PURIFICATION OF DD-CARBOXYPEPTIDASES FROM *STREPTOMYCES* STRAINS R61 AND K15 BY ANTIGEN-ANTIBODY AFFINITY CHROMATOGRAPHY

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ABSTRACT

The exocellular R61 DD-carboxypeptidase and the lysozyme-releasable K15 DD-carboxypeptidase were purified using anti-exocellular R61 enzyme IgG-Sepharose as immunoadsorbent. The specific activity of R61 enzyme was increased 70 fold (yield 90 %) and that of K15 enzyme was increased 550 fold (yield 50 % of the adsorbed material).

INTRODUCTION

The cellular enzyme system involved in peptide crosslinking during synthesis of the bacterial cell wall peptidoglycan consists of at least 1) a transpeptidase activity which catalyzes peptide bond formation, a reaction through which the carboxyl group of the penultimate D-Ala residue of a peptide donor ending in D-Ala-D-Ala is transferred to the free ω-amino group of another peptide acceptor, and 2) a DD-carboxypeptidase activity which simply hydrolyzes the C-terminal D-Ala-D-Ala sequences of the peptide donors without performing any transfer reaction. Both activities are sensitive to ß-lactams, but the inhibition of either of them may be the cause for cell lysis depending on the species investigated.

The DD-carboxypeptidase-transpeptidase enzyme system in Streptomyces sp. is relatively well known (1). In strains K15 und R61, it consists of: 1) a membrane-bound transpeptidase capable of performing low DD-carboxypeptidase activity and 2) a set of DD-carboxypeptidases: a) membrane-bound, b) lysozyme-releasable and c) exocellular, having low transpeptidase activity (2). The relationship between the exocellular, lysozyme-releasable and membrane-bound DD-carboxypeptidase-transpeptidase enzyme system remains to be determined.

The exocellular DD-carboxypeptidase can be isolated from the culture filtrates. At present, the enzyme excreted by strain R61 is the only one which has been purified to homogeneity (3). The procedure makes use of conventional techniques, it is lengthy and the final yield is small.

The lysozyme-releasable DD-carboxypeptidase can be released from the cells through the enzymatic transformation of the mycelium into protoplasts by lysozyme in a sucrose medium (2). This enzyme represents a very small proportion of the total protein present in the

original cell extracts and therefore its isolation is not easy. Previous studies have shown that the purified exocellular DD-carboxypeptidase of strain R61 and the lysozyme-releasable DD-carboxypeptidase of this and other Streptomyces strains were immunologically related (4). On this basis, the purification of the lysozyme-releasable enzyme of strain K15 and of the exocellular R61 enzyme was attempted by affinity chromatography using the IgG fraction of an anti-R61 enzyme antiserum as ligand.

PURIFICATION OF THE IgG FRACTION

The antibodies directed against R61 exocellular DD-carboxypeptidase were purified by affinity chromatography using R61 enzyme Sepharose 6B as adsorbent. The Sepharose was activated according to Sundberg and Porath (5). Bisoxyran- rather than CNBr-activated Sepharose was used because very alkaline conditions were required to dissociate the antigen-antibody complex.

Epoxy-activated Sepharose (2 g - 900-1000 µequiv. of epoxide groups/g dry gel) was added to a solution (4 ml) of the R61 exocellular enzyme (18 mg; i.e. 0.5 µmol of purified enzyme) in 0.5 M carbonate buffer pH 9.5 and the suspension was stirred by rotation for 72 hrs at 28°C. Under these conditions about 30 % of the enzyme was bound to the support. The enzyme-Sepharose complex was successively washed with 1) 250 ml 0.5 M carbonate pH 9.5 - 0.5 M NaCl; 2) 250 ml 0.1 M glycine·HCl pH 3.0 - 0.5 M NaCl; 3) H_2O; 4) 250 ml 30 mM Tris·HCl pH 7.5 - 0.5 M NaCl and 20 mM $CaCl_2$. The remaining free epoxide

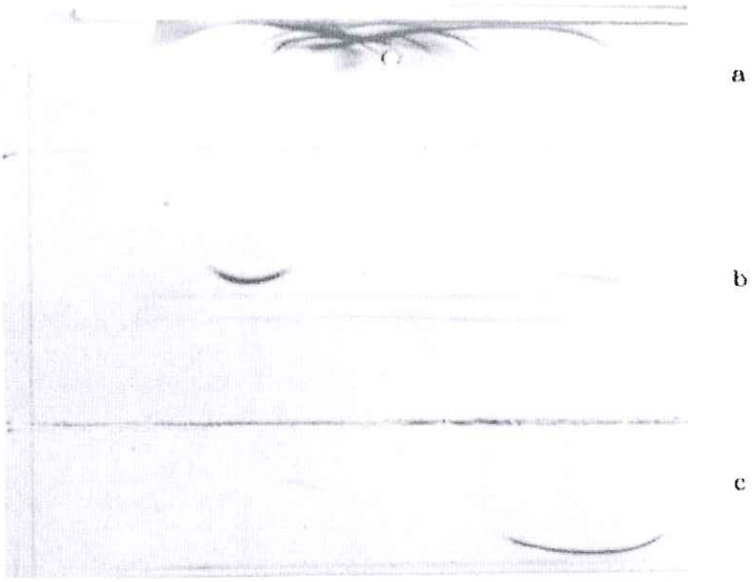

Fig. 1. Immunoelectrophoresis of a) complete exocellular R61 antiserum, b) 0.5 M NaCl eluted material and c) 0.2 M carbonate buffer pH 11.4 eluted IgGs from R61 enzyme Sepharose adsorbent, tested against antiserum to complete rabbit serum.

