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Continuous Spectrometric Assays for Glutaminyl Cyclase Activity

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The enzymatic conversion of one chromogenic substrate, L-glutamine-*p*-nitroanilide, and two fluorogenic substrates, L-glutaminyl-2-naphthylamide and L-glutaminyl-4-methylcoumarinylamide, into their respective pyroglutamic acid derivatives by glutaminyl cyclase (QC) was estimated by introducing a new coupled assay using pyroglutamyl aminopeptidase as the auxiliary enzyme. For the purified papaya QC, the kinetic parameters were found to be in the range of those previously reported for other glutaminyl peptides, such as Gln-Gln, Gln-Ala, or Gln-*tert*-butyl ester. The assay can be performed in the presence of ammonia up to a concentration of 50 mM. Increasing ionic strength, e.g., potassium chloride up to 300 mM, resulted in an increase in enzymatic activity of about 20%. This is the first report of a fast, continuous, and reliable determination of QC activity, even in the presence of ammonium ions, during the course of protein purification and enzymatic analysis.

Several bioactive peptides and proteins (e.g., TRH,[2] IgG, GnRH) contain a pyroglutamate residue at their N-terminal position. This feature was assumed to result from a spontaneous cyclization reaction of the N-terminal glutamine residue. However, enzymatic conversion by glutaminyl cyclase (QC; EC 2.3.2.5) could be shown (1–3).

Up to now, papaya latex has been the only plant source, in which this enzyme is found to be abundant, whereas several mammalian tissues have been shown to express QC (4, 5).

Little is known about the biological role of QC. It has been suggested that QC is responsible for modification of storage proteins during seed germination (6), as well as *in vivo* modification of bioactive peptides such as glucagon, MCP-2, TRH, and GnRH (4, 7, 8). These findings support the idea that the QC-catalyzed reaction might be important for protection of the N-terminus of bioactive peptides against exopeptidases. Moreover, this enzymatically catalyzed N-terminal formation of a pyroglutamic acid residue could be important in developing the proper receptor binding conformation of such peptides.

Interestingly, a number of pyroglutamate peptides are formed in tissues, although no QC activity has been detected there thus far, indicating a need for more detailed investigations (9). As an initial step in elucidating the function of QC in plants or animals, expression studies for various tissues and enzyme purification for further characterization are essential, and both require a reliable assay. In previously applied methods, QC activity was determined either by analyzing the products formed using HPLC (3, 10) or radioimmunoassay (11) or by detecting the release of ammonia spectrophotometrically (12). In the latter assay, the QC-catalyzed cyclization of the N-terminal glutamine residue is quantified by coupling the reaction with the conversion of $NADH/H^+$ to NAD^+ by glutamate dehydrogenase. Avoidance of any ammonium traces is an essential prerequisite, making this assay difficult to handle in some enzyme purification steps. Although the aforementioned methods are sensitive, they are all

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[2] Abbreviations used: Boc, *tert*-butyloxycarbonyl; Gln-AMC, L-glutaminyl-4-methylcoumarinylamide; Gln-βNA, L-glutaminyl-2-naphthylamide; GnRH, gonadotropin-releasing hormone; MCP-2, monocyte chemotactic protein-2; QC, glutaminyl cyclase; *p*NA, *p*-nitroaniline; TEA, triethylamine; THF, tetrahydrofuran; TRH, thyrotropin-releasing hormone; IgG, immunoglobulin G.

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discontinuous and therefore time consuming and laborious. As a result of these disadvantages, we developed new continuous assays which allow determination of QC activity during purification and characterization. In contrast to the assays mentioned above, these new methods use the glutaminyl derivatives of *p*-nitroaniline, 7-amino-4-methylcoumarin, and 2-naphthylamine as substrates. Once cyclized into the respective pyroglutamic acid derivatives by QC, they are subsequently cleaved by pyroglutamyl aminopeptidase. The resulting liberation of *p*-nitroaniline, 7-amino-4-methylcoumarin, or 2-naphthylamine allows the reliable and convenient determination of QC activity.

MATERIALS AND METHODS

Materials

Lyophilized papaya latex and *S*-methylmethane thiosulfonate were purchased from Fluka (Seelze, Germany). Pyroglutamic acid *p*-nitroanilide and molecular mass standards for SDS–PAGE were provided by Sigma (Deisenhofen, Germany). Gln-AMC and Gln-βNA were from Bachem (Bubendorf, Switzerland). SP–Sepharose Fast Flow and Butyl–Sepharose 4 Fast Flow were obtained from Pharmacia Biotech (Uppsala, Sweden). Boc-L-glutamine was supplied by Bachem (Heidelberg, Germany). Pyroglutamyl aminopeptidase from *Bacillus amyloliquefaciens*, recombinantly expressed in *Escherichia coli*, was purchased from Unizyme Laboratories (Hørsholm, Denmark) and Tris as well as Tricine from Serva (Heidelberg, Germany).

