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Synthesis of kyotorphin precursor by an organic solvent-stable protease from *Bacillus licheniformis* RSP-09-37

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Abstract

The synthesis of the analgesic dipeptide kyotorphin precursor (Bz-Tyr-Arg-NH_2) was studied under kinetically controlled conditions in 10–90% (v/v) aqueous-acetonitrile media at −20 °C using a novel protease obtained from the cell free supernatant of a *Bacillus licheniformis* mutant strain (RSP-09-37). α-Chymotrypsin (CT) was used for comparison. The conditions for maximum yield of kyotorphin precursor synthesis were optimized using CT by varying the type of nucleophile (amide and ester), nucleophile concentration and nucleophile to acyl donor ratio. The nucleophile (Arg-NH_2) at a concentration 400 mM and nucleophile to acyl donor ratio 1:40 was found to be optimum for kyotorphin precursor synthesis. The protease from *B. licheniformis* RSP-09-37 was stable even at 90% acetonitrile concentration and allowed for a significantly higher synthesis over hydrolysis ratio (S/H ratio) of 15.6 compared to only 3.0 found for CT at −20 °C.
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Keywords: Protease; Acetonitrile; Peptide synthesis; Kyotorphin; S/H ratio

1. Introduction

Protease-catalyzed peptide synthesis has several advantages over chemical methods as it shows absence of racemization, lack of requirement of side-chain protection and uses milder non-hazardous reaction conditions [1–3]. A number of important dipeptides such as the analgesic dipeptide kyotorphin (Tyr-Arg) [4–6] and aspartame precursors [7] have been synthesized using proteases. As the presence of water is required to maintain high catalytic activity of proteases, strategies must be used to avoid undesired hydrolysis of peptides and to promote peptide formation [8]. This can be achieved by performing the peptide synthesis in a kinetically controlled reaction, in the presence of water-miscible cosolvents [9–11] and by lowering the temperature even to sub zero ranges of −20 °C [5,12,13].

Most studies on protease-catalyzed peptide synthesis were performed with α-chymotrypsin (CT) [4,5,9,14] or subtilisin [11,15]. In this report, we used a protease secreted by a mutant strain, *Bacillus licheniformis* RSP-09-37, in aqueous-acetonitrile media at −20 °C, in order to study its potential to synthesize kyotorphin precursor under kinetically controlled reaction conditions.

2. Experimental

2.1. Screening and mutagenesis of B. licheniformis

A thermophilic strain was isolated from a hot water spring of Sohna, Haryana, India and was subjected to nitrosoguanidine (NTG, 1.5%) mutagenesis as described elsewhere [16]. The thermophilic strain was characterized as *B. licheniformis* RSP-09 based on its partial 16S rDNA sequence (GenBank accession no. AY648981) and the culture has been deposited

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in Bacillus Genetic Stock Center, The Ohio State University, Columbus, Ohio, USA (accession no. 5A37). The mutant obtained from it was named *B. licheniformis* RSP-09-37.

2.2. Production of B. licheniformis RSP-09-37 protease

A cell free supernatant, containing the organic solvent tolerant protease was obtained after growing *B. licheniformis* RSP-09-37 for 4.5 h (late log phase) in yeast extract ($3\,g\,l^{-1}$) peptone ($10\,g\,l^{-1}$), dextrose ($5\,g\,l^{-1}$) (YPD) medium and centrifuging at 8000 rpm for 20 min. The specific proteolytic activity of cell free supernatant was 32 U/mg protein as determined by the method of Fujiwara et al. [17].

