

# Biochemical Characterization of a Trypanosomatid Isolated from the Plant *Amaranthus retroflexus*

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*A protozoan flagellate has recently been isolated from Amaranthus retroflexus. This plant grows near economically important crops in southeastern Spain, which are known to be parasitized by Phytomonas spp. The present study focuses on the characterization of the energy metabolism of this new isolate. These flagellates utilize glucose efficiently as their primary energy source, although they are unable to completely degrade it. They excrete ethanol, acetate, glycine, and succinate in lower amount, as well as ammonium. The presence of glycosomes was indicated by the early enzymes of the glycolytic pathway, one enzyme of the glycerol pathway (glycerol kinase), and malate dehydrogenase. No evidence of a fully functional citric-acid cycle was found. In the absence of catalase activity, these flagellates showed significant superoxide dismutase activity located in the glycosomal and cytosolic fractions. These trypanosomes, despite being morphologically and metabolically similar to other Phytomonas isolated from the same area, showed significant differences, suggesting that they are phylogenetically different species.*

Key words: *Amaranthus retroflexus* - *Phytomonas* - energy metabolism - superoxide dismutase

It is known that plants can be parasitized by members of the Trypanosomatidae family, artificially grouped into the genus *Phytomonas* by Donovan (1909) to differentiate plant from animal trypanosomatids. Our group has isolated plant flagellates from different economically important fruit crops in southeastern Spain (Sanchez-Moreno et al. 1995), and they were consequently classified as members of the genus *Phytomonas* (Uttaro et al. 1997). In another study, we demonstrated that flagellates from different fruits have a common origin and that the same flagellates parasitize several species of plants (Fernandez-Becerra et al. 1997). Now, we sampled wild plants near the crops to determine whether these could serve as reservoirs for the parasites. In this way, we isolated two new flagellates, one in the stems of clover (*Trifolium glomeratum*) and another in the stems of amaranth (*Amaranthus retroflexus*). Both isolates were characterized by isoenzymatic analysis and by kDNA analysis using five different restriction endonucleases, verifying these with the isolates from tomato, cherimoya and mango fruits in the same zone (Sanchez-Moreno et al. 1998).

The isolates from clover were identical to those isolated from the fruits, but the isolates from amaranth were completely different. These isolates presented activity of iso-propanol dehydrogenase, the marker enzyme of the genus *Phytomonas* (Uttaro et al. 1997). In the present work, we analyse these isolates from a metabolic standpoint to test whether they constitute a species different from that found in tomato, cherimoya and mango fruits and in clover stems.

## MATERIALS AND METHODS

*Organisms and in vitro culture* - Vegetative samples from *A. retroflexus* were collected from the southern coast of Spain, in the hydrogenic crops. Small sections of the roots, stems and leaves were homogenized in phosphate buffered saline and examined by phase-contrast microscopy. The samples found to be infected with flagellates (stems) were cultivated in 25 ml of Grace's medium supplemented with 10% heat-inactivated foetal calf serum, as previously described Sanchez-Moreno et al. (1998). Aliquots of 5 ml were taken every 24 h for four days during the experiment. The number of parasites was determined in a Neubauer haemocytometric chamber, after which the cells were removed by centrifugation at 600 x g for 10 min. The pH of the parasite-free supernatants was measured before they were frozen at -80°C for subsequent measurements of glucose consumption, using the enzyme method of Bergmeyer (1988).

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**<sup>1</sup>H NMR spectroscopy and metabolite identification** - For the spectroscopic studies, 5 ml of a four-day-old culture were centrifuged at 1,500 g for 10 min in at 4°C. The pellet was discarded, and the supernatant, freed of parasites, was stored at -20°C until use for <sup>1</sup>H NMR. The <sup>1</sup>H NMR spectra were obtained at 300 Mhz on a Bruker AM-300 spectrometer, which was operated at pulse mode in Fourier transformation with quadrature detection (Gilroy et al. 1988). The temperature of the probe was maintained at 27°C. The acquisition parameters were pulses of 90° in radius and a wavelength of 3,287.5 Hz, 8 sec recycle time and 160 accumulations. The chemical displacements were expressed as part per million (ppm) relative to a tetramethyl xylene signal.

The chemical displacements used to identify the respective metabolites were consistent with those described in the literature (Sánchez-Moreno et al. 1992). The resonance of the peaks observed in the spectra were assigned to a given metabolite by the addition of standards to the fresh medium and the measurement of their chemical shifts.

