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Synthesis of amidrazones using an engineered papain nitrile hydratase

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Abstract To demonstrate the usefulness of an engineered papain nitrile hydratase as a biocatalyst, a peptide amidrazone was prepared by incubation of the nitrile MeOCO-Phe-Ala-nitrile with the Gln[19]Glu papain mutant in the presence of salicylic hydrazide as a nucleophile. The amidrazone results from nucleophilic attack by salicylic hydrazide at the imino carbon of the thioimidate adduct formed between the enzyme and the peptide nitrile substrate. Compared to wild-type enzyme, the engineered nitrile hydratase causes a better than 4000-fold increase in the rate of amidrazone formation and yields a product of much higher purity. The advantages over other nitrile-hydrolyzing enzymes and current limitations of the papain nitrile hydratase are discussed.

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Key words: Papain; Cysteine protease; Nitrile hydratase; Nitrilase; Biocatalysis; Protein engineering

1. Introduction

Organonitriles are important intermediates in organic synthesis. They can readily be prepared by a number of methods [1,2] and then further transformed into various functional groups. In most cases, the chemical transformations of nitriles require relatively severe conditions such as strongly acid or basic solutions and generate toxic by-products [3,4]. In recent years, nitrile-hydrolyzing enzymes (nitrilases and nitrile hydratases) have been recognized as useful biocatalysts to perform many of these reactions. Various applications of nitrile-hydrolyzing enzymes include production of high value amides and acids (acrylamide, nicotinic acid and lactic acid) from their corresponding nitriles [5], conversion of α-aminonitriles to optically active amino acids [6], enantioselective hydrolysis of α-hydroxynitriles (cyanohydrins) [7] and production of optically active 2-arylpropionic acids ibuprofen [8] and naproxen [9,10]. Several nitrile-hydrolyzing enzymes have been isolated from natural sources. However, these enzymes are in general rather unstable, and most applications reported so far make use of whole cell systems. In addition, many of these enzymes cannot be used in the presence of organic solvents, which precludes their use in situations where the organonitrile substrate is poorly soluble in water.

Recently, the cysteine protease papain has been successfully converted into a nitrile hydratase by protein engineering [11]. This was achieved by replacing Gln[19] in the oxyanion hole of papain by a glutamic acid residue. The nitrile substrate forms a covalent thioimidate adduct with the active site thiol group of the enzyme and the role of the Glu[19] residue is to participate in the acid-catalyzed hydrolysis of the thioimidate to the amide by the provision of a proton to form the more reactive protonated thioimidate. Hydrolysis of peptide nitriles proceeds in a step-wise manner: the nitrile is first hydrolyzed to the corresponding amide, and since the Gln[19]Glu mutant retained most of the natural amidase activity of papain, the amide is then further hydrolyzed to the acid. The enzyme can thus be considered also as a nitrilase, since it possesses both nitrile hydratase and amidase activities. Papain is an ideal candidate for use in biocatalysis. It is a very well-studied enzyme, stable in a wide range of conditions of pH and temperature, and active in organic solvents [12,13]. In this paper we report on a synthetic methodology which makes use of the thioimidate adduct formed between the enzyme and a peptide nitrile substrate. Thioimidates are intermediates in several organic synthesis reactions but are not easy to make by traditional methods. With the papain nitrile hydratase the thioimidate is readily made and it will be shown that, with the addition of appropriate nucleophiles, the enzyme can be used to synthesize amidrazones. We also report on the use of the *Pichia pastoris* system to improve the expression level of the papain Gln[19]Glu mutant.

2. Materials and methods

2.1. Materials

Restriction endonucleases, T4 DNA ligase and T4 DNA polymerase were purchased from New England Biolabs (Mississauga, Ont., Canada). Taq DNA polymerase was from Promega (Madison, WI, USA). The vector (pPIC9) and *Pichia pastoris* strain GS115 were purchased from Invitrogen Corporation (San Diego, CA, USA). Subtilisin (Nagarse) was obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ont., Canada). Papain was obtained from Sigma Chemical Co. and was purified and activated as described previously [14]. The peptide nitrile MeOCO-Phe-Ala-nitrile (methoxycarbonyl-L-(phenylalanyl)-L-alanine nitrile) was synthesized as described previously [15]. Salicylic hydrazide and benzoic hydrazide were purchased from Aldrich and the irreversible inhibitor E-64 (1-[[(L-*trans*-epoxysuccinyl)-L-leucyl]amino]-4-guanidino-butane) was obtained from IAF Biochem International Inc., Laval, Que., Canada.

