Kininogenase Activity by the Major Cysteinylo
Proteinase (Cruzipain) from Trypanosoma cruzi*

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The major isozyme of Trypanosoma cruzi cysteinylo proteinase (cruzipain) has generated Lys-bradykinin (Lys-BK or kallidin), a proinflammatory peptide, by proteolysis of kinogen. The releasing of this peptide was demonstrated by mass spectrometry, radioimmunoassay, and ileum contractile responses. The kinin-releasing activity was immunoabsorbed selectively by monoclonal antibodies to the characteristic COOH-terminal domain of cruzipain. To determine the hydrolysis steps that account for the kininogenase activity of cruzipain, we synthesized a fluorogenic peptide (o-aminobenzoyl-Leu-Gly-Met-Ile-Ser-Leu-Met-Lys-Arg-Pro-Pro-Gly-Phe-\(\text{-aminobenzoyl}|-\text{Leu}^{373}-\text{Ile}^{393}\)-hKng-NH\(_2\)), based on the sequence Abz-LGMISLMKRPPGFSPFRSSRI-NH\(_2\); MALDI-TOF, matrix-assisted laser desorption ionization time of flight; Z, benzoylxy carbonyl; MCA, methylcoumarin amide; E-64, trans-epoxysuccinyl-L-leucylamido-4-guanidinobutane; EDDnp, \(\text{N}^-\text{[2,4-dinitrophenyl]ethylenediamine;} \) HMWK, high molecular weight kininogen; LMWK, low molecular weight kininogen; HPLC, high performance liquid chromatography.

amastigotes. The parasitized host cells collapse, releasing newly transformed trypomastigotes to tissue fluids, and then they return to the bloodstream.

In the past few years, there has been significant progress in the characterization of cysteinylo proteinases from T. cruzi (8–12). Encoded by approximately 130 closely related genes (13), cruzipain(s) are synthesized as preproteins that undergo postprocessing by autocatalytic mechanisms (10). The mature form of these enzymes contains a papain-like catalytic domain in addition to a long and structurally unique carboxyl-terminal extension whose function remains unknown (14, 15). Despite the structural similarity to mammalian cathepsin L (8–10), the substrate-specificity properties of cruzipain are somewhat reminiscent of cathepsin B (15). The finding that cruzipain expression is increased markedly in replicating forms of this intracellular parasite (12, 16) has stimulated efforts to develop synthetic inhibitors as anti-parasite drugs (17–20).

Despite the wealth of structural and biochemical information on cruzipain(s), their biological role remains unclear. Recent studies revealed that some polymorphic genes are transcribed by the parasite, suggesting that the parasite may express several isozymes at different stages of development and/or stress conditions (21). Sequence analysis of some of these variant genes revealed that non-conservative amino acid substitutions tend to cluster in the catalytic domain, some of the changes being localized to positions that could conceivably influence the substrate specificity.

In the course of studies aimed at characterizing the substrate specificity of parasite-derived cruzipain (22, 23), we noticed that this enzyme shared some interesting properties with the human tissue kallikrein (24, 25). The ability to hydrolyze efficiently substrates containing Arg or a hydrophobic amino acid at the P1 position. In the present work we demonstrate that cruzipain releases bioactive kinins from human kininogen as well as from human plasma, even though kininogen, a member of the cystatin superfamily of inhibitors of cysteinylo proteases (26), has the ability to inactivate cruzipain (27, 28). Lys-bradykinin (Lys-BK) \(^1\) was demonstrated by mass spectrometry to be the released kinin. The cruzipain kininogenase activity was depleted by affinity chromatography using a monoclonal antibody to the characteristic COOH-terminal domain of the protease. In addition, the sites of cleavage were systematically confirmed, using a synthetic fragment of human

\(^1\) The abbreviations used are: Lys-BK, Lys-bradykinin; Abz, o-aminobenzoic acid; Abz-(Leu\(^{373}\)-Ile\(^{393}\))-hKng-NH\(_2\), human kininogen segment Abz-LGMISLMKRPPGFSPFRSSRI-NH\(_2\); MALDI-TOF, matrix-assisted laser desorption ionization time of flight; Z, benzoylxy carbonyl; MCA, methylcoumarin amide; E-64, trans-epoxysuccinyl-L-leucylamido-4-guanidinobutane; EDDnp, \(\text{N}^-\text{[2,4-dinitrophenyl]ethylenediamine;} \) HMWK, high molecular weight kininogen; LMWK, low molecular weight kininogen; HPLC, high performance liquid chromatography.