Metabolites were quantified enzymatically as described by Bergmeyer (1988). All the experiments and determinations were repeated five times.

**Cell fractionation** - Homogenates of trypanosomatids were prepared by grinding the cell paste with silicon carbide abrasive grain. After cell lysis, centrifugation was carried out following the method described by Steiger et al. (1980). The different subcellular fractions were obtained as follows: unbroken cells and nuclei were sedimented at 1,500 g for 10 min (nuclear fraction: N). From the resulting cytoplasmatic extract (post-nuclear: PN), a granular fraction was obtained by centrifugation at 5,000 g for 10 min (large organelles: LG). The supernatant was centrifugated at 14,500 g for 10 min, and the sediment kept as the small-organelle fraction (SG), while the supernatant was again centrifugated at 140,000 g for 60 min. The pellet was resuspended and kept as the microsomal fraction (M). The remaining supernatant was kept as the soluble fraction (S). For each of the different fractions obtained, the concentration of proteins was determined by the Bio-Rad test, based on the method of Bradford (1976) and aliquots were stored at -80°C until used. All the centrifugations and manipulation of the samples were performed at 4°C.

**Enzyme determinations** - hexokinase (HK), pyruvate kinase (PK), phosphofructokinase (PFK), phosphoglucose isomerase (PGI), glyceraldehyde-phosphate dehydrogenase (GADPH), triosephosphate isomerase (TIM), fructose 1,6-biphosphate aldolase (ALD) and the enzyme of glycerol metabolism, glycerol kinase (GK) were determined according to Missel et al. (1986). Pyruvate kinase

(PK) activity was measured after the addition of fructose 2,6-bisphosphate as described by Van Schaftingen et al. (1985). Malate dehydrogenase (MDH) was assayed according to Opperdoes et al. (1981). Fumarase (FUM) was measured according to Klein et al. (1975) and superoxide dismutase (SOD) activity was assayed by Paoletti and Moccali (1990).

**Electrophoresis** - SOD activity was also demonstrated using an on gel assay. The proteins were separated on 8-25% native polyacrylamide gradient gels and on pH 3 isoelectric focusing gels using a Pharmacia Phast-system. SOD activity staining was carried out with the same reagents as the NBT assay (Beyer & Fridovich 1987). Gels were soaked in 10 ml of the stock solution and illuminated with UV light until enzyme activity appeared as a colourless band on a blue background.

To distinguish between Cu-Zn and Mn or Fe dependent SOD, activity was measured on gel assay in the presence of 20 mM potassium cyanide and after treatment with 5 mM H<sub>2</sub>O<sub>2</sub>, 1 mM EDTA for 45 min at 37°C. Molecular weight was visualized by silver staining (Pharmacia).

## RESULTS AND DISCUSSION

Flagellates isolated from *A. retroflexus* and adapted to the *in vitro* culture presented a growth curve similar to that described for other flagellates isolated from tomato and cherimoya fruits (Sánchez-Moreno et al. 1995). They exhibited an exponential growth phase during the first four days of culture (Fig. 1A) followed by the stationary phase, reaching a cell density of 2.10<sup>7</sup> cells/ml by day 4 of the culture.

The flagellates included in the genus *Phytomonas*, like other trypanosomatids, depend primarily on glycolysis to obtain energy (Sánchez-Moreno et al. 1992). This capacity to utilize glucose is a characteristic that allows them optimal adaptation to the host, especially in view of the fact that these parasites lack a system for carbohydrate storage. In the flagellates isolated from amaranth, glucose consumption from their medium proved inversely proportional to their growth (Fig. 1B). Parallel to this consumption was a significant drop in the pH of the medium (Fig. 1B), reaching the strongest acidification at the beginning of the stationary phase. This was because the glucose was not completely degraded to CO<sub>2</sub> and part of the carbon skeleton was excreted as fermentative metabolites, as demonstrated in other flagellates (Sánchez-Moreno et al. 1992, Blum 1993).

