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Digestive amylase of a primitive animal, the scorpion: Purification and biochemical characterization

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ABSTRACT

Scorpion, one of the most ancient invertebrates was chosen, as a model of a primitive animal, to purify and characterize an amylase located in the hepatopancreas. The scorpion digestive amylase (SDA) was purified. Pure SDA was obtained after heat treatment followed by ammonium sulfate fractionation and three steps of chromatography. The pure amylase is not glycosylated and has a molecular mass of 59,101 Da determined by MALDI-TOF MS analysis. The maximal amylase activity was measured at pH 7.0 and 50 °C, in the presence of Ca^{2+} and using potato starch as substrate. The enzyme was able to hydrolyze also, glycogen and amylose. The 23 NH_2-terminal amino acid SDA residues were sequenced. The sequence obtained is similar to those of mammalian and avian pancreatic amylases. Nevertheless, polyclonal antibodies directed against SDA failed to recognize classical digestive amylases like the porcine pancreatic one.

1. Introduction

α-Amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) are widely distributed in plants, animal tissues and micro-organisms. They catalyze the hydrolysis of α-(1 → 4) glucosidic linkages of polysaccharides such as starch, glycogen and the related polysaccharides from different sizes of oligosaccharides.

The presence of an amylase has been described for many arthropoda including members of the subphylum of crustaceans (crab [1], shrimp [2,3] and lobsters [4]) and also in the subphylum of Hexapods such as insects [5–11]. However, relatively little information is available concerning the eventual presence and the properties of amylases in the subphylum of chelicerate and including the class of Arachnids. Scorpion, one of the ancient arachnids, was chosen in this work as a model of primitive animal to study the enzyme implicated in polysaccharides digestion.

Within the phylum of arthropods, scorpions are ancient chelicerates that have changed little since the Silurian (450 million years), and are considered as the oldest known terrestrial species. The food of scorpions is composed exclusively of a live arthropods, insects and myriapods. It is well known that scorpions can survive several weeks without food or water. During this time, glycogen, which is stored in high concentrations in the digestive glands and muscles, serves as a nutrient reserve [12]. The glycogen is broken down into glucose which is released into the blood so that even during extended starvation periods blood sugar levels remain nearly constant [13]. The scorpion digestive glands, which represent our starting tissue in this work, occupy most of the space in the preabdomen and are conspicuous, clumped together and cannot be distinguished as separate glands. The main part of digestion occurs in these glands (http://www.lander.edu/rsfox/310vaejovisLab.html). The scorpion digestive glands were studied at the ultrastructure level [14]. The digestive diverticula of a scorpion, *Androctonus australis*, are composed of two differentiated cells: basophilic cells and digestive cells. Whereas basophilic cells produce exoenzymes, digestive cells ensure intracellular digestion of nutrients absorbed by pinocytosis and store lipids, glycogen and mineral salts. The wastes of the digestive process are concentrated in brown body vacuoles, and then ejected into the lumen of the diverticulum. According to Goyffon and Martoja [14], the digestive mode of the scorpion associates a primitive intracellular process with an advanced extracellular one. The scorpion hepatopancreas consists of digestive diverticula and interstitial tissue. A digestive diverticulum is composed of two differentiated cell types: the secretory zymogene-like cells and the digestive cells which are the most abundant. The immunocytolocalization of the scorpion digestive lipase (SDL), which has been previously purified from scorpion hepatopancreas [15], indicates

Abbreviations: ELISA, enzyme linked immunosorbent assay; FPLC, fast protein liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SDA, scorpion digestive amylase; BLA, *Bacillus liqueniformis* amylase; BSA, bovine serum albumine; PPA, porcine pancreatic amylase; DNS, 3,5-dinitrosalicilic acid; EDTA, ethylene diamine tetra-acetic acid; G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose; HPLC, high presure liquid chromatography; kDa, kilodalton.

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that lipid digestion might occur in specific granules inside the digestive cells, as suggested by previous studies on the scorpion digestive process [16].

Many studies have attempted to purify serine proteinase-like [17], phospholipase [18] and many toxins from scorpion venom. However, in our knowledge, no digestives enzymes from the scorpion digestive glands have been purified so far except for the scorpion digestive lipase (SDL) which was the first enzyme purified and characterized from the scorpion hepatopancreas [15]. This paper reports the purification to homogeneity of an active amylase from the scorpion digestive glands. This amylase, tentatively named: scorpion digestive amylase (SDA) was characterized with respect to its biochemical properties.

2. Materials and methods

2.1. Animals

Scorpions (chelicerate, scorpionidae, *Scorpio maurus*) were collected alive from the area of Agareb (Sfax, Tunisia) and frozen until death.

2.2. Enzymes and oligosaccharides

Pure porcine pancreatic amylase (PPA) was purchased from Sigma and pure *Bacillus liqueniformis* amylase (BLA) was a generous gift, from Mr. Hmidet N. (Ecole Nationale d'Ingénieurs de Sfax (ENIS), Tunisia).

Oligosaccharides composed of glucose with a polymerisation degree varying from 2 (G 2; maltose) to 7 (G 7; maltoheptaose) were purchased from Sigma.

