

# Induction of Synthesis of the Rat Cystatin S Protein by the Submandibular Gland During the Acute Phase of Experimental Chagas Disease

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*Rats experimentally infected with Trypanosoma cruzi Y strain exhibited hypertrophy of the submandibular gland at 18 days after infection. SDS-PAGE of infected rats saliva revealed the presence of an additional band with an apparent molecular weight of about 13KDa. Electrophoresis of protein saliva and immunochemical analysis with antibody against rat cystatin S confirmed that the protein was identical to that induced by beta adrenergic stimulation.*

Key words: submandibular gland – Chagas disease – rat cystatin S

The observations that in Chagas disease, there occurs enlargement of the submandibular gland in rats at 18 and 32 days after infection with *Trypanosoma cruzi* was first reported by Alves (1977). At histologic level, the main changes observed were acinar development and delayed ductal maturation that are more evident in the developing granular ducts (Alves & Machado 1980). The enlargement induced by *T. cruzi* infection (acute phase) is mediated through  $\beta$ -receptors since it is blocked by propranolol (Alves & Machado 1986). The histological changes observed in submandibular glands during the acute phase of Chagas disease are similar to those caused by administration of sympathomimetic drugs especially isoproterenol (Schneyer & Shackelford 1963, Ekfors et al. 1972, Yamashina & Barka 1972).

Ultrastructural observations of the enlarged glands under isoproterenol (IPR) treatment revealed that the content of acinar secretory granules was altered (Takahana & Barka 1967, Kanda et al. 1968) suggesting that the secretory products of these glands may be chemically different (Robinovitchi et al. 1977).

IPR not only stimulates overall protein synthesis in the submandibular gland, but also induces the synthesis of specific proteins that are absent or occur in very low concentration in unstimulated glands. In fact, a specific protein (rat cystatin S) has been identified in the submandibular gland of IPR treated-rats (Menaker et al. 1974, Naito 1981, Barka et al. 1986, Shaw

et al. 1988, Shaw & Barka 1989, Shaw et al. 1990), after teeth amputation (Abe et al. 1979, Yagil & Barka 1986) and after papain administration (Naito et al. 1992). Measurements of the inhibitory activity of purified rat cystatin S revealed that this protein is a potent inhibitor of cysteine proteinases (Shaw et al. 1988).

Recently, Alves (1990) reported in the acinar cells of 18 days infected-rats, secretory granules with an unusual fine structure, similar to that described by Kanda et al. (1968) in IPR-treated rats. Such finding has suggested that those granules could represent secretion of rat cystatin S (inhibitor of cysteine proteinases) or another protein similar to those described under  $\beta$ -adrenergic stimulation.

In the present paper, biochemical methods were used in order to identify an inhibitor of cysteine proteinases as an effort to understand the role of this inhibitor in the rat submandibular gland during the acute phase of Chagas disease.

## MATERIALS AND METHODS

*Animals inoculation* – Sixteen male Holtzman rats aged 27-29 days were inoculated intraperitoneally with 0.15 ml of blood containing about 300.000 trypomastigotes of Y strain of *T. cruzi* (Silva & Nussenzweig 1953). Living trypomastigotes were detected in the blood stream of all inoculated animals on the day 10 after infection.

*Saliva collection* – 18 days after inoculation, the infected rats (9) and no infected controls (7) were anesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg), placed in supine position and tracheotomized. After dissecting out, the sublingual glands and ligating its duct, the left submandibular duct was cannulated with a glass cannule. Stimulation of

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secretion was achieved by an intraperitoneal injection of isoproterenol (16 mg/kg). Saliva was collected during 1 hr into Ependorf tubes kept on ice. Total protein was determined according to Bradford (1976).