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kininogen labeled at the NH₂-terminus with α-aminobenzoic acid (Abz-Leu³⁷⁷-Ile³⁸⁵)-hKNG-NH₂ and related internally quenched fluorogenic peptides. We then used synthetic substrates based on the NH₂-terminal and COOH-terminal flanking regions of bradykinin in human kininogen to compare the substrate specificity requirements of two recombinant isoforms, namely cruzain and cruzipain 2, with the parasite-derived cruzipain. Finally, we also demonstrated that cruzipain was able to act via contact phase activation cascade by converting plasma prekallikrein into active kallikrein.

**EXPERIMENTAL PROCEDURES**

**Enzymes**—Cruzipain (GP57/51) was isolated from crude aqueous extracts of *Dm28c* strain epimastigotes as described previously (15). A single band of 51 kDa was observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis performed under denaturing conditions. As already reported by Murta et al. (9), the presence of the 57-kDa band can only be distinguished when the electrophoresis is carried out under native conditions. MALDI-TOF mass spectrometry yielded a mass of 43 kDa at the center of a wide peak typical of glycosylation. Two wide peaks of low intensity (60 kDa, corresponding to GP57 and 15 kDa corresponding to a degradation product) were also noticed. We subjected the enzyme to immunoabsorption with a monoclonal antibody to cruzipain (JO1), IgG, which binds to the epitope located in the COOH-terminal extension of cruzipain (29). The immunoabsorption was carried out by treating an agarose-protein G resin (Pharmacia Biotech Inc.) with 100 μl of either JO1 ascites or an unrelated ascites as control. The antibody-coated beads were washed with phosphate-buffered saline, 0.05% Tween, and 0.5 mg/ml bovine serum albumin and thereafter incubated with 2 volumes of cruzipain solution at 120 μg/ml. The supernatants were collected and assayed for hydrolytic activity using the fluorogenic substrate Z-Phe-Arg-MCA as already described (17).

Cruzan, the recombinant protein expressed in *Escherichia coli* without the COOH-terminal domain (10), was kindly supplied by Drs. J. H. McKerrow and J. C. Engel, from the University of California, San Francisco. Recombinant cruzipain 2 was expressed in *Saccharomyces cerevisae* essentially as described by Vernet et al. (30). Briefly, the final construct consisted of a chiمرة containing the prepregorin of the *S. cerevisiae α*-factor gene and the prorucuzapin 2 gene where the carboxy-terminal sequence region was deleted. Transformation and culture conditions were carried out as described in the aforementioned study. The transformed yeast cells were lyzed in phosphate-buffered saline with glass beads in a Brown homogenizer. After removing the cellular debris by centrifugation at 15,000 × g for 20 min, the supernatant was treated with 1% Triton X-100 to improve the solubilization of active recombinant protease. After precipitation with 1 volume of 100 mM sodium acetate buffer, pH 5, the supernatant was extracted with 1 volume of saturated butanol. Recovered in the aqueous phase, the active protease was precipitated further by affinity chromatography on a Sepharose 6B (Pharmacia). Partially purified cruzipain 2 migrated on sodium dodecyl sulfate-polyacrylamide gels as a 29-kDa band.

