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# ENZYMATIC COUPLING OF TWO D-AMINO ACID RESIDUES IN AQUEOUS MEDIA

Björn Ekberg, Christer Lindbladh, Maria Kempe and Klaus Mosbach*

Department of Pure and Applied Biochemistry, University of Lund,

P.O. Box 124, 221 00 Lund, Sweden

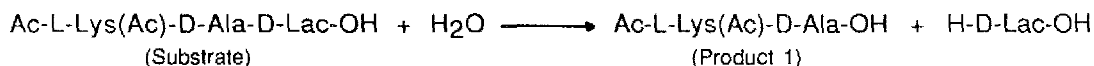
Abstract: The formation of a D-Ala-D-Ala containing tripeptide in aqueous solution, catalysed by muramoylpentapeptide carboxypeptidase (E.C. 3.4.17.8), is described.

The ability of papain to catalyze the formation of peptide bonds was first described in 1909[1]. Until recently however, the application of enzymes to the synthesis of peptides has been neglected but is now considered as an alternative to conventional chemical approaches[2]. One problem still remaining in the enzymatic synthesis of peptides is the incorporation of D-amino acids. A few reports on the synthesis of dipeptide derivatives of the L-D-configuration using a D-amino acid derivative as the nucleophile have appeared[3-5]. More recently, the synthesis of a dipeptide derivative of the D-D-configuration was reported[6]. In this report formyl-D-alanyl-D-alanine amide was prepared by the action of subtilisin on formyl-D-alanine chloro ethylester in the presence of D-alanine amide. However, this reaction was performed in tert-amyl alcohol to change the stereospecificity of subtilisin. Here we wish to describe the formation of the same dipeptide unit (D-Ala-D-Ala) in totally aqueous media using D-alanine carboxypeptidase (muramoylpentapeptide carboxypeptidase), which catalyses the cross-linking of a peptidoglycan involved in the formation of bacterial cell-walls[7].

## Materials and methods.

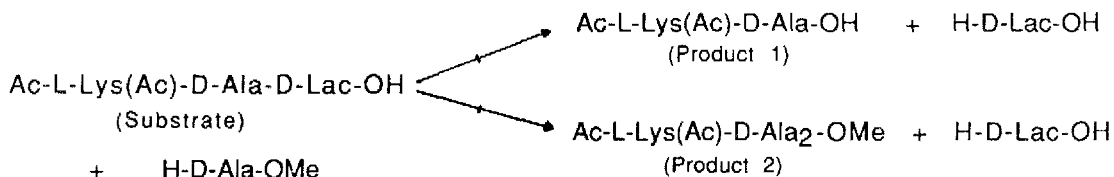
Isolation of the enzyme: The enzyme was isolated from Bacillus subtilis, grown in Luria-Bertani media to late log phase. The cells were harvested by centrifugation and resuspended in 20% (w/v) sucrose in 30 mM Tris-HCl pH 8.0 . EDTA (1 mM) and lysozyme (1mg/ml) were added, the suspension incubated for 30 min and finally clarified by centrifugation. The resulting supernatant was applied onto a column of DEAE-Sepharose equilibrated in 50 mM phosphate buffer, pH 7.5 and eluted with a sodium chloride gradient to 1.0 M. About 5% of the applied activity (see below), equivalent to 0.75% of the total protein loaded onto the column, was recovered and pooled. SDS-PAGE of the pooled fractions showed a major band with a molecular weight corresponding to 53 000[7].

Enzyme activity. Enzyme activity was determined by following the hydrolysis of Ac-L-Lys(Ac)-D-Ala-D-Lac-OH[8] as shown:




Typically, 5-45 µg of protein from the pooled fractions was incubated with substrate (0.8 mM, 0.48 ml) in 0.1 M sodium phosphate buffer, pH 7.5 for 5 hours at 37°C. The incubation mixture was analysed by HPLC (LKB 2150-52) on a Spherisorb C(18)-column using acetonitrile/50mM potassium phosphate, pH 4.0 (6.5/93.5; v/v) as the mobile phase. Elution was monitored at 220nm.

