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Deglycosylation of proteins for crystallization using recombinant fusion protein glycosidases

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Abstract

Obtaining high quality protein crystals remains a rate-limiting step in the determination of three-dimensional X-ray structures. A frequently encountered problem in this respect is the high or heterogeneous carbohydrate content of many eukaryotic proteins. A number of reports have demonstrated the use of enzymatic deglycosylation in the crystallization of certain glycoproteins. Although this is an attractive tool, there are some problems that hinder the more widespread use of glycosidases in crystallization. First, commercially available glycosidases are relatively expensive, which virtually prohibits their use on a large scale. Second, the glycosidase must be removed from the glycoprotein of interest following deglycosylation, which is not always straightforward. To circumvent these problems we have cloned the two most generally useful glycosidases, peptide-N-glycosidase F and endoglycosidase F_1 from *Flavobacterium meningosepticum*, as fusion proteins with glutathione S-transferase. The fusion not only allows rapid purification of these enzymes from *Escherichia coli* cell extracts, but also permits rapid removal from target proteins following deglycosylation. We have used these enzymes to obtain crystals of phytase from *Aspergillus ficuum* and acid phosphatase from *Aspergillus niger* and to obtain a new crystal form of recombinant human renin.

Keywords: endoglycosidase F_1; enzymatic deglycosylation; glycoproteins, crystallisation of; peptide-N-glycosidase F

Proteins of eukaryotic origin are often glycosylated. It has been shown that the glycan moiety of glycoproteins influences crystallogenesis (McPherson, 1982; Davis et al., 1993; Baker et al., 1994). Crystal formation might be hindered if the oligosaccharide(s) shield the protein surface and prevent or reduce favorable crystal contacts. Microheterogeneity might also limit the order and therefore the quality of crystals. Deglycosylation prior to crystallization may offer the key to obtaining high quality crystals.

The most important source of glycosidases is the bacterium *Flavobacterium meningosepticum* from which the peptide N-glycosidase, PNGase F, and the three β-N-acetylglycosidases, Endo F_1, Endo F_2, and Endo F_3 have been isolated. PNGase F and the EndoFs can be prepared easily and in large amounts from *F. meningosepticum* (Tarentino & Plummer, 1994). Despite this, and the fact that all four enzymes from this source have been cloned (Lemp et al., 1990; Barsomian et al., 1990, Tarentino et al. 1990, 1992, 1993), the commercially available enzymes remain very expensive. Indeed, their high cost virtually precludes their routine use for crystallization experiments.

Deglycosylation of target proteins under native conditions, as is required for crystallography, generally requires the addition of larger amounts of glycosidase than under denaturing conditions. This is presumably because the oligosaccharide attachment sites are less readily accessible in the folded protein. Because more than trace amounts of glycosidase must be added, efficient removal of the enyzme prior to crystallization of the target protein becomes important.

Having experienced difficulties obtaining crystals of phytase from *Aspergillus ficuum*, we decided to use commercially available PNGase F to remove the N-linked glycans. Initial results were encouraging and crystals were obtained but the high cost of the glycosidase led us to consider purifying it ourselves from *F. meningosepticum*. However, our crude glycosidase extract yielded qualitatively poorer crystals than those obtained with the commercially available PNGase F. In addition, *F. meningosepticum* is classified as belonging to Risk Group 2 (SKBS[3] guidelines) and needs to be handled accordingly. For these reasons and those noted above we cloned PNGase F and Endo F_1 as fusion proteins with glutathione S-transferase. The fusion proteins were each expressed in

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Table 1. *Phytase preparations for crystallography*

Glycosidase treatment	rh[a] (Å)	Molecular weight (kD) [light scattering]	Molecular weight (kD) [SDS-PAGE]	Crystals
None	35	60–62	65	no
PNGase F	29	39–41	48	yes
Flavobacterium extract	31–32	48–51	48	no
GST-PNGase F	29–30	38–42	48	yes

[a]Hydrodynamic radius.

Escherichia coli and subsequently purified in a single step using glutathione-Sepharose.

