | 80 |
| Neu5Ac2en (0.1 mM) | 0.103 | 51 |
| Neu5Ac2en (0.5 mM) | 0.052 | 25 |

[a] 1 mU catalyzes the hydrolysis of 1 nmol MUF-Neu5Ac per min at 37°C, pH 5.5.

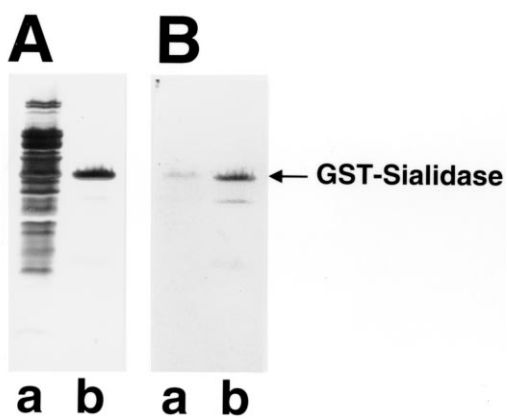


Fig. 5. SDS-PAGE (A) and Western blot immunodetection (B) of affinity chromatography purified GST-sialidase with polyclonal anti-GST antibody. Lanes a, 3.6 μg of *E. coli* M15 pGEX-2T-Scl1 total protein extract; lanes b, 0.4 μg of GST-sialidase after one-step Glutathione Sepharose affinity chromatography purification from the same clone. Proteins were detected with silver stain reagent after Western blotting (A) and with NBT/BCIP solution after membrane transfer and incubation with primary and alkaline phosphatase labeled secondary antibodies (B).

by these cells has been isolated and purified to near homogeneity by Warner et al.[16] The amount of 174 μg of enzyme was isolated from 100 L of cell culture fluid using conventional purification techniques including ion-exchange, hydrophobic interaction, heparin-agarose and chromatofocusing chromatography. The removal of sialic acid from recombinant glycoproteins in cell culture supernatant by a sialidase endogenous to CHO cells has been reported by several groups. The extracellular sialidase was active toward a variety of CHO-cell produced glycoproteins. The enzyme was found to hydrolyze preferentially sialic acid predominantly linked as Neu5Ac(α2–3)Gal(β1–4)GlcNAc-R, where R represents the remaining oligosaccharide, on gp120, soluble CD4, and DNAse.[17] The same preferential hydrolysis of sialoglycoproteins has been reported for cytosolic sialidase from rat liver.[13] Those substrates possessing (α2–3)-sialyl linkage were hydrolyzed much faster than those with (α2–6) or (α2–8) linkage. The degradation by sialidase is diminished through addition of the sialidase inhibitor 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid in the culture supernatant as shown by Gramer et al.[17] CHO-studies, using seven sialidase inhibitors, revealed differential effects of certain inhibitors toward (at least partly) purified sialidases: the four types of rat sialidase (cytosolic and intralysosomal sialidase, membrane associated sialidase I and II) could be discriminated from one another and furthermore from viral and bacterial sialidases.[22]

Genetically manipulated CHO cells are used for the production of recombinant human proteins on bioreactor scale. Changes in glycoprotein oligosaccharide structures, particularly the hydrolysis of terminally linked sialic acid residues, effects, for example, their circulation halftime, biodistribution, or biological activity as shown for erythropoietin.[23] CHO-cell derived antithrombin III, the most important physiological regulator of thrombin and other coagulation factors, carries (α2–3)-sialylated glycans. The rising number of dead cells during bioreactor scale production correlates with increasing neuraminidase activity of CHO cell culture supernatant, resulting in oligosaccharide desialylation and reduced biological activity of recombinant antithrombin III.[18,24]

