Short Communication

Characterization of extracellular γ-glutamyl transpeptidase from *Aspergillus nidulans*

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Abstract

Aspergillus nidulans γ-glutamyl transpeptidase (AnγGT, EC 2.3.2.2) was partially purified from the fermentation broth of carbon stressed cultures. Its temperature and pH optimum was 45 ºC and pH 8.0, respectively. AnγGT had little hydrolase activity. It utilized Gln, glutathione and less efficiently oxidized glutathione as γ-glutamyl donors (beside of γ-glutamyl-*p*-nitroanilide) and amino-acids and peptides (including Glu, Cys, Met, Gly-Gly and Cys-Gly) but not hydroxylamine as γ-glutamyl acceptors. We propose that the function of this enzyme is not to degrade, but to produce, γ-glutamyl compounds which may be related to the utilization of extracellular peptides and amino-acids in carbon stressed cultures.

Key words: Carbon stress, γ-Glutamyl transpeptidase substrates, Glutathione metabolism, Utilization of peptides

γ-Glutamyl transpeptidase (γGT; EC 2.3.2.2) has until now been regarded as an enzyme responsible for the degradation of glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine) (Pócsi et al. 2004). Recent data demonstrate that other enzymes including the cytosolic γ-glutamyl cyclotransferase of mammals (Kumar et al. 2012) and plants (Ohkama-Ohtsu et al. 2008), and glutamine amidotransferase, a member of the DUG (Deficient in Utilization of Glutathione) pathway in yeasts (Kumar et al. 2003), rather than γGT catalyze the degradation of GSH in the cytosol. Hence, the physiological role of γGT is questionable.

Previously, we found that *Aspergillus nidulans* showed high intra- and extracellular γGT activities under carbon stressed conditions and addition of peptides to the cultures enhanced these activities (Spitzmüller et al. 2015). Deletion of the *ggtA* (AN10444) encoding a putative γGT decreased the γGT activities below to the detection limit suggesting that GgtA is the only enzyme possessing significant γGT activity under carbon stressed conditions in *A. nidulans* (Spitzmüller et al. 2015). Surprisingly, deletion of the *ggtA* gene did not influence the continuous decrease in GSH pools which was typical for carbon stressed cultures (Emri et al. 2008; Spitzmüller et al. 2015).

Here we partially purified *A. nidulans* γGT (AnγGT) from the fermentation broth of carbon stressed cultures. We demonstrated that it had high activity at slightly alkaline pH, typical for carbon starving cultures. It had weak hydrolase activity and could utilize Gln, GSH and, less efficiently, oxidized glutathione (GSSG) as γ-glutamyl donors, and several amino-acids and peptides (but not hydroxylamine) as γ-glutamyl acceptors. Our data supports the view that the function of the extracellular AnγGT is not the degradation but the production of γ-glutamyl compounds which may be related to the utilization of extracellular peptides and amino-acids in carbon stressed cultures.

Aspergillus nidulans FGSC A26 (*biA; veA1*) was cultured in minimal-nitrate medium containing 2 w/v % lactose as a carbon source at 37 ºC and 200 rpm for 3 d as described earlier (Spitzmüller et al. 2015). A protein fraction of 100 mL mycelium-free fermentation broth obtained by filtration was precipitated with 60 % $(NH_4)_2SO_4$ at 4 °C for 1 h. Precipitated proteins were removed by centrifugation (20 min, 20000 g, 4 °C) and the $(NH_4)_2SO_4$ content of the supernatant was increased up to 80 % to precipitate the γGT containing fraction. After 1 h incubation at 4 °C, the precipitated proteins were collected again by centrifugation (20 min, 20000 g, 4 °C), dissolved in 5 mL 0.1 M K-phosphate buffer (pH 8) and dialyzed overnight at 4 °C against the same buffer. Ion exchange chromatography was carried out on DEAE Sephadex column (2 cm × 20 cm) using 0.1 M K-phosphate buffer (pH 8) for equilibration and 0.1 M K-phosphate buffer (pH 8) containing 0–1 M NaCl for gradient elution. Fractions containing the highest γGT activity were collected and used throughout the experiments. The applied purification method resulted in 30 × increase in the specific extracellular γGT activity (with 3.4 % yield), although the preparation contained more than one protein band on SDS-PAGE (data not shown).

