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Use of aqueous two-phase systems in sample preparation for polymerase chain reaction-based detection of microorganisms

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Abstract

An aqueous two-phase system, consisting of poly(ethylene glycol) (PEG) and dextran, was employed to separate polymerase chain reaction (PCR)-inhibitory substances from bacterial cells. The PCR inhibition of four soft cheeses was examined and three of them were found to be strongly PCR-inhibitory. Extraction of the PCR-inhibitory soft cheeses inoculated with *Listeria monocytogenes* in an aqueous two-phase system containing 8% (w/w) PEG 4000 and 8% (w/w) dextran 500, was found to lower the PCR detection level of *L. monocytogenes* by more than four orders of magnitude in two of the cheeses compared to the case where no such sample pretreatment was performed. Depending on the type of cheese used, the PCR-inhibitory factors were found to be enriched in either the top or bottom phase in the aqueous two-phase system. These results show that different soft cheeses contain different types and amounts of PCR-inhibitory substances.

Keywords: Aqueous two-phase systems; Microorganisms; Polymerase chain reaction; Cheese; PCR-inhibitory factors; Two-phase systems, aqueous; Sample preparation method; *Listeria monocytogenes*

1. Introduction

Within the space of a few years, the polymerase chain reaction (PCR) has become a widely employed method for the rapid detection of microorganisms in food and clinical samples. Because of its specificity and sensitivity, the technique is well suited for the detection of low numbers of specific microorganisms. Although PCR can be extremely effective when analysing pure microbial cultures, the sensitivity can be dramatically reduced when it is applied directly to complex biological samples, such as food, clinical and environmental samples. The major reason for this is the presence of substances that interfere with the PCR [1]. It has been suggested that proteinases and chelating substances, for example, inhibit the PCR [2,3]. The inhibitors may affect the heat-stable DNA polymerase or interfere with the target DNA sequence that is to be amplified.

Much effort is being devoted to the development of sample preparation methods which facilitate PCR detection, by separating the microorganisms from the PCR inhibitors and/or by concentrating the microorganisms to detectable concentrations (see, for review, Ref. [4]). A traditional method is to extract the sample in phenol and then to precipitate DNA [5]. However, a major fraction of the DNA is usually lost during this procedure. Another technically elegant sample treatment method is to selectively enrich a particular bacterium in a sample by using immunomagnetic separation techniques [6]. A disadvantage of this method is that the specificity is determined by

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the antibodies rather than the specificity of the PCR. Another approach is to separate DNA from a PCR-inhibitory sample with DNA affinity columns [7]. Common to all these sample preparation techniques is that the bacteria and the DNA are separated from the PCR-inhibitory sample matrix. In contrast, we have recently described a method based on an aqueous two-phase system where PCR-inhibitory compounds were separated from the bacteria in a food sample, rather than vice versa [8].

The aim of the present study was to investigate the PCR detection level of *Listeria monocytogenes* in different cheeses after their extraction in an aqueous two-phase system. We have added increasing sample volumes to the aqueous two-phase system to investigate the tolerable load of PCR inhibitors in the system. Different PCR-inhibitory soft cheeses were extracted in aqueous two-phase systems to study the partitioning of PCR inhibitors. The distribution of *L. monocytogenes* in aqueous two-phase systems was determined by recording viable counts.

2. Experimental

2.1. Bacterial strain and culture conditions

L. monocytogenes 167vet was obtained from the Swedish Meat Research Institute (Kävlinge, Sweden). The number of colony-forming units (CFU) was determined by serial dilution, followed by subsequent plating on Listeria-selective agar base (Oxoid Formulation CM 856; Unipath, Basingstoke, UK) supplemented with Listeria-selective supplement (Oxoid SR 140). The plates were incubated at 37°C for 48 h.

2.2. Preparation of food samples

Soft cheeses (Bavaria Blue, Feta, Cambozola and Camembert) were bought at a local store. A 25-g sample of each cheese was mixed with 225 ml sterile physiological saline solution, homogenised in a Stomacher for 2 min, inoculated with *L. monocytogenes*, homogenised again for 30 s, and distributed in 1-ml test tubes, which were stored in a deep-freeze (−20°C). One portion of the Bavaria Blue cheese was homogenised, inoculated and stored at −20°C directly after purchase, while another portion was stored at +4°C for 10 weeks prior to inoculation and homogenisation. Before use, the samples were thawed at room temperature.