The cDNA encoding the soluble sialidase from CHO cells has then been cloned and expressed as an enzymatically active protein in insect cells by Ferrari et al.[19] High levels of the enzyme were expressed in *Spodoptera frugiperda* cells infected with a modified *Autographa californica* nuclear polyhedrosis virus harboring the sialidase cDNA. The nucleotide and predicted amino acid sequences of the cloned enzyme have been reported in detail. Two minor bands with higher molecular masses than native sialidase from CHO cells were detected by immunoblot analysis of SDS–polyacrylamide gels. These higher molecular mass products were suggested to result from glycosylation of the recombinant sialidase, although the original cytosolic CHO cell sialidase is not normally glycosylated.[16] Obviously, the baculovirus protein expression utilizes the host–insect cell Golgi apparatus. The specific activity of the enzyme in the crude cell homogenate at maximal expression was ca. 0.9 U/mg protein. This specific activity is similar to that of the hamster enzyme after six steps of purification from CHO cell supernatant [16] and almost identical with that of one step purified GST-sialidase cloned in *E. coli* (see Table 3). However, the CHO cell sialidase expressed in insect Sf9 cells was not produced and purified on preparative scale and therefore, no data on enzyme yields could be provided.[19]

Due to its low abundance in the cytosol and/or cell culture supernatant and to facilitate laborious purification, we cloned the CHO cell sialidase, which represents a non-glycosylated protein,[16] into the pGEX-2T plasmid vector encoding for GST (see also Miyagi et al.[25]). GST-fusion proteins are easily purified from bacterial lysates by affinity chromatography using Glutathione Sepharose. This approach has been also successfully used in various applications, e.g., for biochemical studies of lectins, enzymes, and membrane proteins.[26–28] Total protein extracts of *E. coli* transformands revealed the GST-sialidase fusion protein with an apparent molecular weight of about 70 kDa, matching the molecular weight of a fusion protein composed of

GST (26 kDa) and cloned cytosolic CHO cell sialidase (43 kDa). The GST-sialidase fusion protein was purified from *E. coli* homogenates derived from a 25 L scale batch fermentation by one-step affinity chromatography on Glutathione Sepharose. The amount of 26.46 mg of a soluble and enzymatically active fusion protein was obtained corresponding to 40-fold purification and overall enzyme yield of 23.3%. This enzyme preparation was used for exploring its ganglioside-hydrolyzing specificity demonstrated in the accompanying publication.[20]

To overcome the sialidase deleterious effect on the quality of recombinantly expressed sialoglycoproteins, cell lines expressing sialidase antisense RNA have been made resulting in significantly lower sialidase levels and consequently in enhanced sialic acid content of recombinant glycoproteins.[29,30] Recently, considerable effort has been expended on maximization of sialic acid content of recombinant glycoproteins by means of over-expression of galactosyl- and sialyltransferases.[31] Metabolic engineering of animal cell protein glycosylation promises to address this challenge by substantially enhancing recombinant protein quality, productivity, and biological activity.

## 4. Experimental

*Cultivation of CHO-K1 cells and RNA isolation.*—Chinese hamster ovary cells (CHO-K1, CCL 61, American Type Culture Collection, Rockville, MD, USA) were cultivated in a 1:1 mixture of Dulbeccos's minimum essential medium and Ham's F12 basal medium (both Gibco BRL, Eggenstein, Germany), supplemented with 10% fetal calf serum (Gibco BRL). The cultures were incubated in conventional tissue grade culture flasks (Nunc, Wiesbaden-Biebrich, Germany) at 37°C and 5% $CO_2$–air atmosphere. Confluent grown cells of two 80 $cm^2$ tissue flasks were washed twice with phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3) and trypsinized with 0.05% trypsin and 0.02% EDTA in Puck's saline A (Gibco BRL). The cells were collected by centrifugation at 500*g* for 5 min (Labofuge M, Heraeus, Kendro Laboratory Products GmbH, Hanau, Germany) and washed with medium and PBS. The supernatant was discarded and the cell sediment was used for the RNA isolation with the RNeasy Total RNA Kit (Qiagen, Hilden, Germany). The cells were lysed and homogenized with QIAshredder™ homogenizers (Qiagen). The isolation was performed according to the suppliers protocol.

*Reverse transcriptase polymerase chain reaction (RT-PCR).*—Single strand cDNA was synthesized by reverse transcription of CHO-K1 cell total RNA with oligo (dT) primers using the GeneAmp® RNA PCR Kit (Perkin–Elmer, Weiterstadt, Germany).

