Study of the Formation of Caseinomacropeptides in Stored Ultra-High-Temperature-Treated Milk by Capillary Electrophoresis

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The proteolytic activity of enzymes of psychrotrophic bacteria on casein and raw and ultra-high-temperature (UHT)-treated milk was studied by capillary electrophoresis (CE) in order to characterize the degradation products and evaluate the possibility of distinguishing them from those due to the action of chymosin on κ-casein. Casein hydrolysates with *Pseudomonas fluorescens* B52 proteinase, milk inoculated with *P. fluorescens*, and commercial UHT milks were studied. The degradation products included caseinomacropeptide (CMP), pseudo-caseinomacropeptide (pseudo-CMP; CMP lacking the 106 Met residue) and an unidentified third peak. These peaks were already present in some freshly prepared UHT milk samples, and a progressive increase in peak area was observed upon storage at 22 °C. This might give rise to false positive results when the presence of rennet whey is investigated by CE. However, low area ratios of pseudo-CMP to CMP might allow the presence of rennet whey solids to be suspected.

Keywords: *Caseinomacropeptide; capillary electrophoresis; UHT milk; proteolysis; psychrotrophic proteinases*

INTRODUCTION

Thermostable proteases of psychrotrophic origin cause extensive degradation of κ- and β-casein in milk, while α_{S1}- and α_{S2}-casein and, in particular, whey proteins are more resistant to the action of these enzymes (Law, 1979). This leads to physical and organoleptic defects, especially in ultra-high-temperature (UHT)-treated milk, such as bitter, unclean, and putrid flavors and gelation, thereby posing a great problem for the dairy industry (Miranda and Gripon, 1986).

In addition, the proteolytic activity of psychrotrophic proteinases on milk interferes with the determination of the fraudulent addition of rennet whey solids, which is based upon the detection and quantification of caseinomacropeptide (CMP), the hydrophilic fragment of κ-casein (106−169) released by chymosin during milk clotting (López-Fandiño and Ramos, 1993). Thus, when the presence of rennet whey is investigated by HPLC on a gel permeation column, psychrotrophic proteinases that progressively split κ-casein in milk might give rise to degradation products similar to CMP, leading to false positive results (Olieman and van den Bedem, 1983). In 1989, Olieman and van Riel published an HPLC separation of CMP on a wide pore reversed-phase column, which allowed the separation of closely related species, and concluded that only with unusually high psychrotrophic counts was a minor shoulder with the retention time of CMP found (Olieman and van Riel, 1989). However, it has been shown that peptides produced by *Pseudomonas* proteinases are formed during storage of UHT milk (López-Fandiño et al., 1993a), depending on the storage conditions (Corzo et al., 1994) and the composition of milk (López-Fandiño et al., 1993b).

Recently, van Riel and Olieman (1995) have published a very selective capillary electrophoresis method to detect the presence of rennet whey solids, which allows the separation of CMP from pseudo-CMP (CMP lacking the N-terminal Met residue) and can prevent false positive results in milk powder and buttermilk powder. However, the suitability of this methodology in the case of liquid milk has not been investigated.

UHT milk consumption is expanding, and in some European countries such as Spain, Germany, France, and Belgium, the market is dominated by this type of milk (Sorensen, 1992). UHT milk is preferred by dairy companies and retailers over other milk products due low labor and refrigeration costs, and thus, it has been increasingly made available to the consumers. Rennet wheys are available at low prices, and thus, it is economically attractive to use them for the adulteration of dairy products (Olieman and van den Bedem, 1983).

The objective of this work has been to follow the proteolytic activity of psychrotrophic enzymes on raw and UHT milk, with the aim of characterizing the degradation products and evaluating the possibility of distinguishing them from those due to the action of chymosin on κ-casein.

MATERIALS AND METHODS

Samples. Isoelectric casein was treated with *Pseudomonas fluorescens* B52 proteinase and crystalline chymosin (EC 3.2.23.4) (Sigma Chemical Co., St. Louis, MO) as previously described (López-Fandiño et al., 1993a,b).

