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Stability of *Araujiain*, a Novel Plant Protease, in Different Organic Systems.

Evelina QUIROGA [1], Nora PRIOLO [2], José MARCHESE [3] and Sonia BARBERIS [1]*

[1] *Laboratorio de Bromatología. Facultad de Química, Bioquímica y Farmacia. Universidad Nacional de San Luis. Chacabuco y Pedernera. (5700) San Luis. Argentina.*
[2] *Laboratorio de Investigación de Proteínas Vegetales (LIPROVE). Facultad de Ciencias Exactas. Universidad Nacional de La Plata. 47 y 115. (1900) La Plata. Argentina.*
[3] *Laboratorio de Ciencias de Superficies y Medios Porosos. Universidad Nacional de San Luis. Chacabuco y Pedernera. (5700) San Luis. Argentina.*

SUMMARY. The effect of different organic solvents on the stability of *araujiain* (the crude enzyme preparation obtained from the latex of fruits of *Araujia hortorum* Fourn., Asclepiadaceae) in mixtures of buffer and different water-miscible or immiscible organic solvents, and in continuous systems (organic solvents with low water activity) after 4 h at 40 ºC was studied in this paper in order to select the most adequate media for peptide enzymatic synthesis. *Araujiain* showed the highest stability in 50% hexane, 50% propanone, and N,N-dimethylformamide (a_w: 0.5 in the enzyme), and those values were remarkably greater than in buffer. In addition, the presence of those organic solvents reduced the autolysis degree. In general, *araujiain* was not inactivated in aqueous-miscible organic systems and the enzyme showed higher activities in those media than in buffer. In biphasic systems, the partition of the organic solvents into the aqueous phase activated the enzyme in several cases. These results did not agree with those obtained in continuous systems, because many of them inactivated the enzyme. Nevertheless, in N,N-dimethylformamide (a_w: 0.5 in the enzyme) *araujiain* showed a remarkable stability after 4 h, and it displayed a higher activity in this organic solvent than in aqueous medium. From these results, it is evident that *araujiain* in the presence of most of the studied organic systems did not suffer unfolding and it was able to retain its native or native-like configuration, though with altered characteristics or properties. This fact was demonstrated by means of comparative FTIR spectroscopy studies for *araujiain* in buffer and non-aqueous systems.

RESUMEN. "Estabilidad de *Araujiaína*, una Nueva Fitoproteasa, en Diferentes Sistemas Orgánicos". Con el objeto de seleccionar los medios más adecuados para la síntesis enzimática de péptidos, estudiamos el efecto de diferentes solventes orgánicos sobre la estabilidad de *araujiaina* (la preparación enzimática no purificada obtenida del látex de frutos de *Araujia hortorum* Fourn., Asclepiadaceae) en mezclas de buffer y diferentes solventes orgánicos miscibles o inmiscibles con el agua y en sistemas continuos (solventes orgánicos con baja actividad de agua), luego de 4 h a 40 °C. *Araujiaina* mostró la mayor estabilidad en hexano (50%), propanona (50%) y en N,N-dimetilformamida (a_w: 0,5 en la enzima), siendo aquellos valores marcadamente más elevados que en buffer. Además, la presencia de aquellos solventes orgánicos redujo el grado de autólisis. En general, en sistemas miscibles *araujiaina* no fue inactivada, mostrando actividades más altas que en buffer. En sistemas bifásicos, la partición de los solventes orgánicos en la fase acuosa produjo en numerosos casos una considerable activación de la enzima. Por el contrario, la mayoría de los sistemas continuos inactivaron la enzima. Sin embargo, en N,N-dimetilformamida (a_w: 0,5 en la enzima) araujiaina fue estable luego de 4 h y más activa que en medio acuoso. De los resultados aquí presentados hay evidencias de que *araujiaina* no sufrió desdoblamiento en presencia de la mayoría de los sistemas orgánicos estudiados y fue capaz de retener su conformación nativa o semejante a la nativa pero con características o propiedades alteradas. Este hecho fue demostrado por medio de estudios comparativos de espectroscopía FTIR para *araujiaina* en buffer y en sistemas no acuosos.

