Chapter 37

Purification and Characterization of a β-Lactam-Resistant Penicillin-Binding Protein from *Enterococcus hirae (Streptococcus faecium)*

Philippe Jacques
Aboubaker El Kharroubi
Bernard Joris
Bernard Dussenne
Fabienne Thonon
Graziella Piras
Jacques Coyette
Jean-Marie Ghuysen

The natural β-lactam resistance of enterococci is a critical problem in clinical chemotherapy. It is thus of interest to better understand the biochemical basis of this resistance.

The resistant strain R40, provided by R. Fontana (2), was isolated in vitro from *Enterococcus hirae* (*Streptococcus faecium*) ATCC 9790 by successive selections in the presence of increasing concentrations of benzylpenicillin. This strain overproduces PBP 5, the PBP most resistant to benzylpenicillin. Thus, we planned to purify and characterize the PBP 5 to better understand the resistance phenomenon.

Philippe Jacques, Aboubaker El Kharroubi, Bernard Joris, Bernard Dussenne, Fabienne Thonon, Graziella Piras, Jacques Coyette, and Jean-Marie Ghuysen • Service de Microbiologie, Université de Liège, Institut de Chimie, B6, B-4000 Sart Tilman (Liège 1), Belgium.

Table 1. Characterization of the tPBP 5 derivatives

Derivative	Molecular mass (daltons)	Yield (%)[a]
tPBP 5a	68,000	79
tPBP 5b	63,000	7.4
tPBP 5c	61,000	1.1
tPBP 5d	44,000	3.5

[a]Related to the total amount of PBP 5 in the membrane.

The R40 mutant and the parent strain, like most of other *E. hirae* strains, contain seven membrane proteins able to form stable complexes with benzylpenicillin (penicillin-binding proteins [PBPs]) (1). These PBPs are numbered in the order of decreasing molecular weights.

If the solubilization of the native protein was easy (90% was extracted from the cells with 0.05% Triton X-100), the purification of the soluble material was very difficult. Three chromatographic techniques were tested to separate the PBPs from the detergent extracts: hydrophobic interaction, gel filtration, and anion exchange. In all three experiments, the PBPs behaved as mixed lipid-protein micelles. Another approach was thus necessary to obtain great quantities of these membrane proteins with a high degree of purity.

Treatment of a membrane preparation from *Enterococcus hirae* R40 with 0.125% (wt/wt) trypsin for 15 min at 37°C released four soluble derivatives

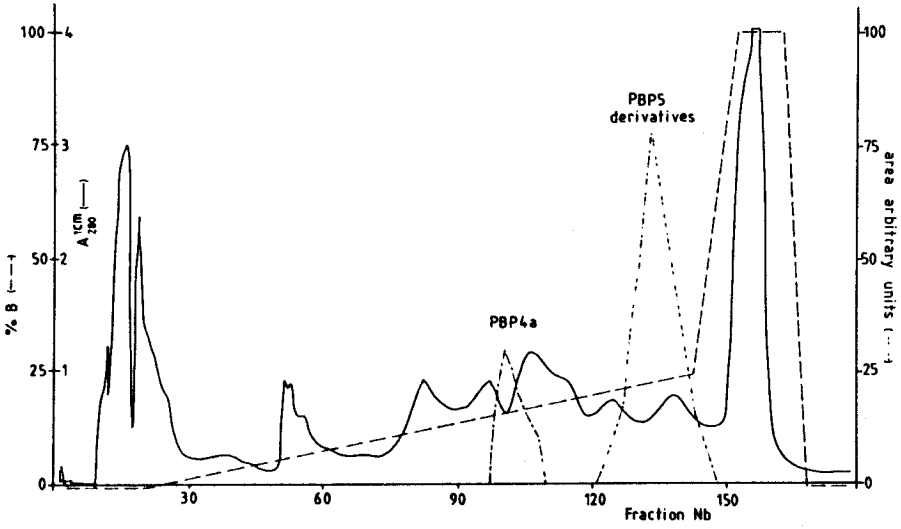

Figure 1. Anion-exchange chromatography. The supernatant obtained after trypsin treatment (371 mg of proteins) was fractionated on an anion-exchange column (Q Sepharose FF) with the FPLC system. The buffer used was 25 mM Tris-borate (pH 8), and the elution was done with increasing amounts of the Tris-borate buffer containing 1 M NaCl.

