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A first order experimental design to assess soluble proteins released by a new keratinase from Doratomyces microsporus on human substrates

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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 an experimental design assisted by a simplex method. Only 16 spectrophotometric analyses were required. This study was carried out by measuring, according to Smith's method, the concentration of soluble proteins released by the enzyme on two human substrates: stratum corneum and nails. Results give optimum conditions for the keratinase to release the soluble proteins: Temperature, 28°C; pH, 9.5; keratinase concentration, 0.94% (weight of keratinase lyophilisat/final volume), substrate concentration, 4.9% (weight of skin powder/final volume); and a 5 h 50 min incubation for the stratum corneum. Temperature, 37°C; pH, 9.2, keratinase concentration, 0.11% (weight of keratinase lyophilisat/final volume); substrate concentration, 4.9% (weight of nail powder/final volume) and a 6 h 10 min incubation time for nails.

Keywords: Keratinase; Soluble proteins; Experimental design; Stratum corneum keratins; Nail keratins

1. Introduction

Keratins are insoluble proteins from feathers, wool, hooves, scales, hair, nails and stratum corneum. These proteins which belong to the scleroproteides group are compounds that are extremely resistant to the action of physical, chemical and biological agents. Characteristics of keratins are their high mechanical stability and resistance to proteolytic degradation which depends on the disulfide and hydrogen bonds, salt linkages and other crosslinkings (Kunert, 1973; Kaluzewska et al., 1991; Friedrich and Antranikian, 1996). Keratins as well as other insoluble proteins, are generally not recognized as

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substrates for common proteases (Letourneau et al., 1998).

Nevertheless, structural modification of keratins is more often obtained by physicochemical processes, than by microbiological processes, although it is known that many bacteria, fungi and actinomyces are able to produce active proteolytic enzymes (Noval and Nickerson, 1959; Kunert, 1989; Mukhopadyay and Chandra, 1990; Böckle et al., 1995; Friedrich and Antranikian, 1996; Santos et al., 1996; Böckle and Müller, 1997).

These keratinolytic enzymes are called keratinases. They have demonstrated strong proteolytic activity toward keratins and other insoluble or soluble protein substrates (Lin et al., 1996). Keratinases accomplish degradation of keratin by some manner other than cleavage of disulfide bonds (Nickerson et al., 1963). Until now, there has not been a description of the reduction of the disulfide bonds in any keratinolytic enzymes (Böckle et al., 1995).

This report describes the activity of a keratinase from Doratomyces microsporus on two keratinous substrates: human stratum corneum and nails, and measures using Smith et al's method (Smith, 1985), the release of soluble proteins.

The keratinolytic activity depends on many parameters. In this report, five parameters have been chosen to assess the keratinase capacity to release the soluble proteins: pH, temperature (T_0), keratinase concentration (C_k), substrate concentration (C_s) and incubation time. The aim of this research was to use an experimental design to determine the optimum parameters for the formation of soluble proteins.

2. Experimental

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) sulfate pentahydrate (Sigma, Saint Quentin Fallavier, France).

The stratum corneum obtained from a chiropodist, was healthy human foot callus. The interest of this collecting method is to obtain pure stratum corneum without chemical separation of stratum corneum from living epidermis. The differential scanning calorimetry thermograms of the collected samples being identical to those taken from different part of the body, described by Van Duzee (1975), Golden et al. (1986), Khan and Kellaway (1989). Nail clippings were collected from the fingers of normal individuals.

The 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 0.05M, hydrochloric acid or sodium hydroxide was added to bring the solution to the chosen pH. The enzyme solution was prepared by dissolving the keratinase lyophilisat in Tris buffer.

Stratum corneum and nails were reduced to powder in a mortar with liquid nitrogen, and sieved through a 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-degradate 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 sulfate 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 was obtained by adding a 1 mg/ml solution of bovine serum albumin (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 assay 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. Values of absorbance were converted into mg of protein/ml of solution by calculations from the standard curve.

