Bioorganic & Medicinal Chemistry Letters, Vol.3, No.4, pp. 727-733, 1993
Printed in Great Britain

0960-894X/93 $6.00 + .00

PROBING THE S-1' SUBSITE SELECTIVITY OF AN INDUSTRIAL ALKALINE PROTEASE IN ANHYDROUS t-BUTANOL.

Shui-Tein Chen[1]*, Shiah-Yun Chen, Hsiu-Jen Chen, Hsuan-Cheng Huang, Kung-Tsung Wang[1,2]*.

1. Laboratory of Biocatalyst, Institute of Biological Chemistry, Academia Sinica, Taiwan. P. O. Box 23-106, Taipei, Taiwan. 10098.
2. Department of Chemistry, National Taiwan University.

(*Received in USA* 10 *December* 1992)

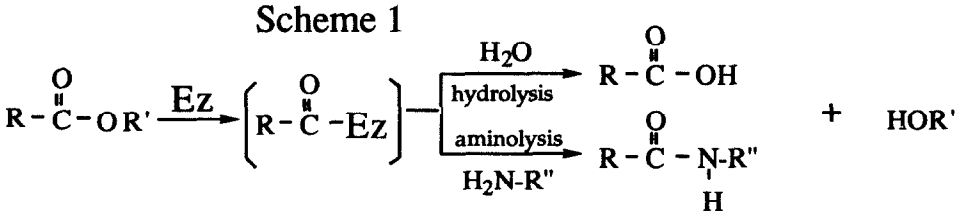
Abstract

Study of the selectivity of s-1'-subsite of alcalase catalyzed reaction in t-butanol shows that the rate of product formation depends on the size of the nucleophiles. D- and L-amino acids as nucleophile form the product with the same reaction rate. Interpretation of this selectivity by molecular modeling using crystal structure of subtilisin Carlsberg complexed with it's substrate gives clear evidence.

Recently, the search for proteases that are stable in organic solvents for peptide synthesis has been the subject of extensive investigation.[1] Several studies have demonstrated that it is possible to use proteases to catalyse peptide synthesis in organic solvents.[2] In a previous study, we have found that the alcalase is very active and stable in anhydrous alcoholic solvents and usable as a catalyst in a kinetically controlled peptide bond formation[3]. Here we report the s-1' subsite selectivity of alcalase catalyzed peptide bond formation in anhydrous t-butanol using a kinetically controlled approach. Knowledge of the s'-subsite selectivity of alcalase is necessary for planning its use as a biocatalyst in peptide synthesis.

Alcalase is a proteolytic enzyme prepared from the submerged formation of a selective strain of *Bacillus licheniformis*.[4] The major enzyme component of alcalase is the serine protease subtilisin carlsberg (alkaline protease A), which is widely used as an additive in detergents as a digesting enzyme. Alcalase has high protease activity and is inexpensive.[5] Kinetically controlled peptide synthesis catalyzed by serine or cysteine protease is widely used, since short reaction times and low enzyme concentrations are required.[6] Scheme 1 shows a typical reaction path for this approach. The initially formed acyl-enzyme intermediate [R-C(=O)-Ez] can be deacylated by water or by an amine nucleophile [:NH_2-R"]. If the reaction takes place under anhydrous conditions, hydrolysis will not occur. The efficiency of various nucleophiles in the deacylation of acyl enzymes reflects the s-1' subsite selectivity of the alcalase in anhydrous t-butanol. A series of amino acid derivatives and peptides were used as nucleophiles to study the efficiency of peptide bond formation. The X-ray crystall structure of subtilisin Carlsberg was used to interprete the selectivity of the alcalase catalysed reaction by fitting the substrates, which have D- or L-amino acid residue at the p-1' position, into the 3-D structure of the enzyme's active site using the computer program InsightII on a Silicon Graphic work station. The volume of the s-1'subsite of subtilisin carlsberg was measured by InsightII using "Distance Measure".

Scheme 1



Results and discussion.

