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Detection and Assay of Single Cell Protein Products in Blends with Animal Feeds

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Electrophoretic patterns of protein extracts obtained with different procedures from the BP-yeast product named Toprina (a *Candida lipolytica* strain grown on n-alkanes) have been compared with protein electrophoretic patterns of a number of different microbial species. The gel electrophoretic pattern of the 0.15 M NaCl extract from Toprina was specific enough to allow an easy differentiation of Toprina from the other microorganisms tested.

To detect and assay Toprina in blends with animal feeds, an immune serum containing antibodies reacting specifically with Toprina has been prepared by immunising rabbits with an antigen preparation extracted from Toprina with 0.15 M NaCl and precipitated by salting out the extract at 4.0 M $(NH_4)_2SO_4$. Three main antibodies reacting with Toprina antigens have been found in the anti-Toprina immune serum, but only one was specific of Toprina. The resistance of the Toprina antigens to peptic digestion and their behaviour on extraction, electrophoresis and gel filtration suggest that they might be acidic polysaccharide in nature.

Immunodiffusion analyses with the anti-Toprina immune serum of extracts obtained with 0.15 M NaCl from very heterogeneous animal feeds added with different amounts of Toprina, allowed the detection of a Toprina amount as low as 2.5%. An accurate assay of Toprina was achieved by submitting the feed extract to radial immunodiffusion with the anti-Toprina immune serum.

1. Introduction

A number of industrial processes for the production from different substrates of protein-rich biomasses are being developed in several countries.[1–10] With the main exception of a method[11] to recognise the carbon origin by measuring the radioactivity of its ^{14}C isotope that might be applied only to biomasses grown on petroleum-derived products, very little work has been devoted to the study of suitable methods to detect and assay single cell products in blends with traditional feeds or other products. In fact, such a control might be very desirable both for safety and commercial reasons.

To develop a general strategy for the solution of this problem we chose as working material the BP-yeast product consisting of dried cells of a *Candida lipolytica* strain grown on a medium containing n-alkanes. The Italian common name of this product is Toprina. Since Toprina was under trial for safety evaluation at the Istituto Superiore di Sanità (ISS), it was available to us in large amounts.

This paper deals with an electrophoretic and immunochemical characterisation of some Toprina components designed to find out at least one protein or antigen fraction specific of Toprina to be used as a molecular marker of Toprina in blends with other materials. We have also studied analytical procedures for the detection and measurement of the specific marker in very heterogeneous mixtures such as crude extracts from Toprina-containing animal feeds.

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As far as we know, although a number of serological studies on yeasts have been carried out,[12–14] no extensive characterisation study of Toprina proteins and antigens has been reported up to now.

2. Experimental

2.1. Strains

Toprina and the *Candida lipolytica* strain used for Toprina production were obtained from Italproteine, Milan, Italy. Liquipron (the Kanegafuchi yeast product consisting of *Candida* strain grown on hydrocarbons) was obtained from Liquichimica, Milan, Italy. Baker's yeast was an unclassified commercial product. All the other microorganisms were cultured at the ISS under the following conditions. The *Escherichia coli* (ISS 8001, isolated from feed) and *Bacillus subtilis* (NCTC 8236) strains were grown in nutrient broth (Difco) for 12–18 h in agitated flasks at 30°C. The *Streptomyces* sp. (ISS 1418, isolated from walnuts) was incubated for 24 h at 30°C in agitated flasks containing a 0.5% autolised yeast and 1% glucose solution. The *Penicillium brevicompactum* (ISS 1439) strain was cultured for 36–48 h at 30°C in agitated flasks containing Czapek–Dox's liquid medium with 3% glucose and 0.5% corn-steep liquor. Two strains of *Candida lipolytica* (5657, isolated from limburg cheese, and 5654, CBS Delft) were obtained from the Istituto di Microbiologia Agraria, Perugia, Italy. They were grown for 18–24 h at 30°C in agitated flasks containing a medium with the following composition: 1% corn-steep liquor, 1% Jamaican molasses, 3% sucrose, 0.05% NH_4NO_3, 0.01% KCl, 0.03% $Na_2HPO_4 \cdot 12H_2O$, 0.01% $MgSO_4 \cdot 7H_2O$. The BP *C. lipolytica* strain was grown at 28°C in a 50-litre stainless steel fermenter for 22 h in a medium containing 0.57% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% NaCl, 0.001% $CaCl_2$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4$, 0.1% yeast extract, 0.3% urea, 3% glucose, 0.0001% silicone. The pH of this medium was 5.7. The fermentation medium (30 litres) was kept under constant mechanical agitation (400 rev/min) and air was admitted at 1 atm. overpressure with a flow rate of 50 litres/min.

