Food Research International 55 (2014) 356–362

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Microbial and endogenous origin of fibrinolytic activity in traditional fermented foods of Northeast India ☆

Thangjam Anand Singh [a,b,1], Khunjamayum Romapati Devi [a], Giasuddin Ahmed [b], Kumaraswamy Jeyaram [a,*]

[a] Microbial Resources Division, Institute of Bioresources and Sustainable Development (IBSD), Takyelpat Institutional Area, Imphal 795001, Manipur, India
[b] Department of Biotechnology, Gauhati University, Guwahati 781014, Assam, India

ARTICLE INFO

Article history:
Received 9 August 2013
Accepted 18 November 2013

Keywords:
Fibrin zymogram
Fibrinolytic to caseinolytic ratio
ARDRA
PCR-DGGE
Bacillus
Proteus mirabilis

ABSTRACT

Traditional fermented foods of Northeast India and their associated microorganisms were studied for fibrinolytic activity. A simple and reliable spectrophotometric method to quantify fibrinolytic activity in comparison to conventional fibrin plate assay was established. Out of 31 different types of fermented foods screened, protein rich fermented foods mostly soybean and fish products possessed fibrinolytic activity. Higher fibrinolytic activity was observed in fermented small cyprid fish (*Puntius sophore* Ham.) products than fermented soybean products. Fibrin zymogram based cluster analysis indicated fibrinolytic activity of endogenous origin in fermented fish products and microbial origin in fermented soybean products. A fermented soybean product *Hawaijar* showed higher specificity towards fibrin as indicated by fibrinolytic to caseinolytic activity ratio of 3.8 ± 0.21. Identification of 85 fibrinolytic isolates by amplified ribosomal DNA restriction analysis and ribosomal DNA sequencing yielded 15 phylotypes, of which 55% were *Bacillus* species. Lactic acid bacteria with fibrinolytic activity namely *Vagococcus carniphilus*, *Vagococcus lutrae*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gallinarum* and *Pediococcus acidilactici* were identified. *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *V. carniphilus*, *V. lutrae* and *Proteus mirabilis* were promising producers of fibrinolytic enzymes with more than 350 plasmin units/mL. The high population of fibrinolytic *P. mirabilis* found in fermented soybean and pork products might be a serious health concern. Fibrinolytic activity found in fermented food could be partially dependent on the isolated microbes. PCR-DGGE analysis detected uncultivated proteolytic bacteria in both raw material and fermented fish products. These fibrinolytic foods and associated microbial resources could be further explored for novel fibrinolytic therapy.

1. Introduction

Cardiovascular diseases (CVDs) are a rapidly emerging health concern in the modern world. It was estimated that more than 23 million deaths by 2030 would be due to CVDs alone (WHO report, 2013) and these deaths would be mostly from India, Southeast Asia and the Mediterranean region. The increase in the incidence of CVDs is often related to a change in food habits. In clinical practices external fibrinolytic enzymes are administered to the patients with CVDs (Bode, Runge, & Smalling, 1996). The most common commercially available as well as FDA approved products include plasmin activators *viz*. streptokinase, urokinase, alteplase (t-PA), anistreptase, releplase (r-PA) and tenecteplase (TNK-t-PA) (Duggal & Harger, 2011). These products are expensive, have limitations in their specificity towards fibrin; and have very short half life. They may also create other complications like internal haemorrhage and allergic reactions (Blann, Landray, & Lip, 2002). Hence, a search for a safe, efficient and cheaper fibrinolytic enzyme is of paramount necessity.

Many fibrinolytic enzymes were reported from various sources like snake venom (Randolph et al., 1992), earthworm (Wu, Wu, & He, 2002), algae (Matsubara, Matsuura, Sumi, Hori, & Miyazawa, 2002), polychaete (Park et al., 2013) and insect (Ahn et al., 2004). The fibrinolytic enzymes from microbial sources are favoured due to the enormous diversity and convenience in industrial production. Recently, many fibrinolytic enzymes were discovered from fungi (Choi et al., 2011), actinomycetes (Uesugi, Usuki, Iwabuchi, & Hatanaka, 2011) and bacteria (Chang, Wang, Hung, & Chung, 2012). Traditional fermented foods consumed all over the world for centuries have microbial resources which are generally regarded as safe (GRAS) category. Nattokinase from *Bacillus subtilis* isolated from *natto* (a traditional fermented food of Japan) was the first success story of a microbial source of fibrinolytic enzyme from fermented food. Since then many fibrinolytic enzymes from traditional fermented foods and their associated microbes had been characterised (Mine, Wong, & Jiang, 2005). *Myulchikinase* (Jeong et al., 2004) from Korean *myul-chi-jeot-gal*, *katsuwokinase* from

☆ Address of the institution where the work was performed: Microbial Resources Division, Institute of Bioresources and Sustainable Development (IBSD), Takyelpat Institutional Area, Imphal 795001, Manipur, India.

