Continuous assays of glutaminyl cyclase: from development to application

Stephan Schilling and Hans-Ulrich Demuth*

Probiodrug AG, Weinbergweg 22, 06120 Halle/Saale, Germany

Abstract. Glutaminyl cyclase (QC, EC 2.3.2.5) catalyses the formation of pyroglutamyl residues from glutamine at the N-terminus of peptides and proteins. In previously applied assays, QC activity was determined by either analysing the products formed using HPLC coupled with photometric or fluorometric detection, radioimmunoassay, or by detecting the release of ammonia spectrophotometrically. Although these methods are sensitive, they are all discontinuous and therefore time-consuming and laborious. To conduct a detailed kinetic investigation of QC catalysis, we developed coupled continuous assays suitable for microplates which allow now convenient determination of QC activity. The methods either use pyroglutamyl aminopeptidase or glutamate dehydrogenase as auxiliary enzymes, which results in the liberation of chromophores or fluorophores such as *p*NA, AMC, β NA or in the conversion of the chromophore NADH/H⁺ into NAD⁺, respectively.

The assays were applied in various enzyme isolation and characterisation studies, using crude protein solutions as well as purified enzyme in pH-dependence, substrate and inhibitor specificity investigations. Depending on the respective analytical task, both assays complement each other. Therefore, different enzymatic properties could be explored in more detail. Since the employed strategy of assay development could be of interest also for the analysis of other enzymes, the methods are described here in a comprehensive manner.

1. Introduction

Several peptides and proteins contain pyroglutamic acid at their N-terminus. Initially, pyroglutamyl formation was assumed to result from a spontaneous cyclisation reaction of a N-terminal glutamine or glutamic acid residue. However, specific enzymatic conversion of glutamine by glutaminyl cyclase (EC 2.3.2.5) has been discovered in plant and animal tissues [1–4].

Although a QC was first explored in papaya latex, its physiological function in the plant is still enigmatic. More recently, however, QCs were also identified in several other plant species, suggesting a general physiological significance of this protein [5]. In contrast to plant QC, several physiological substrates and products of mammalian QC activities could be identified. Pyroglutamic acid is present, for instance, in the hormones thyrotropin releasing hormone (TRH), gonadoliberin (GnRH) and gastrin, neurotensin and chemokines of the monocyte chemotactic protein (MCP) family. The formation of this N-terminal 5-oxoproline residue has shown to cause the bioactive structure of the hormones and to improve their stability towards N-terminal proteolysis [6–8]. Interestingly, plant and animal QCs seem to be very similar at a first glance. Both enzyme forms are expressed via the secretory pathway, carry carbohydrates and are monomeric proteins with similar molecular masses of 33–40 kDa [5,9,10]. Furthermore, all QCs are strictly specific for L-glutamine in the N-terminal position of the substrates and

*Corresponding author: H.-U. Demuth, Probiodrug AG, Weinbergweg 22, 06120 Halle, Germany. Tel.: +49 345 5559900; Fax: +49 345 5559901; E-mail: Hans-Ulrich.Demuth@probiodrug.de.

their kinetic behaviour was found to obey the Michaelis–Menten equation [10–12]. The primary and secondary structures of the QCs from *C. papaya* and that of the highly conserved QC from mammals, however, did not reveal homology [5,13,14]. Due to this apparent divergence, a detailed comparison of the catalytic properties of the QC forms could be helpful for deepening the understanding of pyroglutamylation and to identify, whether the different QC forms catalyse the pyroglutamylation by the same mechanism.

A detailed enzymatic characterisation of QC catalysis and inhibition, however, was hampered by the lack of handy assays. In previously applied methods, QC activity was determined by either analysing the products formed using HPLC linked to photometric or fluorimetric detection [11,10] or radioimmunoassay [3,15] using antibodies directed against TRH, or by detecting the release of ammonia spectrophotometrically in a coupled enzymatic assay [16]. Although the methods are sensitive, they are all discontinuous and therefore time-consuming and laborious. Furthermore, some of these methods can be only applied for one certain substrate, thus hampering detailed substrate specificity studies. In another approach, the change in absorbance occurring due to the formation of an intramolecular amid bond during N-terminal pyroglutamylation by QC is detected [17]. Although this assay allows a continuous data monitoring, the observed changes in absorbance are very small, making the assay insensitive. Furthermore, due to measuring the absorbance change at 220 nm, at the wavelength characteristic for the $n \rightarrow \pi^*$ electron transition of peptide bonds, enzyme activity in crude samples cannot be determined because of the huge background. Accordingly, also the high initial absorbance of large peptides hinders the determination of catalytic parameters for such QC substrates.