Synthesis of Gln-pNA

Boc-Gln-pNA. Boc-Gln-OH (2.46 g; 10 mmol) was dissolved in THF (20 ml) by adding 0.81 ml (10 mmol) pyridine and 1.39 ml (10 mmol) TEA and solvation was completed by a brief warming of the solution. After the mixture was cooled down to −10°C, 1.23 ml (10 mmol) pivaloyl chloride was added, and the clear solution was stirred for 10 min at 0°C. Upon completed formation of the mixed anhydride, 1.311 g (9.5 mmol) *p*-nitroaniline was added. Subsequently, the mixture was stirred for 1 h at 0°C and left overnight at room temperature. In the usual workup the solvent was removed, and the residue was partitioned between ethyl acetate and aqueous HCl (15% in water), followed by a subsequent washing with brine, saturated aqueous solution of $KHCO_3$, and brine again. The organic layer was separated and the solvent was removed. Chromatography on silica gel using a $CHCl_3$:MeOH (1:3, v/v) gradient resulted in a pure Boc-Gln-*p*NA as a faint yellow oil. The overall yield was 2.93 g (80%).

*H-Gln-pNA*HCl.* Boc-Gln-*p*NA was treated with 20 ml HCl solution (4 N in dioxane) until the starting material was no longer detectable. After solvent removal, the resulting material was recrystallized from methanol/ether. The yield was 2.17 g (90%), HPLC purity 99.35% (water/ACN/TFA). ^{13}C NMR (CD_3OD) δ 24.95 (CH_2), 30.11 (CH_2), 53.27 ($CH—NH_3^+$), 121.55 (CH═phenyl), 122.4 (CH═phenyl), 143.87 (═C═phenyl), 147.23 (═C—NO_2), 161.65 (CONH), 173.88 (CONH).

Purification of QC from Papaya Latex

QC from papaya latex was prepared using the BioCAD 700E (Perceptive Biosystems, Wiesbaden, Germany) with a modified version of a previously reported method (13). Fifty grams of latex was dissolved in water and centrifuged as described (13). Inactivation of proteases was performed with *S*-methylmethane thiosulfonate, and the resultant crude extract was dialyzed (13, 23).

SP–Sepharose Fast Flow. After dialysis, the entire supernatant was loaded onto a (21 × 2.5-cm i.d.) SP–Sepharose Fast Flow column, equilibrated with 100 mM sodium acetate buffer, pH 5.0 (flow rate 3 ml/min). Elution was performed in three steps by increasing the sodium acetate buffer concentration at a flow rate of 2 ml/min. The first step was a linear gradient from 0.1 to 0.5 M acetate buffer in 0.5 column volume. The second step was a linear increase in buffer concentration from 0.5 to 0.68 M in 4 column volumes. During the last elution step, 1 column volume of 0.85 M buffer was applied. Fractions (6 ml) containing the highest enzymatic activity were pooled. Concentration and buffer changes to 0.02 M Tris/HCl, pH 8.0, were performed via ultrafiltration (Amicon; molecular mass cut-off of the membrane 10 kDa).

Butyl–Sepharose 4 Fast Flow. Ammonium sulfate was added to the concentrated papaya enzyme, obtained from the ion-exchange chromatography step to a final concentration of 2 M. This solution was applied onto a (21 × 2.5-cm i.d.) Butyl–Sepharose 4 Fast Flow column (flow rate 1.3 ml/min), equilibrated with 2 M ammonium sulfate, 0.02 M Tris/HCl, pH 8.0. Elution was performed in three steps with decreasing concentrations of ammonium sulfate. During the first step a linear gradient from 2 to 0.6 M ammonium sulfate, 0.02 M Tris/HCl, pH 8.0, was applied for 0.5 column volume at a flow rate of 1.3 ml/min. The second step was a linear gradient from 0.6 to 0 M ammonium sulfate, 0.02 M Tris/HCl, pH 8.0, in 5 column volumes at a flow rate of 1.5 ml/min. The last elution step was carried out by applying 0.02 M Tris/HCl at pH 8.0 for 2 column volumes at a flow rate of 1.5 ml/min. All fractions containing QC activity were pooled and concentrated by ultrafiltration. The resultant homogeneous QC was stored at −70°C. Final protein concentrations were determined using the method of Bradford (14),

compared to a standard curve obtained with bovine serum albumin.