Enzyme kinetics measured in anhydrous t-butanol are very rare. We used Cbz-Phe-OCP (OCP: p-chlorophenol ester) as an acyl-donor[7] and amides of various amino acids or peptides which have different side-chains as a nucleophile. In a typical reaction, to one equivalent of Cbz-Phe-OCP and three equivalents of nucleophile in t-butanol was added alcalase (pre-washed with t-butanol for three times; see experimental part) to start the reaction. The time course was monitored by hplc, and the initial reaction rate was determined from time-dependent plots of the increasing concentration of the product. Figure 1 shows the time course of Cbz-Phe-Gly-NH_2, Cbz-Phe-Gly-Gly-NH_2, Cbz-Phe-Leu-NH_2, Cbz-Phe-Phe-NH_2, and Cbz-Phe-D-Phe-NH_2. The smallest amino acid, Gly, formed the product more rapidly than dipeptide the Gly-Gly did. The amino acids which have bulk side-chains such as Phe, and Leu formed the products slower than did Gly and Gly-Gly. Studying the enantiomeric selectivity of the nucleophile, using L-Phe-NH_2 and D-Phe-NH_2 as substrates, respectively, both nucleopliles deacylation of the acyl-enzyme to form the products, Cbz-L-Phe-L-Phe-NH_2 and Cbz-L-Phe-D-Phe-NH_2, at the same rate.

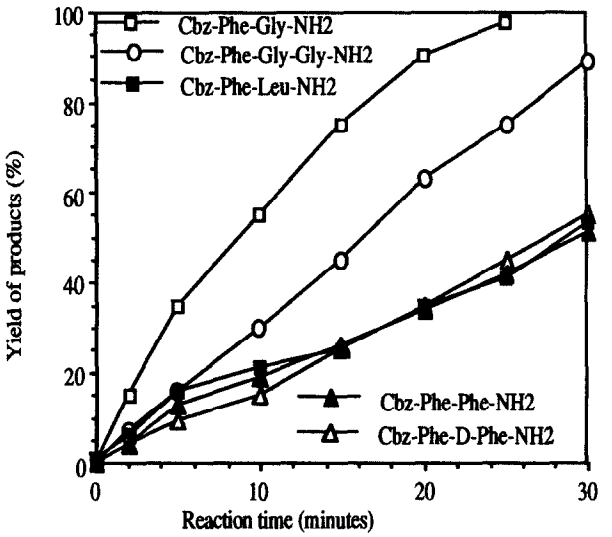


Figure 1. Time course for alcalase catalyzed peptide bond formation in t-butanol.

The s-1' subsite of alcalase could accept both D- and L- form amino acid residues as a nucleopiles.

Several crystall structures of the alkaline protease and of the enzymes complexed with inhibitors have been determined in high resolution.[8] This forms a good basic modeling of different enzyme-substrate intermediates. Figure 2 shows the stereo-view of the 3-D structure of the enzyme-substrate complex.[9] The substrate Pro-Phe-Ala was situated in the corresponding subsites (s-2, s-1, s-1'). The s-1' subsite were surrounded by the amino acids of Thr-220, Gly-219, Asn-218, Leu-217 and His-64, with the Thr-220, and Gly-219 at the bottom of the s-1' subsite, and the Asn-218 and Leu-217 on the lower-left side. L-Ala was put in the s-1' subsite. Figure 3 shows the top-view of the s-1' subsite; the substrate in the s-1' subsite was deleted. Figure 4 shows the side-view of the

s-1 subsite rotated 90° in y-axis of the top view. The volume of the s-1' subsite is denoted by the array (in Fig-3 and Fig-4.). D- or L-nucleophiles could freely attack the Acyl-enzyme intermediate.

In a representive preparative scale reaction, alcalase (2 mL, 5.0 AU, prewashed with 10 mL of t-butanol for three times), acyl-donor (10 mmol), nucleophile (30 mmol), and t-butanol (30 mL) were stirred at 35°C until all the acyl-donors had been consumed (hplc, see table 1). To the reaction mixture was added ethyl acetate (250 mL), and the resulting mixture was washed with 0.1 M HCl (3x30 mL), 0.5 M $NaHCO_3$ (3x30 mL), and then water (3x30 mL). The organic solution was concentrated, and ether was added to it to precipitate the crude product. It was recrystallized with ethyl acetate/hexane (3:1) to give pure product. Amino acid amides and peptide amides, which have different side chains, were used as nucleophiles and reacted. For preparation of poly-amino acids and poly-oligopeptides, esters of N-terminal-free amino acid or peptides were used as substrates and the same procedure was followed. The results are summarized in table 1. The physical properties of the products were confirmed by NMR, amino acid analysis of peptide hydrolysate, Fab mass and its physical properties.