Table 2. Purification of soluble tryptic products

Step	Protein (mg)	Recovery (%) tPBP 5a alone	Recovery (%) Total tPBPs 5	Sp act (%, wt/wt) tPBP 5a alone	Sp act (%, wt/wt) Total tPBPs 5
Membranes	725	100	100	0.92	0.92
Proteolysis	371	79	91	1.42	1.64
Q Sepharose FF	8.29	39	59	31.37	47.47
Phenyl-Superose	2.13	9	32	28.18	100

(tPBPs) of PBP 5 (75,000 daltons) (Table 1). The four tPBP 5 derivatives were purified from the other proteins up to about 100% purity by using two chromatographic steps on a fast polypeptide liquid chromatography system (FPLC). The first step (Fig. 1) was an anion exchange, and the second step (Fig. 2) was a hydrophobic chromatography. The yields of this purification are given in Table 2.

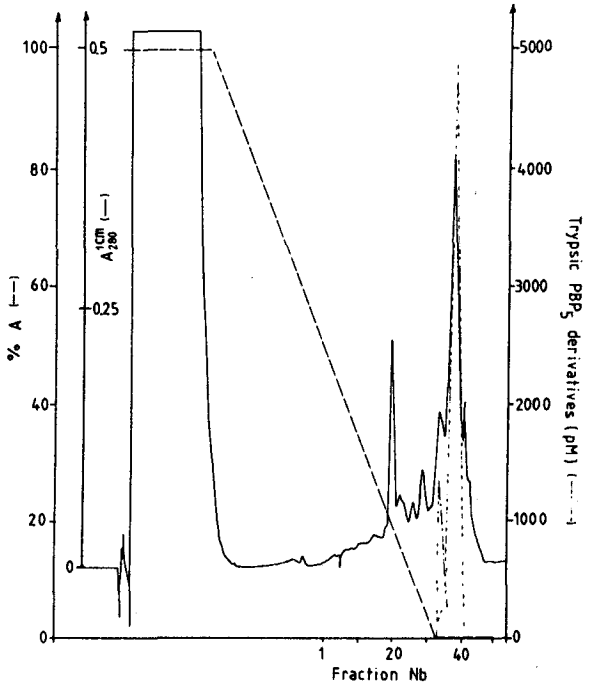

Figure 2. Hydrophobic chromatography. All the fractions collected during the anion-exchange chromatography, containing tPBP 5 derivatives, were pooled, concentrated, and deposited on a hydrophobic column (Phenyl-Superose HR 5/5, FPLC). The buffer used was 25 mM Tris-borate (pH 8), and the elution was done with decreasing percentages of the A buffer (Tris-borate) containing 1.7 M ammonium sulfate.

The improvement of the purity at the different steps of purification is presented in Fig. 3, which shows the Coomassie blue stain and the fluorogram of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis made after the purification. The degradation of tPBP 5a continued during the purification, but after the last step, the relative amount of the tPBP 5 derivatives remained stable.

The pure fraction was used to define the isoelectric point (4.65) and the affinity for benzylpenicillin (3×10^{-4} M) of tPBP 5a. The latter value was identical to that of the native protein. In addition, the active-site tryptic peptide of tPBP 5a was isolated, purified, and sequenced: Gly-Leu-Glu-Ile-(Ser)-Asn-Leu-Lys. The serine was not seen in the results of the Sequenator but could be deduced on the basis of the amino acid composition.