2.3. Preparation of the aqueous two-phase systems

The aqueous two-phase systems were prepared from stock solutions of polymers in water: 20% (w/w) dextran 500 (Pharmacia Biotech Norden, Sollentuna, Sweden), 20% (w/w) dextran 40 (Pharmacia) and 40% (w/w) poly(ethylene glycol) (PEG) 4000 (Merck, Darmstadt, Germany). When preparing aqueous two-phase systems the polymers in question were weighed and mixed with 0.25 ml phosphate buffer (100 μM, pH 7), 0.25 ml NaCl (1.0 M) or 0.25 ml $NaClO_4$ (1.0 M), 0.1–0.4 ml sample and water to a total weight of 2.5 g. The sample and the constituents of the aqueous two-phase system were mixed by inversion approximately 20 times, after which the system was left for 30 min at room temperature to separate.

2.4. PCR conditions

The PCR mixture (50 μl) contained 1.5 U *Taq* DNA polymerase (Boehringer-Mannheim, Mannheim, Germany), 50 mM KCl, 10 mM Tris–HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.1 g/l gelatine, 0.5 mM of each primer and 0.2 mM each of dATP, dCTP, dGTP and dTTP. Unless otherwise stated 10-μl samples were used. The PCR mixture was covered with two drops (≈50 μl) of mineral oil (Sigma, St. Louis, MO, USA) to prevent evaporation, and the tube was placed in a thermocycler (DNA Thermal Cycler, Perkin Elmer Cetus, Norwalk, CT, USA). Each amplification cycle consisted of heat denaturation at 94°C for 1 min, primer annealing at 53°C for 1 min and extension at 72°C for 1 min. The fastest possible transition between temperatures was employed. A final extension was performed at 72°C for 7 min to complete the synthesis of all the strands.

Three primers: LM2 (5′-CCTTTGACCAC-TCTGGAGACAGAGC-3′), LM1 (5′-GGAG-CTAATCCCATAAAACTA-3′) and ru8 (5′-AAG-GAGGTGATC-CA(G/A)CCGCA(G/C)(G/C)TTC-3′) were used in the PCR assay [8], which comprised two DNA amplification steps. The first step involved

amplification with the oligonucleotides LM2 and ru8 for 30 cycles, and the second step, amplification with the oligonucleotides LM1 and ru8 for 30 cycles; the latter after 1:10 dilution of the product obtained from the first PCR incubation with double-distilled, deionized water. The ru8 primer is complementary to a universal eubacterial region at the end of the 16S rRNA gene [9]. The LM2 primer is specific for *Listeria spp.* while the LM1 primer is specific for *L. monocytogenes*. A DNA fragment of 553 bp is produced by the oligonucleotides LM2 and ru8 [10] and the oligonucleotides LM1 and ru8 produces a fragment of 275 bp [10], henceforth referred to as the PCR products. The PCR products were visualised by 1.5% agarose gel electrophoresis, using 1×TBE buffer stained with ethidium bromide [11].

3. Results

3.1. PCR in the polymers

After extraction of the inoculated cheese homogenates in the aqueous two-phase system samples were withdrawn from the top and bottom phases and subjected directly to PCR. Thus, the samples contain polymers as well as bacteria. To investigate whether the polymers affect the PCR, *L. monocytogenes* were added at a concentration of 4×10^5 CFU/ml to three polymer solutions containing 8% (w/w) PEG 4000, 11% (w/w) dextran 40 and 8% (w/w) dextran 500. Water solutions of *L. monocytogenes* containing 4×10^5 CFU/ml served as positive controls. When 10-μl samples of inoculated polymer solution and of water solution were separately subjected to PCR in a final volume of 50 μl, the intensities of the PCR products from the three polymer solutions, as visualised on a ethidium bromide-stained agarose gel, were the same as for the positive controls. Thus, in the concentrations used, none of the polymers inhibited the PCR.

3.2. Variation in PCR inhibition caused by soft cheese homogenates

To investigate the variability in PCR inhibition, cheese homogenates were inoculated with increasing amounts of *L. monocytogenes* cells. The *Listeria* cells could not be detected directly with PCR in any of the Bavaria Blue, Feta or Cambozola cheese homogenates containing 10^6 CFU/ml or less. However, in a homogenate of Camembert cheese, containing 1×10^3 CFU per ml, *L. monocytogenes* was detected directly with PCR. When 0.1 ml of the Bavaria Blue, Feta and Cambozola cheese homogenates were extracted in an aqueous two-phase system (Table 1) the detection level was lowered to 1×10^3 CFU *L. monocytogenes* per ml homogenate for the Cambozola and Bavaria Blue cheeses, while it was lowered to 1×10^5 CFU/ml for the Feta cheese (Table 1).