Enzymatic synthesis of Ac-L-Lys(Ac)-D-Ala-D-Ala-OMe (Product 2):




The same reaction conditions were used as described above for the hydrolysis reaction exept for the addition of H-D-Ala-OMe (67 mM). Aliquots of the reaction mixture were withdrawn at various intervals and subjected to HPLC-analysis as described. Figure 1 shows the results of HPLC analyses of the reaction mixture after 30 min and 3 hours. Product identification was achieved by saponification of the substrate (product 1) and chemical synthesis of Ac-L-Lys(Ac)-D-Ala-D-Ala-OMe[9] (product 2). Figure 2 shows the consumtion of substrate and concommitant formation of products over a 24 hour period, as determined by HPLC analyses.

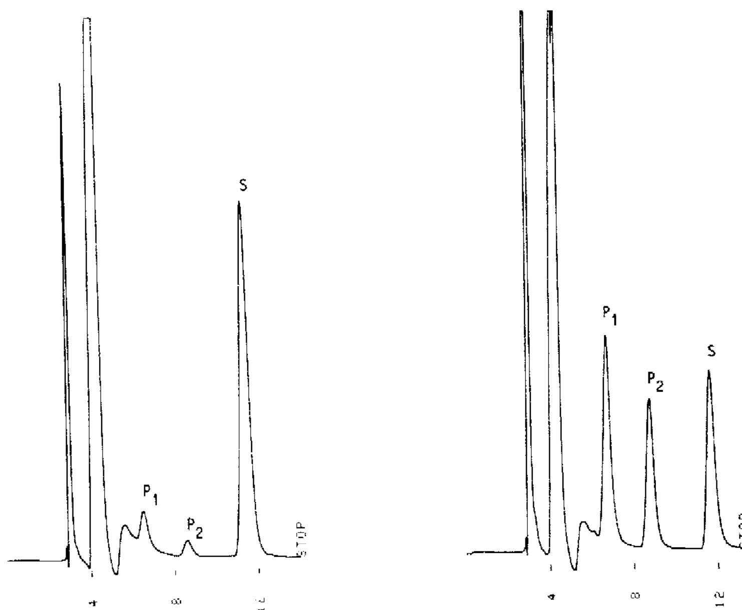



Figure 1. HPLC-analysis of the reaction mixture after 30 minutes (left) and 3 hours (right) of incubation. S=substrate, $P_1$=product 1, $P_2$=product 2.

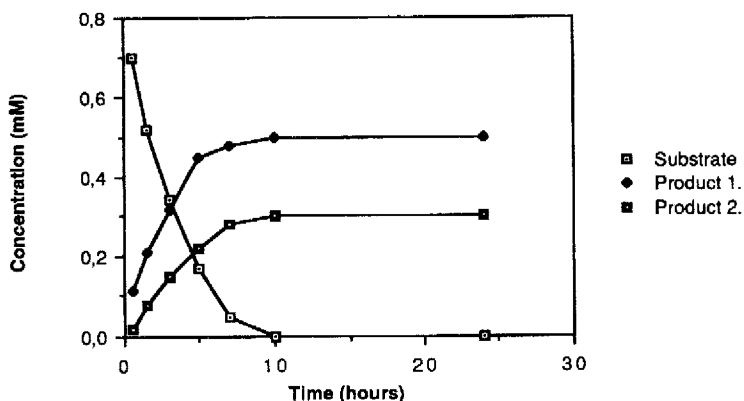



Figure 2. The content of the reaction mixture at different stages of the reaction determined by HPLC-analysis.

## Results and discussion.

The isolated enzyme preparation showed characteristics of muramoyl pentapeptide carboxypeptidase such as the unique ability to hydrolyze D-D peptide substrates or derivatives.

Furthermore, the reaction was inhibited by penicillin[7], in accordance with the effect of penicillin on bacterial growth. In the synthetic reaction catalyzed by the isolated muramoylpentapeptide carboxypeptidase, Ac-L-Lys(Ac)-D-$Ala_2$-OMe (product 2) was obtained in approximately 40% yield after 10 hours (Figure 2). However, if L-alanine methyl ester was used as the nucleophile instead of D-alanine methyl ester, no product formation was observed, thus demonstrating the stereo-specificity of the reaction. In summary, we have demonstrated the enzymatic synthesis of a peptide containing a D-D-configuration in totally aqueous media, obviating the use of organic solvents to alter the stereo-specificity of enzymes[6,10]. We are currently investigating the use of this specific muramoylpentapeptide carboxypeptidase for the synthesis of other D-amino acid containing peptides.

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