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Application of Uricase Isolated from *Bacillus subtilis* SP6 in Uric Acid Assay Diagnostic Kit

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Authors' contributions

This work was carried out in collaboration among all authors. All authors contributed to the experimental design, hands on work, discussions, and commented on the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Uricase enzyme is a major component of the diagnostic kit for the estimation of uric acid for diagnosis, monitoring and treatment of gout and joint inflammations. Uric Acid/Uricase assay kit is a simple assay for measuring uric acid concentrations in biological samples such as serum, plasma, and urine without any need for pretreatment. The level of uricase enzyme activity was detected in the crude extract of some animal liver tissues, plant leaves and a microbial source (*Bacillus subtilis* SP6 bacteria) and expressed as specific activity (unit/mg protein). It was found difficult to produce high yield of uricase in short time from animal or plant tissues and stability of uricase from both sources is still unclear; therefore, it was isolated from *Bacillus subtilis* SP6. The extraction procedure of uricase from *Bacillus subtilis* SP6 involved, isolation and extraction of bacterial cells,

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determining the uricase activity in both intracellular and extracellular fractions, pooling for both fractions and ammonium sulfate precipitation which seemed to be convenient since 74.7 % of uricase activity was recovered. The isolated uricase was applied in the preparation of uric acid diagnostic kit that found sensitive and comparable with commercially available ones.

Keywords: Uricase; mammalian tissues; plant tissues; Bacillus subtilis SP6; diagnostic kit.

1. INTRODUCTION

For humans and higher primates, uric acid is the final oxidation end product of purine nucleotide metabolism. The enzyme xanthine oxidase produces uric acid from xanthine and hypoxanthine, which are derived from purines. Although most animals can metabolize uric acid to the easily excreted product allantoin, humans lack the necessary enzyme, uricase, due to two nonsense mutations in the uricase gene. Uric acid is released in hypoxic conditions and is usually excreted in the urine via glomerular filtration. Approximately 70% of daily uric acid disposal occurs via the kidneys [1-3]. High levels of uric acid have been linked to impaired renal function, polycythemia, leukemia and consumption of foods high in nucleoproteins. Hyperuricemia induces or accelerates the development of gout, kidney stones, hypertension, metabolic syndrome and renal or cardiovascular diseases. Gout is an inflammatory condition that results from uric acid deposits within the body joints [4-6]. Urate oxidase or uricase (urate: oxygen oxidoreductase, EC 1.7.3.3) is an enzyme that catalyze the oxidation of uric acid to allantoin and plays an important role in purine metabolism. It catalyzes the oxidative opening of the purine ring of urate to yield allantoin, carbon dioxide and hydrogen peroxide. This enzyme is widely present in most vertebrates but is absent in humans. It was first found in bovine kidney [7, 8]. Uricase has been produced from various microbial sources such as bacteria, fungi and eukaryotic cells [9-11]. Uricase has been previously isolated from various sources like microorganisms [9, 12], plants like chickpea (*Cicer arietimum* L.), broad bean (*Vicia faba* major L.), wheat (*Triticum aestivum* L.) and animals like porcine and fish [13-15]. An uricase from *Aspergillus flavus* (Uricozyme) was in the market for the treatment of tumor lysis syndrome, hyperuricemia and renal failure [16]. Uricase has also been purified from several microbial sources like *Gliocladium viride* [17], Streptomyces [18] and *Bacillus subtilis* SP6, a strain that was evaluated for its ability to produce uricase [19]. The objective of the present study was screening of uricase activity in the locally available and safe sources as mammalian livers, plant tissues and certain bacterial source. Isolation of uricase enzyme from the source displayed the highest specific activity and using it in the preparation of uric acid diagnostic kit which is mainly used in diagnosis, monitoring and treatment of gout .