When soft cheese homogenates of Bavaria Blue cheese homogenised directly after purchase were added directly to reaction mixtures (50 μl) containing 1×10^3 CFU *L. monocytogenes*, the PCR was totally inhibited at a concentration of 0.1% (v/v) of the reaction mixture. The corresponding concentra-

Table 1
Detection levels obtained with PCR after extraction of cheese homogenates in an aqueous two-phase system composed of 8% (w/w) PEG 4000, 11% (w/w) dextran 40, 100 m*M* NaCl and 10 m*M* phosphate buffer (pH 7.0)

CFU/ml of cheese homogenate	PCR results[a]					
	Bavaria Blue cheese		Feta cheese		Cambozola cheese	
	Top phase	Bottom phase	Top phase	Bottom phase	Top phase	Bottom phase
10^6	+	−	+	+	−	+
10^5	+	−	−	+	−	+
10^4	+	−	−	−	−	+
10^3	+	−	−	−	−	+
10^2	−	−	−	−	−	−

[a] +, PCR product detected on agarose gel; −, no PCR product detected.

tion for the Bavaria Blue cheese that was stored for 10 weeks before homogenisation was 0.01% (v/v).

To see whether the variation in PCR inhibition remained after extraction in an aqueous two-phase system, two Bavaria Blue homogenates were extracted (Table 2). Aliquots from the aqueous two-phase systems were subjected to reaction mixtures containing 1×10^3 CFU *L. monocytogenes*. Twenty-five and 15 μl of top and bottom phase, respectively, inhibited the PCR when the aliquots came from the aqueous two-phase system containing 100 m*M* NaCl when the younger cheese was extracted. The corresponding values when the more ripe cheese was extracted were 20 and 5 μl for the top and bottom phase, respectively.

3.3. Increasing sample volumes in an aqueous two-phase system

To decrease the dilution of target organisms in the aqueous two-phase system, different sample volumes were employed. Increasing amounts of Bavaria Blue homogenate (0.1, 0.2, 0.3 and 0.4 ml) inoculated with 1×10^5 CFU/ml *L. monocytogenes* were added to an aqueous two-phase system containing 8% (w/w) PEG 4000, 8% (w/w) dextran 500, 100 m*M* NaCl and 10 m*M* phosphate buffer (pH 7.0). The system had a total weight of 2.5 g. When 10-μl samples from the top phase were subjected to PCR amplification in a final volume of 50 μl, visible PCR products were obtained in all systems regardless of the sample volume used. No difference in intensity of the PCR products from the different top phases was observed on the ethidium bromide-stained agarose gel. However, samples withdrawn from the bottom phase were all PCR-negative.

3.4. Effect of different salt additives on the aqueous two-phase system

Two aqueous two-phase systems, one containing 100 m*M* NaCl and another containing 100 m*M* $NaClO_4$, were compared regarding their efficiency in distributing PCR-inhibitory substances originating

Table 2
PCR inhibition caused by two different aqueous two-phase systems where water, young and ripe Bavarian Blue cheese were extracted

Sample volume in PCR (μl)	Water		Young cheese[c]		Ripe cheese[c]	
	Top phase	Bottom phase	Top phase	Bottom phase	Top phase	Bottom phase
Aqueous two-phase system I[a]						
5	+[b]	+	+	+	+	−
10	+	+	+	+	+	−
15	+	+	+	−	+	−
20	+	+	+/−	−	−	−
25	+	+	−	−	−	−
Aqueous two-phase system II[a]						
5	+	+	+	+	+	+/−
10	+	+	+/−	−	+	−
15	+	+	−	−	−	−
20	+	−	−	−	−	−
25	−	−	−	−	−	−

Different sample volumes withdrawn from top and bottom phases were added to PCR mixtures (50 μl) containing 1×10^3 CFU *L. monocytogenes*.

[a] The aqueous two-phase systems consisted of 8% (w/w) PEG 4000, 8% (w/w) dextran 500, 10 m*M* phosphate buffer (pH 7.0). System I contained 100 m*M* NaCl, while system II contained 100 m*M* $NaClO_4$; water and cheese homogenate, 0.4 ml, was added to the aqueous two-phase systems. The total weight of the system was 2.5 g.

[b] + and − represent presence and absence of PCR products, respectively. +/− represents a variation in results i.e. one of two amplifications was negative.

[c] Young cheese is cheese homogenized directly after purchase and ripe cheese homogenized after 10 weeks.

Table 3
Partitioning of *L. monocytogenes* in four aqueous two-phase systems

Aqueous two-phase system			log CFU[a] in aqueous two-phase system	
Top-phase polymer	Bottom-phase polymer	Salt (100 m*M*)	Top phase	Bottom phase
PEG 4000 8% (w/w)	Dextran 500 8% (w/w)	$NaClO_4$	0.9	2.3
PEG 4000 8% (w/w)	Dextran 500 8% (w/w)	NaCl	0.9	2.3
PEG 4000 8% (w/w)	Dextran 40 11% (w/w)	$NaClO_4$	0.7	2.2
PEG 4000 8% (w/w)	Dextran 40 11% (w/w)	NaCl	0	1.7

0.4 ml inoculated cheese homogenate (3.2 log CFU) was added to the aqueous two-phase systems to a final weight of 2.5 g. CFU were determined by serial dilution and subsequent plating on supplemented *Listeria*-selective agar base.

from the soft cheese Bavaria Blue (Table 2). The system containing 100 m*M* $NaClO_4$, with the water as sample, resulted in negative PCR amplification when 25 μl of the top phase and 20 μl of the bottom phase were added to the reaction mixtures (Table 2). No such inhibition was observed when the system containing NaCl was employed. Furthermore, the system containing NaCl was shown to be more efficient in the sample preparation of Bavaria Blue cheese (Table 2).

