Effects of processing conditions on the caseinolytic activity of crude extracts of *Cynara cardunculus* L.

Efectos de las condiciones de extracción sobre la actividad caseinolítica de los extractos de *Cynara cardunculus* L.

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Four processing parameters (time of grinding, pH of buffer, salt concentration of buffer, and homogenization time) involved in the liquid extraction of proteinases from flowers of the wild thistle (*Cynara cardunculus*), were studied for their effects on final caseinolytic activity by a surface response method. The caseinolytic activity was assayed spectrophotometrically using *o*-phthaldialdehyde. An empirical quadratic model was applied to experimental data pertaining to the average enzymatic activity and equations describing the optimal conditions were obtained. Simultaneous solution of these equations for the local maxima indicated that, within the range tested, the maximum (estimated) specific caseinolytic activity (around 9.5 μmol of equivalent leucine/min.g of thistle flower) was obtained by grinding the flowers for 36 s, using an extraction buffer with a pH of 5.9 and a salt content of 0% (w/w), and homogenizing the ground flower/buffer suspension for 15 min. These data are of use in the optimization of extraction procedures, which are of relevance to the production of standardized plant rennets suitable for the large scale manufacture of ewe's milk cheese.

Keywords: Cynara cardunculus, wild thistle, plant rennet, proteinases, caseinolytic activity, surface response method

Se ha estudiado el efecto de cuatro variables (tiempo de maceración, pH del tampón, concentración salina del tampón y tiempo de homogeneización) que intervienen en la extracción líquida de proteinasas de flores de cardo silvestre sobre la actividad caseinolítica final, mediante la metodología de superficie de respuesta. Con el ajuste de los valores medios de la actividad enzimática a un modelo empírico cuadrático se obtuvieron las ecuaciones de las zonas óptimas. La resolución simultánea de estas ecuaciones para los máximos locales indicó que dentro del espacio evaluado, el valor máximo (estimado) para la actividad específica caseinolítica (aproximadamente 9,5 μmol equivalentes leucina por minuto y por gramo de flor) se obtuvo con la maceración de las flores durante, aproximadamente, 36 s, con un tampón de pH 5,9 un 0% de concentración salina (w/w) y un tiempo de homogeneización de la suspensión flor/tampón de 15 minutos. Con este trabajo se pone de manifiesto la validez de la optimización de las condiciones de extracción, de relevante importancia para la producción de cuajos vegetales estandarizados útiles para la elaboración a gran escala de queso de oveja.

Palabras clave: Cynara cardunculus, cardo silvestre, cuajo vegetal., proteinasas, actividad caseinolitica, metodología superficie de respuesta

INTRODUCTION

Cynara cardunculus is a prickly variety of thistle (related to the artichoke), which produces big heads and purple flowers throughout summer. This plant variety (of the *Compositae* family) grows wild and abundantly in dry, stony, uncultivated areas of the south and north of Portugal, although it has also been identified in several Mediterranean regions such as North Africa, Canary Islands, Madeira Island, and southern Spain. After collection from the mature plants, the flowers are dried in the shade in the open air, stored in dry places, and sold at local markets. The dried flowers of *C. cardunculus* are extensively employed in the manufacture of traditional cheese varieties such as Serra da Estrela (Vieira de Sá and Barbosa, 1972; Barbosa, 1983; Roseiro, 1991; Macedo *et al.*, 1993a), La Serena (Fernandez del Pozo *et al.*, 1988; Nuñez *et al.*, 1991) and Guía (Fernandez-Salguero *et al.*, 1991). The dried flowers of related varieties, for example *C. humilis*, have been employed in the manufacture of traditional cheese varieties such as Los Pedroches (Velázquez, 1992). Successful experiments have been carried out on their use in the manufacture of French cheeses such as Camembert and Gruyère (Barbosa *et al.*, 1976), and Italian cheeses such as Bel Paese, Grana, and Provolone (Barbosa *et al.*, 1981).

