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Comparison of two hard keratinous substrates submitted to the action of a keratinase using an experimental design

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Abstract

The influence of temperature, pH, keratinase concentration, substrate concentration and incubation time on the soluble proteins released by a new keratinase from *Doratomyces microsporus* was studied with a second-order experimental design. Only 15 or 18 spectrophotometric analyses were required to determine the optimal experimental conditions for this keratinase on nail and hoof. This study was carried out by measuring, according to Smith's method, the concentration of soluble proteins released by the enzyme on two substrates: nails and sheep hooves. Results give optimum conditions for the keratinase to release the soluble proteins: pH 8.2, keratinase concentration 0.14% (weight of keratinase lyophilisate/final volume) and substrate concentration 5% (weight of nail powder/final volume) for nails; temperature 38.8 °C, pH 9, substrate concentration 5% (weight of hoof powder/final volume) and a 5 h 55 min incubation time for hooves. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Keratins are insoluble proteins present at high levels in feathers, wool, scales, hair, stratum corneum, hooves and nails. Their structure is stabilized by disulphide and hydrogen bonds, salt linkages and other crosslinkings (Kaluzewska et al., 1991). Their hydrolysis is, however, effected by specific proteases, so-called keratinases (Noval and Nickerson, 1959; Böckle et al., 1995; Lin et al., 1996; Mohedano et al., 1997; Okamoto et al., 1997).

There are many different uses of keratinases, i.e. the elimination of keratin in acne or psoriasis, elimination of human callus, for the preparation of a vaccine for dermatophytosis therapy, and the elimination of hair in the leather industry (Blank et al., 1972).

The fibrous proteins of hair and nails have a molecular structure similar to that of epidermis, but differ from the latter in that they contain a several fold higher amount of cystine. The term 'hard keratin' has been used to describe the

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fibrous proteins of the appendages while 'soft keratin' is used to denote the fibrous proteins of epidermis. As human nails as a substrate are difficult to obtain, several substrates of hard keratins have been used as substitutes (claws, horns and hooves). The bovine hoof is very similar in its gross anatomic structure to human nail and appears to be an excellent model (Baden et al., 1973; Mertin and Lippold, 1997). Human nails and sheep hooves have both structural and penetration similarities (Hemidy et al., 1994). Sheep hooves were chosen for this study because they are less thick than bovine hooves.

In a previous work, the activity of a new keratinase from *Doratomyces microsporus* on the soluble protein release was studied on two human keratinous substrates: stratum corneum and nails (Vignardet et al., 1999). In this work, data are presented concerning the activity of this keratinase on human nails and sheep hooves by measuring the release of soluble proteins, according to BCA's method (Smith, 1985). The same factors were chosen to assess the keratinase activity on nails and hooves: the temperature, the pH, the keratinase concentration, the substrate concentration and the incubation period. For this study, a second-order experimental design and a Simplex method (Box and Wilson, 1955; Fell et al., 1988) were used to determine activity optima.

2. Materials and methods

2.1. Reagents

All the chemicals used were of reagent grade. The following substances were used: Trizma® base (Sigma, Saint Quentin Fallavier, France), hydrochloric acid (Sigma, Saint Quentin Fallavier, France), sodium hydroxide (Cooper, Melun, France), bicinchoninic acid, bovine serum albumin and Copper(II) sulphate pentahydrate (Sigma, Saint Quentin Fallavier, France).

Nail clippings were collected from the fingers of normal individuals. Sheep hooves were collected from a slaughterhouse.

Keratinase was provided by the Laboratory of Biotechnology of the National Institute of Chemistry of Ljubljana (Slovenia).

2.2. Sample preparation

The Tris buffer was prepared by dissolving Trizma® base in water to obtain a final concentration of 0.05 M; hydrochloric acid or sodium hydroxide was added to bring the solution to the chosen pH. The enzyme solutions were prepared by dissolving the keratinase lyophilisate in Tris buffer.

