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Glycocarbohydrates detected in milk-clotting proteases from *Balanites aegyptiaca* fruits using labeled lectins and gas chromatography

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ABSTRACT

Proteases from *Balanites aegyptiaca* fruits pulp are glycosylated. This work was carried out to identify their glycans linked. Proteases extract was prepared by soaking *B. aegyptiaca* fruits in 50 mM, sodium citrate buffer pH 3 and purified using high cation exchange chromatography. The glycocarbohydrates linked to proteases was detected using both lectins, and gas chromatography coupled with flame ionization detector. The results indicate that the detection of glycocarbohydrates by gas chromatography is better after acetone precipitation. Gas chromatography detection reveal arabinose, rhamnose, xylose, galactose, mannose, glucose and glucuronic acid. Mannose and fucose was detected by labeled lectins.

KEYWORDS

Balanites aegyptiaca;
Protease;
Glycocarbohydrates;
Lectins;
Gas chromatography.

INTRODUCTION

The majority of plant preparations have the capability to clot milk, which is due to the presence of milk-clotting proteases[1]. The stability of milk-clotting enzymes is a fundamental parameter in dairy technology. Many milk-clotting enzymes from plant are glycoproteins, which possess several glycosylation sites[2]. Dairy farmers of sub-saharian area use *Balanites aegyptiaca* fruits extract to thicken raw milk used as ingredient to make special mush. This extract could contain proteases which hydrolyse casein kappa and clot milk used for mush preparation.

B. aegyptiaca is tropical plant which demands and interest is growing by the day. It is a woody tree growing in Northern Cameroon and is distributed in other arid and semi-arid areas. It is used for various needs such as food, fodder, medicines, charcoal and pesticides according to the part of tree used. The fruits of this plant, so-called "*desert date*" are the part which is mostly used. Desert date is described to be rather long in shape, narrow drupe in size, 2.5-7 cm in length and 1.5-4 cm in diameter (Figure 1). Unripe fruits are green, turning yellow and glabrous once ripe. The pulp is bit-

ter-sweet and consumed as sweets[3]. The seed is 1.5-3 cm long; light brown, fibrous and extremely hard. The almond is rich in saturated fatty acids, that fat matter is used as cooking oil. It also contains steroids (saponins, sapogenins, diosgenins) used as raw material for drug[4].

The interest of glycocarbohydrates linked to proteases is important to know both its structure and activity. The glycocarbohydrates moieties are the parts of the functional architecture of enzyme, and are responsible for its thermal stability[5]. Our previous work showed that *B. aegyptiaca* fruits proteases are linked to sugar moieties using gas chromatography coupled to mass spectrometry-electro-ionization[6].

The present study reported the detection of glycocarbohydrates linked to proteases from *B. aegyptiaca* pulp using labeled lectins analysis and gas chromatography flame ionization detection.

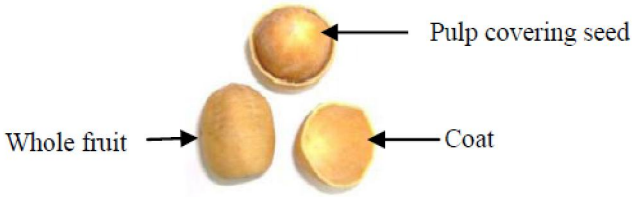

Figure 1 : Desert date (*B. aegyptiaca* fruits).

MATERIALS AND METHODS

Materials

B. aegyptiaca fruits were collected from Pitoa, in the North Region of Cameroon, during dry season. The coat once removed and flesh was used as pulp for the preparation of crude extract. Standard proteins for SDS-PAGE were from Fermentas (Life sciences, European Union). Digoxigenin labeled lectins (GNA, DSA and PNA) were from Roche (Roche Diagnostics GmbH, 68298 Mannheim Germany), biotinylated lectins (WGA, ConA and AAA) were from Vector laboratories (Inc, Burlingame USA). Hydrogen peroxide was from Sigma (Saint Louis, MO, USA). Unless otherwise stated all chemicals used in this work are of reagent grade. All aqueous solutions were prepared using ultra-pure water.