2. MATERIALS AND METHODS

2.1 Collection of Samples

Fresh liver samples of buffalo (*Bubalus bubalis*), camel (*Camelus dromedaries*) and sheep (*Ovis aries*) were obtained from a local slaughter-house and stored at -40 ˚C. The samples were collected from at least six different individuals for each animal. Different plant tissues (Pea, Chard, Clover, Guava, Mango, Wheat and Thermos leaves) were collected from the open fields and stored at -40 ˚C. Soil samples contaminated with chick faecal materials were collected from certain poultry farm for isolation of *Bacillus subtilis* SP6 bacteria.

2.2 Chemicals

Uric acid, glucose, Beef extract, yeast extract, phenol red, BSA (bovine serum albumin), agar powder, peptone, manganese sulfate, 4-aminoantipyrine, peroxidase from horseradish, diaminobenzidine, 2,4,6-tribromo-3-hydroxybenzoic acid (TBHBA) and chemicals for electrophoresis were purchased from Sigma-Aldrich Chemical Co. The other chemicals were of analytical grade.

2.3 Assay of Uricase Activity

“The uricase activity reaction mixture contained 0.6 ml of 2 mM uric acid dissolved in 0.1 M sodium borate buffer (pH 8.5), 0.1 ml of 1.5% phenol, 0.15 ml of 30 mM 4-aminoantipyrine, 0.05 ml of 15 U/ml peroxidase from horseradish, and 0.1 ml of enzyme solution. The mixture was incubated at 25 ˚C for 20 min. The reaction was terminated by the addition of 1 ml of ethanol and the absorbance at 540 nm was read against the blank. One unit of uricase was defined as the

amount of enzyme that produces 1.0 µmol of H_2O_2 per minute under the standard assay conditions. To determine the uricase activity, samples of the culture were withdrawn, and the cells were centrifuged at 3000 xg for 10 min and filtered through 0.2 lm. The filtered supernatant (crude enzyme solution) was used for the analysis of enzyme activity [20].

2.4 Uricase Activity Staining on Polyacrylamide Gels

"Activity staining of uricase was determined after electrophoresis by submerging the gel in 0.1 M Tris-HCl, pH 9.0, 0.1 mM urate, 2.8 mM diaminobenzidine and 3.3 U of horseradish peroxidase and staining was continued till the activity band appears on the gels" [21].

2.5 Extraction of Proteins from Animal and Plant Tissues

All of the procedures were performed at 4˚C unless stated otherwise. 10 grams of animal liver were homogenized in 50 mM Tris-HCl buffer, pH 8.0 using Omni mixer homogenizer. Cell debris and insoluble materials were removed by centrifugation at 8000 xg for 20 min and the supernatant was saved and designated as crude extract. Pea, Chard, Clover, Guava, Mango, Wheat and Thermos leaves were collected and proteins were extracted according to Lanna et al. [22]. "One gram fresh weight was ground in a mortar and pestle containing liquid nitrogen. The resulting powder was macerated for 30 sec in 3 ml extraction buffer [50 mM Tris-HCl buffer, pH 8.0], then centrifuged at 10000 xg for 25 min at 4 °C. The supernatant was divided and kept in ice at -20 °C for the following determination [22].

2.6 Isolation of Bacteria

Bacillus subtilis SP6 formerly isolated in our lab from poultry waste soil samples was used for the production of uricase enzyme [19]. Soil samples contaminated with chick faecal materials were collected from certain poultry farm. The culture was maintained on uric acid agar slants and stored at 4 °C. For uricase production, the composition of pre-culture medium was 1% peptone, 2% glucose, 0.1% K_2HPO_4, 0.05% $MgSO_4.7H_2O$ and 0.5% NaCl. The pH of the medium was adjusted to 8.0. The production medium was prepared by the addition of 0.05% uric acid to the pre-culture medium [19].

2.7 Cell growth and Uricase Production

1% (v/v) of pre-cultured organism was inoculated into a 3000 ml Erlenmeyer flask containing 500 ml of sterilized production medium. The flask was incubated in an orbital shaker incubator for 48 h at 175 rpm maintained at 37 °C. Samples were taken periodically to determine the cell biomass and uricase production.

