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Trypanosoma cruzi: partial characterization of minor cruzipain isoforms non-adsorbed to Concanavalin A–Sepharose

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Abstract

The present paper reports the partial characterization of a subset of atypical cruzipain molecules which do not bind to Concanavalin A–Sepharose column. They are present in different strains of epimastigote forms of *Trypanosoma cruzi* and represent a 2–4% of total cruzipain. They were purified by affinity chromatography on Cystatin–Sepharose, recognized by the polyclonal anti-cruzipain serum, and their activity in gelatin-containing gels was completely abolished by E-64, TLCK, leupeptin, and aprotinin but not by PMSF, pepstatin A, EDTA or 1,10-phenantroline. These cysteine proteinases, as well as cruzipain showed to be endo-proteinases able to hydrolize azocasein, hemoglobin, and bovine serum albumin at acidic pHs. However, evidences are presented indicating that this subset of cruzipain isoforms were also able to use the same blocked chromogenic peptidyl substrates than cruzipain at similar optimal alkaline pH values although with a different order of preference. Moreover, they showed a different oligosaccharide pattern after enzymatic treatment by high pH anion exchange chromatography, suggesting that this structural difference may account for the atypical behaviour in the lectin columm.

Index Descriptors and Abbreviations: *Trypanosoma cruzi*; Cysteine proteinases; Cruzipain. CPs, Cysteine proteinases; Cr, cruzipain; C-T, C-terminal extension; HPLC, high performance liquid chromatography; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; ConA, concanavalin A; NACrI, non-adsorbed cruzipain isoforms; DEAE, diethylaminoethyl; FPLC, fast protein liquid chromatography; TCA, trichloroacetic acid; PMSF, phenyl methyl sulfonyl fluoride; TLCK, tosyl lisyl chloromethyl ketone; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidine) butane; Bz, Benzoyl; CBZ-, *N*-carbobenzoxy; BOC, *N*-*tert*-butoxycarbonyl; OMe, Methoxy-; p-NA, *p*-nitroanilide; β-NAm, β-naphthylamide; BSA, bovine serum albumin; EndoH, EndoH-β-*N*-acetylglycosaminidase; PNGase F, Protein N-glycanase F; HPAEC-PAD, high pH anion exchange chromatography with pulse amperometric detection

1. Introduction

Trypanosoma cruzi, the parasitic protozoan which causes the American Trypanosomiasis, Chagas disease, contains a major cysteine proteinase (CP), cruzipain (Cr) (Cazzulo et al., 2001). The enzyme belongs to the papain family, but as other CPs from Trypanosomatids, shows an unusual C-terminal extension (C-T). This C-terminal domain contains a number of post-translational modifications and is responsible for the immunodominant antigenic character of cruzipain in natural human infections. In addition, this domain is probably the cause of most of the microheterogeneities found in natural cruzipain (Cazzulo et al., 1997).

Natural cruzipain is a complex of isoforms, as judged from ionic exchange chromatography, isoelectrofocusing, reversed-phase HPLC and SDS–PAGE in substrate containing gels (Cazzulo et al., 1995). This heterogeneity is probably due to both the simultaneous expression of several genes encoding amino acid substitutions and to the presence, in different cruzipain molecules, of either high mannose-type, hybrid monoantennary-type, or complex biantennary-type

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oligosaccharide chains at the only N-glycosylation site in the C-T (Parodi et al., 1995). Besides, the presence of non-conservative amino acids substitutions at the C-T, change the predicted isoelectric point of the protein and are likely to result in structural variants at this level (Martinez et al., 1998).

This lysosomal enzyme, also known as cruzain or GP57/51 (Eakin et al., 1992, Murta et al., 1990) has three potential N-glycosylation sites, two in the catalytic domain and one in the C-T domain (Campetella et al., 1992). The only N-glycosylation site in the C-T, as well as the first potential N-glycosylation site in the catalytic moiety, are glycosylated in vivo; the latter bears only high mannose-type oligosaccharides. There is still no evidence on the N-glycosylation status of the second potential site (Asn 169) (Metzner et al., 1996).

Most cruzipain genes completely or partially sequenced so far are highly homologous; the exception, however, is cruzipain 2 (Lima et al., 1994), which has only 86% identity with other sequenced genes (Campetella et al., 1992; Eakin et al., 1992; Lima et al., 1994). The amino acid residue substitutions found in the catalytic domain of cruzipain 2 include substitutions at the S2, S1′, and S2′ subsites and the absence of the first potential N-glycosylation site (Lima et al., 1994). It was shown that truncated recombinant cruzain and cruzipain 2, both lacking the C-T domain, had different substrate specificity when acting on peptides derived from human kininogen (Del Nery et al., 1997) and recent evidences confirmed that both differ in pH stability, substrate specificity and susceptibility to inhibition by natural and synthetic inhibitors of CPs (Lima et al., 2001).