To identify these metabolites, we used <sup>1</sup>H NMR spectroscopy and, when the spectra of the cell-free media in which the parasite had grown for four days (Fig. 2B) were compared with those of fresh

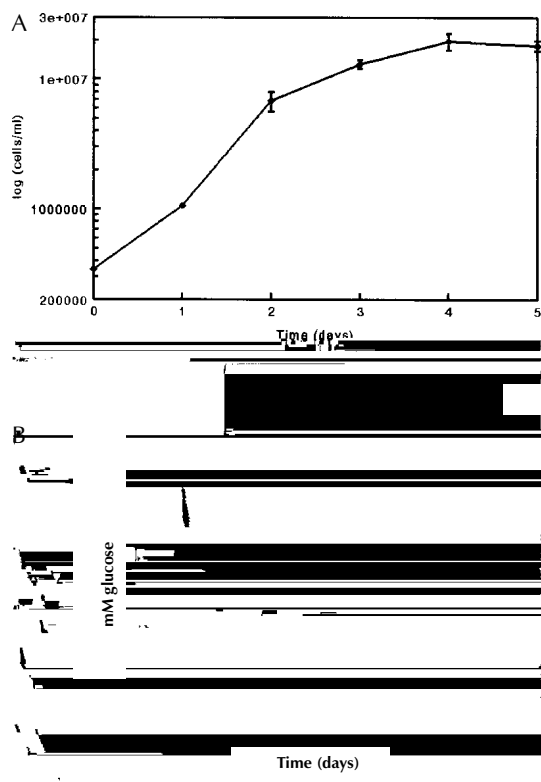


Fig. 1-A: growth curve of flagellates isolated from *Amaranthus retroflexus*, cultured in Grace's medium; B: glucose consumption (-▲-), pH variation of Grace's medium during the growth of flagellates isolated from *A. retroflexus* (-◆-) and excretion of  $\text{NH}_4^+$  (-△-).

and consequently *Phytomonas* cannot produce succinate by the inverse pathway of the Krebs cycle, as demonstrated in other trypanosomes (Fairlamb & Opperdoes 1986). Therefore, succinate is produced as an end product because one part of the glyoxylic cycle is active in these organisms (Sánchez-Moreno et al. 1992). Another aspect of the intermediary metabolism of plant flagellates that has received some attention is ornithine-arginine metabolism. Trypanosomatid genera are differently equipped to promote the interconversion of ornithine-citruline-arginine, a metabolic cycle equivalent to the urea or Krebs cycle of vertebrates (Camargo 1999). Trypanosomatids depending on their enzymatic repertoire require one of these amino acids in their growth medium to grow, and excrete either urea or ammonia, depending on their enzymatic repertoire. Studies on the urea cycle enzymes of latex and fruit flagellates have disclosed that the enzymatic make-up of *Phytomonas* spp. is similar to that of *Herpetomonas* spp. in that they lack arginase and have the arginine deaminase-citrulline hydrolase system for converting arginine to ornithine,  $\text{CO}_2$  and ammonia. This system seems to operate also for the flagellates isolated from *A. retroflexus*, since these are capable of excreting ammonium into the culture medium (Fig. 1B).

The enzymatic study of the total homogenates of these flagellates revealed the presence of the principal enzymes of the glycolytic pathway (Table): HK, PK, PFK, GPI, GAPDH, TIM, ALD, GK and MDH. However, FUM activity was not

medium (Fig. 2A), we found a series of additional peaks which corresponded to metabolites excreted into the medium. Acetate (1.90 ppm), ethanol (1.20 ppm), glycine (3.63 ppm) and succinate (2.40 ppm) were the only metabolites excreted by the isolates from *A. retroflexus*. The production of ethanol, acetate and glycine (Fig. 3) was continuous over all four days of the culture, while the production of succinate increased during the first two days of the culture and remained constant thereafter. Acetate production as the main metabolite may reflect the inability to reoxidize this metabolite (Hellemond et al. 1998) as shown for other species of *Phytomonas* (Fernández-Becerra et al. 1996), although how acetate is produced remains unknown. Ethanol production could be the result either of a partial anaerobiosis in the cell culture during the growth phase or of the inability of the L-a-glycerolphosphate oxidase to eliminate all the reducing equivalents produced during glycolysis. The metabolic capacity of the *Phytomonas* species is quite limited, as the Krebs cycle is not active in the mitochondria (Sánchez-Moreno et al. 1992),

TABLE

Specific activities of enzymes in homogenates of flagellates isolated from *Amaranthus retroflexus*

Enzyme	Specific activity <sup>a</sup> (mU/mg)
GK	740.6
HK	684.0
PK	154.1
PFK	5299.8
PGI	922.2
GAPDH	2323.9
ALD	989.8
TIM	91.7
MDH	1910.6
SOD	40.1
FUM	n.d. <sup>b</sup>

