Identification of free amino acids in several crude extracts of two legumes using Thin Layer Chromatography

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Key words

Amino acids; Aqueous-ethanolic extract; Thin layer chromatography; Organic solvents; *Vigna unguiculata*; *Phaseolus vulgaris.*

1. Introduction

Amino acids are primary metabolites and nutritional organic compounds that are the building blocks of proteins [1]. The standard (proteinogenic) amino acids consist of an amine group (NH2), a carboxylic acid (COOH), alpha hydrogen and a side chain [2]. Thin layer chromatography (TLC) is a simple, qualitative, sensitive and widely used method for the separation and identification of amino acids in plant extracts. Amino acids vary in their solubility in water and organic solvents depending on the nature of their side chains; water soluble amino acids have polar side chains [3].

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In general, the 70% aqueous ethanol is the most preferable solvent to prepare crude extracts prior to amino acid TLC analysis [4].

In this study, the amino acid profiles of seed cotyledons and seed coats from two types of legumes; black-eyed beans (*Vigna unguiculata*) and red kidney beans (*Phaseolus vulgaris*) were determined and compared in order to evaluate their nutritional value as major source of plant amino acids. To improve the separation and detection of free amino acids, the TLC technique was applied on several crude extracts obtained at the primary extraction stage using a range of polar and nonpolar solvents. The obtained crude extracts are:

1. Kidney bean seeds 70% Ethanol (KBS 70% Ethanol)
2. Kidney bean seeds 100% Methanol (KBS100% Methanol)
3. Kidney bean seeds 100% Acetone (KBS 100% Acetone)
4. Kidney bean seed coat 70% Ethanol (KBSC 70% Ethanol)
5. Kidney bean seed coat 100% Methanol (KBSC 100% Methanol)
6. Black-eyed bean seeds 70% Ethanol (BEBS 70% Ethanol)
7. Black-eyed bean seeds 100% Methanol (BEBS100% Methanol)
8. Black-eyed bean seeds 100% Acetone (BEBS 100% Acetone)
9. Black-eyed bean seed coat 70% Ethanol (BEBSC 70% Ethanol)
10. Black-eyed bean seed coat 100% Methanol (BEBSC100% Methanol)
11. Kidney bean seeds 100% Chloroform (KBS 100% Chloroform)

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12. Black-eyed bean seeds 100% Chloroform (BEBS 100% Chloroform)

2. Experimental

2.1. Crude extracts preparation

Two kilograms of black-eyed beans and two kilograms of red kidney-beans were soaked in distilled water for 30 and 45 min respectively to remove the seed coat. Then, seeds and seed coats were left to dry at room temperature. After that, the seeds and seed coats were ground to flour using a coffee grinder and de-fatted using a nonpolar solvent (hexane). The ground seeds and seed coats were extracted using solvent extraction methods [5] in which solvents with different polarity were used in Soxhlet extraction [6]. For both the decorticated seeds and seed coats, 500 ml of solvent were needed for extraction of 100g of flour along the extraction process. The process continued until the resulting filtrate is colourless, which indicates that most of the soluble constituents have been extracted [7]. The following selective aqueous and organic solvents were used to prepare the crude extracts:

i. Aqueous-ethanol (70% ethanol), Polarity = 1.0 for water and 0.65 for ethanol.
ii. Methanol (100%), Polarity = 0.76
iii. Acetone (100%), Polarity = 0.36
iv. Chloroform (100%), Polarity = 0.26

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Extracts were then suction-filtered and evaporated to dryness under vacuum in a rotary evaporator (Rotavapor® R-210/R-215) at the boiling point of the solvent. The dry extracts were preserved at 4°C prior to analysis for amino acid [8].

2.2. Thin Layer Chromatography

The amino acid profiles of all the extracts were determined using the Thin Layer Chromatography technique (TLC). The following fresh solutions were prepared to run the TLC:

i. Mobile phase: Butanol: Acetic acid glacial: Water, mixed in a ratio of 12: 3: 5 respectively [9]

ii. Visualization reagent: Ninhydrin reagent: 0.2g/100ml ethanol.

iii. Amino acid standards: The following Amino acid standards were dissolved in water to a concentration of 2% (w/v) and used for TLC spotting: Glycine, Serine, Leucine, Cysteine, Valine, Aspartic Acid, Tryptophan, Tyrosine, Threonine, Histidine, Proline, Glutamic Acid, Cystine, Arginine, Alanine, Glutamine, Isoleucine, Asparagine, Methionine, Phenylalanine, Hydroxyproline, and Lysine.

Using capillary tubes, small spots of amino acid standards and bean extract solutions were applied 2cm above one edge of silica coated TLC plates (Whatman silica gel 60 A, 20 × 20 cm, 250 µm thickness with fluorescent indicator) with a 1cm space between the spots. The spots were then labeled with a pencil and left to dry at room

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79 temperature before being located vertically in a TLC chamber containing the
80 developing solution (the mobile phase). The plate edge beneath the spots was
81 immersed in the developing solution and the TLC chamber sealed. Then, the plates
82 were left for approximately three hours. The highest point reached by the mobile
83 phase was marked. When the plates were completely dry they were sprayed with the
84 Ninhydrin reagent and left at 80-100°C for 5min to allow the visualizing reagent to
85 react and stain the spots. The distances from the starting point to the spot centers
86 were measured and the Retardation Factor (R_f) value for each standard and unknown
87 spot was calculated (R_f =the distance traveled by the spot / the distance traveled by
88 the mobile phase front).
89 The following symbols were used to describe the results of the detected amino acids:
90 (+) observed; (++) bright; (±) faint [10].