Assays

All measurements were performed with the BioAssay Reader HTS 7000Plus for microplates (Perkin–Elmer) at 30°C.

Spectrophotometric assay for QC. The assay consisted of the chromogenic substrate Gln-*p*NA (1 mM), pyroglutamyl aminopeptidase (0.25 U), and an appropriate amount of QC in a final volume of 0.25 ml buffer (0.05 M Tricine/NaOH, pH 8.0). This pH was reported to be within the optimal range for the catalysis of both QC and pyroglutamyl aminopeptidase (9, 15). Due to the composition of the storage buffer of pyroglutamyl aminopeptidase, assay mixtures contained cysteamine (0.1 mM), sodium chloride (2 mM), EDTA (0.1 mM), and 2% (v/v) glycerol.

Reactions were initiated by addition of QC and preincubated for 2 min, and subsequently absorption was monitored at 405 nm for 8–15 min. QC activity was calculated using an absorption coefficient of 6710 L/mol, which was determined from a standard curve of *p*-nitroaniline under assay conditions.

Fluorometric assays for QC. Glutaminyl cyclase activity was evaluated fluorometrically using either Gln-AMC or Gln-βNA. The samples consisted of the fluorogenic substrate (0.05 mM), 0.25 U pyroglutamyl aminopeptidase in 20 mM Tris/HCl, pH 8.0, containing 200 mM potassium chloride, and an appropriately diluted aliquot of papaya glutaminyl cyclase. Excitation/emission wavelengths were 380/465 nm in the case of Gln-AMC and 320/410 nm in the case of Gln-βNA. The assay reactions were initiated by addition of glutaminyl cyclase. Monitoring of progress curves was started immediately after initiation. QC activity was determined from standard curves of 7-amino-4-methylcoumarin and β-naphthylamine under assay conditions.

Assay for pyroglutamyl aminopeptidase. Measurements of pyroglutamyl aminopeptidase activity were carried out using pGlu-*p*NA as substrate. In order to maintain conditions identical to those used in the QC assay, samples (250 μl) contained 0.1 mM cysteamine, 2 mM sodium chloride, 0.1 mM EDTA, 2 mM pGlu-*p*NA (stock solution in dimethyl sulfoxide), 2% (v/v) glycerol, and an diluted aliquot of pyroglutamyl aminopeptidase in 0.05 M Tricine/NaOH, pH 8.0. The final concentration of 1% (v/v) dimethyl sulfoxide did not interfere with the activity of pyroglutamyl aminopeptidase. One unit is defined as the amount of enzyme that hydrolyzes 1 μmol pGlu-*p*NA per minute under the conditions described. The activity was expressed in units using an absorption coefficient of 6690 L/mol, which was obtained from a standard curve. The specific activity of the enzyme preparation was approximately 4 units/mg. The pyroglutamyl aminopeptidase was stored in a solution consisting of Tris/HCl (6 mM), cysteamine (2 mM), sodium chloride (40 mM), EDTA (2 mM), 50% glycerol, pH 8.0. The enzyme was stable for several months at −20°C.

RESULTS AND DISCUSSION

Spectrophotometric Assay

The spectrophotometric assay is based on the detection of *p*-nitroaniline at 405 nm, one of the products generated in the coupled assay. The first reaction is the conversion of Gln-*p*NA into pGlu-*p*NA, catalyzed by QC. pGlu-*p*NA, in turn, is hydrolyzed in the second reaction by the abundant pyroglutamyl aminopeptidase, which leads to the terminal products pyroglutamate and *p*NA. Thus, QC is the enzyme being analyzed, and pyroglutamyl aminopeptidase represents the auxiliary enzyme. *p*-Nitroaniline is released in equimolar amounts to the Gln-*p*NA converted by QC. Hence, QC activity is directly related to the amount of *p*-nitroaniline released and can therefore easily be quantified. For estimation of the conversion rate from Gln-*p*NA to pGlu-*p*NA, this first step has to be rate determining for the complete assay. Previous theoretical investigations on the kinetics of irreversible coupled enzyme assays showed the possibility of calculating the required amount of an auxiliary enzyme for the development of a reliable method (16). The calculation was performed according to Eq. [1],

