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RESEARCH ARTICLE

PURIFICATION AND PARTIAL CHARACTERIZATION OF CINNAMYL ALCOHOL DEHYDROGENASE (CAD) IN *STRIGA HERMONTHICA* (DEL.) BENTH

[1,2*]Rida DRAIE

[1]Faculty of Agriculture, Idleb University, Syria
[2]Research Work Carried out at LBPV Laboratory, University of Nantes, France

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ABSTRACT

Mannitol is a major compound related to carbon metabolism in the parasitic plant *Striga hermonthica* despite Mannitol 6-Phosphate Reductase (M6PR) that is the key enzyme of mannitol biosynthesis in plants is likely absent in the parasite. Previous studies suggest that M6PR activity is driven by a Cinnamyl Alcohol Dehydrogenase (CAD) in striga. This work aims to characterize the CAD isoforms of the parasite and to check their activity as M6PR. CAD is purified and characterized from the leaves that display simultaneously high M6PR and CAD activities, using a typical protocol for CAD purification from plants. At least two CAD isoforms, called CADa and CADb, is shown in the leaves, CADa being the major isoform. Only one band characterizes both CADa and CADb in SDS-PAGE, at 38 kDa and 40 kDa respectively. Native isoforms display CAD activity in native gels. CADa has a relatively low affinity for cinnamyl alcohol (K_m = 400 µM) and a high stability to heat. Finally, both CADa and CADb do not display M6PR activity and the latter remains unidentified in the protein fraction that does not interact with the 2' 5' ADP-Sepharose affinity gel.

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INTRODUCTION

Considered as the most serious risk in the semi-arid areas, the plant hemi-parasite *Striga hermonthica* (Del.) Benth. (*Scrophulariaceae*) attacks the roots of the food crops whose sorghum, millet and corn and absorbs the nutritive substances (Musselmann and Press, 1995). Because of striga, fields put in fallow even of the whole villages were abandoned in several areas of Africa (Thalouarn and Fer, 1993). It is difficult to prevent and control the expansion of this parasite because of the production of a great number of seeds, perfectly adapted to the climatic conditions met in the arid or semi-arid tropical zones. These seeds are dispersed easily by the wind or the animals (Molau, 1995), and can remain viable in dormancy in the soil several tens of years (Riches and Parker, 1995). Once the seeds of striga are preconditioned, they germinate in the presence of the chemical stimulant exuded by the host's roots.
Different strategies of control are currently proposed, based on the exploitation of trap crops, of predatory specific of striga, herbicides and/or nitrogen fertilizers in the most touched areas (Rousset, 2003). The trap crops emit germination stimulants but not being host cultures, they induce the germination suicide of striga.

Corresponding author: **[1,2*]Rida DRAIE**
[1]Faculty of Agriculture, Idleb University, Syria
[2]Research Work Carried out at LBPV Laboratory, University of Nantes, France

These different strategies aim to reduce the grainer stock in the soil. The impact of striga on the yield is less on tolerant cultivars. The exploitation of these cultivars, preferred a long time, is one of the reasons of the uncontrolled expansion of this parasite. An important effort is currently carried to the search for resistant cultivars and the characterization of the cellular, molecular and biochemical parameters of resistance. This resistance rests primarily on the non-production of germination stimulants. The production of mannitol is a metabolic particularity of striga. This way is effectively absent at the host plants and constitutes of this fact a biochemical target within the framework of a selective chemical control. The mannitol occupies a central place in the physiology and the metabolism of the parasite, not only as an major agent osmotic and compatible aqueous solution (Nour and Todd, 1984; Stewart *et al*., 1984), but also as a principal form of reduction (Simier *et al*., 1998) and of transport to long distance (Fer *et al*., 1994). Few autotrophic plants as celery are producing and accumulating mannitol, the great majority being typically accumulating sucrose and starch. The biosynthetic pathway of the mannitol was for the first time characterized at celery (Rumpho *et al*., 1983; Loescher *et al*., 1992; Everard *et al*., 1994; Everard *et al*., 1997) and implies three cytosolic enzymes (Figure 1) of which the mannose 6-phosphate reductase NADPH-dependent (M6PR, EC. 1.1.1.224).

This enzyme catalyses the NADPH-dependent reduction of the mannose 6-phosphate (M6P) in mannitol 1-phosphate (M1P) and would be the specific enzyme and limiting synthesis of mannitol at the plants. It seems indeed absent at the bacteria, the algae, and mannitol producing fungus, like at the nonproducing plants of the mannitol. The M6PR was characterized at the celery and some other producing autotrophic plants of the hexitol (Loescher *et al*., 1992), and at the parasitic plant *Phelipanche ramosa*, at which the mannitol also occupies a place major (Robert *et al*., 1999a; Delavault *et al*., 2002). As the M6PR misses at the principal host plants of this parasite (Pacaud, 2004), its inactivation by specific inhibitors could constitute the bases of a new strategy of selective chemical control against the broomrape and the striga.

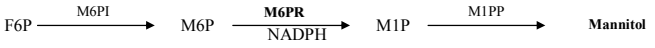

Figure 1. Mannitol production pathway at the celery and the broomrape. F6P: (fructose 6-phosphate); M6PI (mannose 6-phosphate isomerase); M6P (mannose 6-phosphate); M6PR (mannose 6-phosphate reductase); M1P (mannitol 1-phosphate); M1PP (mannitol 1-phosphate phosphatase)

Concerning striga, all the trials to identification of a gene *M6PR* and a protein M6PR are useless to date (Pacaud, 2004). Nevertheless, an activity of the type M6PR is easily detectable at this parasite. Work of Robert *et al*., (1999a) in particular revealed a protein with activity M6PR on non-denaturing gel. The corresponding band of about 35 kDa in SDS-PAGE was taken and analyzed by micro-sequencing in LC-MS/MS (Pacaud, 2004). No peptidic sequence obtained is homologous with a sequence of M6PR (celery, broomrape in reference). Contrary, many peptides have a homology with sequences of cinnamyl-alcohol dehydrogenase (CAD, EC. 1.1.1.195) and of CAD-like. As the M6PR, the CAD are cytosolic enzymes NADP(H)-dependent (Samaj *et al*., 1998). They are also characterized by their low specificity for their substrates. In addition, Pacaud (2004) showed the inhibition of activity CAD of striga by diphenyl-phosphate, an inhibitor specific of the M6PR of broomrape and celery and protein to activity M6PR of striga (Figure 2). Some aromatic compounds prove to be effectively powerful competitive inhibitors of the M6PR (to the mannose 6-phosphate) (Rousset, 2003), which underline the strong affinity of the M6PR to apolar compounds. The whole of these results suggests a structural similarity of the catalytic site of the CAD and protein with activity M6PR and led us to suggest the existence at striga of a "bifunctional" CAD presenting an activity M6PR.