This report shows for the first time, the presence of a new subset of cruzipain molecules in a non-adsorbed to ConA–Sepharose fraction. These isoforms were analyzed by sequential chromatographic methods, SDS–PAGE gelatin containing gels, Western blots, preference of substrates and inhibitory pattern. In addition, the non-adsorbed Cr isoforms (NACrI) were purified by affinity chromatography, treated with PGNase F and its oligosaccharide content was compared with the one obtained from control cruzipain, confirming the presence of a different pattern that may account for the atypical behaviour.

2. Materials and methods

2.1. Preparation of cell-free extract

Epimastigotes of *T. cruzi*, Tulahuen strain, Tul 2 stock or RA strain, were grown and the cell free extract was obtained as previously described (Cazzulo et al., 1985, 1989).

2.2. Purification of cruzipain isoforms non-adsorbed to ConA

2.2.1. Affinity chromatography on ConA–Sepharose and anionic exchange on DEAE and Mono Q columns

The cell free extract was precipitated with saturated ammonium sulfate solution as previously described (Cazzulo et al., 1989). The pellet was dissolved in 10 ml of 50 mM Tris/ClH buffer pH 7.6 containing 150 mM NaCl, dialysed overnight (two changes) against the same buffer. The solution, after the addition of 5 mM final concentration of $CaCl_2$, $MnCl_2$, and $MgCl_2$, was centrifuged at 14,000 rpm during 5 min, and then applied onto a column (10×20 mm) of ConA–Sepharose, equilibrated with buffer 1 (50 mM Tris/HCl buffer, pH 7.6 containing 150 mM NaCl, 3 mM $CaCl_2$, 3 mM $MnCl_2$, and 3 mM $MgCl_2$). The procedure was repeated three times at 4 °C. For comparative purposes, control cruzipain isoforms adsorbed to the column were eluted and purified to homogeneity as described (Labriola et al., 1993). In order to discard that the absence of binding was due to column saturation, the non-adsorbed material was dialysed against 50 mM Tris/HCl buffer, pH 7.6 containing 150 mM NaCl and after the addition of 5 mM $CaCl_2$, $MnCl_2$, and $MgCl_2$ was resubmitted to the same procedure through a fresh ConA–Sepharose column.

The non-adsorbed fraction was dialysed against Tris/HCl 50 mM pH 7.6 and applied onto a DEAE–Sepharose column in the same buffer containing EDTA 1 mM and glycerol 20%. After thorough washing with Tris/HCl–EDTA–glycerol solution (Cazzulo et al., 1989), the NACrI were eluted with a linear gradient from 0 to 0.5 M KCl in the same buffer. The active fractions with Bz-Pro-Phe-Arg-pNA eluting between 0.1 and 0.25 M KCl were pooled, dialysed against 100 vols of 0.05 M Tris/HCl buffer, pH 7.6, containing 20% glycerol (v/v), applied onto a Mono Q HR 5/5 anion exchange column, and analyzed by fast protein liquid chromatography (FPLC), in the following conditions:

(a) Flow: 0.4 ml/min and elution gradient 0–0.5 M NaCl. Fractions of 0.5 ml were collected.
(b) Conditions (a) applying an isocratic step at 0.14 M NaCl for 10 min.

Elution was monitored by absorbance at 280 nm.

2.2.2. Affinity chromatography on Cystatin–Sepharose

The fraction non-adsorbed to ConA, or alternatively, active fractions with Bz-Pro-Phe-Arg-pNA eluting between 0.15 and 0.25 M NaCl from Mono Q column (condition b), (fractions 40–55 from Fig. 2A, Peak III), were dialysed against 0.1 M acetate/acetic acid buffer, pH 5.5, containing 0.3 M NaCl, and affinity chromatography was performed as previously described (Labriola et al., 1993), using 20% propanol for the elution.

2.2.3. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate with or without gelatin

Purification was followed by SDS–PAGE in 10% acrylamide minigels as described by Laemmli (1970), stained with silver nitrate (Oakley et al., 1980). Activity gels were performed including gelatin 0.15% as substrate in the separating gel as previously described (Martinez and Cazzulo, 1992). Protein content was measured by Bradford's method (1976). The inhibition activity assays carried out on gelatin containing gels included the presence of different inhibitors during the gel washing and incubation steps. Controls in the absence of inhibitors were performed. The inhibitors employed were 100 μM pepstatin-A, 100 μM leupeptin, 100 μM antipain, 2 mM PMSF, 0.5 mM TLCK, 100 μM E-64, 10 mM EDTA, and 1 mM 1, 10-phenantroline.