INTRODUCTION

The employment of enzymes for synthesis in organic media has become an attractive alternative to conventional chemical methods [1-4]. This methodology is specially suitable for the modification of precursors of pharmaceutical compounds and fine chemicals, which, in most cases, are insoluble or poorly soluble in water.

It has been demonstrated that enzymes exhibit new important properties in non-aqueous

KEY WORDS: *Araujiain*, Cysteine plant protease, Organic media, Stability.
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* Author to whom correspondence should be addressed. *E-mail:* sbarberi@unsl.edu.ar

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media. These media include the mixtures of water and a water-miscible or immiscible organic solvent, as well as an organic solvent with low water activity [5-11]. Nevertheless, when an enzyme is placed in a non-aqueous medium the organic solvent may alter its structure and its function.

For these reasons, in this paper we studied the effect of the different organic solvents (1-octanol, 1-butanol, trichloroethylene, benzene, ethanol, ethyl acetate, tetrahydrofuran, hexane, propanone, acetonitrile, dichloromethane, dichloroethane, chlorobenzene, N,N-dimethylformamide, acetophenone, diethyl ether, methanol, ethylene glycol, n-dodecane, dioxane and toluene) with different content of water on the stability (residual caseinolytic activity after 4 h) of proteases of *Araujia hortorum* Fourn. *(Asclepiadaceae)* in order to select the most adequate aqueous-organic systems for enzymatic synthesis of peptides.

MATERIALS AND METHODS

Materials

Araujiain is the crude enzyme preparation obtained from the latex of fruits of *Araujia hortorum* Fourn. *(Asclepiadaceae)*. This preparation contains cysteine proteases which belong to the papain family [12,13].

Caseinolytic activity measurement

Proteolytic assays were performed using casein (Hammarsten type, Research Organics, Cleveland, OH, USA) as substrate. The reaction mixture was prepared by mixing 0.1 ml of the enzyme sample with 1.1 ml of 1% casein containing 12 mM Cys, in 0.1 M Tris-HCl buffer (pH 8). The reaction was carried out at 40 °C and it was stopped 10 min later by the addition of 1.8 ml of 5% trichloroacetic acid (TCA). Each test tube was centrifuged at 3,000 x g for 30 min and the absorbance of the supernatant was measured at 280 nm. An arbitrary enzyme unit (caseinolytic unit, Ucas) was defined as the amount of protease, which produces an increment of one absorbance unit per min in the assay conditions [12]. The total content of proteins was determined according to Bradford method [14].

Selection of organic solvents

A statistical design was carried out by clustering 70 organic solvents according to their physicochemical properties (descriptors) extracted from the literature [15-19]. Then, one representative organic solvent of each group was chosen for the study.

Water activity measurement

A constant value of water activity (a_w: 0.5) in the enzyme was established for all trials. 10 mg of enzyme and 10 ml of a solvent containing a given amount of water were incubated for 10 min at 25 °C, and were then centrifuged for 5 min. The liquid was separated from the solid phase by decantation. The amount of water in the liquid phase and in the pellet was measured by the optimized Karl Fisher method using a coulometric KF tritator (model 270, Denver Instrument) [20]. The water content in organic solvents and in the enzyme was determined as volume to volume (v/v) percent and weight to weight (w/w) percent, respectively, and they were expressed as water activity [21].

Stability assays

Enzyme solution of *araujiain* (lyophilized powder) containing 1 mg (total content of proteins) / ml was prepared for stability assays in mixtures of buffer and different water-miscible or immiscible organic solvents at 30:70, 50:50 and 70:30 ratios and in continuous systems (organic solvents with low water activity, a_w: 0.5 in the enzyme). Each trial was performed by incubation of the mixture for 4 h at 40 °C, under controlled magnetic stirring at 160 rpm. 0.1 ml was sampled at periodical intervals of time, and residual caseinolytic activity was quantified. Variation coefficients [(S_d. Mean^{-1}) . 100] of reported values were less than 1.5% for activity assays, calculated in each case from triplicate results.

