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New vegetal sources for milk clotting enzymes

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Abstract

Seven Papilionoideae species (*Eriosema shirense*, *E. ellipticum*, *E. pauciflorum*, *E. gossweilleri*, *E. psoraleoides*, *Adenolichos anchietae* and *Droogmansia megalantha*), the roots of which are traditionally used in the South of Angola for handmade soft dairy products, were studied in order to identify new milk clotting enzymes for industrial use. In general, root and leaf extracts showed higher proteolytic activity than stem, fruit or inflorescence extracts. The root and leaf extracts were screened for clotting activity and curd strength. The highest proteolytic specific activity was observed in leaf extracts of *D. megalantha* and the lowest clotting time was obtained for leaf extracts of *A. anchietae* and *E. psoraleoides*. When stored at −20°C for 2 months the extracts lose 16–40% of proteolytic activity and its clotting time increased 10–40%. Only the extracts from *E. psoraleoides* dried leaves retain clotting activity (90%) after 4 years storage at −20°C.

Keywords: Papilionoideae; Plant enzyme; Dairy product; Protease

1. Introduction

The milk clotting by proteolytic enzymes is very important in dairy technology. In the past, calf rennet was used for cheese making [1]. The worldwide increase of cheese production and the reduced supply of calf rennet has led to search for rennet substitutes. Bovine, chicken, porcine pepsins [2] and fish chymotrypsins [3] as well as acid proteases from fungi, other microorganisms [2,4,5] and transgenics microorganisms [6] have been used, but most of them give rise to unwanted final products or led to ethic and public health problems that discourage their use. Many proteolytic preparations from plants are also known as rennet. In Mediterranean and near Mediterranean countries and in some south America countries (Chile, Argentina) [7] cardoon or curdle thistle rennet from genus *Cynara* have been used to manufacture some types of well-accepted home made cheeses [1,7–9]. In Portugal, this rennet is traditionally used in cheese making of highly appreciated Serra [8] and Serpa cheeses [1,2,8]. Papain, ficin, bromelain [10] and other cysteine proteases from *Dieffenbachia maculata* (Aiton) Aiton [11] and *Calotropis procera* (Lodd.) G.

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Don. f. [12] are some of the tropical species producing clotting extracts but they present too high proteolytic activity which causes bitterness and weakness of the cheese body [13,14]. In the south of Angola, parts of some *Leguminosae*, especially roots, are traditionally used for handmade milk soft clotting products.

The aim of this work is to evaluate alternative plant sources for milk clotting enzymes in order to match the increasing worldwide demand for diversified dairy products like soft cream cheese and desserts, improving the organoleptic properties of final products and competition between dairy industries.

2. Materials and methods

2.1. Biological material

Different organs of individual species of seven *Leguminosae* (Papilionoideae) were collected by J.M. Daniel on Lubango (south of Angola).

The herbarium specimens are preserved in the Jardim-Museu Agrícola Tropical Herbarium (LISJC).

2.2. Reagents

Skim Milk Powder Molico.

Commercial rennet Etalon Boll (chymosin 520 mg + pepsin; 1:10 000 curd strength).

All reagents used are of the recognised analytical grade.

2.3. Methods

2.3.1. Identification of the biological material

The herbarium specimens were analysed for morphological characters with a stereoscopic microscope and taxonomically identified according to the bibliography concerning the species [15–20] and by comparison with type or typified materials.

2.3.2. Extraction

Fresh biological material was washed several times with distilled water and disinfected with sodium hypochloride. After this, each type of organ (root (R), stem (S), leaf (L), inflorescence (I) and fruit (F)) of each species was fragmented into very small pieces, air-dried for 24 h, frozen in liquid nitrogen and milled. The homogenates were made at 5% (w/v) with 50 mM Tris/HCl, pH 8.3, 1 mM EDTA and 3 mM β-mercaptoethanol and stored for 2 h at 4°C with stirring. After centrifugation ($15\,000 \times g$, 15 min, 4°C) the pellet was discarded.

When necessary, extracts were concentrated by ultrafiltration at 4°C and 2 psi, in an Amicon system with PM10 membrane.

2.3.3. Proteolytic activity assay

Proteolytic activity was determined by the standard fluorimetric assay of Twining [21] based on the hydrolysis of the fluorescein isothiocyanate-labelled k-casein (FITC-casein) substrate. One unit of proteolytic activity (1 UA) corresponds to an increase of one emitted fluorescence unit, in the assay conditions (λ_{EX} = 495 nm, λ_{EM} = 525 nm and band pass of 3 nm).