Human nails and sheep hooves were reduced to powder in a mortar with liquid nitrogen, and sieved through 0.200 mm mesh. The substrate powder added to the keratinase solution was placed in flasks. In order to obtain a constant temperature during the incubation, the flasks placed in a double-boiler were stirred. To separate the non-degradated powder from the supernatant, the flasks were centrifuged at 10,000 rpm for 15 min. Then, the soluble proteins released in the solutions were determined by spectrophotometry at a wavelength of 562 nm.

The reagent for soluble protein determination was prepared by adding copper sulphate pentahydrate to bicinchoninic acid (1:50 v/v). 2 ml of reagent were added to 100 μl of serum albumin or samples. The solutions were incubated at 37 °C for 30 min and assayed against a blank.

2.3. Apparatus

Samples were centrifuged with a Jouan centrifuge (Paris, France). The analyses were carried out with an ANA 8 constant spectrophotometer (Odil, Dijon, France). Quartz vats were used for the assays.

2.4. Assay validation

A standard curve representing the bovine serum albumin (BSA) in relation to the absorbancy was obtained by adding a 1 mg/ml solution of BSA to water, to obtain concentrations ranging from 0.2 to 1 mg/ml. Standard BSA solutions were prepared under operating conditions, as described above. The accuracy and precision of the BSA assays were determined by analysing four replicates for each of the five concentrations (0.2, 0.4, 0.6, 0.8 and 1 mg/ml).

2.5. Assessing the soluble proteins released

The quantity of soluble proteins released by both substrates submitted to the enzyme action was assessed according to the BCA method. Values of absorbance were converted into mg of protein/ml of solution by calculations from the standard curve.

For nails, preliminary assays, with a first-order experimental design, enabled the elimination of two non-significant parameters (temperature and incubation time) and the use of three parameters: pH, keratinase concentration (C_k) and substrate concentration (C_s). The temperature and incubation time were fixed at 40 °C and 6 h respectively.

For hooves, preliminary assays, with a first-order experimental design, enabled the elimination of one parameter (keratinase concentration which was fixed at 5% (weight of substrate powder/final volume)) and the use of four parameters: temperature (T), pH, substrate concentration (C_s) and incubation period.

The range of the parameters was chosen in relation to the properties of nails and hooves obtained from previous assays. The temperature varied from 25 to 40 °C, the pH of Tris buffer from 7 to 10, the keratinase concentration from 0.5 to 1% (weight of keratinase lyophilisate/final volume), the substrate concentration from 0.5 to 5% (weight of substrate powder/final volume) and the incubation period from 30 min to 6 h.

2.6. Chemometric methodology

To optimize the keratinase activity, the traditional method would be to study separately each factor which influences the formation of soluble proteins. The chemometric approach is based on the use of a matrix of experiments by which the simultaneous variations of all factors can be studied (Box and Wilson, 1955; Fell et al., 1988).Using this method, the number of experiments can be reduced, compared with the traditional method.

2.6.1. Three factor experimental design

For three factors studied at two levels, there are 2^3 combinations. With two-level factorial designs, the data can be fitted to a first order (Box and Wilson, 1955; Fell et al., 1988). However, to have a more efficient mathematical model, a two-order model was used. This model is obtained using central designs. $2n$ experiments in a distance α on axes $\vec{x}_1$, $\vec{x}_2$, $\vec{x}_3$ were added to the 2^n previous experiments. x_1, x_2, x_3 were added plus one experiment in the centre of the experimental design. Thus, the total number of experiments was $2^n + 2n + 1$, i.e. for $n = 3$, 15 (Table 1). The corresponding two order model is:

$$y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 + a_{11}x_1^2 + a_{22}x_2^2 + a_{33}x_3^2 \quad (1)$$

where y is the response, and x_i values are the logarithm of pH, keratinase concentration and substrate concentration. The a_i, a_{ii}, and a_{ij} terms represent the 10 parameters of the model. All variables were coded to have a variation range from -1.2154 to 1.2154 so that the experimental design matrix contained particular mathematical properties (Box and Wilson, 1955; Fell et al., 1988).

