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The Peptidoglycan Crosslinking Enzyme System in *Streptomyces* R61, K15 and *rimosus*

Immunological Studies

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The exocellular DD-carboxypeptidases from *Streptomyces* R61, K15, the lysozyme-releasable DD-carboxypeptidases from *Streptomyces* R61, K15 and *rimosus*, and the membrane-bound DD-carboxypeptidase of *Streptomyces* K15 are immunologically related to each other.

As many as four enzymes may be involved in the peptidoglycan crosslinking enzyme system in *Streptomyces* sp. [1]. *Streptomyces* K15 excretes an exocellular DD-carboxypeptidase; it has two cell-bound DD-carboxypeptidases, one of which is released by protoplasting the mycelium whereas the other is bound to the plasma membrane; and finally it possesses a transpeptidase which is also membrane-bound. The enzyme system in *Streptomyces* R61 is very similar except for the membrane-bound DD-carboxypeptidase activity which is hardly detectable. Finally, the enzyme system in *Streptomyces rimosus* consists almost exclusively of a membrane-bound transpeptidase. Rabbits were immunized with the purified exocellular enzyme of *Streptomyces* R61 and the antiserum thus obtained was used as a tool for the study of the relationship which might exist between these various enzymes. Two DD-carboxypeptidases unrelated to the preceding ones served as controls. One of them was the exocellular DD-carboxypeptidase from *Actinomadura* R39 [2, 3]. It was selected because of the profound differences that occur in the type of interpeptide bridging in the wall peptidoglycans of the *Streptomyces* strains (a D-Ala-Gly-LL-diaminopimelic acid linkage) and of *Actinomadura* R39 (a C-terminal D-Ala-(D)-*meso*-diaminopimelic acid linkage) [3]. The other control enzyme was the exocellular DD-carboxypeptidase from *Streptomyces albus* G which, in contrast to the other *Streptomyces* exocellular DD-carboxypeptidases, is a cationic protein, does not perform transfer reactions, has a high lytic activity on various wall peptidoglycans and is largely insensitive to β-lactams [3].

MATERIALS AND METHODS

Enzymes

The exocellular enzymes from *Streptomyces* R61 and *Actinomadura* R39 had been purified to protein homogeneity [2]. The exocellular enzyme from *Streptomyces* K15 and the lysozyme-releasable enzymes from *Streptomyces* R61, K15 and *rimosus* were at various stages of purity as described by Leyh-Bouille *et al.* [1]. The cytoplasmic membranes from *Streptomyces* R61, K15 and *rimosus* were also prepared as described in [1]. The final membrane preparations contained about 25 mg protein/ml in 14 mM phosphate buffer. Unless otherwise specified, the DD-carboxypeptidase activity was estimated by using 1.6 mM Ac_2-L-Lys-D-Ala-D-Ala and the transpeptidase activity by using 1.6 mM Ac_2-L-Lys-D-Ala-D-Ala + 1.6 mM Gly-Gly. The techniques are those described previously [1].

Radioactive Benzylpenicillin

[^{14}C]Benzylpenicillin (with the label in the acyl side-chain; 50 Ci/mol) was purchased from the Radiochemical Centre (Amersham).

Antiserum

A male New Zealand white rabbit (4–5 kg) served as the source of antiserum. The rabbit was injected with 2 mg R61 enzyme emulsified in 1.5 ml 10 mM phosphate buffer, pH 7.5, and 1.5 ml of com-

plete Freund's adjuvant (Difco) into the hind foot pads, intramuscularly, and subcutaneously into the dorsum of the neck. Booster injections, each containing 1 mg enzyme, were administered in a similar fashion at 2, 6 and 8 weeks. The rabbit was exsanguinated from the marginal ear vein 10 weeks after the first injection and the serum obtained was decomplemented at 56 °C for 30 min. When the enzyme preparation (16 µg and 32 µg) was reacted against the serum in immunodiffusion analysis [4], a single precipitation band was detected. Normal-rabbit serum obtained prior to any immunization procedures served as the control serum.