The fusion glycosidases were successfully used to produce crystals of two proteins that did not crystallize in their native glycosylated states, namely phytase from *A. ficuum* and acid phosphatase from *Aspergillus niger*. In a third case, recombinant human renin, deglycosylation yielded a new crystal form.

Results

Phytase

Phytase from *A. ficuum* was purified to homogeneity as described in Materials and methods (Fig. 1A). No crystals were observed in an initial sparse matrix. Phytase carries approximately 20% (wt/wt) sugar. It has ten potential N-linked glycosylation sites, eight of which are partially or fully occupied (F. Grüninger and A. Friedlein, unpubl. data). Both the amount of sugar and the heterogeneity due to variable glycosylation site usage could inhibit crystal formation. Therefore, we tested on an analytical scale the effect of removing the N-linked sugars with commercially available PNGase F. Treatment of the phytase with PNGase F in sodium phosphate buffer pH 7.8 at 30 °C for 16 hours led to a shift in molecular weight, as determined by SDS-PAGE, toward 48 kD, the molecular weight expected for unglycosylated phytase (Fig. 1B). A 30% reduction in phytase enzymatic activity occurred concomitantly, which suggests that the glycan chains are either involved directly in the enzymatic reaction or contribute to phytase stability. However, because the loss of activity was not quantitative, crystallization trials were initiated using this deglycosylated phytase. Crystals were obtained that were suitable for X-ray diffraction studies. The crystals were tetragonal, with cell constants $a = b = 92.2$ Å, $c = 101.1$ Å and diffract to 2.5 Å resolution.

For larger scale deglycosylation of phytase, a crude glycosidase fraction was prepared from *F. meningosepticum*, as described in Materials and methods. No attempt was made to resolve the Endo F activities from the PNGase F activity for the reason that the Endo Fs are unlikely to contribute significantly to N-linked deglycosylation at pH 7.8. Phytase treated with the crude glycosidase mixture appeared indistinguishable from PNGase F treated phytase, as judged by SDS-PAGE electrophoresis (Figs. 1B and C), but this material failed to crystallize. Further examination of this phytase by laser light scattering measurements and isoelectric focusing (Fig. 2 and Table 1) indicated that only partial deglycosylation had occurred. Addition of more crude *F. meningosepticum* glycosidase altered neither the molecular weight of the phytase observed in laser light scattering experiments (which was intermediate between fully glycosylated and fully deglycosylated) nor the isoelectric focusing pattern (data not shown).

Because the cloning of PNGase F had already been described by several authors (Lemp et al., 1990; Barsomian et al., 1990; Tarentino et al., 1990), we decided to produce our own stock of pure

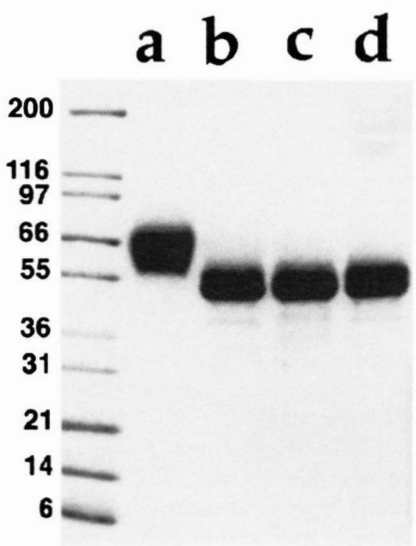

Fig. 1. SDS-PAGE of phytase before and after deglycosylation with various glycosidases. **a**, Purified phytase; **b**, deglycosylated with PNGase F; **c**, deglycosylated with glycosidase extract from *F. meningosepticum*; **d**, deglycosylated with GST-PNGase F.

