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Isolation of (2S,3R,4S)-4-Hydroxyisoleucine from *Trigonella foenum graecum* Seeds

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Antidiabetic compositions derived from botanical sources are well known in traditional medicines in India and other parts of the world. Extensive studies have been carried out on screening the compounds isolated from the fenugreek seeds and other parts of the vegetation for their varied activities. One amongst them is an amino acid *i.e.*, 4-hydroxyisoleucine. This molecule has a potential of not only addressing hyperglycemia amongst diabetic patients but also act in a manner to cure hyperlipidemia, diabetic neuropathy and diabetic nephropathy. Many attempts have been made to isolate this amino acid in pure form but none of the methods were seem to be commercially viable since the content of this amino acid is only 0.3 to 0.4 % in the fenugreek seeds. In this study, an attempt was made to develop a method of isolation of pure form of 4-hydroxyisoleucine followed by scale up to yield a level of kilogram quantity. The isolated pure form of 4-hydroxyisoleucine has been subjected to structural characterization.

Keywords: Isolation, (2S,3R,4S)-4-Hydroxyisoleucine, *Trigonella foenumgraecum* seeds.

INTRODUCTION

Diabetes, a chronic progressive disorder characterized by metabolic abnormalities, has reached epidemic proportions worldwide. Oral hypoglycemic agents used for the treatment of diabetes have side effects on prolonged use. The patients are using herbal medicines which have less side effects, easy availability and economic for them. Even World Health Organization (WHO) permits the use of plant drugs for different diseases, including diabetes mellitus (II). In this study, a known anti diabetic plant, *Trigonella foenumgraecum* Linn., (family: Leguminosae) is used. It is called as 'Fenugreek' and commonly used as a spice in cooking. Fenugreek is cultivated in India, Egypt, Middle East and North Africa. The seeds of the plant have been used as a traditional remedy for conditions including gastrointestinal disorders, gout, wound healing and inflammation, hyperlipidemia and diabetes. In humans, fenugreek seeds exert hypoglycemic effects by stimulating glucose-dependent insulin secretion from pancreatic β-cells, as well as by inhibiting the activities of α-amylase and sucrose. Studies such as glycemic control in a double blind placebo controlled human trials[1], application of fenugreek extract powder in a neonatal model of rats for insulinotropic property[2], usage of reconstituted fenugreek extract powder in ischemic perfusion rat model to address diabetic nephropathy[3], endurance and metabolic syndrome[4] and lipid lowering property of fenugreek powder[5] have revealed the importance of fenugreek in controlling diabetes. Fenugreek contains many bioactive compounds and 4-hydroxyisoleucine (4HI) isolated from the seeds of fenugreek showed hypoglycemic activity. Sauvaire and Ribes[6] demonstrated that 4-hydroxyisoleucine increased glucose-induced insulin release in human and rat pancreatic islet cells. 4-Hydroxyisoleucine was specific to act on pancreatic β-cells, since the levels of somatostatin and glucagon were not altered. It was also found that 4-hydroxyisoleucine stimulated glucose uptake by increasing surface GLUT4 level in skeletal muscle cells *via* phosphatidylinositol-3-kinase dependent pathway[7]. A study by Shah *et al.*[8] exploring the regenerative potential of pancreas in alloxan induced diabetic mice by 4-hydroxyisoleucine in comparison with pioglitazone, a commercially available drug, revealed the importance of 4-hydroxyisoleucine. Therefore, it was considered important to isolate and formulate the pure form of 4-hydroxyisoleucine to address the varied ailments without any side effects. None of the published results seem to be commercially viable from the point of view of economy as the process involves lengthy chromatographic columns resulting in operational difficulties and limited output of quantity. Synthetic routes employed by Sergent *et al.*[9] and Kumaraswamy *et al.*[10] involved several steps which needed expensive reagents. In this present study, a commercially viable method of isolation without involving chromatographic techniques and involving

a batch process was developed to isolate 4-hydroxyisoleucine in the purest possible form. The final isolated product was authenticated by structural elucidation and purity techniques for its use in commercial applications.