For the experiments employing milk inoculated with *P. fluorescens*, raw milk was obtained from a local dairy farm. Milk was skimmed by centrifugation at 3800*g* for 15 min, at 20 °C, transferred aseptically into 250-mL autoclaved storage bottles, and heated in a water bath at 63 °C for 30 min. Milk was inoculated with *P. fluorescens* strain NCIB9046 (Spanish Type Culture Collection, Valencia, Spain), at bacterial counts of 1×10^3, 1×10^5, and 1×10^7 cfu/mL. After inoculation, the flasks were stored at 6 °C for 130 h.

Four batches of UHT milk, two of whole milk (1 and 3) and two of skim milk (2 and 4), were prepared at two commercial direct UHT dairy plants (see below). Batches 1 and 2 were heated at 140 ± 1 °C for 3 s, while batches 3 and 4 were heated at 149 ± 1 °C for 3 s. The batches were stored at 22 °C, and

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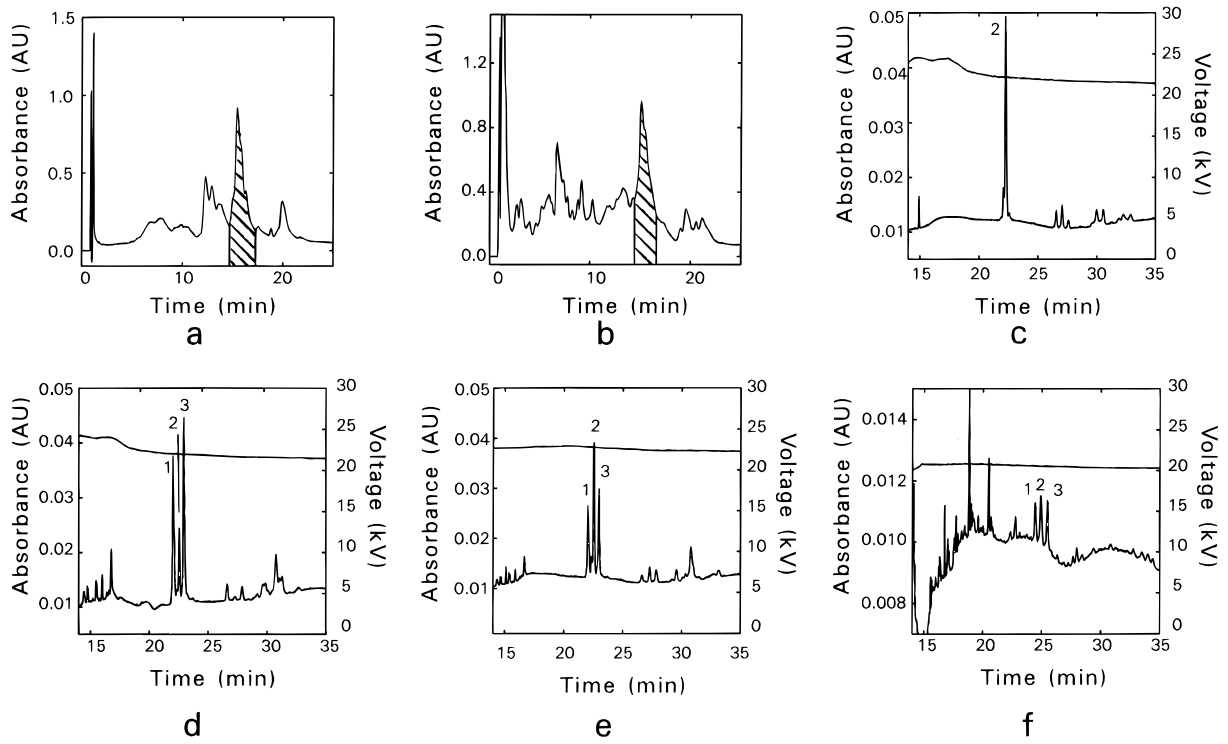

Figure 1. RP-HPLC chromatograms of hydrolysates of casein (the shaded area indicates the collected fraction) with chymosin (a) and *P. fluorescens* B52 proteinase (b) and electropherograms (CE) of the collected fractions of the hydrolysates with chymosin (c) and *P. fluorescens* B52 proteinase (d), coinjection of panels c and d (e), and coinjection of panel d with skim milk powder containing 5% rennet whey solids (f), together with the voltage recordings. Peak 1, tentatively assigned as pseudo-CMP; peak 2, CMP; peak 3, unknown.

samples were taken for analysis by opening two containers each time, at 30-day intervals for 4 months.