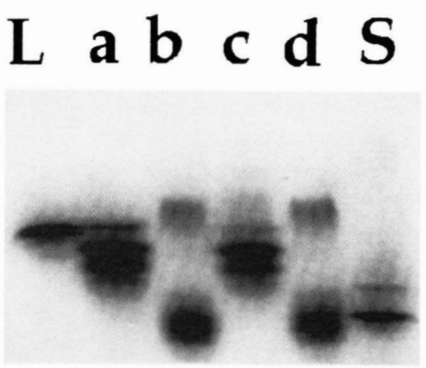

Fig. 2. Isoelectricfocussing of phytase before and after deglycosylation with various glycosidases. **a**, Purified phytase; **b**, deglycosylated with PNGase F; **c**, deglycosylated with glycosidase extract from *F. meningosepticum*; **d**, deglycosylated with GST-PNGase F. **S**, soybean trypsin inhibitor, IEP = 5.2; **L**, lactoglobulin, IEP = 4.55.

Table 2. *Results of crystallization trials*

Protein	Crystallization condition	Suitable for X-ray analysis
Glycosylated phytase	none	—
Glycosylated acid phosphatase	30% Jeffamine 6000 pH 5.5	no
Glycosylated human renin	10% PEG 6000, 0.6 M NaCl pH 4.5	yes
PNGase F or GST-PNGase F treated phytase	1.5 M ammonium sulfate pH 6.5	yes
GST-Endo F_1 treated acid phosphatase	1.6 M ammonium sulfate pH 5.5 or 16% PEG 4000 pH 4.0, 200 mM $MgCl_2$	yes
GST-PNGase F/GST-Endo F_1 treated human renin	50% MPD pH 6.5	yes

enzyme for use on a large scale. However, the protein was cloned as a fusion protein with glutathione-S-transferase (GST-PNGase F) in order to facilitate its purification from *E. coli* extracts. The GST-PNGase F was purified in one step using glutathione-Sepharose (Fig. 3A). The same column material can be used to rapidly remove the GST-PNGase F from target proteins after deglycosylation.

Phytase treated with GST-PNGase F was indistinguishable from PNGase F treated phytase as shown by SDS-PAGE, isoelectric focusing, and laser light scattering measurements (Figs. 1D, 2D, and Table 1). This material also crystallized under the same conditions and the same crystal form was obtained (Table 2).

Acid phosphatase

The pH 2.5 optimum acid phosphatase from *A. niger* is homologous to phytase but has a broader substrate specificity and a single, highly acidic pH optimum (Ullah & Cummins, 1987; Piddington et al., 1993). The protein was purified from recombinant *A. niger* as described in Materials and methods (Fig. 4A). It carries approximately 15% (wt/wt) sugar and has eight N-linked glycosylation sites. No crystals were obtained in an initial sparse matrix. The activity of acid phosphatase decreases rapidly above pH 5.0 (Ullah & Cummins, 1987) and deglycosylation should ideally be performed at or below this pH. PNGase F has a pH optimum of 8.0–8.5 and its activity decreases dramatically below pH 5.5 (Alexander & Elder, 1989). The Endo Fs, however, all have pH optima below pH 6.0 (Tarentino & Plummer, 1994). Analytical scale deglycosylations were performed by incubating acid phosphatase with commercially available Endo F_1 and Endo F_2 in sodium acetate buffer, pH 5.0, at 30 °C for 16 hours. Endo F_2 did not deglycosylate at all (Fig. 4C) but Endo F_1 treatment led to a shift in molecular weight (Fig. 4B). However, only partial deglycosylation occurs because the expected molecular weight for the unglycosylated species is 52 kD. Endo F_1 has a more restricted specificity than PNGase F and cleaves only high mannose type and hybrid type glycans (Trimble & Tarentino, 1991). Partial deglycosylation may therefore be explained either by the presence of other glycan types in acid phosphatase or by the inaccessibility of Endo F_1 to some of the glycans.

To obtain a stock of Endo F_1 for large scale experiments, Endo F_1 was cloned and expressed in *E. coli* as a fusion protein with GST. The fusion protein was purified in a single step using glutathione-Sepharose (Fig. 3B). Acid phosphatase was treated with GST-Endo F_1 at pH 5.0 and 30 °C for 16 hours (Fig. 4D). As with the phytase, a partial loss of enzymatic activity was associated

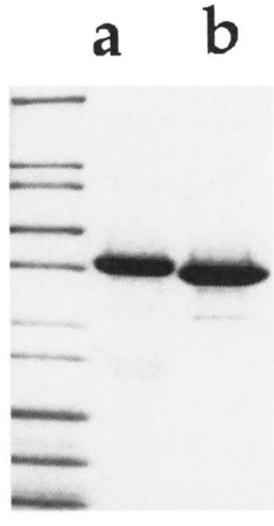

Fig. 3. SDS-PAGE of fusion glycosidases. **a**, GST-PNGaseF/purified on glutathione-Sepharose; **b**, GST-Endo F_1/purified on glutathione-Sepharose.