There are several ways to prepare crude, enzymatically active extracts from thistle flowers for cheesemaking. One of the most popular methods involves soaking a handful of flowers in a bowl of tap water for several hours, mashing with a mortar and pestle, and filtering through a piece of cotton cloth. The brownish liquor thus obtained is added directly to raw ewe's milk in order to bring about coagulation. Salt may be added to the initial aqueous infusion, although it has been argued that no significant differences result on the rates of enzyme extraction (Tsouli, 1974). An alternative way of extraction is grinding the dried flowers with crude kitchen salt, lying the paste on a cotton cloth which acts as a strainer, and solubilizing the enzymes by percolation with warm ewe's milk. The clotting activity of the aqueous extracts of flowers of *C. cardunculus* is due to enzymes which also possess proteolytic activity (Campos *et al.*, 1990; Heimgartner *et al.*, 1990; Morgado, 1990; Faro, 1991; Cordeiro *et al.*, 1992, Macedo, 1993; Macedo *et al.*, 1993b). Three proteinases (formerly termed cynarases or cardosins, and now termed cyprosins) have been isolated, purified and partly characterized in terms of activity (Campos *et al.*, 1990; Heimgartner *et al.*, 1990; Faro, 1991) and selectivity towards pure bovine caseins (Macedo, 1993; Macedo *et al.*, 1993b; Pires *et al.*, 1994; Esteves, 1995), and it has been claimed that one is similar to chymosin whereas another is similar to pepsin. Vieira de Sá and Barbosa (1972) asserted that the milk clotting activity of the flowers of *C. cardunculus* is more dependent on temperature, pH, and substrate concentration than classical animal rennets, and showed that this plant rennet is a good technological substitute for animal rennets especially in the manufacture of ewe's cheese.

Christen and Virasoro (1935a, 1935b) were the first researchers who focused on the extraction process in attempts to establish relationships between its various steps and the enzymatic properties of the crude extract obtained. Although these researchers reported that enzymatic activity existed in the top and middle parts of the flower head, it has been accepted for several years now that only the stylets possess enzymatic activity. This work attempts to complement these studies by assessing the effects of four easily manipulated processing parameters on the caseinolytic activity extracted from flowers of *C. cardunculus*. Research was conducted with the final goal of obtaining conditions that lead to maximum yields of extracted activity, which hopefully could be applied to the optimization of the extraction process, a requirement for industrial scaleup and commercial exploitation of such alternative rennet.

MATERIAL AND METHODS

Material

Dried flowers from *Cynara cardunculus* were obtained from local shops in the Serra da Estrela region (Portugal). Whole sheep casein and leucine were obtained from Sigma (Dorset, UK). Sodium tetraborate (STB), sodium chloride, sodium phosphate, sodium dodecyl sulfate (SDS), citric acid, *o*-phthaldialdehyde (OPA), methanol, and β-mercaptoethanol (β-ME) were all obtained from Merck (Darmstad, Germany).

Preparation of OPA stock solution

The OPA solution was prepared on a daily basis by mixing 50 ml of 100 mM STB solution in water with 10 ml of 10% (w/w) SDS solution in water, 80 mg of OPA previously dissolved in 2 ml of methanol, and 0.2 ml of β-ME, and diluting to a final volume of 100 ml with deionized water.

Preparation of crude extracts of proteinases from *Cynara cardunculus*

Dried *C. cardunculus* flowers (violet part) were divided into 5 g amounts and ground for 0, 0.5 or 1 min (as appropriate) in a laboratory grinder; the powder was extracted with 0.1 M citric acid solution at pH 3, 5 or 7 (as appropriate) containing 0, 1.5 or 3.0% (w/w) sodium chloride as appropriate; the suspension of the powder in the extraction buffer was homogenized in a stomacher for 3, 9 or 15 min (as appropriate). The factorial, three-level experimental layout is depicted in Table 1.