* Corresponding author. Tel.: +91 385 2051278x212; fax: +91 385 2446121.
E-mail address: jeyaram.ibsd@nic.in (K. Jeyaram).
[1] Present address: College of Agricultural Engineering and Post Harvest Technology (Central Agricultural University, Imphal), Ranipool 737135, Sikkim, India.

0963-9969/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodres.2013.11.028

Japanese *shiokara* (Sumi, Nakajima, & Yatagai, 1995), *subtilisin* DFE from *Bacillus amyloliquefaciens* associated with Chinese *douchi* (Peng, Huang, Zhang, & Zhang, 2003), TPase of *B. subtilis* TP-6 from Indonesian fermented soybean *Tempe* and novel metaloprotease of *Bacillus* sp. KA38 from Korean fermented fish *Jeot-Gal* are examples of fibrinolytic enzymes from fermented foods (Kotb, 2012).

People of Northeast India consume more than 100 varieties of traditional fermented foods (Tamang, 2009). These vast resources have not yet been explored for its fibrinolytic activity. The aim of this study was to screen major traditional fermented foods of Northeast India for their fibrinolytic activity and to characterise the related microbial resources.

2. Materials and methods

2.1. Collection of fermented food samples

Samples of traditional fermented foods of Northeast India (Supplementary Table S1) were procured from different markets of 7 states of Northeast India namely Agartala of Tripura state; Silchar, Johrat and Guwahati of Assam state; Aizawl of Mizoram state; Shillong of Meghalaya state; Itanagar and Pashighat of Arunachal Pradesh state and Imphal, Bishnupur, and Kakching of Manipur state. These samples were aseptically collected in sample containers and transported to the laboratory using cooler boxes within 2 days of collection from the markets. The samples were immediately processed for subsequent analyses. 10 g of each sample was transferred to a sterile stomacher bag containing 90 mL of physiological saline (0.1% w/v bacteriological peptone, 0.85% w/v NaCl) and homogenized (Stomacher 400-Circulator, Seward, UK) at 250 ×*g* for 3 min. The homogenate was divided into two portions, one for screening fibrinolytic activity and the other for microbial isolation.

2.2. Screening for fibrinolytic and caseinolytic activities

The homogenate was centrifuged at 10,000 ×*g* for 10 min at 4 °C (5810 R, Eppendorf) and the supernatant was used for studying fibrinolytic activity. Fibrin plate assay was carried out by the Astrup and Mullertz (1952) method with modifications as described by Kim et al. (1997). Briefly, 6 mL of 0.6% fibrinogen (F3879, Sigma-Aldrich) in 0.1 M phosphate buffer, pH 7.4 was mixed with 6 mL of 1.5% low melting agarose (V2111, Promega) in the same buffer containing 10 NIH units of human thrombin (T6884, Sigma-Aldrich). It was mixed gently and poured in a 90 mm diameter Petri plate. It was allowed to stand for 1 h in room temperature before use. Holes of 2 mm diameter were made using a cork borer on the fibrin agar plates in which 4 μL of crude enzymes were loaded and incubated for 2 h at 37 °C. Plasmin (P1867, Sigma-Aldrich) in different concentrations were used as standard. The diameter of the halo of clearing zone was measured for fibrinolytic activity. Five replications of each plasmin concentration were used for preparing a standard graph to measure the fibrinolytic activity by using fibrin plate assay. Spectrophotometric assay of the samples were also carried out with slight modification of the method described by Kim et al. (1997). In 0.2 mL centrifuge tubes 45 μL of 0.6% fibrinogen and 5 μL of 0.01 NIH units/μL of thrombin were mixed and allowed to stand for 1 h to form fibrin clogs. Further, 40 μL of 0.1 M phosphate buffer at pH 7.4 and 10 μL of crude enzyme were added to the fibrin clog and incubated at 37 °C for 30 min in water bath. The reaction was stopped by addition of 10% trichloroacetic acid (T9159, Sigma-Aldrich). Then the mixture was kept at room temperature for 20 min before being centrifuged at 16,000 ×*g* for 15 min. The released tyrosine due to fibrinolytic activity was measured at 280 nm absorbance (A_{280}). A standard graph for plasmin with five replications of different plasmin concentrations against tyrosine released due to fibrinolytic activity was prepared. Fibrinolytic activity was calculated by comparing with the plasmin standard. Fibrinolytic activity equivalent to one plasmin unit (PU) was defined as the A_{280} equivalent of perchloric acid soluble products released from fibrinogen in a reaction volume of 100 μL by 1 NIH unit of plasmin in 30 min at pH 7.4 at 37 °C. One A_{280} was equivalent to 167 plasmin units (PU) in the above defined conditions. Similarly, using 0.6% of casein (C7078, Sigma-Aldrich) solution (in 0.1 M phosphate buffer, pH 7.4) was used for caseinolytic activity. The ratio of fibrinolytic to caseinolytic activity (F/C) was calculated from the above data.