EXPERIMENTAL

Fenugreek seeds were sourced from agricultural market (Nimuch, Madhya Pradesh, India). The seeds were authenticated by Agharkar Research Institute (Pune, Maharashtra, India) and deposited in quality control/quality assurance repository of Indus Biotech Private Limited (Pune, Maharashtra, India) for FDA regulatory purpose (specimen reference ARRM090121 dated 01.02.2010). The following chemicals of reagent grade were used. Dinitrofluorobenzene (SD Fine Chem Limited, Mumbai, Maharashtra, India), *n*-butanol (Reliance Petrochemical Industries, Mumbai, Maharashtra, India) ethyl alcohol (SPY Agro Industries, Nandyal, Andhra Pradesh, India), hexane and liquid ammonia (Yash Shree Chemicals, Pune, Maharashtra, India). Strong acid cation exchange resin (Ion Exchange India, Mumbai, Maharashtra, India) and ADS-adsorbent resin (Thermax India, Pune, Maharashtra, India) were used.

General procedure: One thousand kilograms of fenugreek seeds specifically containing higher level (about 0.4 % predetermined by HPLC) of 4-hydroxyisoleucine was flaked to 5 mm size flakes and soaked in ethyl alcohol water ratio (82:18) mixture at 45 °C for 10 h and were filtered. The clear filtrate was concentrated under vacuum at 40 °C to remove all the alcohol to get a thick paste. The paste was dissolved in deionized water to get a clear solution of extract having total dissolved solids content of 4%. The extract was subjected to a batch treatment of countercurrent solvent extraction using hexane solvent which removes all the fenugreek seed oil and other lipids present in the extract. The extract was treated with ADS-adsorbent resin (750 L). The aqueous layer was filtered out, the resin was separated for recycling and the clear filtrate was treated with strong acid cation exchange resin (800 L of volume) in the form of H^+ form. The gel form of the resin which had 55 % moisture selectively took 4-hydroxyisoleucine and other amino acids present in the extract. The resin was washed sequentially with water (pH = 4.0) followed by water (pH = 5.3) to remove all other amino acids except 4-hydroxyisoleucine. The washed resin was charged in to a reactor having 8 % aqueous ammonia solution and stirred at 10 °C for 8 h. The spent resin was filtered out and taken for regeneration and recycling. The clear brownish yellow liquid was subjected to a batch treatment with *n*-butanol in a reactor for 4 h. The top *n*-butanol layer was distilled and reused. The bottom aqueous layer was separated and concentrated below 40 °C to 200 L volume in a high vacuum distillation reactor followed by passing through a wiping film evaporator at 40 °C to get a thick paste. The resultant paste was dried under vacuum at 45 °C to yield a pale yellow powder weighing 5.8 kg. The purity of 4-hydroxyisoleucine at this stage was about 80 %. The powder was dissolved in aqueous ethyl alcohol and recrystallized twice in hot conditions to yield 2.8 kg of 99 % pure 4-hydroxyisoleucine.

The isolated 4-hydroxyisoleucine (2.8 kg, 0.28 % yield) was subjected to melting point determination (Bio Technics, Mumbai, Maharashtra, India) and optical rotation using digital polarimeter (Jasco International, Japan). The purity of 4-hydroxyisoleucine was determined by using high performance liquid chromatography (HPLC) derivatization with dinitroflurobenzene. To confirm and authenticate the structure of 4-hydroxyisoleucine, mass spectral (MS) and nuclear magnetic resonance (NMR) analyses were carried out. 4-Hydroxyisoleucine was directly injected to a MS instrument (Bruker Daltonics, Switzerland) operating under ESI (positive mode)-Q-TOF mode. NMR analyses were carried out in both liquid and solid state media. Liquid state NMR analysis was carried out using a 500 MHz VNMRS spectrometer (Agilent Technologies, USA) equipped with a 5 mm One NMR probe for ^{1}H, ^{13}C and ^{15}N. NMR experiments which were part of the standard NMR software library of VnmrJ (Agilent Technologies, USA) were employed. 25 mg of 4-hydroxyisoleucine was dissolved in 90 % water and 10 % deuterium oxide to have a final volume of 0.7 mL in a 5 mm diameter NMR sample tube. ^{13}C solid state NMR analysis was carried out using a 400 MHz AVANCEII spectrometer (Bruker Biospin, Switzerland) equipped with a 4 mm MAS probe. NMR experiments which were part of the standard NMR software library of Top Spin (Bruker Biospin, Switzerland) were employed. Adequate amount of 4-hydroxyisoleucine in powdered form was filled into the 4mm rotor of the 4mm MAS probe. The experiment of Cross Polarization with Magic Angle Spinning (CPMAS) with 1H SPINAL decoupling to observe ^{13}C was performed at 30 °C.