Human plasma prekallikrein and activated Hageman factor (factor XIIa) were purchased from Calbiochem. We also used heated human plasma containing 1.8 ml of the substrate solution was placed in the thermostatted cell compartment for 10 min before the enzyme solution was added, and the increase in fluorescence with time was recorded continuously for 10 min. The slope was converted into mol of substrate hydrolyzed/min based on the fluorescence curves for standard peptide solutions before and after total enzymatic hydrolysis. A solution of Abz-Arg-Arg-ArgOH was used as standard for the fluorescence measurements, which was prepared from tryptic hydrolysate of Abz-Arg-Arg-p-nitroanilide (Abz-Arg-Arg-pNA), and its concentration was determined from the absorbance at 405 nm, assuming ε₂₉₅ = 8,900 nM⁻¹ cm⁻¹ for p-nitroanilide. The enzyme concentrations for initial rate determinations were chosen so as to be less than 5% of the Km.

**Kininogenase Activity of Cruzipain**

**Kininogenase Activity on Human HMWK and LMWK**—The ability of cruzipain to cleave the human kininogens was evaluated incubating 1 μl of HMWK and LMWK (100 μg/ml) with active cruzipain (1.8 μM) in reaction mixtures containing 100 mM sodium phosphate buffer, 400 mM NaCl, pH 7.4. The sensitivity of the response was calibrated with standard solutions of bradykinin and Lys-BK (1–10 nm) in buffer and were kept in ice before use. The 1-cm path length cuvette containing 1.8 ml of the substrate solution was placed in the thermostatted cell compartment for 10 min before the enzyme solution was added, and the increase in fluorescence with time was recorded continuously for 10 min. The slope was converted into mol of substrate hydrolyzed/min based on the fluorescence curves for standard peptide solutions before and after total enzymatic hydrolysis. A solution of Abz-Arg-Arg-ArgOH was used as standard for the fluorescence measurements, which was prepared from tryptic hydrolysate of Abz-Arg-Arg-p-nitroanilide (Abz-Arg-Arg-pNA), and its concentration was determined from the absorbance at 405 nm, assuming ε₂₉₅ = 8,900 nM⁻¹ cm⁻¹ for p-nitroanilide. The enzyme concentrations for initial rate determinations were chosen so as to be less than 5% of the Km.

**Kininogenase Activity of Cruzipain on Human HMWK and LMWK**—The ability of cruzipain to cleave the human kininogens was evaluated incubating 1 μl of HMWK and LMWK (100 μg/ml) with active cruzipain (1.8 μM) in reaction mixtures containing 100 mM sodium phosphate buffer, 400 mM NaCl, 10 mM EDTA, pH 6.3, at 37 °C for 2 h. Ethanol (3:1, v/v) was added, and the mixture was centrifuged at 1,000 × g for 15 min. The kinin content in the supernatant was measured by radioimmunoassay, as described previously (35). After 4 h of incubation the kinin released by cruzipain from HMWK (molar ratio 1:40) was identified by MALDI-TOF mass spectrometry, VG-Platform mass spectrometer as a courtesy of Dr. Jörg von Hendel from Fisons Instruments, Mainz-Kastel, Germany, as follows. A 5-μl sample of the enzymatic reaction mixture was applied in a C-4 column (1 × 250 mm) and two solvent systems: (A) trifluoroacetic acid/H₂O (1:2,000) and (B) trifluoroacetic acid/ACN (1:2,000). The column was eluted to a mass spectrometer and UV detector (214 nm) at a flow rate of 20 μl/min with a 5–60% gradient of system B over 90 min and then to 90% of B in 10 min.

