SATISFACTORY SEPARATION AND MS-MS SPECTROMETRY OF SIX SURFACTINS ISOLATED FROM *BACILLUS SUBTILIS NATTO*

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We optimized HPLC conditions for the first time to achieve satisfactory separation of six surfactins, two of which were novel, isolated from *Bacillus subtilis natto*. Peptide sequences were analyzed by sophisticated MS-MS analysis, performed with a pair of two surfactins with different fatty acid substitutions. Fragment peaks were divided into two series; one was a series of the ions which carried fatty acid, and thus were characterized by the fatty acid mass difference, and the other was a series of the ions which consisted of pure peptide and thus showed a superimposable pattern between a pair of surfactins.

KEYWORDS surfactin; peptide; fatty acid; MS-MS; *Bacillus subtilis natto*

Natto is a popular Japanese food made of soybeans by fermentation with *Bacillus subtilis natto*. Japanese researchers[1-7] showed that surfactin (Fig. 1) is built from single heptapeptide carrying a fatty acid with variable chain lengths. Recently, Baumgart et al.[8] has reported that surfactin consisted of three kinds of lipopeptides which differed from each other at the C-terminal amino acid. Surfactin is one of the most efficient biosurfactants[1,9] and has been commercially available in Japan and the USA as a mixture of components without detailed information on the components. To investigate the nutritive value of Natto, separation of surfactins into its constituents may be a prerequisite to further biological experiments.

In this paper, we will describe a novel HPLC procedure for separating surfactins into six pure constituents, two of which were novel, and their MS-MS analysis comparing fragmentation patterns between two peptides carrying different fatty acids.

The methanol soluble fraction of the water extract from the soybean surface mucinoids was mixed with Celite and passed with a mixture of dichloromethane and ethanol (9:1). The eluent was evaporated to afford crude surfactin, which was separated into the six components A to F (yields(%): A 0.004, B 0.012, C 0.006, D 0.030, E 0.017, and F 0.002) by HPLC. Two commercially available surfactins obtained from Wako (Osaka, Japan) and Sigma (St. Louis, MO, USA) showed different constitutional profiles (Fig. 2).

We studied distribution of the major components, surfactin D and E, in Natto. Over 70% of these surfactins were distributed in the methanol soluble fraction of the mucinoids, while the other parts were distributed in seed coats and cotyledon. Every surfactin consisted of colorless crystal showing similar, but apparently different , melting points, as shown in Table 1, where results of the amino acid analysis are also described. FTIR spectra of the six surfactins were almost superimposable (data not shown).

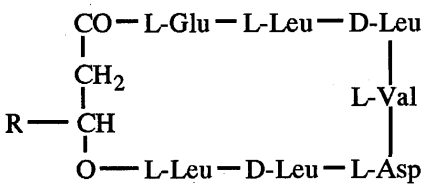
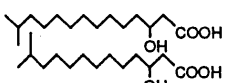
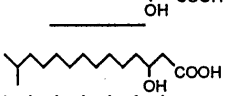
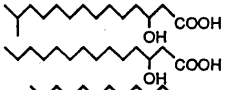
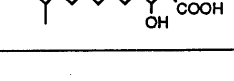


Fig. 1. Structure of Surfactins
R=side chain of fatty acid.

Table I. Physicochemical Properties of Surfactins

Surfactin	m.p. (°C)	Mr	AA comp.					Fatty acid	Ref.
			Asp	Glu	Val	Leu	Ile		
A	132-134	1035	1	1	1	3	1		-
B	138-139	1021	1	1	1	4	0		4
C	135-138	1049	1	1	1	4	0		-
D	136-138	1035	1	1	1	4	0		4,8
E	147-150	1021	1	1	1	4	0		4
F	139-141	1007	1	1	1	4	0		4,8