2.2.4. Western blotting

The samples submitted to SDS–PAGE were electrotransferred to nitrocellulose sheets at 150 mA during 2 h. Anti-Cr polyclonal antibody (1:1000 dilution) (Campetella et al., 1990), and anti-rabbit IgG antibody coupled to alkaline phosphatase (McGadey, 1970) were used.

2.2.5. Determination of enzymatic activity

The enzymatic activity was assayed with the synthetic chromogenic substrate Bz-Pro-Phe-Arg-pNA at pH 8 (Cazzulo et al., 1990a) throughout the purification procedure. The reaction was followed spectrophotometrically, at 410 nm. The NACrI activity on chromogenic peptidyl *p*-nitroanilides (0.15 mM) and β-napthylamides (0.25 mM), was assayed at pH 8.5 and measured spectrophotometrically, at 410 nm or 520 nm, respectively (Torruela et al., 1981). When azocasein, BSA and hemoglobin were used as substrates, the enzyme assays were performed as described (Bontempi et al., 1984) but the reaction was stopped by addition of TCA after incubation for 4 h at 35 °C. In the experiments with E-64, NACrI fraction was preincubated with 0.1 M buffer Tris/acetic 0.1 M pH 5 and the inhibitor for 1 h at 0 °C, the reaction was started by addition of the substrate and 2-mercaptoethanol was omitted.

2.2.6. Endo-β-N-acetylglycosaminidase H treatment and electrophoresis

Purified cruzipain (0.1 mg) was dissolved in (0.2 ml) triethanolamine acetate buffer, pH 5.5, containing 20 μM leupeptin, and 0.1% SDS. Endo-β-*N*-acetylglycosaminidase H (endo H) (10 mU) was added, and the mixture was incubated for 24 h at 37 °C. Samples were then subjected to SDS–PAGE. Western blotting was carried out as described above. Controls without Endo H, incubated under the same conditions, were run simultaneously.

2.2.7. Protein N-glycanase F (PNGase F) digestion

Samples were dissolved in 0.5% SDS, 1% β-mercaptoethanol and boiled for 2 min at 100 °C. Digestion was performed in 0.05 M sodium phosphate buffer pH 7.5, 1% NP-40 containing PNGase F (New England Biolabs) (8 mU) for 18 h at 37 °C. Samples were further reduced with NaB^3H_4 (0.5 mCi) for 3 h, cold $NaBH_4$ (1 mg) was added and after 3 h, excess of reducing agent was destroyed with acetic acid up to pH 5 and samples were freeze-dried.

2.2.8. Gel filtration chromatography

Gel filtration chromatography was performed on a Biogel P-2 column (200–400 mesh, 2×100 cm) using water as eluent. Elution was monitored by liquid scintillation.

2.2.9. High pH anion exchange chromatography with pulse amperometric detection

A DX-300 Dionex BioLC system with pulse amperometric detector was used. Analysis was performed in a Carbopack PA-100 column equipped with a PA-100 precolumn using a gradient elution of 50 mM NaOH and 0–50 mM sodium acetate during 25 min. Flow: 0.6 ml/min.

3. Results and discussion

It is well-known that cruzipain molecules are able to interact with ConA and are active on Bz-Pro-Phe-Arg-pNA (Labriola et al., 1993). However, when a crude epimastigote extract is applied onto a ConA–Sepharose column, a fraction (about 10% of the total activity on the mentioned synthetic substrate) is not adsorbed, even after repeated percolations on a fresh lectin column. This fact prompted us to study the presence of this atypical subset of cruzipain molecules in the non-adsorbed fraction.

The unbound to ConA–Sepharose fraction was passed through a DEAE column and the active fractions were further loaded onto a Mono Q column (conditions a). An active peak with Bz-Pro-Phe-Arg-pNA was evidenced and was analyzed by gelatin containing gels after SDS–PAGE. By gelatinolytic analysis, at least three proteinases activities of about 200, 120, and 45–66 kDa were shown (Fig. 1A, lanes a, b, c, d, and e). Only the latter (fractions c, d, and e) was abolished by E-64 (Fig. 1B lanes c, d, and e) as occurs with control cruzipain (Fig. 1B, lane f) indicating that this fraction corrresponds to CPs (Cazzulo et al., 1990a). When the immunoreactivity of the same fractions towards a specific polyclonal cruzipain antibody was tested, only the lower molecular weight bands were recognized (Fig. 1C, lanes b, c, d, and e).