Extract preparation

The extract was prepared by macerating of 180 g of *B. aegyptiaca* fruits in 500 mL of 50 mM, sodium citrate buffer pH 3 overnight at 4°C. The mixture was then filtered through a folded standard filter paper to remove most of the solid residues. It was centrifuged at 4°C for 30 min at 10,000 g to eliminate the insoluble materials. The supernatant was concentrated approximately three times through a 10,000 Da cut off membrane (Millipore). The concentrated solution was then diafiltered against 50 mM, pH 4.5 acetate sodium buffer; treated for 10 min with 10% (w/v) of activated charcoal and kept on ice for 30 min, and centrifuged at 10,000 g for 60 min at 4 °C. The resulting supernatant was crude extract.

Cation exchange chromatography

The purification of crude extract was carried out by high cation exchange chromatography Sulfopropyle (SP) fast flow column on a FPLC system (*Pharmacia Uppsala, Sweden*). The column was quilibrated and washed with 50 mM acetate sodium buffer, pH 4.5, and bound material eluted (0.5 mL min^{-1}) at 280 nm with a linear gradient of sodium chloride (0.15-1M) in the same buffer. Eluted fractions (2 mL each) were collected. Fractions containing purified extract kept at -20°C until analysis.

Milk clotting activity

The substrate was prepared by dissolving skimmed milk powder in 100 mL of 10 mM $CaCl_2$ to a final concentration of 12% (w/v). The substrate (1 mL) was pre-incubated for 5 min at 37°C and 0.1 mL of extract was added. The milk-clotting unit (MCU) was defined as the amount of protein that coagulates reconstituted milk during 40 min at 37°C. The calculation is MCU = $2400 * V / t * v$, where V equals the volume of milk (mL), v the volume of extract (mL) and t the clotting time in seconds.

Proteolytic activity

Extract was incubated for respectively 1, 3, 4, 5, 8 and 10 hours at 40°C. The activity of the extracts was assayed on azocasein following the method described by Sarath[7] with slight modification. Aliquots (0.15 mL) of crude and purified extracts were added to 0.25 mL of a 2% (w/v) solution of azocasein in different buffers. Each mixture was then incubated at 37°C for 60 min. The hydrolysis was stopped by adding 1.2 mL of 10% trichloracetic acid. After 15 min standing at room

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temperature to ensure complete precipitation of remaining azocasein, the samples were centrifuged at 8,000 g for 5 min. 1.2 mL of the supernatant were mixed with 1.4 mL of 1M NaOH and the absorbance was measured immediately at 440 nm.

SDS-PAGE and lectin blot analysis

Proteins were first electrophoretically separated using 12.5% SDS-PAGE according to Laemmli[8]. SDS-PAGE of enzyme samples was performed in a Miniprotean II cell (Bio-Rad). Samples were treated in denaturing buffer with SDS and β-mercaptoethanol and boiled for 5 min before SDS-PAGE. Electrophoresis was run at 100 V until the bromophenol blue dye marker disappeared from the separating gel. For Western blotting analysis, protein samples (100-300 µg) separated for SDS-PAGE were electroblotted onto nitrocellulose membrane (*Hybond-C Extra, Nitrocellulose, Supported,* 0.45 µ, *30 cm X 3 m, Roll Amersham Biosciences*) and PVDF (*Hybond™ P GE Healthcare Amersham, membrane optimized for protein transfert Roll* 0,300 m x 3 m *removal rating*: 0,45 µ) sheets in methanol/Tris-Glycine (15/85) buffer, at 250 mA for 3h in a Mini Trans-Blot (Bio-Rad) as described by Burnette[9]. Transfer efficiency was checked using Ponceau red staining.

Detection of glycans with digoxigenin labeled lectins

Membranes (nitrocellulose) were saturated in TBS (10 mM Tris, 150 mM NaCl, pH 7.5) for 1 hour and washed 3 times for 10 min. Then the blots were incubated 1 hour with the digoxygenin (DIG) labeled lectins (1 $\mu g.mL^{-1}$ of GNA and DSA, 10 $\mu g.mL^{-1}$ of PNA) for DIG Glycan Differentiation Kit (*Roche*) diluted in TBS, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.5. To control the lectin specificity, experiments were performed simultaneously with a specific competitor for each lectin (0.2 M α-D methylmannopyrannoside for GNA, 0.2 M D-Galactose for PNA and 0.2 M N-Acetyl -D-glucosamine for DSA). The positive control was performed by using carboxypeptidase Y for GNA and asialofetuin for PNA and DSA. The blots were then washed tree times for 10 min in TBS, saturated in the blocking reagent at 0.1% in TBS for 1 hour and incubated for 1 hour in the presence of anti-digoxygenin alkaline phosphatase (diluted in TBS buffer). Blots were washed 3 times in TBS buffer for 10 min and labeled glycoproteins were revealed by NBT/BCIP staining.