Figure 2. Structure of the inhibitors of the CAD and the M6PR. a-Inhibitors of CAD (Kennedy *et al*., 1999), (R^1 =H or OCH_3; R^2=H or OCH_3; R^3=OH or OCH_3; R=OC_2H_5 or OH or $NHCH_2Ph$). b-Inhibitors of the M6PR of broomrape, Diphenyl phosphates (DPP), (Robert *et al*., 1999b; Rousset, 2003).

The CADs are implied in the lignification, which is an essential process in control of the cellular extension (Boudet, 2000), the port of the organs (Monties, 1989), the conduction of the crude sap (Northcote, 1989), and the defense of the plants against different pathogens (Vance *et al*., 1980; Whetten and Sederoff, 1995). Lignins are aromatic complex polymers derived from phenylalanine and represent for 30% of the organic carbon of plant biomass (Goffner *et al*., 1992; Hawkins and Boudet, 1994). These molecules are produced by long chain reactions constituting two principal stages. The first stage corresponds to the pathway of phenylpropanoids biosynthesis, which leads to the production of the cinnamoyl-CoA starting from phenylalanine. This pathway is common at the plants and conduits to different products of which flavonoids and coumarins. The second stage is specific to the production of the monolignols, the precursors monomeric of lignins (Figure 3). In this pathway, the cinnamoyl CoA reductase (CCR; EC. 1.2.1.44) reduces the cinnamoyl-CoA in cinnamaldehyde which is then reduced in corresponding alcohol (the monolignol) by a CAD. From its site at the end of the pathway of the monolignols, the CAD is considered as the biosynthetic indicator of lignins (Walter *et al*., 1988). Nevertheless, the CAD is also expressed in nonproducing cells of lignins (O'Malley *et al*., 1992; Grima-Pettenati *et al*., 1994), and it is induced in response to stress (Galliano *et al*., 1993), to pathogenic elicitors (Campbell and Ellis, 1992) and to wounds. The CAD is thus controlled by developmental and environmental factors and thus seems to be implied in processes other than lignification (Whetten and Sederoff, 1995). For example, the monolignols are also used for lignans biosynthesis (Lewis and Yamamoto, 1990) and of surface polymers (suberins and cutins), (Kolattukudy, 1987). These compounds are implied in the defense of the plants and do not intervene in the port of the organs, nor in the conduction of the crude sap ((Bostock and Stermer, 1989).

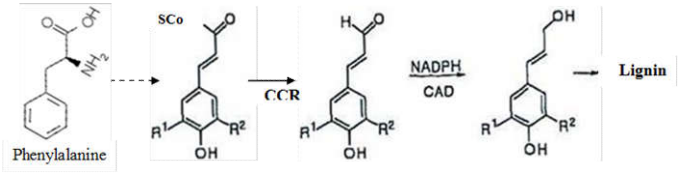

Figure 3. Structure of the substrates of cinnamyl alcohol dehydrogenase CAD. R1=R2=H (4-coumaroyl CoA, 4-coumaraldhyde, 4-coumaryl alcohol); R1=H, R2=OCH3 (feruloyl CoA, coniferaldehyde, coniferyl alcohol); R1=R2=OCH3 (sinapoyl CoA, sinapaldehyde, sinapyl alcohol); CCR: cinnamoyl-CoA reductase, (Lüderitz and Grisebach, 1981)

Actually, the CAD is known as a multigenic family. Many *CAD-like genes* were effectively identified at the plants by their homology with genes *CAD* of tobacco and pine (Kim *et al*., 2004). Thus, several isoforms of the CAD, with a different affinity to their substrates aldehydes, was characterized at many Angiosperms, such as wheat (Mitchell *et al*., 1994), eucalyptus (Hawkins and Boudet, 1994), poplar (Savidge, 1989), tobacco (Halpin *et al*., 1992), bean (Grima-Pettenati *et al*., 1994), willow (Mansell *et al*., 1976) and soya (Wyrambik and Grisebach, 1975), but also at of Gymnosperms like the pine (O'Malley *et al*., 1992) and the spruce (Lüderitz and Grisebach, 1981). More recently, the protein recombining of each of 9 genes *cad* of *A. thaliana* was characterized (Kim *et al*., 2004). This work showed that these isoforms CAD were different clearly by their activities and their affinities to the different substrates aldehydes. Thus, some isoforms present a very feeble activity, which suggests their implication relatively limited in the production of lignins. The analysis of specific mutants of *A. thaliana* will certainly help to understand the metabolic and physiological significance of these CAD.

In this work, we will purify and characterize the CAD(s) of striga by the technique of chromatography. The final objective being to analyze the activity and the affinity of the CAD purified to the mannose 6-phosphate, the natural substrate of the M6PR, and to justify the bifunctionality of the CAD as a CAD and a M6PR.

MATERIALS & METHODS

Vegetable Materials

The seeds of *Striga hermonthica* Del. Benth (*Scrophulariaceae*) were collected in Sudan (1995) in fields of infested sorghum. The parasite is cultivated at the laboratory on a susceptible variety of sorghum (*Sorghum bicolor* L., var. SH4 Arval, *Poaceae*). The seeds of the parasite are sown in a pot of culture (10 cm^3) in a mixture of soil and sand 50/50 (v/v) and are maintained for one week with the darkness, at 25°C (wet soil). After this phase of preconditioning, the sorghum is sown at a rate of a caryopse per pot. The cultures are maintained in greenhouse with 20-25°C (day-night) under 14 hours of photoperiod (300 µmoles m^{-2} S^{-1} "photosyntetically activates radiation", PAR). The cultures are sprinkled three times per weeks. The different organs of the parasite are taken four weeks after emergence of the parasite that is to say approximately 10 weeks after the sowing of the sorghum.

Extraction and purification of striga's CAD

Extraction

The vegetable material (100 g fresh matter FM) is crushed with ultra-turrax at 4°C in cold extraction buffer (ratio 1/10 w/v) composed of 100 mM Tris-HCl (pH 7.5) and of 15 mM β–mercaptoethanol, in the presence of 80% of polyvinylpolypyrrolidone (PVPP). Crude extract is filtered through two layers of Miracloth, centrifuged to 12000*g* during 10 min at 4°C.