**Histological study** – Before stimulation, the right submandibular gland (SBM) was removed and fixed during 12 hr at 4 °C with paraformaldehyde buffered in 0,1M PBS at pH 7.4. It was then dehydrated through a graded series of ethanol, embedded in glycol methacrylate JB-4 (Polysciences); sectioned at 3 µm and stained with 1% toluidine blue dissolved in 1% sodium tetraborate or hematoxylin and eosin.

**Quantification of rat cystatin S** – The concentration of rat cystatin S of SBM saliva was measured by a solid phase enzyme-linked immunoabsorbent assay (ELISA) by using a specific antibody against this protein. This antibody was raised in rabbit against rat cystatin S from saliva of IPR treated rats (Naito 1981). Briefly, wells of multi-well plates were coated with saliva. The wells were washed and the remaining sites were blocked with 1% bovine serum albumin. The incubation of the wells with diluted rabbit anti-rat cystatin S at 1:800, dilution in 1% BSA, was followed by repeated washings and incubation with peroxidase-coupled goat anti-rabbit IgG in 1:2000 dilution (Sigma). After washings, peroxidase activity was measured by the absorption at 492 nm using H<sub>2</sub>O<sub>2</sub>/O-phenylenediamine dihydrochloride as substrate. In the ELISA described above the antigen (rat cystatin S) was quantified under conditions in which the logarithm of the concentration of the protein was linearly related to the optical density (OD). A linear regression analysis of the log protein concentration versus OD 492 indicated that probability of regression was 0.979758 ( $r^2 = 0.96$ ). Statistical analysis of the data was performed by means of a *t* test.

**SDS-PAGE of saliva and western blot** – Saliva samples of each control and infected rats were used for electrophoresis and western blot. Electrophoresis was performed in 12.5% polyacrylamide gels, according to Laemmli (1970). The electrophoretic transfer of proteins from sodium dodecyl sulfate (SDS)-polyacrylamide gels to nitrocellulose papers was carried out by using the conditions described by Burnetter (1981). The western blot were immunostained essentially as described by Barka et al. (1986).

## RESULTS

**Histological observations** – The histological aspect of the normal SBM as seen in the control rats, has already been described (Jacoby &

Leeson 1959, Alves & Machado 1980). The submandibular glands of the infected rats exhibit a histological picture that confirms our early finding (Alves & Machado 1980, 1986): developed acini, as judged by their size, and scanty cytoplasmic basophilia. As the adult appearance of the acini was reached earlier in the infected animals suggesting an acceleration of the acinar development.

**ELISA** – By ELISA we have measured the relative concentration of the rat cystatin S in the submandibular gland saliva of control and infected rats. The results are shown in Figs 1A and B. There was a significant difference between the control and infected rats (Fig. 1B). This results indicate that rat cystatin S is synthesized and secreted in submandibular gland.

**SDS-PAGE and Western blot** – Cystatin S was seen as a single band with an apparent molecular weight of about 13 KD in the silver-stained SDS-polyacrylamide gels of the infected