In addition, 26 commercial samples of direct and indirect, skim and whole UHT milks were studied. In direct heating systems the milk is heated by direct contact with steam under pressure, while in indirect systems the milk and the heating medium are separated by a heat-conducting barrier, usually made of stainless steel. The elapsed time from processing was not the same in all cases, but all the milks were within their use by dates.

Skim milk powder containing 5% (w/w) rennet whey solids (Ni-5) was used to identify the caseinomacropeptide peak. It was prepared at the NIZO experimental dairy by adding pasteurized whey (5%, w/w, dry matter) to skim milk followed by low-temperature spray-drying with a small-scale industrial spray-drier (van Riel and Olieman, 1995).

Microbiological Analysis. Appropriate dilutions of milk were made using sterile 1% (w/v) bactopeptone (Difco Laboratories, Detroit, MI) and spread-plated onto plate count agar (Oxoid, Basinstoke, Hampshire, U.K.). Total aerobic and psychrotrophic counts were obtained after incubation at 31 °C for 48 h and at 7 °C for 7 days, respectively. Determinations were performed in duplicate.

HPLC Analysis. Casein digests were treated with trichloroacetic acid to a final concentration of 4% (w/v) in order to precipitate intact caseins and large peptides. The supernatants were filtered through Durapore 0.45 μm filters (Millipore Corp., Bedford, MA).

A Beckman System Gold HPLC, composed of two Model 115 pumps in combination with a Beckman system organizer and a 168 diode array detector module was used with a System Gold Software data acquisition system (Beckman Instruments Inc., San Ramon, CA). Separations were performed on a reversed-phase Beckman ultrapore column (75 × 4.6 mm) at room temperature. Solvent A was 0.1% trifluoroacetic acid (TFA; Merck, Darmstadt, Germany) in Milli-Q water (Millipore Corp.), and solvent B was 0.1% TFA in acetonitrile (HPLC grade from Scharlau, Barcelona, Spain). The column was initially equilibrated with 80% (v/v) A and 20% (v/v) B. The gradient was formed by increasing solvent B from 20% (v/v) to 35% (v/v) during the first 20 min after the injection, and the elution was stopped after a 5-min hold at this concentration. A flow rate of 1 mL/min was employed with detection at 210 nm (López-Fandiño et al., 1993a).

The main peaks from the chromatograms of the *P. fluorescens* B52 proteinase and chymosin digests of caseins were collected from several injections, lyophilized, and further subjected to CE analysis.

Capillary Electrophoresis (CE). Milk and Ni-5 samples for CE analyses were prepared as described by van Riel and Olieman (1995). Milk samples were acidified with 2 N HCl to a final pH of 4.6 followed by centrifugation at 4500*g* for 15 min at 5 °C, in order to separate casein from whey. Wheys were frozen until analyzed; 1 mL of whey was heated at 90 °C for 6 min, to induce precipitation of whey proteins and avoid their interference, and then cooled to room temperature before the addition of 200 μL of lactate buffer, pH 4.6. After standing for 15 min at room temperature, the sample was centrifuged at 1600*g* for 10 min, and finally, 400 μL of the supernatant was mixed with 600 μL of the sample buffer. Lyophilized fractions collected after RP-HPLC analysis of the *P. fluorescens* proteinases and chymosin digests of casein were dissolved directly in sample buffer diluted with water (6:4).

CE was carried out using a Beckman P/ACE System 2050 workstation controlled by a System Gold Software data system, version 810. The separations were performed using a hydrophilic coated fused-silica gel capillary column, Supelco CElect P1 (Bellefonte, PA), 37 cm × 50 μm i.d., with a slit opening of 100 × 800 μm. Separations were run as described by van Riel and Olieman (1995), except that the final current was 50 μA, with a final voltage of around 21 kV. At least two replicate analyses were performed.

RESULTS AND DISCUSSION

Proteolytic Products Produced by the Action of *P. fluorescens* Proteinases on Caseins and Raw Milk. Initially, model systems composed of casein and either chymosin or the extracellular proteinase of *P. fluorescens* B52 were studied. The hydrolysates were analyzed by RP-HPLC, and although the psychrotrophic enzyme digest gave a more complex elution pattern, the main peak was indistinguishable in both cases (Figure 1a,b).