Proteins precipitation in ammonium sulfate

The crude extract non-desalted is saturated to 40% by addition of ammonium sulfate (226 g L^{-1}) then incubation under slow agitation at 4°C for 1h. The precipitated extract is centrifuged to 12000*g* during 10 min at 4°C. The pellet is resuspended in buffer A (20 mM Tris-HCl, pH 7.5 containing 5 % (v/v) ethylene glycol and 5 mM DTT), then desalted onto PD-10 column pre-equilibrated in this same buffer. The supernatant is fractioned sequentially by successive addition of ammonium sulfate. Different fractions are thus collected: 40-50%, 50-60%, 60-70%, 70-80% and > 80%. Each pellet is resuspended in buffer A then desalted onto PD-10 column pre-equilibrated in this same buffer. Enzymatic activities of CAD and M6PR are measured in each one of these desalted fractions. The fractions presenting an activity CAD and M6PR are combined for later chromatography analysis (BIO-RAD BioLogic LP chromatography System).

NAD(P) Affinity chromatography

The desalted proteomic fraction is loaded at a flow of 0.5 mL min^{-1} onto a HiTrap™ Blue HP column (5 mL, Amersham Biosciences) pre-equilibrated in buffer A. The column is washed at 1.5 mL min^{-1} with 5 volumes of buffer A then a volume of buffer A containing 4 mM NAD. The NADP-dependent enzymes (of which the CAD) are then specifically eluted by a gradient from 0-4 mM $NADP^+$ in buffer A (1.5 mL min^{-1}, 4 volumes of the column) and are collected by fraction of 2 mL. These fractions are desalted and concentrated simultaneously by ultrafiltration (Vivaspin concentrator, cut with 5 kDa, Vivascience), then tested for their activities CAD and M6PR. The active fractions are combined to be analyzed by anions exchanging chromatography.

Anion-exchange chromatography

The proteomic fraction is loaded at a flow of 0.5 mL min^{-1} onto an anions exchanging column (Econo-Pac® High Q Cartridge, BIO-RAD, 1 mL) pre-equilibrated in buffer A. After washing of the column at 1.5 mL min^{-1} with 6 volumes of buffer A, the proteins are eluted by a linear gradient 20-400 mM Tris-HCl (pH 7.5) containing 5 % (v/v) ethylene glycol and 5 mM DTT (1.5 mL min^{-1}, 20 volumes of the column). The proteins are collected by fraction of 2 mL. These fractions are desalted by ultrafiltration, then tested for their activity CAD and M6PR. The active fractions are combined for the last stage of purification.

Affinity Chromatography on 2', 5' ADP-Sepharose gel

According to published protocols (Goffner *et al*., 1992; Halpin *et al*., 1992; Grima-Pettenati *et al*., 1994), this affinity chromatography very frequently constitutes the last stage of purification of the CAD. It is accompanied effectively by an increase in the degree of purification of this protein. The proteomic fraction is desalted onto PD-10 column pre-equilibrated with a buffer B composed of 100 mM Tris-HCl (pH 7.5) containing 5 % (v/v) ethylene glycol and 5 mM DTT. The proteins are eluted from PD-10 column with this same buffer. The desalted fraction is loaded onto a column containing 3 mL of 2' 5' ADP-Sepharose™ 4B gel (Amersham Biosciences) pre-equilibrated in the buffer B. the gel is washed with 5 volumes of the buffer B then 2 volumes of buffer B containing 4 mM NAD. The CAD is then eluted by 2.5 mL of buffer B containing 4 mM of $NADP^+$. Activities CAD and M6PR are measured in this fraction desalted previously and concentrated by ultrafiltration.

Measure *in vitro* of enzymatic activity

Measurement of CAD and M6PR activities

CAD Activity is determined by spectrophotometry by measuring the increase in OD_{400} during the oxidation of coniferyl alcohol (CA) into coniferyl aldehyde in the presence of $NADP^+$ (35°C, Δt of 5 min). The reational medium (1 mL) is composed of 100 mM Tris-HCl pH 8.8, 2 mM of $NADP^+$, 5 mM of CA, and 1-100 protein µg according to the tested proteomic fraction (Halpin *et al*., 1992). A control is carried out in parallel without CA. M6PR Activity is measured by spectrophotometry by measuring the reduction of OD_{340} during the reduction of mannose 6-phosphate (M6P) in mannitol 1-phosphate in the presence of NADPH (35°C, Δt of 10 min). The reactional medium (1 mL) is composed of 100 mM Tris-HCl pH 7.0, 0.3 mM NADPH, 25 mM M6P (potassium salt),

and 10-100 µg of proteins according to the tested proteomic fraction (Robert *et al*., 1999a). A control is carried out in parallel without M6P.

Methods of activities calculation

An enzymatic Unit corresponds to one µmole of substrate consumed per minute. A nanokatal is equivalent to a nanomole of substrate consumed per second. CAD and M6PR activities are calculated according to following formulas:

Specific activity (SA, nkat mg^{-1})

$SA = \{[OD\ (T_2) - OD\ (T_1)] - [OD\ (C_2) - OD\ (C_1)]\} \times 1000 / [60 \times (\varepsilon \times t \times Q)]$

With OD, the optic density at 340 nm for the M6PR and at 400 nm for the CAD

T_1 and T_2, the two different times for which the OD are measured for the test curve

C_1 and C_2, the two different times for which the OD are measured for the control curve

ε, the coefficient of extinction µmolar of $NADP^+$ (CA) or NADPH (M6P)

$\varepsilon = 21.0\ \mu M^{-1}\ cm^{-1}$ ($NADP^+$); $\varepsilon = 6.3\ \mu M^{-1}\ cm^{-1}$ (NADPH)
t, reactional time; Q, quantity of proteins used for the enzymatic test (mg)

The increase in the specific activity during different stages of chromatography testifies the increase in the degree of purification of protein.

Total activity (TA, nkat)

$TA = SA \times Q_{tot}$

With: Q_{tot}, quantity of proteins contained in the analyzed proteomic fraction (mg). The total activities of the collected fractions testify to the yield of the different stages of purification.

Proteins Assays and analyses of SDS-PAGE and PAGE

The proteins are quantified according to the technique of Bradford (1976). The different proteomic fractions collected during the purification of the CAD are analyzed by SDS-PAGE on 12% acrylamide gel (1.5 mm thickness (Laemmli, 1970); System Mini Protean III, Bio-Rad). After migration, the proteins are colored with the blue of colloidal Coomassie (Pacaud, 2004). After electrophoresis, the proteins are fixed during 2 times 1 hour in a mixture 50% (v/v) ethanol - 2% (v/v) phosphoric acid. The gel is washed during 1 hour in a bath of phosphoric acid. The proteins are sensitized to the coloring by a 20 minutes incubation in a mixture 17% (v/v) ethanol - 15% (w/v) ammonium sulfate - 2% (v/v) phosphoric acid, then colored during 24 to 72 hours in this same mixture added with 0,1% (w/v) Blue of Coomassie G250. The gel is rinsed twice 10 minutes in distilled water, 10 minutes in an ethanol 20% bath (v/v), and then stored in distilled water.