Unless otherwise stated, at the end of the fermentation the cells were collected by centrifuging at 12 000 × *g*, washed 4 times with water and lyophilised.

2.2. Preparation of extracts from Toprina and other microorganisms

Unless otherwise stated, Toprina and the other microorganisms were extracted by homogenising 1 g of material in a Potter homogeniser with 10 ml of 0.15 M NaCl at 0°C. Then, the suspension was left for 3 h at 4°C. At the end of the extraction, the suspension was centrifuged at 45 000 × *g* for 30 min and the clear supernatant was freeze-dried. About 50 μl of water solution containing 20 mg/ml of lyophilate were submitted to gel electrophoresis and 15 μl were used for double immunodiffusion analysis as described below.

A larger quantity of Toprina extract was required for injection into rabbits to obtain the antigen preparation and was made by suspending 10 g of material in 100 ml of the salt solution for 3 h with mechanical mixing. The suspension was centrifuged for 30 min at 45 000 × *g*. The clear supernatant was made 4.0 M with $(NH_4)_2SO_4$ and the resulting precipitate collected by centrifuging at 45 000 × *g* for 30 min, redissolved in water, dialysed against water for 5 days at 4°C, and lyophilised.

2.3. Preparation of extracts from feeds

Two types of animal feeds (pellet and flour) containing known amounts of Toprina were analysed. The pellet type contained corn flour (60%), wheat bran (17%), vitamins (3%) and different amounts of peanut flour and Toprina to make the remaining 20% (see Figure 4D). The flour type contained soybean flour (23%), fish meal (6%), milk whey (5%), alfalfa (3%), $CaHPO_4$ (1%), $CaCO_3$ (1%), NaCl (0.5%), vitamins (0.5%) and different amounts of corn flour and Toprina to make the remaining 60% (see Figure 4D).

For extraction, 10 g of finely ground feed were suspended in 100 ml of 0.15 M NaCl and stirred for 3 h under mechanical mixing. At the end of the extraction, the suspension was centrifuged at 45 000 × *g* for 30 min and the clear supernatant was freeze-dried. Fifteen μl of aqueous solution

of the lyophilate (10 mg/ml) were used for double immunodiffusion and 12 μl for radial immunodiffusion analyses.

2.4. Preparation of anti-Toprina immune serum

Ten New Zealand rabbits, each about 3.5 kg body weight, were individually injected with 0.5 ml of antigen solution (10 mg total lyophilate) emulsified with the same volume of Freund's complete adjuvant. About 0.3 ml of this suspension was injected into the foot pad of the posterior limb in each rabbit, and the residual 0.7 ml was given by intramuscular injection. After 21 days a second injection of 2 ml of antigen solution (40 mg total lyophilate) was given intravenously. Following that, the rabbits were injected twice (after each 10 days) with 1 ml of a saline antigen solution containing 1 mg of antigen/ml. After 8 days the rabbits were bled from the marginal vein of the ear to check the antibody levels, then submitted to total bleeding. The sera were heated at 56°C for 30 min to inactivate the complement and stored at −30°C.