2.2. Construction of the yeast expression plasmids

A 970-bp DNA fragment encoding propapain was amplified by PCR using Taq DNA polymerase, the plasmid YpDC222 [16] as a template and oligonucleotides 5′-GCA TCC GCT CGA GAA AAG AGA GGC TGA AGC TGA T-3′ and 5′-GAA TCG CTT TGC GGC CGC TCA GTT CTT AAC TGG GTA GAA AGA AGA GGT G-3′ as primers. The PCR product was subsequently cloned into the *Xho*I/*Not*I site of the vector pPIC9. The Gln[19]Glu mutant of papain was generated by replacing the *Eco*RI/*Apa*I fragment of the wild-type papain gene with the corresponding sequence of the Gln[19]Glu mutant described previously [17]. Positive clones were identified by restriction site analysis and confirmed by DNA sequencing. The Gln[19]Glu mutant of papain was expressed in the yeast *Pichia*

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pastoris as a prepro-α-factor fusion construct using the culture conditions recommended by Invitrogen.

2.3. Enzyme activation and purification

The proenzyme was expressed and secreted at approximately 3–5 mg/l of initial culture medium. Yeast cells in suspension culture (1 l) were centrifuged at 3000×*g* for 10 min and the supernatant concentrated 10-fold using an Amicon YM10 membrane in a stirred cell. The concentrated solution was diafiltered against 50 mM sodium phosphate, pH 6.0. The $Gln^{19}Glu$ mutant autoprocessed slowly and was converted to the mature form at pH 6.0 with 0.1 mg/ml subtilisin at 37°C. The processed enzyme was activated for 1 h with 2 mM DTT, the reducing agent removed by gel filtration (Sephadex G-15) and the enzyme purified by covalent affinity chromatography on a thiopropyl-Sepharose matrix [14]. The purified protein was stored at 4°C in 50 mM sodium phosphate buffer, pH 6.5, containing 100 mM NaCl and 100 μM $HgCl_2$. Prior to its use, the mercury-inhibited papain mutant was reactivated by incubation of a diluted sample in the presence of 5 mM DTT. The concentration of active $Gln^{19}Glu$ papain was determined by titration using E-64 [18].

2.4. Reaction monitoring by HPLC and product identification

Reactions were performed at 22°C in presence of 50 mM citrate buffer, pH 5.0, 0.2 M NaCl, 1 mM EDTA and 10% CH_3CN. Aliquots from the reaction mixture were analyzed by reverse phase analytical HPLC on a C-18 column (Vydac) as described previously [11]. The reaction products were also purified in large amount by semi-preparative reverse phase HPLC and lyophilized. The purified products, other than the amide and acid identified as described previously [11], were dissolved in 10% acetic acid and characterized by mass spectrometry, performed on a triple quadrupole mass spectrometer (API III LC/MS/MS system; Sciex, Thornhill, Canada). Compound concentrations from analytical HPLC were determined by peak integration. Solutions of known concentrations of the purified reaction products and nitrile substrate were used as HPLC standards.

3. Results

In order to investigate the potential utility of the papain nitrile hydratase in organic synthesis, the peptide nitrile MeOCO-Phe-Ala-nitrile was incubated with the $Gln^{19}Glu$ papain nitrile hydratase in the presence of 50 mM salicylic hydrazide as a nucleophile. The reaction was followed by analytical HPLC (Fig. 1). It can be seen that after 21 h of incubation with 200 nM of $Gln^{19}Glu$ papain, approximately 50% of the nitrile (compound **1**) has been consumed and replaced by a new product which appears at a retention time of 25 min on the chromatogram. After 68 h, only 6% of the nitrile remains and compound **2** constitutes the main product of the reaction. Compound **2** results from nucleophilic attack by salicylic hydrazide at the imino carbon of the thioimidate enzyme adduct to form the corresponding amidrazone (Fig. 2a). Since water can also act as a nucleophile and compete for the thioimidate, small amounts of the corresponding amide **3** and acid **4** are also detected in the chromatogram. In addition, side-products were also observed to elute with retention times greater than 25 min (Fig. 1c). Similar results were obtained with benzoic hydrazide as an external nucleophile. In both cases, however, no product is detected when the reaction is carried out at pH 7.2. No significant product formation was observed using

