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L-Selective dipeptide synthesis using novel thermophilic enzyme from *Clostridium* sp.†

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Abstract—A novel, inexpensive, thermophilic protease-type enzyme isolated from *Clostridium thermohydrosulfuricum* was used for dipeptide synthesis. The enzyme showed broad substrate selectivity and enantioselectivity towards L-amino acids in peptide bond formation.

1. Introduction

The design of novel biologically active polypeptides is a new field of protein chemistry. The short chain peptides are known for their biological importance in nutritional, flavoring and pharmacological areas.[2] At present, dipeptides are synthesized by both chemical and enzymatic routes.[3] Chemical routes for peptide synthesis, especially fragment condensation often suffer from problems such as racemization and time consuming protection/deprotection of amino acids.[4] The alternative to chemical synthesis is the use of biocatalysts for peptide bond formation where, the reaction often takes place under mild, aqueous conditions with high degrees of stereo- and regioselectivity.[5] Although enzymes, viz., proteases are becoming increasingly popular as biocatalysts in peptide formation, one of the main drawbacks for industrial application of the enzymes is the intrinsic instability of the enzyme in many of the unnatural environments required for organic reactions.[6] Accordingly, much research has focused on the enhancement of the enzyme stability at higher temperature.[7,8] Microorganisms growing in extreme environments are reservoirs of enzymes that could change the face of biocatalysts. Among these enzymes, the extremozymes like thermolysin (protease) a heat stable enzyme isolated from *Bacillus thermoprotedyticus rokko* are known to catalyze dipeptide formation.[9] However, this method has the drawback of poor availability and feasibility for industrial production.[10] So there is a need to explore new inexpensive extremozymes that can be used in the field of peptide synthesis. Herein, we report an effective method of dipeptide formation using crude enzyme protease isolated from thermophilic anaerobic *Clostridium thermohydrosulfuricum* SV 12.[1]

2. Results and discussion

The crude thermophilic enzyme isolated from the anaerobic microorganism *C. therohydrosulfuricum* has shown protease-type activity and was used in catalyzing dipeptide synthesis (Table 1). It was observed that the isolated enzyme was stable and exhibited specific activity in biphasic media using a number of solvents such as ethyl acetate, chloroform, *t*-butyl methyl ether and hexane. The enzyme was found to be unstable in miscible solvent systems.[1]

The isolated enzyme showed maximum stability and selectivity in saturated ethyl acetate/buffer medium. The optimal conditions of enzyme activity at different temperatures and pH in the synthesis of the dipeptide *Z*-L-Asp-(β-OMe)-L-Phe-OMe (entry 1) is shown in Fig. 1. It was observed that the maximum enzyme activity for dipeptide bond formation is at 45°C with the buffer medium of pH 6.5. No appreciable enzyme activity was observed on pre-incubation of the isolated enzyme with methanesulfonyl fluoride (Sigma chemical,

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Table 1. Protease type enzyme isolated from *C. thermohydrosulfuricum* catalyzed dipeptide synthesis

Entry no.	Substrate		Product[a]	Yield (%)	$[\alpha]_D^{25}$ methanol
	Acyl donor	Nucleophile			
1	*Z*-L-Asp-β-OMe	L-Phe-OMe	*Z*-L-Asp-(β-OMe)-L-Phe-OMe	53	−13.8
2	*Z*-L-Asp	L-Phe-OMe	*Z*-L-Asp-L-Phe-OMe	40	−14.7
3	*Z*-DL-Asp-β-OMe	L-Phe-OMe	*Z*-L-Asp-(β-OMe)-L-Phe-OMe	35[b]	−14.0
4	*Z*-DL-Asp	L-Phe-OMe	*Z*-L-Asp-L-Phe-OMe	39[b]	−14.9
5	*Z*-L-Asp-β-OBz	L-Leu-OMe	*Z*-L-Asp-(β-OBz)-L-Leu-OMe	35	+13.7
6	*Z*-L-Asp-β-OBz	L-Val-OMe	*Z*-L-Asp-(β-OBz)-L-Val-OMe	35	+18
7	*Z*-L-Phe	L-Asp-$(OMe)_2$	*Z*-Phe-L-Asp-$(OMe)_2$	35	
8	Boc-Gly-Gl	L-Phe-OMe	Boc-Gly-Gly-L-Phe-OMe	30	–

[a] To confirm the products, identical physical and spectral data (tlc, NMR, and specific rotation) were found in comparison with independently synthesised materials.[10]
[b] Yields were with respect to L-enantiomer.