2.5. Immunodiffusion analyses

Agarose gel immunodiffusion analyses were carried out according to a slight modification of Wieme's micromethod as described by Piazzi *et al.*[15] Additional immunodiffusion tests were carried out, at different levels of reactions, according to Piazzi.[16] Immunodiffusion analysis performed to localise the position of Toprina antigens on polyacrylamine gel was carried out according to the procedure previously described by Minetti *et al.*[17] Radial immunodiffusion was carried out according to Lietze,[18] using 0.2 ml of antiserum per slide.

2.6. Gel electrophoresis

Disc electrophoresis was carried out in a 0.05 M Tris–0.383 M glycine buffer (pH 8.5) as previously described for wheat albumins.[17] Protein patterns were recorded by a Gilford Spectrophotometer Mod. 2400 (Gilford Instrument Laboratories Inc., Oberlin, Ohio) at 620 nm.

2.7. Gel filtration

About 1 g of the antigen preparation from Toprina was submitted to gel filtration on a Sephadex G-100 column (120 × 6 cm) equilibrated in 0.1 M ammonium acetate buffer (pH 7.8). The absorbance of the eluate at 280 nm was measured continuously (Photochrom flow-analyser, Rastelli, Italy) and recorded (Kompensograph, Siemens, Germany). Twenty × 1 ml fractions were collected and submitted to immunodiffusion analysis as described above. Fractions containing antigenic activity were pooled, lyophilised and submitted to gel electrophoresis and immunodiffusion analysis according to the procedure described by Minetti *et al.*[17]

2.8. Digestion with pepsin

Three hundred μg of pepsin were added to 1 ml of HCl solution (pH 2.2) containing 10 mg of antigen from Toprina. The mixture was incubated for 24 h at 37°C and then the pH was adjusted to 7.0 with 0.1 N NaOH.

2.9. Nitrogen determination

The nitrogen content of the extracts from Toprina and other materials were determined according to Kjeldahl.

3. Results

3.1. Nitrogen extraction

When 100 g of Toprina was dispersed in 0.15 M NaCl (1:10 w/v) with a Potter homogeniser and rapidly centrifuged to clarify the extract, about 0.95 g of nitrogen was extracted. When the homogenate was allowed to stand for 3 h at 4°C or 37°C before centrifuging, 1.1 g of nitrogen was extracted from 100 g of Toprina. No improvement in nitrogen extraction was obtained by allowing

the homogenate to stand at 4°C for periods of time up to 24 h or by using 0.01 M acetic acid, 5 M urea, or 0.01 M NaOH as extraction solvents instead of the salt solution. Light microscopy observation showed that in no case had a significant cell disruption occurred. Therefore, we tried to improve nitrogen extraction by treating the biomass in a press (X-press, AB Biox, Nacka, Sweden) at pressures up to 6 atm. Such treatment, however, was ineffective either in causing cell fragmentation or in improving nitrogen extraction.

About 1.7 g of nitrogen was extracted with 0.15 M NaCl under standard extraction conditions from 100 g of the biomass obtained by growing the BP *Candida lipolytica* strain in the laboratory under the conditions described in the experimental section. After treatment with the X-press about 4 g of nitrogen could be extracted with 0.15 M NaCl from 100 g of this material. In this case light microscopy observation showed extensive cell disruption after the X-press treatment. However, after the biomass had been heated for 15 min at 80°C and then dried overnight at 110°C, only 1.1 g of nitrogen could be extracted from 100 g of the biomass even after treatment with the X-press, which is very similar to the result obtained with Toprina.

We succeeded in obtaining a more effective nitrogen extraction from Toprina (about 7 g/100 g of material) only by suspending the biomass in 1.0 M NaOH at room temperature for 24 h.