Double strand cDNA fragments of the cytosolic sialidase and in-frame restriction sites were amplified using a mixture of KlenTherm and AccuTherm DNA polymerase (Synergy™ DNA polymerase; GeneCraft, Münster, Germany) instead of the kit polymerase (AmpliTaq® DNA polymerase). CHO-SIAL 51 sense primer (5′-T CAG T*GG ATC C*GT CCT GCA GAA GGA GAC GCT ATT CC-3′; BamHI restriction site in italics) and CHO-SIAL 52 antisense primer (5′-TCA GT*G AAT TC*G CAT GCA GCA AGA TCA CTG GGC ACC-3′; EcoRI restriction site in italics; synthesized by Life Technologies, Eggenstein, Germany), based on the known CHO sialidase cDNA sequence,[19] were used for the PCR biosynthesis.

The samples were subjected to a temperature cycle step of 94°C (40 s) and 68°C (2 min) for a total of 35 cycles, followed by a 7 min extension step at 72°C after the final cycle.

*Agarose gel electrophoresis of RT-PCR DNA and restriction fragments.*—The RT-PCR DNA and the restriction fragments were mixed 10:1 with tenfold concentrated loading buffer (50% glycerol, 0.1 M EDTA, 1% SDS, 0.1% bromophenolblue). The reference 1 kb-ladder marker X (Boehringer, Mannheim, Germany) was diluted with loading buffer to concentrations of 100, 50, and 25 ng/μL. Ten μL of the marker mixtures and 10–15 μL of samples were separated on 2% agarose (Life Technologies, electrophoresis grade) gels ($7.3 \times 10.3 \times 0.2$ cm mini gel chambers) in running buffer (0.4 M Tris–acetate, 1 mM

EDTA, pH 8.0) at 100 V. The gels were stained with ethidium bromide (10 μg/mL) for 10 min and washed for 5 min in water. The gels were placed on a transilluminator (312 nm excitation wavelength, Biometra, Göttingen, Germany) and the stained bands were documented with a Polaroid direct screen instant DS 34 camera.

*Sialidase cDNA subcloning*.—The Original TA Cloning® kit (Invitrogen, Groningen, The Netherlands) was applied to subclone the PCR product in the pCR®2.1 vector by direct insertion and transformation of *E. coli* InfαF′. The generated plasmid pMBU6-2, isolated with the Quantum Prep® Plasmid Miniprep Kit (BioRad, München, Germany) from the cultivated subclones, was digested with the restriction enzymes BamHI, EcoRI and ScaI. The inserted fragment was cut off the vector pMBU6-2 with BamHI and EcoRI, isolated by gel extraction (QIAquick Gel Extraction Kit, Qiagen) and used for further cloning into the pGEX-2T expression vector.

*Sialidase cDNA cloning into the pGEX-2T expression vector*.—The pGEX-2T plasmid vector of the GST Gene Fusion System (Pharmacia Biotech, Freiburg, Germany) was used for cloning the CHO cell derived sialidase as glutathione S-transferase (GST) fusion protein. The pGEX-2T plasmid vector encodes for GST at the amino terminus of the generated fusion protein, which is under the control of the tac promoter ($P_{tac}$). The plasmid encoding the cytosolic CHO cell sialidase was constructed by inserting the BamHI–EcoRI cDNA fragment into the multiple cloning site of the predigested and dephosphorylated expression vector (see Fig. 2) resulting in identical plasmids pGEX-2T-Scl1 and pGEX-2T-Scl2 (Scl = sialidase clone). Competent *E. coli* M15 pREP4 cells were transformed with the ligation products. The transformands were screened by restriction analysis with BamHI, EcoRI and PstI.

*Cell cultivation, IPTG induction, and cell disintegration*.—*E. coli* M15 pGEX-2T (control without insert), pGEX-2T-Scl1 and pGEX-2T-Scl2 were cultivated at 37°C overnight in Lennox Broth (LB) medium (Life Technologies) supplemented with 100 mg/L ampicillin and 25 mg/L kanamycin.