FTIR spectroscopy

The infrared spectra were measured at 25 °C with a Nicolet Protégé model 460 Fourier transform infrared spectrophotometer, provided with CsI beam splitter between 4000 and 225 cm^{-1}. The spectral resolution was better than 2 cm^{-1} between 4000 and 2000 cm^{-1}, and better than 1 cm^{-1} in the remaining ranks. *Araujiain* was incubated for 4 h in Tris-HCl buffer (pH 8) and in 50% hexane- buffer Tris-HCl (pH 8). Afterwards, the samples were ultracentrifuged and the obtained pellet was separated from the supernatant and stored in dissecator until analysis. Around 0.5-1.0 mg of dried protein was combined with 600 mg of potassium bromide and ground into a fine powder. The powder was annealed into a disc using a hydraulic press. The correction of the basis line and the quantification was carried out using OMNIC spectrophotometer program.

RESULTS AND DISCUSSION

The stability of *araujiain* was studied in mixtures of buffer Tris-HCl (0.1 M, pH 8) and different water-miscible organic solvents at 30:70, 50:50 and 70:30 ratios, in biphasic systems consisting in buffer Tris-HCl (0.1 M, pH 8) and different water-immiscible organic solvents at 30:70, 50:50 and 70:30 ratios, and in continuous systems (organic solvents with low water activity, a_w: 0.5 in the enzyme).

The comparison of the residual caseinolytic activities of *araujiain* in different systems after 4 h of incubation at 40 °C is shown in Figure 1.

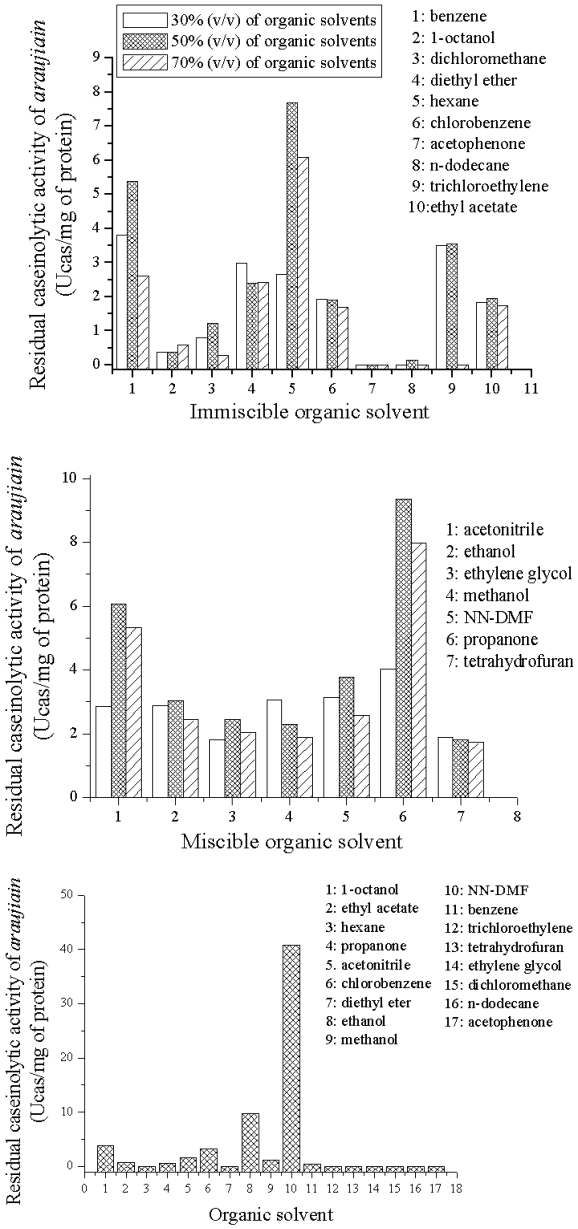

Figure 1. Residual caseinolytic activity (Ucas/mg of protein) of *araujiain* in imiscible (up), mmiscible (medium) and continuous (down) organic systems, after 4 h of incubation at 40 °C.

Araujiain showed the highest residual caseinolytic activity (Ucas/mg of protein) in media containing 50% (v/v) hexane, 50% (v/v) propanone, and N,N-dimethylformamide (a_w: 0.5 in the enzyme), after 4 h of incubation at 40 °C.

