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ARTICLE

# Sodium Caseinates with an Altered Isoelectric Point As Emulsifiers in Oil/Water Systems

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Sodium caseinate was chemically modified in order to alter its isoelectric point (pI). Negatively charged carboxylic groups were introduced to lower the pI, and positively charged amino groups to achieve the opposite. Different chemical amino acid modification approaches were studied and the modified proteins were characterized using free amino group assays, SDS−PAGE, MALDI-TOF mass spectrometry, and zeta potential measurements. Oil-in-water emulsions were prepared using these modified caseinates. The pH stability behavior of the emulsions was monitored, and interestingly, the stability of the emulsion could be modulated through steering the pI of caseinate. Using different modified caseinates, it was possible to create emulsions that were stable in the acid, neutral, and alkaline regions of the pH spectrum. The stability behavior of the emulsions correlated well with the theoretical and experimentally determined pI values of the caseinates. Storage stability of emulsions was also studied at pH values around 7, and emulsions made of modified caseinates showed storage stability similar to that of unmodified caseinate emulsions.

**KEYWORDS: Emulsion; stability; MALDI-TOF; sodium caseinate; amino acid modification**

## INTRODUCTION

Oil-in-water (O/W) emulsions are widely used in various industrial application areas such as in food products, cosmetics, and pharmaceuticals. In general, an O/W emulsion refers to a dispersion of oil in an aqueous phase. Because of the difference in density between oil and water, O/W emulsions are thermodynamically unstable, and phase separation occurs as a function of time. In order to solve this problem, surface-active emulsifiers are utilized to stabilize the oil−water interface, and as a result, the de-emulsification process is retarded. A good emulsifier should have a specific molecular structure with moieties attracted to the water and oil phases, a good solubility in the continuous phase, and capability to adsorb the dispersed droplets quickly and form a condensed film with subsequent reduction of the interfacial tension. The capability to increase the viscosity of the continuous phase of an emulsion is also one of the important characteristics of an effective emulsifier. In terms of practical issues, it should function at low concentration and be inexpensive, nontoxic, and safe to handle (*1*).

Proteins, such as casein and whey protein (*2*), are widely used as emulsifiers. They prefer to localize at the emulsion interface because of their amphoteric nature and thereby lower the surface tension. At the interface, the hydrophobic parts of the protein are in the oil phase, while the hydrophilic parts remain in the water phase, providing an electrostatic and steric repulsive force against coalescence or disproportionation (*3*). A drawback with proteins as emulsifiers is their low solubility due to low net charge at pH values close to their isoelectric points (pI), and as a result of this, their emulsifying activity in that pH range is considerably reduced (*4*). In the case of caseins with an average isoelectric point around 4.5 (*5*), reduction of emulsifying activity at pH values 4−5 has been a severe limitation in casein's applications in the acidic pH range (*6*).

Derivatization of amino acid moieties in proteins by chemical or enzymatic modification is an efficient way to change the physicochemical properties of proteins such as charge, hydrophilicity, and viscosity (*7*). For example, glutaraldehyde mediated cross-linking of $\beta$-casein endows the emulsion with enhanced stability against disproportionation (*8*). Glycol-conjugates such as caseinate−maltodextrin (*6*, *9*) and casein modified with glucose, ribose, fructose, lactose, and fructo-oligosaccharide (*10*) have been reported with improved solubility at the pI of unmodified caseinate, and increased thickness of interfacial layer and a better emulsifying capability were observed. Modifications such as thiolation (*11*), phosphorylation (*12*), acetylation, and succinylation (*13*) have also been studied in terms of changing physicochemical properties of the proteins to optimize their use as emulsifiers.

Succinylation (**Scheme 1**) is one of the most common ways to acylate the amino group of proteins. In this reaction, the positively charged $\varepsilon$-amino groups of lysine residues are modified with negatively charged succinic acid groups through a covalent bond, resulting in a protein with a decreased pI toward the $pK_a$ of the introduced succinic acid group.

