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THE FIRST DEMONSTRATION OF A PROCARYOTIC GLYCOSYLASPARAGINASE+

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SUMMARY: Glycosylasparaginase was purified to near homogeneity from intracellular lysates of *Flavobacterium meningosepticum*. The enzyme is a heterodimer with an estimated molecular weight of 38 kDa and consists of one α-subunit (18 kDa) and one β-subunit (16 kDa). The β-subunit of the *Flavobacterium* enzyme has a direct evolutionary relationship to the β-subunit of mammalian glycosylasparaginases as evidenced by: (1) strong cross-reactivity with antibodies made to the denatured rat β-subunit, (2) a high degree of homology with the amino-terminus of the corresponding eukaryotic enzymes, and (3) irreversible inactivation with 5-diazo-4-oxo-L-norvaline, a reagent known to react with the catalytic amino-terminal threonine residue on the β-subunit of a mammalian glycosylasparaginase.

Glycosylasparaginase, N^4-(N-acetyl-β-glucosaminyl)-L-asparaginase (EC 3.5.1.26) is a well-known lysosomal amidase/amidohydrolase that hydrolyzes the γ-carboxyl amide bond between aspartate and 1-amino N-acetylglucosamine of asparagine-linked glycans (1-4). The 1-amino-N-acetylglucosamine is subsequently converted nonenzymatically to N-acetylglucosamine and ammonia. The enzyme is ubiquitously distributed in mammalian tissues (5) where it plays a central role in the normal ordered catabolism of asparagine-linked glycoproteins in lysosomes (6,7). A genetic deficiency of glycosylasparaginase leads to the pathological condition known

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Abbreviations: AspNHGlcNAc, N^4-(β-N-acetylglucosaminyl)-L-asparagine; AspNH$(GlcNAc)_2(Man)_6$, high mannose-type glycoasparagine; DONV, 5-diazo-4-oxo-L-norvaline; PNGase F, Peptide-N^4-(N-acetyl-β-glucosaminyl)-L-asparagine amidase; dns, 5-dimethylaminonapthalenesulfonyl-; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinyldifluoride; CAPS, (3-[cyclohexylamino]-1-propane sulfonic acid).

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as aspartylglucosaminuria, a lysosomal storage disease in which AspNHGlcNAc and other glycoasparagines accumulate (8,9).

Thus far glycosylasparaginases have only been described in mammalian systems, but like other eukaryotic proteins they may have evolved from more rudimentary life forms. We decided to investigate whether this enzyme might be expressed in the bacterium *F. meningosepticum* since this organism secretes a related enzyme, Peptide-N^4-(N-acetyl-β-glucosaminyl)-L-asparagine amidase (PNGase F), that hydrolyzes the same amide linkage in asparagine-linked glycans (reviewed in: (10,11), but differs from glycosylasparaginase in requiring a peptide backbone.

We have made the novel finding that this bacterium expresses both glycosylasparaginase and PNGase F, although glycosylasparaginase is strictly an intracellular protein whereas PNGase F is secreted into the medium. In this communication we describe the purification and properties of *F. meningosepticum* glycosylasparaginase and compare some of its properties with those of its mammalian counterparts.

MATERIALS AND METHODS

Materials

N^4-(β-N-acetylglucosaminyl)-L-asparagine (AspNHGlcNAc) and H-Asp(pNA)-OH, the p-nitroanilide of L-aspartic acid were from BACHEM (Philadelphia). $AspNH(GlcNAc)_2$ $(Man)_6$ was isolated from ovalbumin as previously described (12). 5-Diazo-4-oxo-L-norvaline (DONV) was provided by Dr. Nancita Lomox of the Drug Synthesis and Chemistry branch of the National Cancer Institute (NSC-117613). Rabbit antisera to native intact rat liver glycosylasparaginase and to the denatured α and β-subunits were generously provided by Dr. Nathan N. Aronson, Jr.