GK: glycerol kinase; HK: hexokinase; PK: pyruvate kinase; PFK: phosphofructokinase; PGI: phosphoglucose isomerase; GAPDH: glyceraldehyde-phosphate dehydrogenase; ALD: aldolase; TIM: triosephosphate isomerase; MDH: malate dehydrogenase, SOD: superoxide dismutase; FUM: fumarase; *a*: n = 5; *s*<sub>n-1</sub> < ± 10%; *b*: not detectable

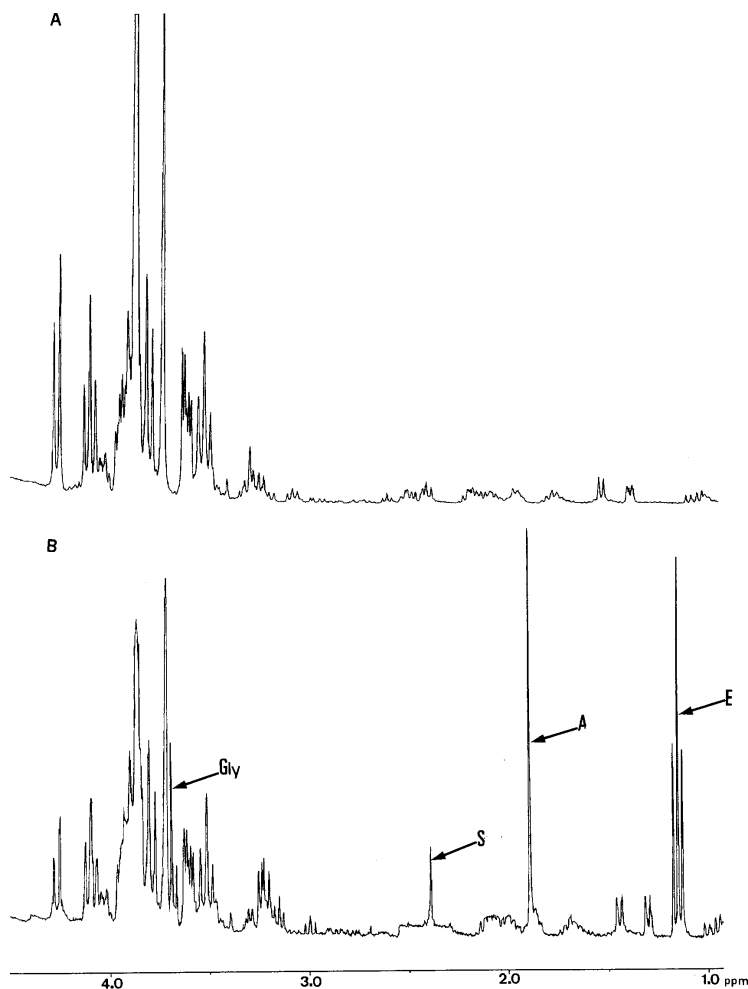


Fig. 2: <sup>1</sup>H NMR spectra of flagellates from *Amaranthus retroflexus* culture. A: fresh culture medium before inoculation of cells; B: spectra for the cell-free culture media, after four days of culture. Metabolites excreted were: E, ethanol (1.12 ppm), A, acetate (1.85 ppm), S, succinate (2.41 ppm) and Gly, glycine (3.32 ppm).

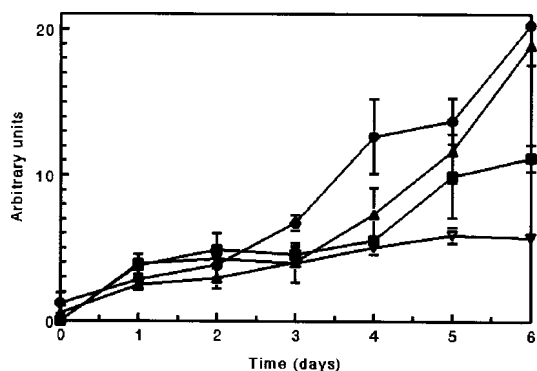


Fig. 3: production and excretion of end metabolites by flagellates isolated from *Amaranthus retroflexus* during the culture in Grace's medium: ethanol (-▲-), acetate (-●-), succinate (-▽-) and glycine (-■-)

detected, in agreement with the non-functionality of the Krebs cycle in these flagellates. After these analyses, determinations were made concerning the association of these enzyme activities with different cell organelles obtained by differential centrifugation of the cell homogenate (Fig. 4). The glycolytic enzymes HK, PFK, PGI, ALD and TIM, together with GK, were associated mainly with the SG. The enzyme involved in CO<sub>2</sub> fixation (MDH) was also associated the SG fraction. The enzyme PK was clearly located in the cytosolic fraction (S). As in other flagellates isolated from plants (Fernández-Becerra et al. 1997), the isolate from *A. retroflexus* presented a clear compartmentalization of the glycolytic pathway, of glycerol metabolism and of CO<sub>2</sub> fixation, the enzymes of which