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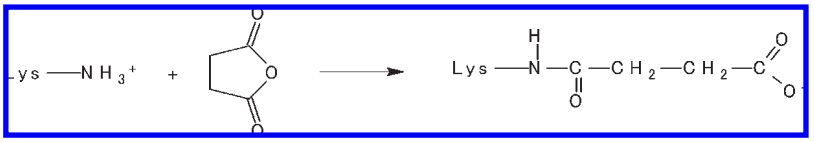
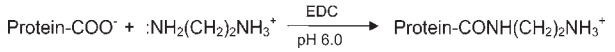
**Scheme 1**



**Scheme 2**




This modification has been applied to various proteins such as hydrolyzed soy proteins (*14*), soy (*15*), mung bean (*16*), fava bean legumin (*17*), lentil (*18*), leaf protein (*19*), peanut protein (*20*), and sunflower protein (*21*). Most of the succinylated products, compared with their native counterparts, exhibited improvement in functional properties such as increased solubility and better emulsifying capacity.

The ethylene diamine (EDA) modification (**Scheme 2**) alters the pI in the opposite way. Carboxyl groups in proteins react with EDA in the presence of a water-soluble carbodiimide, converting the negatively charged carboxyl groups into positively charged amino groups, resulting in a shift of the protein's pI toward the p$K_a$ of an amino group in water (*22*).

So far, there has been no work published on using EDA-modified caseinate as an emulsifier. Ethylenediamine used for this type of modification might not be an ideal chemical for food emulsion applications, but it can be used as a model to validate the impact of an increased pI on emulsion stability.

In this work, physicochemical properties of caseinate were altered through succinylation and EDA modification in order to shift the isoelectric point toward both the acidic and the alkaline regions of the pH spectrum. By shifting the isoelectric point toward alkaline pH, it would possible to use caseinates as emulsifiers in the pH range from 3 to 7, which is an important pH range for food applications. In this study, the suitability of chemically modified sodium caseinates for this purpose were compared.

## MATERIALS AND METHODS

**Materials.** Sodium caseinate was obtained from KasLink-Foods (Finland) (Protein 94%, Lactose 0.1%, Fat 1%, Ash 3.5%) and flaxseed oil was purchased from Elixi Oil Oy (Somero, Finland), where the fatty acid content was the following 4% 16:0, 3% 18:0, 12% 18:1, 15% 18:2, and 66% 18:3. Succinic anhydride (Purity ≥97.0%) and ethylenediamine dihydrochloride (Purity ≥99.0%) were purchased from Sigma. EDC (Purity ≥98.0%) was purchased from Pierce.

**Modification of Na-Caseinate by Succinylation.** A 3 mg/mL sodium caseinate solution was prepared by dissolving the protein in PBS buffer (pH 7.0), and the pH was adjusted to 8.0 using a 1.0 M NaOH solution. Succinic anhydride was added at a concentration of 0.03, 0.06, 0.1, 0.2, 0.3, 0.6, 1, and 4 g/g of protein, and 1.0 M NaOH was used to maintain the pH above 8.0 during the reaction. The mixture was stirred for 2 h at room temperature after all of the succinic anhydride was completely dissolved. Dialysis against distilled water was then applied to remove the unreacted reagents. Succinylated sodium caseinate was recovered by freeze-drying. The succinylated caseinate using 1 g of succinic anhydride per gram of sodium caseinate (SUCA) was made in large scale ( > 300 mg) in order to be used as the emulsifier.

**Modification of Na-Caseinate by EDA.** A 3 mg/mL solution of sodium caseinate, which had been partially succinylated with 0.06 and 1 g of succinic anhydride per gram of protein was prepared by dissolving the protein in 0.1 M MES buffer containing 1 M ethylenediamine dihydrochloride. The pH was adjusted to 4.7 with a 0.1 M NaOH solution, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was added at a concentration of 2 mg/mL. The reaction mixture was stirred for 2 h at room temperature. Dialysis against a 0.02 M $NaH_2PO_4$ and 0.15 M NaCl solution at pH 7.5 was used to quench the reaction, and dialysis against sterilized water was applied afterward to remove the unreacted reagents. The EDA modified caseinate was recovered by freeze-drying. The modification was also performed using bovine serum albumin (BSA) and whey protein as a comparison. EDA modified succinylated caseinate using 0.06 g of succinic anhydride per gram of sodium caseinate (EDCA) was made in large scale ( > 300 mg) in order to be used as the emulsifier.

