Pergamon

Tetrahedron Letters, Vol. 36, No. 3, pp. 475-476, 1995
Elsevier Science Ltd
Printed in Great Britain
0040-4039/95 $9.50+0.00

0040-4039(94)02290-9

A General Method for the Synthesis of Peptidyl Substrates for Proteolytic Enzymes

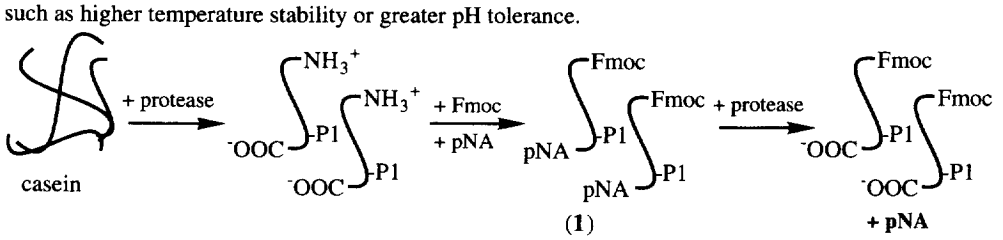
Andrew J. Whitmore, Roy M. Daniel*, and Helen H. Petach

Department of Biological Sciences, University of Waikato, Private Bag 3105, Hamilton, New Zealand

Abstract:. The synthesis of an heterogeneous peptide substrate for the assay of proteases was carried out by cleaving a protein using a protease to generate small peptides which were then coupled to a chromophore, p-nitroaniline. The chromophoric peptide product could be used to assay for the protease which produced the original peptides.

Proteolytic enzymes are known to exhibit a large range of specificities for their preferred cleavage sites along a peptide chain. These specificities can be determined by assaying a given endoprotease with numerous different peptidyl substrates and determining which amino acids are most often present at the site of cleavage. The simplest type of protease substrate is a short chain of amino acids linked to a chromogenic compound (e.g. p-nitroaniline or p-nitrophenol) at the carboxyl terminus. When the protease recognises the terminal amino acid (denoted P1) as a preferred site of hydrolysis, the cleavage takes place and releases the p-nitroaniline (pNA) anion to the solution. The enzyme activity is measured by determining the number of pNA anions (which absorb strongly at 405 nm) produced over a measured time period.

The preparation of small peptidyl substrates by the addition of single amino acids to one another and then to a chromogenic endgroup is a time-consuming, low yield, expensive synthesis. In this manuscript, we describe a general method for synthesising peptidyl substrates by cleaving a protein with the protease to be assayed and subsequently adding the chromogenic group to the carboxyl terminus of each of the peptide fragments in the heterogeneous mixture (Scheme 1). By this procedure, the heterogeneous substrate should, in principle, exhibit a specificity which matches that of the protease which cleaved the original protein since the only exposed C-terminal amino acids were residues selected by the protease as preferred P1 amino acids. Since the substrate was prepared to match the specificity of the protease used to generate the peptides, the substrate could be used to search for proteases with a similar substrate specificity, but different characteristics such as higher temperature stability or greater pH tolerance.



Scheme 1. Method for the preparation of small peptidyl protease assay substrates.

Casein was denatured by heating to 95°C for one hour and subsequently incubated at 70°C for 24 hours with 5 mM $CaCl_2 \cdot 2H_2O$ and 50 mg/l of the broad specificity thermophilic alkaline protease from *Thermus sp.* strain Rt41A (activity 9000 azocasein U/mg)[1]. SDS-PAGE results indicate that the casein is no longer

present after 24 hours of incubation with the protease. The solution was ultrafiltered through a YM3 (Amicon) membrane to collect only peptides with molecular weights below ca. 3000 Da. Size exclusion chromatography indicates that half of the isolated peptides are 2500-3500 Da. The peptides were lyophilized and the resulting yield was 20% based on the original input of casein.

The filtrate was treated with 9-fluorenylmethylchloroformate (Fmoc) to block the N terminus of each peptide and prevent their coupling to one another instead of the chromogenic endgroup[2]. N-protected peptides were coupled to p-nitroaniline following the EEDQ procedure[3] carried out in 70% ethanol. The resulting Fmoc-peptide-pNA (**1**) was purified by separation on a silica gel column eluting with ether/chloroform. Thin layer chromatography suggests that the reactions between Fmoc, peptide, and pNA were successful, but the thoroughness of the reactions was difficult to assess due to the heterogeneity of the product. Confirmation of the pNA derivatization was carried out by hydrolyzing bound pNA with base and measuring the resulting absorbance at 405nm. The substrate (**1**) was used to assay proteases by incubation of 950 µl of substrate (10 mg/ml) with 50 µl of protease (1 mg/ml) in 50% propanol/50 mM Hepes-NaOH buffer, pH 7.5. The substrate was not soluble in aqueous solutions.

Several proteases (Rt41A as described, caldolase from *Thermus sp.* strain ToK3[4],trypsin E.C. 3.4.21.4, and chymotrypsin E.C. 3.4.21.1) with differing P1 specificities were assayed using the heterogeneous synthetic peptidyl substrate (**1**) and the results are shown in Table 1. The difference in activities between the Rt41A protease and caldolase on the substrate (**1**) suggests that the substrate, although heterogeneous, still exhibits P1 sites specific to the type of protease used to cleave the original protein. Both the Rt41A and caldolase enzymes used for the activity assays were highly active in 50% propanol. Rt41A protease retains 87% of its aqueous activity in 90% propanol[1] while the caldolase retains 99% of its aqueous activity in 80% propanol[4]. Both the trypsin and chymotrypsin have limited resistance to organic solvents and thus the lack of any detectable activity when assayed in 50% propanol may partly reflect an effect of the solvent rather than being entirely due to substrate specificity.

Table 1. Relative Protease Activity on the Heterogeneous Substrate (**1**).

Enzyme	**Reaction conditions**	**Relative activity (%)**
Rt41A protease	5 min/70°C	100
caldolase	5 min/70°C	72
trypsin	24 hrs/37°C	0
chymotrypsin	24 hrs/37°C	0

K_m values, obtained from Lineweaver-Burk plots of the activities, for the substrate are indicative of the specificity of the protease for the substrate. The K_m for hydrolysis of the substrate (**1**) by Rt41A protease is 0.75 mg/ml in 50% propanol which is similar to K_m values observed for other synthetic peptide substrates[1]. Under the same assay conditions, the K_m for hydrolysis of the substrate (**1**) by caldolase is 2.85 mg/ml which is much larger than K_m values for other synthetic peptide substrates[4] and is larger than that obtained for Rt41A. This difference in K_m further suggests that the heterogeneous substrate (**1**) is more specific for the protease used to prepare the substrate than for another general protease.

In conclusion, an heterogeneous synthetic peptide substrate can be synthesized for proteases and demonstrates specificity for the protease used to generate the initial peptides. The major drawback to this procedure was broad range of physical properties exhibited by the resulting peptides which limited both the extent of the chemical coupling reactions and the solubility of the final substrate product.

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(*Received in UK* 1 *November* 1994; *accepted* 18 *November* 1994)