The purified fractions of CAD are also analyzed on native gel (PAGE, 12% of acrylamide). The buffer of proteins dissolution, the gel and the buffer of migration do not contain a SDS. CAD activity is detected on native gel by reduction of the nitro-blue of tetrazolium (Halpin *et al*., 1992). After electrophoresis, the gel is incubated at the darkness and room temperature in 10 mL of buffer Tris-HCl 100 mM (pH 8.8) containing 0.2 mM of nitro-blue tetrazolium, 0.03 mM phenazinemethosulfate, 1.4 mM coniferyl alcohol and 0.3 mM of NADP. CAD activity is detectable by formation of a clear brown precipitate.

RESULTS

Purification of striga CAD

Determination of the most active organ face the CAD and the M6PR. CAD and M6PR Activities are measured in the desalted crude extract from different organs of striga (Figure 4). The most significant activity CAD is detected in the stems of the parasite. Nevertheless, it remains high in the adult leaves, which present the most significant activity M6PR. Consequently, the ratio M6PR/CAD is maximum in the adult leaves (55%). The purification of the CAD is thus carrying out starting from the adult leaves of the parasite, which are in addition the richest organs in proteins.

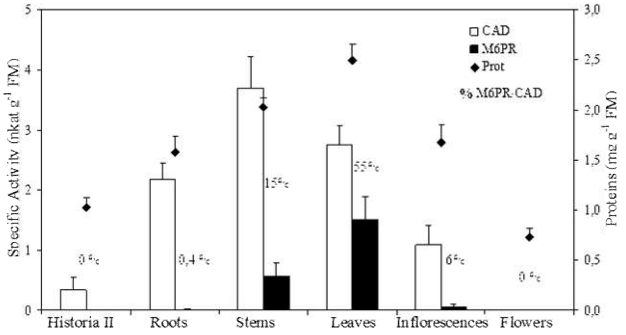

Figure 4. CAD and M6PR activities in the different organs from striga. 100 µg of proteins are utilized for the measure of enzymatic activities

Precipitation and fractionation with the ammonium sulfate

The crude extract of striga is saturated sequentially with the ammonium sulfate. CAD and M6PR Activities are measured in the different fractions obtained and desalted on PD-10 column (Figure 5A). In the same way, the proteomic profile of each fraction is analyzed in SDS-PAGE. CAD and M6PR Activities are mainly recovered in fractions 40 to 80%, with a maximum of CAD and M6PR activities in the fraction 60-70%. In these fractions, the average ratio of CAD/M6PR activities is about 30%. The proteomic profile is similar for the fractions up to 70% of saturation. On the other hand, the proteomic profile of fraction 70-80% appears simpler with mainly three major bands. Nevertheless, this fraction contains only 16% on average activities CAD and M6PR of the crude extract. The fractions from 40 to 80% are combined to recover the maximum of activity. Thus, fraction 40-80% contains 85% on average activities CAD and M6PR of the crude extract, and only 50% of total proteins (Figure 5B). The isolation of the proteomic fraction 40-80% thus constitutes a first stage interesting for the purification of the CAD of the striga.

This stage allows indeed the elimination of 50% of total proteins without significant loss of activity.

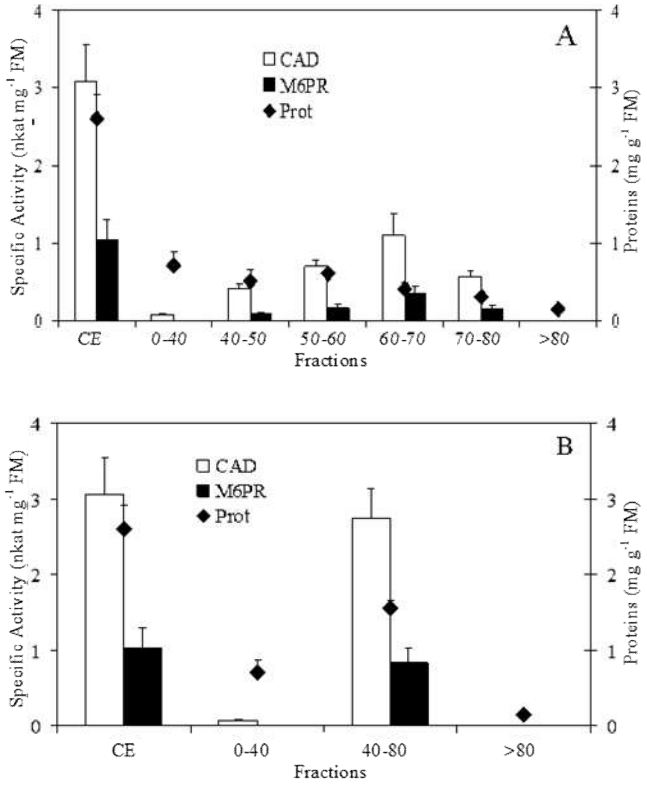

Figure 5. Precipitation and fractionation with the ammonium sulphate. A- activities and proteins in the fractions from 0 to 100%; B- activities and proteins in the combined fraction 40-80%. FM: fresh matter; CE: crude extract. (25 and 100µg of proteins are used for the analysis SDS-PAGE and the measurement of the enzymatic activities respectively).

Affinity and ions-exchange chromatographies

After fractionation with the ammonium sulfate, the CAD is purified by successive chromatographies on columns: Blue Sepharose, Mono Q and 2'5' ADP-Sepharose.

Blue Sepharose column (affinity to NAD(P)$^+$)

The fraction of saturation 40-80% is desalted on PD-10 column then deposited on Bleu Sepharose column. The great majority of deposited proteins (95% on average) is not retained by this gel of affinity. In addition, only 4% of the initial CAD activity is detected in the fraction not retained. One washing of the column with 4 mM NAD^+ is carried out before elution of the CAD. This stage makes it possible to eliminate the NAD-dependent dehydrogenases, and in particular alcohol dehydrogenases (Halpin *et al.*, 1992). Twenty percents on average of proteins fixed on the column are eluted with NAD^+ and the eluate contains only 2% of activity CAD initially deposited. The CAD can be then eluted by a gradient of $NADP^+$ from 0 to 4 mM (Figure 6). CAD activity is detected in the large majority of the collected fractions, with nevertheless activities definitely more significant in fractions 7 to 10, corresponding to a concentration from 1 to 2 mM of $NADP^+$. The whole of the fractions presenting an activity CAD is grouped and concentrated by ultrafiltration. CAD and M6PR specific activities are measured: 56 nkat mg^{-1} for the CAD and 24 nkat mg^{-1} for the M6PR. At most, 55% of total activities CAD and initial M6PR are recovered in this affinity fraction.