**Analysis of the Extent of Modification.** The 2,4,6-trinitrobenzene sulfonic acid (TNBSA) method of Hall et al. (*23*) was used to determine the extent of succinylation and EDA modification. The modified proteins and unmodified controls were dissolved in 0.1 M $NaHCO_3$ at a concentration of 200 $\mu$g/mL. After this, 0.25 mL of 0.01% solution of TNBSA was added to 0.5 mL of protein sample solution and mixed well. The samples were incubated at 37 °C for 2 h, and 0.25 mL of 10% SDS and 0.125 mL of 1 M HCl were added. The absorbance of the solutions was measured at 335 nm in a spectrophotometer (Perkin-Elmer Lambda 45 UV/vis Spectrometer, USA) against a reagent blank. The absorbance of the unmodified sodium caseinate was set equal to 100%. The relative percentage of modified lysine groups was obtained using the following formula:

$$\text{Percentage of modified lysine groups} = 1 - (\text{absorbance of modified caseinate} / \text{absorbance of unmodified caseinate}) \times 100\%$$

Matrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) was applied to determine the extent of the chemical modifications. Sinapinic acid was selected as the matrix and dissolved to saturation in a 1:1 mixture of 0.1% trifluoroacetic acid (TFA) and acetonitrile. The samples of succinylated caseinate and EDA modified caseinate were dissolved in distilled water and 1:1 mixed with the matrix. One microliter of the mixture was spotted on the target plate and dried in air for 10 min. The analysis was conducted using a mass spectrometer (Bruker AutoflexII, Germany). The samples were also analyzed using a Coomassie stained 12% SDS−PAGE gel with low molecular weight markers as standards.

**Measurement of Zeta Potential.** The samples were prepared by dissolving native and modified caseinates (SUCA and EDCA) in 10 mM NaCl solutions at the concentration of 1 mg/mL, and the pH was adjusted using 0.1 M HCl and 0.1 M NaOH. The zeta-potential of 1 mL of each sample was measured using a Zetasizer Nano-ZS instrument (Malvern, UK).

**Preparation of O/W Emulsion.** Freeze-dried sodium caseinate and modified caseinates were solubilized in 100 mL of Milli-Q water at a controlled pH (mentioned in the figure legends and table) at a protein concentration of 0.3% and 1%. Ninety percent w/w protein solutions were homogenized with 10% w/w flaxseed oil in two steps. A pre-emulsion

was prepared using a stirring-type homogenizer (Heidolph Diax 900, Germany) under constant conditions: 2 times, 2 min at 26000 rpm at room temperature. The main emulsification was performed using a pressure homogenizer (Microfluidics M-110Y, USA) using 100 mL of pre-emulsion at 0 °C and 40 psig (500 bar). After the pre-emulsion was applied, it circulates in the homogenizer for 10 min, during which it passes through the chamber 30 times.

**Analysis of Emulsion Properties.** pH stability of the emulsions was analyzed by transferring a 2 mL emulsion sample into an eppendorf tube, where after the pH was adjusted from 2 to 12 by adding 1 M NaOH or 1 M HCl solutions. After this, the sample was centrifuged at 6000 rpm for 5 min. The liquid phase was taken by drilling a hole from the bottom of the tube, diluted 100-fold into Milli-Q water, and the absorbance at 633 nm was measured for each sample. To study the storage stability of emulsions, the particle size distribution of freshly made emulsions and emulsions stored at room temperature for 24, 48, and 72 h was measured by laser diffraction (Beckman Coulter LS230, CA). The volume-weighted geometic mean particle diameter (d33) was calculated from the particle size distribution.

## RESULTS

**Chemical Modification of Sodium Caseinate.** Sodium caseinate was succinylated to different extents using succinic anhydride to protein ratios varying from 0.03 g of succinic anhydride per gram of protein to 4 g of succinic anhydride per gram of protein. The relative extent of the chemical modification was measured using the TNBSA assay, which detects the number of free amino groups in a protein and thus can be used to monitor the covalently modified lysine groups as shown in **Figure 1A**. With an increasing dosage of succinic anhydride, the extent of succinylation increased accordingly. Fifteen percent of the lysine groups in sodium caseinate were succinylated when modified by 0.03 g of succinic anhydride per gram of protein, while the extent of modification increased to 94% when the ratio was increased to 0.3 g/g, and with a weight ratio of succinic anhydride to sodium caseinate over 1:1, sodium caseinate was completely succinylated.