The entire preparative scale reaction was completed within 2 hours, and the yields ranged from 100% to 64%. The shorter the reaction was, the higher was the yield. In synthesis of N-terminal-free poly-amino acids or poly-oligopeptide, the products were precipitated in the reaction solution during chain-elongation. Using Cbz-Ser-OBzl as the substrate, the hydroxy-group of serine could react as a nucleophile; thus the reaction was transesterification.[10] Polyethyleneglycol-modified papain has been used in polymerization of amino acids in low water organic solvents.[11] Using alcalase the modification procedure is not necessary. Several of the peptides prepared are biologically active. For example, $(Ala\text{-}Ala\text{-}Thr)_n$ is a antifreeze peptide of fish,[12] Asp-D-Ala-OPr^n is a novel sweetner,[13] and $(Glu)_n$ is a liquid crystalline material.[14] The subtilisin protease has very broad specificity at its s-1 subsite. Both D- and L- peptides and amino acids are accepted as nucleophiles at the s-1' subsite. Perhaps many biologically active peptide derivatives, particularly those containing unusual amino acids, can be made using this enzymatic method. The use of alkaline proteases to catalyze peptide bond formation in anhydrous solvents has been limited. This new enzymatic process has several advantages: 1) the high turnover rate and low cost of the enzyme make enzyme immobilization unnecessary; 2) the enzyme is stable in alcoholic solvent, thus allowing operation at high substrate concentration; and 3) the reaction is highly selective for ester hydrolysis, and the peptide bonds remain intact.

Experimental Section

Removal of water from the alcalase solution. Alcalase 2.5L (1.0 mL) and anhydrous t-butanol (10 mL) were added to a centrifuge tube (20 mL), and the mixture was agitated on a super-mixer for 10 minutes. The resulting mixture was centrifuged (3000 rpm) for 15 minutes to spin down the enzyme, and the supernatant was decanted. t-Butanol (10 mL) was added again, and the same procedure was repeated three times until the water was removed completely.

Preparative scale Peptide bond formation in t-butanol. Cbz-Phe-D-Leu-NH_2 as an example.

Cbz-Phe-OCP (10 mmol, 4.10g), D-Leu-NH_2 (30 mmol, 3.90g), and alcalase 2.5L (2.0 mL, prewashed with t-butanol three times) were added to anhydrous t-butanol (50 mL). The mixture was shaken at 35°C for 2 hours. Small samples were taken in known time intervals and analyzed by hplc to measure the concentration of

product formation. After the substrate was consumed, the resulting mixture was then diluted with ethyl acetate (300 mL) and washed with 0.5N $NaHCO_3$, 5% citric acid, and water. After the organic solvent was evaporated under reduced pressure, Cbz-Phe-D-Leu-NH_2 was obtained (3.58g, 87%): mp 159-160°C; $[\alpha]_D$ = +19.30, (c 1 MeOH).

Synthesis of poly-amino acids.(poly (Glu)-Bzl as example).

To a mixture of t-butanol (20 mL) containing Glu(Bzl)-Bzl.HCl (5 mmol, 1.82g),triethylamine (5 mmol, 0.71 mL), was added alcalase 2.5L (1.0 mL, prewashed with t-butanol for three times). The mixture was shaken at 35°C. The reaction solution became gradually turbid and the product precipitate after 30 min. Until the substrate was consumed (1.5 h), the resulting solution was centrifuged and the precipitated washed with 0.5 M sodium bicarbonate (3 x 50 mL) and with distilled water (3x 20 mL). The precipitate was dried under vacuum, and the $(Glu\text{-}Bzl)_n$-Bzl was obtained as a white amorphous solid (1.54g, 64%). The product was further characterized by FAB Mass.