2.8 Extraction and Partial Purification of Uricase

"The cells free culture broth was sonicated for 21 sec at max speed then centrifuging at 5000 rpm for 20 min. The uricase enzyme in the cell filtrate was further concentrated by 80% ammonium sulfate precipitation, centrifuged at 10000 xg for 30 min followed by dialysis. Enzyme activity was determined for each separate fraction [23].

2.9 Electrophoretic Analysis

Native gel electrophoresis was carried out with 7% PAGE [24] and proteins were stained with 0.25% Coomassie Brilliant Blue R-250.

2.10 Protein Determination

Protein content was determined by the dye binding assay method using bovine serum albumin (BSA) as a standard protein [25].

2.11 Construction of Uric Acid Diagnostic Kit

The partially purified *Bacillus subtilis* SP6 uricase enzyme was used in the construction of uric acid diagnostic kit. The uric acid kit is composed of two reagents: (R1: 100 mM phosphate buffer pH 7.0 and 1.25 mM TBHBA (2, 4, 6-Tribromo- 3-hydroxybenzoic acid) and (R2: 100 mM phosphate buffer pH 7.0, 1.5 mM 4-aminoantipyrine, 50 mM K_4 [Fe $(CN)_6$], ≥10 kU/L peroxidase (POD) and ≥ 150 U/L bacterial uricase). The standard: 6 mg/dL (357 µmol/L) uric acid [26].

3. RESULTS AND DISCCUSION

3.1 Comparison of Uricase Activity

"In gout, the increased serum levels of uric acid lead to formation of mono-sodium urate crystals around the joints. Further causes of elevated blood concentrations of uric acid are renal diseases with decreased excretion of waste products, starvation, drug abuse and increased alcohol consume as well as use of certain

medicaments. High uric acid levels also constitute an indirect risk factor for coronary heart disease. Uricase catalyzes the oxidation of uric acid to allantoin and plays an important role in purine metabolism. It catalyzes the oxidative opening of the purine ring of urate to yield allantoin, carbon dioxide, and hydrogen peroxide" [27,28,1,2]. The level of uricase activity was detected in the crude extract of different samples and expressed as specific activity (units / mg protein) and the data of uricase specific activity are summarized in (Table 1). The specific activity of the uricase was found to be (0.214 units / mg protein) for buffalo liver, (0.015 units / mg protein) for camel liver, (0.185 units / mg protein) for sheep liver, (0.19 units / mg protein) for Pea leaves, (0.085 units / mg protein) for Chard leaves,, (0.24 units / mg protein) for Clover leaves, (0.87 units / mg protein) for Guava leaves, (0.24 units / mg protein) for Mango leaves, (0.1 units / mg protein) for Wheat leaves, (0.194 units / mg protein) for Thermos leaves and (0.92 units / mg protein) for the *Bacillus subtilis* SP6 bacteria. The specific activity of uricase from Guava leaves and the bacterial source is higher than other sources. Both of the protein and uricase isoenzyme patterns of different samples were compared by analysis on 7 % native PAGE. For comparison, a definite amount of protein (100 µg) was applied for protein and uricase isoenzyme patterns (Fig. 1). The uricase isoenzyme pattern indicated the presence of one isoenzyme in the bacterial crude extract. Different protein patterns were monitored on the native PAGE from different tissues. Uricase has been produced from various microbial sources such as bacteria, fungi and eukaryotic cells [9-11]. Uricase from leaves of chickpea, broad bean and wheat has been purified to electrophoretic homogeneity [14].