The modification of sodium caseinate could also be monitored using SDS−PAGE, and a band shift was detected when sodium caseinate with a different extent of modification was loaded onto the gel. With an increasing amount of succinic anhydride used as modifier, the molecular weight of the products increased, and the caseinate bands shifted upward accordingly (**Figure 1B**, lanes 2−9). From lane 5 to lane 9, the bands have the same relative mobility in the gel, which correlates well with the extent of modification measured using the TNBSA assay. Modification of proteins in general leads to a more heterogeneous population of proteins, which results in a reduced sharpness of the bands in the SDS−PAGE gel and a broadening of the MALDI-TOF MS spectrum (see below).

Succinylation of sodium caseinate was further studied using MALDI-TOF MS by which it was possible to directly measure the extent of modification. It is noteworthy that sodium caseinate is a complex mixture of different casein variants (α, $\beta$, and $\kappa$ casein), therefore a spectrum that is obtained from native or modified caseinates represents the overall population of unmodified or modified variants rather than homogeneous products. The average molecular mass of the unmodified caseinate mixture was detected to be 23966 Da, and after succinylation to different extents, the mass increased gradually as can be seen in **Figure 2A**. The average

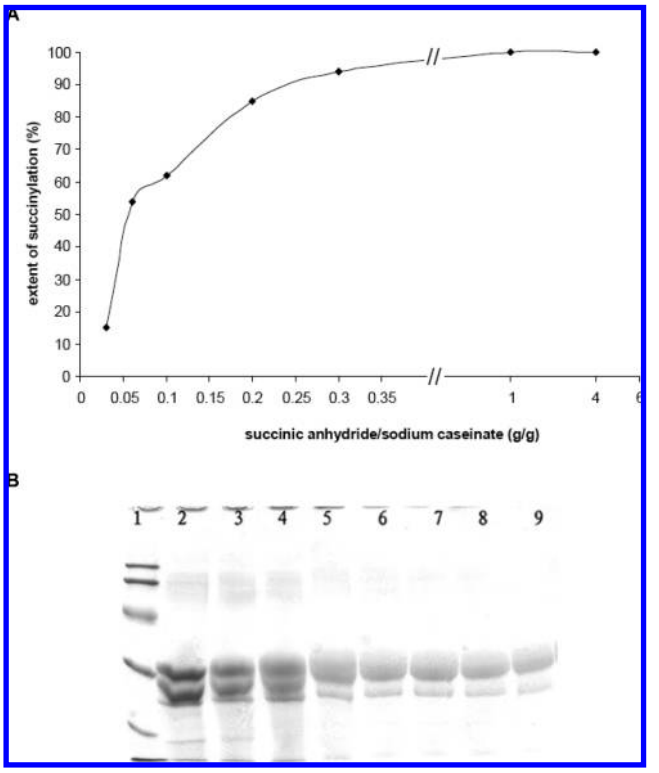



**Figure 1.** (**A**) Extent of caseinate succinylation measured using the TNBSA method. (**B**) SDS−PAGE analysis of succinylation of sodium caseinate. Lane 1, low molecular weight marker proteins of 97, 67, 45, 30, and 20 kDa; lane 2, sodium caseinate control; lane 3, succinylated caseinate by 0.03 g of succinic anhydride per gram of sodium caseinate; lane 4, succinylated caseinate by 0.06 g of succinic anhydride per gram of sodium caseinate; lane 5, succinylated caseinate by 0.2 g of succinic anhydride per gram of sodium caseinate; lane 6, succinylated caseinate by 0.3 g of succinic anhydride per gram of sodium caseinate; lane 7, succinylated caseinate by 0.6 g of succinic anhydride per gram of sodium caseinate; lane 8, succinylated caseinate by 1 g of succinic anhydride per gram of sodium caseinate (SUCA); lane 9, succinylated caseinate by 4 g of succinic anhydride per gram of sodium caseinate.

