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THE *ENTEROCOCCUS FAECALIS* EXTRACELLULAR METALLOENDOPEPTIDASE (EC 3.4.24.30; COCCOLYSIN) INACTIVATES HUMAN ENDOTHELIN AT BONDS INVOLVING HYDROPHOBIC AMINO ACID RESIDUES

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SUMMARY: The extracellular metalloendopeptidase (EC 3.4.24.30; coccolysin) from *Enterococcus faecalis* (Strain OG1-10) inactivates human endothelin-1 by hydrolyzing the peptide primarily at the Ser^{5}-Leu^{6} and the His^{16}-Leu^{17} bonds and the human big endothelin at several bonds involving hydrophobic amino acid residues. The big endothelin fragment 22-38 was also hydrolyzed at a high rate. The degradation of endothelin by coccolysin resembles the peptidolytic processing of endothelin by thermolysin. Because *E. faecalis* is associated with a large number of infectious diseases, it is possible that the manifestation of inflammatory conditions in the presence of this organism is related to the coccolysin-catalyzed inactivation of endothelin.

INTRODUCTION: The 21-amino acid peptide endothelin has been considered to be a potent vasoconstrictor both *in vivo* and *in vitro* (1-4) and contributes to a variety of physiologic and pathologic phenomena in the body. The possible role of ET-1 in a large number of pathological conditions, including sepsis (5) and cardiovascular disease (6,7), and in diseases affecting the ophthalmic artery (8), and in other conditions has been elucidated. ET-1 may also play a role in osteoclast regulation (9). ET-1 occurs in human saliva and salivary glands where it may contribute to the integrity of the oral and gastrointestinal tract mucosa (10), and in dental pulp where it may participate in the regulation of blood pressure and flow (11). Against the background of these and other diverse ET-associated functions (12), it is understandable that the enzymatic formation of ET-1, and also its enzymatic breakdown *in vivo*, have become a subject of enormous interest. The mode of enzymatic hydrolysis (inactivation?) of ET-1 can be divided into two main categories: breakdown as a result of tissue peptidases and breakdown catalyzed by peptidases produced by pathogenic organisms. Among the first mentioned reactions, the various enkephalinases (neutral endopeptidase 24.11) have received attention (13,14). Related to the bacteriolytic reactions, it is possible that bacterial extracellular peptidases contribute to inflammatory tissue reactions by affecting the concentration levels of ET-1. We have earlier characterized and purified an extracellular metalloendopeptidase (formerly EC 3.4.24.4; "gelatinase") from *Enterococcus faecalis* (strain OG1-10), an organism that has been found to be associated with a large number of infectious diseases (15). This enzyme has been characterized as a strongly hydrophobic molecule

Abbreviations used: Big ET-1 (1-38), big endothelin-1 (human); Big ET-1 (22-38), big endothelin-1 fragment (22-38) (human); ET-1, endothelin-1 (human).

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that cleaves the substrate at sites involving pronounced hydrophobicity (15). The most recent Enzyme Nomenclature terms this enzyme "coccolysin" (EC 3.4.24.30) (16). We have now demonstrated that the purified *E. faecalis* enzyme hydrolyzes human ET-1 at specific peptide bonds, a reaction whose possible role in the initiation and propagation of *E. faecalis*-associated inflammations deserves consideration.

MATERIALS AND METHODS: ET-1 was a synthetic product obtained from Sigma. The extracellular metalloendopeptidase from *E. faecalis* was purified into homogeneity as previously described (17). The enzyme used in the present experiments was homogenous according to the criteria previously mentioned (15).

The activity of the enzyme was routinely checked using Azocoll as substrate (17), while the hydrolysis of peptides was based on the separation of the products of hydrolysis by reversed phase chromatography (17). The peptide substrates were first incubated for various periods of time (4 to 100 min) in 50 mM Mes (pH 6.5) which contained 2 μg of enzyme and a suitable quantity of the peptide (0.1 mM) at 30°C. Aliquots of the mixtures were withdrawn at desired reaction times, chilled to 0°C, and immediately subjected to reversed phase chromatography on a PepRPC R 5/5 column, using the Pharmacia FPLC system. Eluent A was 0.1% trifluoroacetic acid in water, and eluent B was 0.05% trifluoroacetic acid in 2-propanol. The gradient was from 0 to 30% eluent B in 60 min and from 30 to 100% eluent B in 10 min at a flow rate of 1.0 ml/min. The separation of the peptide fragments was monitored at 214 nm. The fractions containing these fragments were combined, and the resulting solutions were evaporated to dryness using a SpeedVac evaporator. The dry residues were used for compositional amino acid analyses, and for mass spectrometric and N-terminal studies (Table 1). For amino acid analyses, the dry residues were hydrolyzed for 4 h at 145°C in 6 N HCl, and the resulting hydrolysates were evaporated to dryness, dissolved and analyzed on a Beckman Model 6300 High Performance Analyzer. The molar ratios of the individual amino acids were used to determine the structure of the peptide fragments involved.