**Kinin Bioassays**—The biological activity of the released kinin was measured as isotonic contraction on isolated guinea pig terminal ileum. The isolated organ was suspended in a bath of 2-ml capacity in Tyrode’s solution at 37 °C containing 135 mM NaCl, 2.7 mM KCl, 1.36 mM CaCl₂, 0.5 mM MgCl₂, 0.36 mM NaH₂PO₄, 11.9 mM NaHCO₃, 5.04 mM glucose, pH 7.4. The sensitivity of the response was calibrated with standard solutions of bradykinin and Lys-BK (1–10 nm). Heat-treated human plasma (100 μl) and human HMWK (100 μl, 1 mg/ml) were added to the ileum preparation bath. After the equilibration period of 3 min, the activated cruzipain or recombinant cruzain solutions (0.4–40 nm) were added and the isotonic contraction recorded. The amounts of enzyme and substrates were adjusted in a manner that the released kinin fit into the dose-response curve for bradykinin. Similar experiments were performed in the presence of the bradykinin b₁-receptor antagonist (Hoe-140, N-Arg-Arg-Pro-Hyp-Gly-β-(2-thienyl)Ala-Ser-tetrahydrodipinquinoline-3-carboxylic acid-octahydroindole-2-carboxylic acid) (10 nm) and with E-64-pretreated cruzipain.

**Kinetic Analysis of the Activation of Purified Human Prekallikrein**—Purified human prekallikrein (2 μg) was incubated with active cruzipain (0.6 mM final concentration) in 100 mM NaCl, pH 7.4, 10 mM EDTA, pH 6.3, for 30 min to 1 h at 37 °C. After the incubation period cruzipain was inactivated irreversibly with E-64 (10 μM), and the kallikrein activity was measured using 20 μM N-Pro-Preo-Arg-MCA in 50 mM Tris-HCl buffer containing 0.015 mM NaCl, pH 7.5, at 37 °C. As experimental controls of the employed preparation of human plasma prekallikrein, it was also activated for 1 h with 0.6 mM human factor XIIa.
Hydrolysis was carried out for 10 min at 57 °C in 100 mM sodium phosphate buffer, 400 mM NaCl, pH 6.3, containing 10 mM EDTA. Values are means ± S.D.; †, Cleavage site; ND, not determined.

| Peptide Substrates                      | 1 (Cruzipain (GP 57/51)) | 2 (Cruzipain) | 2* (Cruzipain 2) |
|-----------------------------------------|--------------------------|---------------|------------------|
|                                         | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (μM.s$^{-1}$) | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (μM.s$^{-1}$) | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (μM.s$^{-1}$) |
| 1 Abz-LGMISLMKRPPQ-EDDnp                | 0.3 ± 0.05 | 1.5 ± 0.07       | 5,000             | 0.8 ± 0.09 | 0.4 ± 0.01       | 500              | ND              |
| 2 Abz-MISLMKRPPQ-EDDnp                  | 1.3 ± 0.4   | 0.6 ± 0.05       | 462               | 3.5 ± 0.8   | 0.1 ± 0.02       | 29               | 353             |
| 3 Abz-GFSPPRSSIQ-EDDnp                  | 2.7 ± 0.2   | 5.9 ± 0.4        | 2,185             | 2.7 ± 0.9   | 8.1 ± 1.4        | 3,000            | 9               |

*Determined under pseudo first-order conditions as described under “Experimental Procedures.”

**RESULTS**

**Hydrolysis of Fluorogenic Peptides Containing Met$^{379}$, Lys$^{380}$ or Arg$^{385}$-Ser$^{390}$ Bonds of Human Kininogen**—Internally quenched fluorescent peptides with residues flanking amino and carboxyl sides of the Met-Lys (peptides 1 and 2) and Arg-Ser bonds (peptide 3), as in the bradykinin region of human kininogen, were synthesized to study the behavior of the parasite-derived cruzipain as well the two recombinant isoforms, namely cruzain and cruzipain 2 (Table I). The HPLC analysis using authentic synthesized fragments demonstrated that all of the peptides were hydrolyzed only at Met-Lys or Arg-Ser bonds, except for the peptide 1, which was at low peptide concentrations hydrolyzed at the Gly-Met and subsequently at the Met$^{379}$-Lys$^{380}$ bond. A similar pattern was observed when these peptides were hydrolyzed by two recombinant isoforms. As demonstrated by the $k_{cat}/K_m$ values, Met-Lys or Gly-Met bonds were not hydrolyzed as efficiently by cruzain as by parasite-derived cruzipain. In contrast to the behavior of this isoform, recombinant cruzipain 2 has hydrolyzed the Met-Lys bond quite efficiently, being similar to the parasite-derived enzyme in this respect. On the other hand, the Arg-Ser bond in peptide 3 was hydrolyzed efficiently by cruzain, whereas cruzipain 2 displayed a very low $k_{cat}/K_m$ value. These results revealed that these two recombinant isoforms have distinct requirements for the $S_1-P_1$ interaction, suggesting that their substrate specificities might not be identical.

