CHAPTER 1

INTRODUCTION

We all strive for an optimum health. All of us eat healthy diets, take nutritional supplements and try to exercise regularly. But there is a key factor that every one of us may be missing. It may not matter how many good things we put into our body or how many bad things we avoid – if our blood circulation is not flowing the way it should, then critical nutrients may not be getting into our cells. Blood circulation is the critical factor here. If blood circulation is optimised, then the body has a better chance of being able to do its own job. Blood clot fibrins (Figure 1.1) are formed from fibrinogen by thrombin (E.C. 3.4.21.5) and are lysed by plasmin (E.C. 3.4.21.7), which is activated by plasminogen by tissue plasminogen activator [1].

![Blood Clot Diagram](image)

**Figure 1.1 Blood Clot – a schematic representation**

Now the question arises: Why Can’t the Body Take Care of Clots on Its Own?? During injury, our body produces one main enzyme to breakdown clots –
“plasmin”, produced in the endothelial cells. In order for the body to stop excess bleeding when necessary and to increase the blood flow when needed, there must be very delicate balance of naturally occurring thrombolytic enzymes. But many of the populations do not have the proper balance because of poor diet, genetic defects, aging etc [1].

Many people just learn to live with varying degrees of impaired circulation and just chalk it up to aging, while others may experience a sudden impairment in circulation. The causes of impaired circulation are multifaceted, with many contributing factors. It is important to understand that while using blood-thinning substances whether they are natural or drugs- are really just a “Band Aid” and we still are not treating the condition at its root cause [1].

The typical thrombolytic agents for therapeutic purposes include urokinase and tissue-type plasminogen activator. These are plasmin activators and convert plasminogen to plasmin, which degrades fibrin. These activators are of human origin and generally safe but expensive [2]. The biological activity of intravenous fibrinolytic agent like streptokinase is short lived in the circulation after intravenous administration and there is a significant risk of hemorrhagic complications with a pronounced activation of fibrinolytic activity in whole blood in some circumstances [3]. Therefore, microbial fibrinolytic enzymes attracted much more medical interest lately.

1.1 NATTOKINASE : THE NATURAL SOLUTION

Nattokinase is one of the most powerful new dietary supplements to be introduced to the market place in recent years. It is, in general, isolated from fermented Soybean (Figure 1.2) A few nattokinase researchers feel that nattokinase gets close to the root of the problems associated with impaired circulation [4].
The enzyme nattokinase offers a completely natural means of helping prevent and dissolve blood clots. It closely resembles plasmin, our own natural clot-dissolver, and actually enhances our body’s production of plasmin. Nattokinase cleaves fibrin (the protein that helps our body form the ‘mesh’ of a clot from a wound or trauma). It is like our natural plant kingdom source of plasmin, and is the most potent fibrinolytic enzyme of nearly 200 foods studied for their clot-dissolving abilities [4].

It can even outperform our own body: in one remarkable in-vitro study, nattokinase, urokinase and plasmin (all capable of dissolving clots) were placed on a plate of fibrin. A clear halo showed degraded fibrin. The halo around nattokinase was over twice the size of the halo created by the other two enzymes, which our body manufactures. It is also more potent than garlic, bromelain or ginseng (Figure 1.3) [1].
According to the reports by Milner [1], nattokinase is truly a multidimensional supplement, useful in each of the following conditions:

- Arterial wall thrombi formation with atherosclerosis
- Atherosclerosis
- Coronary artery disease (CAD) - heart attack prevention
- Pulmonary embolism
- Atrial chamber thrombi present in chronic atrial fibrillation
- Thrombi in the eyes - known as vena Centralia retinæ acresia
- Diabetes, which often leads to excess platelet aggregation
- Hypertension - a natto rich diet or nattokinase supplements have been shown to lower blood pressure. The microscopic trauma to a vessel wall under high pressure increases platelet aggregation and the need for blood thinning in the long term management of the prevention of CAD and strokes in the presence of hypertension.
- Peripheral vascular disease - arterial atherosclerosis or venous thrombosis. Nattokinase almost always improves spider veins and varicose veins. Hemorrhoids are improved as well.
- Senile dementia in which there is poor circulation and blood supply or cerebral thrombi formation
- Ischemic stroke - prevention
- Chronic migraine - where platelet aggregation releases vasoactive chemicals implicated in migraines
• Fibromyalgia, CFS and Lyme Disease - where chronic infection produces antibodies that cross-react with endothelial cells, leading to fibrin deposition

• Dysmenorrhea - where excessive clotting causes painful cramping

• Excessively fast clotting times due to platelet aggregation

Functional clotting problems do steady, silent damage long before obvious disease shows itself. If we can reverse silent, functional clotting problems, we can offer a profound healing tool to patients. This is where the real treasure of this enzyme lies and allows us to treat cardiac disease in a way that puts the patients at the forefront of preventive medicine. In fact, by breaking down fibrin, increasing blood flow and thus tissue oxygen levels, we are lowering a risk factor that is implicated in almost all chronic disease [1].

1.2 Why Nattokinase is Unique

Medical science has synthesized various compounds to help thin blood, from aspirin to warfarin, urokinase and streptokinase. Each has their role. Warfarin, for example, blocks factors in the Vitamin K clotting cascade. However, warfarin does not help a patient lower their platelet aggregation or dissolve their fibrinogen or existing clots.

A patient on warfarin is only treating one part of the clotting cascade and dietary Vitamin K toxicity has not been shown to be a significant etiology in cardiovascular disease. These patients with high fibrinogen and persistent platelet aggregation are still a walking time bomb [1].