2.3. Fibrin zymogram based cluster analysis

With some minor modifications, the method of Kim and Choi (2000) was followed for fibrin zymogram analysis. A native polyacrylamide gel of 4% stacking gel and 12% separating gel copolymerized with 0.12% fibrinogen and 10 μL of thrombin 5 NIH units/mL was used. Four microlitres of the crude enzyme was mixed with an equal volume of sample loading buffer without β-mercaptoethanol and loaded in the acrylamide gel without heating. Electrophoresis (Mini protean, BioRad) was carried out initially for 15 min at 60 V followed by 100 V for 90 min. The gel was washed with sterile 2.5% Triton-X solution (Sigma-Aldrich, T8787) for 1 h in a platform rocker. It was followed by 3 washing steps of 15 min each by 25 mL of distilled water to remove traces of the detergent. It was followed by incubation of the gel at 37 °C for 3 h in the incubating buffer (50 mM phosphate buffer, pH 7.4 and 0.2% NaN_3). The gel was stained with a Coomassie brilliant blue (Merck) solution (40% methanol, 10% acetic acid and 0.5% Coomassie brilliant blue-R250) for 2 h and de-stained. The colourless clearing band indicated the enzyme and its isozymes. In this study, we compared the zymograms of different fermented foods. The zymogram profile was scored manually and cluster analysis was done based on simple matching similarity co-efficient and unweighted pair group method with arithmetic mean (UPGMA) using NTSYSpc version 2.20f software.

2.4. Microbial isolation

The homogenate was serially diluted using physiological saline and spread plated in triplicates over plate count agar (PCA) for mesophilic aerobes, de Man Rogosa and Sharpe (MRS) agar supplemented with 1% $CaCO_3$ for facultative anaerobes and yeast extract peptone dextrose (YEPD) agar supplemented with 100 μg/mL each of filter-sterilized ampicillin and tetracycline for yeast. PCA and YEPD plates were incubated for a period of 24–48 h. The MRS plates were incubated in anaerobic Jars (Merck) with anaerobic gas packs (Anaerocult C, Merck) for maintaining a micro-aerobic environment for 48–120 h. From each plate 10–15 colonies were subcultured after selecting on the basis of colony and cell morphology and their relative abundance. These microbial isolates were maintained as agar slants on respective agar media at 4 °C up to two weeks for further studies.

2.5. Screening of microbial isolates for fibrinolytic activity

For rapid and economic screening, a direct colony spotting method was employed. In this method, the previously maintained subcultured single colonies were spotted on the fibrin plates prepared as described in Section 2.2. by using sterile toothpicks. The inoculated fibrin plates were incubated at 37 °C for up to 16 h with regular observation for every 1 h of incubation. The colonies showing clearing zones on the fibrin plates were selected as fibrinolytic isolates. The selected fibrinolytic bacterial isolates were grown on 5 mL Luria Bertani (LB) broth and yeast isolate on YEPD broth at 30 °C using shaking incubator at 250 ×*g* for 24–72 h depending on its growth rate. The culture broth was divided into three portions; one portion (1 mL) was stored in 25% glycerol stock in −80 °C, the second portion (2 mL) was utilised for DNA extraction and the third portion (2 mL) was used for fibrinolytic assay. For this assay the broth culture was centrifuged at 10,000 ×*g* for 8 min at 4 °C. One mL of the culture supernatant was mixed with