The proteomic profile of the affinity fraction reveals in SDS-PAGE the presence of three principal groups of polypeptides, around 30, 40 and 50 kDa.

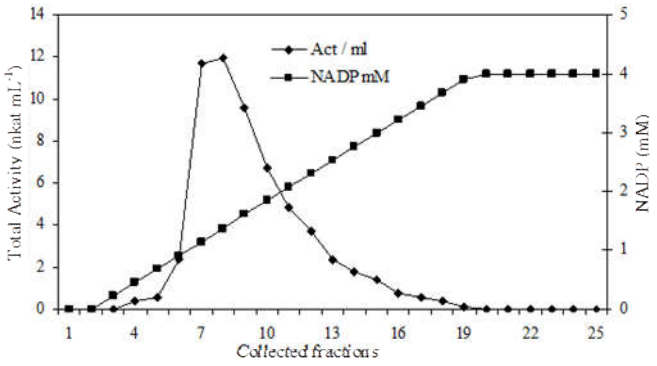

Figure 6. Purification of the CAD of striga, affinity chromatography on the Blue-Sepharose column

Mono Q column (Anion-exchange)

The proteins are deposited on the Mono Q column, which finally retains only 55% on average of these proteins. In addition, only a very feeble CAD activity is detected in the proteomic fraction not retained (less than 0.01% of CAD activity initially deposited). The fixed proteins are sequentially eluted by a gradient of Tris-HCl from 20 to 400 mM (Figure 7). After concentration by ultrafiltration of the collected fractions, two peaks with CAD activity are detected: A majority peak of activity eluted at 100 mM of Tris and a second peak of activity definitely weaker eluted at 200 mM of Tris. By regrouping of the active fractions, two proteomic fractions, known as MQ_1 (fractions 4 to 8) and MQ_2 (fractions 9 to 11), are thus differentiated. Their activities CAD and M6PR respectively are measured: MQ_1: 72 nkat mg^{-1} for CAD activity and 8 nkat mg^{-1} for M6PR activity, MQ_2: 44 nkat mg^{-1} for CAD activity and 72 nkat mg^{-1} for M6PR activity. The proteomic profile of these two fractions is analyzed in SDS-PAGE. The polypeptide composition of MQ_1 appears similar to that of the fraction of affinity eluted on the Bleu Sepharose column. On the other hand, the polypeptide composition of MQ_2 is definitely simpler and is characterized by a major band with 40 kDa approximately.

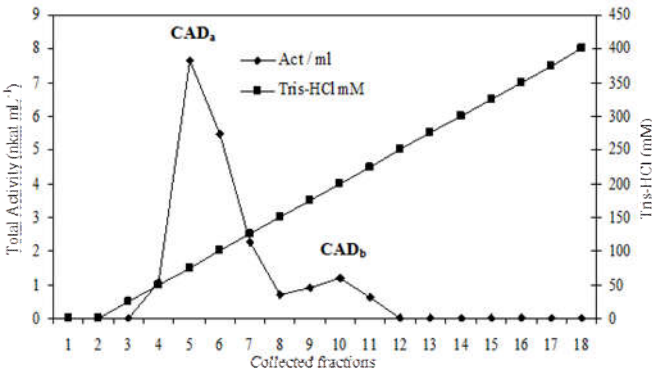

Figure 7. Purification of the CAD of striga, anions-exchange chromatography on the Mono-Q column. Two peaks of CAD activity are detected MQ_1 and MQ_2

2'5' ADP-Sepharose column (affinity to NADP$^+$)

The two active fractions MQ_1 and MQ_2 are concentrated by ultrafiltration then desalted on PD-10 column equilibrated previously with a 100 mM Tris buffer containing ethylene glycol 5% and DTT 5 mM.

The fixing of proteins on 2' 5' ADP-Sepharose gel is effectively improved with this buffer (compared to the 20 mM Tris buffer, results not presented). The two fractions MQ_1 and MQ_2 are deposited separately on one volume of 2' 5' ADP-Sepharose gel. The affinity of the gel appears better to proteomic fraction MQ_2. Nevertheless 10% and 30% only of proteins of fractions MQ_1 and MQ_2 respectively are retained by affinity. In addition, the fraction not retained of MQ_1 contains 90% and 100% of activities CAD and M6PR measured after this last stage of purification (Tables 1 and 2). On the other hand, the fraction retained of MQ_2 contains 80% of activity CAD measured after this last stage of purification. The M6PR activity of this fraction 2' 5' ADP-Sepharose is also void. We note that the fractions eluted by the 4 mM NAD are relatively low in proteins (1-2% of proteins initially deposited on the gel) and that they do not contain any activity CAD, nor M6PR. The two active fractions, collected after elution of the 2' 5' ADP-Sepharose gels with 4 mM NADP, are arbitrarily called CADa and CADb. Their specific activity CAD is close, 187 nkat mg^{-1} and 143 nkat mg^{-1} for CADa and CADb respectively. The proteomic profile of these two fractions in SDS-PAGE is similar and is limited to only one band to the environs of 40 kDa.

Table 1. Purification of the CAD starting from 100 g FM of adult leaves of striga

Steps	Total Protein *(mg)*	Specific Activity *(nkat mg^{-1} protein)*	Total Activity *(nkat)*	Yield *(%)*	Purification *(Fold)*
Crude Extract	328	1.4	459	100	1
Fraction 40-80%	126	3	378	82	2
Blue Sepharose CADa	3.4	56	190	41	40
Mono Q_1	1.2	72	87	19	52
2'5' ADP-Sepharose CADb	0.4	137	55	12	98
Mono Q_2	0.50	44	22	5	31
2'5' ADP-Sepharose	0.06	163	9.8	2.2	116

Table 2. Purification of protein with activity M6PR starting from 100 g FM of adult leaves of striga

Steps	Total Protein *(mg)*	Specific Activity *(nkat mg^{-1} protein)*	Total Activity *(nkat)*	Yield *(%)*	Purification *(Fold)*
Crude Extract	328	0.6	197	100	1
Fraction 40-80%	126	0.9	113	64	2
Blue Sepharose M6PR (CADb)	3.4	24	81	40	43
Mono Q_2	0.50	52	26	13	87
2'5' ADP-Sepharose	0.06	113	7	4	188

Estimation of the purification of striga $CAD_{(s)}$

The total analysis of the active fractions collected during the purification is presented in Figure 8 and Tables 1 and 2. The first affinity chromatography (Blue Sepharose) makes it possible to eliminate 97% from total proteins of the fraction 40-80%. This stage is accompanied by a fall of CAD and M6PR activities by 50% and 30% respectively, but accentuates the degree of purification of the CAD and the M6PR of a factor of about 40 (Tables 1 and 2). The second chromatography on anion exchange column does not improve the degree of purification of the CAD and the M6PR but reveals the existence of at least two peaks of activity CAD (MQ_1 and MQ_2).