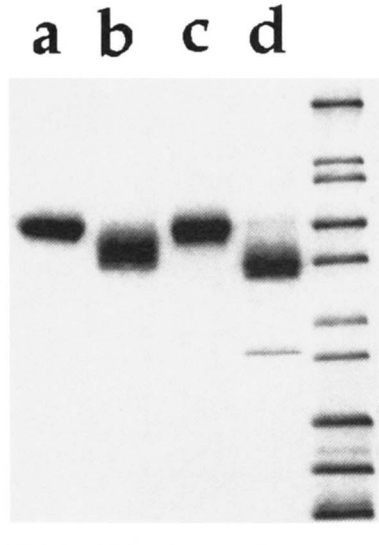

Fig. 4. SDS-PAGE of acid phosphatase before and after deglycosylation with various glycosidases. **a**, Purified acid phosphatase; **b**, deglycosylated with Endo F_1; **c**, deglycosylated with Endo F_2; **d**, deglycosylated with GST-Endo F_1.

with deglycosylation (M. Wyss, pers. comm.). The additional 32 kD band seen in Figure 4D derives from the fusion glycosidase and could be removed along with the glycosidase by subsequently passing the deglycosylated material over glutathione-Sepharose. The material was then concentrated and submitted for crystallization. This preparation produced crystals in the initial sparse matrix screen, which were then optimized for X-ray analysis (Table 2). The crystals diffract to 2.5 Å and belong to the trigonal space group either $P3_12_1$ or $P3_22_1$ with cell constants $a = 114.2$, $b = 114.2$, $c = 190.29$ Å.

Renin

Renin has two N-linked glycosylation sites, and a total of nine different glycan structures have been identified for recombinant human renin expressed in Chinese hamster ovary (CHO) cells (Aeed et al., 1992). Renin has been crystallized in its native as well as in a partially deglycosylated state (Sielecki et al., 1989; Rahuel et al., 1991). Crystals of the partially deglycosylated renin diffract better then those of native renin (2.5 Å compared with 2.8 Å). For our renin inhibitor program, renin was required for co-crystallization with new lead structures. Because high quality crystal data were needed, we decided to deglycosylate renin prior to crystallization. In the protocol described by Carilli et al. (1988b), renin was deglycosylated with "Endo F" at pH 6.5 for at least 5 days at 37 °C. We observed some proteolysis of renin using such extended incubation times and therefore deglycosylations were always carried out for 16 hours maximum at 30 °C. The "Endo F" used by Carilli et al. (1988b) and Sielecki et al. (1989) was presumably a mixture of Endo F_1, Endo F_2, and Endo F_3, since the first description of the purification of three distinct Endo Fs appeared only in 1991 (Plummer & Tarentino, 1991). Older Endo F preps were sometimes also contaminated with PNGase F. Aeed et al. (1992) showed that approximately 60% of the glycan structures on CHO renin are of the complex type, which cannot be cleaved by Endo F_1. However, PNGase F should be able to completely deglycosylate renin. We therefore compared commercially available Endo F (a mixture of Endo F_1 and Endo F_2), PNGase F, and our recombinant fusion glycosidases on the deglycosylation of renin. Figure 5 shows that both Endo F and PNGase F can be used for the (partial) deglycosylation of renin and that similar results can be obtained with a mixture of GST-PNGase F and GST-Endo F_1. As with acid phosphatase (using Endo F_1), a proportion of glycan structures are not cleaved by PNGase F, presumably because the potential cleavage sites are buried in the native protein.