The lower MW band (Fig. 1A) inhibited by E-64 (Fig. 1B) and recognized by the polyclonal anti-cruzi-

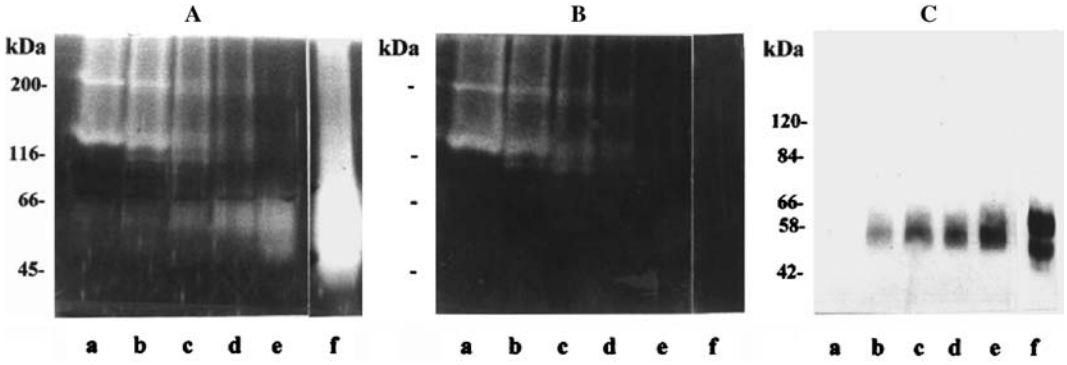

Fig. 1. Analysis by activity gels (A and B) and Western blot (C) of fractions obtained from the non-adsorbed to ConA–Sepharose column after Mono Q chromatography. The non-adsorbed to ConA–Sepharose column fraction was passed through DEAE–Sepharose column, the active fractions towards Bz-Pro-Phe-Arg-pNA were pooled, dialysed and loaded onto a Mono Q column under conditions a. Fractions were monitored by SDS–PAGE gelatin containing gels in the absence or presence of E-64 (Figs. 1A and B, respectively) and Western blot with specific polyclonal anti-Cr serum (Fig. 1C). The samples for Western blot were separated in 7.5% SDS–PAGE gels. Cruzipain isoforms were used as control (Cr).

pain rabbit antisera (Fig. 1C) would represent a subset of a typical cruzipain molecules called non-adsorbed cruzipain isoforms (NACrI).

In an attempt to further purify these NACrI, different Mono Q eluting conditions were tested. Three protein fractions were separated applying an isocratic step at 0.14 M on the linear gradient from 0 to 0.5 M NaCl (condition b in Section 2) (Fig. 2A): the first (Peak I), eluting between 0 and 0.14 M NaCl did not show activity on synthetic substrate; fractions eluting with 0.14 M NaCl (Peak II) contained a gelatinolytic proteinase corresponding to the band of 120 kDa in SDS–PAGE (Figs. 1A and B, lanes a, b, and c), and the third one (Peak III), eluting between 0.15 and 0.25 M NaCl, corresponded to the NACrI.

The gelatinolytic activity of the latter was completely abolished by the presence of E-64 and TLCK during incubation of the gel after electrophoresis (Fig. 2B, lane b and d, respectively); antipain, and leupeptin gave identical results (not shown), however the gel lanes incubated with PMSF (lane c) as well as 1,10-phenantroline, EDTA or pepstatin (not shown) were identical to that of the control (lane a) indicating that these inhibitors did not affect the enzyme activity. The inhibitory pattern obtained confirmed the nature of CPs of NACrI (Fig. 2A, peak III).

