DOCTORAL THESIS

# Investigations of the industrial compatible aqueous purification techniques and intrinsic stabilizing factors for nattokinase

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## Abstract

Nattokinase is a potent fibrinolytic enzyme produced by *Bacillus sutbilis,* which is one of the most impotant sources of fibrinolytic enzymes. Nattokinase has a dual function of hydrolyzing blood thrombin directly and indirectly. It was found to be active between pH 6-12 and lose fibrinolytic activity dramatically when pH is lower than 5. Currently nattokinase preparations are not pure enough to get rid of the unique smell that is repulsive to the non-Japanese. These two reasons severely blocked nattokinase to be developed for food and pharmaceutial industial useages as supplements and active ingredients. The objective of this study was to isolate the bacteria which have ability to produce nattokinase-like fibrinolytic enzyme, purify and characterize these enzymes to determine the actual biochemical proteries. An industrial compatible anqueous protocol can be applied not only for the purificication of nattokinase but also for other fibrinolytic enzymes from *Bacillus* source was estabilished to purify the enzymes to homogeneous. The intrinsic stabilizing factors in raw nattokinase fermented broth were also identified in order to make natural stable nattokinase preparation for acidic environment. 71 strains belonging to 13 different genus was determined as the fibrinolytic enzyme producing bacteria. The fibrinoytic enzyme produced by *Bacillus tequilensis, Bacillus amyloliquefaciens,* and *Bacillus cereus* was purified and characterized. One of these three enzymes was determined to be a new fibrinolytic enzyme that never be reported. The purification protocol established here cotained 3 operation units that including one chromatographic separation step followed by membrane polishing after nattokinase was extracted with 70% ammonium sulphate from the fermeted broth. The purified

nattokinase and other fibrinolytic enzymes showed single band as tested by SDS-PAGE and already pure enough to determine the N-terminal sequence directly by Edman degration. A purification efficiency of 476.1 fold enzymatic activity increase with 48.3% recovery was obtained by using this protocol to purity nattokinase at lab level. The efficiency was 428.1-fold with 42.6% recovery at industrial compatible pilot scale (60-fold amplification). The efficiency was 329.7-fold with 42.7% recovery for purification of fibrinolytic enzyme produced by *Bacillus tequilensis*, 221.7-fold with the recovery of 32.5% for purification of fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* and 288.5 fold with 38.7% recovery for purification of fibrinolytic enzyme produced by *Bacillus cereus*. The purification fold of most current protocols used for nattokinase and other fibrinolyitc enzymes purification was lower than 100 and the recovery fall in between 6.28% to 80%. Thus, the protocol established in this study has a very high purification efficiency. The result of intrinstic stabilizing factor identification shown both starch and levan have the stabilizing effect on nattokinase at low pH environment. The stabilizing ability of starch is much higher than levan, and was retained even hydrolyzed by amylase. The active concentraion range of starch was from 20 and up to 500 μg/ml at the pH range of 4-5.

Key words: Nattokinase; Fibrinolytic enzyme; *Bacillus*; Purification; Stabilizing factor

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