For small scale analysis, a 0.5 mL aliquot of an overnight culture was mixed with 3 mL of medium and incubated under shaking for 60 min at 37°C. The cell suspension was adjusted to 25°C and induced with 0.5 to 2 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) for 3 h. The cells (1.5 mL) were collected by 30 s centrifugation at 13,000*g* using a 5415 C centrifuge (Eppendorf GmbH, Hamburg, Germany). The supernatant was removed and the sediment stored at −20°C until use. For cell disintegration, the sediments were gently thawed at +4°C and dissolved in 980 μL PBS containing 1% Triton X100. Twenty μL lysozyme solution (50 mg/mL $H_2O$, from chicken egg white, 50 kU/mg protein; Sigma, Deisenhofen, Germany) were added and the sample was incubated for 30 min at +4°C. To diminish the viscosity of the solution, the cell homogenate was drawn 15 times through a cannula of 0.9 mm diameter and then centrifuged for 20 min at 13,000*g* in a 5415 C centrifuge (Eppendorf). The supernatant was removed and saved at +4°C for immediate use or stored at −20°C.

For flask scale production, 50 mL of LB medium with antibiotics were inoculated with 0.5 mL cell suspension and cultivated overnight at 37°C. From this overnight culture, 2.5 mL aliquots were used to inoculate 250 mL LB medium (glass flasks, Braun Biotech International, Melsungen, Germany) and incubated at 37°C on a rotary shaker (Certomat®U, Braun Biotech International; 220 rpm). The cell suspensions with an optical density between 0.4 and 1.2 (measured at 600 nm wavelength) were adjusted to 25°C and induced with 0.1 to 2 mM IPTG for 3 h. The cells were collected by 10 min centrifugation at 8000*g* and +4°C in a RC5C centrifuge (Sorvall, Kendro Laboratory Products GmbH) with a GS-3 rotor. The supernatants were removed and the sediments stored at −20°C until use. For cell disruption, the sediments were gently thawed at +4°C, resuspended each in 12.5 mL PBS containing 10 mM EDTA and 1 mM phenylmethylsulfonyl fluoride and transferred into a Rosett Cooling Cell® (Branson, Carouge-Geneve, Switzerland). The cells were disrupted on ice by four cycles of 10 s constant sonication intervals

(Sonifier 250, Branson; output control set on position '3') with a microtip (5 mm diameter). The addition of 1% Triton X100 (final concentration) and gentle rotation (REAX2, Heidolph, Kelheim, Germany) for 40 min at rt completed the cell lysis. The cell debris was removed by centrifugation in a RC5C centrifuge (Sorvall, Kendro Laboratory Products GmbH) with a SS-34 rotor for 10 min at 12,000*g* and +4°C. The supernatant was removed and saved at +4°C for immediate use or stored at −20°C.

*Large scale production of the GST-sialidase fusion protein.*—LB medium (25 L) supplemented with antibiotics were inoculated with *E. coli* M15 pGEX-2T-Scl1. The cells were cultivated at 37°C, 80% air saturation and pH 7.0 in a 30 L bioreactor (LAB 30L bioreactor, New MBR, Zürich, Switzerland). Induction with 0.1 mM IPTG for 3 h was initiated after chilling the bioreactor to 25°C. The culture broth was dialyzed against PBS and the volume was reduced by ultra- and diafiltration. Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM and the cells were disrupted using a French Press (high pressure laboratory homogenizer, Mini-Lab Type 8.30H, 800 bar, two cycles; APV Rannie Inc., St. Paul, MD, USA). The cell debris was removed from the bacterial lysate by centrifugation (Sorvall GS-3 rotor; 12,000*g*, 20 min, +4°C).

*Glutathione Sepharose 4B affinity purification of the GST-sialidase fusion protein.*—Crude bacterial lysate supernatant of small scale cultures were mixed with 50 μL Glutathione Sepharose 4 B slurry (Pharmacia Biotech), which has been twofold washed with 1 mL PBS and 1 mL 1% Triton X100 in PBS before use. The mixture was gently moved for 40 min at +4°C, centrifuged for 1 min at 500*g*, and the supernatant was discarded. The GST-sialidase loaded Glutathione Sepharose 4B was washed three times with 1% Triton X100 in PBS and twice with 50 mM Tris buffer (pH 8.0). The resin was resuspended in 1 mL 50 mM Tris buffer supplemented with freshly prepared 20 mM reduced glutathione and incubated for 10 min at rt. After 5 min centrifugation at 500*g*, the GST-sialidase containing supernatant was collected and saved at +4°C or stored at −20°C.