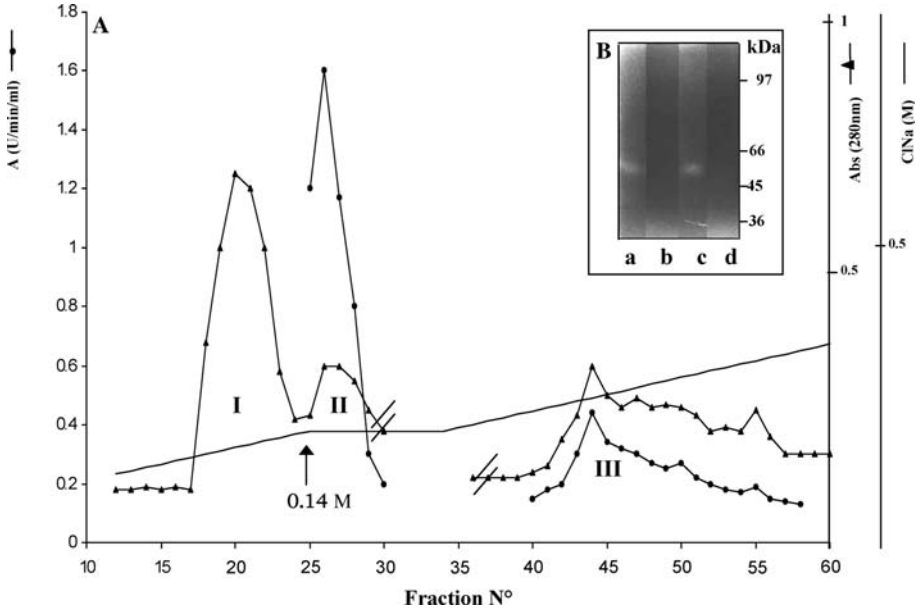

Fig. 2. Purification by Mono Q column chromatography of NACrI. (A) Protein profile of the non-adsorbed to ConA fraction after DEAE column as previously described, when applied to a Mono Q column in conditions b. Enzymatic activity towards Bz-Pro-Phe-Arg-pNA was monitored. (B) Analysis of Peak III (from A) in gelatin containing gels (a) in the absence of inhibitors, (b) in the presence of E-64, (c) PMSF, and (d) TLCK. Molecular weight of markers is indicated (in kDa) at the right side of the figure.

NACrI showed an endoproteinase activity. They were able to digest proteins such as azocasein, with an optimal pH 5 (Fig. 3A), BSA and denatured hemoglobin at pH 4 (Figs. 3B and C, respectively) and a blocked synthetic substrate Bz-Pro-Phe- Arg-pNA at optimal pH of 8.5 (Fig. 3D). Similar pH profiles were reported for cruzipain (Bontempi et al., 1984) (Cazzulo et al., 1990a). The activities were abolished by E-64, confirming it was due to CPs.

In another experiment, the non-adsorbed to ConA fraction was submitted to Cystatin–Sepharose affinity column chromatography and eluted with 20% *n*-propanol. The eluted fractions were analyzed by SDS–PAGE, (Fig. 4A, lane a), Western blot with the specific polyclonal anti-cruzipain serum (Fig. 4B, lane a) and gelatinolytic activity gels (Fig. 4C). NACrI and Cr were loaded in growing amounts according to similar activity on Bz-Pro-Phe-Arg-pNA. In order to eliminate excess of

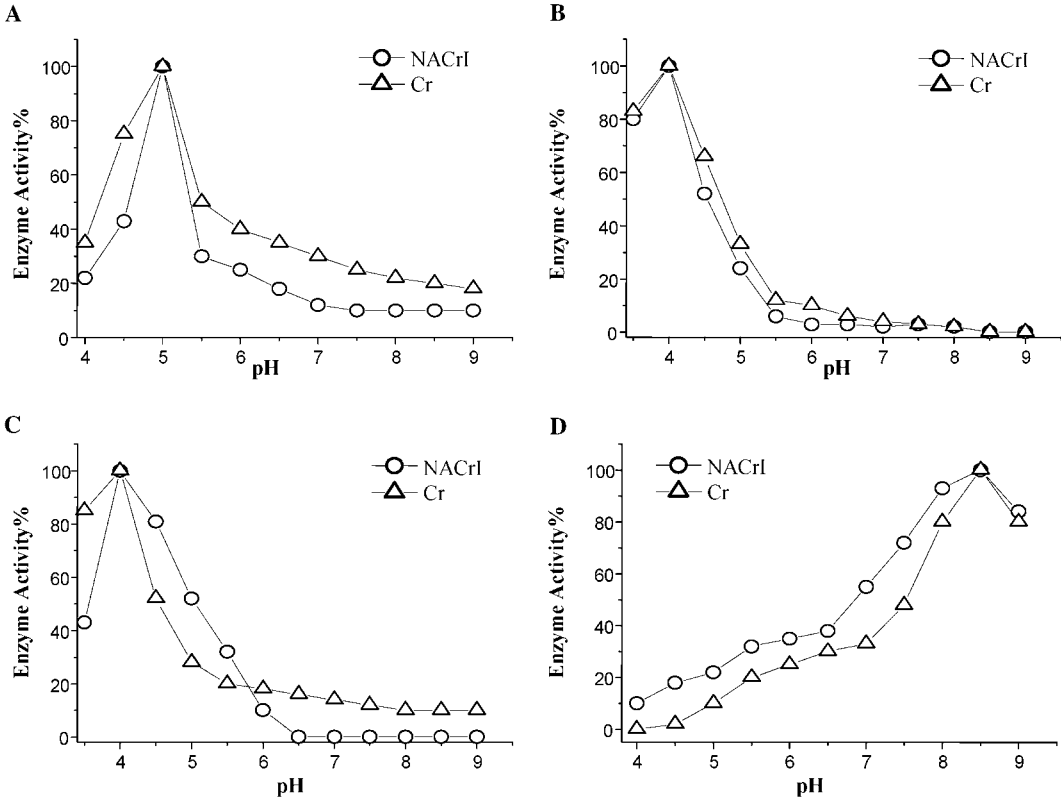

Fig. 3. NACrI activity on protein or a blocked peptidyl chromogenic substrate at different pHs. pH profiles for NACrI and cruzipain with azocasein (A), BSA (B), and hemoglobin (C) or with the chromogenic peptidyl substrate Bz-Pro-Phe-Arg-pNA (D). % activity values were calculated considering 100% to the highest activity obtained in each case.