3.2. Gel electrophoresis

When submitted to polyacrylamide gel electrophoresis in a Tris–glycine buffer system (pH 8.5), the extracts obtained from the same batch of Toprina under the different experimental conditions described in section 3.1, with the exception of the 0.1 M NaOH extract, gave electrophoretic patterns all closely related to that shown in Figure 1(a). No distinct electrophoretic pattern could be obtained from the 1.0 M NaOH extract from Toprina even after neutralisation and exhaustive dialysis against distilled water, indicating extensive denaturation of the protein in this case. A few minor qualitative differences and some significant quantitative differences were evident among the electrophoretic patterns of extracts obtained with 0.15 M NaCl from different lots of Toprina [Figure 1(a), (b), (e)]. As shown in Figure 1, large qualitative differences were evident among the electrophoretic patterns of Toprina and those of all the other microorganisms tested [Figure 1(d), (f), (g), (h), (i), (l)], including the two strains of *Candida lipolytica* 5657 and 5654 which gave identical patterns [Figure 1(c)]. The electrophoretic pattern of the BP *C. lipolytica* strain grown on the synthetic laboratory medium was more closely related to the electrophoretic pattern of the two *C. lipolytica* strains than to those of the three lots of Toprina tested.

3.3. Immunochemical studies

An anti-Toprina immune serum was prepared by injecting a salt-soluble extract from Toprina into rabbits. By submitting the anti-Toprina immune serum to immunodiffusion analysis at different levels of reaction with the 0.15 M NaCl extract from Toprina (Figure 2), we showed the presence in the immunodiffusion pattern of three main precipitation lines. This result was further confirmed by comparing (Figure 3) the electrophoretic pattern of the protein extract from Toprina [already reported in Figure 1(b)] with the immunodiffusion pattern of the same extract submitted to gel electrophoresis and then reacted with anti-Toprina immune serum (see experimental section). With this more sensitive technique, in addition to the three main precipitation lines, we showed the presence of four very faint precipitation lines not detectable in the double immunodiffusion system. No direct correspondence was found between the protein bands and the precipitation lines (Figure 3), showing that Toprina antigens cannot be identified with protein components. This interpretation was supported by the fact that, after peptic digestion of the antigen preparation, protein bands were no longer detectable in the electrophoretic pattern, whereas the number and the position of the precipitation lines in both the immunodiffusion and immunoelectrophoretic patterns were unaffected. To evaluate apparent molecular weights of Toprina antigens, the 0.15 M NaCl extract from Toprina was submitted to gel filtration on a Sephadex G-100 column and antigenic activities of the eluted fractions were determined. The absorbance at 280 nm of the same fractions was measured as well. With this procedure we showed that the three main antigens indicated in Figures 2