2.3. Delipidation of scorpion hepatopancreases

After defreezing, preabdomens were cleared from the cuticle and delipidated according to the method described previously [19]. After delipidation, 15 g of powder were obtained from 60 g of fresh tissue isolated from 200 scorpions.

2.4. Determination of protein concentration

Protein concentration was determined as described previously [20], using bovine serum albumin ($E^{1\%}_{1\,cm} = 6.7$) as reference.

2.5. Amylase activity determination

Amylase activity was measured with the dinitrosalicylic acid method using soluble starch as substrate [21]. The reaction mixture contained 0.5 ml of appropriately diluted enzyme and 0.5 ml of buffer A (100 mM MOPS pH 7,3 mM $CaCl_2$ and 10 mM NaCl) containing 0.5% (w/v) of soluble starch. The amylase activity was calculated using maltose solutions as reference. One amylase unit (U) is defined as the amount of amylase releasing 1 μmol reducing sugar as maltose per minute.

2.6. Purification steps of SDA

Delipidated powder (10 g) of scorpion hepatopancreases was suspended in 100 ml of buffer B (10 mM Tris–HCl pH 8, 10 mM NaCl and 3 mM $CaCl_2$). The mixture was stirred during 30 min at 4 °C, then centrifuged for 30 min at 12,000 rpm.

2.6.1. Heat treatment

The supernatant was incubated for 5 min at 55 °C. After rapid cooling, insoluble proteins were removed by centrifugation during 30 min at 12,000 rpm. The recovery of amylase activity in the treated crude extract was about 98% of the initial activity (Table 1). This step is important to eliminate proteases (data not shown).

2.6.2. Ammonium sulfate precipitation

The supernatant was subjected to ammonium sulfate fractioned precipitation. SDA precipitated in a saturation range of [50–80%] ammonium sulfate added under stirring conditions at 4 °C. After centrifugation (30 min at 12,000 rpm) the pellet was resuspended in 12 ml of buffer B. Insoluble material was removed by centrifugation for 10 min at 12,000 rpm.

2.6.3. Filtration on Sephadex G-100

The supernatant issued from ammonium sulfate precipitation, was loaded on a gel filtration Sephadex G-100 column (2.8 cm × 90 cm) equilibrated with buffer B. Elution of amylase was performed with the same buffer at 30 ml/h. The fractions containing the amylase activity (eluted at 1.2 void volume) were pooled (fractions from 86 to 104) (Fig. 1A).

2.6.4. FPLC anion exchange Mono-Q Sepharose

The pooled fractions of Sephadex G-100 column were concentrated and injected onto an FPLC Mono-Q Sepharose column equilibrated with buffer B. The column (1.6 cm × 15 cm) was rinsed with the same buffer. Then, proteins were eluted with a linear gradient of NaCl prepared in buffer B. SDA was eluted at a salt concentration of 50–70 mM NaCl (Fig. 1B).

2.6.5. Phenyl-sepharose chromatography

Active fractions eluted from Mono-Q Sepharose column were pooled and loaded onto a phenyl-sepharose column (1.6 cm × 6 cm) equilibrated with buffer B. Under these conditions, SDA was adsorbed onto the gel. After washing the column with buffer B, the enzyme was eluted by running a linear gradient of acetonitryl ranging from 0 to 20% prepared in buffer B. The amylase activity was not affected by the presence of acetonitryl (data not shown).

2.7. Chromatographic analysis of the starch or oligosaccharides hydrolysis products

The reaction mixture contained 10 U of amylase and 0.5% of soluble starch or oligosaccharides in buffer A at 50 °C. The starch hydrolysis products were analysed by HPLC method using an Aminex HPX-42A saccharide analysis column. Water was used as mobile phase at a flow rate of 0.3 ml/min. Eluted peaks were

Table 1
Flow sheet of SDA purification.

Purification step	Total activity[a] (units)	Protein (mg)	Specific activity (U/mg)	Activity recovery (%)	Purification factor
Extract of SDA (pH 8)	10,204 ± 563	1884.6 ± 367	5.41 ± 0.2	100	1
Heat treatment (55 °C)	10,057 ± 498	1509 ± 330.8	6.66 ± 0.23	98.6 ± 5.9	1.23 ± 0.09
$(NH_4)_2SO_4$ precipitation (50-80%)	5618 ± 75	150.5 ± 2.8	37.42 ± 1.89	55 ± 2.7	6.91 ± 0.32
Sephadex G-100 chromatography	3923 ± 104.4	73.3 ± 0.8	53.5 ± 5.65	38.4 ± 1.56	9.89 ± 0.28
Mono-Q-Sepharose chromatography	3276 ± 89.5	13.24 ± 0.47	247.43 ± 7.8	32.1 ± 0.78	45.73± 1.89
Phenyl-sepharose chromatography	1652 ± 94.34	1.18 ± 0.35	1400 ± 58	16.19 ± 0.24	258.79 ± 8.5

[a] 1 unit, 1 μmol of maltose released per min at 50 °C and pH 7 using soluble starch as substrate.