$$V_2 = -K_{m2} \times \ln(1 - [\text{pGlu-}p\text{NA}]_t/[\text{pGlu-}p\text{NA}]_{SS})/t, \quad [1]$$

where K_{m2} is the Michaelis constant and V_2 the maximal velocity of the reaction catalyzed by the auxiliary enzyme, pyroglutamyl aminopeptidase. $[\text{pGlu-}p\text{NA}]_t$ represents the concentration of pGlu-*p*NA at time t after initiation of the reaction. $[\text{pGlu-}p\text{NA}]_{SS}$ describes the steady-state concentration of pGlu-*p*NA under these conditions. Equation [1] is valid if the initial substrate concentration does not change significantly during the considered time and the second reaction follows a first-order rate law ($[\text{pGlu-}p\text{NA}]_{SS} \ll K_{m2}$). Using the known K_{m2}, the required amount of auxiliary enzyme can be calculated from V_2. We determined a K_{m2} value of 710 ± 50 μM for pyroglutamyl aminopeptidase and pGlu-*p*NA under the assay conditions, which corresponds to data obtained from the literature (17). Using this value and assuming that the definition of 1 unit of auxiliary enzyme refers to saturated substrate concentrations, we estimated 0.25 U pyroglutamyl aminopeptidase to be required in the sample volume to reach 95% of the steady-state concentration of pGlu-*p*NA after 130 s. Thus, linear progress curves

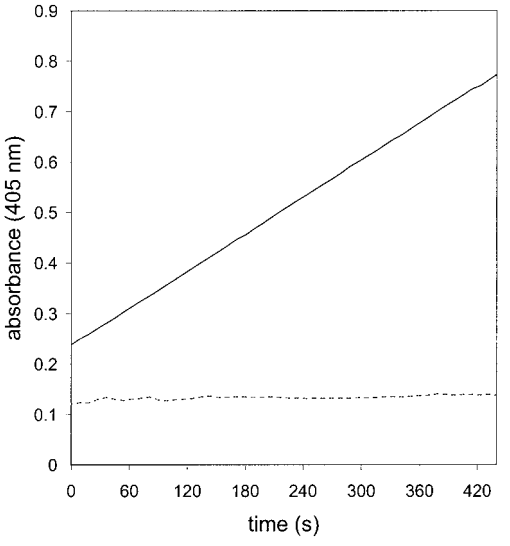

FIG. 1. Progress curves of *p*NA formation from Gln-*p*NA, monitored by the increase in absorption at 405 nm. No increase in absorption was detected in the absence of QC (dotted trace). Linear product formation was observed in the presence of QC (solid trace). Reactions were initiated by the addition of QC, preincubated for 2 min, and subsequently the absorption was monitored. The assay conditions were Gln-*p*NA (1 mM), pyroglutamyl aminopeptidase (0.25 U), and QC (3×10^{-3} U) in 50 mM Tricine/NaOH, at pH 8.0, 30°C. The final sample volume was 250 μl.

were expected after a lag time of 2 min, independent of the concentration of Gln-*p*NA.

As shown in Fig. 1, Gln-*p*NA is recognized as a substrate for QC, demonstrated by an increase in absorption at 405 nm. There was no increase in absorption without QC, indicating that formation of *p*-nitroaniline is dependent on the presence of QC. Linear product formation was observed at 0.25 U pyroglutamyl aminopeptidase and a preincubation time of 120 s, verifying the reliability of the assay and the calculation made above regarding the excess of pyroglutamyl aminopeptidase required. To counteract rapid substrate consumption during preincubation, the concentration of QC was adjusted precisely by dilution. Based on this, the assay was carried out at an activity concentration of 1 unit/ml pyroglutamyl aminopeptidase, giving reproducible progress curves of glutaminyl cyclase activity under the conditions used. Increasing the amount of pyroglutamyl aminopeptidase shortened the lag time, but had no influence on the slope of the progress curves.

In addition, there was a linear relationship between the initial velocity and the concentration of QC (Fig. 2). This confirms that the rate of catalysis is dependent on the QC-mediated conversion of Gln-*p*NA to pGlu-*p*NA.