Table 1. Alcalase catalyzed synthesis of peptides and polyamino acids.

acyl donor	nucleophile	product[a]	reaction time (h)	yield %	mp: °C	$[\alpha]^{20}_D$: c 1, MeOH
CBz-Phe-OCP[+]	Gly-NH_2	Cbz-Phe-Gly-NH_2 (1)	1	100	127-128	-3.53
"	Gly-Gly-NH_2	Cbz-Phe-Gly-Gly-NH_2(2)	1.5	96	187-188	-3.33
"	Leu-NH_2	Cbz-Phe-Leu-NH_2(3)	1	100	186-187	-20.95
"	D-Leu-NH_2	Cbz-Phe-D-Leu-NH_2(4)	1.5	87	159-160	+19.30
"	Phe-NH_2	Cbz-Phe-Phe-NH_2 (5)	1	79	225-227	-8.46
"	D-Phe-NH_2	Cbz-Phe-D-Phe-NH_2(6)	2	81	202-204	+8.57
"	Gly-Leu-NH_2	Cbz-Phe-Gly-Leu-NH_2(7)	1	59	196-198	-13.48
"	Gly-Phe-NH_2	Cbz-Phe-Gly-Phe-NH_2(8)	1	57	193-195	+1.74
Cbz-Asp(Bzl)-Bzl	D-Ala-OPr[n]	Cbz-Asp(Bzl)-D-Ala-OPr[n](9)	2	67	92-93	+1.69
Ala-Ala-Thr-Bzl	Ala-Ala-Thr-Bzl	$(Ala\text{-}Ala\text{-}Thr)_n$-Bzl n=4-6 (10)	1.5	68[#]	-	-
Glu(Bzl)-Bzl	Glu(Bzl)-Bzl	$[Glu(Bzl)]_n$-Bzl n=6-10(11)	1.5	64[#]	-	-
Cbz-Ser-Bzl	Cbz-Ser-Bzl	poly(Z-Ser ester) n=5-8 (12)	1.5	76[#]	-	-

a: No acylation-product was found under the reaction condition without the addition of alcalase.

+: OCP =p-chlorophenol. [#]: yield was calculated using the highest MW peptides as basis.

^{1}H-nmr (DMSO-d_6) of products for: (1), δ, 2.69-3.05 (m 2H), 3.62-3.66 (m 2H), 4.20-4.26 (m 1H), 4.92 (s 2H), 7.09 (s 2H), 7.17-7.31 (m 10H), 7.55-7.57 (d 1H), 8.22 (t 1H); (2), δ, 2.70-3.07 (m 2H), 3.62-3.64 (m 2H), 3.74-3.76 (m 2H), 4.22-4.30 (m 1H), 4.98 (s 2H), 7.90 (s 2H), 7.17-7.35 (m 10H), 7.56-7.59 (d 1H), 8.00 (t 1H), 8.35 (t 1H); (3), δ, 0.82-0.84 (d 3H), 0.86-0.88 (d 3H), 1.43-1.47 (m 2H), 1.54-1.61 (m 1H), 2.68-3.03 (m 2H), 4.20-4.25 (m 2H), 4.93 (s 2H), 6.97 (s 2H) 7.18-7.31 (m 10H), 7.47-7.50 (d 1H), 7.94-7.97 (d 1H); (4), δ, 0.71-0.74 (d 3H), 0.77-0.80 (d 3H), 1.20-1.31 (m 1H), 1.31-1.38 (m 2H), 2.69-2.96 (m 2H), 4.08-4.36 (m 2H), 4.94 (s 2H), 7.00 (s 2H), 7.19-7.41 (m 10H), 7.54-7.58 (d 1H), 8.12-8.16 (d 1H); (5), δ, 2.63-3.04 (m 4H), 4.18-4.26 (m 1H), 4.42-4.51 (m 1H), 4.92 (s 2H), 7.09 (s 2H), 7.18-7.37 (m 15H), 7.45-7.48 (d 1H), 7.99-8.02 (d 1H); (6), δ, 2.64-2.80 (m 2H), 2.97-3.07 (m 2H), 4.16-4.29 (m 1H), 4.43-4.58 (m 1H), 4.90 (s 2H), 7.13-7.33 (m 15H), 7.37-7.41 (d 1H), 8.28-8.32 (d 1H);(7), δ, 0.81-0.83 (d 3H), 0.85-0.87 (d 3H), 1.43-1.48 (m 2H), 1.51-1.59 (m 1H), 2.70-3.05 (m 2H), 3.39-3.48 (m 1H), 3.64-3.83 (m 2H), 4.18-4.25 (m 1H), 4.93 (s 2H), 7.00 (s 2H), 7.17-7.35 (m 10H), 7.56-7.59 (d 1H), 7.77-7.80 (d 1H), 8.26-8.30 (t 1H); (8), δ, 2.67-3.05 (m 2H), 3.55-3.81 (m 2H), 4.18-4.27 (m 1H), 4.38-4.47 (m 1H), 4.92 (s 2H), 7.10 (s 2H), 7.14-7.31 (m 15H), 7.54-7.57 (d 1H), 8.23-8.24 (t 1H); (9), δ, 0.91 (t 3H), 1.34 (d 3H), 1.57-1.68 (m 2H), 2.70 (dd 1H), 2.88 (dd 1H), 3.76 (s 3H), 3.99-4.08 (m 2H), 4.35 (q 1H), 4.58 (q 1H), 5.00 (s 2H), 5.08 (s 2H), 6.86 (d 2H), 7.26-7.33 (m 7H). Fab Mass of products for: (10), MW^{+1}=1657.3 (11), MW^{+1}=2298.7, (12), MW^{+1}=1989.7.