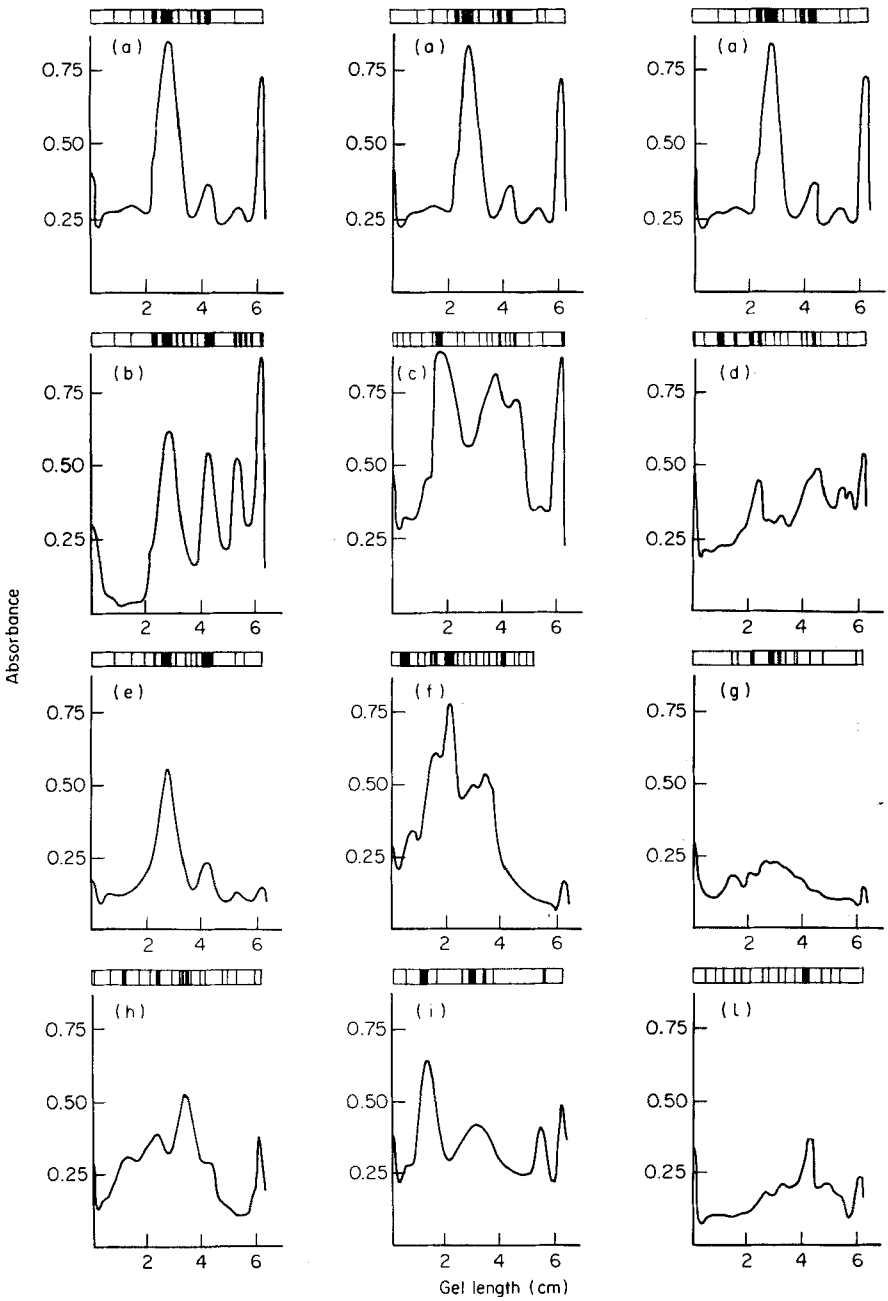

Figure 1. Gel electrophoretic patterns of 0.15 M NaCl extracts from Toprina, Liquipron, and other biomasses. (a) Toprina batch A; (b) Toprina batch B; (c) *Candida lipolytica*; (d) *Bacillus subtilis*; (e) Toprina batch C; (f) commercial baker's yeast; (g) *Bacterium coli*; (h) Liquipron; (i) *Penicillium brevicompactum*; (l) *Streptomyces* sp.

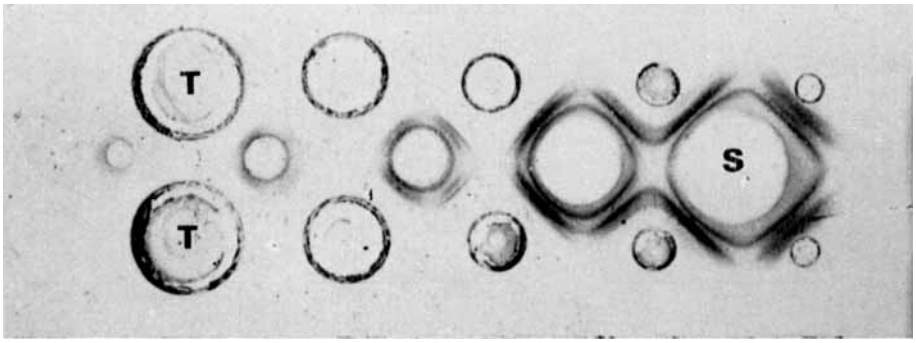

Figure 2. Double immunodiffusion analyses at different levels of reaction of 0.15 M NaCl extract from Toprina (T) with anti-Toprina immune serum (S).