Extracellular γGT activity was determined as described earlier (Spitzmüller et al. 2015) using 0.1 M Tris/HCl (pH 8.0) buffer containing 1 mM γ-glutamyl-*p*-nitroanilide (γGpNA; as a γ-glutamyl donor) and 20 mM Gly-Gly (as a γ-glutamyl acceptor) at 37 ºC. To find pH or temperature optima the pH and the temperature were changed between pH 5–11 and 20–80 ºC, respectively using the following buffers: 0.1 M K-phosphate buffer (pH 5–7), 0.1 M Tris/HCl buffer (pH 7–9) and 0.1 M Gly buffer (pH 9–11). In some experiments Gly-Gly was omitted from the reaction mixture or was replaced with hydroxylamine, Cys-Gly, or different amino-acids. In other experiments

γGpNA was replaced with Gln, GSH or GSSG and the consumption of these γ-glutamyl donors was detected. The decrease in GSH or GSSG concentration was followed by an enzymatic assay as described earlier (Spitzmüller et al. 2015), while Gln concentrations were determined with a LC-ESI-MS/MS (High-performance liquid chromatography electrospray ionization tandem mass spectrometry) method. LC-ESI-MS/MS was also used to detect and identify the products of GgtA. In these experiments a Thermo Accela HPLC attached to a Thermo LTQ XL Linear Ion Trap MS (using Hypersil Gold 50 mm × 2.1 mm × 1.9 μm column) system was used. ESI ionization parameters were as follows: heater temperature was 300 °C; N_2 was used as sheath gas, flow rate and aux gas flow rate were 22 and 10 arbitrary units (arb), respectively; spray voltage was 4 kV; capillary temperature was 300 °C; capillary voltage was +3.00 V (positive ion mode). Gradient components were water with 0.1% (v/v) formic acid (A) and acetonitrile with 0.1% (v/v) formic acid (B). The time program was 10 % B (0–6 min), 10 → 90 % B (6–9 min), 90 % B (9–10 min), 90–10 % B (10–10.1 min), 10% B (10.1–12 min). Flow rate was 250 μL/min. CID parameters were: normalized collision energy 35, activation Q 0.250, activation time 30 ms.

The temperature optimum of AnγGT was at 45 °C (at pH 8.0) and no enzyme activity was detected above 65 °C. Its pH optimum was at pH 8.0 (at 37 °C) and no significant activities were detected below pH 7.0 or above pH 10. The enzyme showed Michaelis-Menten kinetics with γGpNA as a substrate (Km = 0.2 mM; at 37 °C and pH 8.0). Similar enzymatic properties were found with the γGT of *Penicillium roqueforti* by Tomita et al. (1990). Similarly to other fungal γGTs (Tomita et al. 1990) and in contrast to prokaryotic enzymes (Lin et al. 2006), NaCl (0.05–1 M) could not activate but inhibited the enzyme. NaCl at 1 M concentration decreased the enzyme activity by 40 %

(at 37 °C and pH 8.0 in the presence of 1 mM γGpNA and 25 mM Gly-Gly as substrates). In contrast, 2.7 M NaCl inhibited the γGT activity from *P. roqueforti* by 15 % (Tomita et al. 1990)

AnγGT had minor hydrolase activity (Table 1), similar to the γGT of *P. roqueforti* (Tomita et al. 1990), but in contrast to the extracellular glutaminase A of *A. oryzae* which has significant hydrolase and transpeptidase activities (Tomita et al. 1988). Remarkably, Glu was as efficient γ-glutamyl acceptor as Gly-Gly which is generally used in enzyme assays. Cys, Met, Cys-Gly and Ala could also significantly increase the liberation of *p*-nitroanilide from γGpNA, but less efficiently than Glu did (Table 1).

AnγGT could utilize Gln, GSH and (less significantly) GSSG as γ-glutamyl donors (Fig. 1). The formation of γGlu-Gly-Gly in the presence of 1 mM Gln, GSH or GSSG and 20 mM Gly-Gly during the γGT reaction was also detected by LC-ESI-MS/MS (Table 2). Moreover, formation of γGlu-Cys-Gly (GSH) in the presence of 1 mM Gln and 20 mM Cys-Gly was also detected (Table 2).

