A microtiter plate assay for the determination of the synthetic activity of protease

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Proteases have several industrial applications due to their hydrolytic activity [1] and have attracted considerable interest due to their ability to synthesize peptides in organic media [2,3]. Protease-catalyzed peptide synthesis occurs either by a thermodynamically controlled process (condensation of the acid and amine groups) or by a kinetically controlled process (aminolysis of an ester). In the kinetically controlled process the rapidly formed covalent acyl enzyme can transfer the acyl moiety either to the nucleophilic amino component, resulting in the formation of the desired peptide, or to water, resulting in hydrolysis [3]. The hydrolysis step can be suppressed by performing the reaction at subzero temperature [4-6].

Serine proteases have been employed in the synthesis of many biological active oligopeptides [2], including kyotorphin, which is an analgesic dipeptide (Tyr-Arg). This dipeptide has been synthesized using L-Tyr-OEt or Bz-L-Tyr-OEt and H-Arg-OH or L-Arg-OEt as the nucleophile in organic solvent [6,7] and analyzed using high-performance liquid chromatography (HPLC).[1] The presence of water-miscible organic solvents in enzyme-catalyzed reaction increases the solubility of hydrophobic substrates and shifts the thermodynamic equilibrium of hydrolytic reaction to synthetic reaction [8,9]. In addition water-miscible organic solvents such as dimethylformamide (DMF) when added to a subtilisin BPN^0-catalyzed reaction have shown preference toward aminolysis as His on the active site flips in and a strong hydrogen bond observed between His and Asp in water is disrupted [10]. However, increasing solvent concentration has led to enzyme inactivation [11,12]. Hence, techniques such as site-directed mutagenesis and directed evolution have been employed to improve the stability of enzymes in organic solvents. Directed evolution has been used as an elegant approach to generate and identify new enzyme variants [13]. A range of methods for generation of enzyme libraries is available [14], but the major problem is the lack of a rapid and reliable assay system for the screening of improved enzyme variants out of a pool of 10^4-10^6 mutants. To overcome such difficulty, hydrolase variants were identified using chromogenic esters to identify an active esterase [15], to increase the stereoselectivity of a lipase [16], and to identify active esterases using pH indicators [17]. Due to the high number of clones generated during directed evolution techniques, the use of HPLC or gas chromatography for screening is often a cumbersome and time-consuming method. It can, however, be used as the tool to finally confirm the results. In the present study, a microtiter plate (MTP) assay based on *p-nitro*-anilide (pNA) release has been developed for the first time for rapid analysis of synthetic activity of protease.

Materials and methods

Kyotorphin, Bz-L-Tyr-pNA, and H-Arg-NH_2 were purchased from Bachem, Switzerland. DNase I was purchased from Boehringer Mannheim. IPTG was purchased from Gerbu, Germany. Lysozyme was purchased from Sigma, USA and chymotrypsin from Fluka, USA.

A PCR kit was obtained from Bangalore Genei, India. Luria Bertoni (LB) broth was procured from Himedia, India. Acetonitrile and water were of HPLC grade and were procured from Merck, India.

Determination of synthetic activity of protease. Protease-catalyzed kyotorphin (Bz-Tyr-Arg-NH_2) synthesis was carried out using the method of Meos et al. [6] with some modifications. The synthetic activity of protease was demonstrated in a reaction mixture of 200 *ll* using 10mM Bz-L-Tyr-pNA and 400 mM H-Arg-NH_2 in 90% acetonitrile—water mixture. The reaction was performed at kinetic maximum (2.5h) at -20 °C in MTP at pH 10.0. The reaction was stopped by addition of 100 *ll* of 1M HCl. Analysis of kyotorphin was carried out using Agilent 1100 series HPLC system equipped with Novapak Qg column and G1315B diode array detector. Acetonitrile-water 50-80% was used as mobile phase in a gradient system and a flow rate of O.Smlmin$^{\wedge 1}$ was maintained. Alternatively, the amount of p-nitroanilide released during the protease-catalyzed synthetic reaction was monitored at 410 nm spectrophotometrically. As the coloration of pNA is pronounced at basic pH, 10 *ll* of 10N NaOH was added at the end of the reaction. In both microtiter plate assay and HPLC measurement controls were taken without addition of enzyme and no activity was observed in both experiments.

Error-prone polymerase chain reaction (ep-PCR) for generating mutant libraries of protease. The protease gene from *Bacillus licheniformis* RSP-09-37 was cloned in *Escherichia coli* JM109 competent cells using the pUC 19 vector and the clones were selected on $LBAm_p$ and casein plates. Screening of 1000 clones on the basis of their protease activity resulted in the selection of RSP-46. The clone RSP-46 was later sequenced and to improve the synthetic protease activity, ep-PCR was performed using a method modified from Chen and Arnold [18]. PCR was carried out at 94°C for 2min, 48 °C for 30 s, and 68 °C for 4min and 30 cycles were performed. The last chain extension was carried out at 68°Cfor 8min.