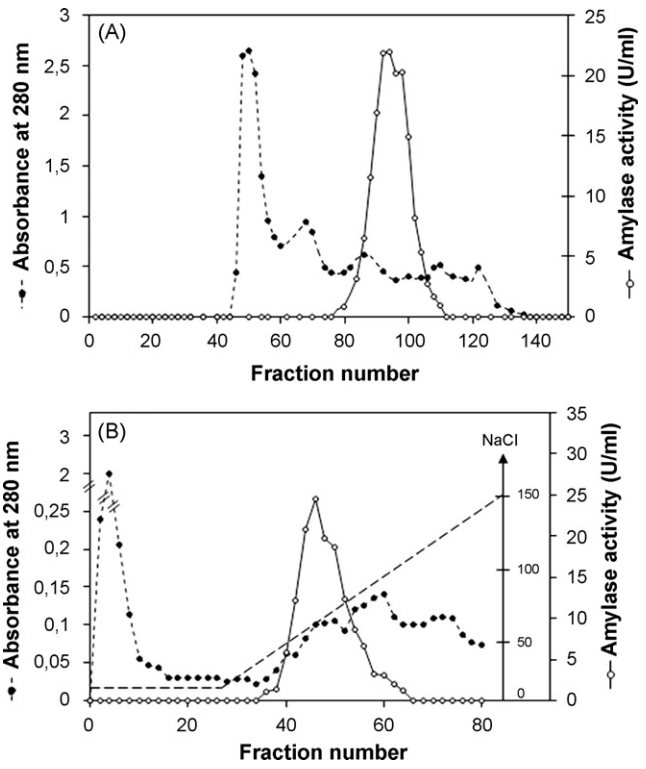

Fig. 1. (A) Chromatography of SDA on Sephadex G-100. The column (3.2 cm × 100 cm) was equilibrated with buffer B (10 mM Tris–HCl pH 8, 10 mM NaCl and 3 mM $CaCl_2$). The elution of amylase was performed with the same buffer at a flow rate of 30 ml/h and 4.6 ml by fraction. SDA activity was measured as described in Section 2 using potato soluble starch as substrate. Active fractions (86–104) were pooled. (B) Chromatography of SDA on FPLC Mono-Q Sepharose. The column was equilibrated with buffer B; a linear gradient was applied from 10 to 150 mM NaCl in buffer B; the elution of amylase was performed with the same buffer at a flow rate of 2 ml/min and 3 ml by fraction.

detected using a differential refractometric detector (Shimadzu). Furthermore, the starch or various oligosaccharides (from G 2 to G 7) hydrolysis products were subjected to a thin-layer chromatography (TLC) on silica gel 60 (Merck) using a solvent system composed of chloroform/acetic acid/water (60:70:10; v/v/v). The spots were visualized by spraying TLC plates with H_2SO_4/methanol (5:95; v/v) followed by a heating step at 105 °C for 10 min.

2.8. Zymogram

Zymogram for amylase activity was carried out on polyacrylamide native gel. After separation, the polyacrylamide gel was placed on the top of an agarose–starch gel and incubated for 10 min at 50 °C [22]. The agarose–starch matrix was stored in close iodine chamber and amylase activity was revealed by clear bands on a dark background.

2.9. SDS-PAGE, isoelectric focusing gel and Western blotting

Analytical polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed as described by Laemmli [23]. Isoelectric focusing gel was stained, using Coomassie brilinant blue in the same way as the SDS-PAGE gel after removing the ampholitic compounds. Samples for sequencing or immunoblotting were electrotransferred onto polyvinylidene difluorid and a nitrocellulose membrane respectively. Protein transfer was performed during 2 h at 4 mA/cm^2 at room temperature.

2.10. MALDI-TOF mass spectrometry analysis

The molecular mass of the purified SDA was determined by matrix-assisted laser desorption ionization time-of-flight analysis (Applied Biosystems, Foster City, CA, USA) in positive ion linear mode with a delayed extraction. A solution of sinapinic acid in water and acetonitrile (40:60, v/v) was used as a matrix. One microliter of enzyme solution was mixed on the plate target with an equal volume of the above matrix, and the spot was allowed to air-dry. Ions were accelerated with an extraction voltage of 25 kV. Spectra were obtained by performing 256 successive laser shots. It is worth noting that when proteins are being studied, an error of less than 0.5% can be expected to occur with MALDI-TOF analysis.

2.11. Amino acid sequencing

The NH_2-terminal end of scorpion digestive amylase (SDA) was sequenced by automated Edman's degradation, using an Applied Biosystems Protein Sequencer Procise 492 cLC [24].

2.12. Oligosaccharides content

The glycan chains content of the purified SDA was estimated by anthrone-sulfuric acid method using glucose as a standard [25].

2.13. Production of polyclonal antibodies

Polyclonal antibodies directed against purified SDA were produced on rabbits after subcutaneous and intra-muscular injections every 3 weeks of 0.5 mg of pure amylase. The first injection included complete Freund's adjuvant, while the last two injections contained incomplete adjuvant.