3.2 Extraction of *Bacillus subtilis* SP6 Bacteria Uricase

In this study, it was found difficult to produce high amounts of uricase enzyme in short time either from animal or plant tissues. Therefore, we isolated the desired amount of uricase from the intracellular and the extracellular fractions of *Bacillus subtilis* SP6. The two fractions were pooled together, brought to 80% saturation by gradually adding solid $(NH_4)_2SO_4$, stirred for 30 min at 4 °C and centrifuged at 10000 xg for 20 min. The pellet of this step was dissolved in 0.05 M sodium phosphate buffer pH 7.0 and dialyzed extensively against the same buffer. Most of the uricase activity was precipitated in the ammonium sulfate fraction that 74.7 % of the activity was recovered. The uricase specific activity was found to be 1226.4 m units / mg protein (Table 2). Various uricase specific activities were reported; 0.007 U/mg for broad bean, 0.11 U/mg for chickpea and 0.009 mU/mg for wheat [14], 32 U/mg for *Bacillus subtilis* [8], 0.131 U/ml for *Halobacillus sp* [29], 0.05 U/mg for *Candida sp.* [30] and the intracellular uricase ranged from 0.09 to 0.5 U/ml and the extracellular ranged from 0.08 to 0.43 U/ml for *Streptomyces exfoliates* UR10 [23].

Table 1. Uricase specific activity of different animal, plant and microbial tissues

Sample	Uricase specific activity*
Buffalo liver	0.214
Camel liver	0.015
Sheep liver	0.185
Pea leaves	0.19
Chard leaves	0.085
Clover leaves	0.24
Guava leaves	0.87
Mango leaves	0.24
Wheat leaves	0.1
Thermos leaves	0.194
Bacillus subtilis SP6 bacteria	0.92

** The specific activity is expressed as units / mg protein*

** Each value represents the mean of at least 4 tissues*

Table 2. Purification scheme of uricase from *Bacillus subtilis* SP6 bacteria

Purification step	Total protein (mg)	Total Activity (m unit)	Specific Activity (mU/mg protein)	Yield (%)	Fold Purification
Bacillus subtilis SP6 intracellular fraction	6.7	6300	940.3	100.0	1.0
Bacillus subtilis SP6 extracellular fraction	3.5	2400	685.7	100.0	1.0
The pooled fraction	10.2	8700	852.9	100.0	1.0
80% $(NH_4)_2SO_4$ fraction	5.3	6500	1226.4	74.7	1.4