Because tryptophan is destroyed in the hydrolysis of peptides for compositional amino acid analysis, it may be uncertain whether Trp^{21} is indeed present in the peptide fragments formed by various enzymes from ET-1. For this purpose, we determined Trp^{21} directly using a sensitive spectrophotometric method for free and bound tryptophan, based on the amino acid's reaction with D-fructose in the presence of H_2SO_4 and cysteine (18).

RESULTS: Incubation of ET-1 in the presence of coccolysin resulted in a rapid hydrolysis of the peptide primarily at the Ser^5-Leu^6 bond, with the formation of a two-chain ET-1 (peak 9; Fig. 2), followed by cleavage at His^{16}-Leu^{17} with the formation of fragments Cys^1-His^{16} and Leu^{17}-Trp^{21} (peaks 4 and 8; Fig. 2). During prolonged incubation of ET-1 in the presence of coccolysin additional cleavage sites were observed (Table 1; Fig. 1 and Fig. 2). The disappearance of intact ET-1 from the reaction mixture was rapid; measurable hydrolysis at His^{16}-Leu^{17} was observed already after one min reaction time. The reaction was more clearly demonstrable after 4 min (Fig. 3) and most of ET-1 had lost its important C-terminal end within 30 min.

The *E. faecalis* coccolysin also readily hydrolyzed the human Big ET 1-38 into several products. The Big ET fragment 22-38 was hydrolyzed by coccolysin at a fast rate forming Val^{22}-Val^{28}, Leu^{33}-Ser^{38} and Val^{29}-Gly^{32}. The formation of all these oligopeptides presumed the cleavage of the Big ET fragment at sites involving pronounced hydrophobicity.

Table 1. Hydrolysis of human ET-1 by coccolysin: Identification of products of hydrolysis

Peak (Fig. 2)	Residues present in end product	Cleavage sites*	Number of chains†	Molecular mass‡	
				Measured	Theoretical
1	1-11 14-16§	**Ser^5-Leu^6; Tyr^{13}-Phe^{14};** His^{16}-Leu^{17}	3	1633	1659.8
2	1-16	**Ser^5-Leu^6; Cys^{11}-Val^{12};** His^{16}-Leu^{17}	3	1904	1887.1
3	1-18	**Ser^5-Leu^6; Cys^{11}-Val^{12};** Asp^{18}-Ile^{19}	3	2143	2138.4
4	1-16	**Ser^5-Leu^6;** His^{16}-Leu^{17}	2	1881	1869.1
5	1-18	N.d.§§		2107	
	1-16	N.d.		1876	
7	N.d.	N.d.		2129	
8	17-21	His^{16}-Leu^{17}		682	658.9
9	1-21	**Ser^5-Leu^6**	2	2529	2510.0
10	1-21	No cleavage#	1	2492	2492.0

*The cleavage sites were verified with N-terminal amino acid analyses (3 cycles). †The peptide chains are held together by two S-S bridges (Cys^3-Cys^{11} and Cys^1-Cys^{15}). ‡Determined by means of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. The compositional amino acid analyses were carried out using Beckman Model 6300 Amino Acid Analyzer. §These peptides are held together by S-S bridges; the dipeptide Val^{12}-Tyr^{13} was enzymatically removed. §§Not determined. The peptide bonds shown in bold are present in the loop structure. #Intact ET-1.

DISCUSSION: The coccolysin's action on ET-1 resembles that of a neutral endopeptidase (EC 3.4.24.11; enkephalinase) in initially hydrolyzing the Ser^5-Leu^6 bond. However, in the C-terminal end of ET-1, enkephalinase's primary target has been reported to be the Asp^{18}-Ile^{19} bond, the His^{16}-Leu^{17} bond representing only a secondary site of cleavage. Coccolysin clearly hydrolyzed His^{16}-Leu^{17} primarily, thereby resembling more closely thermolysin (EC 3.4.24.27) in its action on ET-1. Coccolysin also shows high homology