Assay of caseinolytic activity of crude extracts

The caseinolytic activities of crude extracts were determined using a modified form of the OPA method (Church *et al.*, 1983) with whole sheep casein as substrate. This method is based on the reaction of OPA and β -Me with the primary amines of proteins, peptides, and free amino acids to form adducts that absorb strongly at 340 nm. Inclusion of sodium dodecyl sulfate in the assay provides a convenient way to terminate proteolysis and ensure full exposure (and thus complete reaction) of the α-amino groups.

Aliquots (0.3 ml) of crude extracts (CE) of *C. cardunculus* prepared as described above were added to 2.7 ml of a 0.2% (w/w) solution of whole sheep casein in 100 mM sodium phosphate (pH 7.0), and incubated at 37 °C in a shaking bath for 30 min. Samples (200 µl) were withdrawn at various intervals (1, 3, 5, 10, 15, 20, 25 and 30 min) and added to 2 ml of the OPA stock solution; the absorbance measured after 2 min of reaction at 340 nm in 10 mm quartz cuvettes (which corresponds roughly to the peak absorbance) was recorded. Three blanks were used: a substrate control (SC), consisting of 450 µl whole sheep casein and 50 µl sodium phosphate buffer (pH 7.0); an enzyme control (EC), consisting of 50 µl of CE and 450 µl of sodium phosphate buffer (pH 7.0); and a reagent control (RC), consisting of 1000 µl OPA and 450 µl sodium phosphate buffer (at the appropriate pH). The corrected absorbance, A^*, was then calculated from

$$A^* = A - \frac{(0.2/3)(0.3A_{EC} + 2.7A_{SC}) + 2A_{RC}}{2.2} \qquad (1)$$

where the experimental absorbance of the crude extract 2 min after additon of OPA is denoted as A, the absorbance of the substrate control as A_{SC}, the absorbance of the enzyme control as A_{EC}, and the absorbance of the reagent control as A_{RC}. An example

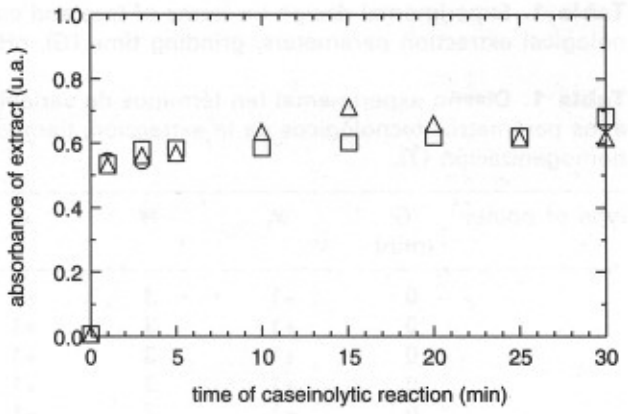

Figure 1. Evolution of the corrected absorbance, A^*, of three replicated samples containing extracts of flowers of *Cynara cardunculus* L. (obtained at $G = 0.5$ min, $H = 5$, $S = 1.5\%$ (w/w), and $T = 9$ min) versus time of reaction in the presence of pure sheep casein.

Figura 1. Evolución de la absorbancia corregida, A^*, de tres repeticiones de un extracto de *Cynara cardunculus* L. (obtenido a $G = 0{,}5$ min, $H = 5$, $S = 1{,}5\%$ (p/p) y $T = 9$ min) frente al tiempo de reacción en presencia de caseína pura de oveja.

of a typical evolution of A^* with time is shown in Figure 1.

A calibration curve of the corrected absorbance of the crude extract as a function of the mass concentration of Leu, C_{Leu}, was prepared with 18 data points spanning the range 0.0 to 0.8 g/L, and a linear fit to such data yielded $A^* = 0.09011 + 1.985C_{Leu}$ (correlation coefficient: 0.996).