Detection of glycans with biotinylated lectins

After electroblotted onto PVDF membranes and checking efficiency transfer by Ponceau red staining, the sheets were incubated overnight at room temperature in blocking solution and then washed 3 times with Tween TBS (TTBS) 20 at 0.1% for 30 min. The blots were incubated 1 hour with the biotinylated lectins (Vector Laboratories, Inc Burlingame). Lectins use for this step was 5 $\mu g.mL^{-1}$ for ConA and WGA, 2 $\mu g.mL^{-1}$ for AAA. Simultaneously specific competitor for each lectin (0.1 M fucose for AAA, 0.2 M αD-methylmannoside for ConA and 0.2 M N-Acetyl -D- glucosamine for WGA) were used to performed experiments. Then the blots were incubated 3 times for 10 min in TTBS and 1 hour with horseradish peroxidase avidin D. After washing 10 min in TBS, peroxidase activity was visualized with 3,3'-diaminobenzidine in the presence of 0.1% H_2O_2. Carboxypeptidase Y and asialofetuin were used respectively as control for ConA and WGA. Control for AAA was not found.

Gas chromatography analysis

The aliquots of 100 µg protein of crude and purified extract were methanolysed with 0.5 mL methanolic HCl 0.5M for 16h at 80°C. After evaporation under a stream of nitrogen, the released methyl glycosides were dissolved in 200 µL anhydrous acétonitrile and peracylated by adding 25 µL heptafluorobutyric anhydride, the reaction being conducted at 150°C for 30 min. After evaporation under a stream of nitrogen, the perheptafluorobutyryl-1-*O*-methylglycosides were dissolved in anhydrous acetonitrile and then analyzed by gas chromatography on a Trace GC Ultra Thermo Electron Flame Ionization Detection instrument, equipped with a 30 m x 0.25 mm *Alltech Econo-Cap EC-1*/MS capillary column and 0.25 µm film phases. The samples were analyzed using a linear gradient of 1.2°C.min^{-1} from 100 to 140°C, followed by a gradient of 4°C.min^{-1} until 240°C[10].

RESULTS AND DISCUSSION

Proteases activities

Such as other milk-clotting proteases, clotting ac-

tivity was affected by *B. aegyptiaca* pulp extracts concentration[11]. Milk-clotting time decreased with concentration of extract; meanwhile milk-clotting activity increased in the same conditions (Figure 2). Milk-clotting time decreased until 0.05 mg/mL concentration, and the form of curve became asymptotic. However, clotting activity of *B. aegyptiaca* extract had the opposite behavior. There was an intersection point at 0.02 mg/mL between curves of milk-clotting time and milk-clotting activity. This intersection point may imply that this concentration could be used for milk coagulation in cheese-making.

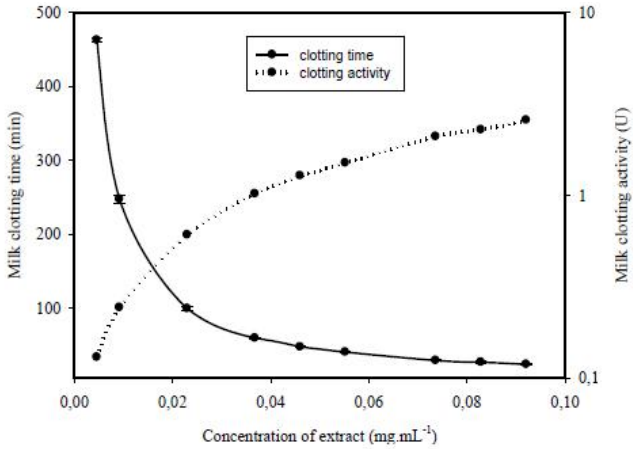

Figure 2 : Variation of milk-clotting activity and milk-clotting time of *B. aegyptiaca* pulp extract. A volume of 0.1 mL of extract was added to substrate (1 mL) at 37°C. One milk-clotting unit (MCU) was defined as the amount of protein that coagulates 1 ml of reconstituted skimmed milk powder at 37°C in 40 min.