2.3. Kyotorphin precursor synthesis

2.3.1. Materials

All solvents used were of HPLC grade. Benzoyl L-tyrosine-*p*-nitroanilide (Bz-L-Tyr-pNA), L-argininamide dihydrochloride (H-Arg-NH_2·2HCl), the methyl ester of arginine dihydrochloride (H-ArgOMe·2HCl) and Kyotorphin were obtained from Bachem, Switzerland. α-Chymotrypsin (CT) (EC 3.4.21.1) type II from bovine pancreas with a specific activity of 60 U/mg was obtained from Fluka, USA. H-Arg-NH_2·2HCl (30 mmol) and 33 mmol of NaOH were dissolved in distilled water and L-argininamide was extracted with 200 ml dichloromethane using a rotary evaporator at room temperature for a period of 30 min. The resulting evaporated salt with partial water was dried at 40 °C for 4–5 h to obtain the fully dried salt of H-Arg-NH_2. For the preparation of H-ArgOMe from its hydrochloride, a similar protocol was used.

2.3.2. Reaction conditions for the synthesis of Kyotorphin precursor

The standard reaction mixture (200 μl) contained 10 mg CT (60 U/mg), the acyl donor Bz-L-Tyr-pNA (10 mM) and the nucleophiles L-Arg-NH_2 or L-ArgOMe (400 mM). This was dissolved in 50 mM Tris–HCl buffer (pH 8.0) containing acetonitrile ranging from 40–90% to make up a final reaction volume of 200 μl. After addition of protease, the reaction mixture was incubated for 8 h either at −20 °C or in a water bath maintained at 25 °C. The effect of the ratio of the acyl donor to the nucleophile was studied by varying ratios from 1:10 to 1:50. Reactions were stopped by the addition of 100 μl of 1 M HCl and the solution was later filtered through 0.20 μm filters to remove the enzyme and insoluble fractions before the samples were analyzed by HPLC (Agilent 1100 series) using a C_{18} Novapak column (4 mm × 250 mm) (Waters, USA) and detected by a G1315B diode array detector at 257 nm. The flow rate of $0.8\,ml\,min^{-1}$ with a linear gradient of acetonitrile/water from 50% to 80% over 10 min was maintained with a G1311A quaternary pump. The temperature was maintained at 20 °C by a G1316A temperature controller. The yield was calculated from the peak areas of substrates, kyotorphin precursor and by-products. The S/H ratio was calculated from the amount of kyotorphin precursor formed divided by the amount of Bz-L-Tyr-OH formed. Experiments with protease from *B. licheniformis* RSP-09-37 were performed using 17.2 mg (32 U/mg protein) of the enzyme. All experiments were performed in duplicates. Reactions without enzyme served as blanks.

2.4. Stability of B. licheniformis RSP-09-37 in aqueous-acetonitrile media

The enzymes obtained from *Bacillus*, and CT were incubated for 10 h in acetonitrile ranging from 10 to 90% (v/v) concentration, and the synthetic activity for kyotorphin precursor was determined. The stability of these two enzymes was also checked by incubating in 50% (v/v) acetonitrile-aqueous medium for given time intervals. The activity was calculated as mM kyotorphin precursor produced per second at −20 °C. The relative activity of the enzyme was measured by incubating the enzymes with different percent concentrations (v/v) of aqueous-acetonitrile media for a given time interval and then determining the activity at −20 °C. All the protease activity determinations were carried out at a time when maximum amount of kyotorphin precursor synthesis was obtained for *B. licheniformis* RSP-09-37 protease (i.e. 2.5 h) and for CT (i.e. 2.0 h), respectively.

3. Results and discussion

3.1. Synthesis of kyotorphin precursor

Synthesis of kyotorphin precursor was carried out using the solvent tolerant protease obtained from the cell free supernatant of a mutant *B. licheniformis* RSP-09-37. The kinetic maximum of the reaction was determined by performing the reaction in the presence of 50% (v/v) aqueous-acetonitrile medium. The mechanism of the kinetically controlled synthesis of kyotorphin precursor by protease is depicted in Fig. 1. The maximum concentration of kyotorphin precursor (7 mM) was observed at 2.5 h after which, due to the complete consumption of the acyl donor, the reaction shifted to the thermodynamic route (Fig. 2). Hence all further reactions using this protease were performed for 2.5 h. For CT, the maximum concentration of kyotorphin precursor (7.5 mM) was observed at 2 h (Fig. 2). Hence the reactions with CT were performed for 2 h.