Total activity MQ_1 is 4 times higher than that of MQ_2. On the other hand, the fraction MQ_2 presents an activity M6PR 3-4 times higher than the fraction MQ_1 (Table 2). An additional affinity chromatography on 2' 5' ADP-Sepharose column clearly improves the degree of purification of the CAD (Table 1); it approximately reduces effectively the proteomic profile of these fractions to only one band of 40 kDa (Figure 8), which presents *in vitro* only one activity CAD. This gel, similar to the majority of activity CAD of fraction MQ_1, does not retain the M6PR activity of MQ_1 and MQ_2 fractions. On a native gel, CADa and CADb present only one band. Their respective CAD activity is detectable on gel by reduction of the tetrazolium nitroblue. These results are not presented, the resolution of the gel not being suitable.

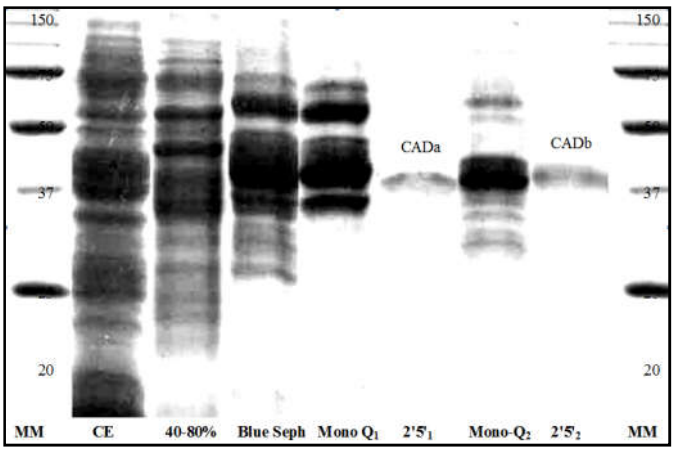

Figure 8. Different stages of purification of the CAD starting from the adult leaves of striga. 25 µg of proteins are deposited (wells 1,2) and 10 µg of proteins are deposited (well 3-7). MM: mass molecular marker (KDa); DCE: desalted crude extract; 40-80%: precipitated fraction 40-80% with the ammonium sulphate; Blue Seph: proteins eluted on the Blue-Sepharose affinity column; Mono-Q: proteins eluted on the anions-exchange column; 2' 5': proteins eluted on the 2' 5' ADP-Sepharose affinity column.

Characterization of the CADa major isoform

The major isoform is partially characterized starting from MQ_1 fraction. Its affinity to the cinnamyl alcohol is graphically given according to the representation in double reverse: K_m = 400 µM. The sensitivity of CADa to the temperature is given on a wide range of temperature going from 25°C to 70°C. The optimal CADa activity is recorded for temperatures of 40°-55°C, the activity at 25°C accounting for only 30% of this maximum activity.

DISCUSSION

Distribution of CAD and M6PR activities at striga

The mannitol occupies a central place in the carbonaceous metabolism of *Striga hermonthica*. For as much, striga seems to be different from the other producing plants of mannitol (celery, broomrape…) by the absence of a typical M6PR. Work of Pacaud (2004) suggests at striga a substitution of the M6PR by a CAD (or alcohol dehydrogenase) little specific to its substrates. An action program was thus defined and aims at characterizing isoforms CAD and the genes *cad* at this parasite, with a final aim to reveal or not the existence of a "bifunctional" CAD presenting an activity M6PR.

Initially, this work made it possible to define the profile of CAD and M6PR activities in the different organs of parasite at one month old after emergence (Figure 4). The M6PR activity of the nonchlorophyllian organs (haustoria, roots and flowers) is extremely weak (even null) by comparison with that of the stem and especially of the adult leaves. The adult leaves are effectively the most active organs to the M6PR, which suggests the close link between M6PR activity and the photosynthetic intensity at this stage of development of the parasite. Similar results were deferred at celery where protein M6PR is confined in the mesophylle of the leaves (Everard *et al*., 1993). At celery (Rumpho *et al*., 1983) and striga (Rousset, 2003), the mannitol is thus the major photosynthetic product. With regard to the CAD, it is not surprising to find a strong activity in every lignified organ of the parasite, such as the roots and especially the stems (Figure 4). These results underline most probably the implication of the CAD in the lignification of tissues of these organs. In parallel, the presence of an activity CAD relatively high in organs very little lignified (haustoria, adult leaves, inflorescences), in particular in the adult leaves, is to be underlined at striga and suggests the role of the CAD in these organs in processes other than constitutive lignification. In spite of a significant documentation on the CAD, the search of work presenting the distribution of this activity in the different organs of a plant, and in particular in the leaves, proved unfruitful to date. Specific activity CAD measured in the crude extracts of the leaves or the stems of striga is about 2 to 3 nkat mg^{-1} proteins, that is to say a value definitely higher (3 to 30 times) than specific activity CAD measured in the crude extracts of the majority of lignified tissues analyzed to date (stems, xylem purified, pods…), (Lüderitz and Grisebach, 1981; Goffner *et al*., 1992; Halpin *et al*., 1992; Grima-Pettenati *et al*., 1994; Hawkins and Boudet, 1994). Only O'Malley *et al*., (1992) deferred an activity specific CAD similar to that of the leaves of striga in the crude extracts of xylem of the pine (3 nkat mg^{-1} proteins). These observations translate a metabolic specificity of striga.