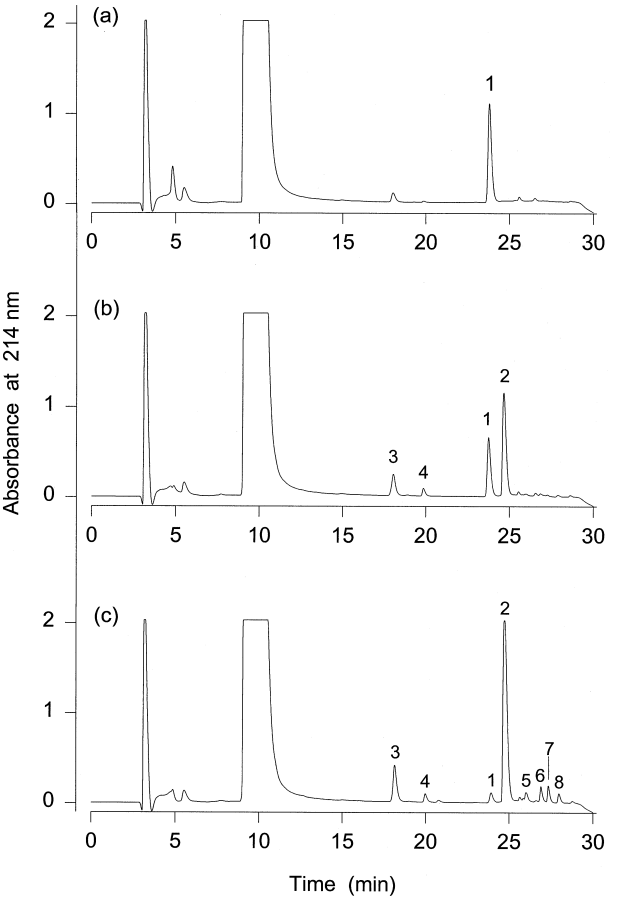

Fig. 1. Analytical HPLC of the products generated by the reaction of $Gln^{19}Glu$ papain (200 nM) with the peptide nitrile MeOCO-Phe-Ala-nitrile (1 mM) in the presence of the nucleophile salicylic hydrazide (50 mM). Peak labels correspond to the compound numbering in Fig. 2. Incubation times: 0 (a), 21 h (b) and 68 h (c). The large peak with a retention time of approx. 10 min corresponds to salicylic hydrazide.

ethanolamine, 2-aminophenol, alanine-*t*-butyl ester, tryptophanamide or benzylhydrazide as nucleophile.

If the reaction is carried out at lower concentration of the $Gln^{19}Glu$ papain (30 nM), the side-products become very important (Fig. 3). The amidrazone **2** is unstable in solution and slowly transformed into products **5**–**8** [19,20]. Upon incubation of the purified amidrazone **2** under reaction conditions but in the absence of enzyme, compounds **5**–**8** were generated which confirms that these further transformations are not enzyme-catalyzed (data not shown). The molecular weights of compounds **2** and **5**–**8** were determined by mass spectrometry and the results are presented in Table 1. The values obtained are in agreement with predicted values based on the structures presented in Fig. 2. The relative amounts of products obtained for the different enzyme concentrations are reported in Table 2. At 200 nM of $Gln^{19}Glu$ papain, the amidrazone **2** consti-

Table 1
Molecular mass of products from the reaction of MeOCO-Phe-Ala-nitrile with $Gln^{19}Glu$ papain in the presence of salicylic hydrazide

Compound number	**2**	**5**	**6**	**7**	**8**
Observed (Da)	428.2	429.3	410.3	545.2	411.3
Predicted[a] (Da)	428.5	429.5	410.5	545.6	411.4

[a]Predicted molecular masses are based on the corresponding structures proposed in Fig. 2.