Due to these disadvantages, the development of new assays was triggered that allow (a) the convenient and fast determination of QC activity, making it suitable during protein purification and characterisation and (b) to easily determine the specificity of QCs for an assortment of substrates of different size and structure. This flexibility was obtained by developing coupled continuous assays that utilise different auxiliary enzymes. The inability of detecting the intramolecular amid transferase reaction in buffered systems could be compensated by well-observable coupled reactions, enabling detailed QC-characterisation studies such as substrate and inhibitor specificity, influence of ionic strength and pH-dependence of the kinetic parameters.

2. Coupled enzymatic assays – theoretical considerations

Enzyme catalysed reactions are most often analysed using spectrophotometric or fluorimetric detection, since the detectors, i.e. the photometers or fluorimeters, are relatively inexpensive and present in nearly all life science laboratories. However, many enzyme catalysed reactions cannot be monitored directly, since substrate conversion does not result in a change of the absorbance or fluorescence characteristics, as for instance the case in kinase-, phosphatase- or many transferase-catalysed reactions. Therefore, coupled enzymatic assays were established, using an auxiliary reaction that results in a change in absorbance or fluorescence. In the coupled reaction, one of the products of the reaction that should be analysed is consumed. The simplest case, which is also valid for the assays described below, can be represented schematically

For a reliable assay, the following assumptions have to be fulfilled [18]: (a) k_1 represents a zero-order rate constant, i.e., the concentration of A does practically not change during the observed reaction time,

(b) the second reaction is irreversible, and (c) k_2 is a rate constant of first order, which requires that the concentration of B is always much lower than the Michaelis constant of the auxiliary enzyme for B ($[B] \ll K_B$). Based on these assumptions, the rate equation focussed on formation and consumption of B, which is the prerequisite of the observed spectroscopic changes, is

$$\frac{d[B]}{dt} = k_1 - k_2[B] \quad (1)$$

which provides after integration

$$[B] = \frac{k_1}{k_2} (1 - e^{-k_2 t}). \quad (2)$$

As can be seen from this equation, if time runs to infinity ($t \rightarrow \infty$), a constant concentration of B is reached, the so-called steady state concentration $[B_{ss}]$, characterised by a linear progress of the formation of C. In a practical view, progress curves are usually indistinguishable from linearity, if 95% of the intermediate steady-state concentration is reached, which is sufficient for providing reliable results. The time to reach this state is characterised by a “lag phase”, the progress curve shows an exponential increase. After rearrangement of Eq. (2), substitution of $-V_{max2}t/K_B$ for $-k_2t$ and $[B_{ss}]$ for k_1/k_2 , the following equation is obtained, which offers the opportunity to calculate the time until reaching assay conditions that provide progress curves indistinguishable from linearity

$$V_{max2} = \frac{-K_B \ln(1 - [B]/[B_{ss}])}{t^*}. \quad (3)$$

In this equation, t^* denotes the time to reach a certain fraction of $[B_{ss}]$ (i.e., $[B]/[B_{ss}]$), which is dependent on the concentration and specificity of the auxiliary enzyme for its substrate, indicated by V_{max2}/K_B . Therefore, with knowledge of the specificity of the respective auxiliary enzyme, one can calculate the amount of protein required to obtain a reliable assay, without consuming excessive protein quantities. As follows, Eq. (3) was applied for the development of two different continuous assays for determination of QC activity.

3. Coupling QC to pyroglutamyl aminopeptidase (pGAP) catalysis – development

Coupling the cyclising activity of QC to a peptidase was accomplished by use of dipeptide surrogates that are prone to cleavage after conversion by QC. Accordingly, potential assay substrates possessing N-terminal glutamine are Gln-*p*NA, Gln- β NA or Gln-AMC. After cyclisation by QC, the respective intermediates pGlu-*p*NA, pGlu- β NA or pGlu-AMC are hydrolysed by pGAP, liberating a chromophoric or fluorogenic group. Since the spectrophores are liberated in equimolar amounts to the glutaminy-substrate converted, QC activity can be calculated from standard curves. The reactions are exemplified for the turnover of Gln- β NA in Fig. 1.