groups were blocked by reaction with 2 M ethanolamine at pH 9.5 for 8 hrs at 25°C. This adsorbent was washed and finally equilibrated with 10 mM Tris·HCl pH 7.7. 2 ml of complete anti-R61 enzyme antiserum were adsorbed onto a column of the above mentioned adsorbent (1 mg of ligand/ml of gel). The non-adsorbed material had completely lost the capability of inhibiting the DD-carboxypeptidase activity of the R61 exocellular enzyme, which showed that fixation of the specific IgGs had occurred with high efficiency. The column was thoroughly washed with 0.5 M NaCl + 0.01 M Tris·HCl pH 7.7. The IgGs were eluted from the adsorbent with 0.2 M carbonate buffer pH 11.4, rapidly dialysed against 10 mM Tris·HCl buffer pH 7.7 and concentrated to 2 ml by ultrafiltration. Through a series of several runs performed under identical conditions, 30 mg of purified IgG's (Fig. 1) were obtained and used as ligand for the purification of the lysozyme-releasable enzyme.

ENZYME PURIFICATION

Sepharose 6B was activated as described above and the purified IgG fraction was coupled onto it. The reaction was performed in 0.5 M carbonate buffer pH 10.5 for 48 hrs at 34°C. In this step the yield was 84 %. The IgG-Sepharose 6B, washed and treated with ethanolamine as described above, was equilibrated with 30 mM Tris·HCl pH 7.5.

In order to check the behaviour of the immunoadsorbent, the purification of the crude exocellular R61 enzyme was first attempted. The enzyme was applied to the column, eluted with 0.2 M carbonate buffer pH 11.4, dialysed immediately against 10 mM Tris·HCl pH 7.7 and concentrated by ultrafiltration. Measurements of enzyme activity showed a 70 fold increase in specific activity. The yield was 90 % (Table 1).

TABLE 1. Purification of exocellular R61 DD-carboxypeptidase on anti-exocellular R61 DD-carboxypeptidase IgG-Sepharose 6B immunoadsorbent.

	Total activity* (µmoles/min)	Yield	Specific activity** (µ-moles/min/mg)
Crude enzyme	1790	100	0.74
Non-adsorbed material	0	0	
NaCl 0.5 M pH 7.9 eluted material	0	0	
Na_2CO_3 0.2 M pH 11.4 eluted material	1658	92	52

* Substrate: 1.6 mM Ac_2-L-Lys-D-Ala-D-Ala

** Specific activity measured at substrate concentrations of 10 times the Km value (12 mM) as described by J.M. Frère et al. (3).

The same technique was then applied to a crude lysozyme-releasable DD-carboxypeptidase preparation of Streptomyces K15, obtained after ammonium sulphate precipitation. The K15 enzyme was adsorbed on the IgG-Sepharose column, which was then washed with 0.5 M NaCl, 0.2 M carbonate buffer H 10.5. The enzyme was eluted with 0.2 M carbonate pH 11.4, resulting in a 550 fold increase in specific activity with

a yield of 50 % of the adsorbed material (Table 2).

TABLE 2. Purification of K15 lysozyme-releasable DD-carboxypeptidase on anti-exocellular R61 DD-carboxypeptidase IgG-sepharose 6B immunoadsorbent.

	Total activity* (µmoles/min)	Yield	Specific activity** (µmoles/min/mg)
Crude enzyme	0.255	100	0.002
Non-adsorbed material	0.139	55	
NaCl 0.5 M pH 10.5 eluted material	0.145	57	
Na_2CO_3 0.2 M pH 11.4 eluted material	0.060	23.5	1.15

* Substrate: 1.6 mM Ac_2-L-Lys-D-Ala-D-Ala

** Specific activity measured at substrate concentration of 10 times the Km value (9 mM) as described by M. Leyh-Bouille *et al.* (2).

CONCLUSION

The anti-exocellular R61 enzyme antiserum was found to inhibit the DD-carboxypeptidase activity of the exocellular and lysozyme-releasable enzymes from other *Streptomyces* strains (4). On this basis, the exocellular R61 DD-carboxypeptidase and lysozyme-releasable K15 DD-carboxypeptidase were purified by using anti-exocellular R61 enzyme IgG-Sepharose 6B as an immunoadsorbent. The results reported here are still preliminary. However, they are very promising and when the technique will be refined, it may well give rise to procedures through which the various enzymes of the Streptomyces peptidoglycan crosslinking enzyme system can be isolated in a specific manner, with high yields and with a minimum of steps involved.

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