Enzyme assay

Glycosylasparaginase was assayed colorimetrically by measuring the release of N-acetylglucosamine from AspNHGlcNAc using the Reissig modification (13) of the Morgan-Elson reaction. A 100 μl reaction mixture contained 10 mM AspNHGlcNAc, 20 μl, 500 mM sodium Hepes, pH 8.8, 20 μl, and enzyme + water, 60 μl. One unit of glycosylasparaginase releases 1 μmole of N-acetylglucosamine per minute at 37°C under the conditions defined above. Protein was determined spectrophotometrically at 280 nm assuming a value of 10 for a 1% solution.

Enzyme Purification

Step I - Cell Lysate

Flavobacterium meningosepticum (Elder Strain) ATCC 33958 was grown aerobically at 30°C for 48 hours in four 2-liter flasks containing 1 liter of medium (1% tryptone-0.5% yeast extract - 0.5% NaCl). The cells were harvested by centrifugation at 10,000 x g for 20 minutes and lysed by sonication as follows: approximately 120 g wet weight of cell paste was resuspended in about 200 ml of lysis buffer (20 mM Tris-Cl, pH 7.5 - 50 mM NaCl - 10 mM EDTA) and sonicated six times in an ice-water bath using a Sonics and Materials Vibra-Cell with a 70% duty cycle. The cell lysate was centrifuged at 12,000 x g for 30 minutes and the insoluble pellet was resonicated two more times as described above. The combined supernatants (550 ml) were treated with 1% protamine sulfate (92 ml) for 30 minutes to precipitate nucleic acids, and the turbid extract was clarified by centrifugation at 10,000 x g for 30 minutes. The supernatant was dialyzed exhaustively against two 6-liter changes of 10 mM sodium acetate, pH 5.8.

Step II - Phosphocellulose chromatography
All subsequent operations are performed at room temperature. The dialyzed cell lysate (606 ml) was applied at a flow rate of 2 ml/min to a 2.5 x 10 cm column of phosphocellulose (Schleicher & Schuell) equilibrated in 10 mM sodium acetate, pH 5.8. The column was washed with the same buffer for 1 hour to purge non-retarded proteins, and then developed with a linear gradient to 0.45 M NaCl for 19 hours at 1.5 ml/min. Fractions of 9 ml were collected. Glycosylasparaginase was tightly bound to the phosphocellulose matrix and eluted near the end of the gradient. The active fractions (tubes 80-105) were combined and dialyzed against two 6-liter changes of 12 mM sodium Hepes (pH 8.8).

Step III - Q-Sepharose Chromatography
The dialyzed extract from Step II (269 ml) was applied at a flow rate of 2 ml/min to a 1.5 x 13.5 cm column of Q-Sepharose (Pharmacia) equilibrated in 12 mM sodium Hepes, pH 8.8. The column was washed for 30 minutes with starting buffer and developed with a linear gradient to 0.5 M NaCl over a 6 hour period. Fractions of 5 ml were collected. The fractions containing glycosylasparaginase activity (tubes 41-46) were combined, concentrated by ultrafiltration to about 4.5 ml with a YM-10 membrane in an Amicon cell, and dialyzed against two 2-liter changes of 10 mM sodium acetate, pH 6.5

Step IV - Protein Pak Sulfopropyl HR 15-Chromatography
Glycosylasparaginase from Step III was applied at a flow rate of 0.6 ml/min to an 0.6 x 10 cm Protein Pak sulfopropyl HR15 (Waters) column equilibrated in 10 mM sodium acetate, pH 6.5. The column was washed with starting buffer for 10 minutes, and then developed with a linear gradient to 0.25 M NaCl over 90 minutes. Fractions of 0.6 ml were collected. Glycosylasparaginase eluted sharply in fractions 39-42 and was concentrated to about 1 ml using an Amicon P-10 spin column.