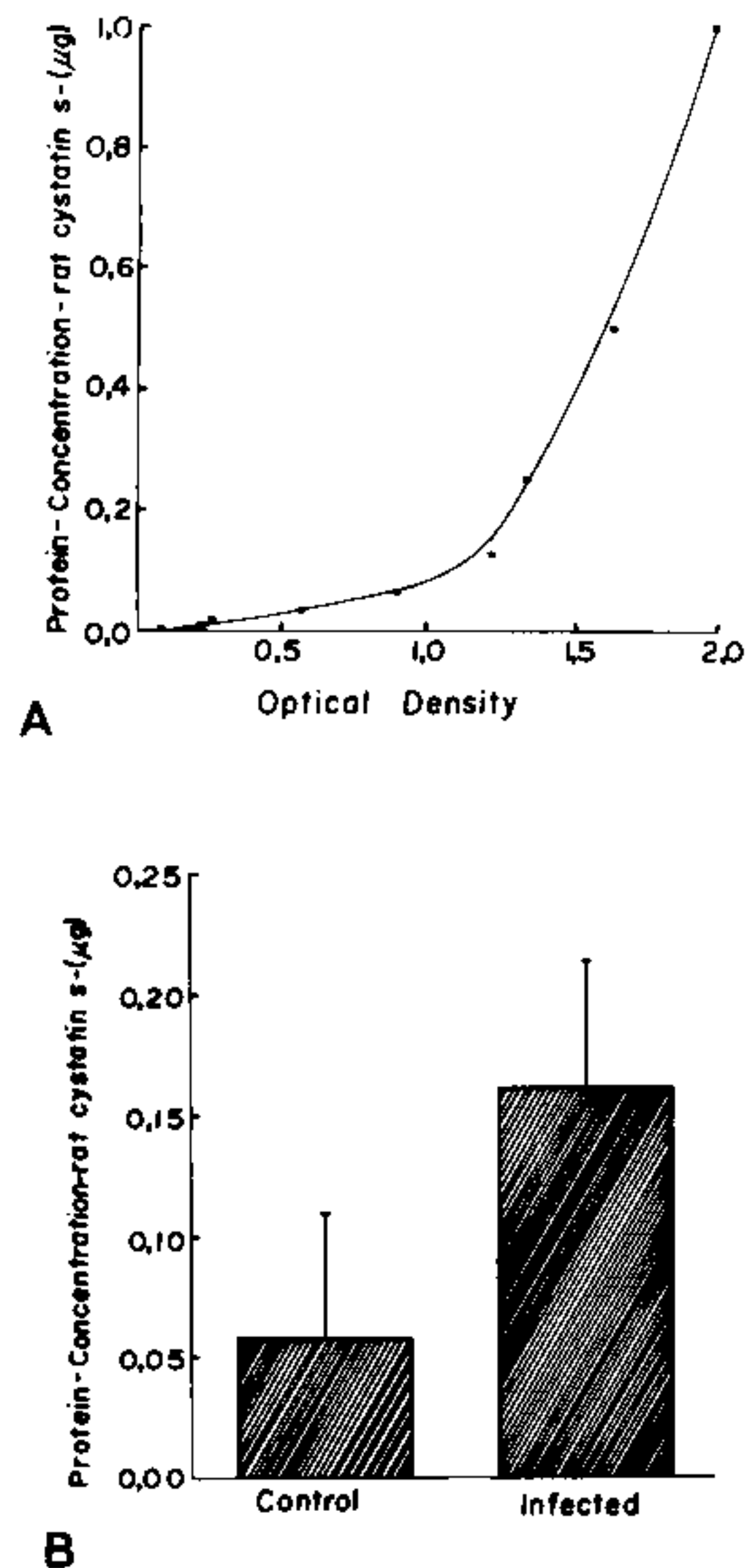


Fig. 1: protein concentration, as determined by the ELISA value of a standard curve of cystatin S (A) and in the saliva of control (7) and infected rats (9) as measured by ELISA ( $t = -2,80 - P < 0.05$ ) (B). The linear regression analysis between the log of the protein concentration and the optical density indicated that the probability of regression was 0,979758 ( $r^2 = 0.96$ ).