RESULTS AND DISCUSSION

Melting point determination yielded a value of 223.5 °C. Optical rotation measurement yielded a value of $[\alpha_D]$ +30.5 (c = 1, H_2O) [literature[11], $[\alpha_D]$ +31 (c = 1.1, H_2O)] which revealed that the isolated 4-hydroxyisoleucine was (2S,3R,4S)-4-hydroxyisoleucine. The HPLC derivatization method revealed the purity of 4-hydroxyisoleucine as 99 %. The MS result revealed the molecular weight of 4-hydroxyisoleucine as 147.1 (*m/z*: 148.1). 4-Hydroxyisoleucine (Fig. 1) has three exchangeable protons, *viz*. -OH, -COOH, $-NH_2$ and the observation of $-NH_2$ signal in ^{1}H NMR spectrum is very crucial to determine the structure unambiguously. Observation of $-NH_2$ signal was not possible at room temperature without adjusting pH. Ionization studies (pKa) have been carried out to give an appropriate picture about pH which can be set to observe the $-NH_2$ signal. Accordingly, the pH was set to 3.3 and NMR data were collected at a pH of 3.3 and at a temperature of 10.6 °C. All NMR experiments involving ^{1}H and ^{13}C have been carried out at a pH of 6 and at a temperature of 25 °C employing both one dimensional and two dimensional NMR methods and it included 1D survey of ^{1}H and ^{13}C, $^{1}H^{1}H$-gDQCOSY, $^{1}H^{13}C$-ASAPHMQC. Since the $-NH_2$ signal was not seen at pH of 6

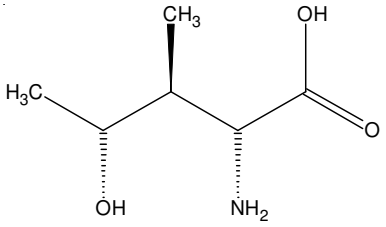

Fig. 1. Structure of 4-hydroxyisoleucine

and at 25 °C, the pH was lowered to 3.3 and the temperature was lowered to 10 °C. Under these conditions, the 1D survey of ^{1}H showed -NH_2 signal and the 2D experiment of ^{1}H ^{15}N-ASAPHMQC was performed to assign the chemical shift of ^{15}N. WET based PRESATURATION to suppress the water signal was employed in all the experiments. Further, solid state ^{13}C CPMAS spectrum also clearly revealed the nature of carbon atoms present in 4-hydroxyisoleucine. Table-1 lists the chemical shift assignments of ^{1}H, ^{13}C and 15N which were determined from both liquid and solid state NMR data.

TABLE-1
LIQUID AND SOLID STATE NMR CHEMICAL SHIFT VALUES FOR 4-HYDROXYISOLEUCINE

Position	^{1}H (δ)[a]	^{13}C (δ)[b]	^{13}C (δ)[c]	^{15}N (δ)[d]
1		176.32	177.08	
2	3.78 (1H, s)	59.38	59.14	
3	1.72 (1H, m)	43.84	46.81	
4	3.51 (1H, m)	72.22	72.96	
5	0.92 (3H, d, J = 6.22Hz)	23.15	26.83	
6	0.66 (3H, d, J = 7.02Hz)	14.47	17.02	
7	7.82 (1H, s)			–340.21

[a] ^{1}H chemical shifts were obtained from liquid state ^{1}H NMR spectrum recorded at pH of 2.6 and at 10°C, [b] ^{13}C chemical shifts were obtained from liquid state ^{13}C and $^{1}H^{13}C$-ASAPHMQC NMR spectra, [c] ^{13}C chemical shifts were obtained from solid state ^{13}C NMR spectrum. [d] ^{15}N chemical shifts were obtained from liquid state ^{1}H ^{15}N-ASAPHMQC NMR spectrum

Conclusion

A commercially viable method of isolation without involving chromatographic techniques and involving a batch process was developed to isolate 4-hydroxyisoleucine was presented. One final step, involving two stages crystallization, was introduced to yield a fairly pure form of 4-hydroxyisoleucine. The isolated 4-hydroxyisoleucine was characterized by analytical techniques especially liquid and solid state NMR experiments to authenticate purity and structure. The efficacy of the presented method holds potential to isolate the pure form of 4-hydroxyisoleucine in large scale.

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