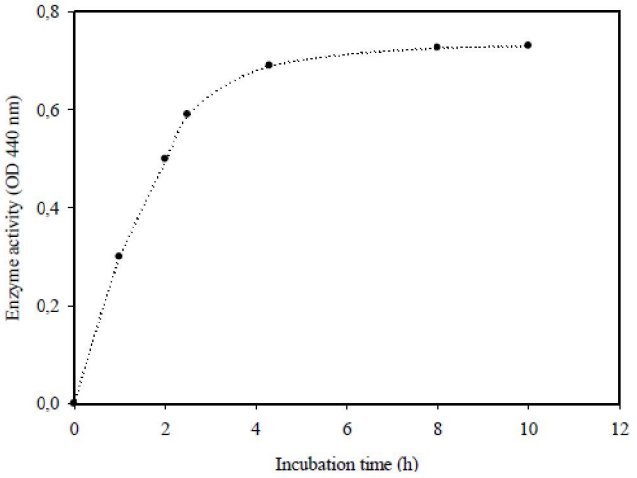

Figure 3 : Variation of proteolytic activity of *B. aegyptiaca* pulp extract. Extract was incubated for respectively 1, 3, 4, 5, 8 and 10 hours at 40°C. The activity was assayed on azocasein 2% at 37°C for 60 min.

The proteolytic activity of *B. aegyptiaca* pulp extract was highly increased with incubation time (Figure 3). The variation of proteolytic activity indicated that extract of *B. aegyptiaca* was very active during the first 4 hours of incubation at 40°C. After this duration, the activity of *B. aegyptiaca* protease remained constant. The results indicated that extract contains proteases which having proteolytic activity and high stability. These proteases, as other milk-clotting enzymes such *cynarases*[12], *dubiumin*[5] may possess glycosylated part; which should be interesting to identify by lectins and gas chromatography.

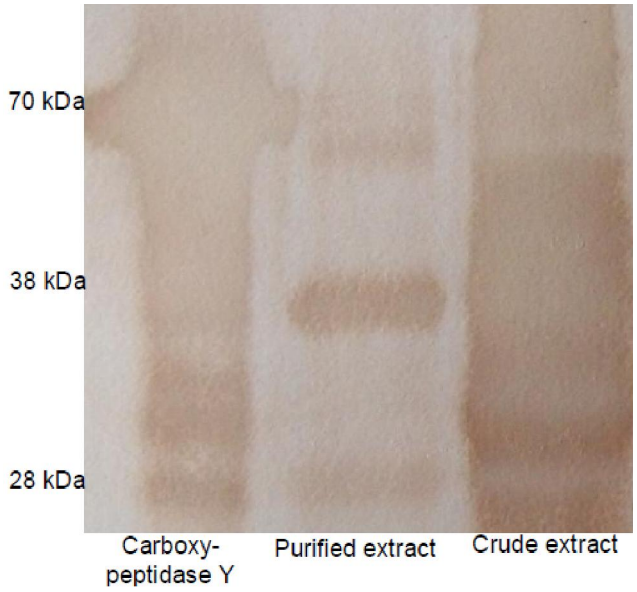

Figure 4a : Immunodetection with ConA on PVDF (polyvinylidene difluoride) membrane. The positive reaction of *B. aegyptiaca* proteases with ConA indicates the presence of oligomannosidic glycan type.

Identification of glycans with lectins

(a) Reaction with ConA

The immunodetection using ConA (Concanavalin A) agglutinin on PVDF membrane is shown in Fgure 4a. ConA agglutinin is a plant which binds to high mannose-type and hybrid-type with high affinity; while N-glycans complex-type are weakly bound to this lectin[13]. The positive reaction of *B. aegyptiaca* extract with ConA indicated the presence of oligomannosidic glycan type. The 38 kDa band was marked and indicated the presence of mannose; whereas the 70 and 28 kDa bands had more or less positive reaction for the purified sample (TABLE 1).

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TABLE 1 : Immunodetection of glycans from crude and purified proteases using lectins.

Sample		GNA[1]	ConA[2]	AAA[3]	WGA[4]	PNA[5]	DSA[6]
Crude extract		+ (70 kDa)	+++	+++	-	-	-
Purified extract	28 kDa	-	±	+	-	-	-
	38 kDa	-	++	++	-	-	-
	70 kDa	-	±	+	-	-	-
Control		Carboxypeptidase Y		NF		Asialofetuin	
		++	++		±	+	+
Specificity		Terminal Mannose	Mannose	Fucose	Terminal Glc-NAc	Terminal Galactose	Galβ(1-4)Glc-NAc