Screening of mutant libraries on MTP. For the screening of mutants each well of a MTP was filled with 200 *ll* LB broth supplemented with ampicillin (lOOngul$^{\wedge 1}$). Each well was inoculated and incubated for 24h at 37 °C. From this 100 *ll* was added to a fresh $LBAm_p$ MTP, cultivated, and further induced with 200 mM IPTG. The culture was harvested and later the pellet was lysed with 250 *ll* lysis buffer comprising lysozyme and DNase I. The lysate was centrifuged to obtain the enzyme solution which was then assayed using the MTP test described above. The protein content was determined by the Bradford's [19] method.

Results and discussion

In this study pNA was used for the detection of synthetic activity of a protease using Bz-L-Tyr-pNA and Arg-NH_2 as the substrates, which resulted in the formation of Bz-Tyr-Arg-NH_2 and concomitant release of pNA (Fig. 1). In the synthesis of kyotorphin with protease, normally HPLC, which is a time-consuming and lengthy procedure, is used for detection. In earlier studies pNA was employed for the detection of hydrolytic activity of protease in microtiter plates using succinyl-Ala-Ala-Pro-Phe-pNA as the substrate and the hydrolytic activity was tested based on the release of pNA in this particular reaction [20]. To verify the reliability of this method, samples were also tested using conventional HPLC analysis. First, the protease synthetic activity was tested with the commercially available chymotrypsin and the usefulness of this method was then verified in the screening of libraries generated from a protease produced by *B. licheniformis* by using ep-PCR. Of the 200 clones obtained after the first round of ep-PCR, RSPep-60 showed the best synthetic activity of protease in the presence of acetonitrile. After the second round of ep-PCR was performed, RSPep-60-1 was selected from 100 colonies. To further improve the synthetic activity, a third round of ep-PCR was performed and from another 100 colonies RSPep-60-1-2 was selected. It is evident from Table 1 that the activity of the enzyme as detected using the MTP assay was very close to the HPLC data. Although the release of pNA must not imply synthesis of the peptide but can also be due to hydrolysis of Bz-L-Tyr-pNA, the amounts of pNA released and kyotorphin formed were very similar. We assume that performing the reaction at subzero temperature under kinetic control quantitatively suppressed the nondesired hydrolysis. This procedure gave reproducible results when carried out for a large number of

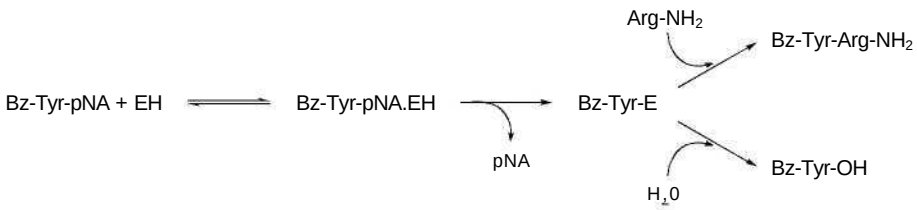

Fig. 1. Mechanism of the kinetically controlled peptide synthesis by protease.

Table 1
Synthetic activity of protease using Bz-Tyr-pNA and H-ArgNH_2 as substrate at pH 10.0

	Specific activity (lmol/min/mg protein)	
	MTP	HPLC
Chymotrypsin	0.197 ±0.01	0.205 ± 0.07
Bacillus licheniformis		
RSP-09-37	0.467 ± 0.02	0.472 ± 0.09
RSP-46	0.259 ± 0.02	0.271 ± 0.04
RSPep-60	0310 ± 0.05	0.310 ±0.05
RSPep-60-1	0.395 ± 0.07	0.395 ± 0.07
RSPep-60-1-2	0.563 ± 0.03	0.575 ± 0.04

The reaction was conducted at — 20 °C for 2.5 h. The reaction was then terminated using 1M HCl. Samples were analyzed spectrophotometrically for the amount of p-nitroanilide released and by HPLC to determine the amount of kyotorphin (data are the mean of four experiments ± SD).

protease-producing *B. licheniformis* colonies. The protease obtained from the selected clones was also found to have a better synthetic activity than the commercially available chymotrypsin.

Thus this method can be used as a reliable screening method for rapid detection of synthetic activity. In principle, this method can also be easily applied to the synthesis of other bioactive peptides using the release ofp-nitroanilide. Thus, we have developed a new rapid assay procedure for synthetic activity of protease that can be further used for screening of libraries.

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