For scale-up experiments we worked with a mixture of GST-PNGase F and GST-Endo F_1. The partially deglycosylated renin obtained by treatment with the two fusion glycosidases crystallized under different conditions and in a crystal form distinct from that described by Sielecki et al. (Table 2). These tetragonal crystals diffracted to 2.7 Å and have cell constants $a = 152.6$, $b = 152.6$, $c = 84.4$ Å.

Discussion

Baker et al. (1994) have suggested the general use of deglycosylation for the crystallization of glycoproteins. The work with phytase, acid phosphatase, and renin presented here confirms the notion that deglycosylation should be used as a routine method in screening for crystallization conditions. It is not necessary that deglycosylation be complete; removal of a subset of glycans, as shown here for acid phosphatase and renin, may already bring an improvement of crystallizability or crystal quality.

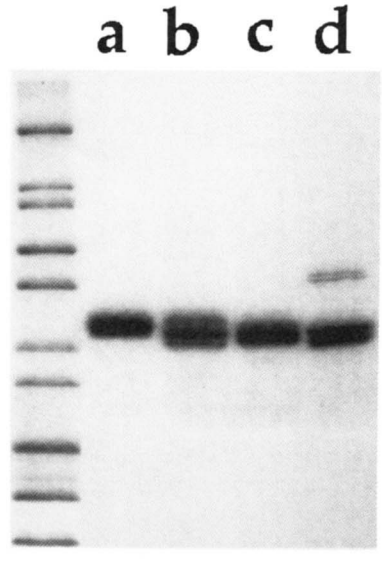

Fig. 5. SDS-PAGE of renin before and after deglycosylation with various glycosidases. **a**, Purified renin; **b**, deglycosylated with "Endo F"; **c**, deglycosylated with PNGase F; **d**, deglycosylated with GST-PNGase F and GST-Endo F_1.

The use of recombinant fusion glycosidases circumvents many of the problems otherwise associated with these enzymes such as cost, purity, and subsequent removal. In addition, the well-defined specificities of recombinant PNGase F and Endo F_1 make them more attractive for crystallization studies than the *Flavobacterium* extract used by Baker et al. (1994). Because the enzymes can be readily produced in large amounts, they could be routinely employed to test for the effect of deglycosylation on glycoproteins where high or heterogeneous carbohydrate content may influence crystallization.

Materials and methods

Purification of phytase, acid phosphatase, and renin

Phytase

Phytase was purified from the commercially available feedstuff Natuphos® (Gist-Brocades, Delft, The Netherlands), which contains recombinant phytase derived from *A. ficuum*. Natuphos® was dialyzed against 10 mM sodium acetate-acetic acid pH 4.5 (Buffer A) and then loaded onto a column of S-Sepharose FF (Pharmacia, Duebendorf, Switzerland). The column was washed with Buffer A and developed with a gradient of Buffer A + 1 M NaCl. The phytase-containing fractions were pooled and dialyzed against 10 mM sodium acetate-acetic acid pH 5.0 (Buffer B). The pool was loaded onto a column of Q-Sepharose FF (Pharmacia). The column was washed and eluted with a gradient of Buffer B + 1 M NaCl. Phytase containing fractions were pooled and dialyzed against Buffer B and stored at −20 °C.

Acid phosphatase

Acid phosphatase was purified from recombinant *A. niger* containing additional copies of the acid phosphatase gene (K. Vogel et al., unpubl. data). The molecule is secreted into the fermentation

medium. Fermentation broth was concentrated 15-fold by ultrafiltration and the concentrate desalted on Sephadex G-25 (Pharmacia) into Buffer B. The material was then loaded onto a column of Q-Sepharose, which was washed with Buffer B and developed with a gradient of Buffer B + 1 M NaCl. Acid phosphatase containing fractions were pooled and dialyzed against 25 mM glycine pH 3.0 and stored at −20 °C.

Renin

Recombinant human renin was purified from CHO cells essentially according to Carilli et al. (1988a).

Cloning, purification, and activity of recombinant glycosidases

Isolation of genomic DNA

Genomic DNA from *F. meningosepticum* ATCC 33958 (Elder & Alexander, 1982) was prepared essentially according to Marmur (1961).