(+++): Total detection (without electrophoretic band specificity); (++): Good detection; (+): Average detection; (±): Poor detection; (-): No detection; NF: No Found; [1]GNA: *Galanthus nivalis* agglutinin, positive reaction with GNA indicates mannose, terminally linked; [2]ConA: *Concanavalin A* agglutinin, positive reaction with ConA indicates oligomannosidic type glycan; [3]AAA: *Aleuria Auriantia* agglutinin, positive reaction with AAA indicates fucose linked; [4]WGA: *Wheat Germ* Agglutinin, positive reaction with WGA indicates N-acétylglucosamine terminally linked; [5]PNA: *Peanut* agglutinin, positive reaction with PNA indicates galactose terminally linked; [6]DSA: *Datura stramonium* agglutinin, positive reaction with DSA indicates disaccharide Gal-β(1-4)GlcNAc.

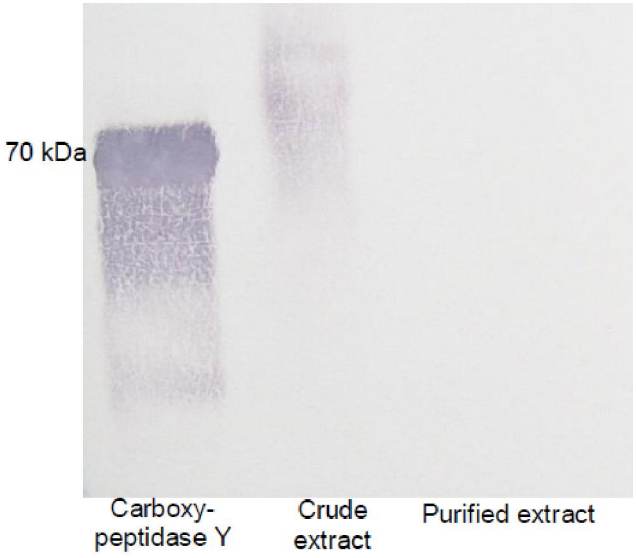

Figure 4b : Immunodetection with GNA on nitrocellulose membrane. Reaction with GNA identifies high mannose N-glycan chains or O-glycosidically linked mannoses in *B. aegyptiaca* proteases.

The total reaction of the crude extract is justified by the abundance or the presence of glycoproteins and polysaccharides badly solved in SDS-PAGE during the migration. The negative reaction with α D-methylmannoside (results not shown) confirmed the presence of mannose in both crude and purified *B. aegyptiaca* extracts. Moreover, a given lectin is sometimes able to recognize various oligosaccharidic sequences. For example, ConA exhibits a strong affinity for oligomannosidic type, biantennary N-acetyllactosamine type as well as for some hybrid-type glycans which has been successfully used to identify N-glycoproteins from many biological complex samples[14,15].

(b) Reaction with GNA

Detection of glycan using GNA (*Galanthus nivalis* agglutinin) on nitrocellulose membrane is exhibited in Fgure 4b. GNA recognizes terminal mannose, α (1-3), α (1-6) or α (1-2) linked to mannose; thus it is suitable for identifying "high mannose" N-glycan chains or O-glycosidically linked mannoses in yeast glycoproteins[16]. Lectin GNA reacted positively with the crude extract (TABLE 1). These results suggested that a small proportion of mainly polysaccharides group containing the mannosyl chains or high molecular N-glycoprotein terminally mannoses. However, the purified sample was not reacted. The Non-reactivity with sugar candidate (α-D methylmannopyrannoside) confirmed the presence of mannose in crude extract. Moreover, the carboxypeptidase Y signal used as pilot was not completely extinguished by carbohydrate. A lectin with an exclusive specificity towards mannose may be isolated from snow drop (*Galanthus nivalis*) bulbs by affinity chromatography on immobilized mannose[17]. Immobilized GNA only interacted with oligomannosidic-type glycopeptides containing a terminal non-reducing Man (α1-3) Man sequence which was retarded on the lectin column. The interaction with the lectin also depends on the number of these disaccharidic units. However, glycopeptides hybrid-type with this Man (α1-3) Man sequence, together with a bisecting GlcNAc residue do not interact with the immobilized lectin[16].

(c) Reaction with AAA

Figure 4c shows Immunodetection using AAA (*Aleuria Aurantia* Agglutinin) on PVDF membrane.