Mathematical and statistical analyses

As shown by Figure 1, a plateau in the absorbance of the buffered solutions of whole sheep casein containing CE was reached typically after about 15 min. The ratio of the absorbance at 15 min to this time period was thus computed as an estimate of the average specific caseinolytic activity for each set of experimental conditions. These results are tabulated in Table 1. The whole data set was then fitted to the following empirical model using linear regression (Box *et al.*, 1978):

$$\hat{a} = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{44}x_4^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{34}x_3x_4 \qquad (2)$$

Table 1. Experimental design (in terms of true and coded variables) and associated experimental data for the technological extraction parameters, grinding time (*G*), pH (*H*), salt content (*S*), and homogenization time (*T*).

Tabla 1. Diseño experimental (en términos de variables reales y codificadas) y los datos experimentales asociados a los parámetros tecnológicos de la extracción, tiempo de maceración (*G*), pH (*H*), contenido salino (*S*) y tiempo de homogenización (*T*).

type of points	*G* (min)	x_1	*H*	x_2	*S* (% wt/wt)	x_3	*T* (min)	x_4	*a* (μmol_{Leu} *per* $min.g_{thistle}$)
	0	+1	3	+1	0	+1	3	+1	7.827
	0	+1	3	+1	0	+1	15	−1	5.983
	0	+1	3	+1	3	−1	3	+1	7.827
	0	+1	3	+1	3	−1	15	−1	7.827
	0	+1	7	−1	0	+1	3	+1	6.982
	0	+1	7	−1	0	+1	15	−1	4.831
	0	+1	7	−1	3	−1	3	+1	6.214
	0	+1	7	−1	3	−1	15	−1	4.601
corner	1	−1	3	+1	0	+1	3	+1	6.828
	1	−1	3	+1	0	+1	15	−1	4.601
	1	−1	3	+1	3	−1	3	+1	7.059
	1	−1	3	+1	3	−1	15	−1	5.599
	1	−1	7	−1	0	+1	3	+1	4.140
	1	−1	7	−1	0	+1	15	−1	3.679
	1	−1	7	−1	3	−1	3	+1	4.370
	1	−1	7	−1	3	−1	15	−1	3.218
	0.5	0	5	0	1.5	0	9	0	6.828
centre	0.5	0	5	0	1.5	0	9	0	6.982
	0.5	0	5	0	1.5	0	9	0	6.828
	0	−1	5	0	1.5	0	9	0	4.447
	1	+1	5	0	1.5	0	9	0	5.292
	0.5	0	3	−1	1.5	0	9	0	5.215
	0.5	0	7	+1	1.5	0	9	0	5.292
axial	0.5	0	5	0	0	−1	9	0	6.137
	0.5	0	5	0	3	+1	9	0	8.134
	0.5	0	5	0	1.5	0	3	−1	8.134
	0.5	0	5	0	1.5	0	15	+1	6.982

Note: x_1, normalized grinding time, defined as (*G*–0.5)/0.5; x_2, normalized pH, defined as (*H*–5)/2; x_3, normalized salt content, defined as (*S*–1.5)/1.5; x_4, homogenization time, defined as (*T*–9)/6; *a*, specific caseinolytic activity.

where $\hat{a}$ is the estimated specific caseinolytic activity of the thistle flower, the b_is are ajustable (empirical) parameters, and the x_is are coded variables as defined in Table 1. The best estimates (and associated probability values) for the parameters are listed in Table 2.