The conditions using α-chymotrypsin (CT) were optimized for maximum selectivity towards kyotorphin precursor synthesis over hydrolysis which included temperature, type of nucleophile and ratio between the nucleophile and the acyl donor, as these parameters play an important role in kyotorphin synthesis [4,15,18,19]. The CT-catalyzed synthesis of kyotorphin precursor was studied at 25 °C and at −20 °C and the conditions were standardized for the highest synthesis over hydrolysis ratio (S/H ratio). The S/H ratio for kyotorphin precursor synthesis was 0.8 at 25 °C and increased to 2.0

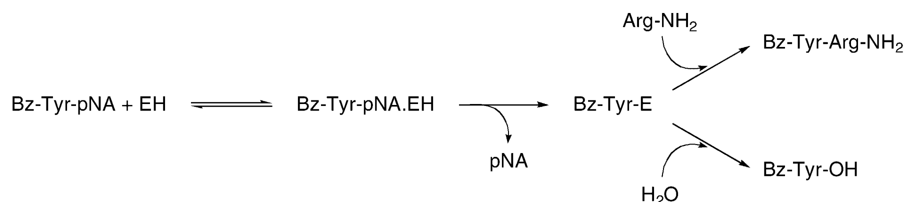

Fig. 1. The mechanism of the kinetically controlled synthesis of kyotorphin precursor by protease.

(2.5-fold) by lowering the temperature to −20 °C (Fig. 3A). This increased nucleophile selectivity at low temperature may be attributed to lowering of the *P*-value. The *P*-value is defined as the nucleophile concentration at which the rate of the aminolysis reaction equals the rate of hydrolysis at given reaction conditions [9,20,21] and thus describes the competition between nucleophile and water for the acyl enzyme. Thus, at lower temperature a lower concentration of nucleophile is required for a higher yield of peptide. These results are in agreement with an earlier report where a 15% increase in peptide yield was obtained when the peptide synthesis was carried out at −20 °C instead of 25 °C [15].

The choice of nucleophile that exhibits higher efficiency is an important parameter for increasing peptide synthesis. It has been reported that hydrophobic amino acids act as poor nucleophiles in the presence of organic solvents as compared to charged amino acids like arginine and lysine towards CT [5]. Hence, the kyotorphin precursor synthesis was conducted at −20 °C using Arg-NH_2/ArgOMe as nucleophiles until the kinetic maximum [22] was reached. With Arg-NH_2, a 2.3-fold higher S/H ratio was found compared to ArgOMe (Fig. 3B). The lower S/H ratio found using ArgOMe could be attributed to lower nucleophilic efficiency of the amino acid ester [18,19], as these derivatives have a reduced capacity to form eutectic mixtures with the acyl donors compared to the corresponding amides [23]. Further, the improved S/H ratio observed in the case of Arg-NH_2 can be attributed to greater hydrogen bonding capacity of the amide, which enhances the interaction of this derivative with the acyl donor facilitating the formation of a eutectic mixture [18,19]. This efficiency of Arg-NH_2 was further improved by lowering the reaction temperature, which decreased the *P* value resulting in a better synthesis of kyotorphin precursor. The ratio of acyl donor (Bz-Tyr-pNA) (S′) and acyl acceptor (Arg-NH_2) (N) plays an important role in peptide synthesis. Hence the selectivity of the kyotorphin precursor synthesis was determined by the [N]/[S′] ratio in the form of a saturation curve as depicted in Fig. 3C. It is apparent from the saturation curve that more than a 20-fold excess of the nucleophile over the acyl donor results in a S/H ratio towards kyotorphin precursor synthesis of ∼1.7. Interestingly, it was observed that the S/H ratio of the reaction depends upon the [N]/[S′] ratio rather than the concentration of the nucleophile (Fig. 3C). The maximum S/H ratio (2.0) was obtained at a 1:40 ratio of [N]/[S′] and an acyl donor concentration of 10 mM. These findings are in agreement with Meos et al., who reported that a [N]/[S′] ratio >20 gave a saturation in the yield of kyotorphin [4]. Further experiments were carried out using a 1:40 N/S′ ratio with 10 mM concentration of acyl donor.