**Hydrolysis of Abz-(Leu$^{373}$-Ile$^{383}$)-hKng-NH$_2$: Characterization of the Cleavage Products**—Table II shows all the fragments resulting from the hydrolysis of Abz-(Leu$^{373}$-Ile$^{383}$)-hKng-NH$_2$ by cruzipain, following analysis by mass spectroscopy. The HPLC profiles (Fig. 1) show the time course of enzymatic hydrolysis of the peptide Abz-(Leu$^{372}$-Ile$^{383}$)-hKng-NH$_2$. The fragment Abz-LGMISLMKRPPGFSPFR was observed to accumulate, indicating that the initial hydrolysis occurs at the Arg-Ser bond. The fragments Abz-LG and KRPPGFSPFR (Lys-bradykinin) were detected as the reaction proceeded, suggesting that Gly-Met and Met-Lys bonds have similar susceptibility to hydrolysis by cruzipain under these conditions. Only traces of bradykinin were detected after a 45-min incubation, indicating that the Lys-Arg bond is not cleaved readily by cruzipain. It is noteworthy that Lys-BK and bradykinin were resistant to hydrolysis by cruzipain (20 nM) for up to 6 h of incubation. The same fragments were detected upon hydrolysis of Abz-(Leu$^{373}$-Ile$^{383}$)-hKng-NH$_2$ by recombinant cruzain; but in contrast to the activity of the parasite-derived protease the Met-Lys bond was only slowly hydrolyzed by the recombinant enzyme. This observation is consistent with the data presented in Table I, which also indicate the lower susceptibility of Met-Lys bond in the peptide Abz-(Leu$^{373}$-Ile$^{383}$)-hKng-NH$_2$. This peptide was resistant to hydrolysis by cruzipain 2.

**Cruzipain-released Bioactive Kinins**—The releasing assay of kinin was carried out with heat-treated human plasma (to inactivate preferentially the aminopeptidases that readily inactivate kinins) and purified form of bovine LMWK. The kinin released was detected by guinea pig ileum contraction assay, as shown in Figs. 2 and 3. At the concentrations used, human heat-treated plasma or cruzipain alone did not induce detectable smooth muscle contractile activity. The reaction was only observed at enzyme concentrations above 5 nM; no contraction was seen when the human plasma was incubated with E-64-treated cruzipain, confirming the reaction dependence on a thiol proteinase. Importantly, the addition of Hoe-140 has abrogated the ileum contractile response. Similar results were observed with human and bovine kininogen. The HPLC profile (Fig. 3) of the reaction mixture of purified bovine or human LMWK with cruzipain shows that Lys-BK was the major kinin released. Under the same assay conditions, recombinant cruzain and cruzipain 2 did not show any detectable kininogenase activity when incubated with human plasma or purified kininogens. Radioimmunoassay experiment confirmed that cruzipain releases kinin from human HMWK and LMWK as shown in Table III. MALDI-TOP mass spectrometry analyses of the material collected from the HPLC of the reaction mixture of human HMWK in the same conditions of Fig. 3 has shown that the Lys-BK was the major kinin released. The agarose gel affinity column containing a monoclonal antibody to the COOH-terminal domain of cruzipain depleted completely the proteolytic activity of the enzyme solution compared with the agarose gel column devoid of the antibody (Table III).