The necessary condition for a maximum caseinolytic activity in the crude extract is given by

$$\left(\left(\frac{\partial \hat{a}}{\partial x_i}\right)_{x_{j\neq i}}\right)_{x_i = x_{i,\mathrm{opt}}} = 0, \quad i = 1,2,3,4 \tag{3}$$

A true local critical point exists (for $x_i = x_{i,\mathrm{opt}}$, $i = 1,2,3,4$) if all four equations are solved simultaneously for all four variables. Such a critical point ($x_{1,\mathrm{opt}} = -1.559$, $x_{2,\mathrm{opt}} = -1.164$, $x_{3,\mathrm{opt}} = +0.718$, and $x_{4,\mathrm{opt}} = +1.140$) is, in mathematical jargon, a saddle point, that is it yields a maximum in the x_1- and x_2-directions (because the second order derivatives of $\hat{a}$ with respect to x_1 and x_2 are –3.118 and –2.328, and thus negative) but a minimum in the x_3- and x_4-directions (because the second order derivatives of $\hat{a}$ with respect to x_3 and x_4 are +1.436 and +2.228, and thus positive); therefore, variables x_3 and x_4 were deliberately left arbitrary and only the two first equations were solved with respect to x_1 and x_2, respectively. The results obtained from such algebraic manipulation are :

$$\begin{aligned} x_{1,\mathrm{opt}} &= 0.2078 + 0.0061x_3 + 0.0056x_4 \\ x_{2,\mathrm{opt}} &= 0.3595 - 0.1029x_3 - 0.0034x_4 \end{aligned} \tag{4}$$

Table 2. Estimated parameters (and *p*-values) in the quadratic model describing the specific caseinolytic activity of proteinases from *Cynara cardunculus* as a function of the technological extraction parameters. Residual standard deviation: 0.828. Correlation coefficient: 0.848.

Tabla 2. Parámetros estimados (y valores p) del modelo cuadrático que describe la actividad caseinolítica de proteinasas de *Cynara cardunculus* en función de los parámetros de la extracción. Desviación estándar residual: 0,828. Coeficiente de correlación: 0,848.

Effect	Parameter	Estimate
Average	b_0	6.5887
G	b_1	0.6529**
H	b_2	0.8577**
S	b_3	−0.2134
T	b_4	0.6700**
$G \times G$	b_{11}	−1.5590*
$H \times H$	b_{22}	−1.1640*
$S \times S$	b_{33}	0.7180**
$T \times T$	b_{44}	1.1140*
$G \times H$	b_{12}	−0.1153
$G \times S$	b_{13}	0.0095
$G \times T$	b_{14}	0.0193
$H \times S$	b_{23}	−0.2689
$H \times T$	b_{24}	0.0096
$S \times T$	b_{34}	0.1536

* *p*-value > 99%; ** *p*-value > 95%.

for the loci of the maxima in terms of x_3 and x_4, respectively, and

$$\hat{a}_{opt} = 6.807 - 0.308x_3 + 0.677x_4 + 0.153x_3x_4 + 0.758x_3^2 + 1.114x_4^2 \quad (5)$$

for the corresponding value of the maxima. Equation (5) is graphically depicted in Figure 2.

DISCUSSION AND CONCLUSIONS

It should be emphasized here that maximization of the specific caseinolytic activity of extracts of *Cynara cardunculus* means maximization of the product of the total amount of enzyme extracted by the catalytic activity per unit of enzyme extracted; hence the processing conditions determined above, which are associated with the maximum enzymatic activity of the extract, are not necessarily those that give the maximum yield of enzyme or those that give the maximum enzyme specific activity.