The main peaks were collected from several HPLC runs and further subjected to CE analysis. CE gave a better resolution of the peptides and showed that, while chymosin hydrolysis was limited to the formation of CMP (Figure 1c), the profile of the RP-HPLC peak from the psychrotrophic enzyme digest could be separated by CE into three different hydrolysis products (Figure 1d). One of them (peak 2) was identified as CMP, arising from the cleavage of the Phe(105)−Met(106) bond of κ-casein, by coinjection with the isolated peak from the chymosin hydrolysate (Figure 1e). By comparison with purified CMP samples characterized by amino acid analysis and sialic acid content, van Riel and Olieman (1995) assigned that peak to the nonglycosylated monophosphorylated variant of CMP.

When the isolated peak from the *Pseudomonas* enzyme digest was coinjected with skim milk powder containing 5% (w/w) rennet whey solids (Ni-5) (Figure 1f), a variation in the migration times was observed. This could be due to the presence of different ions in the sample matrix the absence of lactate in the sample corresponding to the collected peak being particularly relevant.

The peak migrating before the CMP (peak 1) was tentatively identified as pseudo-CMP (CMP lacking the 106 Met residue) by coinjection with an acid buttermilk sample prepared as explained in van Riel and Olieman (1995). CMP and pseudo-CMP can be adequately separated by this method despite the fact that there is no difference in their net charge at pH 3 (van Riel and Olieman, 1995).

In order to investigate whether these degradation products were also produced during psychrotrophic growth in milk, raw milk was inoculated with *P. fluorescens* at bacterial counts of 1×10^3, 1×10^5, and 1×10^7 cfu/mL. Figure 2 shows the electropherograms obtained after 130 h of incubation at 6 °C (bacterial counts had reached values of 6.8×10^7, 1.2×10^8, and 1.4×10^8 cfu/mL, respectively), compared with a sample of skim milk powder containing 5% (w/w) rennet whey solids (Ni-5). Proteolysis increased with the initial bacterial levels and gave rise to pseudo-CMP, CMP, and the unidentified third peak.

Although the possibility arises that these enzymes do not produce "real" CMP but a closely related degradation product, indistinguible by the CE method, it should be noted that acid proteases are highly specific toward the Phe(105)−Met(106) position of κ-casein, due to the particular structure of the protein. Nevertheless, the general proteolytic activity of psychrotrophic proteinases is greater than that toward the rennet sensitive bond of κ-casein (Dalgleish, 1993).