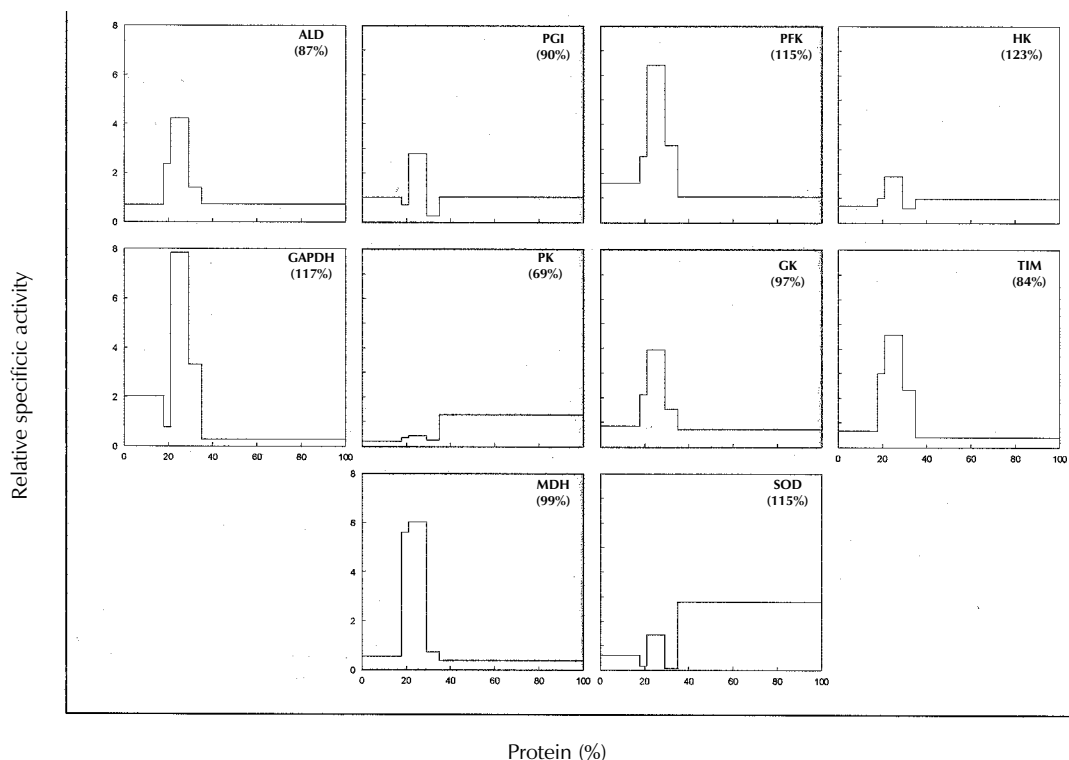


Fig. 4: distribution profiles of the enzymes from flagellates isolated from *Amaranthus retroflexus* assayed in the different fractions obtained by differential centrifugation. The fractions are plotted in the order of their isolation, from left to right, nuclear (N), large organelles (LG), small organelles (SG), microsomal (M) and final supernatant (S); ALD: aldolase; PGI: phosphoglucose isomerase; PFK: phosphofructokinase; HK: hexokinase; GAPDH: glyceraldehyde-phosphate dehydrogenase; PK: pyruvate kinase; GK: glycerol kinase; TIM: triosephosphate isomerase; MDH: malate dehydrogenase, SOD: superoxide dismutase

were all present in the glycosomal fraction. Most of these enzymes showed activities substantially higher than those determined in other *Phytomonas* spp. from either tomato, cherimoya or coconut fruits, as well as in the isolates of *Euphorbia characias* and *Euphorbia hyssopifolia* (Fernández-Becerra et al. 1997), indicating that the isolates of *A. retroflexus* have a high capacity to metabolize glucose to pyruvate.