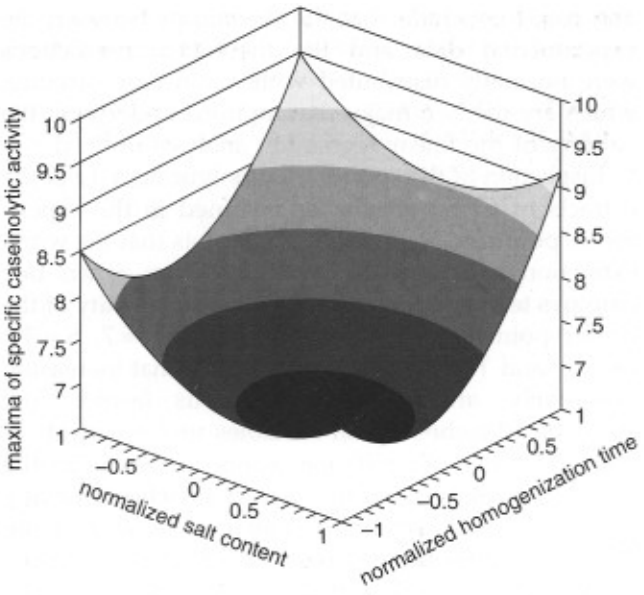

Figure 2. Variation of the maxima of the estimated specific caseinolytic activity versus the technological (coded) variables salt content (x_3) and homogenization time (x_4).

Figura 2. Variación de los máximos de los valores estimados de la actividad caseinolítica frente a las variables tecnológicas relativas al contenido salino, x_3 y al tiempo de homogenización, x_4.

Postulation of the second order polynomial model represented by Equation (2) resulted from the outcome of preliminary statistical analyses based on the assumption that a first order polynomial model was applicable to the data corresponding to corner and center points; such analyses indicated that interactions between factors as well as quadratic terms were important. From such preliminary analyses (not shown), it became apparent that a simple polynomial model was unable to provide a good approximation to the data, and so further experimentation at axial points was entertained for correct estimation of those second-order effects (pure quadratic terms and quadratic interaction terms). Requirement of a third order polynomial model was not, however, warranted in view of the negligible third-order effects estimated from the whole data set generated thus far (analyses not shown). The statistical adequacy of fitting a second order degree polynomial model to the data was further assessed through diagnostics of residuals (not shown); since neither major deviations from linearity in normal plots of residuals, nor funnel-shaped (or other biased) tendencies in plots of residuals against $\hat{a}$ and against x_1, x_2, x_3, x_4 were observed,

one could conclude that the deviations between the experimental data and the theoretical predictions were normally distributed with a constant variance, which are the two major assumptions underlying the validity of the linear regression analysis utilized.

Inspection of the specific caseinolytic activity of the extracts of *C. cardunculus* as obtained in the experiments performed (see Table 1) suggests that the worst extraction of enzymatic activity occurs when one employs technological conditions in the vicinity of the corner point (+1,+1,+1,+1); $G = 1$ min, $H = 7$, $S = 3\%$ (w/w), and $T = 15$ min. This indicates that increasing excessively, and in a simultaneous fashion, the levels of all technological variables under consideration is a bad option if one wants to maximize the caseinolytic activity of the extract for cheesemaking purposes. Conversely, from Equation (4) and Figure 2, one concludes that the best extraction of enzymatic activity occurs when one employs technological conditions corresponding to the highest value of the homogenization time that is practically feasible (15 min, or $x_4 = +1$) coupled with the lowest value of salt in the extraction buffer that is practically feasible (0%, or $x_3 = -1$); in such a limiting case, the normalized grinding time and the normalized pH should be varied according to the relationship denoted as Equation (4).

Since true local maxima exist for the normalized variables x_1 and x_2 but not for x_3 or x_4, then the overall maxima of the enzymatic activity extracted lies on constraints imposed upon x_3 and x_4. If the constraints imposed on (x_3,x_4) are set equal to the boundaries of the experimental range considered, i.e. (–1,–1), (–1,+1), (+1,–1), and (+1,+1), then one obtains 9.5 μmol of equivalent leucine/min.g of thistle flower as the overall maximum, and $x_1 = 0.207$, $x_2 = 0.459$, $x_3 = -1$, and $x_4 = 1$ as the coordinates of such optimum; this corresponds to grinding the flowers for about 0.60 min, extracting the ground flowers at a pH of around 5.92 and with a salt concentration of 0%, and homogenizing the suspension of the ground flowers in the buffer for 15 min. The maximum value obtained as a result of the optimization statistical analysis is considerably above the maximum experimentally obtained (8.134 μmol/min.g); this underlies the usefulness of the experimental design and the modelling analysis developed as opposed to a (traditional) merely empirical analysis that requires random and comprehensive experimental layouts when searching for a maximum.