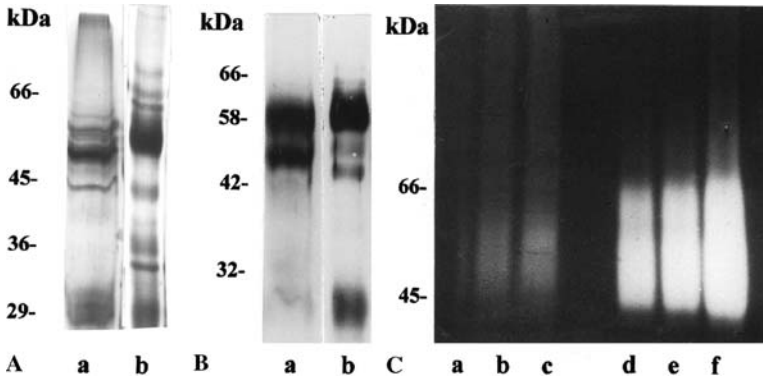

Fig. 4. Purification of NACrI by affinity chromatography on Cystatin–Sepharose column. (A) Silver stained 10% SDS–PAGE of purified NACrI (lane a) and cruzipain (lane b). (B) Western blot of purified NACrI (lane a) and control cruzipain (lane b) using anti-cruzipain antibody. (C) Gelatinolytic activity pattern of purified NACrI (a, b, and c) and cruzipain (d, e, and f) in a 7.5% SDS–PAGE. Similar amounts were loaded according to their activity on Bz-Pro-Phe-Arg-pNA (0.0025 U/min/ml (lanes a and d); 0.005 U/min/ml (lanes b and e); and 0.01 U/min/ml (lanes c and f).

SDS which could interfere in the enzyme activity, the gels were submitted to three washes in the presence of Triton-X-100 before incubation assay. The gelatinolytic activity of the non-adsorbed to ConA fraction after Cystatin–Sepharose, (lanes a, b, and c) resulted considerably lower than that of cruzipain (lanes d, e and f). This fact suggested the presence of isoforms with different peptidase activity. In both cases, the activity bands disappeared in the presence of E-64 (data not shown).

In order to investigate possible differences in the peptidase activity of NACrI and cruzipain, several blocked synthetic chromogenic peptides were tested (Table 1). NACrI and cruzipain hydrolyzed the same substrates but their order of preference was considerably different. CBZ-Arg-Arg-OMe-βNAm resulted the best substrate, among tested, for NACrI, cruzipain (Cazzulo et al., 1990a), as well as for the CP of *T. rangeli* (Labriola and Cazzulo, 1995) and for cathepsin B (Barrett and Kirschke, 1981).

The substrates following in order of preference were: CBZ-Ala-Arg-Arg-OMe-βNAm and Bz-Phe-Val-Arg-pNA for NACrI (Table 1), CBZ-Phe-Arg-OMe-βNAm and Bz-Pro-Phe-Arg-pNA for cruzipain, as well as for other trypanosomatid cysteine proteinases (Lonsdale-Eccless and Grab, 1987; North et al., 1983; Pupkis and Coombs, 1984). However, it should be noted that NACrI hydrolysed CBZ-Ala-Arg-Arg-OMe-βNAm and CBZ-Phe-Arg-OMe-βNAm with low percentages of activity (Table 1) while cruzipain digested CBZ-Phe-Arg-OMe-βNAm with 86%, indicating that NACrI present a lower preference for hydrophobic residues in P2 position than cruzipain.

Table 1
Utilization of peptidyl *p*-nitroanilides and peptidyl β-naphthylamides by NACrI

Substrate	Activity (U/min/ml)	Enzyme activity (%)	
		NACrI	Cr[a]
CBZ-Arg-Arg-OMe-βNAm	0.265	100	100
CBZ-Ala-Arg-Arg-OMe-βNAm	0.096	36	19
CBZ-Phe-Arg-OMe-βNAm	0.081	30	86
CBZ-Gly-Gly-L-Leu-βNAm	0	0	0
Bz-Phe-Val-Arg-pNA	0.095	100	14
CBZ-Gly-Pro-Arg-pNA	0.063	66	0
Bz-Val-Gly-Arg-pNA	0.049	52	0.6
Bz-Pro-Phe-Arg-pNA	0.039	41	100
Boc-O-Benzyl-Ser-Gly-Arg-pNA	0.037	39	0
Tosyl-Gly-Pro-Arg-pNA	0.024	25	0
Boc-Val-Leu-Gly-Arg-pNA	0.023	24	15
Tosyl-Gly-Pro-Lys-pNA	0.008	9	0
Bz-Arg-pNA	0.002	2	0.5
Gly-pNA, Lys-pNA	0	0	0

NACrI fraction activity was determined in the presence of 0.15 mM β-naphthylamides or 0.25 mM *p*-nitroanilides at pH 8.5.