Five parameters were chosen: the incubation temperature which varied from 25 to 40°C, the pH of tris buffer from 7 to 10, the keratinase concentration from 0.05 to 1% (weight of keratinase lyohilisat/final volume), the substrate concentration from 0.5 to 5% (weight of powder/final volume) and the incubation period from 30 min to 6 h. The range of parameters was chosen in relation to the properties of stratum corneum and nails obtained from previous assays.

2.6. Chemometric methodology

To optimize the soluble protein formation, the traditional method would be to study separately each factor which influences the formation. The chemometric approach allows passing from experiments to a model or from a model to experiments by a quantitative mathematical treatment such as experimental designs. This approach is based on the use of a matrix of experiments by which the simultaneous variation of all factors can be studied (Box and Wilson, 1955; Cochran and Cox, 1957). This significantly reduces the number of experiments, as compared with the traditional method. A mathematical model was used which linked the observed response Y and the influencing factor (x_i). Variables were coded to have a variation from -1 to $+1$. The experimental quantitative factors which controlled the quantity of soluble proteins released by the keratinase included the logarithm of the temperature (x_1), pH (x_2), keratinase concentration (x_3), substrate concentration (x_4) and incubation time (x_5). The model can be expressed as:

$$Y = \alpha_0 + \sum_{i=1}^{5} \alpha_i x_i + \sum_{i=1}^{5}\sum_{j=1}^{5} \frac{(\alpha_{ij} x_i x_j)}{2} \quad (i \neq j) \qquad (1)$$

where $\alpha_0,\ldots\alpha_{ij}\ldots$ are the coefficients of the model. $\forall(i, j)\ \alpha_{ij} = \alpha_{ji}$ and their values were determined using a basic program. Y is the quantity of soluble proteins released:

$$Y = MP \qquad (2)$$

$$Y = \begin{bmatrix} y_1 \\ \cdot \\ \cdot \\ y_{16} \end{bmatrix} \qquad P = \begin{bmatrix} \alpha_0 \\ \cdot \\ \alpha_i \\ \cdot \\ \alpha_5 \\ \alpha_{12} \\ \cdot \\ \alpha_{ij} \\ \cdot \\ \alpha_{45} \end{bmatrix}$$

$$M = \begin{bmatrix}
1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\
1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & -1 & -1 & -1 & -1 & -1 & -1 & -1 & -1 \\
1 & 1 & 1 & 1 & -1 & -1 & -1 & -1 & 1 & 1 & 1 & 1 & -1 & -1 & -1 & -1 \\
1 & 1 & 1 & 1 & -1 & -1 & -1 & -1 & -1 & -1 & -1 & -1 & 1 & 1 & 1 & 1 \\
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1 & -1 & -1 & 1 & -1 & 1 & 1 & -1 & -1 & 1 & 1 & -1 & 1 & -1 & -1 & 1
\end{bmatrix}$$

where y_1,... y_{16} are the experimental values of the quantities of soluble proteins released recorded for each combination of parameters. The parameter vector P was calculated as:

$$P = M^{-1}Y \tag{3}$$

where M^{-1} is the reverse matrix of M.

3. Simplex optimization process

To optimize the mathematical y model given by the experimental design, a simplex method was used. This way, the y value was calculated for m sets of starting conditions where m was given by 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 continued to be selected.

4. Results

4.1. 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 $a = 0.9430y + 3.130.10^{-2}$ ($r^2 = 0.996$) 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 variation coefficient: CV.) and 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%.

4.2. Soluble proteins released

Using the chemometric methodology, the effect of the temperature, pH, keratinase concentration,

Table 1
Calculated y_c and measured y_m soluble protein concentration in ten experimental conditions