In conclusion, we have established an original procedure to obtain, in good yield, almost pure, soluble tryptic products of PBP 5, which confers β-lactam resistance in *E. hirae*.

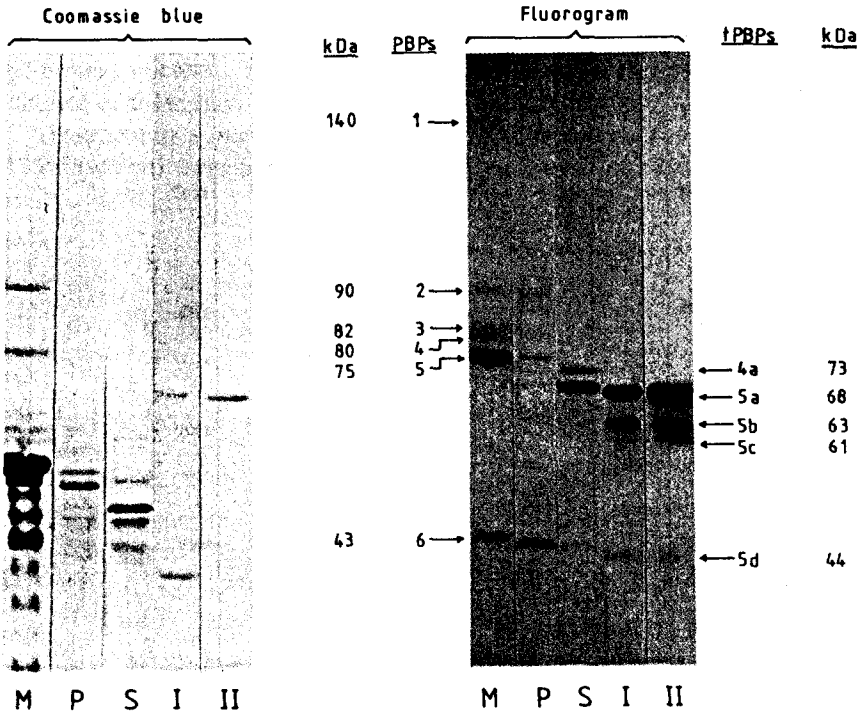

Figure 3. Coomassie blue stain and fluorogram of the gel showing the different steps in the purification of tPBP5 derivatives. M, Membrane preparation; P, pellet after ultracentrifugation of the membrane preparation treated with trypsin; S, supernatant after ultracentrifugation of the membrane preparation treated with trypsin; I, tPBP 5 derivatives after the anion-exchange chromatography; II, tPBP 5 derivatives after the hydrophobic chromatography. kDa, Kilodaltons.

The membranes of *E. hirae* were first treated with trypsin to give tPBPs 5a, 5b, 5c, and 5d, four soluble products of the native PBP which still had the capacity to bind benzylpenicillin. The tPBP 5 derivatives were purified to a level of 100% purity, and the purification yield determined on the basis of the membrane amount was 32%. The tPBP 5a isoelectric point was determined by isoelectrofocusing, and its affinity for benzylpenicillin was found to be identical to that of PBP 5.

ACKNOWLEDGMENTS. This work was supported by the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (grant to P.J.), the Gouvernement belge (action concertée 86/91-90), the Fonds de Recherche de la Faculté de Médecine, the Fonds de la Recherche Scientifique Médicale, Brussels (contracts 3.4522.86 and 3.4507.83), and the Région wallonne (C2/C16/conv. 246/20428).

LITERATURE CITED

1. **Coyette, J., J. M. Ghuysen, and R. Fontana.** 1980. The penicillin-binding proteins in *Streptococcus faecalis* ATCC9790. *Eur. J. Biochem.* **110**:445–456.
2. **Fontana, R., R. Cerini, P. Longoni, A. Grossato, and P. Canepari.** 1983. Identification of a streptococcal penicillin-binding protein that reacts very slowly with penicillin. *J. Bacteriol.* **155**:1343–1350.