The feasibility of the novel assay was tested during the QC purification from papaya latex, demonstrated here by determination of QC activity in fractions of the first purification step (Fig. 3). Highly purified QC was generated by only two separation steps. A rapid purification was reached by implementing a multilevel gradient in the initial ion-exchange chromatography followed by a hydrophobic interaction chromatography. Similar to the purification procedure described by Zerhouni *et al.* (13), QC was eluted among the last enzymes that can be purified from papaya by cation-exchange chromatography. This is somewhat surprising since the proteins should be eluted in the order of their increasing isoelectric points (p*I*). However, the QC of our preparation showed a p*I* of 9.4–9.6, determined by isoelectric focusing in agarose gels (data not shown). This value is more alkaline than the reported isoelectric point of papain (p*I* 8.75), but noticeably more acidic than that of papaya proteinase A (p*I* 11) (18), the most basic of the papaya proteinases (19). The reasons for the anomalous order of elution were not examined in detail, but the phenomenon might be explained by assuming that highly basic regions on the surface of QC may account for strong electrostatic interactions with the cation exchange resin. The high hydrophobicity reported previously (13) might be attributed to other regions, established in the correctly folded protein. Although the overall yield of the purification presented here was approximately 25%, three times less than that previously published (13), our strategy was much less time consuming. Hence, the reduced overall yield of the purification can be easily compensated by the yield of QC in the latex of *C. papaya*. Thirty-five milligrams of homogeneous QC was obtained from

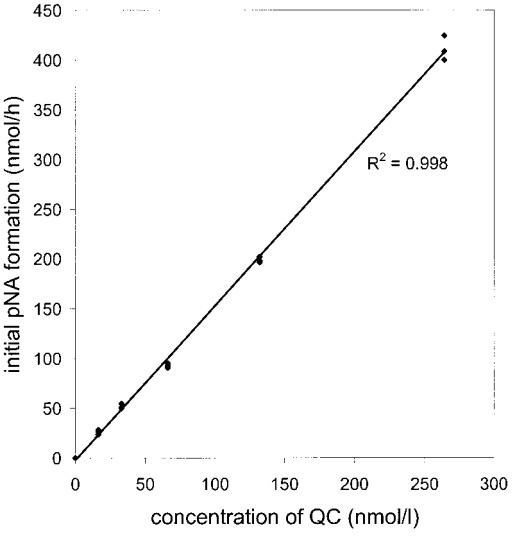

FIG. 2. Dependence of *p*NA formation on the concentration of QC in the sample. The samples contained various amounts of QC between 16.5 and 264 nmol/L, prepared by the dilution of a stock solution. The protein concentration was assayed using the method of Bradford (14) and calculated using a molecular mass of 33 kDa for QC. For assay conditions, see Materials and Methods.

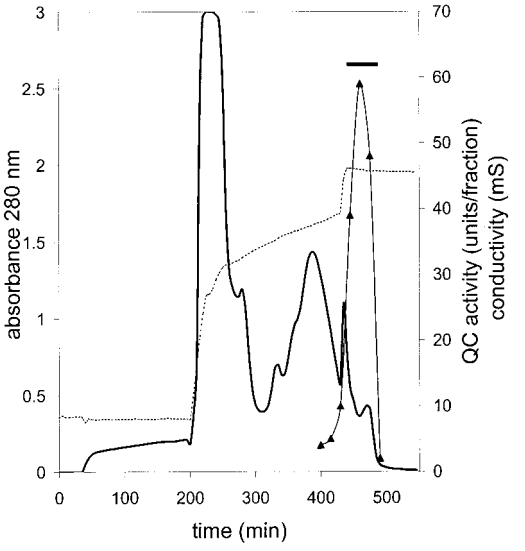

FIG. 3. Elution profile of QC during ion-exchange chromotography. Lyophilized papaya latex was dissolved, and proteases were inactivated using *S*-methylmethane thiosulfonate and centrifuged as described elsewhere (13, 23). The supernatant after centrifugation was applied onto a SP–Sepharose Fast Flow column and proteins were eluted as described under Materials and Methods. Absorption at 280 nm (solid trace) and conductivity (dotted trace) were measured directly in the column eluate. Assays of QC activity contained an appropriately diluted aliquot of the column eluate and were performed using conditions given under Materials and Methods. Enzymatic activity was determined and plotted as units/chromatographic fraction (▲). Pooled fractions, indicated by the bar, were concentrated and further purified by hydrophobic interaction chromatography.

150 g of lyophilized papaya latex using the new procedure.

Although Gln-*p*NA is a dipeptide surrogate, the kinetic parameters for its conversion by QC correspond to those of other dipeptides. Using the continuous spectrophotometric assay presented here, a Michaelis constant of 102 ± 4 μM was determined (Fig. 4). This corresponds to 90 ± 20 μM detected for Gln-Gln and 210 ± 40 μM for Gln-Ala under comparable conditions (6). Based on the assumption of a monomeric protein with a molecular mass of 33 kDa, determined by gel electrophoresis (13), a first-order rate constant for breakdown of the enzyme/substrate complex to the products (k_{cat}) of 28 ± 1 s^{-1} at 30°C was determined. This corresponds to the value of 50 s^{-1} at 37°C reported for Gln-*tert*-butyl ester (13).