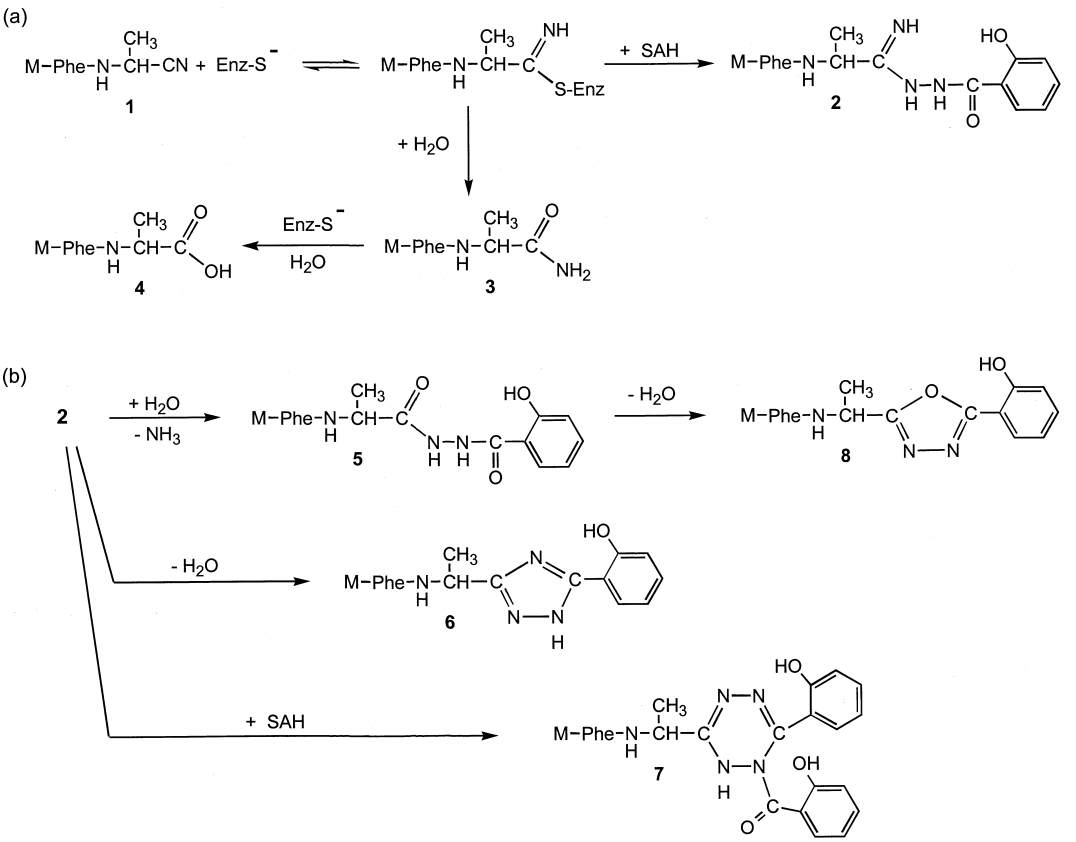

Fig. 2. Schematic representation of proposed reactions between $Gln^{19}Glu$ papain and MeOCO-Phe-Ala-nitrile in the presence of the nucleophile salicylic hydrazide. a: Enzymatic reactions. b: Non-enzymatic reactions of the amidrazone **2** [19,20]. Only one tautomeric form of each compound is presented to simplify the figure. SAH: salicylic hydrazide; M: methoxycarbonyl.

tutes nearly 70% of total products and the main side products are the amide and acid **3** and **4**. At 30 nM enzyme, the proportion of amidrazone drops to only 30%, and compounds **5**–**8** now constitute near 50% of the products. The larger amount of side-products **5**–**8** in Fig. 3 is attributable to the fact that a longer incubation time was required to achieve near 95% completion of the reaction. This shows the importance of using an efficient enzyme to obtain the amidrazone. If wild-type papain is used, no product is detected after 93 h incubation at a concentration of enzyme of 200 nM (Table 2). Products are obtained when a very high concentration of enzyme is used (100 μM). This has been observed previously, using Ac-Phe-Gly-nitrile and aniline as a nucleophile, but the reaction was extremely slow, necessitated 180 μM of papain, and generated large amounts of side-products [21]. In the present work, with 100 μM of wild-type enzyme, side-products constitute 60% of the total products, and only 86% completion is achieved after 478 h incubation. It must be noted that in the absence of added nucleophile, the thioimidate adduct is extremely stable and no hydrolysis can be detected after more than 200 h incubation in the presence of 200 μM (5 mg/ml) of papain.

4. Discussion

The results of this study demonstrate that the papain nitrile hydratase can be used to synthesize amidrazones which are themselves intermediates in the synthesis of key components of biologically active molecules [20]. Amidrazones can be prepared chemically by reaction of hydrazide with a nitrile. However, the reaction is limited only to certain nitrile derivatives and secondary reactions can be a problem [20]. Alternatively, amidrazones can be synthesized by addition of hydrazide derivatives to imidates and thioimidates. Even though this is one of the best methods for preparing amidrazones, side products are also a problem and in many cases the amidrazone is not the major product of the reaction [20]. In addition, non-en-

Table 2
Distribution of products for the reaction of $Gln^{19}Glu$ and wild-type papain with MeOCO-Phe-Ala-nitrile in the presence of salicylic hydrazide

Enzyme	Concentration (nM)	Incubation time (h)	Percentage completion[a]	Percentage of product in reaction mixture		
				2	**3** and **4**	**5**–**8**
$Gln^{19}Glu$	200	68	94	67	25	8
$Gln^{19}Glu$	30	480	96	31	23	46
Wild type	200	93	<1	0	<1	0
Wild type	100 000	478	86	43	20	39

[a]Percentage of nitrile substrate converted to products.