2.3.4. Protein determination

Protein content was estimated with Bradford method [22] in 0–10 μg sensitivity range. Calibration curve was obtained with BSA.

2.3.5. Proteolytic specific activity (PSA)

Proteolytic specific activity was calculated by the formula: PSA = U proteolytic activity/mg protein.

2.3.6. Milk clotting activity

The time spent in milk clotting at 30°C, after the enzyme addition, was determined using the Norma FIL-IDF 110A [23].

The curd strength (F) is given by the formula $F = (1800 \cdot \mathrm{Mv} \cdot d)/t \cdot \mathrm{Cv}$ were Mv is the milk volume, d is the dilution of the enzyme

Table 1
Identification of Herbarium specimens

Species number	Collection number	Taxonomic identification [14–19]
1	4	*E. shirense* Bak. f.
2	12	*E. ellipticum* Bak.
3	3, 8	*E. pauciflorum* Klotzsch
4	7	*E. gossweilleri* Bak. f.
5	2, 9	*E. psoraleoides* G. Don.
6	6, 10	*A. anchietae* Harms
7	11	*D. megalantha* De Wild

solution in the milk and Cv is the curd volume obtained after milk clotting.

3. Results and discussion

Material's disinfection prevented the growth of symbiotic or contaminant microorganisms. The specimens from south of Angola were taxonomically identified as shown in Table 1.

The method used for the assay of proteolytic activity is very sensitive, in the range of ng for almost all proteinases [21]. With this method, the proteolytic activity for the standard commercial curd used was 12 500 UA/ml. The results of screening for proteolytic specific activity (PSA) are shown in Fig. 1. Each value was the average of two independent sets of triplicate (with $r = 0.95$). In general, it was observed higher PSA in leaves (L) and roots (R) than in stems (S). The proteolytic activity was very low or absent in inflorescences (I) and fruits (F). The highest PSA was found in leaf extracts of *D. megalantha*. The clotting time (CT) is an operational definition, depending on the technique which was monitoring the clotting [5]. In order to compare the milk clotting time from leaf and root extracts, the protein contents were adjusted to the same value (35 μg) and the clotting assays were followed for 5 h. As shown on Fig. 2, the clotting time for *A. anchietae*, *E. psoraleoides* and *D. megalantha* varies between 75 and 120 min (except for *E. ellipticum*

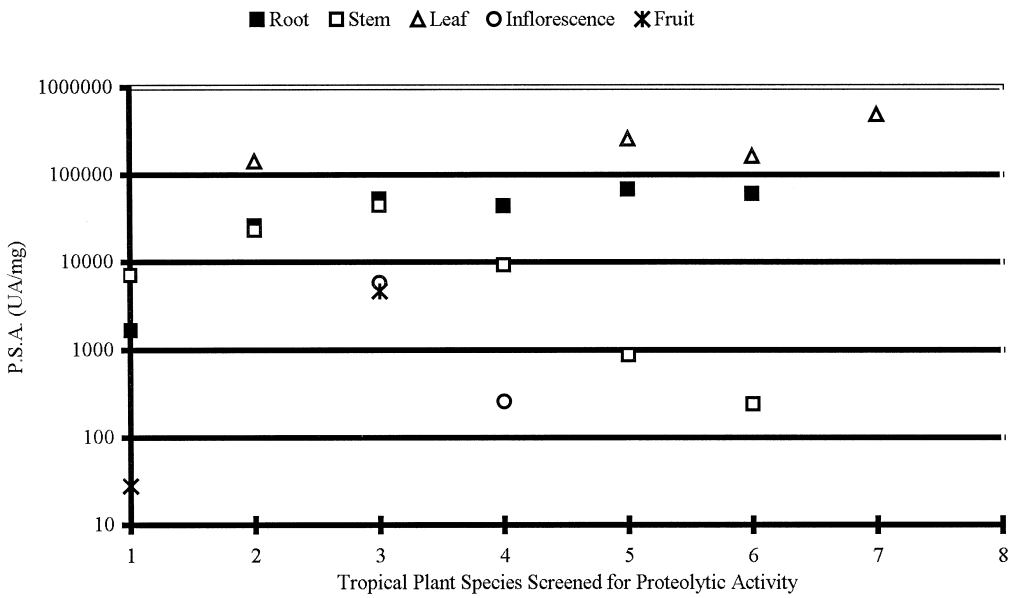

Fig. 1. Screening of proteolytic activity of some Tropical Leguminosae used for milk clotting. (1) *E. shirense*, (2) *E. ellipticum*, (3) *E. pauciflorum*, (4) *E. gossweilleri*, (5) *E. psoraleoides*, (6) *A. anchietae*, (7) *D. megalantha*. P.S.A., proteolytic specific activity (UA/mg). In the assay conditions, the change by 1 unit of the emitted fluorescence corresponds to one unit of proteolytic activity.