In order to verify these parameters, they were evaluated by an alternative method (12). In this assay, detection of QC activity was accomplished by conversion of the ammonia formed into glutamate, catalyzed by glutamate dehydrogenase in the presence of α-ketoglutaric acid and NADH/H^+. In the subsequent reaction, consumption of NADH/H^+ can be monitored spectrophotometrically at 340 nm. Due to the overlapping absorption of the substrate Gln-*p*NA at 340 nm, a detection wavelength of 355 nm was found to be optimal when using this substrate. Kinetic parameters computed for the conversion of Gln-*p*NA correlate well with those obtained using the novel assay described above, e.g., K_m and k_{cat} were calculated to be 99 ± 6 μM and 27 ± 1 s^{-1}, respectively, thus indicating the validity of the continuous method.

In order to substantiate the feasibility of the new assay in more detail, the effect of ammonia concentration on the conversion rate of Gln-*p*NA to pGlu-*p*NA was recorded at constant ionic strength (μ = 0.071 M). No change in activity could be observed in the range of ammonia concentration analyzed, indicating that the assay can be performed up to 50 mM ammonia. This suggests that QC lacks product inhibition by ammonia. Obviously, the release of ammonia is not the rate-limiting step in the catalysis by papaya QC.

Ionic strength was tested as another important parameter in establishing the new QC assay in enzyme characterization. As a prerequisite, constant activity of the auxiliary enzyme is necessary under the chosen conditions, e.g., time to reach steady-state conditions (Eq. [1]) is unaltered. This prerequisite was fulfilled, since activity of pyroglutamyl aminopeptidase was enhanced by increasing ionic strength (data not shown). As demonstrated in Fig. 5, activity of QC increased steadily up to 300 mM KCl and was almost constant up

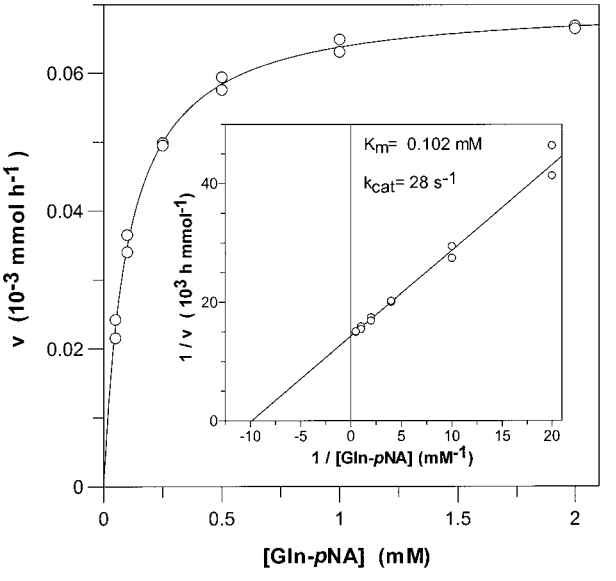

FIG. 4. Dependence of initial *p*NA formation on the concentration of Gln-*p*NA due to conversion to pGlu-*p*NA by QC. Reactions were performed in a final volume of 250 μl at 30°C. The following component concentrations were used: 0.05–1 mM Gln-*p*NA, 0.25 U pyroglutamyl aminopeptidase, and 1 mU QC in 50 mM Tricine/NaOH, pH 8.0. For the determination of the rate constant, k_{cat}, a molecular mass of 33 kDa of the monomeric papaya glutaminyl cyclase was assumed (13).

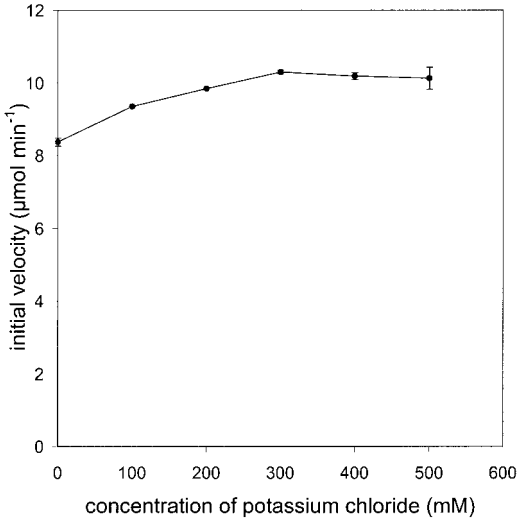

FIG. 5. Dependence of QC activity on ionic strength. Reactions were performed in 50 mM Tricine/NaOH, pH 8.0, at different concentrations of potassium chloride between 0 and 500 mM. This corresponds to an ionic strength of 0.02 to 0.52 M. Determinations were performed in triplicate. Error bars indicate the standard deviation. Other assay conditions were as described under Materials and Methods.