Experiment No.	x_1 (°C)	x_2	x_3 (%)	x_4 (%)	x_5 (h)	y_m (skin) (mg/ml)	y_c (skin) (mg/ml)	y_m (nail) (mg/ml)	y_c (nail) (mg/ml)
1	40	10	1	5	6	4.30	4.15	0.71	0.79
2	40	10	1	0.5	30 min	1.90	2.05	0.18	0.11
3	40	10	0.05	5	30 min	5.08	4.95	1.55	1.61
4	40	10	0.05	0.5	6	0.45	0.42	0.09	0.08
5	40	7	1	0.5	6	0.80	0.88	0.14	0.16
6	25	7	1	5	6	6.52	6.70	1.05	1.00
7	25	7	1	0.5	30 min	0.59	0.64	0.07	0.08
8	25	7	0.05	5	30 min	0.92	1.00	0.75	0.71
9	25	7	0.05	0.5	6	0.46	0.41	0.12	0.11
10	25	10	1	5	30 min	6.47	6.99	1.11	1.18

substrate concentration and incubation time were studied and the soluble protein concentration released assessed. The experimental y values were determined. All experiments were repeated twice. The coefficient of variance of the values was less than 2% in most cases, indicating high reproducibility and good stability for the system. The results were processed by computer and the parameters of Eq. (1) were obtained. The fitting of the model to the results was respectively 95 and 94% for stratum corneum and nails. The Student's t-test was used to provide the basis for precision to determine the significance of the model coefficients. The results of the test showed that two parameters for nails (incubation time and temperature) but none for the stratum corneum could be excluded from the model. The predicted and measured y values for ten experiments are given in

Table 2
Results of the simplex process for the stratum corneum

Experiment No.	x_1 (°C)	x_2	x_3 (%)	x_4 (%)	x_5 (h)	y
1	40	10.0	1.00	5.0	6.0	4.15
2	40	10.0	1.00	0.5	0.5	1.90
3	40	10.0	0.05	5.0	0.5	5.08
4	25	7.0	1.00	5.0	6.0	6.52
5	25	7.0	1.00	0.5	0.5	0.59
6	25	10.0	1.00	5.0	0.5	6.47
7	26	7.0	0.05	1.5	1.0	1.00
8	24	7.5	0.20	2.0	1.5	1.12
9	23	8.5	0.21	2.5	2.0	1.12
10	30	9.5	0.29	3.0	2.4	4.08
11	32	10.0	0.50	3.5	2.3	4.08
12	40	10.0	0.52	4.0	3.4	3.99
13	37	7.5	0.05	4.0	1.0	5.01
14	35	7.5	0.10	3.9	1.0	6.01
15	35	8.0	0.10	3.7	2.5	7.01
16	35	8.4	0.52	2.7	3.0	4.01
17	25	8.5	0.60	1.0	4.0	3.01
18	25	9.1	0.70	3.4	4.5	5.01
19	25	9.2	0.80	4.9	2.9	6.01
20	28	9.3	0.71	4.8	3.8	5.01
21	29	9.4	0.85	3.8	3.7	4.01
22	30	10.0	0.90	3.8	4.0	4.15
23	31	7.6	0.91	4.9	4.0	5.02
24	32	7.6	0.80	4.9	5.2	7.02
25	25	9.4	0.82	3.8	5.2	8.02
26	25	9.6	0.75	2.6	1.0	7.02
27	40	9.5	0.80	2.5	0.7	5.02
28	40	9.7	0.82	2.5	0.8	6.02
29	38	10.0	0.60	3.0	2.0	4.02
30	37	10.0	0.55	4.1	3.5	4.02
31	35	10.0	0.90	4.5	3.4	3.02
32	34	9.5	0.90	4.4	4.0	7.02
33	25	9.5	0.94	4.0	5.2	8.15
34	28	7.0	0.94	4.8	5.0	5.10
35	29	9.8	0.85	4.9	5.6	8.42
36	30	9.7	0.92	4.9	5.7	9.02
37	28	9.8	0.94	4.9	4.02	8.52
38	28	9.5	0.94	4.9	5.06	9.15
39	28	9.5	0.94	4.9	5.87	9.20
40	28	9.5	0.94	4.9	5.80	9.20