2.14. Immunoblotting technique

The reactivity of anti-SDA serum with amylases (SDA, PPA or BLA) was checked using immunoblotting technique. After protein transfer, membranes were rinsed three times with PBS (phosphate buffer saline: 10 mM phosphate pH 7.2, 150 mM NaCl), then saturated with 3% of milk powder in PBS (saturating buffer) for 1 h at room temperature. Thereafter, anti-SDA serum diluted at 1:1000 with PBS containing 0.05% Tween-20 (PBS/Tween-20) were incubated with the membranes for 1 h at room temperature. Afterwards, membranes were washed three times with PBS/Tween-20 then incubated for 1 h at room temperature with a 1:2000 dilution of alkaline phosphatase-conjugated anti-rabbit immunoglobulin (Sigma). After washing as mentioned above, membranes were incubated with a phosphatase substrate solution containing 0.3 mg/ml of nitroblue tetrazolium chloride (Sigma), 0.2 mg/ml of 5-bromo-4-chloro-3 indolyl-phosphate (Sigma) and 0.2 mg/ml of $MgCl_2$ to reveal the specific immunoreactivity.

2.15. Enzyme linked immunosorbent assay (ELISA) analysis

The immunoreactivity of anti-SDA polyclonal antibodies with amylases (SDA, PPA or BLA) was checked, using the ELISA technique. Purified amylases (SDA, PPA or BLA) were diluted using coating buffer (PBS) to obtain a final concentration of 1 μg/ml. Aliquots (100 μl) were coated onto polyvinyl chloride microtiter wells and incubated overnight at 4 °C. The wells were then saturated by adding 100 μl of saturating buffer (3% of powder milk in PBS) for 2 h at 37 °C. Thereafter, 100 μl of serum, diluted at 1:500 with saturating buffer, were added to each well and the plates were incubated for 1 h at 37 °C. Afterwards, 100 μl of peroxidase-conjugated anti-rabbit immunoglobulin (Sigma) diluted at 1:2000 with saturating buffer were added to each well and the plates were kept at 37 °C

for an additional hour. Then, 100 μl of freshly prepared peroxidase substrate solution (an o-phenylenediamine tablet (Sigma) was solubilized in 50 mM sodium phosphate/citrate, pH 5 containing 0.4% of fresh hydrogen peroxide) were added to each well. The plates were incubated in the dark for 30 min at room temperature. The enzymatic reaction was then stopped by adding 50 μl of 0.5 M H_2SO_4. The absorbance was read at 490 nm in a micro-ELISA reader (Dynatech).

3. Results and discussion

3.1. Purification of SDA, and NH_2-terminal sequence determination

SDA was purified according to the procedure described in Section 2. The purification flow sheet is shown in Table 1. After phenyl-sepharose chromatography, the purification factor reached 259 with a recovery yield of 16% of the initial amylase activity. At this stage, the SDA specific activity was found to be 1400 U/mg using soluble starch as substrate at 50 °C and pH 7.

After hydrophobic chromatography, the fractions containing SDA were pooled and analysed on SDS-PAGE. Fig. 2A shows that after the last step of chromatography, the enzyme appears as one band having an apparent molecular mass of 59 kDa. This value is in agreement with that determined by MALDI-TOF analysis 59,101 Da (data not shown).

The activity staining of purified enzyme under native conditions given in Fig. 2B revealed a clear zone of amylase activity indicating that the purified protein corresponds to the scorpion amylase.

Amylases molecular masses are generally in the range of 45–67 kDa (Table 2). However, SDA has a smaller molecular mass than that recorded for tick α-amylase [7]. This molecular mass is however higher than that of *Morimus funereus* larvae α-amylase (33 kDA [10]) or *Paneiuss vannamei* (30 kDa [26]). The isoelectric point of the SDA was 7.0 as determined by isoelectric focusing (data not shown).

The NH_2-terminal sequencing of native SDA allowed the unambiguous identification of 26 residues. It is noteworthy that the sequencing of the transferred SDA band gave the same aminoacids sequence (Table 3). The alignment of SDA sequence with those of ostrich [27], human [28], porcine [29] and shrimp [30] amylases is shown in Table 2. SDA NH_2-terminal sequence exhibits more than 54% identity with those of mammalian, bird or crustacean amylases.

3.2. General characteristics

3.2.1. Substrate specificity

Based on their mode of action [31], amylases can be classified into various groups: α-amylases, exoamylases (β-amylase, glucoamylase) and debranching enzymes (pullulanase). To establish the specificity of the purified SDA toward some substrates, the enzyme was incubated, under the same conditions, with various substrates as shown in Table 4. Except for pullulan, SDA hydrolysed efficiently all tested substrates (starch, amylose, amylopectine