Microsequence Analysis
Glycosylasparaginase samples were electrophoresed at 200 V in commercial 15% acrylamide minigels (Bio-Rad) under standard reducing conditions. The proteins were electroblotted to PVDF (Bio-Rad Trans-Blot transfer medium) at 50 V for 45 min at room temperature in a 25 mM CAPS buffer, pH 11, containing 10% methanol and 4 mM dithiothreitol. The membrane was lightly stained with 0.01% Coomassie blue R-250 in 20% methanol, destained with 40% methanol containing 5% acetic acid, and washed overnight with several changes of distilled water. The protein bands corresponding to the α and β subunits were excised and automated Edman degradation was performed with a Model 477A Applied Biosystems pulsed liquid sequencer equipped with a Model 120 amino acid analyzer. The efficiency of microsequencing from PVDF was estimated at about 30% based on a molecular mass of approximately 38,000 daltons for the intact *Flavobacterium* glycosylasparaginase.

Substrate Specificity
Reaction mixtures contained 200 nmoles of $AspNH_2$, AspNHGlcNAc, $AspNH(GlcNAc)_2(Man)_6$, or Asp(pNA); 10 μmoles of sodium Hepes, pH 8.8, and glycosylasparaginase (19 milliunits) in a final volume of 100 μl. Appropriate controls minus enzyme were included. The tubes were incubated at 37°C for 10 minutes and the reactions terminated with 700 μl of sodium diluent pH 2.2 (Pickering Laboratories). 200 μl from each reaction was filtered through an Amicon microcon spin filter, and 100 μl was analyzed for aspartic acid using a Pickering amino acid resolution column at 51°C in conjunction with a Waters 625 LC system.

RESULTS AND DISCUSSION

Purification of glycosylasparaginase from crude intracellular lysates was achieved in three steps that are summarized in Table 1. Classical chromatography using phosphocellulose at pH

Table 1. Purification of *F. meningosepticum* glycosylasparaginase

Step	Volume ml	Total Activity Units (μmoles/min)	Total Protein mg	Yield %
1. Crude lysate	605	24.9	N.D.[a]	100
2. Phosphocellulose	242	20.1	95	80.6
3. Q-Sepharose	30.5	13.8	1.1	55.5
4. Sulfopropyl HR	0.95	7.0[b]	0.28	28.1

[a]Not determined
[b]The specific activity at step 4 corresponds to 25 μmoles AspNHGlcNAc hydrolyzed per min/mg protein.

5.8 afforded a very large purification with an excellent recovery of enzyme activity. The actual purification factor could not be directly determined because of heavily pigmented materials in the lysate that interfered with protein determinations, but it is estimated that at least 90% of non-enzyme protein was removed at this stage. Crude intracellular lysates also contained significant asparaginase activity, which was completely removed on phosphocellulose chromatography. Subsequent fractionation on Q-Sepharose followed by high-resolution chromatography on sulfopropyl HR provided a very reproducible protocol for obtaining nearly homogeneous glycosylasparaginase. The *Flavobacterium* enzyme had a final specific activity of about 25 μmoles/min/mg protein with AspNHGlcNAc as substrate, which is roughly 4 to 5 fold higher than most mammalian glycosylasparaginases (14,15). The *Flavobacterium* enzyme preferred substrates with a glycosylated asparagine moiety as shown in Table 2. AspNHGlcNAc was hydrolyzed completely within 10 min (actual time for 100% cleavage was less than 6 min as determined by N-acetylglucosamine release). By comparison, the longer glycoasparagine, $AspNH(GlcNAc)_2(Man)_6$ was still hydrolyzed at a very fast rate, with 90% conversion in 10 min. Interestingly, the bacterial enzyme also hydrolyzed non-glycosylated asparagine analogues such as the p-nitroanilide of aspartic acid, Asp(pNA), and asparagine, $AspNH_2$, albeit at a much slower rate. The mammalian glycosylasparaginases vary in their ability to hydrolyze $AspNH_2$: the hog kidney enzyme (16) and the rat and bovine enzyme (Tollersrud and Aronson, personal

Table 2. Specificity of *F. meningosepticum* glycosylasparaginase

Substrate	% hydrolysis/10 minutes
AspNHGlcNAc	100
$AspNH(GlcNAc)_2(Man)_6$	90
Asp(pNA)	20
$AspNH_2$	9
$dnsAspNH(GlcNAc)_2(Man)_6$	trace[a]

[a]Incubation time, 6 hours with five times the concentration of enzyme.