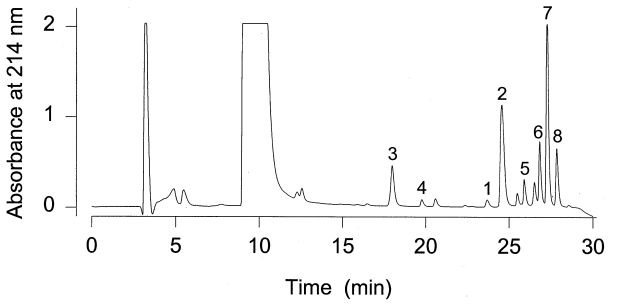

Fig. 3. Analytical HPLC of the products generated at low concentration (30 nM) of $Gln^{19}Glu$ enzyme after an incubation time of 480 h. Concentrations of MeOCO-Phe-Ala-nitrile and salicylic hydrazide were the same as in Fig. 1.

zyme bound imidates and thioimidates hydrolyze rapidly at neutral pH values and are unstable at elevated temperatures [19,22]. Therefore, anhydrous reactants have to be used and the temperature must be kept low (e.g. 0–5°C). By comparison, peptide nitriles form stable thioimidate adducts with papain, due in part to the lower pK_a of the thioimidate bound to the enzyme [23]. Hydrolysis can occur when assisted by the protonated Glu^{19} with the $Gln^{19}Glu$ mutant at lower pH [11]. The present results indicate that reaction of the thioimidate adduct with an external nucleophile is also greatly accelerated by the Gln^{19} to Glu mutation, and that side products formation can be minimized. From the data in Table 2, the mutation can be estimated to cause a better than 4000-fold increase in the rate of amidrazone formation and to yield a product of much higher purity.

The papain nitrile hydratase presents several advantages over other nitrile-hydrolyzing enzymes. Some reactions were carried out with incubation times as long as 20 days, a period over which the enzyme remained very active, demonstrating once again the great stability of papain. Substrate inhibition, often encountered with natural nitrile-hydrolyzing enzymes [24], is a serious drawback towards the exploitation of these enzymes in the industrial processing of chemicals. With the papain nitrile hydratase, there is no inhibition for hydrolysis of the nitrile or synthesis of the amidrazone, only the formation of the acid is inhibited by the nitrile which acts as a competing substrate. It must be noted also that contrary to natural nitrile hydratases and nitrilases, papain is active in organic solvents [12,13,25], an important feature for use with poorly soluble substrates. There are, however, certain limitations to the use of the papain nitrile hydratase. First of all, the enzyme is selective or specific for peptide based nitriles. Nitrile-containing compounds will be screened to obtain a specificity profile for the enzyme against non-peptidyl nitriles. It might also be possible to alter the specificity of the papain nitrile hydratase by site-directed mutagenesis, a task which might prove to be challenging depending on the substrate considered. A second limitation for certain applications is the fact that the enzyme is active mainly at low pH (i.e. pH 4–7). The synthetic approach described in this study was successful only when using compounds with good nucleophilicity at $pH < 7$. Salicylic and benzoic hydrazides have a low pK_a (ca. 3), and the deprotonated hydrazides can act as good nucleophiles even at pH 5 where the enzyme is most active. Again, site-directed mutagenesis or solvents other than water could be used to alter the pH-dependency of the reaction. It must be noted, however, that the activity of the papain nitrile hydratase at low pH can prove to be advantageous in several situations where the substrate is unstable at neutral or high pH, like in the case of cyanohydrins, important synthetic intermediates that are unstable at pH values greater than 4. This also differentiates the papain nitrile hydratase from natural nitrile-hydrolyzing enzymes, which generally display an optimum pH of activity at pH 7–8. Finally, for synthesis of compounds other than amides or acids, the hydrolysis reaction can be a further problem. The main side-product observed in the present study for preparation of the amidrazone is the amide **3**. To favor formation of the amidrazone **2** over the amide, the nucleophile concentration can be increased (data not shown). An alternative approach presently under evaluation is to carry out the reaction in organic solvent.

There has been very little work done on the use of nitrile-hydrolyzing enzymes to synthesize compounds other than amides or acids. The present work demonstrates the feasibility of the process with the papain nitrile hydratase. A more detailed evaluation of this potential application will involve careful investigation of the nature of nitrile compounds that are recognized by the papain mutant, as well as the reaction in solvents other than water. With the proper selection of nucleophiles, this methodology could lead to the synthesis of useful compounds that might be difficult to obtain otherwise.

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