For assay development, the bacterial pyroglutamyl aminopeptidase from *Bacillus amyloliquefaciens* was chosen. This well-characterised cysteine protease shows a broad substrate specificity, suitable stability and is commercially available. With regard to specificity, the potential intermediates in QC assay have shown to be among the best substrates of this enzyme [19–22]. The time to reach virtual steady

Fig. 1. Representation of the coupled QC assay using Gln-βNA as substrate. In the initial reaction, Gln-βNA is converted by QC into pGlu-βNA. Subsequently, the intermediate is cleaved by the abundant pyroglutamyl aminopeptidase into pyroglutamic acid and the fluorophore 2-naphthylamine, resulting in an increase in the observed fluorescence.

Table 1

Times to reach virtual steady state conditions in QC assay by coupling to pyroglutamyl aminopeptidase (1 U/ml), calculated according to Eq. (3). The kinetic data for pGAP were obtained from references [20] and [21]^a

Substrate	K_B (mM)	$V_{\max 2}$ ($\mu\text{mol mg}^{-1} \text{min}^{-1}$)	Time to reach 95% [B _{ss}] in QC assay at 1 U/ml
pGlu- <i>p</i> NA	0.69	0.56	2 min
pGlu-AMC	0.33	5.7	6 s
pGlu-βNA	0.13	20.0	<1 s

^aThe unit definition refers to pGlu-*p*NA. One unit of pGAP is defined as 1 μmol substrate converted per min under the described conditions.

state conditions in the QC assay, if 1 U/ml auxiliary enzyme is applied, were calculated according to Eq. (3) using the available specificity data of pGAP (Table 1). Due to the relatively low specificity of pGAP towards the intermediate pGlu-*p*NA, the time until observation of linear progress curves is approximately 2 min for the chromophoric substrate Gln-*p*NA. In contrast, when using Gln-βNA as QC substrate, virtual steady state conditions are observed within one second after initiation of the reaction caused by the high specificity of pGAP for the intermediate pGlu-βNA.

In fact, linear progress curves were observed according to such calculation. They are exemplified for Gln-βNA and Gln-AMC in Fig. 2. For all substrates, there was a linear relationship between the QC concentration and the observed rate, indicating the linear dependence of the assay on conversion of the QC substrate (not shown). Finally, the assay could be applied for recombinant human or mouse and purified papaya QC. The now possible characterisation studies enabled the comparison of the QC forms concerning differences and similarities of their catalysis. Due to the shorter lag times observed with the fluorogenic substrates (Table 1), the assays using Gln-AMC or Gln-βNA provide a higher flexibility. Small alterations in the activity of the auxiliary enzyme do not affect the assay, because the auxiliary enzyme activity is still excessive to provide reliable results, i.e., the “lag times” are always shorter than the time required for starting the reaction and mixing of the samples. Therefore, most of the characterising studies shown below were carried out using the fluorogenic substrates.

Fig. 2. Progress curves of the conversion of Gln- β NA and Gln-AMC by QC, investigated by coupling the reaction to pGAP catalysis. According to a calculation (Eq. (3)), linear progress curves were observed directly after initiation of the reaction. Assays were carried out in 0.05 M Tris/HCl, pH 8.0 at 30°C. The substrate and QC concentrations were 0.25 mM and 0.9 nM, respectively.

4. Coupling QC to pyroglutamyl aminopeptidase (pGAP) catalysis – application

Applications of spectroscopic enzyme assays range from the identification of enzyme activity in tissues, quantification of enzymatic activity during protein purification, protein characterisation in terms of pH-dependence of catalysis, substrate specificity and for inhibitor screening. Recently, we applied the assay during purification of papaya QC [23]. Although the continuous data monitoring already accelerated the enzyme determination during the purification procedure, its application is much more important during characterisation studies, since many assay reactions have to be performed, thus favouring the continuous assays. Moreover, only the analysis of kinetic parameters investigating a wide substrate concentration range makes it possible to detect differences in kinetic mechanisms or models.