Nattokinase is unique in profoundly lowering fibrinogen levels and degrading branched fibrin. It has three different mechanisms of action. It lyses fibrin directly, changes prourokinase to urokinase, and increases tissue plasminogen activator, which increases our own plasmin (Figure 1.4) [2]. At the
same time, nattokinase does not appear to actually destroy the fibrinogen molecule, as streptokinase and urokinase do. It is in a unique class of fibrinolytic agents.

Figure 1.4 Mode of action of Nattokinase

As fibrin is being dissolved, levels of EFA (euglobin fibrinolytic activity) and FDP (fibrin degradation products) increase in the blood. Endothelial cells and/or the liver release TPA antigen (tissue plasminogen activator). The increase in these parameters allowed researchers to confirm the action of Nattokinase and also to determine that the activity lasts from eight to twelve hours [5].

Nattokinase lessens excessive coagulation and thus improves circulation, increasing oxygen to tissues. That is one reason disorders such as fibromyalgia, chronic fatigue syndrome, and chronic infections such as Lyme disease and inflammatory bowel disease may respond to Nattokinase.

To make a long story short, clotting is a key, often overlooked factor in chronic illness, and with nattokinase, we know how to reverse it. Nattokinase is actually superior to conventional clot dissolving drugs [6], which have many benefits including convenient oral administration, known efficacy, prolonged
effects, cost effectiveness, and prevention of clot formation. Nattokinase has been

demonstrated to have pH and temperature stability and so can be found in the
gastrointestinal tract [7].

As a member of the subtilisin family of serine proteases, Nattokinase

has the same conserved catalytic triad (Asp 32, His 64, Ser 221) and oxyanion hole
(Asn 155) [8]. Nattokinase has a molecular weight of 27.7 kDa with a pI value of 8
and it has no disulfide bonds in its secondary structure [9]. Nattokinase is

composed of 275 amino acids and the gene sequence is homologous to those of

other members of the Subtilisin family (99.5% homology with Subtilisin E, 86%
with Subtilisin BPN, 72% with Subtilisin Carlsberg) [9].

The serine protease subtilisin is an important industrial enzyme, as well

as a model for understanding the relationship between enzyme structure and

function. [10]. Sumi et al [4] further demonstrated that oral administration of

Nattokinase capsules enhanced fibrinolysis in canine plasma in an experimental

thrombosis model.

Fibrinolytic agents, such as, Streptokinase, Urokinase, t-PA are

previously used for therapy, but because of their expensive prices and undesirable

side effects prompted researchers to search for cheaper and safer resources [11].

Urokinase and t-PA are expensive, moreover t-PA in particular has a short life [12].

Streptokinase is a non-human protein and is immunogenic. The most severe defect

of these blood clot dissolving agents is the possibility of causing hemorrhagic side
effect, which is fatal [13]. In particular, oral intake of Natto or its enzyme has
effectively augmented the release of an endogenous plasminogen activator for
thrombus degradation in both animal models and human subjects [4,14].

The activity of the enzyme is enhanced in the plasma for a longer half

life with oral administration [15]. Nattokinase not only dissolves blood clot but
also degrades amyloid fibrils [16]. In extensive studies on oral fibrinolytic therapy,

Nattokinase, a strong fibrinolytic agent, has been found in commercially available
Natto products (Figure 1.5) and cultured media of Natto, *Bacillus* [17,18,19,20]. Since the enzyme triggers a mild but sustainable fibrinolytic effect by oral administration, the Natto products have drawn attention from the health food industry and several clinical organisations [21,22]. In the field of ophthalmology, Nattokinase has been used as a fibrinolytic agent, especially for the treatment of retina, central vein thrombosis [23].

![Figure 1.5 Commercially available brands of Nattokinase](image)

Nattokinase can play a key role in treating hypertension, as well as preventing the long-term sequelae of damaged, inflamed blood vessel walls. Scientists at Yonsei University in Korea tested 86 individuals aged 20 to 80 whose blood pressure ranged from 130 to 159 mmHg. Each received either nattokinase at 2000 FU (fibrinolytic units) per capsule daily or a placebo [1]. After eight weeks, those on nattokinase had significantly lower systolic and diastolic blood pressure. The researchers concluded that increased intake of nattokinase might play an important role in preventing and treating hypertension.

The use of oral administration of Nattokinase in fibrinolytic therapy for thrombosis and the prevention of CVD, atherosclerosis is, therefore, of interest.
1.3 **AIMS AND OBJECTIVES**

The major objectives of this research work were:

(a) **Isolation and Screening of Microorganisms**

Isolation of microorganisms from different sources; Preliminary Screening for Proteolytic Activity through Casein Plate Assay; Blood Plate Assay; Blood Clot Dissolution Assay

(b) **Characterization and Identification of Shortlisted Isolates**

Microscopic observation; Biochemical Tests; Genomic DNA Isolation, Amplification (16s rRNA) and Phylogenetic Dendrogram for Identification

(c) **Standardization of Growth Parameters**

Optimization of production media for the production of proteolytic enzyme; Optimization of Carbon and Nitrogen Sources; Optimization of Temperature, pH and Agitation; Fibrinolytic activity

(d) **Purification Studies**

Enzyme purification by Ammonium sulphate precipitation and Gel filtration methods; SDS-PAGE; Protein Estimation

(e) **In-Silico Approach**

Molecular modelling and structural refinement studies; Docking and Molecular Simulation studies; Homology modelling

(f) **Scale Up Studies**

Scale up trials in a 7 L fermentor with *B. amyloliquefaciens*; Concentration of the enzyme through Ultrafiltration; Formulation of the enzyme; Comparison with other fibrinolytic enzymes