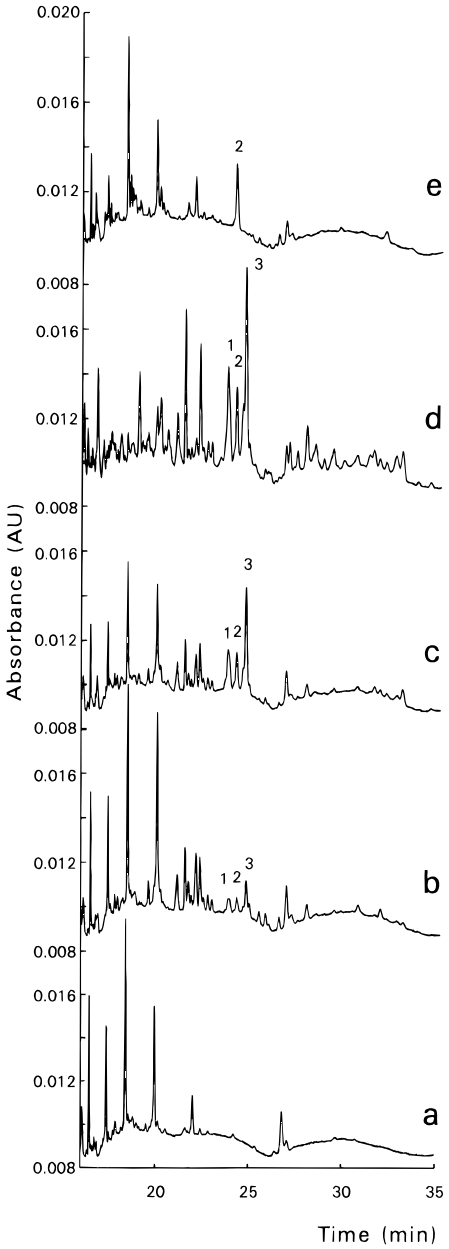

Figure 2. CE of raw milk (a), and milk inoculated with *P. fluorescens* at bacterial counts of 1×10^3 (b), 1×10^5 (c), and 1×10^7 (d) cfu/mL, after 130 h of incubation at 6 °C (final counts of 6.8×10^7, 1.2×10^8, and 1.4×10^8 cfu/mL, respectively), and a sample of skim milk powder containing 5% (w/w) rennet whey powder (e). Peak 1, tentatively assigned as pseudo-CMP; peak 2, CMP; peak 3, unknown.

Proteolysis due to Psychrotrophic Enzymes upon Storage of UHT Milk. Commercial UHT milks may contain proteinases with different heat stabilities, pH and temperature optima, or substrate specificities. Thus, experiments with UHT milks of different origins were carried out to evaluate whether the degradation pattern obtained with crude psychrotrophic enzymes and pure bacterial strains could also be found in processed milk.

Formation of CMP, pseudo-CMP, and peak 3 was studied in four batches of direct UHT milk, stored for 4 months at 22 °C, as shown in Table 1. Freshly prepared UHT milks from the different batches differed with respect to the presence of proteolytic degradation products. In batch 1, no peptides were detected, whereas in milks from the other batches, the peaks corresponding to CMP, pseudo-CMP, and peak 3 were already present immediately after processing. The presence of certain peptides in UHT milk has been related to the bacteriological quality of the raw material employed (Mottar et al., 1985).

A progressive increase in the peak areas was observed upon storage, which was clearly influenced by the initial peptide content. Milk for batch 4 experienced the greatest proteolytic degradation on storage, while batch 1 showed the lowest formation of peptides. In all batches, pseudo-CMP was formed upon storage more rapidly than CMP, leading to an increase in the peak area ratio of pseudo-CMP to CMP from 0.8 to 1.9, for CMP areas ranging from 0.042 to 0.5. Electropherograms of milks from batches 3 and 4, after 90 days of storage, showed a CMP peak which was equivalent to the addition of 4% (w/w) rennet whey solids. However,

Table 1. Integrated Areas of CE Peaks of Pseudo-CMP, CMP, and Peak 3, during Storage of Direct UHT Whole and Skim Milk Samples

	whole milk					skim milk				
time (days)	batch	pseudo-CMP	CMP	peak 3	pseudo-CMP/CMP	batch	pseudo-CMP	CMP	peak 3	pseudo-CMP/CMP
0	1	0.000	0.000	0.000	0.00	2	0.015	0.026	0.020	0.59
30	1	0.045	0.042	0.051	1.06	2	0.062	0.063	0.074	0.98
60	1	0.072	0.046	0.082	1.56	2	0.116	0.077	0.106	1.50
90	1	0.120	0.064	0.106	1.88	2	0.165	0.089	0.130	1.83
120	1	0.146	0.067	0.130	2.17	2	0.260	0.175	0.175	1.49
0	3	0.062	0.058	0.084	1.08	4	0.085	0.101	0.210	0.84
30	3	0.340	0.275	0.300	1.24	4	0.350	0.285	0.590	1.23
60	3	0.475	0.325	0.270	1.46	4	0.470	0.300	0.530	1.57
90	3	0.715	0.460	0.320	1.55	4	0.780	0.453	0.685	1.72
120	3	0.818	0.500	0.305	1.64	4	0.910	0.474	0.620	1.92

Table 2. Mean Values and Variability of the Integrated Areas of CE Peaks of Pseudo-CMP, CMP, and Peak 3 in Commercial UHT Milks

	pseudo-CMP	CMP	peak 3	pseudo-CMP/CMP
X	0.253	0.192	0.347	1.3
min	0.048	0.033	0.084	0.39
max	1.023	0.667	1.648	2.50
n	26	26	26	26
SD	0.240	0.153	0.312	0.47

in the case of batch 1, even after 120 days of storage, the amount of CMP formed was lower than that corresponding to milk with 0.3% (w/w) whey solids added.

