Modification of Two Amino Groups with Polyethylene Glycol Causes a Unique Activity Change of an Alkaline Proteinase from Alkalophilic Bacillus sp.

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Abstract. An alkaline proteinase from Bacillus sp. NKS-21 (ALPase I) was modified by activated polyethylene glycol (PEG2) with an Mr of 10,000. The modified preparation was purified with the isoelectric focusing method. Two amino groups out of the total five amino groups of ALPase I were modified. This modification gave rise to an 85% loss of the activity towards milk casein as a substrate, while the modification caused a 20% loss of binding ability towards anti-ALPase I antiserum. The kinetic parameters of PEG2-ALPase I towards peptidyl-p-nitroanilides (pNA-substrates) and peptidyl-7-methyl-coumaryl-4-amides (MCA-substrates) suggested that at least one of the two amino groups modified with PEG2 was near the active site of ALPase I. The stability of the modified enzyme against heat treatment was increased. The PEG2-ALPase I could express enzymatic activity in the organic solvent benzene.

Materials and Methods

An alkaline proteinase from Bacillus sp. (ALPase I) was prepared by the method of our previous paper [17]. Activated polyethylene glycol (PEG2; Mr = 10,000) was purchased from the Seikagakukogyo Co. (Tokyo). Peptidyl-p-nitroanilides (pNA-substrates) and peptidyl-7-methyl-coumaryl-4-amides (MCA-substrates) were purchased from Peptide Institute, Inc. (Minoshi, Osaka) and Sigma Chemical Co. (St. Louis, Missouri). Peptidyl-4-methyl-coumaryl-7-amides (MCA-substrates) were from Peptide Institute, Inc.

Enzyme assay. Proteolytic activities in ALPase I and PEG2-ALPase I at pH 10.0 were assayed as in our previous paper [17]. The chromogenic and fluorogenic assays (with pNA-substrates or MCA-substrates) were conducted under the same conditions of the previous paper [5].

Protein concentration. Protein concentration was usually estimated as follows. The protein was hydrolyzed by the method of McGrath [13], and this sample was reacted with 2,4,6-trinitrobenzene sulfonic acid by the method of Fields [4]. The colors of 2,4,6-trinitrophenyl (TNP) amino acids were measured at 420 nm.

Preparation of PEG2-ALPase I. One gram of PEG2 was reacted with 10 mg of ALPase I in 10 ml of 0.1 M Atkins and Pantin’s buffer, pH 8.5, at room temperature overnight. The mixture was applied to an isoelectric focusing chromatography. Isoelectric focusing [18] was carried out on an LKB column (100 ml) containing 1% carrier ampholite (Ampholine) with a pH gradient of 3.5–10.0. The potential gradient of 300 V was applied for 72 h at 4°C.
Fig. 1. Isoelectric focusing chromatogram of PEG2-ALPase I. The pH range of used ampholine was 3.5–10.0.

The amount of PEG2 attached to the amino groups of ALPase I was estimated by the reaction with 2,4,6-trinitrobenzene sulfonic acid, as described by Fields [4].

**FPLC-Superose 12 of PEG2-ALPase I.** FPLC-Superose 12 (gel filtration LC) was performed with 0.1 M phosphate buffer at pH 7.2 containing 0.3 M Na2SO4. Flow rate was 0.5 ml/min.

**Polyacrylamide gel electrophoresis.** Polyacrylamide gel electrophoresis (PAGE) at pH 2.3 was carried out by the method of previous paper [9].

**Immunodiffusion study.** ALPase I and PEG2-ALPase I were set up for immunodiffusion slides against rabbit anti-ALPase I antiserum. Ouchterlony double immunodiffusion and single radial immunodiffusion methods were carried out [3].

**Enzyme assay in benzene.** Benzene (1.25 ml) and 5 µl of 10 mM Suc-Ala-Ala-Pro-Phe-MCA in dimethylsulfoxide (DMSO) solution were mixed. Ten µl of enzyme solution was added to this mixture. Fluorescence of the released 7-amino-4-methylcoumarin (AMC) was measured with a Hitachi fluorescence spectrometer, model F-3000, equipped with recorder, excitation at 345 nm and emission at 445 nm.

**Kinetic parameter determination.** The values of Km and kcat were determined from Lineweaver-Burk plots.

**Results**

**Preparation and properties of PEG2-ALPase I.** Figure 1 shows an isoelectric focusing chromatogram, with two peaks of the absorbance at 280 nm. The isoelectric point of the modified PEG2-ALPase I was 9.3. The peak at pH 5.0 was considered as non-reacted PEG2. The active fraction was pooled and stored at 4° C.

The modification rate of the amino groups of ALPase I was determined to be 40%. This result showed that two amino groups out of five of ALPase I were modified by PEG2.

Figure 2 shows polyacrylamide gel electrophoresis at pH 2.3 for PEG2-ALPase I (lane 2) and native ALPase I (lane 1). The modified enzyme showed a broad band, and it was less movable than the native one.

The molecular weight of the modified enzyme was estimated to be about 40 K by the gel filtration method of FPLC-Superose 12 (Fig. 3).

The residual activities of the modified enzyme towards Suc-Ala-Ala-Pro-Phe-MCA and casein were 25% and 15% of the native enzyme.

Thermostability of the PEG2-ALPase I was improved by attaching PEG2 to ALPase I (Fig. 4). The native ALPase I was completely destroyed with the treatment at 50°C for 10 min, but the PEG2-ALPase I still had 40% of the activity of the control. At 55°C