91 **3. Results and Discussion**

92 The organic solvents used to produce the crude extracts varied in their ability to
93 extract amino acids; no amino acids were detected in Chloroform fractions. Sixteen
94 amino acids were extracted by different polar solvents. The calculated R_f value for
95 each standard is shown in Table 1. These R_f values were used as markers to identify
96 the amino acids in each extract (Table 2).
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98 Table1. Essential and nonessential amino acid standards; abbreviation, polarity and
99 R_f values obtained from the TLC on silica plates, eluted with n-butanol: acetic acid:
100 water mixture 12:3:5 (by volume) respectively and visualized by Ninhydrin stain.

Amino acids	Abbreviations	Side-chain polarity	R_f
Histidine	His	polar	0.162
Isoleucine	Ile	nonpolar	0.515
Leucine	Leu	nonpolar	0.562
Lysine	Lys	polar	0.131
Methionine	Met	nonpolar	0.462
Phenylalanine	Phe	nonpolar	0.615
Threonine	Thr	polar	0.262
Tryptophan	Try	nonpolar	0.615
Valine	Val	nonpolar	0.439
Alanine	Ala	nonpolar	0.277
Arginine	Arg	polar	0.162
Asparagine	Asn	polar	0.215
Aspartic acid	Asp	polar	0.24
Cysteine	Cys	polar	0.362
L-Cystine	L-Cys	polar	0.139
Glutamic acid	Glu	polar	0.277
Glutamine	Gln	polar	0.223
Glycine	Gly	nonpolar	0.246
Proline	Pro	nonpolar	0.262
Serine	Ser	polar	0.254
Tyrosine	Tyr	polar	0.539
Hydroxyproline	Hyp	polar	0.426

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102 Table2. Amino acids detected in extracts of seed cotyledon and seed coat of kidney-
103 beans and black-eyed beans identified by their R_f values referring to the amino acid
104 standards' R_f values.

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Extracts► Amino acids▼	KBS 70% Ethanol	KBS 100% Methanol	KBS 100% Acetone	KBSC 70% Ethanol	KBSC 100% Methanol	BEBS 70% Ethanol	BEBS 100% Methanol	BEBS 100% Acetone	BEBSC 70% Ethanol	BEBSC 100% Methanol
Ala		++	+				+	±	±	+
Arg	+		+							
Asn	±									
Asp			±			+				
Cys				+					+	
Glu			+					±	±	+
Gly	+	+								
Pro			+				+			
Tyr	±	+	+				++	++	++	+
His	±	+	±							
Ile	+	+	+			+				
Leu		++				+	±			
Lys	+	+	±			+	±			
Phe		+		+				±		
Thr	+	±		+			+			
Val	+	++	+			+	+	±	+	+

105

106

107 The results obtained from the TLC (Table 1and Table 2) indicate that the amino acid
108 profile of the kidney-beans and the black-eyed beans varied with different amino acids
109 detected in different crude extracts. Some amino acids such as Ala, Asp, Glu, Pro, Leu
110 and Val were poorly or not detected in the crude extracts prepared with the 70%
111 aqueous ethanol. All of the essential amino acids with the exception of Met were found
112 in the kidney-bean extracts whilst Arg, Asn, Met, Gly and His were absent from the
113 black-eyed bean extracts. Most of the amino acids were detected in the seed

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cotyledon fractions, with only a few detected in the seed coat extracts. More amino acids were detected in the black-eyed bean seed coat than the kidney bean seed coat; the 70% Ethanol fraction of black-eyed bean seed was found to contain Ala, Cys, Glu, Tyr, Val and the100% Methanol fraction contains Ala, Glu, Tyr and Val. Only one fraction of the kidney bean seed coat was found to have amino acids; the 70% Ethanol that found to contain Cys, Phe, Thr. Amino acids have different functions in different legume seeds; Cys is a sulfur containing amino acid that plays an important role in plant defense mechanisms against predators and is found to be toxic to cowpea beetles *Callosobruchus maculatus* [11]. This may explain why seed coats help protect legumes against stored seed pests and minimize losses of stored products. Moreover, both types of beans seem to lack the following amino acids: Hyp, Ser, Gln, L-Cys, Try, and Met which reduces the nutritional value of these beans. Combining beans with other legumes or grains is recommended to provide a meal that is rich in all essential amino acids.

4. Conclusion

TLC is an efficient method for the analysis of amino acids from plant materials. Amino acids vary in their polarity according to their side chains. Producing several crude extracts is a vital first step for the further separation steps. Using several solvents, with different polarity in crude extracts preparation improves the efficacy of

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the TLC technique, enhances the separation and simplifies the identification of polar and non-polar amino acids.

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