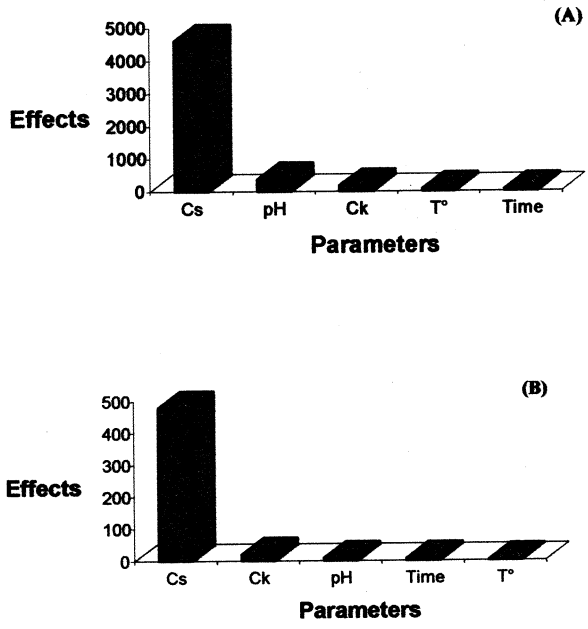

Fig. 1. Effects of five parameters on the enzymatic degradation of human stratum corneum (A) and nails (B), where C_k represents the keratinase concentration; C_s, the substrate concentration and T_0, the temperature.

Table 1. Using Eq. (1), the y values were calculated for the different values of the five factors.

40 iterative processes for stratum corneum and nails were performed by the computer. Results for the stratum corneum are set out in Table 2. The highest quantity of soluble proteins was 9.20 mg/ml for the stratum corneum and 2.70 mg/ml for nails indicating the best values. The optimum conditions were respectively 28°C, pH 9.5, 0.94% for keratinase concentration, 4.9% for substrate concentration and 5 h 50 min (5.8 h) for incubation time for the stratum corneum and 37°C, pH 9.2, 0.11% for keratinase concentration, 4.9% for substrate concentration and 6 h 10 min (6.17 h) for incubation time for nails.

5. Discussion

The composition of mammalian stratum corneum is similar to hair and nails with regard to its X-ray diffraction pattern and insolubility in neutral buffers. Stratum corneum and nails are derived from the ectodermal cells, but show significant structural differences in their fully differentiated form. They contain an α-fibrous protein with the same molecular dimensions, but Baden et al. (1973, 1980) observed important differences in their chemical and physical properties.

The main constituents of stratum corneum and nails are keratins. The stratum corneum contains dead anucleate keratinized cells, constituted by 75–80% proteins, 5–15% lipids and 5–10% unidentified materials. The protein fraction predominantly encloses α-keratins (approximately 70%), β-keratins (10%) and the cell envelope (5%) (Williams and Barry, 1992). Nails principally contain keratins with some mineral constituents, water and lipids (0.5–0.76%). Keratins are proteins characterized by their high sulphur, cystine and arginine content (Sayag and Jancovici, 1980).

These substrates belong to two different types of keratins: soft keratin for stratum corneum, hard keratin for nails. The main difference between these two keratins is their amino acid composition: hard keratins having a higher half-cystine content (7.4%) and a lower glycine content (6.6%) than soft keratins which have a low half-cystine content (2.9%) and a high glycine content (11.6%) (Fraser, 1972). The half-cystine concentration is responsible for the compact and rigid structure of the hard keratins. These cysteine groups form disulphide bonds, which provide molecular stability. The compact structure of hard keratins limits the number of sites accessible to the keratinase action. This difference explains the results obtained for both substrates: five factors were significant for the stratum corneum while only three (the substrate concentration, the keratinase concentration and the pH) were significant for nails. Moreover, the five parameters were classified differently for each substrate (Fig. 1).

The difference in the composition between the two substrates was demonstrated by the quantity of soluble proteins released; it was higher for the stratum corneum than the nails. Moreover, the quantity of soluble proteins released by the keratinase in relation to the quantity of substrate varied from 1.8 to 37.9% for the stratum corneum and from 1.4 to 4.2% for the nails.

The results demonstrated the role of the substrate concentration on the quantity of the soluble proteins released. The other factors were less important but nevertheless significant.

The experimental design connected with the simplex method can be considered to be a fast technique to classify with a limited number of experiments the respective role of five parameters on the degradation of two keratinized substrates. Indeed, this method allowed us to carry out 16 experiments instead of 40 if the simplex method was the only used.

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