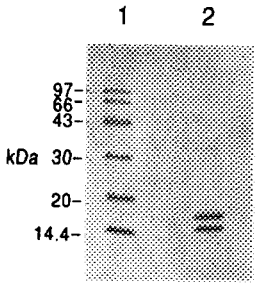

Figure 1. Electrophoresis of purified *F. meningosepticum* glycosylasparaginase. Protein samples were subjected to electrophoresis under denaturing conditions on a 15% SDS-polyacrylamide gel. Lane 1, standard molecular weight markers (Bio-Rad); lane 2, step 4 purified glycosylasparaginase (A_{280}/ml = 0.188), (approximately 1.6 μg,9 μl) applied.

communication) also hydrolyze $AspNH_2$ at less than 10% the rate of AspNHGlcNAc, but the human leukocyte glycosylasparaginase does not utilize this substrate (17). As expected, a dansylated glycoasparagine, $dnsAspNH(GlcNAc)_2(Man)_6$, was not appreciably hydrolyzed (Table 2). Four different preparations subjected to SDS-PAGE at step 4 gave the typical result shown in Fig. 1. The enzyme migrated as two protein bands with an apparent molecular weight of approximately 18 kDa and 16 kDa, respectively, that have been designated the α and β subunits in accordance with the terminology adopted for the non-identical subunits of the mammalian glycosylasparaginases (18). The evidence that the two major protein bands represent non-identical subunits of a corresponding bacterial glycosylasparaginase was provided by molecular-weight determination of the intact protein using HPLC/LKB Blue chromatography. In the absence of detergent the glycosylasparaginase activity eluted sharply at a molecular mass of about 38 kDa. SDS (0.1%) completely inhibited the enzyme and shifted the molecular mass to a broad peak which SDS-PAGE resolved into the typical 18- and 16-kDa subunits.

Microsequence analysis of the α and β subunits trans-blotted to PVDF revealed in three independent runs the following amino-termini:

α-subunit	T N K P I V L S T T N F G L (X) A N V E A (X) K
β-subunit	T I G M I A L D A Q G N L S G A (X) T T S G M

(X) = an undetermined residue.

The minor band migrating at about 32 kDa (Fig. 1) represents a trace impurity in the glycosylasparaginase preparation: it was variable in amount, sometimes completely absent, and its amino-terminus did not correspond to either the α or β subunit.

To determine whether there was any structural similarity between the bacterial and mammalian glycosylasparaginases, the *Flavobacterium* enzyme was separated into its α and β

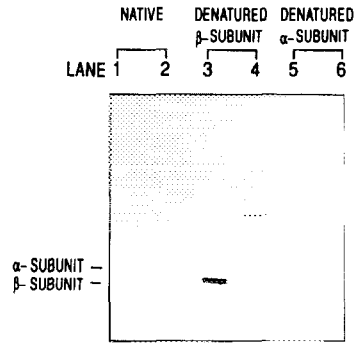

Figure 2. Western blot analysis of the cross-reactivity of the *F. meningosepticum* glycosylasparaginase subunits against antibodies to rat liver glycosylasparaginase. Protein samples were subjected to 15% SDS-PAGE and Western-blotted to PVDF using standard protocols. *F. meningosepticum* glycosylasparaginase (A_{280}/ml = 0.436), 3 μl applied to lanes 1,3,5; *F. meningosepticum* PNGase F (A_{280}/ml = 0.480), 3 μl applied to lanes 2,4,6. 1° Antibody: lanes 1,2-antibody to native rat glycosylasparaginase (1/500 dilution); lanes 3,4-antibody to denatured rat glycosylasparaginase β-subunit (1/1000 dilution); lanes 5,6-antibody to denatured rat glycosylasparaginase α-subunit (1/300 dilution). 2° antibody and color development were conducted according to a Promega Western blot kit.