3.5. Partition of L. monocytogenes in four different aqueous two phase systems

The distribution of *Listeria* cells from cheese homogenate in the aqueous two-phase systems was determined in four different systems (Table 3). An inoculated cheese homogenate was added to the aqueous two-phase systems, samples being withdrawn from the top and bottom phases for viable count determination. Most of the *L. monocytogenes* detected were partitioned to the dextran phase. However, the number of *L. monocytogenes* recovered in the aqueous two-phase systems with the polymers PEG 4000 and dextran 40 were 0.9 to 1.5 logs below the added amount of *L. monocytogenes*.

4. Discussion

The use of aqueous two-phase systems lowered the PCR detection level of *L. monocytogenes* in two soft cheeses by more than four orders of magnitude, compared with the detection level in cheese homogenates without sample treatment (Table 1). After extraction in an aqueous two-phase system, 1×10^3 CFU per ml cheese homogenate were detected with a semi-nested PCR strategy, which gives approximately 10 CFU per reaction tube. However, the cells were enriched together with the PCR inhibitors in the bottom phase when the Bavaria Blue cheese was extracted, which gives an even lower number of CFU per reaction tube. A detection level of 1×10^4 CFU/ml has earlier been achieved with the same semi-nested PCR strategy when using cheese homogenate of Danish Blue Castello [8]. Bessesen et al. [12], in turn, have reported a detection limit of 10^5 CFU/ml for an infected milk sample following a centrifugation and washing procedure.

The partitioning of cells and particles in aqueous two-phase systems depends on their size and surface properties [13,14]. In these systems, cells generally partition to the dextran-rich bottom phase and the inter phase, whereas low molecular mass substances are more evenly distributed between the phases [15]. Hydrophobic substances show a slight preference for the PEG-rich top-phase. $NaClO_4$ has been reported to increase the partitioning of hydrophobic compounds to the PEG phase [16]. We investigated whether using $NaClO_4$ instead of NaCl in the aqueous two-phase systems resulted in a more advantageous distribution of PCR inhibitors. However, the system containing 100 m*M* $NaClO_4$ showed negative results in comparison with systems containing 100 m*M* NaCl. None of the single-polymer solutions containing the respective polymers in the concentrations in which they appear in the aqueous two-phase systems inhibited PCR. Nor did the aqueous two-phase system with 100 m*M* NaCl. The system containing 100 m*M* $NaClO_4$, on the other hand, did inhibit PCR when 20–25 μl aliquots were added to reaction mixtures. However, the

sample volume in a PCR mixture of 50 μl is usually 5–10 μl.

The detection level has earlier been reported to be strongly dependent on the soft cheese used [5,17]. Rossen et al. [1] stated that the inhibitory effect of soft cheeses may, at least in part, be explained by the presence of proteases. The variation in starter cultures when manufacturing different soft cheeses may result in a variety of proteases which may explain the different partitioning behaviour of PCR inhibitors seen when Cambozola and Bavaria Blue cheese were partioned in an aqueous two-phase system. The young Bavaria Blue cheese inhibited the PCR, and PCR inhibitors were also found to be formed during the ripening of the Bavaria Blue cheese. The *Listeria* cells were detected in the dextran phase when inoculated Cambozola cheese was extracted, and in the PEG phase when the Bavaria Blue cheese was extracted. However, the *Listeria* cells partitioned to the dextran phase, as determined by viable counts, when the Bavaria Blue cheese was extracted in an aqueous two-phase system. All the bacteria added were not recovered with the viable count method, as some bacteria probably partitioned to the inter phase [18]. Another conceivable explanation of the loss is that the bacteria were stressed by the conditions employed in the aqueous two-phase systems thus becoming nonculturable. It has been reported that some pathogens exposed to stress remain viable but nonculturable [19]. When the majority of the PCR inhibitors partition to one phase, but both phases still contain bacteria, the PCR is sufficiently sensitive that detection and identification are possible. This is especially useful when PCR is employed to simultaneously detect several bacteria, which may partition differently using aqueous two-phase systems for the preparation of samples prior to PCR [9].

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