Column affinity chromatography purification was performed with the FPLC system (Pharmacia Biotech, consisting of the Controller LCC-501 Plus, two pumps P-500, two motor valves MV-8, injection motor valve MV-7, conductivity monitor, optical unit UV1, and the fraction collector Frac 100). The column (3 mL bed volume of Glutathione Sepharose 4 B, 2.6 cm internal diameter; Pharmacia Biotech) was equilibrated with PBS containing 1% Triton X100, loaded with the bacterial lysate supernatant of a 250 mL culture and washed with the equilibration buffer followed by 50 mM Tris buffer. The GST-sialidase was eluted with 50 mM Tris buffer containing 20 mM reduced glutathione with a flow rate of 0.5 ml/min. Protein absorbance was monitored at 280 nm and fractions of 0.5–2 mL were collected. Protein containing fractions (6–8 mL) were pooled and saved at +4°C or stored at −20°C.

The supernatant of lysed cells from the bioreactor cultivation (1.2 L dialysate) was mixed with 20 mL equilibrated Glutathione Sepharose 4 B slurry in a 2 L flask. The suspension was gently shaken overnight (Certomat U, Braun Biotech, 40 rpm) at 25°C. The loaded resin was separated from the supernatant by filtration and filled into a 2.6 cm diameter column. Elution from the column and fractionation of the GST-sialidase was performed with the FPLC system (Pharmacia Biotech) as described above. The Glutathione Sepharose 4 B was regenerated by washing the column (three cycles) with 40 mL 0.1 M Tris, 0.5 M NaCl, pH 8.5 and 40 mL 0.1 M AcONa, 0.5 M NaCl, pH 4.5. After the regeneration procedure, the resin was equilibrated, loaded again with the supernatant and the procedure was repeated twice as described (first repeat 30 min loading incubation; second repeat 60 min loading incubation). Eluted fractions containing the GST-sialidase were pooled and stored in 1 mL portions at −80°C.

*SDS-PAGE of total protein extracts and purified GST-sialidase.*—SDS gradient PAGE of total protein extracts was performed with the PhastSystem™ (Pharmacia Biotech) [32]. Before electrophoretic separation, cell sediments of 1–2 mL culture aliquots were dissolved in

100 μL SDS-PAGE buffer (3% β-mercaptoethanol, 3% SDS, 0.3% bromophenol blue, 10% glycerine in water) and mixed at 37°C for 30 min. Low molecular weight (LMW) calibration kit proteins (Pharmacia Biotech; 575 μg/vial) were used as standards and one vial was dissolved in 100 μL SDS-PAGE buffer. The samples and the standard protein mix were incubated at 95°C for 10 min in a block heater followed by centrifugation (13,000*g*, 10 min). The sample supernatants and the reference protein mixture, the latter 1:2 diluted with SDS-PAGE buffer, were loaded onto a PhastGel Sample Applicator 8/1 ($8 \times 1$ μL volumes) and automatically applied to a 8–25% PhastGel Gradient gel fitted with PhastGel SDS buffer strips. The gel was subjected to electrophoresis according to the suppliers protocol (PhastSystem™ application file no. 370). The separation is complete when bromophenol blue band reaches the anodal buffer strip. Protein staining was performed with Coomassie Blue. The gels were incubated for 5 min at 50°C in staining solution (one tablet PhastGel Blue R 350 (Pharmacia), dissolved in 300 mL 16% AcOH, 33% isopropanol in $H_2O$) and washed for 30 min at 50°C with destaining solution (10% AcOH, 20% isopropanol in $H_2O$).