Inspection of the estimates of the linear, cross, and quadratic effects of the four extraction parameters studied (see Table 2) indicates that: (i) the linear effects of parameters G, H, and T are statistically significant at the 5% level, (ii) the quadratic effect of parameter S is statistically significant at the 5% level, (iii) the quadratic effects of parameters G, H, and T are statistically significant at the 1% level and (iv) the linear effect of parameter S and the interaction effects of all parameter combinations are not statistically significant at the 5% level. Therefore, in attempts to simplify the model denoted as Equation (2) all such parameters that are not statistically significant may be dropped out for a given level of significance.

Of all technological parameters tested, the pH of the aqueous solution utilized in the extraction has the most important linear effect on the final specific activity of the extracted enzyme. The maximum specific activity of the crude extracts from *C. cardunculus*, obtained at pH 5.92, is also in reasonable agreement with the optimum pH for the activity of the proteinases from the same plant variety which have been characterized; 5.1 (Heimgartner *et al.*, 1990), 5.7 (Campos *et al.*, 1990), or 6.0 (Faro, 1991). In view of these considerations, it may be suggested that the large linear effect of pH on the final specific activity of the enzyme extracted arises primarily from the change in the intrinsic activity of the enzyme as a response to pH rather than from changes in the extraction yield brought about by pH.

Both the grinding time of the flowers and the homogenization time of the suspension are associated with positive linear effects on the final specific activity of enzyme extracted (see Table 2). Increasing the time of grinding of the dry flowers is expected to improve extraction due to more extensive disruption of cell membrane with concomitant easier and faster release of proteinases into the extraction medium. Furthermore, increases in the homogenization time of the suspension are expected to lead to increases in the rate and extent of proteinase extraction due to the more intimate contact promoted between the solid and the liquid phases. However, since the quadratic effect of the former parameter is negative, then the extent to which increases in G lead to increases in $\hat{a}$ becomes smaller as G becomes higher, whereas the conclusion is reversed when the latter parameter is considered (i.e. higher values of T lead to higher and higher increases of $\hat{a}$).

With respect to the pH and the salt content of the extraction buffer, Table 2 indicates that increases in the former affect the value of $\hat{a}$ positively (but to a lesser extent as H increases) whereas increases in the latter affect the value of $\hat{a}$ negatively (but to a lesser extent as S increases). It should be emphasized here that in the traditional extraction process crude kitchen salt is often used for its abrasive features in addition to its contribution to the ionic strength and the osmotic pressure of the extraction solution; however,

only the contributions of salt towards the ionic strength and osmotic pressure of the extraction buffer were (implicitly) considered in this research effort. It is well established that in cheese hydrolysis of α_{S1}-casein by rennets (which occurs well before hydrolysis of β-casein) is greatly influenced by the concentration of NaCl: although such proteolytic activity in the case of chymosin is stimulated by increasing NaCl concentrations up to an optimum at 6% (w/w), proteinases from *C. cardunculus* appear to become inhibited only at higher NaCl levels, for example 20% NaCl (Fox and Walley, 1971; Sousa, 1993). Therefore, the observed decrease in $\hat{a}$ arising from an increase in S should in principle be more directly attributed to the extraction yield of enzyme rather than to a decrease in its enzymatic activity. Although proteolysis of β-casein by chymosin and proteinases from *C. cardunculus* is strongly inhibited at 5%, and completely inhibited at 10 %(w/w) NaCl (Fox and Walley, 1971; Sousa, 1993), it is not expected that this observation will fully account for our results since hydrolysis of this form of casein usually occurs much later in the ripening period (and our experimental times were limited to several minutes). The effect of salt upon $\hat{a}$ is pH-dependent (i.e. as pH increases the effect of salt becomes more and more negative) as can be concluded from realization of the relatively high value of the cross effect b_{23} (Table 2). Although it appears that the effect of salt lies more on the side of the extraction yield, it should be noted that the inhibitory effect of NaCl on proteolysis is also pH-dependent which may result from the alteration of the specificity of the rennet (Mulvihill and Fox, 1977, 1978).