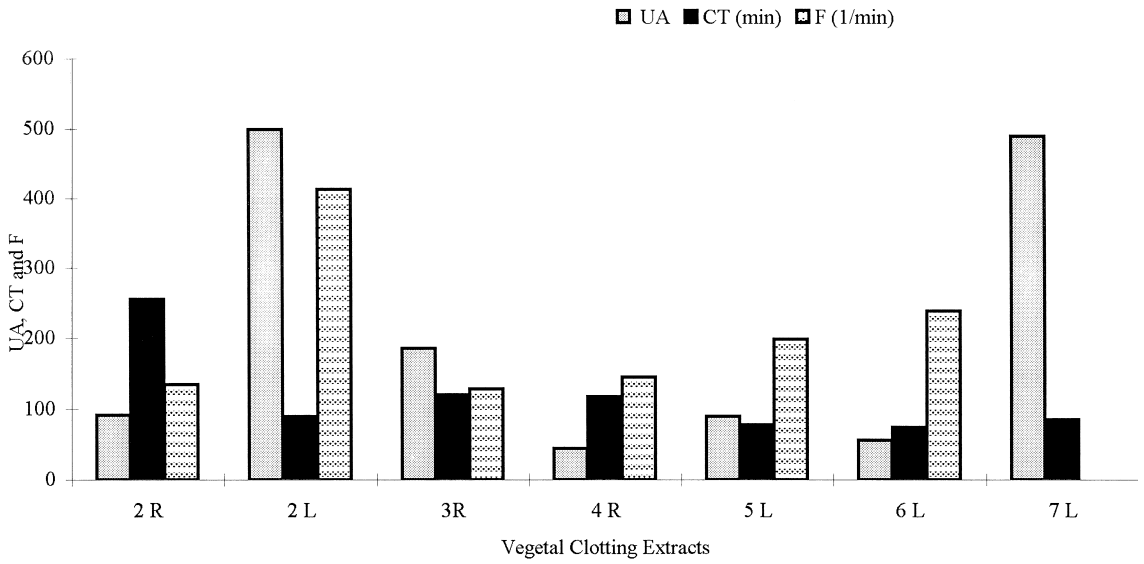

Fig. 2. Characteristics of the vegetal clotting extracts. The assays were made with 0.035 mg of protein. CT, time (min) spent to clot skim milk Molico at 30°C. *F*, curd strength. R, root; L, leaf. (2) *E. ellipticum*, (3) *E. pauciflorum*, (4) *E. gossweilleri*, (5) *E. psoraleoides*, (6) *E. psoraleoides*, (6) *A. anchietae*, (7) *D. megalantha*.

root extracts) and in general, is lower for leaf extracts than root extracts. These values were lower than those observed for cynarase 1 [1] and for *Pseudomonas* extract [5], closer to that obtained for *Mucor bacilliformis* [4] but higher than those observed for the extracts of *Centaurea calcitrapa* [24]. The highest proteolytic activity obtained with *D. megalantha* leaf extracts has the disadvantage of giving a poor and disperse curd that may indicate a non-specific ac-