RESULTS

Effects of Antiserum on the Activities of the Exocellular R61 Enzyme

Enzyme (6 ng) and various amounts of either antiserum or control serum (0.001 – 5 µl) were incubated together in 30 µl, final volume, of 8 mM Tris-HCl buffer pH 7.5 for 1 h at 37 °C. The DD-carboxypeptidase activity was then estimated by incubating the reaction mixtures with 1.6 mM Ac_2-L-Lys-D-Ala-D-Ala for 4 h at 37 °C. The control serum had no effect. Maximal inhibition of the enzyme activity by the antiserum was of 80 – 85% (Fig. 1). In other experiments the enzyme activity was estimated by using larger concentrations of Ac_2-L-Lys-D-Ala-D-Ala up to 24 mM (*i.e.* 2 × the K_m value) and by decreasing accordingly the incubation times in order to maintain initial velocity measurements. The extents of enzyme inhibition effected by the various antiserum dilutions remained unchanged. Finally, the inhibition of the transpeptidase activity (as measured with 1.6 mM Ac_2-L-Lys-D-Ala-D-Ala + 1.6 mM Gly-Gly) was found to parallel the inhibition of the DD-carboxypeptidase activity.

Effects of Antiserum on the Penicillin-Binding Capability of the Exocellular R61 Enzyme

Enzyme (8.4 µg), various amounts of antiserum (2 – 140 µl) and various amounts of control serum (0 – 138 µl, with the result that all the reaction mixtures contained 140 µl antiserum + control serum) were incubated together in 1040 µl, final volume, of 5 mM Tris-HCl buffer pH 7.5, for 1 h at 37 °C. The ratios of enzyme protein to antiserum were identical to those used in the preceding experiments but in the present case there was a 40-fold increase in the absolute concentrations of the two reagents. Samples (2 µl) were removed and tested for residual enzyme activity by incubating with 1.6 mM Ac_2-L-Lys-D-Ala-D-Ala in 8 mM Tris-HCl buffer pH 7.5 (final volume: 30 µl) for 90 min at 37 °C. All reaction mixtures were then supplemented with 0.6 nmol [^{14}C]benzylpenicillin and incubated for 15 min at 37 °C. Trichloroacetic acid (5 ml of an 11% solution) was added to each mixture at 0 °C to precipitate bound-penicillin-labelled protein. The precipitates were collected by centrifugation, washed with 8 ml cold trichloroacetic acid solution, dried at 60 °C, solubilized with 1 ml of Soluene and the radioactivity was counted (in 10 ml Bray scintillation liquid). Non-specific trapping of radioactivity was estimated from solutions containing the same amounts of serum and antiserum as above but without enzyme. Fig. 1 shows that the loss of enzyme activity paralleled the loss of the ability of the enzyme to bind penicillin.