CAD_s: heterogeneous proteins on the structural and functional plans

In the beginning of the 1990s, many endeavored work to show the implication of the CAD in lignification and to analyze the impact of a limitation of activity CAD on the capacity of mutants to produce the lignins. Nowadays, research turns mainly to study the "plurifunctionality" of the CAD. The CAD was purified starting from a great number of plants (Boucher *et al*., 1998). At the majority of the studied plants, work was limited to the characterization of only one isoform which is closely related to the process of lignification constitutive (Feuillet *et al*., 1993; Walter *et al*., 1994; Samaj *et al*., 1998). The CAD is a small multigenic family, *Arabidopsis thaliana* presenting for example 9 genes *cad* (1-9) coding really for a protein with activity CAD (Raes *et al*., 2003). In a limited number of cases, two isoforms CAD (known as CAD1 and CAD2) were characterized, in particular at *Eucalyptus gunni*, *Phaseolus vulgaris* and *Medicago sativa* (Goffner *et al*., 1992; Grima-Pettenati *et al*., 1994; Hawkins and Boudet, 1994; Hawkins *et al*., 1997; Goffner *et al*., 1998; Brill *et al*., 1999).
At *E. gunni*, although CAD1 and CAD2 present a similar catabolic function, i.e. a reversible reduction activity of coniferaldehyde and 4-coumaraldehyde in their corresponding alcohol, CAD1 differs from CAD2 by its incapacity to use sinapaldehyde as like as substrate (Goffner *et al*., 1992). Moreover, antibodies targeted against CAD2 do not react with CAD1 and conversely, the anti-CAD1 antibodies do not recognize CAD2. These two isoforms thus present structural and functional differences. DNAc coding for CAD1 shows a stronger homology with of DNAc coding for dihydroflavonol 4-reductases (DFR, EC. 1.1.1.219) or cinnamoyl-CoA reductases (CCR) that for of DNAc coding for typical CAD2 (Goffner *et al*., 1998). To date, this CAD1 is identified like a single aromatic alcohol dehydrogenase on the structural and functional levels. If the role of CAD2 in constitutive lignification is clearly established, the role of CAD1 is not elucidated yet at *E. gunni*. At *M. sativa*, *MsaCAD1* and *MsaCAD2* code both for a CAD. The isoform MsaCAD2 is active only in the presence of the cinnamyl aldehydes as substrates (typical CAD) whereas the affinity of MsaCAD1 extends to benzaldehydes and aliphatic aldehydes (Brill *et al*., 1999). In addition, DNAc coding for MsaCAD1 shows a stronger homology with of ADNc coding for ELI-3 proteins than with of DNAc coding for typical CAD2. The ELI-3 proteins correspond to a defense protein class inductibles in the cells not lignified by pathogen, bacteria or fungus (or by the salicylic acid).

Basing only on the homology of sequences of the genes *eli3* with the *Agmtd* gene coding for the mannitol dehydrogenase from celery, Williamson *et al*., (1995) associated the genes *eli3* with genes coding for a mannitol dehydrogenase. Since, Logemann *et al*., (1997) showed that the *Pceli3* gene of parsley coded in fact for a CAD whose specificity to its substrates is definitely larger than that of the typical CAD2. In the same way, the gene *Ateli-3* (*cad5*) of *A. thaliana* code for a benzyl alcohol dehydrogenase (aromatic alcohol dehydrogenase very specific to benzaldehyde) (Somssich *et al*., 1996). *Pceli3* and *Ateli-3* are overexpressed in the defense reactions, just like *MsaCAD1* of the alfalfa in response to the wound or the acid salicylic (Brill *et al*., 1999). The isoform MsaCAD1 would thus be implied in the mechanisms of defense, rather than in constitutive lignification. The whole of this work underlines the existence at the plants of multiple CAD, structure and function varied. At *A. thaliana*, 8 of the 9 genes *cad* integrate three different classes of CAD: *cad2* and *cad6*, pertaining to a class I, are very close to the typical genes *cad* implied in lignification; *cad3*, *cad4* (*Ateli3-1*) and *cad5* (*Ateli3-2*) belong to a class II and have a homology with a certain number of genes coding for alcohol dehydrogenases whose specificity to the substrates is varied (mannitol dehydrogenase of celery, sinapyl alcohol dehydrogenase of poplar, and proteins ELI3/CAD of parsley); *cad1*, *cad7* and *cad8* belong to a class III with *MsaCad1* de *Medicago sativa* (Raes *et al*., 2003).

With regard to striga, the maintenance in the adult leaves of a high activity CAD (Figure 4) thus suggests the presence of at least an atypical isoform CAD in these organs. At *A. thaliana*, the 9 genes *cad* are more or less strongly expressed in the leaves, the *Atcad4*, *Atcad5*, *Atcad6* and *Atcad9* genes is different from the others by a strong expression in these organs. It is also the case of *Fxacad1* at strawberry (*Fragaria* x *ananassa)* (Blanco-Portales *et al*., 2002), and of *cad1* of *E. gunni* (Goffner *et al*., 1998). Among the whole of these genes, alone *Atcad6* and *Fxacad1* code for a typical CAD implied in constitutive lignification. The role of other genes in the leaves is unspecified.

CADa and CADb: two CAD isoforms of striga

In the leaves of striga, the hypothesis of a link between the presence of an atypical isoform CAD and activity M6PR

specifically high in these organs are posed. If this assumption appeared exact, the specific expression of an isoform CAD presenting a nonspecific activity M6PR (large specificity to its substrates) would characterize the photosynthetic organs of the parasite. The purification of the CAD thus was logically carried out starting from the leaves of striga, which to our knowledge constitutes a first. Indeed, until this work, the CAD was purified exclusively starting from organs or of strongly lignified tissues (stems, xylem, pods…), (Halpin *et al*., 1992; Grima-Pettenati *et al*., 1994). The tissues of striga have the characteristic to be extremely rich in polyphenols. The addition of a significant quantity of PVPP in the buffer of extraction (80% of the weight of used FM) is thus essential to avoid the tanning of the extracts and the inactivation of the enzymes. The presence of 15 mM mercaptoethanol in the buffer of extraction also contributes to the maintenance of the enzymatic activities (redactor agent, proteases inhibitor). The residual polyphenols, not trapped by the PVPP, are eliminated during desalting on PD-10 column of the fraction of saturation 40-80%. In the same way, the measurement of activities CAD and M6PR in the crude extracts requires a preliminary desalting of the extracts on PD-10 column (results not presented).