The specific activity is expressed as m units / mg protein

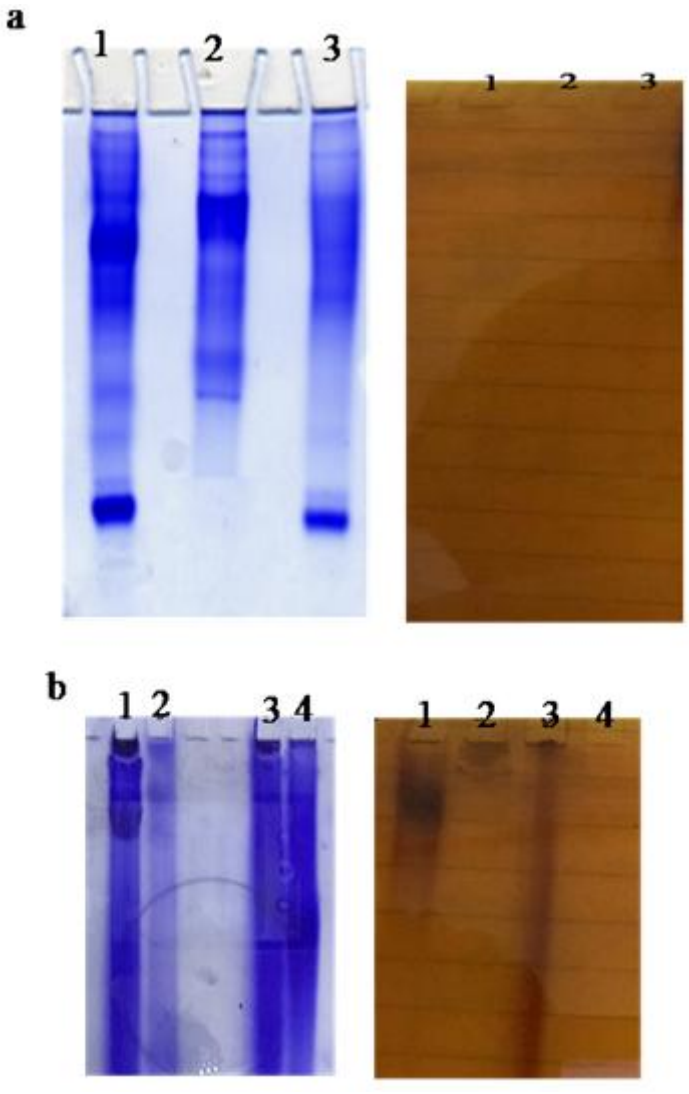

Fig. 1. (a) Electrophoretic analysis of protein and uricase isoenzyme patterns on 7 % native PAGE: (1) buffalo, (2) camel and (3) sheep. (b) Electrophoretic analysis of protein and uricase isoenzyme patterns on 7 % native PAGE: (1) *Bacillus subtilis* SP6 extract, (2) Guava leaves extract, (3) Mango leaves extract (4) Thermos leaves extract

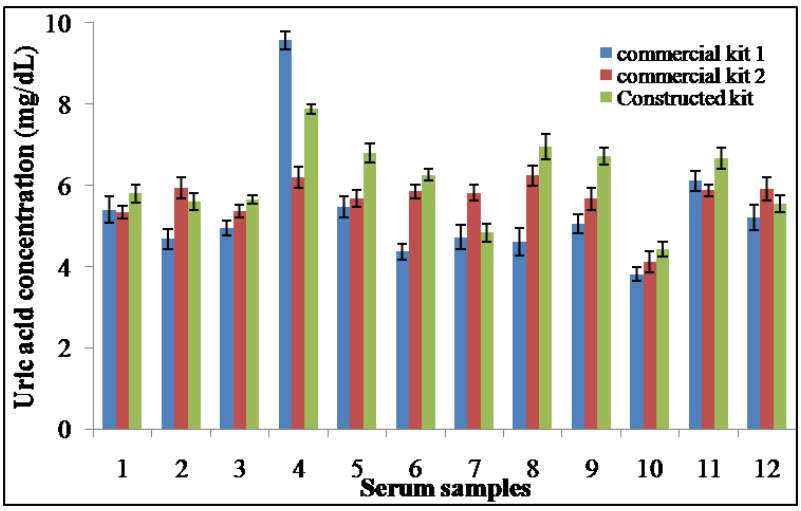

Fig. 2. Comparison of the constructed uric acid kit with commercially available kits

Table 3. Comparison of the constructed uric acid kit with commercially available kits

Serum samples	Uric acid concentration		
	commercial kit 1	commercial kit 2	Constructed kit
1	5.4	5.34	5.8
2	4.68	5.94	5.6
3	4.96	5.37	5.66
4	9.57	6.2	7.88
5	5.48	5.67	6.8
6	4.37	5.85	6.26
7	4.73	5.82	4.84
8	4.61	6.24	6.96
9	5.05	5.67	6.73
10	3.82	4.11	4.43
11	6.11	5.88	6.67
12	5.22	5.92	5.56

3.3 Comparison of the Constructed Uric Acid Kit with Commercially Available Kits

The isolated *Bacillus subtilis* SP6 uricase is used in the construction of uric acid diagnostic kit. The constructed kit (prepared uric acid diagnostic kit with the isolated uricase enzyme) has been compared with commercially available kits utilizing different individual samples (Table 3 and Fig. 2). Uric acid diagnostic kit is very sensitive in the measurement of uric acid concentration and mainly used in diagnosis, monitoring and treatment of gout and joint inflammations. The prepared kit was found to be comparable to the commercial kits. The variance between the constructed and commercial kits was found more or less within the experimental error.

4. CONCLUSION

In conclusions, the uricase enzyme has been isolated from *Bacillus subtilis* SP6 bacteria as a newer source of uricase which is cost effective as well as having more specificity that makes it suitable for various medical applications. The isolated bacterial uricase enzyme was used in the preparation of uric acid diagnostic kit which found comparable to commercially available kits.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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