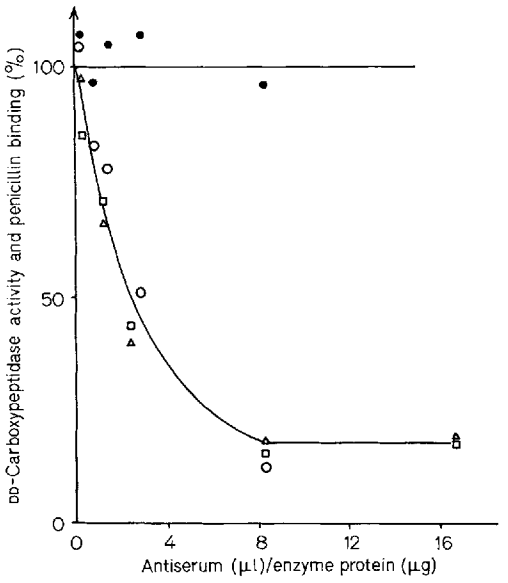

Fig. 1. *Effects of the antiserum to exocellular R61 enzyme on the DD-carboxypeptidase activity and penicillin-binding capability of the exocellular R61 enzyme.* (●——●) Effect of control serum on the DD-carboxypeptidase activity; (○——○) inhibition of DD-carboxypeptidase activity under conditions where 6 ng enzyme and samples of antiserum (from 0.001 to 0.1 µl) were incubated together in 30 µl, final volume; (□——□) inhibition of DD-carboxypeptidase activity under conditions where 8.4 µg enzyme and samples of antiserum (from 2 to 140 µl) were incubated together in 1040 µl, final volume; (△——△) effect of the antiserum on the ability to bind [^{14}C]benzylpenicillin. For other experimental conditions, see text

Effects of Antiserum on the Activities of Enzymes Other than The Exocellular R61 Enzyme

The same microscale technique was used to assay inhibition of other soluble (exocellular or lysozyme-releasable) enzymes. The antiserum had no effect on the exocellular enzymes from both *Actinomadura* R39

Table 1. *Effects of the antiserum to exocellular R61 enzyme on the* DD-*carboxypeptidase and transpeptidase activities of the exocellular and lysozyme-releasable enzymes*
The activities are expressed as percentages of the tripeptide Ac_2-L-Lys-D-Ala-D-Ala hydrolysed (DD-carboxypeptidase activity) or transformed into Ac_2-L-Lys-D-Ala-Gly-Gly (transpeptidase activity). All the enzyme/antiserum incubations were carried out at 37 °C and in 30 µl, final volumes. The buffers used were 5–10 mM Tris-HCl, pH 7.5, except for the exocellular enzyme from *Streptomyces albus* G (50 mM Tris-HCl pH 8.3 + 5 mM $MgCl_2$) and for the exocellular enzyme from *Actinomadura* R39 (30 mM Tris-HCl pH 7.5 + 3 mM $MgCl_2$). The amounts of enzyme used were such that the activities measured in the absence of antiserum necessitated 4 h of incubation at 37 °C with the substrates, except for the lysozyme-releasable enzyme of *Streptomyces rimosus* in which case the incubation time was of 18 h

Strain	Enzyme	DD-Carboxypeptidase activity: 1.6 mM Ac_2-L-Lys-D-Ala-D-Ala			Transpeptidase activity: (a) 1.6 mM Ac_2-L-Lys-D-Ala-D-Ala + 1.6 mM Gly-Gly (b) 1.6 mM Ac_2-L-Lys-D-Ala-D-Ala + 8 mM Gly-Gly		
		activity in the absence of antiserum	antiserum present in 30 µl final volumes	activity in the presence of antiserum	activity in the absence of antiserum	antiserum present in 30 µl final volumes	activity in the presence of antiserum
		%	µl	%		µl	%
Streptomyces R61	exocellular	23	0.05	3.0	(a) 4	0.17	0.6
					(b) 10	0.17	2.9
Streptomyces R61	lysozyme-releasable	20	0.05	2.6	(a) 2.14	0.17	0.6
					(a) 2.25	1	0.5
					(b) 5.47	1	1.1
Streptomyces K15	exocellular	22	1	15.8			
Streptomyces K15	lysozyme-releasable	20	0.05	1.8	(a) 3.35	0.17	0.5
					(a) 2.50	1	0.7
					(b) 6	1	1.1
Streptomyces rimosus	lysozyme-releasable	10	1	2.5			
Streptomyces albus G	exocellular	24	1	24			
Actinomadura R39	exocellular	23	1	23			

and *Streptomyces albus* G (Table 1). The lysozyme-releasable enzymes from strains R61, K15 and *rimosus* were inhibited by 70–90% both in the DD-carboxypeptidase and the transpeptidase assays. The exocellular enzyme from strain K15 was only inhibited by about 28%. This low extent of enzyme inhibition might be easily explained if one assumes that the second penicillin-binding component of M_r 62000 present in the K15 enzyme preparation [1] is another DD-carboxypeptidase not related immunologically to the DD-carboxypeptidase of M_r 38000.