It is important to point out that residual caseinolytic activity of *araujiain* in water medium (0.1 M Tris-HCl buffer pH 8) was 0.560 (Ucas/mg of protein), after 4 h of incubation at 40 °C. This value was 90, 94 and 98% lower than those obtained in 50% (v/v) hexane, 50% (v/v) propanone and in N,N-dimethylformamide (a_w: 0.5 in the enzyme), respectively.

In addition, the initial caseinolytic activity of that enzyme in buffer was much lower than those values observed in the organic media already mentioned. When comparing the initial and residual caseinolytic activity in water and in those organic media, a decrease of the autolysis degree was observed.

In general, *araujiain* in aqueous-miscible organic systems was not inactivated and the enzyme showed higher activities in those media than in buffer.

A similar behaviour to the one showed in a buffer solution was expected in studied biphasic media, because of the enzyme had been dissolved in an aqueous phase. However, the partition of the organic solvents into the aqueous phase reduced the autolysis degree and produced a considerable activation of the enzyme with the studied immiscible organic solvents.

Nevertheless, in the presence of some exceptional immiscible organic solvents, like acetophenone, *araujiain* was inactivated. The enzyme inactivation can be due to the toxicity of the organic solvent molecules dissolved in the aqueous phase (molecular toxicity) and/or the presence of a separate organic phase (phase toxicity) [22]. As the concentration of organic solvent dissolved in the aqueous phase was low to extremely low, the molecular toxicity must have had smaller effects on inactivation.

The amount of water associated with the enzyme is considered a key determinant of the properties (e.g. activity, stability, specificity) exhibited by the enzyme in non-aqueous media [21,23-28]. A general conclusion of the effect of water percentage in the biphasic or miscible mixture on the residual enzymatic activity was impossible to achieve in our trials, because it depended on the organic solvent used in each particular case.

The water effect in the continuous systems was analyzed by means of its thermodynamic activity because the total water concentration

does not say much about the hydration of enzymes (necessary to maintain the active catalytic conformation) [5,6,29-32]. This parameter governs the degree of hydration of the enzyme and thus permits to establish the amount of necessary water to maintain the three-dimensional enzyme conformation, independently of the hydrophobicity of the solvent used.

Several continuous systems (organic solvents with low water activity, a_w: 0.5 in the enzyme) led to the inactivation of the enzyme. Nevertheless, *araujiain* in N,N-dimethylformamide (a_w: 0.5 in the enzyme) was stable after 4 h, and the enzyme was more active in this organic solvent than in an aqueous medium. A similar effect was observed in ethanol.

We thought that the changes in the stability of *araujiain* could be related to the changes in the conformation that the studied enzyme suffered in each medium.

By means of FTIR spectroscopy studies, we demonstrated that the organic solvents modified the secondary structure of the studied enzyme.

Strictly, the correlation between enzyme structure and catalytic activity requires direct measurement of active-site structure and of the effect of the reaction medium on the transition state of the reaction. Nevertheless, according to FTIR spectra, it is clear that the non-covalent forces (hydrogen bonding, and ionic, hydrophobic and Van der Waals interactions) which maintain the native secondary and tertiary structures of enzymes were modified by the organic solvents.

As an example, Fig. 2a shows the overlay of primary infrared spectra of *araujiain* in both Tris-HCl buffer (0.1 M, pH 8) and 50% hexane-Tris-HCl buffer (0.1 M, pH 8), at 25 °C.

The amide I band (1700-1600 cm^{-1}) arises primarily from the in-plane C=O stretching vibration of the peptide linkage that constitutes the backbone structure and is known to be sensitive to protein secondary structure and conformational changes, weakly coupled with C–N stretching and in-plane N-H bending.

According to Fig. 2a, *araujiain* in buffer exhibits a strong low-wavenumber β-sheet band at 1635 cm^{-1} with a shoulder near 1627 cm^{-1} and a weak high-wavenumber β-sheet band at 1698 cm^{-1}. On the other hand, the maximum frequency of the amide I band by *araujiain* in hexane-buffer was 1698 cm^{-1}.