to 500 mM. The overall activation by increasing ionic strength was approximately 20%. A very similar dependence was observed for NaCl (not shown). Thus, within the QC assay a constant ionic strength is important to avoid activating effects of abundant ions. The effect of ionic strength on QC activity might be caused by (i) weakened salt bridges, leading to structural changes and higher enzymatic activity, and by (ii) altered dissociation constant of catalytic groups of the enzyme. Such altered constants at increasing ionic strength were reported previously for lysozyme (20).

Fluorometric Assays

Based on the results obtained with the spectrophotometric assay, two further assays using fluorometric detection were developed. For convenience, the commercially available fluorogenic substrates Gln-βNA and Gln-AMC were purchased and tested as substrates of papaya glutaminyl cyclase. As expected from data in the literature, pyroglutamyl-βNA and pyroglutamyl-AMC are potent substrates for the auxiliary enzyme (21, 22). In agreement with these reports, we determined V_{max}/K_m values for the fluorogenic substrates that were approximately 20-fold higher than that of pGlu-*p*NA. This potency of the auxiliary enzyme for hydrolysis of the potential intermediates emerging in the QC assay results in drastically reduced lag times when using the same concentration (1 U/ml) of pyroglutamyl aminopeptidase compared to the spectrophotometric assay based on release of *p*NA. The times required to reach steady-state conditions were calculated according to the method mentioned above and were found to be less than 5 s for Gln-AMC as well as Gln-βNA. Thus, steady-state conditions were reached within the time required for mixing and beginning of data monitoring. In fact, linear progress curves were obtained in the QC assay for the conversion of both fluorogenic substrates, immediately after initiation of the reaction (Fig. 6A). In the case of both substrates, the observed change in fluorescence was found to increase linearly during the time of incubation. There was no increase in fluorescence detected in the absence of QC or pyroglutamyl aminopeptidase.

As shown in Fig. 6B, linearity was observed between the reaction rate and the enzyme concentration when 0.05 mM substrate was used under these conditions, indicating that the compounds can be used as sensitive substrates for glutaminyl cyclase. Furthermore, in comparison to the chromogenic substrate Gln-*p*NA, the assays described here seem to be approximately 1000 times more sensitive and are capable of detecting enzyme concentrations as low as 0.4 ng/ml.

Kinetic analyses of the enzymatic conversion of both substrates revealed that the enzyme reactions fit Lineweaver–Burk plots (data not shown). The K_m values for conversion of the substrates were 43 $\pm$ 4 μM for Gln-βNA and 52 $\pm$ 5 μM in the case of Gln-AMC. Interestingly, these are the lowest values that were determined for payaya glutaminyl cyclase, and they are noticeably less than that of the chromogenic substrate Gln-*p*NA. The values of k_{cat} for conversion of Gln-βNA (46 s^{-1}) and Gln-AMC (31 s^{-1}) by QC are similar to that of Gln-*p*NA. As a consequence, it can be concluded that the chromogenic and fluorogenic substrates described here can be regarded as the best for papaya QC with respect to their k_{cat}/K_m values. Both fluorogenic substrates seemed to show weak substrate inhibition at concentrations higher than 10 K_m. However, at the concentrations used, this inhibition was minimal and did not interfere with the determination of the kinetic parameters.

A limitation of feasibility, also in the case of other assays of QC, is the susceptibility of the chromogenic and fluorogenic substrates to aminopeptidase attack. Thus, in some applications, addition of aminopeptidase inhibitors will be necessary. Accordingly, we tested three peptidase inhibitors, i.e., bestatin (100 μg/ml), aprotinin (50 μg/ml), and EDTA (5 mM), concerning their influence on the assay, using Gln-βNA as substrate. None of the three substances interfered with the assay, making them useful as additives to inhibit peptidases in crude samples.