Acknowledgment.
Support for this research provided by the National Science Council, Taiwan, (NSC 81-0208-N-001-70) is gratefully acknowledged.

Figure 2. The X-ray crystal structure of subtilisin Carlsberg was modeled on a Silicon Graphic work station using the computer program Insight II. The model of the substrate, Pro-Phe-Ala, was built using standard bond lengths and bond angles and was fitted into the active site to make the three amino acid residues of the substrate inargulated in the subsites of s-2, s-1, and s-1' of subtilisin Carlsberg. The nucleophile of oxygen atom of the Ser-221 was labelled with yellow color. The amino acids that aruound the S-1' subsite, was labelled with white ribbon on backbond.

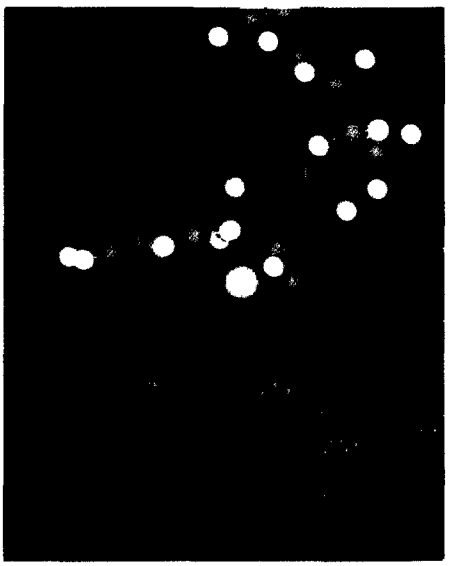


Figure 3. Top view of the s-1' subsite of subtilisin Carlsberg.

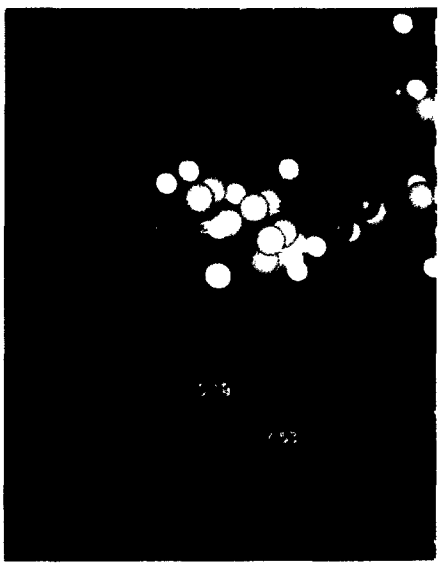


Figure 4. Side-view of the s-1' subsite of subtilisin Carlsberg that rotated 90° in y-axis from the top view.

References.

1) Wong, C. H.; Wang, K. T., Experientia, **1991**, 47, 1123.
Schellenberger, V.; Jakubke, H. D. Angew Chem, Int. Ed. Engl. **1991**, 30,1437.
Klibanov. A. M. Chemteck. **1986**, 16. 354.
Morihara, k., TIBTECH. **1987**, 5. 164-170.