In general, trypanosomatids lack catalase, although these protozoa possess other enzyme systems such as SOD. The presence of this enzyme in species belonging to the genus *Phytomonas* has been reported previously by us (Fernández-Becerra et al. 1996), but in the present work its activity has been quantified and located within the cell. The enzymatic determination was performed following the method of Paoletti and Mocali (1990), very high enzyme activity being detected in homogenates of *A. retroflexus* (Table). This activity was found to be mainly associated with the cytosolic fraction (S) (Fig. 4).

This activity and its location were confirmed by isoelectric focusing in polyacrylamide gels (pI 3-9). The activity appears as two isoenzymatic forms with different pI, one of the forms having a pI of 4.8, while the other form presented a pI of 7.5 (Fig. 5). The SOD activity detected was insensitive to cyanide but sensitive to hydrogen peroxide, and thus is a Fe-SOD (Fig. 6). By polyacrylamide-gradient electrophoresis of 8 to 25%, the Mr of these two bands was determined, at approximately 30 and 51 kDa, respectively (Fig. 7).

On the basis of our data, we conclude that the flagellates isolated from *A. retroflexus*, as those previously isolated from tomato, cherimoya, mango fruits and clover stems, all collected from the same geographical area, present strong morphological and metabolic similarities. Nevertheless, the characterization of the *A. retroflexus* isolates by isoenzyme and kDNA analysis (Sánchez-Moreno et al. 1998) has revealed that these flagellates are sufficiently dissimilar from the others to suggest that the ones analysed here must be a different species.

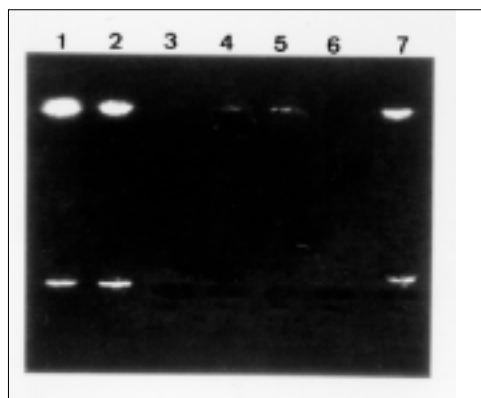


Fig. 5: superoxide dismutase activity in the different fractions obtained from homogenates of *Amaranthus retroflexus*, on the isoelectrofocusing gels (pl. 3-9). lane 1: homogenate, lane 2: post-nuclear fraction, lane 3: postnuclear fraction, lane 4: large-organelle fraction, lane 5: small-organelle fraction, lane 6: microsomal fraction and lane 7: cytosolic fraction

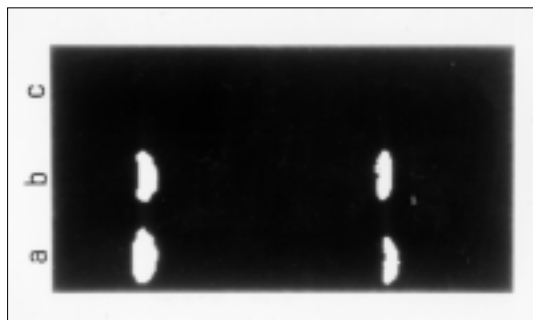


Fig. 6: isoenzymatic profile in IEF 3-9 of the superoxide dismutase activity in the cytosol fraction obtained from *Amaranthus retroflexus*. a: cytosol fraction; b: cytosol fraction incubated with 5 mM KCN; c: cytosol fraction incubated for 45 min with  $H_2O_2$  5mM, 0,1 mM disodium EDTA.

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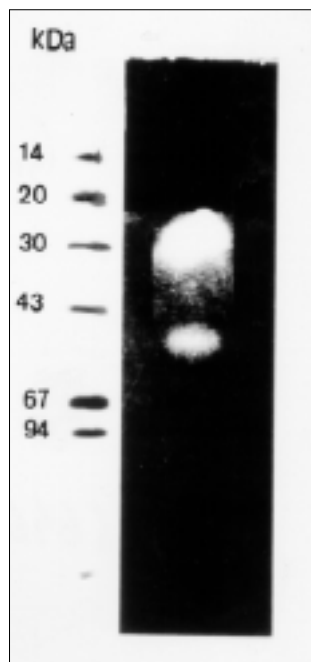


Fig. 7: isoenzymatic profile by polyacrylamide-gradient electrophoresis of 8 to 25% of the superoxide dismutase activity in the cytosol fraction obtained from *Amaranthus retroflexus*.

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