Hence, the plots of substrate concentration versus the respective velocities obtained for Gln-AMC and Gln- β NA follow different kinetic laws. Whereas the kinetic data for Gln-AMC readily resembled Michaelis–Menten kinetics in the concentration range limited by substrate solubility, Gln- β NA showed discernible substrate inhibition (Fig. 3). Interestingly, papaya QC showed a higher specificity for Gln- β NA, but was inhibited by the substrate with similar potency. To our knowledge, Gln- β NA is the only QC substrate showing deviations from Michaelis–Menten-kinetics, which could be indicative for a similar catalytic action of both enzymes.

Subsequently, the pH-dependence of the catalytic parameters k_{cat} and K_{M} for conversion of Gln-AMC by human QC was investigated. The pH-range used was restricted to 5.5–8.5, due to the limited stability of QC and the auxiliary enzyme at more basic and acidic pH. Similar experiments were already performed with papaya QC using Gln-OtBu as substrate [17], revealing that the catalytic activity depends only on changes of substrate binding. Apparently, the substrate having a protonated amino group was not bound by the active site. The rate constant k_{cat} did not change in the investigated pH-range. Also human QC catalysis exhibited only a dependence in terms of substrate binding, reflected by a pH-dependent change of K_{M} , and a pH-independent k_{cat} (Fig. 4). Fitting of the pH-dependent kinetic data of K_{M} to an equation that accounts for two dissociating groups revealed a $\text{p}K_{\text{a}}$ -value that is very close to the $\text{p}K_{\text{a}}$ of the substrate amino group and a second $\text{p}K_{\text{a}}$, probably representing a dissociating group of the enzyme. Thus, human and papaya QC bind only N-terminally unprotonated substrate molecules in a catalytically

Fig. 3. Dependence of the conversion-rate of Gln- β NA from the substrate concentration, determined for human ($\circ$) and papaya QC ($\square$). The resulting graphs were obtained by fitting the data to the general equation of substrate inhibition. Human QC ($K_M = 70 \pm 3 \mu\text{M}$, $k_{\text{cat}} = 21 \pm 1 \text{ s}^{-1}$, $K_i = 1.21 \pm 0.07 \text{ mM}$) showed a reduced specificity compared to papaya QC ($K_M = 36 \pm 2 \mu\text{M}$, $k_{\text{cat}} = 49 \pm 1 \text{ s}^{-1}$, $K_i = 1.14 \pm 0.05 \text{ mM}$), but the catalysis was inhibited to similar extends by the substrate. Reactions were carried out in 0.05 M Tris/HCl, pH 8.0 (human QC) or 0.05 M Tricine/NaOH, pH 8.0 (papaya QC) at 30°C.

Fig. 4. The pH-dependence of Gln-AMC conversion by human QC. At varying pH-values, the kinetic parameters K_M and k_{cat} were determined, and the logarithmic values plotted. Whereas the k_{cat} -value ($\square$) was independent from pH, the K_M -values ($\bullet$) increased in the acidic and basic pH-region. Fitting the data to an equation that accounts for two dissociating groups resulted in $\text{p}K_a$ -values of 6.81 ± 0.04 and 8.6 ± 0.1 . The former value is in good agreement with the $\text{p}K_a$ of the substrate whereas the latter probably reflects a dissociating group of the enzyme. Reactions were carried out in a buffer system providing a constant ionic strength over the entire pH-range, consisting of 0.06 M acetic acid, 0.06 M Mes and 0.12 M Tris [25] at 30°C.

productive manner, indicating some general similarity in the catalytic mechanism, in spite of a lack of structural homology.

Differences, however, were observed in the binding of inhibitory compounds. Whereas papaya QC was inhibited by peptides bearing N-terminal proline [12], human QC was not. We found, however, competitive inhibition of human QC by peptides bearing N-terminal γ -glutamyl-hydrazide residues (Fig. 5). Furthermore, human QC was also inhibited by imidazole derivatives which contrasts with plant QC (not shown). These results suggest differences of plant and human QC concerning substrate conversion, apparently due to differences in the substrate and inhibitor recognition modes [26].

Fig. 5. The array of curves obtained for the conversion of Gln-AMC by human QC in presence of varying concentrations of the inhibitory active peptide H-Glu(NH-NH₂)-Ser-Pro-Thr-Ala-NH₂. Data points were fitted according to the general equation for competitive inhibition. The resulting K_i -value was 0.697 ± 0.003 mM. The assay was carried out in 0.05 M Tris/HCl, pH 8.0, containing 5 mM EDTA. The substrate concentrations ranged from 1 mM to 0.125 mM.