2.6.2. Four factor experimental design

For four variables, the total number of experiments is $2^4 + 2 \times 4 + 1 = 25$. As this was too high, another two-order experimental design was developed that did not diverge from the optimal properties (i.e. the independence of effect estimations and the minimization of the bias errors of the model).

The following design structure was thus adopted. The first design fraction was constructed with a factorial design at two levels. The second fraction was built up by using one experiment in the centre of the experimental design. The third fraction was developed using the Fedorov exchange method. Among experiments of a complete factorial design at three levels, those which minimize the generalized variance of the chosen model parameters were selected. The previously determined points were fixed.

The result of this construction is given in Table 1. The total number of experiments was 18. This two-order experimental design provided sufficient data for the fitting of a quadratic model to the data set.

The two-order model has the following form:

$$y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{14}x_1x_4 + a_{23}x_2x_3 + a_{24}x_2x_4 + a_{34}x_3x_4 + a_{11}x_1^2 + a_{22}x_2^2 + a_{33}x_3^2 + a_{44}x_4^2 \quad (2)$$

where y is the response, and x_i values are the logarithm of temperature, pH, keratinase concentration and incubation time. The a_i, a_{ii}, and a_{ij} terms represent the 15 parameters of the model. Variables were coded to have a variation from −1 to +1, so that the experimental design matrix also contained particular mathematical properties (Box and Wilson, 1955; Fell et al., 1988).

2.7. Simplex optimization process

To optimize the mathematical model (y) representing the soluble protein concentration given by

Table 1
Two-order experimental design for three factors (A) and four factors (B)

Experiment number	x_1 (pH)	x_2 (keratinase concentration)	x_3 (substrate concentration)
(A) Three factors			
1	−1	−1	−1
2	1	−1	−1
3	−1	1	−1
4	1	1	−1
5	−1	−1	1
6	1	−1	1
7	−1	1	1
8	1	1	1
9	−1.2154	0	0
10	1.2154	0	0
11	0	−1.2154	0
12	0	1.2154	0
13	0	0	−1.2154
14	0	0	1.2154
15	0	0	0

	x_1 (temperature)	x_2 (pH)	x_3 (substrate concentration)	x_4 (incubation time)
(B) Four factors				
1	1	1	1	1
2	−1	1	1	1
3	−1	−1	1	1
4	1	−1	−1	1
5	−1	1	−1	−1
6	1	−1	1	−1
7	1	1	−1	1
8	−1	−1	−1	−1
9	0	0	0	0
10	−1	0	−1	1
11	1	−1	0	−1
12	−1	0	1	−1
13	1	0	1	1
14	0	−1	−1	0
15	0	−1	0	1
16	0	−1	1	−1
17	−1	−1	1	0
18	1	0	−1	−1

Table 2
Student t-test used to study the effects of variables for nails and hooves

Independent variables	Calculated t^{a} (for nails) (y)	Calculated t^{a} (for hooves) (y)
Intercept	16.10	
x_1	7.11	15.42
x_2	6.84	38.40
x_3	5.82	42.50
x_4	/	45.40
x_1^2	4.48	8.18
x_2^2	3.52	10.14
x_3^2	3.12	11.15
x_4^2	/	18.24
x_1x_2	3.89	6.18
x_1x_3	2.11	7.24
x_1x_4	/	3.25
x_2x_3	2.11	6.28
x_2x_4	/	7.42
x_3x_4	/	9.44

the experimental design, a Simplex method was used. This way, the y value was calculated for m sets of starting conditions where m represented the number of factors to be optimized, plus one. The point corresponding to the lowest value of y was then reflected in relation to the surface that was defined by the other points. This gave a new set of starting conditions. Once again, the point with the lowest value of y was reflected and the process was repeated until the same conditions were obtained.