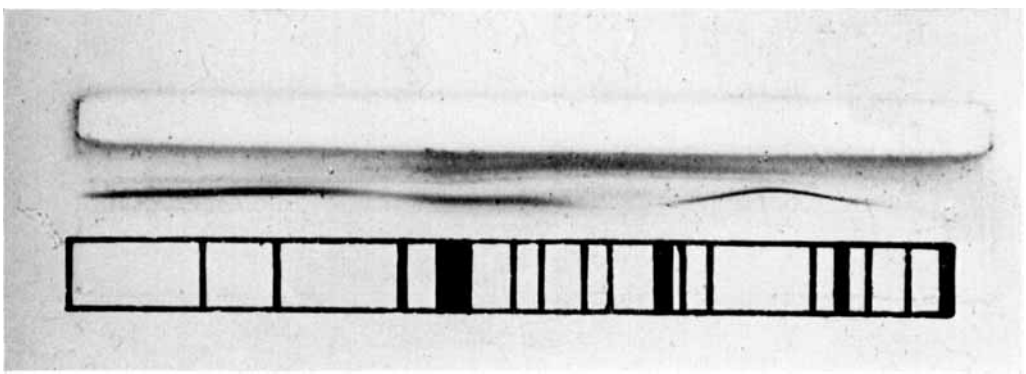

Figure 3. Comparison of gel electrophoretic protein pattern (bottom part) and immunoelectrophoretic pattern (upper part) of 0.15 M NaCl extract from Toprina.

and 3 have different apparent molecular weights. The two antigens moving slower during the gel electrophoretic fractionation (Figure 3) were eluted in the void volume of the Sephadex column, whereas the retention volume of the fast moving one corresponded to that of cytochrome *c*. Moreover, no correspondence was observed between the elution pattern of the antigens and that of the material absorbing at 280 nm.

In order to evaluate the specificity of anti-Toprina immune serum, we submitted the salt extracts from all the microorganisms of Figure 1 to immunodiffusion analysis with the immune serum. As shown in Figure 4, identical immunodiffusion patterns were given by the extracts from two lots of Toprina (Figure 4A, 2 and 4), whereas the extracts from the two strains of *C. lipolytica* only gave two precipitation lines, lacking the one closer to the antigen hole (Figure 4A, 6 and 7). The extracts from all the other microorganisms, including *Candida tropicalis* (not shown in the Figure), only gave the precipitation line closest to the antiserum hole (Figure 4B and C). Immunodiffusion analyses with anti-Toprina immune serum of salt extracts from animal feeds containing different amounts of Toprina are shown in Figure 4D. It appears that intensities of precipitation lines increased with the amount of Toprina added. Identical immunodiffusion patterns were obtained with both pellet and flour animal feeds. Only one precipitation ring was obtained when the extracts from animal feeds with added Toprina were tested with anti-Toprina immune serum by radial immunodiffusion (Figure 5A). As shown in Figure 5B, there was a linear relationship between the areas of the precipitation rings of Figure 5A and the amounts of Toprina added to the feed samples.