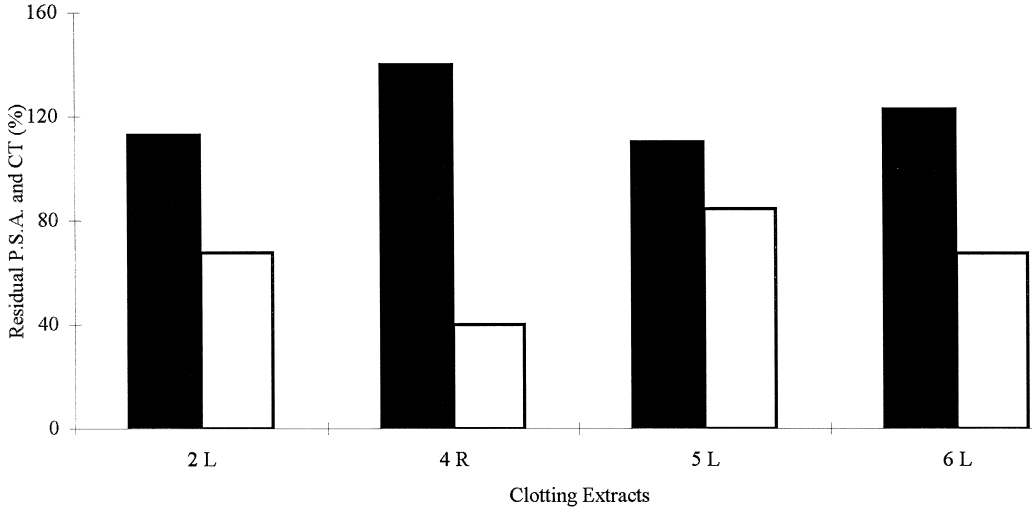

Fig. 3. Effect of two months storage at −20°C on some clotting extracts. The residual values were obtained from considering the initial values as 100%. CT, Time (min) spent to clot skim milk Molico at 30°C. R, root; L, leaf. (2) *E. ellipticum*, (4) *E. gossweilleri*, (5) *E. psoraleoides*, (6) *A. anchietae*.

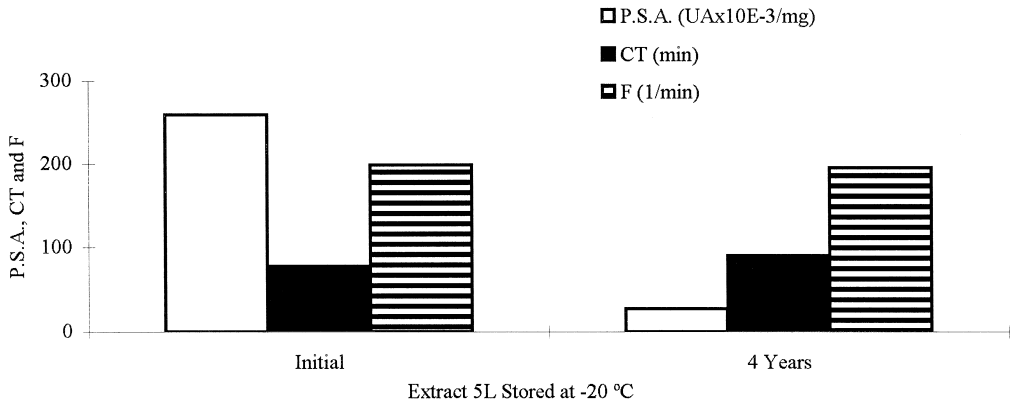

Fig. 4. Effect of four years storage at −20°C on the *E. psoraleoides* (5 L) extract. P.S.A., proteolytic specific activity (UA/mg). CT, time (min) spent to clot skim milk Molico at 30°C. *F*, curd strength. L, leaf. (5) *E. psoraleoides*.

tion of these proteolytic enzymes upon milk caseins [1].

The pH of milk whey ranged between 5 and 6 (not far from pH 6.35 for the milk substrate) and is close to milk whey of other rennets. The curd strength was significatively low (within 1/128 e 1/413); however, some soft dairy products are made with rennets of moderate curd strength (1/500; 1/250) and higher clotting time (24–48 h) [25].

When stored at −20°C for two months, the clotting extracts lost 16–40% of their proteolytic activities and the clotting time increased 10–40%, as shown in Fig. 3. After 4 years storage at −20°C, the clotting time of *E. psoraleoides* leaves was increased 14% and the residual proteolytic activity was 10% (Fig. 4). In the other tested biological material, proteolytic activity decreased by more than 80% and the clotting activity was completely lost.

4. Conclusion

This study shows the presence of proteolytic and clotting activity in the extracts from Papilionoideae species, collected in south of Angola and locally used for traditional handmade soft milk products.

As far as stability and clotting time are concerned, leaves from *E. psoraleoides* proved to be better than roots. The high proteolytic activity of leaves of *D. megalantha* and dispersed clots formed must discourage its use as milk coagulant.

For the reasons mentioned above, we can suggest further and deeper studies on *E. psoraleoides*, *E. ellipticum* and *A. anchietae*, in order to face these plants as new sources for industrial soft dairy products.

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