2.8. Reduction of disulphide bonds

The substrate powder was added to 1% (w/v) of a mercaptoacetic acid solution. The mixture was incubated and stirred for 30 min, then the powder was filtered and dried.

The reduced powder was added to the keratinase solution in the optimum conditions determined by the experimental design. Then, the soluble proteins were determined using the BCA method.

3. Results

3.1. Linearity—assay validation—detection limit

The calibration curve for the bovine serum albumin was linear over the concentration range of 0.2–1 mg/ml. The equation determined from five different concentrations (experiments repeated four times) was $a = 0.9430y + 3.130 \times 10^{-2}$ ($r^2 = 0.996$) where a is the absorbance, y the concentration of BSA in mg/ml, and r the correlation coefficient. The detection limit of the method was 0.05 mg/ml. The precision (expressed as the coefficient of variation:CV) and the accuracy of the calibration curve were determined from the variation of the standard solutions. The within day coefficient of variation was less than 6% ($n = 4$) over the concentration range 0.2–1.0 mg/ml. The accuracy was between 95.1 and 104%.

3.2. Soluble protein study

Using a second-order experimental design, the optimum of the temperature, pH, keratinase concentration, substrate concentration and incubation period were determined for the soluble protein formation on both substrates by the keratinase. The experimental y values were determined. All experiments were repeated twice. The coefficient of variation of the values was less than 3% in most cases, indicating a high reproducibility and a good stability for the system. The results were processed by computer and the parameters of Eqs. (1) and (2) were obtained. The correlation coefficients of the soluble protein concentration (y) were greater than 0.98.

From the full regression models, it was interesting to exclude those variable terms that had no significant effect on the soluble protein released. Student's t-test was used to provide the basis for precision to determine the significance of the model coefficients. These results of this test are given in Table 2 for nails and hooves. The results of the test show that all variables were significant for both models. The predicted and measured y values for all the experiments are given in Table 3 for nails and in Table 4 for hooves. Using Eqs. (1) and (2), the y values were calculated for the

different values of the factors. A computer was used to perform 35 or 30 iterative processes for both nails and hooves.

The optimum conditions were pH 8.2; 0.14% (w/v) for the keratinase concentration and 5% (w/v) for the substrate concentration for the nails

Table 3
Calculated y_c and measured y_m soluble protein concentrations in experimental conditions for nails (with temperature 40 °C and incubation time 6 h)

Experiment number	x_1 (pH)	x_2 (keratinase concentration) (%)	x_3 (substrate concentration) (%)	y_m	y_c	e (%) [a]
1	7.3	0.134	0.9	0.26	0.28	7.1
2	9.7	0.134	0.9	0.19	0.20	5.0
3	7.3	0.916	0.9	0.39	0.40	2.5
4	9.7	0.916	0.9	0.18	0.19	5.2
5	7.3	0.134	4.6	2.01	2.10	4.3
6	9.7	0.134	4.6	1.11	1.08	2.7
7	7.3	0.916	4.6	0.96	0.99	3.0
8	9.7	0.916	4.6	1.20	1.16	3.3
9	7	0.525	2.75	0.95	1.00	5.0
10	10	0.525	2.75	0.65	0.73	10.9
11	8.5	0.05	2.75	1.01	0.90	10.9
12	8.5	1	2.75	0.47	0.49	4.1
13	8.5	0.525	0.5	0.14	0.13	7.1
14	8.5	0.525	5	1.14	1.10	3.5
15	8.5	0.525	2.75	0.62	0.71	12.7

[a] Relative difference between calculated and measured values.