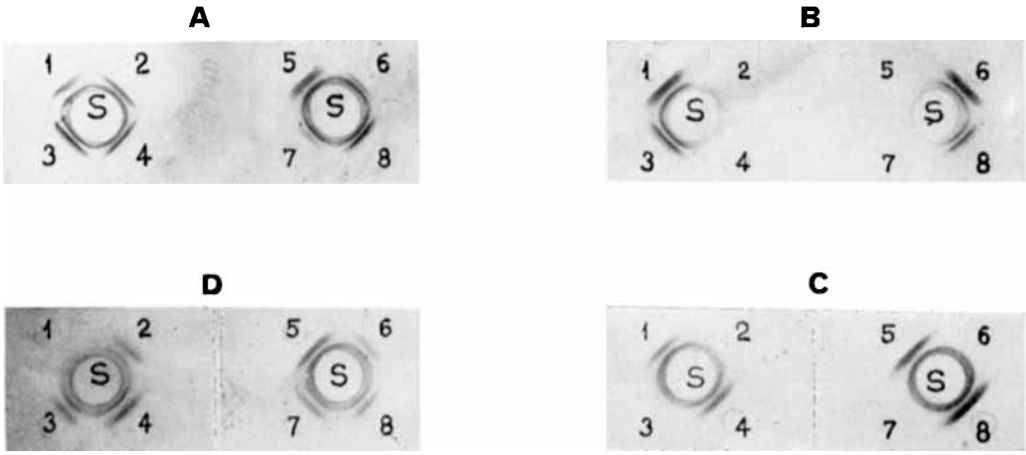

Figure 4. Double immunodiffusion analyses of 0.15 M NaCl extracts from different microbial species, Toprina, and Toprina-containing feeds with anti-Toprina immune serum.

A: 1, 3, 5, 8, controls (Toprina antigen); 2, Toprina batch A; 4, Toprina batch B; 6, *Candida lipolytica* (strain 5654); 7, *Candida lipolytica* (strain 5657).

B: 1, 3, 6, 8, controls (Toprina antigen); 2, *Penicillium brevicompactum*; 4, *Bacterium coli*; 5, *Bacillus subtilis*; 7, *Streptomyces* sp.

C: 1, 4, 5, 8, controls (Toprina antigen); 2, Liquipron; 3, commercial baker's yeast; 6, 7, mixtures of equal parts of *Penicillium brevicompactum*, *Bacterium coli*, *Bacillus subtilis*, *Streptomyces* sp. and commercial baker's yeast.

D: 1, feed without Toprina; 2, feed containing 2.5% Toprina; 3, 6, feed containing 5% Toprina; 4, 8, feed containing 10% Toprina; 5, control (Toprina antigen); 7, feed containing 15% Toprina.

4. Discussion

The data reported show that only a small amount (about 10%) of the total nitrogen content of Toprina could be extracted with a number of different procedures. The poor nitrogen extraction from Toprina appeared related to the high resistance of Toprina cells to mechanical disruption. The cells obtained by growing the BP *C. lipolytica* strain on a synthetic medium containing glucose did not show such high mechanical resistance and much higher nitrogen yields could be obtained when they were extracted under the same conditions as used for Toprina. However, the nitrogen extraction pattern of this biomass after short heating in water suspension at 80°C and drying overnight at 110°C became very similar to that of Toprina. This result suggests that the industrial processing of Toprina, which also includes a relatively high temperature drying, causes an increase of cell resistance to mechanical damage. Disrupting the cell walls might help to increase nitrogen availability when feeding Toprina to animal species not able to digest microbial cell walls.

The qualitative composition of the proteins extracted from Toprina was found to be very specific and allowed an easy differentiation of Toprina from the other microorganisms tested. Some quantitative differences found between the protein extracts from the different lots of Toprina tested might be the result of some lack of standardisation of the conditions used for biomass culture or processing. Although useful for checking the constancy of Toprina production, the gel electrophoretic patterns were too complex to be of practical help for the detection of Toprina in mixture with other feeds. In this respect, our serological investigations gave more conclusive results. We have shown that Toprina has at least one specific antigen that allows its differentiation from the other microorganisms tested, including the two strains of *C. lipolytica* that appeared most closely related to Toprina. Immunodiffusion analysis with anti-Toprina immune serum obtained by injecting a salt-soluble fraction from Toprina into rabbits allowed the detection of Toprina added to very heterogeneous animal feeds in amounts as low as 2.5%. Moreover, as long as the content of the specific antigen is constant in different lots of Toprina, a quantitative determination of Toprina can be