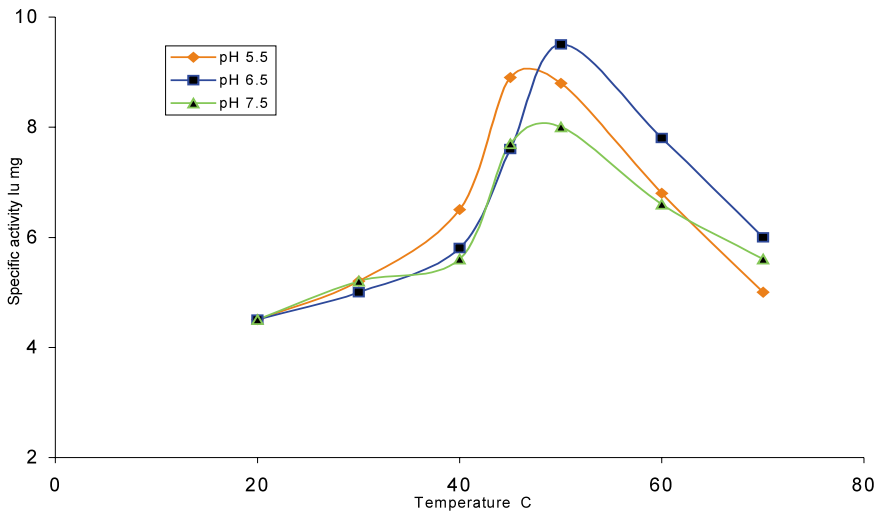

Figure 1. Enzyme activity at different pH and temperature in the synthesis of dipeptide (*Z*-L-Asp-(β-OMe)-L-Phe-OMe).

USA), thus confirming the isolated enzyme has protease-type activity. We have been able to separate the dipeptide and the unreacted amino acid. We did not isolate any acid component of the protected amino acid. So we presume that the enzyme only catalyzed dipeptide formation and not the hydrolyzed amino acid due to esterase or amidase. The product formed was precipitated out from the biphasic solvent mixture (ethyl acetate 40%) depending upon the partition coefficient of the product in the solvent used, this seems to be the decisive factor of the reaction towards favorable peptide synthesis.[11]

Compared with the earlier reports,[14] the important feature of the isolated enzyme protease is the stereoselectivity and enantiomeric purity in the peptide bond formation towards L-amino acids (entries 3, 4). It was observed that the reaction of a carboxyl donor (*Z*-L-asp) with hydrophobic *C*-amidated nucleophiles (L-leu and L-val) occurred in moderate yield. The rate of peptide formation was also observed by kinetic parameters i.e. on V_{max} and k_m. (Table 2). The variation of rate of product formation V_{max} correlates with the substrate binding constant K_m, which in turn depends on the solubility of the product in biphasic media (log *P*).

Table 2. Kinetic parameters of the thermostable enzyme[a] in synthesis of dipeptides

Entry no.	Dipeptide	Log *P*[b]	t_{max} (h)	K_m (mol min^{-1})	V_{max} (mol min mg^{-1})	$[\alpha]_D^{25}$ methanol
1	Boc-L-Asp-L-Phe-OMe	0.18	8	0.16	1.65	−14.7
2	*Z*-L-Phe-L-Asp-$(OMe)_2$	0.08	8	0.15	1.08	−13.6
3	*Z*-L-Asp-(OBz)-L-Leu-OMe	0.11	12	0.19	1.10	+13.7
4	*Z*-L-Asp-L-Phe-OMe	0.19	8	0.15	1.80	−15.0

[a] Kinetic parameters were determined from a Lineweaver Burk plot.[12]
[b] Values of (log *P*) were calculated as described by Laroute et al.[13]

The peptide bond formation of *Z*-L-asp with various hydrophobic nucleophiles was carried out using different thermostable enzymes thermolysin and protease type extremozyme isolated from *C. thermohydrosulfuricum* (Table 3). Peptide bond formation with protease isolated from *C. thermohydrosulfuricum* proceeded stereoselectively with broad substrate specificity when compared to thermolysin obtained from *B. thermoprotedyticus rokko*.