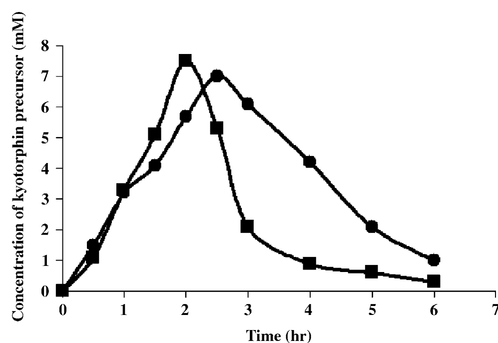

Fig. 2. Reaction profile and determination of the kinetic maximum of kyotorphin precursor synthesis catalyzed by CT (■) and protease from *B. licheniformis* RSP-09-37 (●). Reactions were performed at 50% acetonitrile concentration. The reaction was carried out at pH 8.0 in the case of CT and pH 10.0 in the case of protease obtained from *B. licheniformis* RSP-09-37 with 10 mM of the acyl donor and 400 mM of the nucleophile (Arg-NH_2) and at −20 °C.

3.2. Determination of the selectivity of the synthetic reaction catalyzed by α-chymotrypsin and the protease obtained from B. licheniformis RSP-09-37

Next, the S/H ratio of both enzymes for kyotorphin precursor synthesis was studied at increasing concentrations of acetonitrile. In the case of CT, the S/H ratio increased 1.4-fold by increasing the acetonitrile concentration from 40% to 50% (v/v). The highest S/H ratio 3.0 was observed at 50% (v/v) acetonitrile. A further increase of the concentration of acetonitrile probably led to denaturation of the enzyme, as no reaction was observed (Fig. 4). On the contrary, the protease obtained from *B. licheniformis* RSP-09-37, tolerated higher acetonitrile concentrations (up to 90%) and this resulted in a significantly higher S/H ratio of 15.6 (Fig. 4). Thus, a ∼13-fold increase in selectivity was observed with protease from *B. licheniformis* RSP-09-37, when the acetonitrile concentration was increased from 40% to 90% (v/v). Even though a higher increase in S/H ratio was found for CT compared to the protease from *B. licheniformis* RSP-09-37 at 50% (v/v) aqueous acetonitrile media, the final S/H ratio was five-fold higher for the latter enzyme. The S/H ratio observed in the present study using CT (S/H = 3) is significantly higher than similar experiments performed on immobilized CT (CT-celite)