The pH of the extraction buffer and the homogenization time of the suspension are virtually independent parameters as can be inferred from the negligible value of the cross factor b_{24} in Table 2. Similar inference can be made about mutual interaction between the grinding time and the salt content of the extraction buffer from inspection of the value of factor b_{13} in Table 2. Finally, although intense stirring (or homogenization) usually promotes denaturation and, thus, deactivation of dissolved enzymes via large interfacial area associated with high interface tension or via large times associated with high shear rates (Thomas and Dummill, 1979), this does not appear to occur in our case as can be concluded from the negligible value of factor b_{14} in Table 2.

Due to the OPA method selected to assay for enzyme activity, the experimental results generated in terms of caseinolytic activity of the enzyme extracted from flowers of *C. cardunculus* encompass all catalytic activities upon peptide bonds of casein, i.e. coagulant, proteolytic, peptidase and aminopeptidase activities. Hence, the sharp increase in absorbance of the medium where the caseinolytic reaction occurs (Figure 1) might be due to a combination of the aforementioned activities, although it is expected that the coagulant activity will dominate. It is also apparent from Figure 1 that the analytical method utilized is quite reproducible. The nature of the experimental layout followed did not allow one to ascertain which are the major components of the overall caseinolytic activity. However, Faro (1991) and Faro *et al.* (1987, 1992) have provided evidence that the clotting activity of extracts from *C. cardunculus* are due to the presence of an acid protease which cleaves κ-casein specifically at the Phe_{105}-Met_{106} bond. Macedo *et al.* (1993b) proved that the turnover number is of the same order of magnitude as those obtained for animal rennets, but the Michaelis-Menten parameter is significantly lower, thus reflecting a higher affinity of the rennet from *C. cardunculus* to κ-casein (Morgado, 1990; Cordeiro *et al.*, 1992; Sousa, 1993; Macedo *et al.*, 1993b). Macedo (1993) asserted that, in addition to κ-casein, the proteinases from the thistle flower also hydrolyze other proteins (including α_{S1}-casein, β-casein and at least one of the γ-caseins) in cow's milk. Cordeiro *et al.* (1992) suggested that the plant proteinases exhibit higher specificity towards hydrolysis of proteins in ewe's than in cow's milk, and Nuñez *et al.* (1991) showed that proteinases in crude plant extracts cleave casein into high-molecular weight peptides but possess very low peptidase activity.

Converting the maximum specific caseinolytic activity calculated above to mass of κ-casein cleaved (using the fractional composition of this type of casein in cow's milk and the assumption that only one labile peptide bond per molecule is cleaved for each primary amine generated) and to mass of total protein in the thistle flower, one obtains around 696 μg of casein hydrolysed per min and mg protein. This figure is of the same order of magnitude (especially knowing that only one labile bond per molecule was assumed) of the value reported by Cordeiro *et al.* (1992) for assays of hydrolysis of fluorescein isothiocyanate-labelled κ-casein for similar extracts of *C. cardunculus*, 144 $\mu g_{\kappa\text{-casein}}/min.mg_{protein}$; this value, which in turn is higher than that obtained for chymosin (94 $\mu g_{\kappa\text{-casein}}/min.mg_{protein}$), is probably due to lower selectivity of the enzymes in the plant rennets towards milk proteins than chymosin, which exhibits a high specificity towards the peptide bond Phe_{105}-Met_{106}.

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