subunits by SDS-PAGE, Western blotted, and tested for cross-reactivity against antibodies made to the native (intact) rat liver glycosylasparaginase (lane 1) or the corresponding denatured β (lane 3) or α (lane 5) subunits. As shown in Fig. 2 (lane 3) only antibodies to the denatured rat liver glycosylasparaginase β-subunit cross-reacted with the *Flavobacterium* glycosylasparaginase, and specifically "lit-up" the β-subunit of the bacterial enzyme. It is noteworthy that antibodies to the denatured rat β subunit cross-reacted weakly with the PNGase F control (lane 4), suggesting that these catalytically related enzymes share some common determinants.

A recent review by Mononen *et al.* (19) clearly shows that the amino-terminus of the β-subunit is a highly conserved feature among mammalian glycosylasparaginases as well as in a plant asparaginase (20). As shown in Table 3 the β-subunit of the *Flavobacterium* glycosylasparaginase (C) shows a remarkable degree of homology with its mammalian counterparts (A) and even more so to a *Lupin arboreus* asparaginase (B), suggesting an ancestral link between the procaryotic and mammalian enzymes. The amino-terminal threonine residue is found on all glycosylasparaginases (Table 3) and evidence is mounting that it is an active-site residue. Kaartinen *et al.* (15) recently demonstrated that 5-diazo-4-oxo-L-norvaline(DONV), a potent irreversible inhibitor of asparaginase (21) and glycosylasparaginase (22) specifically reacts with the amino-terminal threonine of the β-subunit of the human leukocyte enzyme via an ether linkage with the hydroxyl side chain. We have found that the *Flavobacterium* glycosylasparaginase is also very sensitive to inhibition by DONV (100% inactivation in 30 min at 200 μM DONV). Attempts to sequence the β-subunit of DONV-inhibited

Table 3. Structural comparison of the β-subunit of four mammalian glycosylasparaginases and a plant asparaginase with *F. meningosepticum* glycosylasparginase

	Source[1]	amino-terminal sequence
A.	glycosylasparaginase	
	human	T I G M V V I H K T G H I A A G T S T N G I
	rat	T I G M V V I H K T G H T A A G T S T N G L
	pig	T I G L V V I H K M G Y T A A G T S T N G I
	cow	T I G M V V I H K T G N I A A G T S T N G I
B.	lupin arboreus asparginase	T V G C V A V D S Q G N L A S A T S T G G L
C.	*F. glycosylasparaginase*[2]	T I G M I A L D A Q G N L S G A X T T S G M

[1]The amino acid sequences in A and B were adapted directly from Mononen *et al.* (19).
[2]X is an undetermined residue, most probably cysteine.

glycosylasparaginase were uniformly unsuccessful and the peptide behaved as though the amino-terminus was completely blocked. We take this as tentative evidence that the amino-terminal threonine of the β-subunit was reacted with DONV but mass spectrometry as demonstrated by Kaartinen *et al.* (15) will be needed to conclusively demonstrate this point.

Mammalian glycosylasparaginases are derived from a single gene that encodes a catalytically inactive precursor polypeptide. Post-translation cleavage of this precursor polypeptide generates the α and β subunits that associate to form active enzyme (23-25). This processing mechanism positions a threonine residue from an internal locus in the precursor to the amino-terminus of the β-subunit. Since bacteria are generally thought to lack the elaborate post-translational processing mechanisms common to eukaryotic cells (26), the question of the origin of the α- and β-subunits of the *Flavobacterium* glycosylasparaginase becomes important. We are currently cloning the *Flavobacterium* glycosylasparaginase gene and should be able to determine whether post-translational processing is involved in the formation of active enzyme.

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