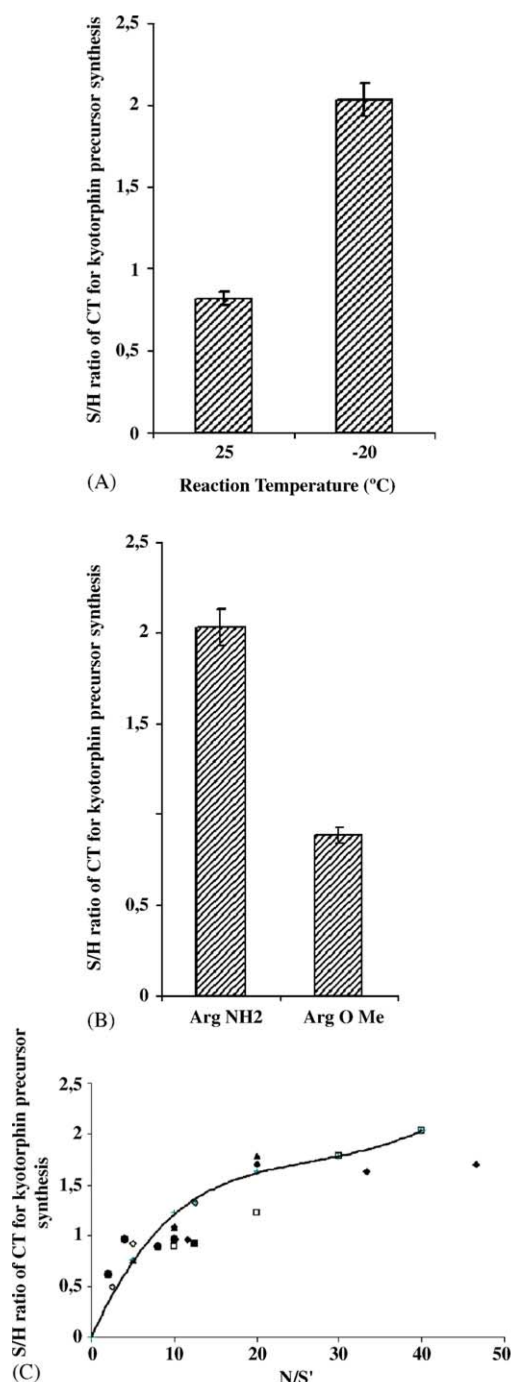

Fig. 3. Standardization of the reaction conditions with CT for maximum S/H ratio towards kyotorphin precursor. (A) Effect of temperature on the selectivity of the reaction towards kyotorphin precursor synthesis. The reactions were performed at 25 °C and −20 °C using 400 mM Arg-NH_2 and 10 mM of the acyl donor in the presence of 30% acetonitrile in the reaction mixture. (B) Effect of the nucleophile on the synthesis of the reaction towards kyotorphin precursor. The reactions were performed at −20 °C using 10 mM of the acyl donor and 400 mM of the nucleophile (Arg-NH_2 and ArgOMe) in the presence of 30% acetonitrile in the reaction mixture. (C) The effect of nucleophile/acyl donor substrate (N/S) ratio on the synthesis of kyotorphin precursor in the presence of 30% acetonitrile in the reaction mixture. The pH of the reaction mixture was 8.0 and the reaction was carried out for 2 h. The concentration of acyl donor were 2 mM (■), 3 mM(⧫), 5 mM (▲), 10 mM (□), 20 mM(◊), 50 mM (●).

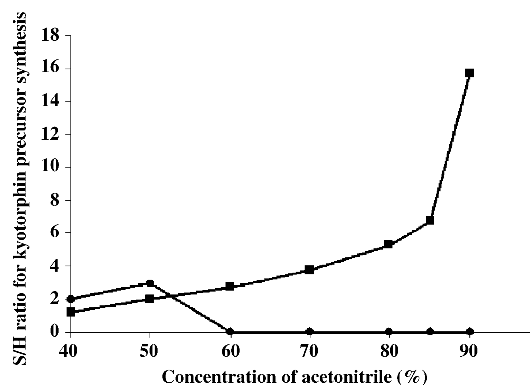

Fig. 4. Effect of acetonitrile concentration on the kyotorphin precursor synthesis. The reaction was performed with protease from *B. licheniformis* RSP-09-37 (■) and CT (●) with 40–90% (v/v) aqueous-acetonitrile media until the kinetic maximum with 10 mM of the acyl donor and 400 mM of the nucleophile (Arg-NH_2) at −20 °C. The values are mean of three separate experiments conducted using the protease from *B. licheniformis* RSP-09-37, and CT.

(S/H = 0.4) with dimethylformamide (DMF) as organic solvent, although in the latter case the stability of the enzyme was higher, i.e. the enzyme was stable at even 60% DMF [24].