Physiological functions of extracellular γGTs frequently belong to the hydrolysis of different γ-glutamyl compounds. Cells can utilize the hydrolytic products of these enzymes; as an example, γGT of *Helicobacter pylori* hydrolyzes GSH and Gln and cells take up only the released Glu, Cys-Gly and ammonia (Shibayama et al. 2007), while *Bacillus subtilis* γGT functions as a exo-γ-glutamyl hydrolase acting on capsule poli-γ-Glu-Glu to gain free Glu (Kimura et al. 2004). In other cases, degradation of GSSG can maintain a redox milieu as it was found in plant apoplast (Ohkama-Ohtsu et al. 2008). The low hydrolase activity of AnγGT and its weak GSSG preference (Table 1; Fig. 1)

6

do not support the existence of similar physiological roles in *A. nidulans*. It is possible that the main function of this enzyme is not to degrade but to produce γ-glutamyl compounds. Based on the regulation of AnγGT as well as on the properties of *ΔggtA* strains not producing γGT activity, we previously described a hypothesis which suggests that extracellular AnγGT is involved in the amino-acid and peptide utilization of carbon stressed cultures by producing γ-glutamyl compounds (Spitzmüllet et al. 2015). The low hydrolase activity of AnγGT as well as the observation that it can utilize Gln as γ-glutamyl donor and works well at slightly alkali pH, typical for carbon starved cultures (Emri et al. 2008), supports this idea. However, further experiments are needed to justify or reject this hypothesis.

There is little data on the production and physiological function of ascomycetal extracellular enzymes possessing γGT activity. Our data support the view that these enzymes have real biotechnological significance in the production of different γ-glutamyl compounds and may influence the fermentations in food industry (Tomita et al. 1988, 1990).

Disclosures

Authors declare that they have no conflict of interest. All the experiments undertaken in this study comply with the current laws of the country where they were performed.

Acknowledgments

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Figure legends

Fig. 1 – Utilization of γ-glutamyl donors by AnγGT.

γGT activities were followed at 25 ºC (pH 8.0) in the presence of 1 mM GSH (closed squares), GSSG (open squares), Gln (closed circles) or γGpNA (open circles) as γ-glutamyl donors and 20 mM Gly-Gly as a γ-glutamyl acceptor. (Substrate consumptions were less than 10 % in the absence of Gly-Gly.) Means calculated from 3 parallel experiments are presented. The standard deviation (S.D.) values were less than 12 % of the means.

The decrease in γGpNA concentration was calculated according to the liberated *p*-nitroanilide.

Table 1 − Comparison of AnγGT activities in the presence of different γ-glutamyl acceptors.

γ-Glutamyl acceptor[b]	Relative enzyme activity[a] (%)
20 mM Gly-Gly	100 ± 6[c]
20 mM Glu	98 ± 6[c]
20 mM Cys-Gly	36 ± 5[c]
20 mM Met	36 ± 6[c]
20 mM Cys	28 ± 5[c]
20 mM Aln	25 ± 5[c]
20 mM NH_2-OH[d]	9 ± 3
H_2O (control)	10 ± 2

[a] Enzyme activities were determined by measuring the liberation of *p*-nitroanilide from γGpNA (1 mM) at 37 ºC (pH 8.0). Enzyme activity measured with Gly-Gly was regarded as 100 % (25 nkat). Mean ± S.D. calculated from 4 parallel experiments are presented.

[b] In case of 20 mM Gln, Asp, Gly, Ser, Thr and Orn the relative enzyme activities did not differ significantly (Student's t-test, $p < 0.05$, n = 4) from the control.

[c] Significantly higher relative enzyme activity (Student's t-test, $p < 0.05$, n = 4) than the control.

[d] No γ-glutamylhydroxamate formation was detected by the method of Imaoka et al. (2010).

Table 2 – Formation of peptides by AnγGT in the presence of different γ-glutamyl donors.

γ-Glutamyl donor	γ-Glutamyl acceptor	Identified peptide[a]
Gln	Cys-Gly	Glu-Cys-Gly[b]
Gln	Gly-Gly	Glu-Gly-Gly[b]
GSH	Gly-Gly	Glu-Gly-Gly[c]
GSSG	Gly-Gly	Glu-Gly-Gly[c]

[a] Peptides were identified by LC-ESI-MS/MS system.

[b] A high product peak with m/z 308 ($[M+H]^+$) was detected in the reaction mixture containing the enzyme, Cys-Gly and Gln. The peak had identical mass spectrometric properties to that of the authentic standard GSH. It also yielded the following product ions in MS^2 (CID), supporting the structure of the compound: 179 (Y_2^+), 205 (X_2^+ or A_2^+), 162 (Z_2^+ or Y_2^{*+}), 233 (B_2^+) and 291 ($[M-NH_3+H]^+$).

[c] In reaction mixtures containing the enzyme, Gly-Gly and Gln or GSSG a product peak was detected at m/z 262 ($[M+H]^+$). It was successfully identified as $[Glu\text{-}Gly\text{-}Gly+H]^+$ after fragmentation in MS^2. The product ions supporting the structure were: m/z 187 (B_2^+) and 133 (Y_2^+).

Fig. 1