All surfactins consisted of a common amino acid composition except that surfactin A contained a replacement of Leu at the C-terminal position by Ile. Thus the peptide moieties of the surfactins showed an equal mass so that the differences in the molecular weights must have been due to the fatty acid substitutions. ^{1}H-NMR in $CDCl_3$ revealed marked differences in methyl substitutions on the fatty acid. Our interest was focused on the ^{1}H-NMR at the highest magnetic field. Isopropyl substitutions at the side chain terminal in surfactin A, B, D, and F were identified by the appearance of two doublets at 0.82-0.85 ppm, where a vicinal coupling constant of 6 Hz and a chemical shift difference between the two methyl groups of 0.005 ppm were observed owing to the free rotation-inhibition of the isopropyl group. These results and reports[10,11] on mass analysis, afforded the structures of the side chain branching as illustrated in Table I, except that surfactin C may involve an unknown side chain structure.

We applied a new technique of sequence analysis to the surfactins. One shortcoming of the up-to-date MS-MS technique is how to differentiate one series of sequential fragment peaks from others. This difficulty arose from variable fragmentation mechanisms due to the different sites and directions of cleavage. We compared a pair of surfactins, both of which had the same peptide sequence but different mass of fatty acids. Figure 3 shows a typical pair of the MS-MS spectra of two major surfactins, D and E. One of the sequential fragmentations was found in the higher molecular mass region showing differences in mass numbers of 14 between D and E, and another was found in lower molecular weight regions where superimposable fragmentation was observed. Thus we were able to identify the sequence in D and E as RCO-Glu-Leu-Leu-Val-Asp-Leu-Leu.

Natto is a popular soybean product made by fermentation with *B. subtilis natto*. Our results showed that Natto contains a larger number of surfactins than reported. We found two novel surfactins, A and C. The commercially available surfactins produced in Japan and the USA differed from each other in composition of the major constituents, D and E (Fig. 2), owing possibly to differences between the bioorganisms *B. subtilis* IAM 1213[1] and ATCC 21332,[12] respectively.

Pharmacological properties of surfactin have been defined; the most potent biosurfactant,[1,9] cytolytic activity against fungi and yeasts,[13-15] antifungal antibiotic,[16] antitumor effect against Ehrlich ascites carcinoma,[17,18] fibrin clot inhibition,[1] hypocholesterolemic effects,[19] and cAMP phosphodiesterase inhibiton.[10] These properties have been studied using crude mixtures of surfactins with little knowledge of the constituents. Our results provide a satisfactory method for preparation of pure surfactins, and should be used in the study of their pharmacological activities. Otherwise, by using easily available mixtures, various activities of surfactins cannot be clearly understood. Even little changes in the molecular structures of such amphiphiles as the surfactin and iturin groups may result in considerable differences in biological properties.[11]