Although the described assays have shown to be suitable for many applications, there is one major disadvantage. Only QC substrates can be used, whose conversion yields finally chromophoric or fluorogenic groups. Thus, the substrate spectrum is limited to variations of the chromophores and fluorophores, which hampers a detailed substrate specificity investigation. Therefore, alternative assays were needed, that overcome this drawback without waiving a continuous data monitoring.

5. Coupling QC to glutamic dehydrogenase (GDH) catalysis – development and application of an alternative assay

This assay is based on quantification of ammonia, that is liberated by cyclisation of glutamine. The auxiliary enzyme in this assay is glutamic dehydrogenase, converting ammonia, α -ketoglutaric acid and NADH/H⁺ into glutamic acid and NAD⁺. Since the absorbance characteristics of NADH/H⁺ changes by oxidation, its conversion can be followed at 340 nm. QC activity can be subsequently quantified by calculation of the liberated ammonia from standard curves of ammonia under assay conditions. The assay reactions are illustrated in Fig. 6.

Originally, the assay was developed as a discontinuous method [16], probably due to the relatively low affinity of GDH towards ammonia. In turn, this low reactivity leads to very high auxiliary enzyme concentrations necessary to implement conditions that enable a continuous data monitoring according to Eq. (3). The usage of common cuvettes for spectrophotometers requiring sample volumes of 1–2 ml, however, causes the consumption of tremendous amounts of GDH, making the assay prohibitive. A calculation of the required auxiliary enzyme amount (Eq. (3)) resulted in 30 U/ml GDH needed to reach a virtual steady state 20 s after initiation of the reaction (assuming a K_M of 3.2 mM of GDH for ammonia [24] and that one unit of GDH refers to saturating concentrations of all substrates). Due to implementation of microplate readers for assay development, it was possible to reduce the assay volume to 250 μ l, thus keeping the required auxiliary enzyme amount low, but still providing convenient volumes for pipetting. Furthermore, the use of the microplates, that were already applied in the fluorometric assays,

Fig. 6. Representation of the QC-assay using GDH as auxiliary enzyme and an *N*-terminal glutaminyl peptide as substrate. In the initial reaction, the respective pyroglutamyl peptide and ammonia are formed. Subsequently, ammonia, α -ketoglutaric acid and NADH/H^+ are converted into glutamic acid and NAD^+ catalysed by GDH. The consumption of NADH/H^+ can be observed at 340 nm.

Fig. 7. Linear dependence of the initial rate of conversion on concentration of human QC using GDH as auxiliary enzyme. The inset shows two progress curves, in the sample containing human QC (12 nM), a linear decrease of absorbance was observed. Without added QC, the decrease in absorbance was negligible. Reactions were carried out in 0.05 M Tris/HCl pH 8.0, containing 5 mM EDTA.

enable a fast determination of QC activity in many samples at the same time, thus accelerating the determinations enormously. Finally, due to the detection of ammonia that is liberated from the QC substrates the assay can be implemented for a fast examination of a variety of glutaminyl-peptides.

In fact, linear progress curves were obtained according to the predicted conditions, and most importantly there was a linear relationship between QC concentration and initial velocity, indicating that the assay provides reliable results (Fig. 7). Subsequently, a detailed substrate specificity investigation was performed using about 40 newly synthesised substrates [27], showing that the assay is applicable in-

Fig. 8. The influence of ionic strength on the specificity constant $k_{\text{cat}}/K_{\text{M}}$ for conversion of various substrates by human and papaya QC. For most peptides, there was little effect of changes in ionic strength detected. However, human and papaya QC specificity towards positively charged peptides increased significantly by addition of 0.5 M KCl. Without additional salt added, the ionic strength was 0.029 M, corresponding to a 0.05 M Tris- or Tricine buffer.

dependently from changes of substrate amino acid composition and peptide size. Moreover, since the auxiliary enzyme was not influenced significantly by potassium chloride concentrations up to 0.5 M, the method was also implemented to investigate the influence of ionic strength on conversion of different substrates. Interestingly, the observed changes of the specificity constant $k_{\text{cat}}/K_{\text{M}}$ were substrate dependent (Fig. 8). Neither papaya QC, nor human QC revealed altered activity by increasing ionic strength towards peptides with uncharged backbone and residues. Both enzymes, however, displayed a significant increase in activity towards peptides comprising positively charged amino acid residues. Thus, besides the similar pH-dependence of catalysis for both enzymes, also the behaviour of the different QC-forms in environments with differing dielectric constants is similar, which could be also indicative for analogous catalytic mechanisms. Finally, this conclusion was also corroborated by the ability of both, human and papaya QC, to cyclise N-terminal β -homoglutaminyl residues with the same catalytic efficiency (not shown).