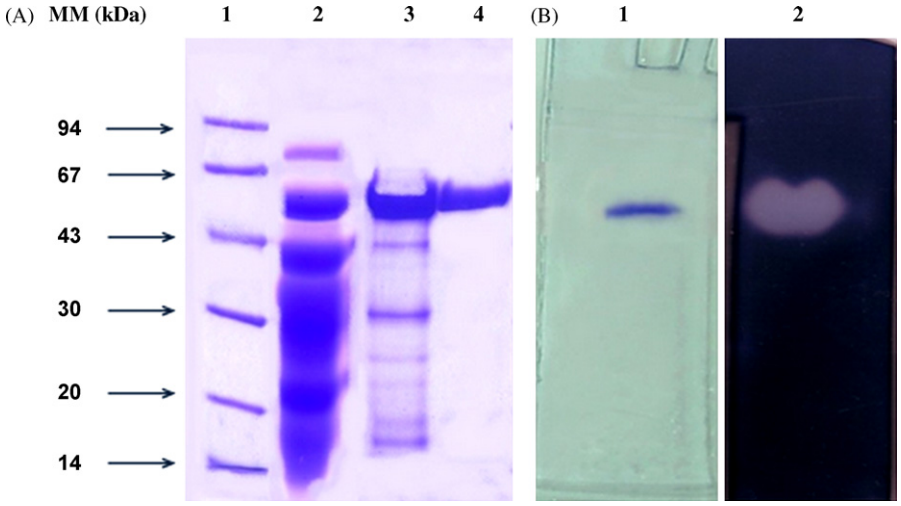

Fig. 2. (A) Analysis of purified SDA by SDS-PAGE (10%). Lane 1, molecular mass markers (Pharmacia); lane 2, SDA solution (100 μg) obtained after Sephadex G-100 chromatography; lane 3, SDA solution (20 μg) obtained after Mono-Q Sepharose chromatography; lane 4, purified SDA (10 μg). The gel was stained with Coomassie blue. (B) Native-PAGE of the purified enzyme, protein stained by Coomassie blue (lane 1); zymogram of the purified α-amylase SDA (lane 2).

Table 2
Properties of purified digestives amylase from different invertebrates.

Origin	Subphylum class	Molecular mass (kDa)	Optimum pH (activity–stability)	Optimum T° (activity–stability)	Metal ion requirement	References
Scorpion *S. maurus*	Chelicerata arachnida	58	7, 6–11	50 °C, 50 °C	$CaCl_2$	This study
Snail *A. glabratus*	Mollusca	ND	6.5, 5–10	37 °C	ND	[34]
Scallop *P. maximus*	Bivalvia	50	ND	ND	ND	[4]
H. bacteriophora	Nematode	46	7	40 °C, up to 40 °C	$CaCl_2$, NaCl	[32]
Erthworm *E. foetida*	Annelida	60	5.5; 7–9	50 °C, 50–60 °C	$CaCl_2$	[11]
P. vannamei	Crustacean decapoda	30	7.6	37	ND	[26]
Silkworm *A. mylitta*	Hexapoda insect	58	9.5	45 °C, 45–60 °C	$CaCl_2$	[6]
Camel tick *H. dromedarii*	Hexapoda insect	106	7	40 °C, up to 50 °C	$CaCl_2$	[7]
Large grain borer *P. truncatus*	Hexapoda insect	60.2	6	30 °C, 40 °C	ND	[8]
M. funereus	Hexapoda insect	33	5.2	45 °C, 45–60 °C	$CaCl_2$, NaCl	[10]

ND: not determined.

Table 3
Alignment of the NH_2-terminal sequence of scorpion digestive amylase with those of ostrich [27], human [28], porcine [29] and shrimp [2] amylases. Residues in bold indicate the identical amino acids.

Amylase origin	NH_2-terminal sequence 1 5 10 15 20 25	Identity (%)	Reference
Scorpion digestive glands	SFHE**PNT**VA**GR**SVL**VHLFEWRW**KD**IA**	–	This study
Ostrich pancreas	$^{-1}$QYN**PNT**QP**GR**TSI**VHLFEWRW**AD**IA**	61	[27]
Human pancreas	^{1}AQYS**PNT**QQ**GR**TSI**VHLFEWRW**VD**IA**	54	[28]
Porcine pancreas	$^{-1}$QYA**P**Q**T**QS**GR**TSI**VHLFEWRW**AV**IA**	54	[29]
Shrimp digestive glands	$^{-1}$QWD**PN**SSN**G**QV-I**VHLFEW**K**WSDIA**	54	[2]