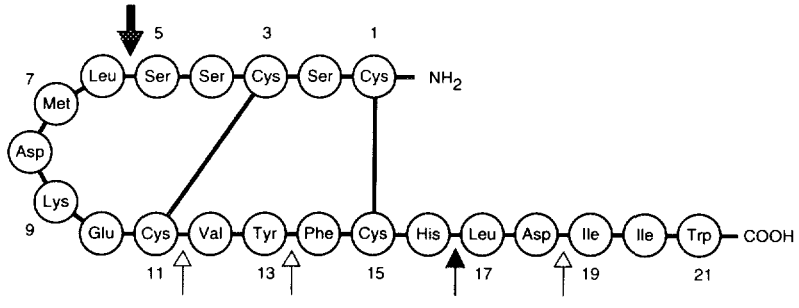
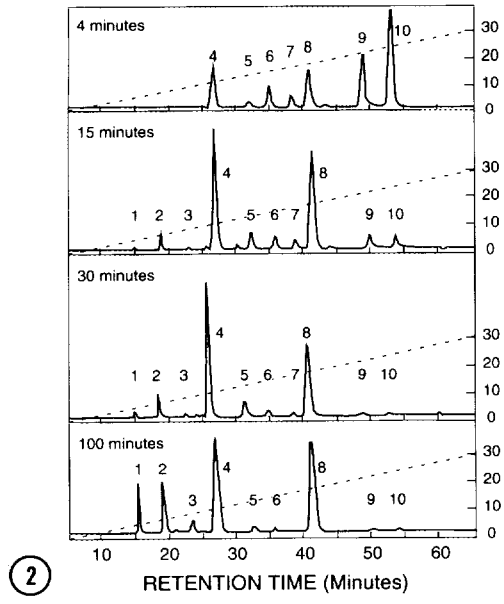


Fig. 1. The primary cleavage site of ET-1 by coccolysin (bold arrow). Another important site of hydrolysis is the His^{16}-Leu^{17} bond. Additional cleavage sites are indicated with smaller arrows. The fragments Cys^1-Ser^5 and Leu^6-Trp^{21} do not separate because of the presence of the -S-S- bonds. This structure was subsequently hydrolyzed at His^{16}-Leu^{17}.



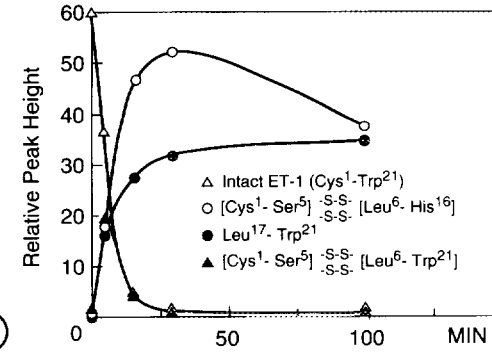


Fig. 2. Reversed phase chromatography of the products of hydrolysis of ET-1 by coccolysin determined after 4, 15, 30 and 100 min reaction time. The right axis gives the percentage concentration of eluent B in elution (see Materials and Methods); the gradient is shown in dashed lines. The ET-1 fragments were numbered as shown in Table 1. Peak No.10 represents intact ET-1.

Fig. 3. Hydrolysis of ET-1 by coccolysin. The fast disappearance of intact ET-1 (open triangles) is accompanied by an initial formation of another form of ET-1 (solid triangles) where all of its 21 constituent amino acids are still present, but which consists of peptides Cys^1-Ser^5 and Leu^6-Trp^{21}, held together by -S-S- bridges. The relative amount of this structure reduced rapidly as a result of its hydrolysis at His^{16}-Leu^{17}.

with thermolysin (19). During prolonged incubation Cys^1-Asp^{18} also appeared, indicating slow cleavage of the Asp^{18}-Ile^{19} bond. This is a major cleavage site of ET-1 by neutral endopeptidase (13). The preferential hydrolysis of the Ser^5-Leu^6 bond can perhaps be chemically explained by a close apposition of the Lys^9-Cys^{15} region with the C-terminal hexapeptide, a confirmation which has been suggested for ET-3 (20).

Previous studies have shown that ET-1 loses its biological activity upon the hydrolysis of a peptide that contains the C-terminal tryptophyl residue, suggesting that this residue is very important for biological activity (21). The isolated C-terminal hexapeptide His^{16}-Trp^{21} has been shown to be biologically inactive. In the isolated guinea pig ileum system, ET fragment 16-21 was suggested to contain the message for the biphasic response, whereas the N-terminal portion was suggested to be responsible for the strong binding to the receptor and for the tachyphylactic properties of ET-1 and ET-3 (22). The peptide bond between Ile^{19}-Ile^{20} is vital to receptor binding (21). The biological activities of the endothelins are dependent on the presence of intact C-terminal hexapeptide and in particular the C-terminal tryptophan residue. Removal of the C-terminal tryptophan lowers the biological activity by 3 orders of magnitude (23). Hence coccolysin, by removing a

pentapeptide from intact ET-1, destroys the biological activity of ET-1. Based on the importance of the C-terminal tryptophan-bearing end, it can be assumed that the significance of the formation of fragment 17-21 by coccolysin is associated with the pathogenicity of *E. faecalis*. Coccolysin hydrolyzes readily also big ET-1. Therefore, coccolysin's role may be considered to be that of an endothelinase rather than that of an endothelin-converting enzyme.

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