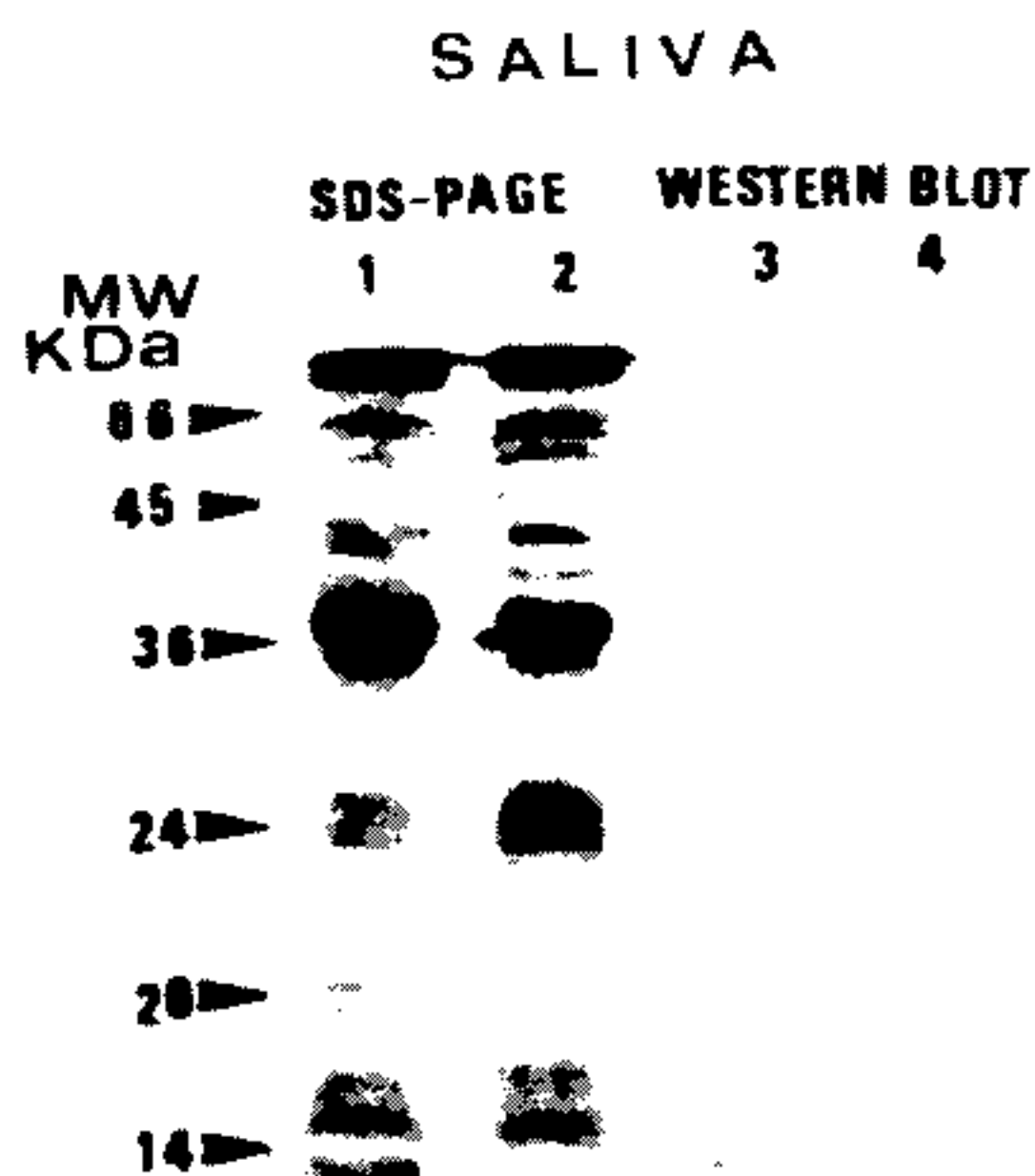


Fig. 2: SDS-PAGE and western blots of saliva of control (7) and infected rats (9). Lanes 1 and 3, saliva of infected-rats; lanes 2 and 4, saliva of control rats. Saliva samples were electrophoresed on 12.5% SDS-polyacrylamide gels; the proteins were transferred electrophoretically onto nitrocellulose paper and immunostained using anti-cystatin S antiserum. Arrows indicate molecular weight markers (from Albumin bovine: 66 KD; ovalbumin 45 KD; Glyceraldehyde-3-P-dehydrogenase: 36 KD; Trypsinogen, Bovine pancreas: 24 KD; Trypsin Inhibitor, Soybean: 20 KD;  $\alpha$ -Lactalbumin, Bovine milk: 14 KD).

rats saliva. Immunoblot using anti-rat cystatin S antiserum revealed a single protein band with the same electrophoretic mobility in the saliva obtained from infected rats. No trace of this protein was observed in the control saliva. SDS-PAGE and immunoblots of such gels corroborated the findings obtained by the ELISA.