Finally, the substrates were also tested upon conversion by human glutaminyl cyclase, recombinantly expressed in the yeast *Pichia pastoris* (published elsewhere). All three assays also worked with this enzyme

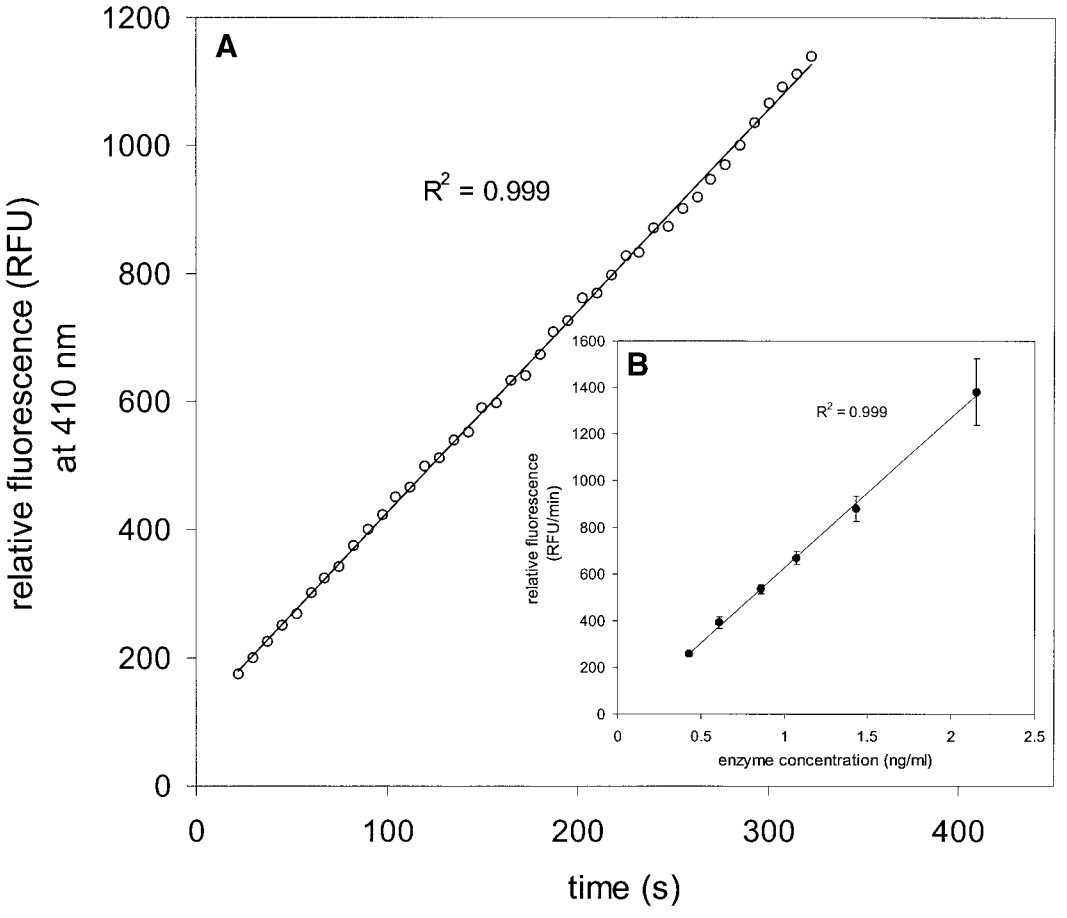

FIG. 6. (A) Progress curve of 2-naphthylamine formation from L-glutamine-2-naphthylamide (Gln-βNA) by catalysis of QC and pyroglutamyl aminopeptidase, monitored by measurement of the relative fluorescence at 410 nm. No increase in fluorescence was detected in the absence of QC (not shown). Product formation obeyed with high accuracy a straight line during the time of analysis. (B) Dependence of initial velocity on the concentration of QC, using Gln-βNA as the fluorogenic substrate. Determinations were carried out in the high sensitivity scale of the spectrofluorometer. Every data point is the mean value of four determinations, while error bars indicate the standard deviations. QC concentrations were achieved by dilution of a stock solution. The assay composition is described under Materials and Methods.

(data not shown), making the procedures applicable for plant and animal sources of QC.

To our knowledge, these are the first continuous assays described for QC that can be implemented during protein purification. Among the advantages of the presented methods, the continuous measurement and less time consumption make the methods more favorable compared to previously developed methods.

The most important advantage of the new methods, in comparison to a previously described assay (12), is the fact that these methods can be performed in the presence of ammonia. Thus, various protein purification steps using ammonium sulfate can be easily monitored.

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