AAA strongly interacted with all N-glycosylpeptides or related glycans containing a fucose residue in α-1,6-linkage to the innermost GlcNAc residue of the core. Immobilized AAA weakly reacted with glycans containing a fucose residue in α-1,3-linkage to one of the outer GlcNAc residue of the N-acetyl-lactosamine units. The presence of both α-1,6-linked and α-1,6-linked fucose residues enhances the affinity of the lectin for the glycans[18]. The 38 kDa band gave a highly positive reaction (Figure 4c), which indicated the presence of α-L-fucose on its proteins (TABLE 1). he important reactivity of the crude extract could indicate fucosylated glycoprotein and polysaccharides. Fucose-binding lectin can be purified from fruiting bodies of the mushroom *Aleuria aurantia*[18,19]. The specificity of these lectins towards L-fucose containing oligosaccharides was determined by analysis on the behavior of fucosylated oligosaccharides on immobilized lectins. Immobilized AAA represents a very valuable tool for the resolution of microheterogenity of glycopeptides and glycans due to the presence of different L-fucose substitutes.

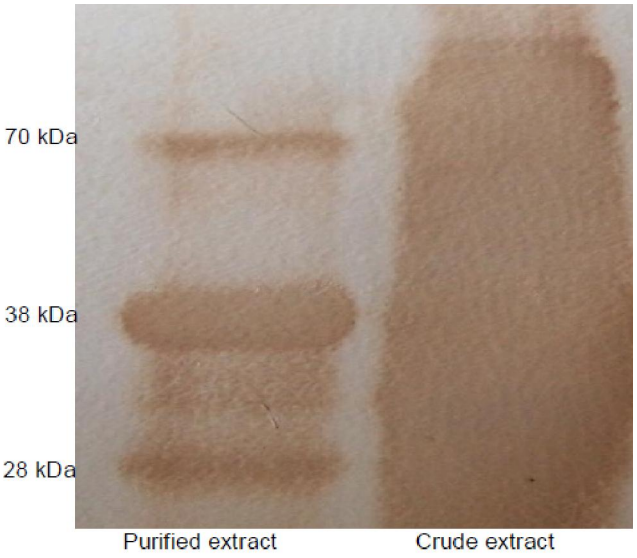

Figure 4c : Immunodetection with AAA on PVDF membrane. Reaction with AAA indicates the presence of glycans containing a fucose residue in α-1,6-linkage to GlcNAc within *B. aegyptiaca* proteases.

(d) Reaction using WGA, PNA and DSA lectins

TABLE 1 showed that there was no reaction using WGA (*Wheat Germ* Agglutinin), PNA (*Peanut* agglutinin) and DSA *(Datura stramonium* agglutinin). WGA showed a stronger affinity for N-glycosylpeptides than for the glycans released from these glycopeptides either by chemical or enzymatic cleavages. This can be explained by the fact that the attachment of a glycan to asparagines (glycoasparagine) imposes to the trisaccharide core sequence Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N)Asn a rigid structure[20,21] favoring the recognition of the oligosaccharidic sequence on the glycan by the lectin. PNA lectins have been reviewed in a classification according to their specificity towards Gal or GalNAc[22]. However, the carbohydrate-binding specificity of PNA lectin isolated from *Arachis hypogaea*[23] has been studied on the immobilized lectins of mucin-type glycopeptides and related oligosaccharides of known structures. For PNA, substitutions of the Gal and/or the GalNAc residue by sialic acid decreased the interaction with the lectins. PNA recognized the core disaccharide galactose β (1-3)N-acetyl-galactosamine. Thus, it was suitable for identifying O-glycosidically linked carbohydrate chains (with exception of yeast glycoproteins). DSA lectin recognizes Gal β (1-4) GlcNAc in complex and hybrid N-glycans, in O-glycans and GlcNAc in O-glycans[24]. DSA did not react with "high mannose" structure like those found in carboxypeptidase Y. Furthermore, DSA is suitable for identifying individual N-acetyl-glucosamine residues with O-glycosidic links to serine or threonine in certain proteins from cell nuclei or cytoplasm.

It is obvious that there was total lake of terminally N-acetyl-glucosamine type (specific of WGA), galactose in β(1-3)Gal-NAc, the O-glycan type (specific of PNA) and galactose in β(1-4)Glc-NAc, N-acetyl-lactosamine type of N-glycans (specific of DSA) in both crude and purified extract.