The microscale system was modified to assay the effects of the antiserum on the membrane-bound enzymes. In this modification, membranes (1.8 mg protein), antiserum or control serum (dilution range: 1/6 to 1/300) were incubated together in 14 mM phosphate buffer pH 7.5 (final volume: 72 µl) for 1 h at 37 °C and then for 18 h at 4 °C. Samples were removed and tested for DD-carboxypeptidase and transpeptidase activities. The experimental conditions and results are given in Table 2. Other than a slight inhibition (19%) of the membrane-bound DD-carboxypeptidase activity of strain K15, the antiserum had no effect on the other membrane-bound enzymes tested.

DISCUSSION

The antiserum to exocellular R61 enzyme was found to inhibit the DD-carboxypeptidase and transpeptidase activities as well as the penicillin-binding capability of the exocellular R61 enzyme. This same antiserum was also found to inhibit the exocellular and lysozyme-releasable DD-carboxypeptidases from *Streptomyces* R61, K15 and *rimosus*. This observation suggests a certain structural relatedness for these enzymes. Whether the observed antigenic cross-reactivity is due to a similarity of the active sites or other areas of the enzyme molecules warrants further study. The antiserum to exocellular R61 enzyme had no effect on the activity of the membrane-bound transpeptidases of the three strains examined. It is known that a temperature as low as −30 °C is required to prevent the membranes of *Streptomyces* R61 from performing

Table 2. *Effects of the antiserum to exocellular R61 enzyme on the* DD-*carboxypeptidase and transpeptidase activities of the membranes of* Streptomyces *R61, K15 and* rimosus

DD-Carboxypeptidase activity was estimated by incubating for 20 h at 37 °C, 25-µl samples of the membrane/antiserum suspensions in 30 µl, final volume, of 11 mM phosphate buffer, pH 7.5, in the presence of 4.7 mM Ac_2-L-Lys-D-Ala-D-Ala. Depending upon the membranes, 4–10% of the tripeptide was hydrolysed in the control samples. Transpeptidase activity was estimated by incubating for 4 h at 37 °C, samples of the membrane/antiserum suspensions in 30 µl, final volume, of 9 mM phosphate buffer pH 7.5, in the presence of 1.6 mM Ac_2-L-Lys-D-Ala-D-Ala and 1.6 mM Gly-Gly. The volumes of the samples used were: (1) for strain R61: 2.5 µl (a), 1.25 µl (b), 3 µl (c); (2) for strain K15: 10 µl; and (3) for strain *rimosus:* 2.5 µl (d) and 2 µl (e). Depending upon the cases, 1.5–6.2% of the tripeptide was transformed into Ac_2-L-Lys-D-Ala-Gly-Gly in the control samples. The average value for all the residual activities in the presence of antiserum, except for the membrane-bound DD-carboxypeptidase of strain K15, is 104% with a standard deviation value of 10. The average value for the K15 residual DD-carboxypeptidase activity is 81% with a standard deviation of 4

Strain	Serum dilution	Residual activity	
		DD-carboxy-peptidase	trans-peptidase
		% control samples	
R61	1/6	92–104	125(a)–107(b)
	1/30	102	91(c)
	1/300		108(c)
K15	1/6	79–76–81	90–114
	1/30	86.5	97
	1/300	81	124
rimosus	1/6	103–104	107(d)
	1/30	93	111(e)
	1/300	98	96(c)

transpeptidation reactions [5]. Such a property suggests that the enzyme is deeply buried within the lipid phase of the membrane and for this reason, antigenic sites may be inaccessible to antibodies. Part of the DD-carboxypeptidase activity of the membranes of strain K15, however, was inhibited by the antiserum to exocellular R61 enzyme. It can thus be attributed to an enzyme which is distinct from the transpeptidase and is immunologically related to the exocellular and lysozyme-releasable enzymes.

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