6. Conclusions

The application of theoretical deductions [18] facilitated the development of the first coupled enzymatic assays for glutaminyl cyclase (QC) activity. Due to the use of different enzymatic reactions for coupling to QC catalysis, many characterisation studies could be performed including pH-dependence, inhibitory and substrate specificity. In this regard, the general differences in the coupling strategy, i.e., the consumption of the QC products, either the pyroglutamyl peptide by pGAP or the liberated ammonia by GDH, led to a compensation of respective disadvantages of both assays. For instance, traces of ammonia in a sample hamper the QC determination in the GDH-coupled assay, but show no effect in the assays using pGAP, resulting in the preferred usage of the latter enzyme for assays during enzyme purification. When investigating different peptide substrates, however, only the coupling to ammonia production provided satisfying results since a large substrate spectrum that can be investigated. Thus, the use of the different assay coupling strategies enabled the convenient determination of QC activity in different fields of protein characterisation.

Finally, the demonstrated strategy to develop continuous assay techniques could also be used to modify discontinuous assays for other enzymes or to develop new ways for their catalytic characterisation by implementing different coupling strategies.

Acknowledgements

We thank K. Zunkel, H. Cynis and J. Zwanzig for valuable technical assistance.

References

- [1] M. Messer, Enzymatic cyclization of L-glutamine and L-glutaminyl peptides, *Nature* **4874** (1963), 1299.
- [2] M. Messer and M. Ottesen, Isolation and properties of glutamine cyclotransferase of dried papaya latex, *Biochim. Biophys. Acta* **92** (1964), 409–411.
- [3] W.H.J. Busby, G.E. Quackenbush, J. Humm, W.W. Youngblood and J.S. Kizer, An enzyme(s) that converts glutaminyl-peptides into pyroglutamyl-peptides. Presence in pituitary, brain, adrenal medulla, and lymphocytes, *J. Biol. Chem.* **262** (1987), 8532–8536.
- [4] W.H. Fischer and J. Spiess, Identification of a mammalian glutaminyl cyclase converting glutaminyl into pyroglutamyl peptides, *Proc. Natl. Acad. Sci. USA* **84** (1987), 3628–3632.
- [5] S.W. Dahl, C. Slaughter, C. Lauritzen, R.C. Bateman, Jr., I. Connerton and J. Pedersen, Carica papaya glutamine cyclotransferase belongs to a novel plant enzyme subfamily: cloning and characterization of the recombinant enzyme, *Protein Expr. Purif.* **20** (2000), 27–36.
- [6] E. Van Coillie, P. Proost, I. Van Aelst, S. Struyf, M. Polfliet, I. De Meester, D.J. Harvey, J. Van Damme and G. Opdenakker, Functional comparison of two human monocyte chemotactic protein-2 isoforms, role of the amino-terminal pyroglutamic acid and processing by CD26/dipeptidyl peptidase IV, *Biochemistry* **37** (1998), 12672–12680.
- [7] G.N. Abraham and D.N. Podell, Pyroglutamic acid. Non-metabolic formation, function in proteins and peptides, and characteristics of the enzymes effecting its removal, *Mol. Cell. Biochem.* **38** (Spec. No) (1981), 181–190.
- [8] S.A. Hinke, J.A. Pospisilik, H.-U. Demuth, S. Manhart, K. Kühn-Wache, T. Hoffmann, E. Nishimura, R.A. Pedersen and C.H.S. McIntosh, Dipeptidyl peptidase IV (DPIV/CD26) degradation of Glucagon, *J. Biol. Chem.* **275** (2000), 3827–3834.