3. Conclusion

In summary, the extremoprotease enzyme isolated from anaerobic *Clostridium* species is a novel biocatalyst in synthesis of dipeptides. In comparison with thermolysin, the protease enzyme isolated from anaerobic bacterium is stable at higher temperature with broad substrate specificity and high stereoselectivity towards L-amino acids. Further work on application of the enzyme in synthesis of tri- and tetrapeptides is currently in progress.

4. Experimental

An anaerobic extremophilic bacterium strain *C. thermohydrosulfuricum* SV 12 was isolated from local soil samples (deposited in microbial culture center OU). The bacterium was identified on the basis of cell morphology growth characteristics and DNA/DNA hybridization. The strains were grown under anaerobic conditions (0.002 in inert (nitrogen) atmosphere in serum vials containing bacterium strain grew in pre-reduced PYE (peptone yeast extracts) medium and incubated 0.1 M phosphate buffer for 8 days, at optimal conditions of pH 6.8 at 45°C. The thermostable protease type enzyme was produced as extracellular enzyme in the medium of the extremophilic organism *C. thermohydrosulfuricum*. The fermented medium containing protease-type enzyme activity was partially purified. Briefly, the medium was centrifuged to remove cellular debris and protein in the culture medium was precipitated by adding 30% ammonium sulfate solution. The protein precipitate was dissolved in 0.1 M phosphate buffer pH 6.5 and enzyme present was partially purified on passing the protein solution through Sephadex G-50. The fraction having protease-type activity was freeze-dried and was used as the source of enzyme. The specific activity of the protease enzyme isolated was found to be 28 IU/mg protein (Bradford method) using casein as substrate.[15] All the organic solvents used were of analytical grade. Standard protease enzyme thermolysin (*B. thermoprotedyticus*) obtained from Sigma Chemicals Co, USA.

The biocatalytic peptide bond condensations were performed as follows: the acid component (10 mmol) and the amine component (20 mmol) were taken in phosphate buffer solution (0.1 M, pH 6.5, 10 ml), which was pre-saturated with ethyl acetate (40%). To this reaction medium isolated protease enzyme/thermolysin (40 mg, crude) was mixed and then incubated at 40°C in an orbital shaker at 200 rpm for different time intervals. The product formation was followed by TLC and HPLC using an analytical reversed phase (C18) column.[16] the reaction was terminated by adding 50% aqueous ethanol containing 8% trifluoroacetic acid, the protein precipitate was filtered off and the product formed was dissolved in a minimum amount of methanol for crystallization. The dipeptides obtained were conformed by spectral data.

For the compounds in entries 5 and 6 (Table 3), the substrates used are unprotected. The low rate of synthesis may be due to the higher hydrophilicity of the substrates/products. The isolation of the products requires the separation of the biphasic system, lyophilization of the aqueous layer and crystallization in methanol to give dipeptides.

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Table 3. Comparison of peptide bond formation using thermostable protease

Entry	Product	Rate of synthesis[a] (peptide bond formation)	
		Protease[b]	Thermolysin[c]
1	*Z*-L-Asp-(β-OMe)-L-Phe-OMe	110	80
2	*Z*-D-Asp-(β-OMe)-L-Phe-OMe	>5	35
3	*Z*-L-Phe-L-Asp(OMe)$_2$	40	20
4	*Z*-L-Asp(β-Obz)-L-Leu-OMe	55	20
5	L-Asp-(β-OMe)-L-Phe-OMe[d]	30	16
6	L-Phe-L-Asp(OMe)$_2$[d]	15	12

[a] Rate of synthesis of peptide were expressed in μM of product formed per minute per mg of protein (quantitated by r.p. HPLC).
[b] Isolated from *C. thermohydrosulfuricum* SV12.
[c] Isolated from *B. thermoproteolitions*.
[d] See experimental procedure.

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16. Samples are analyzed in HPLC of Shimadzu-10A using analytical RP18 column (4.6×250 mm particle size 5 μM, 300 Å) and eluted, using water and acetonitrile containing 0.1% trifluoroacetic acid under gradient conditions at 254 nm.