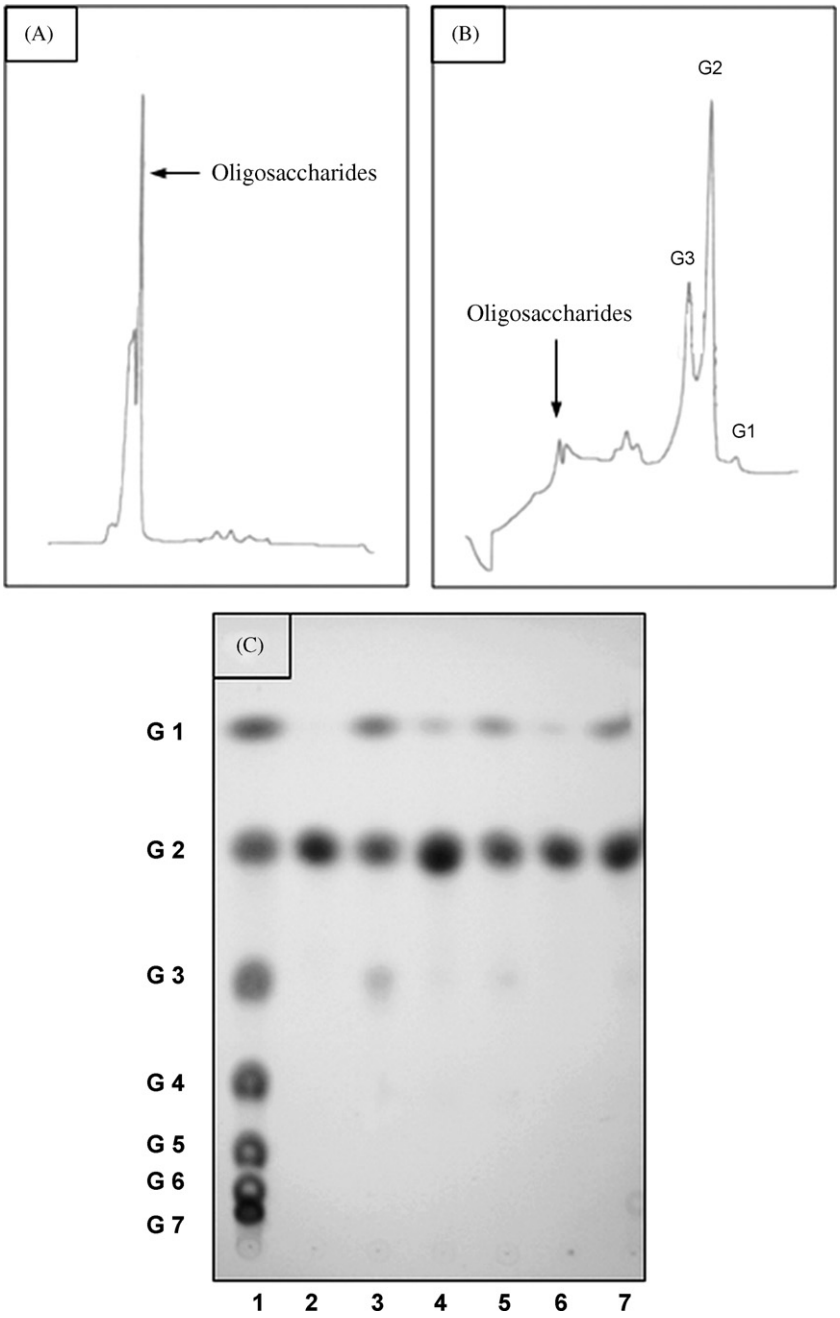

Fig. 3. High performance liquid chromatography of starch hydrolysate produced by the purified amylase SDA. (A) Soluble starch (1%), (B) soluble starch (1%) after incubation with purified SDA (10 U) at 50 °C for 60 min and (C) thin-layer chromatography analysis of the main products from hydrolysis of maltooligosaccharides (G2–G7) by the purified SDA. The reaction mixture contained 10 U of amylase enzyme and 1% substrate in 0.1 M MOPS buffer (pH 7) at 50 °C. Lane 1: a mixture of maltooligosaccharides containing glucose (G 1) was used as standards. Lanes 2–7 represent the products after 1 h incubation as reacted with maltose (G 2) (lane 2), maltotriose (G 3) (line 3), maltotetraose (G 4) (lane 4), maltopentaose (G 5) (lane 5), maltohexaose (G 6) (lane 6) and maltoheptaose (G 7) (lane 7).

Table 4
Substrate specificity of the purified SDA and PPA.

Substrates (1%)	Main linkage/monomer	Specific activity (U/mg)	
		SDA	PPA
Starch	α(1 → 4)/α(1 → 6) glucose	1357	1810
Amylose	α(1 → 4) glucose	1065	1661
Amylopectine	α(1 → 4)/α(1 → 6) glucose	934	1263
Glycogen	α(1 → 4)/α(1 → 6) glucose	434	630
Pullulan	α(1 → 6) glucose	0	0

or glucogen). The enzyme is unable to attack the α-(1 → 6) glucosidic linkage. The kinetic of hydrolysis remains linear for more than 20 min (data not shown). The SDA maximal activity was recorded on starch, followed by amylose, amylopectine and glycogen. PPA, taken as a model of pancreatic α-amylase, hydrolyses at comparable levels the same substrates (Table 4).

To confirm the action mode of SDA, potato soluble starch was hydrolyzed at various time durations, and the products were analyzed by HPLC column. After 15 min of hydrolysis, the sugars produced were maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5) and maltooligosaccharides of higher polymerization degrees (data not shown). Nevertheless, the products analysis of 1 h hydrolysis reaction showed essentially an accumulation of G2 with small amounts of glucose (G1), G3 or G4 (Fig. 3A and B). Furthermore, when using various intermediate oligosaccharides of starch hydrolysate (from G2 to G7) as substrates, maltose remained the major hydrolysis product with a minor amount of glucose (Fig. 3C). It can be concluded that scorpion digestive amylase SDA is an endoamylase presenting the same behaviour than an α-amylase.