- [9] S. Zerhouni, A. Amrani, M. Nijs, N. Smolders, M. Azarkan, J. Vincentelli and Y. Looze, Purification and characterization of papaya glutamine cyclotransferase, a plant enzyme highly resistant to chemical, acid and thermal denaturation, *Biochim. Biophys. Acta* **1387** (1998), 275–290.
- [10] T. Pohl, M. Zimmer, K. Mugele and J. Spiess, Primary structure and functional expression of a glutaminyl cyclase, *Proc. Natl. Acad. Sci. USA* **88** (1991), 10059–10063.
- [11] A.P. Consalvo, S.D. Young, B.N. Jones and P.P. Tamburini, A rapid fluorometric assay for N-terminal glutaminyl cyclase activity using high-performance liquid chromatography, *Anal. Biochem.* **175** (1988), 131–138.
- [12] M.Y. Gololobov, W. Wang and R.C.J. Bateman, Substrate and inhibitor specificity of glutamine cyclotransferase (QC), *Biol. Chem. Hoppe Seyler* **377** (1996), 395–398.
- [13] K.A. Oberg, J.M. Ruysschaert, M. Azarkan, N. Smolders, S. Zerhouni, R. Wintjens, A. Amrani and Y. Looze, Papaya glutamine cyclase, a plant enzyme highly resistant to proteolysis, adopts an all-beta conformation, *Eur. J. Biochem.* **258** (1998), 214–222.
- [14] S. Schilling, T. Hoffmann, F. Rosche, S. Manhart, C. Wasternack and H.U. Demuth, Heterologous expression and characterization of human glutaminyl cyclase: evidence for a disulfide bond with importance for catalytic activity, *Biochemistry* **41** (2002), 10849–10857.
- [15] J.B. Koger, J. Humm and J.S. Kizer, Assay of glutaminylpeptide cyclase, *Methods Enzymol.* **168** (1989), 358–365.
- [16] R.C.J. Bateman, A spectrophotometric assay for glutaminyl-peptide cyclizing enzymes, *J. Neurosci. Methods* **30** (1989), 23–28.
- [17] M.Y. Gololobov, I. Song, W. Wang and R.C.J. Bateman, Steady-state kinetics of glutamine cyclotransferase, *Arch. Biochem. Biophys.* **309** (1994), 300–307.
- [18] W.R. McClure, A kinetic analysis of coupled enzyme assays, *Biochemistry* **8** (1969), 2782–2786.
- [19] K. Fujiwara, R. Kobayashi and D. Tsuru, The substrate specificity of pyrrolidone carboxyl peptidase from *Bacillus amyloliquefaciens*, *Biochim. Biophys. Acta* **570** (1979), 140–148.
- [20] K. Fujiwara and D. Tsuru, New chromogenic and fluorogenic substrates for pyrrolidonyl peptidase, *J. Biochem. (Tokyo)* **83** (1978), 1145–1149.

- [21] D. Tsuru, K. Fujiwara and K. Kado, Purification and characterization of L-pyrrolidonecarboxylate peptidase from *Bacillus amyloiliquefaciens*, *J. Biochem. (Tokyo)* **84** (1978), 467–476.
- [22] P.M. Cummins and B. O'Connor, Pyroglutamyl peptidase: an overview of the three known enzymatic forms, *Biochim. Biophys. Acta* **1429** (1998), 1–17.
- [23] S. Schilling, T. Hoffmann, M. Wermann, U. Heiser, C. Wasternack and H.-U. Demuth, Continuous spectrometric assays for glutaminyl cyclase activity, *Anal. Biochem.* **303** (2002), 49–56.
- [24] C. Frieden, Glutamic dehydrogenase. III. The order of substrate addition in the enzymatic reaction, *J. Biol. Chem.* **234** (1959), 2891–2896.
- [25] K.J. Ellis and J.F. Morrison, Buffers of constant ionic strength for studying pH-dependent processes, *Methods Enzymol.* **87** (1982), 405–426.
- [26] S. Schilling, S. Manhart, T. Hoffmann, H.-H. Ludwig, C. Wasternack and H.-U. Demuth, Substrate specificity of glutaminyl cyclases from plants and animals, *Biol. Chem.* **384** (2003), 1583–1592.
- [27] S. Schilling, A.J. Niestroj, J.-U. Rahfeld T. Hoffmann, M. Wermann, K. Zunkel, C. Wasternack and H.-U. Demuth, Potent and specific inhibition of human glutaminyl cyclase by imidazole derivatives reveals it as metalloenzyme, *J. Biol. Chem.* **278** (2003), 49773–49779.