Cloning of PNGase F and Endo F_1 genes as GST fusions

For construction of the bacterial expression plasmid pGEX-PNGase F, the PNGase F gene was amplified by polymerase chain reaction (PCR) from genomic *F. meningosepticum* DNA with the following primers: 5′-ATTCAAGGGATCCAGGCTCCGGCAGA TAATAC-3′ and 5′-CTACAAGGATCCTTATTAGTTTGTAACTA CCG-3′. The following conditions were used for the PCR: 100 ng of genomic DNA was amplified with 25 pmoles of each primer using 2.5 units of Pfu polymerase (Stratagene, La Jolla, California) in 50 μl total reaction volume. Primer annealing was carried out at 55 °C for 1 minute and extension at 72 °C for 1 minute; 25 cycles were performed. The 0.8 kb PCR product was purified on a low melting agarose gel, digested with BamH1 and ligated to BamHI-cleaved pGEX3 (Pharmacia), which was dephosphorylated with calf intestinal phosphatase. The Endo F_1 gene was obtained using the primers 5′-ATCAATCCCGGGGCGGTAACTGGTACAAC-3′ and 5′-CTACAAGAATTCTTATTACCAGTCCTTAGAAT-3′ and inserted as a SmaI/EcoRI fragment into the SmaI and EcoRI sites of pGEX3, resulting in the expression plasmid pGEX3-Endo F_1. The gene sequences of the endoglycosidase moieties were confirmed by DNA sequencing (Sanger et al., 1977). In both fusion proteins the endoglycosidase moiety begins with the first amino acid of the mature protein; this is Ala 41 for PNGase F and Ala 51 for Endo F_1 in the respective sequence numbering (Barsomian et al., 1990; Tarentino et al., 1992). Additional residues are inserted for cloning purposes, namely, Ile and Gln in the GST-PNGase F construct and Gly, Ile, Pro and Gly in the GST-Endo F_1 fusion.

Expression and purification of GST-PNGase F and GST-Endo F_1 fusion proteins

For expression of GST-PNGase F, 2 L of LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin were inoculated 1:100 with an overnight culture of *E. coli* M15/pREP4 (Stüber et al., 1990), transformed with pGEX3-PNGase F. After growing the cells at 37 °C to an OD550 of 0.8 the culture was cooled to room temperature. Expression was induced with 0.2 mM isopropyl-1-thio-β-D-galactoside (IPTG) and the culture was incubated for 4 hours at 22 °C. The cells were harvested by centrifugation. Essentially the same procedure was followed for the expression of GST-Endo F_1 except that IPTG induction was performed at 37 °C.

Purification of GST-PNGase F and Endo F_1

The *E. coli* biomass obtained from 2 × 1 L shake flasks (approximately 1 L culture) was resuspended in 25 volumes of 50 mM TrisHCl pH 8.0. The suspension was then broken in a bead mill (Bead Beater, Biospec Products, Bartlesville, Oklahoma) using an equal volume of 0.1 mm zirconium beads for 3 × 1 minutes with 5-minute pauses between (to minimize heating). The beads were removed by filtration and washed with one volume of chilled Tris buffer. The lysate and wash were pooled and centrifuged at 20,000 × *g* for 30 minutes. The supernatant was removed and passed over a 10 ml column of glutathione-Sepharose (Pharmacia) that had been equilibrated in Tris buffer. The column was washed with 100 ml of 50 mM Tris buffer and then eluted with a gradient of Tris buffer containing 10 mM reduced glutathione (gradient volume = 50 ml). Fractions containing GST-PNGase F were pooled, concentrated to 4 ml, and loaded onto a Superdex 75 column (Hiload 16/60, Pharmacia) that had been equilibrated in 50 mM TrisHCl pH 8.0, 200 mM NaCl. Active fractions were pooled, concentrated, and dialyzed against 50 mM TrisHCl, pH 8.0, 2.5 mM EDTA, 50% glycerol. The material was stored at −20 °C. Approximately 10 mg of fusion protein could be recovered from a 2 L culture. The procedure was the same for GST-Endo F_1 except that the final pool was dialyzed against 10 mM sodium acetate-acetic acid, pH 5.5, 50% glycerol. The yield of GST-Endo F_1 was approximately 50 mg/2 L culture.