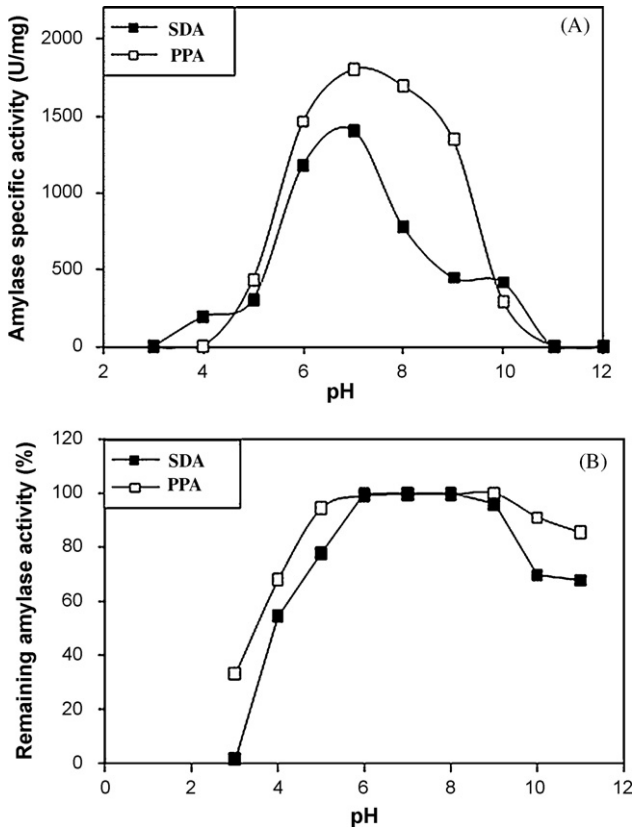

Fig. 4. Effects of pH on SDA or PPA activity (A) and stability (B). The enzyme activity was tested at various pH using soluble starch as substrate in the presence of 3 mM $CaCl_2$ at 50 °C. The pH stability of the amylase was determined by incubating the enzyme in different buffers for 1 h at 4 °C and the residual activity was measured at pH 7 and 50 °C. The activity of the enzyme before incubation was taken as 100%.

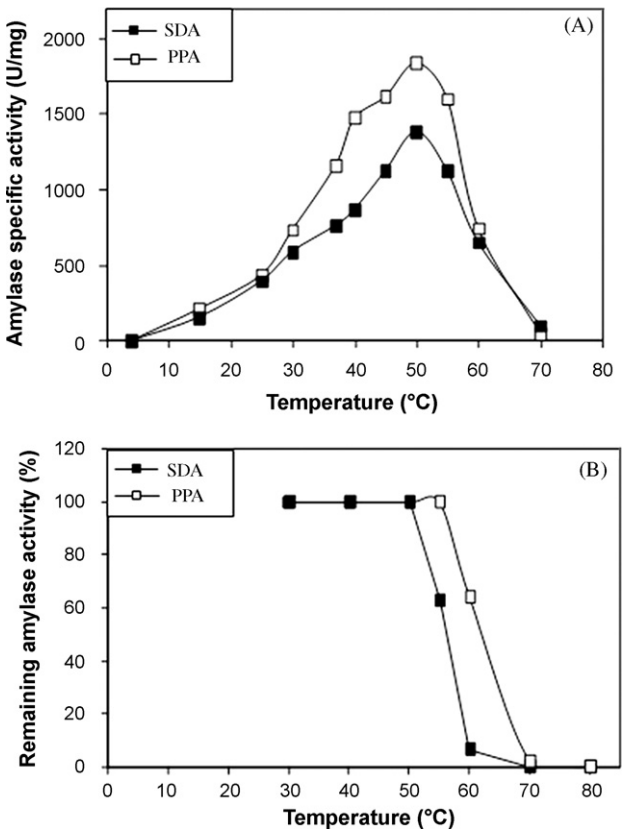

Fig. 5. Effects of temperature on SDA or PPA activity (A) and stability (B). The enzyme activity was tested at various temperatures using soluble starch as substrate at pH 7 in the presence of 3 mM $CaCl_2$. Residual enzyme activity was determined from 0 to 15 min. The initial activity before pre-incubation was taken as 100%.

3.2.2. *Effect of pH, temperature and metal ions on SDA activity*

The effect of pH and temperature on the activity and stability of the purified SDA was examined and compared to that on the activity of a pancreatic model (PPA) using soluble potato starch as substrate. Our results showed that maximal SDA activity was measured at pH 7 (Fig. 4A). The enzyme was stable in a pH range of 6–11 at 4 °C for 1 h. Similar results were obtained with PPA which is stable in a

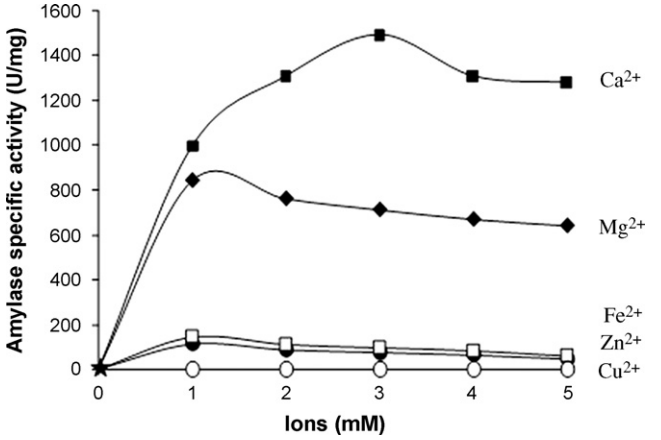

Fig. 6. Effect of increasing Ca^{2+} (■), Mg^{2+} (♦), Zn^{2+} (□), Fe^{2+} (●) or Cu^{2+} (○) ions concentrations on the SDA activity using starch as substrate, (⋆) indicates the value of amylase activity measured in presence of 5 mM EDTA. SDA activity was measured at 50 °C and pH 7.