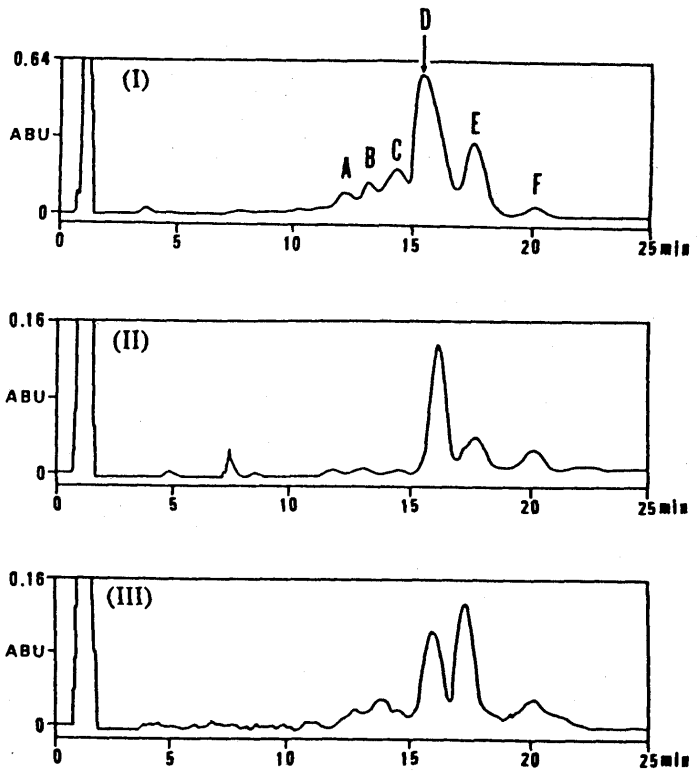


Fig. 2. Chromatogram of Crude Mixture of Surfactins:(I) obtained from Natto; (II and III) commercially available in Japan and the USA, respectively. HPLC was carried out by a Model 880 LC system, using an analytical column (4 mm i.d. × 250 mm) packed with silica gel (Lichrospher Si-60, Merck, Germany). The mobile phase solvent was a mixture of distilled water, acetic acid, ethanol, and n-hexane (0.2:0.2:7.0:92.6) at a flow rate of 2.0 ml/min.

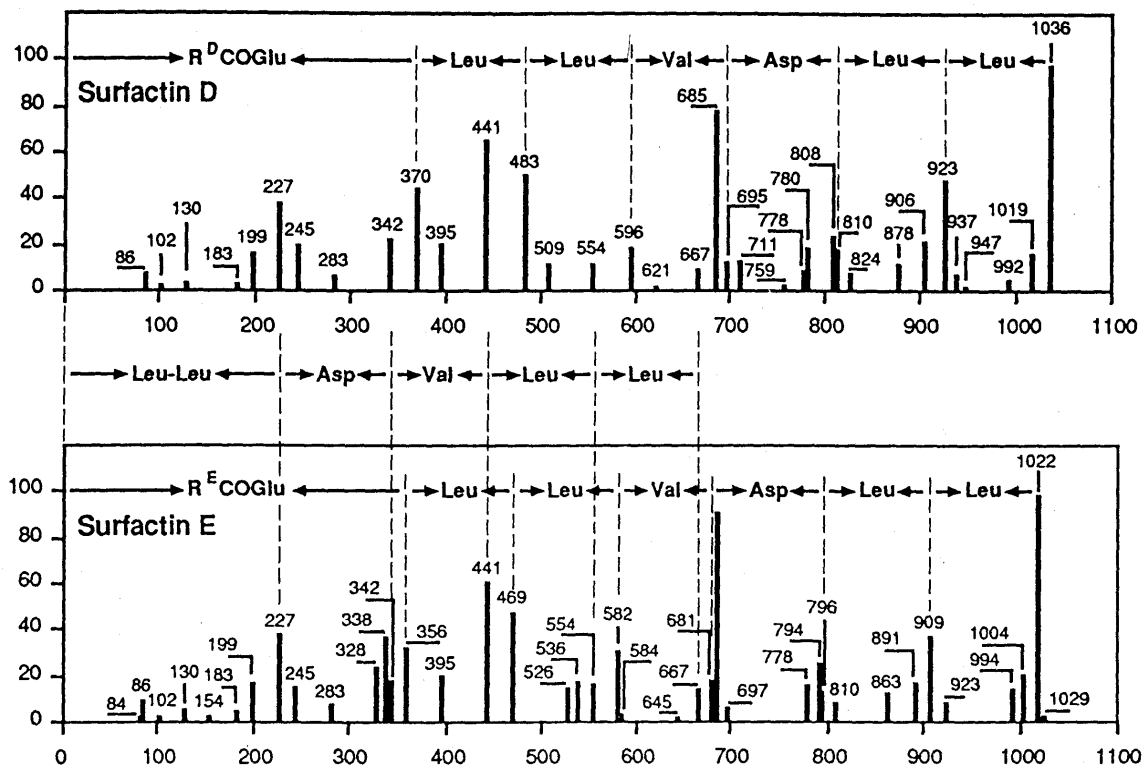


Fig. 3. A pair of MS-MS Spectra of Surfactins D and E
Two series of fragmentations, acyl peptides and pure peptides, were easily differentiated.

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