Gas chromatography detection

Chromatograms obtained by gas chromatography coupled with flame ionization detection (GC/FID) showed different profiles for crude extract before and after acetone protein precipitation (results not show). The intensity of peaks indicated impact of acetone precipitation; reason being that it increaseed carbohydrates concentration of the sample. It was noticed that after precipitation, a strong presence of glycoprotein and polysaccharides was detected in the sample. Lysine used as an internal standard has permitted to identify various glycocarbohydrates present in purified extract according to retention time. Galactose was in abundance after

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acetone precipitation (Figure 5). However, negative reaction has been observed using PNA lectins. This result may be explained by the presence of galactose in the purified extract; however, it was neither N-terminally nor implied in the classically described both for O-glycans mucins types and N-glycans N-Acetyllactosamine type. On the other hand, GC also revealed the presence of the glucuronic acid, arabinose, rhamnose, xylose, mannose and glucose. Generally, glycosylation was detected in most of the isolated plant protease such as cynarases, cucumisin, taraxalisin, cryptolepain, milin, wrightin and dubiumin[5,25-29]. This study demonstrates that proteases isolated from *B. aegyptiaca* fruits extract are glycoprotein. Further work should be necessary to quantifying the glycocarbohydrates in the protease extracted from *B. aegyptiaca*.

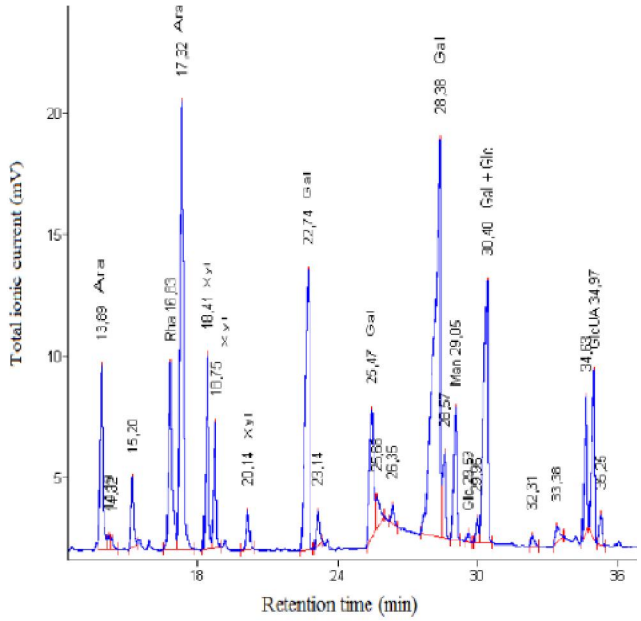

Figure 5 : Chromatogram showing detection of carbohydrates within *B. aegyptiaca* proteases. Analysis was performed using gas chromatography/flame ionization detection after aceton precipitation. Ara: Arabinose; Rha: Rhamnose; Xyl: Xylose; Gal: Galactose; Man: Mannose; Glc: Glucose; GlcUA: Glucuronic Acid.

CONCLUSION

The most important findings of this study can be resumed as follows:

- Proteases extracted from *B. aegyptiaca* possess both clotting and proteolytic activity.
- Regarding detection of glycans by lectins, a positive reaction has been observed when ConA, GNA and AAA used; whilst it was negative with PNA, WGA and DSA.
- Mannose and fucose are detected using labeled lectins;
- Acetone precipitation has a positive effect on the detection of glycans using GC/FID.
- Galactose, glucuronic acid, arabinose, rhamnose, xylose, mannose, and glucose are monosaccharides which were detected using GC/FID.

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ABBREVIATIONS

AAA: *Aleuria Auriantia* agglutinin, BCIP: 5-Bromo-4-chloro-3-Indolyl-Phosphate, ConA: *Concanavalin A* agglutinin *Concanavalin A* agglutinin, DSA: *Datura stramonium* agglutinin, FID: Flame Ionization Detection, FPLC: Flow Pressure Liquid Chromatography, GC: Gas Chromatography, GNA: *Galanthus nivalis* agglutinin, NBT: 4-Nitro bleu Tetrazolium Chloride, PNA: *Peanut* agglutinin, PVDF: polyvinylidene difluoride, SDS-PAGE: Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis, TBS: Tris Buffer Saline, TTBS: Tween Tris Buffer Saline, WGA: *Wheat Germ* Agglutinin.