Larger amounts of antiparallel β-sheet residues indicate the formation of tight intermolecular hydrogen bonds and the enzymatic aggregates in larger quantities in buffer than in hexane-buffer.

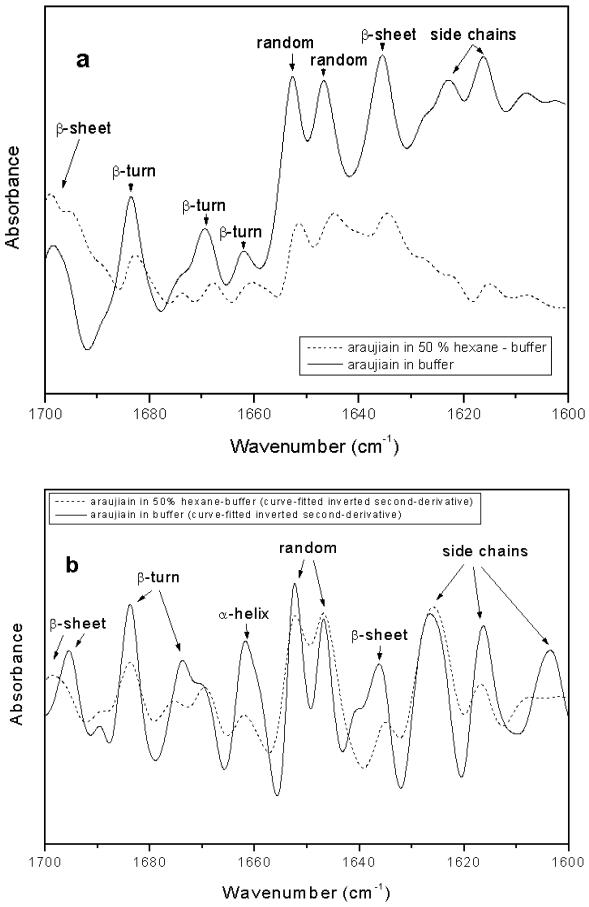

Figure 2. (**a**) Primary infrared spectra of *araujiain* in buffer and 50% hexane - buffer pH 8, at 25 °C; (**b**) Curve fitted inverted second-derivative infrared spectra of *araujiain* in buffer and 50% hexane - buffer pH 8, at 25 °C

The original infrared spectrum of *araujiain* in both media shows random bands near 1647 and 1653 cm^{-1}. Nevertheless, *araujiain* showed a secondary structure of more random character in buffer.

According to the literature, bands located at 1622 and 1615 cm^{-1} are assigned to side chains while those at 1684, 1669 and 1662 cm^{-1}, with a shoulder near 1674 cm^{-1}, belong to various types of β-turn structures [33-35]. The frequencies of vibration of all of them were lower in 50% hexane-buffer than in buffer.

Although the informational content of the primary spectra is rather low, the flexibility and conformational differences of *araujiain* between buffer and 50% hexane-buffer are readily detectable.

Second-derivative analysis of the original spectrum was carried out in order to gain more detailed structural information and to resolve the overlapping band components under the

amide I contour. Fig. 2b shows the curve-fitted inverted second-derivative spectra of *araujiain* in buffer and 50% hexane-buffer pH 8, at 25 °C. The α-helical character of *araujiain* was greater in buffer than in 50% hexane-buffer.

According to our results, the activity and stability of *araujiain* is higher in 50% hexane-buffer than in buffer. We thought that this fact is related to the low folding of the secondary structure of the enzyme in the biphasic medium. There is evidence that *araujiain* dissolved into most of the studied organic media did not unfold and it was able to retain its native or native-like configurations, though with altered characteristics or properties.

CONCLUSIONS

This work allowed to select the best non-aqueous media for the peptide enzymatic synthesis based on the effect of a great number of organic solvents on the *araujiain* stability, in different aqueous-organic systems. We believe that this work is a contribution on this respect because most of the work reported in the literature used a limited amount of organic solvents in the peptide enzymatic synthesis reactions and they were selected with no previously established criteria.

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