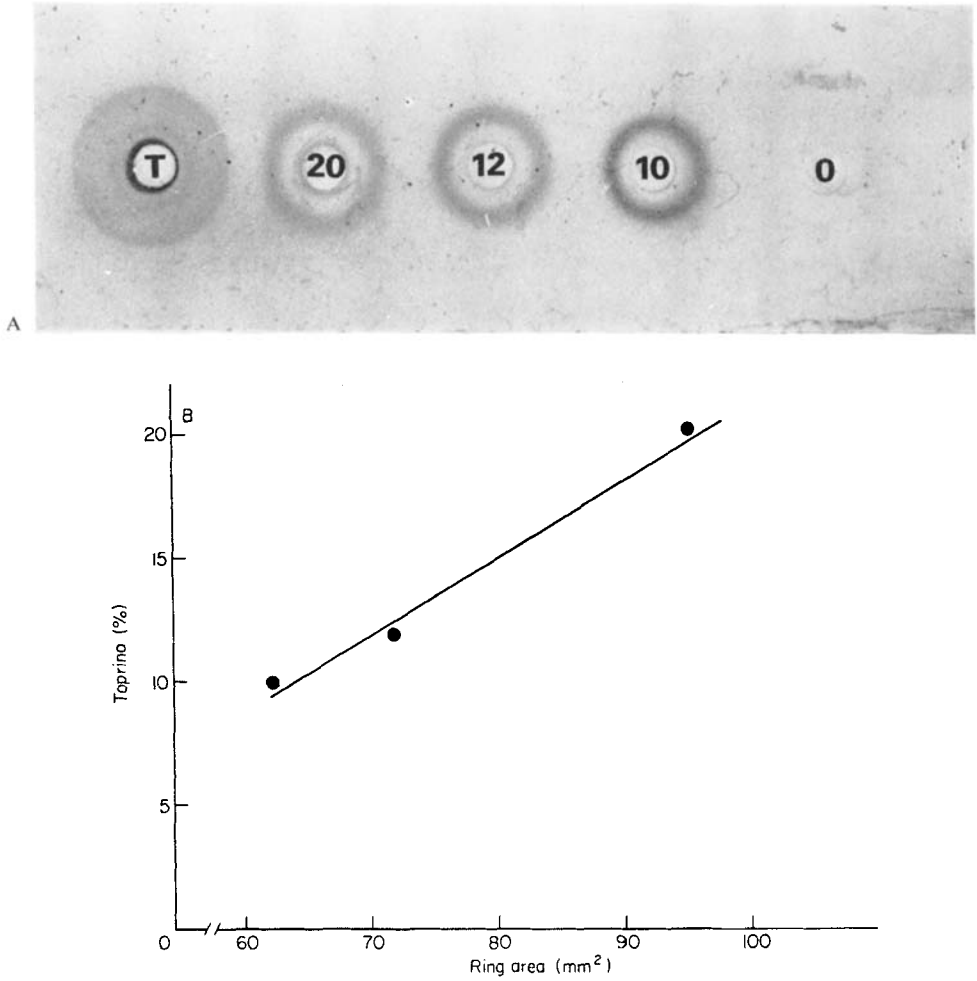

Figure 5. Radial immunodiffusion analyses of 0.15 M NaCl extracts from Toprina-containing feeds with anti-Toprina immune serum. A (from left to right), Toprina and animal feeds containing 20, 12, 10% and no Toprina, respectively. B, areas of the precipitation rings of Figure 5A are plotted against Toprina percentage of the corresponding samples.

achieved by submitting feed extracts to radial immunodiffusion with anti-Toprina immune serum.

Even though there are a number of aspects that militate against a protein nature of Toprina antigens, at this moment we have not yet established their chemical nature. The extraction, electrophoretic, and gel filtration behaviours of the Toprina antigens suggest that they might be acidic polysaccharides. This possibility is also supported by the results of Fukazawa *et al.*[13] who showed that acidic soluble polysaccharides of *Candida albicans* have a high degree of specificity and immunogenicity and that IgG produced by rabbits after immunisation with soluble polysaccharides contained a specific antibody factor capable of differentiating *C. albicans* from *C. tropicalis*.

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