[a] The data of percentage of cruzipain activity were calculated from Cazzulo et al. (1990a).

In addition, among the *p*-nitroanilides, Bz-Phe-Val-Arg-pNA resulted the best substrate for NACrI in contrast to cruzipain whose best sustrate was Bz-Pro-Phe-Arg-pNA but similar to cathepsin B-like cysteine proteinases of *Leishmania mexicana*, that do not bind to ConA, are sensitive to inhibitors specific for CPs and also prefer peptidyl substrates with the Phe-Val-Arg moiety (Robertson and Coombs, 1993).

It is noteworthy that NaCrI were able to hydrolyze a synthetic substrate containing as terminal aminoacid Lys instead of Arg (tosyl-Gly-Pro-Lys-pNA); on the other hand, the replacement of Arg by Leu terminal aminoacid (CBZ-Gly-Gly-L-Leu-OMe-βNA) abolished their activity. Similar results were reported for cruzipain and cysteine proteinases of *Leishmania* (Cazzulo et al., 1990a; Pupkis and Coombs, 1984).

Non-amino-blocked substrates, such Lys-pNA were neither hydrolyzed by NACrI nor by cruzipain, showing that both lack aminopeptidase activity. Similarly to cruzipain, and at variance to oligopeptidase B, specific for peptide substrates with basic aminoacid residues in the P_1 position (Ashall et al., 1990b), the activity of NACrI on Bz-Arg-pNA was almost nil.

In summary, NACrI degrade similar substrates than cruzipain but with different order of preference. The requirement for small peptide hydrolysis by NACrI includes a basic amino acid, preferently Arg to Lys in the P_1 position and at least one or more amino acids between P_1 and the amino-blocking group. These subclass of enzymes seem also to prefer a basic (Arg) or hydrophobic (Phe, Val) amino acid at the P_2 position. However, NACrI, in contrast to cruzipain, prefer Val in P_2 (Bz-Phe-Val-Arg-pNA, 100% for NACrI and 14% of activity for cruzipain) instead of Phe (Bz-Pro-Phe-Arg-pNA, 41% of activity for NACrI and 100% for cruzipain). Besides, the presence of Gly in the P_2 position did not decrease nor abolished the enzyme activity (Bz-Val-Gly-Arg-pNA, 52% and Boc-Val-Leu-Gly-Arg-pNA, 24% of activity, Table 1). Interestingly, the presence of Pro at the P_2 position, either with Arg or Lys in P_1, (CBZ-Gly-Pro-Arg-pNA 66% and Tos-Gly-Pro-Arg/Lys-pNA, 25 and 9% of activity, respectively in Table 1) did not abolish the activity as it was reported for cruzipain.

Taking into account that the non-adsorbed fraction corresponds to 10% of the total Bz-Pro-Phe-Arg-pNA activity (36 U), that NACrI were not the only enzymes present in the unbound to ConA fraction (Ashall, 1990a; Burleigh and Andrews, 1995; Santana et al., 1992), acting on this substrate and that this fraction was also active on Bz-Arg-pNA (21.6–28.8 U) (between 6 and 8%, among different experiments) while NACrI presented an almost negligible activity on the latter (Table 1), it was calculated that NACrI represent at least 2–4% of the total cruzipain isoforms present in epimastigotes of *T. cruzi*.

Cruzipain is a high mannose-type glycoprotein containing about 10% carbohydrate (Cazzulo et al., 1990b). This property has been applied to devise a purification method using ConA–Sepharose affinity chromatography (Labriola et al., 1993). In order to compare NACrI glycosylation pattern, both proteins were subjected to Endo H digestion and analysed by SDS–PAGE followed by immunoblotting. In contrast with cruzipain (Cazzulo et al., 1990b) (Fig. 5, lanes a and b), no change in the treated purified NACrI was observed (Fig. 5, lanes c and d) suggesting that NaCrI are either not glycosylated or they present a different glycosylation pattern.

To address this point, samples of purified NACrI and control cruzipain were digested in parallel with PNGase F and the released oligosaccharide fractions were labelled by reduction with NaB^3H_4. Analysis on Biogel P-2 (Fig. 6A) showed that both proteins released labelled oligosaccharides indicating that NACrI, as well as cruzipain, are glycosylated. In addition, while digested cruzipain showed two labelled peaks, NACrI presented only an included peak. When both included peaks were compared by HPAEC-PAD, differences were observed (Fig. 6B). While NACrI presented a major labelled component eluting between 15 and 20 min that co-migrated with a biantennary complex type oligosaccharide standard, the major components of cruzipain corresponded to high-mannose type oligosaccharides as already described (Parodi et al., 1995). Complex type N-linked oligosaccharide chains have been already reported as minor components of cruzipain (Barboza et al., 2003), however they would represent the major type glycans in NACrI.