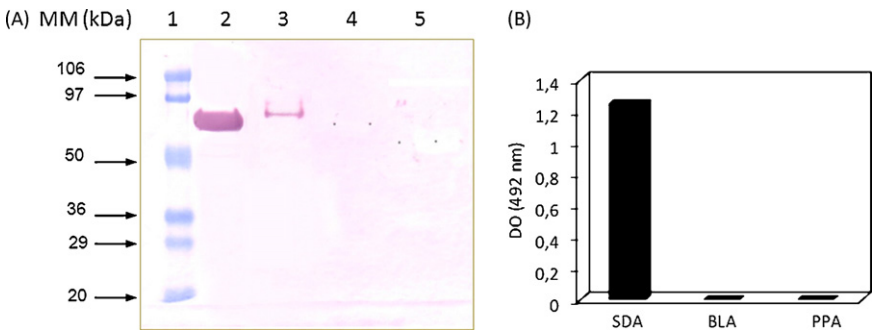

Fig. 7. (A) Immunoblot analysis, molecular mass markers (Bio-Rad) (lane 1), pure SDA (10 μg) (lane 2), SDA solution (200 μg) (lane 3), BLA (50 μg) (lane 4) and PPA (50 μg) (lane 5) using anti-SDA serum at 1:1000 dilution. (B) Immunocross-reactivity of SDA, BLA, and PPA with anti-SDA serum using a simple sandwich ELISA. Each experiment was performed by direct coating of one amylase (100 ng/well) with a fixed dilution of 1:500 for the serum tested.

pH range of 5–11 at 4 °C for 1 h and showing an optimal activity at pH 7 (Fig. 4B). The optimum pH values of SDA and PPA were also similar to those of *Heterorhabditis bacteriophora* [32] and Camel tick *Hyalomma dromedarii* [7] α-amylases (Table 2).

The effect of the temperature on SDA and PPA activities were also checked. As shown in Fig. 5A, the SDA activity increased with the temperature to reach an optimum at 50 °C and decreased sharply at higher temperatures. However, the enzyme activity was rapidly lost after 15 min incubation at 60 °C (Fig. 5B). Similar results were obtained for PPA (Fig. 5A). The enzyme remains stable up to 50–60 °C during an incubation of 15 min (Fig. 5B). The maximal PPA activity was measured at 50 °C (Fig. 5A). One can say that SDA and PPA are moderately thermostable α-amylases as those isolated from various insects (Table 2).

The effect of various metal ions on SDA activity was also investigated. Our results showed that no SDA activity can be detected in the presence of 5 mM of ions chelator such as EDTA (Fig. 6). To identify the required ion, increasing concentrations of various ions (Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+} or Zn^{2+}) were added to the reaction medium. The addition of Fe^{2+}, Zn^{2+} or Cu^{2+} ions failed to trigger significantly the SDA activity. The SDA activity was reached its maximal activity in the presence of 3 mM Ca^{2+}. It can be claimed that the presence of Ca^{2+} is mandatory to detect the enzyme activity [33].

3.2.3. Immunochemical properties

The supernatant of the scorpion hepatopancreas homogenate containing 200 μg of total proteins was subjected to SDS-PAGE analyses followed by immunoblotting using anti-SDA serum. Our results showed that anti-SDA serum reacts with a single band (59 kDa) corresponding to the SDA present in the crude extract (Fig. 7A, lane 3). No other protein bands were recognized by this serum. This result suggests a good specificity of this serum toward SDA. Anti-SDA serum was used to carry out cross-reactivities between SDA, PPA and BLA using the ELISA and the Western blotting techniques. SDA was strongly recognized by anti-SDA serum and no cross-immunoreactivity was detected with pancreatic or bacillus amylases (Fig. 7A). For the ELISA technique used for the sake of better sensibility, microtitration plates were coated with a fixed amount of pure amylases and incubated with an antiserum diluted 50 times. Only SDA reacted strongly with its corresponding antiserum (Fig. 5B). These results might be explained by the fact that SDA may not share common antigenic determinants with classical pancreatic amylases. Although the 26 residues of SDA NH_2-terminal end showed significant homology with those of pancreatic amylases, the absence of immunoreactivity between PPA and anti-SDA serum strengthen the idea that SDA could be structurally different from mammalian pancreatic amylases. This hypothesis needs further structural and biochemical investigations.

4. Conclusion

In this paper we report for the first time in our knowledge the purification of an amylase from a primitive chelicerate, the scorpion. The enzyme was purified to homogeneity from delipidated hepatopancreas. SDA has a molecular mass of 59,101 Da and it was not glycosylated. The maximal SDA activity was measured at pH 7 and at 50 °C. The enzyme remained stable up to 60 °C of incubation during 15 min and its thermostability can be enhanced by the presence of Ca^{2+}. The SDA NH_2-terminal sequence exhibits more than 54% identity with those of mammalian, bird and crustacean amylases. Our results indicate that, despite the primitive character of the scorpion, similar properties have been observed between SDA and known pancreatic amylases. However, the absence of cross-immunoreactivity between porcine pancreatic amylase and anti-SDA serum strengthens the idea that SDA could be structurally different from mammalian pancreatic amylases. Further investigations are needed to better establish the structure–function relationship of this class of enzyme.

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