**Cruzipain Activity on Prekallikrein**—After being converted to α-kallikrein by factor XIIa, plasma prekallikrein possesses the ability to generate the chemical mediator bradykinin from high-molecular-weight kininogen (HMWK). To test the possibility of cruzipain indirectly influencing kininogenase activity via contact phase activators, human plasma prekallikrein was incubated with active cruzipain, and the releasing assay of kinin was carried out with heat-treated human plasma (to inactivate preferentially the aminopeptidases that readily inactivate kinins) and purified form of bovine LMWK. Bothrops kininogenase activity when incubated with human plasma or purified kininogens. Radioimmunoassay experiment confirmed that cruzipain releases kinin from human HMWK and LMWK as shown in Table III. MALDI-TOP mass spectrometry analyses of the material collected from the HPLC of the reaction mixture of human HMWK in the same conditions of Fig. 3 has shown that the Lys-BK was the major kinin released. The agarose gel affinity column containing a monoclonal antibody to the COOH-terminal domain of cruzipain depleted completely the proteolytic activity of the enzyme solution compared with the agarose gel column devoid of the antibody (Table III).

**DISCUSSION**

The recognition that proteolytic enzymes, other than kallikreins, were also capable of releasing kinins upon incubation with blood plasma was reported long ago by Rocha e Silva et al. (36), in their enzymatic studies with trypsin and Bothrops...
jararaca venom proteases. Subsequent work indicated that ficin, papain, and a cysteine-proteinase secreted by Clostridium histolyticum could also generate bradykinin-like peptides from plasma (37). More recently it has been reported that cysteine proteinases from Streptococcus pyogenes (38) and from Porphyras gingivalis, the major causative agent in the devel-

| Fragments | Molecular weight (calculated) | Observed ion (m/z) |
|-----------|-------------------------------|-------------------|
| Abz-Leu Gly | 307.2 | MH+ 308.2 |
| M-I-S-L-M | 593.3 | MH+ 594.3 |
| K-R-P-P-G-F-S-P-F-R | 1,187.7 | (MH2H)2+ 594.3 |
| Abz-Leu Gly-M-I-S-L-M-K-R-P-P-G-F-S-P-F-R | 2,052 | MH+ 2,053.0 |
| S-S-R-I-NH2 | 461.3 | MH+ 462.3 |

**Fig. 1.** HPLC profile of the hydrolysis of Abz-[Leu373-Ile397]-hKng-NH2 by cruzipain. The HPLC profile after incubation of the substrate (40 μM) with cruzipain (final concentration 4 nM) at 37 °C in 100 mM sodium phosphate buffer, 400 mM NaCl, pH 6.3, containing 10 mM EDTA is shown. The elution profiles were determined at 220 nm. a and b correspond to reaction times of 15 and 45 min, respectively.

**Fig. 2.** Guinea pig ileum contraction induced by kinin generated by cruzipain using heat-treated human plasma. Guinea pig ileum contraction was carried out with heat-treated human plasma (Pl). Solutions of 100 μl of human plasma and 20 nM cruzipain (Cz) were held in Tyrode’s solution bath for at least 1 min, and the response of guinea pig ileum was recorded to certify if the protein substrates themselves present contractile activity. Cruzipain solutions (5–40 nM), preactivated with 5 mM dithiothreitol for 10 min, were added directly to the bath containing 100 μl of heat-treated human plasma, and the isotonic contraction was recorded. Hoe-140 (10 nM) was supplemented to the bath 3 min before adding the enzyme and heat-treated plasma. To assess the participation of cysteine proteinases in the reaction, 5 nM dithiothreitol-treated cruzipain was treated with 1,000-fold excess of E-64 for 10–30 min at 37 °C immediately before use in the ileum contraction assay.
abolished by treating the enzyme with E-64, thus confirming bovine LMWK. The kininogenasic activity of cruzipain was Lys-BK as the major kinin released from human HMWK and which hardly cleaved Arg-Ser bond. In agreement with the bond is hydrolyzed poorly by cruzain, in contrast to cruzipain 2, received protease. However, it is noteworthy that the Met-Lys bonds are susceptible to hydrolysis by parasite-derived cruzipain, since the biological activity was specifically depleted by immunoaffinity columns prepared with a monoclonal antibody that reacts with their characteristic COOH-terminal domain.