With a view to finding a relationship between the different degradation products, we analyzed 26 individual samples of UHT milks with different fat contents, which had been subjected to either direct or indirect heat treatments. As shown in Table 2, there was a considerable variability among the samples, with some of them showing an intense proteolytic degradation. However, in most of these cases the area ratio of pseudo-CMP to CMP ranged from 0.86 to 2.50, for CMP area values similar to the ones found in the four controlled batches. This suggests that the presence of CMP could be attributed to the degradation of κ-casein by heat-resistant bacterial proteases during storage. Only in the case of one milk sample, which showed a low peak area ratio of 0.39, with a CMP area of 0.558, can the possibility of the fraudulent addition of rennet whey solids be suspected, as the area of the CMP peak is too high in comparison with the areas of the other two degradation products.

The present results show that the proteolytic activity of psychrotrophic proteinases in raw and UHT milk leads to the appearance of CMP, as well as to pseudo-CMP and an unidentified third peak, when using the CE method of van Riel and Olieman (1995). This was confirmed not only with crude proteinases and pure bacterial strains but also in commercial UHT milk samples which could contain proteases with various specificities from many different bacterial strains. Although this might give false positive results for the presence of rennet whey, the detection of low amounts of rennet whey solids added to milk is possible in the case of milks manufactured from raw materials of good microbiological quality.

In the case of UHT milks with high proteolytic activity, low area ratios of pseudo-CMP to CMP allow the presence of rennet whey solids to be suspected. In addition, the electropherograms provide an indication of excessive proteolytic degradation, pointing to bad storage conditions or poor bacterial quality of the milk used.

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LITERATURE CITED

Corzo, N.; López-Fandiño, R.; Delgado, T.; Ramos, M.; Olano, A. Changes in furosine and proteins of UHT-treated milks stored at high ambient temperatures. *Z. Lebensm.-Unters. Forsch.* **1994**, *198*, 302−306.

Dalgleish, D. G. The enzymatic coagulation of milk. In *Cheese: chemistry, physics and microbiology. Vol. 1. General Aspects*; Fox, P. F., Ed.; Chapman & Hall: London, 1993; pp 63−96.

Law, B. A. Reviews of the progress of dairy science: enzymes of psychrotrophic bacteria and their effects on milk and milk products. *J. Dairy Res.* **1979**, *46*, 573−588.

López-Fandiño, R.; Ramos, M. El caseinomacropéptido bovino. II. Detección de la presencia de suero de quesería en productos lácteos (Bovine caseinomacropeptide. II. Detection of cheese whey in dairy products). *Rev. Esp. Cienc. Tecnol. Aliment.* **1993**, *33*, 1−12.

López-Fandiño, R.; Olano, A.; San José, C.; Ramos, M. Application of reversed-phase HPLC to the study of proteolysis in UHT milk. *J. Dairy Res.* **1993a**, *60*, 111−116.

López-Fandiño, R.; Olano, A.; Corzo, N.; Ramos, M. Proteolysis during storage of UHT milk: differences between whole and skim milk. *J. Dairy Res.* **1993b**, *60*, 339−347.

Miranda, G.; Gripon, J. C. Origine, nature et incidences technologiques de la protéolyse dans le lait (Origin, nature and technological significance of proteolysis in milk). *Lait* **1986**, *66*, 1−18.

Mottar, J.; van Renterghem, R.; de Vilder, J. Evaluation of the raw material for UHT milk by degermining the degree of protein breakdown through HPLC. *Milchwissenschaft* **1985**, *40*, 717−721.

Olieman, C.; van den Bedem, J. W. A sensitive HPLC method of detecting and estimating rennet whey total solids in skim milk powder. *Neth. Milk Dairy J.* **1983**, *37*, 27−36.

Olieman, C.; van Riel, J. A. M. Detection of rennet whey solids in skim milk powder and buttermilk powder with reversed-phase HPLC. *Neth. Milk Dairy J.* **1989**, *43*, 171−184.

Sorensen, H. H. The liquid milk market. *Bull. IDF* **1993**, *280*, 46−64.

van Riel, J.; Olieman, C. Determination of caseinomacropeptide with capillary zone electrophoresis and its application to the detection and estimation of rennet whey solids in milk and buttermilk powder. *Electrophoresis* **1995**, *16*, 529−533.

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