Activity of recombinant glycosidases

The activity of the fusion proteins was checked using α_1-acid glycoprotein (GST-PNGase F) and/ or RNAse B (GST-PNGase F, GST-Endo F_1) according to Alexander and Elder (1989). In neither case did factor Xa treatment of the fusion proteins, which efficiently removes the GST fusion tail, affect activity against these standard substrates.

Preparation of Flavobacterium extract

F. meningosepticum (ATCC 33958) was obtained from the American Type Culture Collection and grown as described by Elder and Alexander (1982). The cells were removed by centrifugation and the culture supernatant was then filtered through a 0.2 micron filter, all under sterile conditions. The filtrate was made 50 mM in EDTA and the pH adjusted to 6.5 before being concentrated 50-fold by ultrafiltration. Ammonium sulfate was then added to 90% saturation. The resulting precipitate was collected by centrifugation, washed with 90% ammonium sulfate, and taken up in 10 ml (for 10 L culture filtrate) of 50 mM sodium phosphate, pH 7.8, 20 mM EDTA. The concentrate was then loaded onto a 1.6 × 67 cm Sephacryl S-100 column (Pharmacia) that had been equilibrated in the same buffer. Fractions containing glycosidase activity were pooled, concentrated to 5 ml by ultrafiltration, mixed 1:1 with glycerol, and stored at −20 °C as aliquots.

Deglycosylation

Deglycosylation was carried out overnight (approximately 16 hours) at 30 °C in 20 mM sodium phosphate, pH 7.8 (phytase), 20 mM sodium acetate-acetic acid, pH 5.0 (acid phosphatase), or 20 mM sodium acetate-acetic acid, pH 5.5 (renin). Purified PNGase F, Endo F, Endo F, and Endo F, were all obtained from Boehringer-Mannheim (Mannheim, Germany). The amount of each glycosidase used for deglycosylation of the three target proteins was

optimized empirically using SDS-PAGE analysis to give maximal deglycosylation. For crystallization trials the ratio of glycosidase:glycoprotein on a weight basis was 1:25 (GST-PNGase F: phytase), 1:15 (GST-EndoF$_1$: acid phosphatase) and 1:1.5:20 (GST-PNGase F:GST-EndoF$_1$:renin).

Protein determination

Protein adsorption was measured at 280 nm and concentrations were calculated using E_{280} (1 cm, 1 mg/ml) = 0.86 (GST-Endo F$_1$), 1.34 (GST-PNGase F), 1.05 (phytase), 2.0 (acid phosphatase), and 1.42 (renin). These extinction coefficients were calculated from the respective amino acid sequences according to Pace et al. (1995) but omitting the contribution of cystine.

SDS-PAGE and IEF

SDS-PAGE and IEF were carried out using pre-cast gels purchased from Novex (San Diego, CA).

Light scattering

To determine whether the deglycosylation had caused aggregation, phytase samples were examined using dynamic light scattering before and after treatment with the various PNGase preparations. The protein sample was diluted to approximately 2 mg/ml in 25 mM acetate buffer at pH 5.5, and prefiltered through Whatman Anatop 0.1 mm filters. A Dyna Pro 801 molecular size detector (Protein Solutions, Charlottesville, Virginia) was used and analysis performed with the software supplied by the manufacturer. Acid phosphatase and renin also were also examined following PNGase F treatment.

Crystallization

For crystallization all three protein samples were concentrated to between 15 and 20 mg/ml using 10 kD cut-off "Ultrafree" concentration units (Millipore, Bedford, Massachusetts). A sparse matrix screen similar to the original described by Jancarik and Kim (1991) was used to screen for initial crystallization conditions in a hanging drop system (McPherson, 1982). For typical experiments 3 ml of protein was mixed with 3 ml of reservoir solution on silanized microscope slides and sealed over the same reservoir. Results of crystallization trials are described in Table 2.

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