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Extraction, Purification and Identification of Vitamin K2 from Flavobacterium Fermentation Broth (Postprint)

Authors: Wei Hongfei, Wang Peng, Zhao Genhai, Liu Hui, Wang Li, Wang Han, Wu Hefang, Fang Xue, Zheng Zhiming, Zheng Zhiming

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Abstract

Vitamin K2 (VK2) is a class of fat-soluble menaquinone compounds, which can be represented as Menaquinone-n (MK-n, n=1~14) according to the number of isopentenyl units in its side chain. Studies have shown that VK2 possesses physiological functions such as promoting blood coagulation, preventing and treating osteoporosis, Parkinson' s disease, and cardiovascular diseases. VK2 produced through microbial fermentation offers advantages including an all-trans side chain and high biocompatibility, making it more readily accepted by consumers as food and pharmaceutical products. However, its downstream separation and purification process suffers from numerous issues including low product concentration, complex composition, cumbersome purification procedures, and low product yield, with few reports on high-purity VK2 preparation processes currently available. Flavobacterium can synthesize VK2 homologs such as MK-5 and MK-6. In this study, a complete set of corresponding separation and purification processes was developed based on the characteristics of its fermentation products. First, Flavobacterium cells were rapidly obtained through membrane concentration and centrifugation. After drying the cells, solid-liquid extraction was performed using methanol at a solid-liquid ratio of 4:1 (ml/g) for 20 minutes, repeated three times consecutively, achieving a VK2 methanol extraction yield of over 99.1%. Subsequently, through macroporous resin adsorption chromatography using methanol/dichloromethane = 1/1 (V/V) as the eluent, a VK2 crude product with approximately 15% purity was obtained. After molecular sieve chromatography at a height-to-diameter ratio of 255:15 with dichloromethane as the mobile phase, a low-purity VK2 product of approximately 57% purity was obtained. Thereafter, through reversed-phase silica gel column chromatography with sequential gradient elution using methanol/dichloromethane = 9:1, 6:1, 3:1 (V/V), various VK2 homologs were separated and purified, all achieving HPLC purities above 90%. Finally, a series of light-yellow crystals were

prepared using cooling crystallization. Through mass spectrometry, infrared spectroscopy, and proton nuclear magnetic resonance detection, all conformed to the corresponding spectral characteristics of VK2, confirming them as MK-5 and MK-6 crystals. HPLC analysis revealed that the purities of MK-5 and MK-6 crystals reached 98.0% and 99.3%, respectively. After multiple repeated experiments, the complete process demonstrated stability, with product recovery rates exceeding 88%. After 15 repeated uses of each packing material, no significant effects on VK2 purity or recovery rate were observed. The method established in this study for extracting and purifying VK2 from Flavobacterium fermentation broth offers advantages including simple process, large processing capacity, and high product purity and yield, laying an optimized foundation for the biological preparation and industrialization of VK2. Throughout the entire extraction, purification, and crystallization process, only two organic solvents were employed, which is more conducive to organic solvent recycling and large-scale VK2 production.

Full Text

Preamble

Extraction, Purification, and Identification of Vitamin K2 from *Flavobacterium* Fermentation Broth

Hongfei Wei[1,2,#], Peng Wang[1], Genhai Zhao[1], Hui Liu[1], Li Wang[1], Han Wang[1,2], Hefang Wu[1], Xue Fang[1,2], Zhiming Zheng[1,*]

[1]University of Science and Technology of China
[2]Institute of Technical Biology and Agriculture Engineering, Hefei Institutes of Physical Science, Chinese Academy of Sciences

#First Author, E-mail: hong.fei.wei@163.com
*Corresponding Author, E-mail: zmzheng@ipp.ac.cn

Vitamin K2 (VK2) is a class of lipid-soluble menaquinone compounds that can be represented as Menaquinone-n (MK-n, n=1~14) based on the number of isoprenyl units in their side chains. Research has demonstrated that VK2 possesses various physiological functions, including promoting blood coagulation and preventing or treating osteoporosis, Parkinson' s disease, and cardiovascular disease. VK2 produced through microbial fermentation offers advantages such as an all-trans side chain and high biocompatibility, making it more readily accepted by consumers as a food and pharmaceutical ingredient. However, the downstream separation and purification process faces numerous challenges, including low product concentration, complex composition, cumbersome purification procedures, and low product yield, with few reports currently available on the preparation of high-purity VK2.

Flavobacterium can synthesize VK2 homologues such as MK-5 and MK-6. In

this study, we developed a complete separation and purification process tailored to the characteristics of its fermentation products. First, *Flavobacterium* cells were rapidly obtained through membrane concentration and centrifugation. After drying the cells, solid-liquid extraction was performed using methanol at a solid-liquid ratio of 4:1 (ml/g) for 20 minutes, repeated three times, achieving a VK2 extraction yield of over 99.1% in the methanol extract. Subsequently, macroporous resin adsorption chromatography using methanol/dichloromethane (1:1, V/V) as the eluent yielded a VK2 crude product with approximately 15% purity. This was followed by molecular sieve chromatography at an aspect ratio of 255:15 using dichloromethane as the mobile phase, producing a low-purity VK2 product of about 57% purity. Finally, reverse-phase silica gel column chromatography with gradient elution using methanol/dichloromethane ratios of 9:1, 6:1, and 3:1 (V/V) successfully separated and purified each VK2 homologue, with HPLC purities exceeding 90%. Cooling crystallization was then employed to obtain a series of light-yellow crystals. Analysis by mass spectrometry, infrared spectroscopy, and proton nuclear magnetic resonance confirmed spectral characteristics consistent with VK2, identifying them as MK-5 and MK-6 crystals. HPLC analysis revealed purities of 98.0% and 99.3% for MK-5 and MK-6 crystals, respectively. Repeated experiments demonstrated that the entire process is stable, with product recovery rates exceeding 88%.

After 15 repeated uses of each chromatographic medium, no significant effects on VK2 purity or recovery rate were observed. The method established in this study for extracting and purifying VK2 from *Flavobacterium* fermentation broth offers advantages including process simplicity, large processing capacity, and high product purity and yield, laying an optimized foundation for the biological preparation and industrialization of VK2.

Throughout the entire extraction, purification, and crystallization process, only two organic solvents were utilized, which facilitates solvent recycling and the scaled-up production of VK2.

Note: Figure translations are in progress. See original paper for figures.

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