REFERENCES

[1] L.B.Roseiro, M.Barbosa, J.M.Ames, A.Wilbey; Int.J.Dairy Technol., **56**, 76–85 **(2003)**.
[2] J.Costa, D.A.Ashford, M.Nimtz, I.Bento, C.Frazao, C.L.Esteves, C.J.Faro, J.Kervinen, E.Pires, P.Verissimo, A.Wlodawer, M.A.Carrondo; Eur.J.Biochem., **243**, 695–700 **(1997)**.
[3] R.Hardman, E.A.Sofowora; Planta Med., **20**, 124-130 **(1971)**.
[4] B.P.Chapagain, Z.Weisman; Phytochem.Anal., **18**, 354–362 **(2007)**.

[5] I.A.Mohamed Ahmed, I.Morishima, E.E.Babiker, N.Mori; Phytochem., **70**, 483–491 **(2009)**.

[6] R.G.Beka, V.D.Guiama, A.Delmont, P.Donn, M.-C.Slomianny, D.G.Libouga, C.M.Mbofung, D.Guillochon, D.Vercaigne-Marko; Int.J.Biol. Macrom., **49**, 397–401 **(2011)**.

[7] G.Sarath, R.S.Motte, F.W.Wagner; Protease assay methods, in R.J.Beynon, J.S.Bond (Ed); Proteolytic enzymes – A practical approach Oxford: IRL Press, 25–55 **(1989)**.

[8] U.K.Laemmli; Nat., **227**, 680-685 **(1970)**.

[9] W.N.Burnette; Anal.Biochem., **112**, 195-203 **(1981)**.

[10] J.P.Zanetta, P.Timmerman, Y.Leroy; Glycob., **3**, 255-266 **(1999)**.

[11] D.J.McMahon, R.J.Brown; Dairy Sci., **68**, 628-632 **(1985)**.

[12] S.Chazarra, L.Sidrach, D.Lopez-Molina, J.N.Rodriguez-Lopez; Int.Dairy J., **17**, 1393-1400 **(2007)**.

[13] V.Faid, G.Evjen, O.K.Tollersrud, J.C.Michalski, W.Morelle; Glycob., **16**, 440-461 **(2006)**.

[14] D.Ghosh, O.Krokhin, M.Antonovici, W.Ens, K.G.Standing, R.C.Beavis, J.A.Wilkins; J.Prot.Res., **3**, 841-850 **(2004)**.

[15] Z.Xu, X.Zhou, H.Lu, N.Wu, H.Zhao, L.Zhang, W.Zhang, Y.L.Liang, L.Wang, Y.Liu, P.Yang, X.Zha; Proteomics, **7**, 2358-2370 **(2007)**.

[16] N.Shibuya, I.J.Goldstein, E.J.Van Damme, W.J.Peumans; J.Biol.Chem., **263**, 728-734 **(1988a)**.

[17] E.J.M.Van Damme, A.K.Allen, W.J.Peumans; FEBS Let., **215**, 140-144 **(1987)**.

[18] H.Debray, J.Montreuil; Carbohyd.Res., **185**, 15-26 **(1989)**.

[19] N.Kochibe, K.Furukawa; Biochem., **19**, 2841-2846 **(1980)**.

[20] J.Montreuil; Adv.Carbohyd.Chem.Biochem., **37**, 157-223 **(1980)**.

[21] J.Montreuil; P.Appl.Chem., **56**, 859-877 **(1984a)**.

[22] A.M.Wu; Mol.Cel.Biochem., **61**, 131-141 **(1984)**.

[23] R.Lotan, N.Sharon; Meth.Enzymol., **50**, 361-367 **(1978)**.

[24] J.F.Crowley, I.J.Goldstein, J.Arnap, J.Lönngren; Arch.Biochem.Biophys., **231**, 524-533 **(1984)**.

[25] L.Sidrach, F.Garcia-Canovas, J.Tudela, J.N.Rodriguez-Lopez; Phytochem., **66**, 41–49 **(2005)**.

[26] G.N.Rudenskaya, A.M.Bogacheva, A.Preusser, A.V.Kuznetsova, Y.E.Dunaevsky, B.N.Golovkin, V.M.Stepanov; FEBS let., **473**, 237–240 **(1998)**.

[27] M.Pande, V.K.Dubey, S.C.Yadav, M.V.Jagannadham; J.Agric.Food Chem., **54**, 10141–10150 **(2006)**.

[28] S.C.Yadav, M.Pande, M.M.V.Jagannadhan; Phytochem., **67**, 1414–1426 **(2006)**.

[29] R.Tomar, R.Kumar, M.V.Jagannadham; J.Agric. Food Chem., **56**, 1479–1487 **(2008)**.