In the present study, we demonstrate that the major cysteine protease from the pathogenic parasite T. cruzi also displays kinin-releasing activity. The biochemical characterization of this reaction was initially carried out with the fluorescent labeled peptide, Abz-(Leu373-Ile393)-hKng-NH2, the data indicating that Arg-Ser, Gly-Met, and Met-Lys bonds were successively cleaved, thus releasing Lys-BK. The same pattern of cleavage was observed using a series of internally quenched synthetic peptides. The data showed that all enzymes have comparable substrate specificity requirements of recombinant cruzain, cruzain 2, and parasite-derived cruzipain were compared using synthetic peptides. The data showed that all enzymes have hydrolyzed the same peptide bonds. The recombinant cruzain has a marked preference for Arg over Met at the P1 position of the synthetic substrates (Table I), in contrast to cruzipain 2, which hardly hydrolyzes Arg-Ser bond but cleaves Met-Lys bond with almost the same efficiency of the parasite-derived cruzipain. Accordingly, the relative endurance of Met-Lys or Arg-Ser bonds to the action of recombinant cruzain or cruzipain 2, respectively, might explain their failure to develop significant kallikreinase activity in assays with human plasma. Notwithstanding the above arguments, it should be pointed out that the genetically engineered cruzain (10) and cruzipain 2 do not contain the highly glycosylated COOH-terminal domain (130 residues) present in the native form of cruzipain. The functional role of this long and unique extension remains unknown, but it is conceivable that its presence in the intact proteinase might sterically hinder the binding. As a consequence, it decreases the susceptibility to inhibition by the cystatin-like inhibitory domains of human/bovine kininogen slowing the rate of association to the inhibitory domain; however, the kinetics of the kinin-releasing reaction might be favored. It

TABLE III

| Kinin released | HMWK | LMWK |
|----------------|------|------|
| Null           | 190  | 65   |
| Monoclonal antibody-anti-cruzipain | 0   | ND*  |
| Agarose-gel    | 156  | ND*  |
| Cystatin       | 42   | ND*  |

* Not determined.

FIG. 4. Cruzipain activity on prekallikrein. Plasma prekallikrein (2 μg) was incubated with factor XIIa for 1 h (●) and activated cruzipain (final concentration of 0.6 mM) for 30 min (□) and 1 h (■) in the same buffer described under “Experimental Procedures.” After the incubation period cruzipain was inhibited completely with 10 μM E-64. Aliquots of the reaction mixtures were removed, and the kallikrein activity was identified using the fluorogenic substrate d-Pro-Phe-Arg-MCA by the production of 7-amino-4-methylcoumarin registered continuously at 37 °C for 10 min at λ ex 360 nm and λ em 480 nm. Prekallikrein activity (△) was also tested as a control experiment.
is worthwhile mentioning that reversible conformational changes are thought to affect the enzymatic properties of cruzipain, this phenomenon being tentatively attributed to the COOH-terminal extension (15). Independently from the role, if any, of the COOH-terminal domain, the ability of cruzipain to cleave the flanking sequences of BK must be the primary requirement for the expression of a kininogenase activity. The identification of polymorphic variants of cruzain in the parasite genome (21) suggests that cruzipain isoforms displaying different substrate specificities and/or organelle compartmentalization may exist. Furthermore, we have demonstrated that cruzipain activates human plasma prekallikrein; therefore cruzipain can act directly on the physiological kininogenase system generating active plasma kallikrein to target the kallikrein-mediated processing cascade. The capability to generate vasoactive kinins in the bloodstream or interstitial fluids may qualify the structurally diverse T. cruzi cysteine proteinases as factors of virulence in Chagas’ disease.

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