The protocol of purification defined in this study, is a precipitation of proteins with the ammonium sulfate and two stages of affinity chromatography (Blue-Sepharose and 2' 5' ADP-Sepharose) separated by a stage from anions exchange chromatography (Mono Q), was employed before successfully to purify different isoforms of CAD starting from the stems of tobacco ((Halpin *et al*., 1992), from the xylem of *E. gunni* (Goffner *et al*., 1992), or from the pods of *Phaseolus vulgaris* (Grima-Pettenati *et al*., 1994). The yields of fixing of activity CAD of striga on these different columns appeared satisfactory by comparison with those deferred in work previously quoted.
As at *Phaseolus vulgaris* (Grima-Pettenati *et al*., 1994), *E. gunni* (Hawkins and Boudet, 1994), the stage of anions-exchange chromatography on gradient of buffer Tris-HCl reveals the existence at striga at least two isoforms CAD, known as CADa and CADb, (Figure 7). These two isoforms could be purified separately and their activity CAD shown on native gel. CADa is the major isoform in the leaves of the parasite, its activity being 4 times higher than that of CADb (Table 1). The affinity chromatography 2' 5' ADP-Sepharose is effective for the purification of CADb and only one band of 40 kDa is visible in SDS-PAGE (Figure 8). On the other hand, this gel appears not very closely linked to CADa (Table 1). Nevertheless, only one band of 38 kDa is also visible in SDS-PAGE (Figure 8). CADa and CADb are thus of slightly different size. The CAD purified starting from the spruce (Lüderitz and Grisebach, 1981) and of the poplar (Savidge, 1989) are homodimers, made up of two identical subunits of 40 kDa approximately. On the other hand, the CAD2 of *E. gunni* (Hawkins and Boudet, 1994) and of tobacco (Halpin *et al*., 1992) is a hetero-dimer of 42 and 44 kDa, and 42.5 and 44 kDa respectively. The polypeptide composition of CADa and CADb of striga was not precisely given in this work. Nevertheless, if these proteins proved to be dimeric, their subunits would be then of the same molecular weight since in SDS-PAGE only one band characterizes CADa and CADb (Figure 8). This work also made it possible to start the study of the physicochemical properties of the CAD of striga. CAD activities collected after affinity on gel 2' 5' ADP-Sepharose are too weak to allow such an analysis. On the other hand, this one is possible starting from less purified fractions, such as MQ_1 and MQ_2. Only the isoform majority CADa was partially characterized in this work. From fraction MQ_1, CADa presents a K_m to the coniferyl alcohol of about 400 µM, that is to say an affinity of the same order than that deferred for CAD1 and CAD2 of *P. vulgaris* (520 and 140 µM respectively), (Grima-Pettenati *et al*., 1994), and of CAD1 of *E. gunni* (Goffner *et al*., 1992). Some CAD is characterized by an affinity definitely stronger for their substrates (in particular the coniferyl alcohol), such as a CAD of tobacco (1.2 µM), (Halpin *et al*., 1992) and CAD2 of *E. gunni* (6 µM), (Goffner *et al*., 1992). Moreover, at striga CADa has a strong stability at heat, its activity being optimal with 40-55°C. Although the response of the CAD to such temperatures is not given at other plants, our results represent most probably the adaptation of the metabolism of striga to the subtropical regions where it is propagated (Thalouarn and Fer, 1993).

The genes cad of striga

A work undertaken in parallel (specific participation) aims at characterizing the genes *cad* of striga and corresponding proteins. To date, two DNAc coding for putative CAD were characterized at the parasite. Named CAD1 and CAD2, the two sequences translated into proteins present the characteristic motifs of the CAD (Kim *et al*., 2004). CAD1 and CAD2 have a homology between them only 40%. The strongest homology of CAD2 was found with a CAD of poplar (85%). On the other hand, CAD1 is homologous with dehydrogenases with various sensitivity to alcohols (hydroxygeraniol oxydoreductase of *Catharanthus roseus* (70% of homology) and a putative mannitol dehydrogenase of strawberry (67% of homology)) that with typical CAD. It has nevertheless a homology with a CAD of strawberry of about 65%. These results attest existence, as at many other plants, of at least two genes *cad* at striga, one typical (*cad2*) most probably implied in constitutive lignification, and the other, atypical (*cad* 1), which seems to belong to class II of the CAD of Angiosperms (Raes *et al*., 2003). The correspondence between these two genes and both isoforms purified CADa and CADb is not established to date.

Behavior of protein with M6PR activity during the purification of CADa and CADb

The protein with M6PR activity is mainly co-eluted with MQ_2 (CADb) fraction during the anions-exchange chromatography, but contrary to CADb it is not retained by the 2' 5' ADP-Sepharose affinity gel (Table 2). Two hypothesis can thus be posed:

- The protein with M6PR activity corresponds to an isoform CAD not retained by the affinity gel.
- The M6PR activity is not ensured by a CAD.

Conclusion and Perspectives

Two isoforms CADa and CADb were purified starting from the leaves of striga. They differ in particular by their molecular weight and their affinity to 2' 5' ADP-Sepharose gel affinity. CADa partially characterized (K_m to the cinnamyl alcohol and optimal temperature) starting from semi-purified fraction MQ_1 collected by ions-exchange chromatography.

The study of the physicochemical characteristics of CADa and CADb will have to be supplemented, to define in particular:

- Their degree of purity by bidimensionally electrophoresis.

- Their respective affinity to the three substrates aldehydes (coumaryl, coniferyl and sinapyl aldehydes) and their corresponding alcohol.
- The molecular weight of the native forms by gel-filtration chromatography (for example: Sephacryl S-200 HR, Pharmacia) and their polypeptide composition by denaturing electrophoresis.

With the sight of strong CAD activity of the not fixed fraction of MQ_1 by 2' 5' ADP-Sepharose gel (Table 1), we cannot dismiss the hypothesis that the band observed in SDS-PAGE characterizing the CADa fraction (Figure 8) corresponds in fact to another isoform minority. This one presents in SDS-PAGE a molecular weight slightly different from that of CADb, and thus does not come from a contamination of fraction MQ_1 by CADb. 2' 5' ADP-Sepharose gel does not allow a complete analysis of fraction MQ_1. An intermediate stage (hydrophobic column or gel-filtration for example) could be useful to identify or not the presence of several isoforms CAD in this fraction. The molecular approach carried out in parallel should contribute to the characterization of isoforms CAD of striga, *via* the production of recombining proteins CAD1 and CAD2. Their affinity to the mannose 6-phosphate, substrate of the M6PR, could be in addition directly given. The production of anti-CAD1 and anti-CAD2 antibodies must be considered for a characterization supplements these isoforms. They will be used in particular for the localization of CAD1 and CAD2 in the various organs and tissues of the parasite (immunolocalization). Finally, this work did not make it possible to underline the existence of a CAD presenting a M6PR activity. It did not lead either to the purification and the identification of protein with M6PR activity. This protein remains unknown and confined in the pool of polypeptides of MQ_2 not retained by 2' 5' ADP-Sepharose gel. The effort will have to be related to the analysis of this proteomic fraction.

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