Table 4
Calculated y_c and measured y_m soluble protein concentrations in experimental conditions for hooves (with keratinase concentration 1%)

Experiment number	x_1 (temperature in °C)	x_2 (pH)	x_3 (substrate concentration) (%)	x_4 (incubation time)	y_m (mg/ml)	y_c (mg/ml)	e (%) [a]
1	40	10	5	30 min	0.20	0.19	5
2	25	10	5	6 h	1.65	1.71	3.5
3	25	7	5	6 h	0.78	0.72	7.6
4	40	7	0.5	6 h	0.27	0.30	10
5	25	10	0.5	30 min	0.23	0.21	8.6
6	40	7	5	30 min	1.22	1.18	3.2
7	40	10	0.5	6 h	0.17	0.14	17.6
8	25	7	0.5	30 min	0	0.007	100
9	32.5	8.5	2.75	3 h 15	1.05	0.99	5.7
10	25	8.5	0.5	6 h	0.31	0.34	8.8
11	40	7	2.75	30 min	0.92	0.87	5.4
12	25	8.5	5	30 min	0.72	0.68	5.5
13	40	8.5	5	6 h	1.98	2.04	2.9
14	32.5	7	0.5	3 h 15	0.09	0.11	18.2
15	32.5	7	2.75	6 h	1.04	1.00	3.8
16	32.5	7	5	30 min	1.05	1.02	2.8
17	25	7	5	3 h 15	0.93	0.96	3.1
18	40	8.5	0.5	30 min	0.45	0.50	10

[a] Relative difference between calculated and measured values.

Table 5
Comparison of normal substrate powder and reduced substrate powder (values averages from five experiments)

	Normal nails	Thioglycolated nails	Normal hooves	Thioglycolated hooves
Released soluble proteins (in mg/ml)	1.47	3.76	0.81	3.01
Released soluble proteins/substrate powder (%)	2.89	7.37	1.6	6.0

and 38.8 °C, pH 9, 5% (w/v) for the substrate concentration and 5 h 55 min incubation time for hooves (Table 5).

3.3. Results after reduction of disulphide bonds

The quantity of soluble proteins released by the keratinase was determined using both the normal substrate and the reduced substrate powders. All experiments were performed under the optimum conditions and were repeated three times. The quantity of soluble proteins released increased: for nails, it was multiplied by 2.5 and for hooves by 3.7. The results are reported in Table 5.

4. Discussion

Many authors have studied the protease or keratinase activity of enzymes of the *Streptomyces* or *Aspergillus* species (Kaluzewska et al., 1991; Ginter, 1996; Lin et al., 1996).In these studies, each factor or parameter was assessed separately to determine their optimum of activity. In this work, a keratinase from *Doratomyces microsporus* was studied with an experimental design in order to limit the number of experiments.

The study of five parameters responsible for the soluble protein release showed that for hooves all the factors were significant, while for nails only three factors were important, the two others being fixed. However, a comparison of the optimal values obtained for the two substrates showed that the only very different value was the keratinase concentration; all the other values were similar. Moreover, the quantity of soluble proteins released from nails was higher than that from hooves: it was up to 2.89% (soluble protein concentration/substrate concentration) for nails while it was up to 1.6% for hooves.

These results can be related to the compact structure of those hard keratinous substrates which contain a disulphide bond that assures their stability. Indeed, hooves have a more compact structure than nails. The cut of these bonds changes the conformation of keratins and more easily liberates attackable sites for the enzyme. Thus, the quantity of soluble proteins released by the keratinase is larger following treatment with mercaptoacetic acid solution. The higher increase in the soluble proteins for the hooves than for the nails can be explained by the more compact structure of the sheep hooves due to a higher number of disulphide bonds.

The use of an experimental design provided optimum determinations for five factors responsible for the soluble protein release and a comparison of two hard keratinous substrates, with a minimal number of experiments. This method is a quick technique for the precise assessment of enzymatic substrate degradation. Although harder, the sheep hooves can be considered to be substrates able to mimic human nails.

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