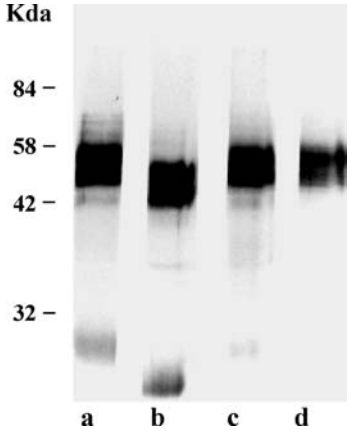

Fig. 5. Western blot analysis of purified NACrI subjected to EndoH treatment. (a) cruzipain; (b) Endo H treated cruzipain; (c) Cystatin–Sepharose purified NACrI; and (d) Endo H treated Cystatin–Sepharose purified NACrI.

A different subset of isoforms named cruzipain 2, encoded by multicopy genes, lacking the first potential N-glycosylation site have also been described (Lima et al., 1994). They were expressed in *Saccharomyces cerevisiae* without the C-T domain finding differences in activity and substrate specificity with truncated *r*-cruzipain (Lima et al., 2001) but no data of the glycosylation pattern of the natural protein have been reported in epimastigotes of *T. cruzi*. Unfortunately, the low amount of NACrI available after Cystatin–Sepharose purification precluded to date, a peptide sequence analysis in order to compare its possible homology to cruzipain 2.

In summary, this study reports the presence of a group of atypical cruzipain molecules which do not bind to ConA–Sepharose columns (NACrI), that represent a minor sub-class with a different oligosaccharide pattern and different preference on chromogenic substrates. Similar isoforms were also obtained from the RA strain, suggesting that their presence might be ubiquitous in *T. cruzi* strains and then, comparative studies might be extended to different stages of the parasite. Up to date, structure and functional studies on cysteine proteinases of *T. cruzi* were limited to cruzipain, the major cysteine proteinase of the parasite. But, under the light of our results, the presence of NACrI must be taken into

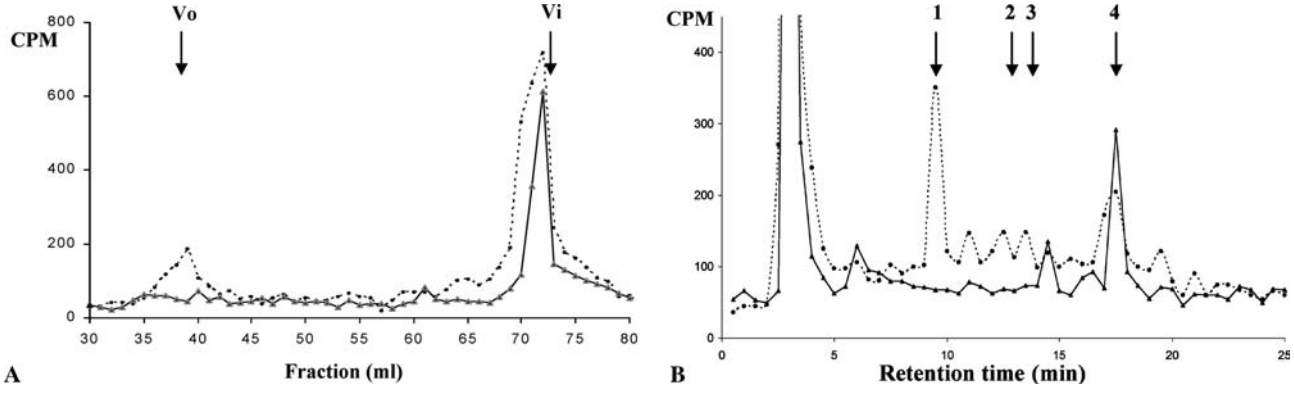

Fig. 6. Analysis of NACrI glycosylation after PNGase F digestion. (A) Fractionation on Biogel P-2 of the enzymatically released oligosaccharides labelled with NaB^3H_4. (B) Analysis on HPAEC-PAD of the oligosaccharides released after PNGase F digestion and NaB^3H_4 reduction. 1, $NAcGlc_2Man_3$; 2, $NAcGlc_2Man_5$; 3, $NAcGlc_2Man_6$; 4, $NAcGlc_4Man_3Gal_2$. –▲– purified NACrI - -●- - cruzipain.

consideration in future studies particularly regarding that the use of ConA has been a common practice to deplete cruzipain activity from *T. cruzi* homogenates when working on signaling pathways processes.

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