#### DISCUSSION

Acinar hypertrophy accompanied by ductal atrophy, as observed in the submandibular gland of rats affected by *T. cruzi* (Alves & Machado 1980) is similar to those observed in glands enlarged after treatment with adrenergic agents, especially isoproterenol (Schneyer & Shakelford 1963, Ekfors et al. 1972, Yamashina & Barka 1973) or teeth amputation (Yagil & Barka 1986). The hypertrophic enlargement of the submandibular glands produced by chronic administration of pharmacological doses of IPR is also accompanied by the expression of a number of proteins and glycoproteins that are absent or occur in very low concentrations in unstimulated glands. The best characterized substances are those of the family of proline-rich proteins (Mirels et al. 1987) and a 13 KD protein (rat cystatin S) inducible by IPR injection (Naito 1981, Naito & Suzuki 1981) or teeth amputation (Yagil & Barka 1986, Abe et al. 1979) or papain administration (Naito et al. 1992).

It has been suggested that in the acute phase of Chagas disease increased levels of plasma catecholamines could accelerate acinar maturation or stimulate protein synthesis (Alves & Machado 1986). Increase of the circulating levels of catecholamines would then be expected as a result of large scale destruction of adrenergic nerve terminals in the acute phase, as does occur in the heart (Machado et al. 1984) and in the parotid gland (Alves 1990). It appears, therefore, that the circulating catecholamines would act as a  $\beta$  adrenergic agonist directly on the submandibular acina cells and, like IPR, could regulate the expression of gene coding for specific proteins (Ziemer et al. 1982).

Our data obtained by ELISA, SDS-PAGE and western blots are indicative of the presence in the rat submandibular gland with Chagas disease of a protein which is electrophoretically and immunologically identical with rat cystatin S induced by IPR (Naito 1981). Although no quantitative study has been performed in order to compare levels of rat cystatin S in Chagas disease, IPR and teeth amputation, morphological data suggest that in infected rats the process was less effective than in the others. Also, as the changes produced by IPR and teeth amputation are reversible, the same happens in Chagas disease, since the enlargements returns to normal, as shown by Alves and Machado (1980).

The role played by rat cystatin S and its relationship to the alterations of the submandibular gland during the acute phase of Chagas disease are yet unknown. However, increases in cystatin S concentration within tissue have been associated with pathophysiological changes, including inflammation (Brzin et al. 1984, Hopsu-Havu et al. 1983), myelin destruction (Kopitar et al. 1983), suggesting that the production of cystatin S may be a response to tissue injuries.

In fact, it has been reported in rat submandibular gland, induction of cystatin S by a variety of systemic agents that are capable of causing tissue damage by a different mechanism (Cohen et al. 1993). Furthermore, in rats with Heymann nephritis, a chronic autoimmune disease cystatin was expressed within the cells of renal proximal tubules (Cohen et al. 1990). On the other hand, during acute phase of Chagas disease in spite of the absence of appreciable inflammatory process in the submandibular gland (Alves & Machado 1980), these glands undergo severe reduction of sympathetic (Machado et al. 1984) and parasympathetic innervation (Alves & Machado 1986).

Together with our previous findings the present data indicate that the submandibular

glands respond to denervation caused by Chagas disease in a manner similar to that observed in isoproterenol treated rats. Thus, cystatin S production may represent one possible physiological response to tissue injury. Our findings also support the hypothesis of Choen et al. (1993), in which detection of inducible cystatin may serve as a sensitive biological marker of tissue damage in salivary glands. Further studies are needed to clarify the significance of cystatin S induction in the pathogenesis of salivary glands during acute phase of Chagas disease.

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