SDS gradient PAGE of the Glutathione Sepharose 4 B bound GST-sialidase was performed as described above with minor modifications. A 50 μL aliquot of Glutathione Sepharose 4B slurry, loaded with the GST-sialidase from small scale culture lysate (see above), was mixed with 20 μL SDS-PAGE buffer and incubated for 10 min at 95°C in a block heater followed by centrifugation as described above. A 1:8 dilution of the calibration kit proteins was used as reference. Silver staining of gel separated proteins was performed with the PhastSystem according to Heukeshoven and Dernick.[33]

*Western blot and immunodetection of GST and GST-sialidase.*—SDS gradient PAGE separated proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham, Freiburg) by diffusion blotting according to the suppliers protocol (Phast System™, Development Technique File no. 220). The membrane, equilibrated with distilled water for 10 min, was placed onto the gel and the Peltier element of the separation bed was heated to 70°C. After 20 min blotting, the membrane was detached from the gel by rinsing with water, transferred into blocking buffer (3% bovine serum albumin (BSA), fraction V, Serva, Heidelberg, Germany) and incubated at 4°C overnight. After removal of the blocking buffer, the membrane was overlayed with polyclonal goat anti-GST antibody (Pharmacia) in blocking buffer (1:1000 dilution) for 1 h, washed three times for 10 min with washing buffer (0.5% BSA, 0.3% Tween 20 in PBS) and incubated for 1 h with secondary alkaline phosphatase-conjugated affinity pure rabbit anti-goat IgG antibody (Dianova, Hamburg, Germany) in blocking buffer (1:1000 dilution). Then the membrane was washed (see above) and the immunoblot was developed with 200 μL nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate-*p*-toluidine salt (NBT/BCIP stock solution, Boehringer, Mannheim, Germany) in 10 mL substrate buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH9.5). Finally, the reaction was stopped with distilled water, the blot fixed with 3% trichloroacetic acid, washed with distilled water and dried.

*Determination of sialidase activity.*—The sialidase activities of the bacterial lysate supernatants and the purified GST-sialidase were determined fluorometrically with 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid substrate (MUF-Neu5Ac, Sigma) according Potier et al.[34] and modified by Gramer and Goochee.[21] The standardized assay contained 25 μL of a 4 mM MUF-Neu5Ac solution, 10 μL of 1 M AcONa buffer (pH 5.5), and 65 μL cell free supernatant or purified GST-sialidase (both diluted 1:10 in water). In the negative control the sialidase solution was replaced by water. The inhibitory effect of Neu5Ac (Biomol, Hamburg, Germany) and Neu5Ac2en (Sigma) was tested in assay mixtures containing original sialidase activities of 0.203 mU (nmol/min) and specified concentrations of the inhibitors (see Table 4). The samples were placed in a block heater for 1 h at 37°C and the reaction was stopped with 900 μL 0.2 M glycine–NaOH buffer (pH 10.4). The mixtures were centrifuged at 16,000*g*

(Biofuge Pico, Heraeus) for 15 min at rt and sialidase activity was quantified with a spectrofluorometric detector RF-551 (Shimadzu, Kyoto, Japan) in 1 mL spectrophotometer quartz cuvets, operating with 362 nm excitation and 448 nm emission wavelengths. Hydrolyzed MUF-Neu5Ac was calculated with a reference curve of different 4-methylumbelliferone concentrations. One unit is defined as the amount of enzyme that will catalyze the hydrolysis of 1.0 μmol of MUF-Neu5Ac per min at 37°C, pH 5.5. The hydrolysis of 1 μmol of MUF-Neu5Ac per min and mg protein is defined as the specific activity of 1 U/mg protein. Protein quantification was performed according to Bradford.[35]

## 5. Abbreviations

Enzymes: Deoxyribonucleases or restriction enzymes BamHI, EcoRI, ScaI, and PstI (EC 3.1.21.4); sialidase or neuraminidase or *N*-acylneuraminosyl glycohydrolase (EC 3.2.1.18); BSA, bovine serum albumin; CHO, Chinese hamster ovary; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; MUF-Neu5Ac, 4-methyl-umbelliferyl-α-D-*N*-acetylneuraminic acid; Neu5Ac, *N*-acetylneuraminic acid; Neu5Ac2en, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (IUPAC-IUB recommendations[36]); PBS, phosphate buffered saline; RT-PCR, reverse transcriptase polymerase chain reaction.

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