2) Zhong, Z., Liu, J. J.C., Dinterman, L.M., Finkelman, M.A.J., Mueller, T.W., Rollence, M.L., Whitlow, M., Wong, C.H., J. Amer. Chem. Soc. **1991**, 113, 683.
Wong, C. H.; Chen, S. T.; Hennen, W. J.; Bibbs, J. A.; Wang,Y. F.; Liu, J. L. C.; Pantoliano, M. W. Whitlow, M.; Bryan, P. N. J. Amer. Chem. Soc. **1990**, 112, 945.
Kuhl, P.; Halling, P. J.; Jakubke, H. D. Tetrahedron Lett. **1990**, 31, 5213.
West, J. B.; Wong, C. H. Chem. Commun. **1986**, 417.
Margolin, A. L., Tai, D. F., Klibanov, A. M., J. Am. Chem. Soc. **1987**, 109, 7885.
Stahl, M.; Mansson, M. O.; Mosbach, K. Biotechnology Letters, **1990**, 12(3), 161.
Barbas, C. F. III.; Wong, C. H., Chem. Commun. **1987**, 532.
Kise, H. Bioorg. Chem. **1990**, 18, 107.
Nagashima, T., Watanabe, A., Kise, H., Enzyme Microb. Technol., **1992**, 14, 842.

3) Chen, S. T., Hsiao, S. C., Wang, K. T., Bioorg.. Med. Chem. Letters, **1991**, 1(9), 445.
Chen, S. T. Chen. S. Y. , Wang, K.T. J. Org. Chem. **1992** (inpress)

4) Philipp, M., M. L. Bender: Molecular & Cellular Biochem. **1983**, 51. 5. and references, cited herein.

5) For related references see: Chen, S. T.; Wang, K. T.; Wong, C. H. Chem. Commun. **1986**, 1514.
Chen, S. T.; Wang, K. T. J. Chem. Research, **1987**, 308.
Chen, S. T.; Chen, S. Y. Hsiao, S. C.; Wang, K. T. Biotech. Lett. **1991**, 13(11), 773.
Chen, S. T.; Wu, S. H.; Wang, K. T. Int. J. Peptpide Protein Res. **1991**, 37, 347.

6) Brubacher, L. J.; Bender, M. L. Biochem. Biophys. Res. Commun. **1967**, 27, 176.
Fastrez, J.; Fersht, A. R. Biochem. **1973**, 12, 2025.
Polgar,L., Bender, M. M., Biochem. **1967**, 6, 610-620.

7) Amino acid p-chlorophenol derivatives are good acyl-donor for protease-catalyzed peptide bond formation. No background acylation occurred under our reaction condition. see the following references:
Nakatsuka, T., Sasaki, T., Kaiser, E. T., J. Amer. Chem. Soc. **1987**, 109, 3808.
Tanizawa, K., Sugimura, A., Kanaoka, Y., FEBS Letters, **1992**, 296, 163.

8) Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, Jr.,E. F., Brice, M. D Rodgers., J. R., Kennard, O., Shimanouchi, T., and Tasumi, M., J. Mol. Biol., **1977**, 112, 535-542.
Abola, E.E., Bernstein, F.C., Bryant, S. H., Koetzle,T. F., and Weng, J. , "Protein Data Bank" in Crystallographic Databases-information Content, Software Syetem, Scientific Applications, eds. F.H. Allen, G. Bergerhoff, and R. Sievers, Data Commission of the International Union of Crystallography, Bonn/Cambridge/Chester, **1987**, pp107-132.

9) Well J. M., Estell, D. A. TIBS **1988**, 13. 291.

10) Gelbin, M.E. Kohn, J. J. Amer. Chem. Soc. **1992**, 114, 3962.

11) Anne, F., Antoine, P., Hubert, G., Biotech. Lett, **1991**, 13, 161.

12) Devries, A. L. Ann Rev. Physiol **1982**, 45, 245.

13) Fuller, W. D.; Goodman, M.; Verlander, M. S. J. Am. Chem. Soc. **1985**, 107, 5821.
U.S. Patent 4,411,925; **1983**, Oct.

14) Horton, J. C., Donald, A.M., Hill, A. Nature, **1990**, 346, 44.