3.3. Stability of CT and protease obtained from B. licheniformis RSP-09-37 in aqueous acetonitrile media

The effect of the acetonitrile concentration on enzyme stability was studied by incubating the enzymes for a period of 10 h in aqueous-acetonitrile at concentrations between 10 to 90% and the residual activity was checked at the respective kinetic maximum. Initially at 10% (v/v) aqueous-acetonitrile *B. licheniformis* RSP-09-37 protease showed a 1.2-fold increase in its residual activity as compared to CT. This improvement in the tolerance for *B. licheniformis* RSP-09-37 increased to 3.8-fold at 50% acetonitrile and was at its maxima at 60% with a 60-fold increase, while no activity could be observed for CT under similar conditions (Fig. 5A).

The stability of the enzyme in aqueous-acetonitrile was also checked as a function of time whereby the enzymes were incubated in 50% acetonitrile for the given time interval and the residual activity was checked at the respective kinetic maximum (Fig. 5B). *B. licheniformis* RSP-09-37 protease showed a 1.5-fold increase in its stability compared to CT, when the two enzymes were exposed to 50% (v/v) acetonitrile for 2 h. However, on increasing the exposure to acetonitrile it was observed that the residual activity in both cases decreases. However, the decrease is less pronounced in the case of *B. licheniformis* RSP-09-37 protease, which retained 70% of its activity even after 10 h of exposure to acetonitrile, which corresponds to a six-fold higher tolerance compared to CT. This suggests that the protease obtained from *B. licheniformis* RSP-09-37 has a substantially

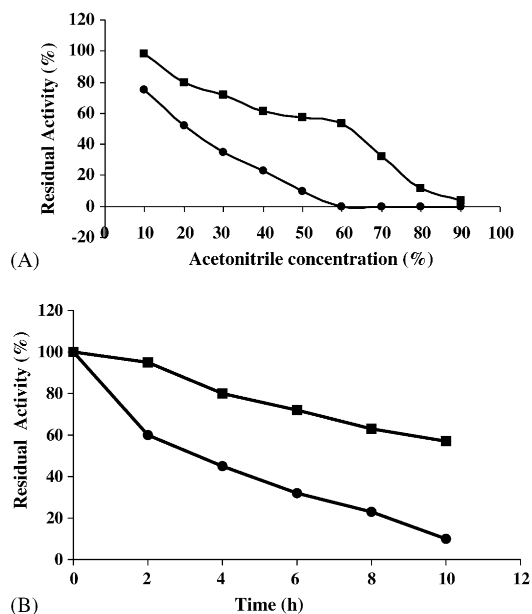

Fig. 5. Effect of acetonitrile concentration on the residual activity of protease from *B. licheniformis* RSP-09-37 (■) and CT (●). (A) The enzymes were incubated for 10 h in aqueous-acetonitrile media ranging from 10 to 90% concentration of acetonitrile and the synthetic activity for kyotorphin precursor was determined at their kinetic maximum. (B) Protease activity towards kyotorphin precursor synthesis was determined after incubating in 50% (v/v) aqueous-acetonitrile medium for given time intervals and subsequently, the activity at the kinetic maximum was determined.

better tolerance for organic solvents than CT and thus is better suited for kyotorphin precursor synthesis. One possible reason for the higher stability of *B. licheniformis* RSP-09-37 protease could be its thermophilic origin. This hypothesis is supported by the reports citing better stability of thermophilic enzymes in organic solvents compared to mesophilic enzymes [25,26].

4. Conclusions

The protease obtained from *B. licheniformis* RSP-09-37 shows significantly better performance in the synthesis of a kyotorphin precursor than the commercially available α-chymotrypsin. As the *B. licheniformis* RSP-09-37 protease tolerates higher concentrations of acetonitrile, this results in higher conversions, less hydrolysis and increased stability and thus makes this biocatalyst suitable for the synthesis of peptides.

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