number of lysine residues, which had been modified, was calculated by dividing the difference between the molecular mass of modified caseinate and unmodified caeinate obtained from MALDI-TOF MS by the mass of the chemical modifier. The average number of succinylated lysine groups in sodium caseinate, which had been modified with 0.03, 0.06, 0.2 g of succinic anhydride per gram of protein, was 2.5, 5.7, and 11.5, respectively (**Figure 2A**, peaks B, C and D). Interestingly, more and more laser power was required to generate mass spectra from succinylated caseinates with an increasing extent of modification, as can be seen from the reduction of the peak size in **Figure 2A** when succinylation is more extensive. After the succinic anhydride/protein ratio exceeded 0.2 g of succinic anhydride per gram of protein, it was no longer possible to obtain reliable spectra from the modified protein.

EDA modification of a protein converts the carboxyl groups of protein to amino groups. According to Burkey and Gross (*22*), the optimum pH condition for this reaction is at pH 6, while in the case of sodium caseinate, the largest extent of modification was reached at pH 4.7. In our work, partially succinylated caseinate was used as the starting material for the EDA modification because of its improved solubility at the optimum pH of the reaction and more

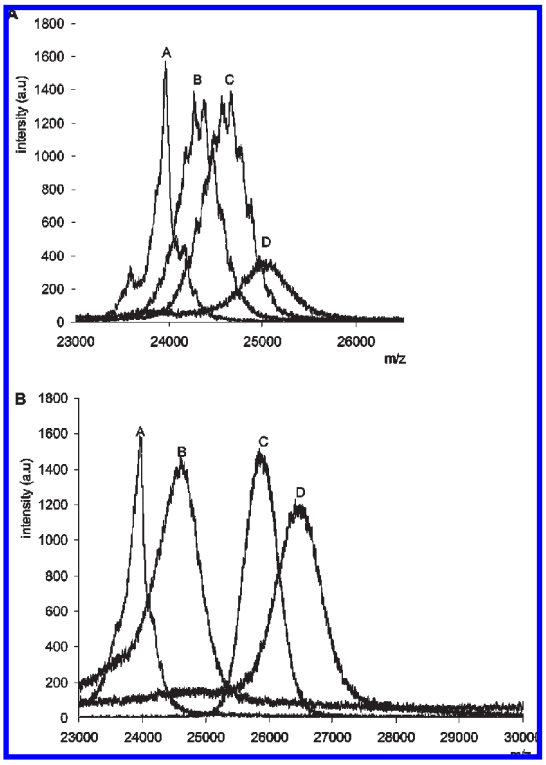



**Figure 2.** MALDI-TOF mass spectra of modified caseinates. (**A**) Succinylation of sodium caseinate. Spectrum A, sodium caseinate control with molecular weight of 23966 Da; spectrum B, succinylated caseinate by 0.03 g of succinic anhydride per gram of sodium caseinate with molecular weight of 24211 Da; spectrum C, succinylated caseinate by 0.06 g of succinic anhydride per gram of sodium caseinate with molecular weight of 24529 Da; spectrum D, succinylated caseinate by 0.2 g of succinic anhydride per gram of sodium caseinate with molecular weight of 25089 Da. (**B**) EDA modification. Spectrum A, sodium caseinate control with molecular weight of 24148 Da ; spectrum B, succinylated caseinate by 0.06 g of succinic anhydride per gram of sodium caseinate with molecular weight of 24584 Da; spectrum C, EDCA with molecular weight of 25853 Da; spectrum D, EDA modified succinylated caseinate by 1 g of succinic anhydride per gram of sodium caseinate with molecular weight of 26467 Da.

importantly, a much larger extent of modification than that of the modification based on native sodium caseinate. An increase in the amount of free amino groups was observed when the product of the modification reaction was compared with the unmodified sodium caseinate using the TNBSA assay. EDA modified 0.06 g/g succinylated caseinate (EDCA) contained 78% more free amino groups than the native sodium caseinate.

Besides amino acid modification, EDA seemed to have an effect on the mobility of the protein in an SDS−PAGE gel (**Figure 3**, lane 5). The sodium caseinate monomer band had a higher mobility in the gel compared to the succinylated starting material, and treatment with EDC generated cross-linked products, which is due to the formation of intra molecular cross-links. Attempts such as modification using smaller amounts of EDC and lower temperature were made to avoid the cross-linking reaction. With a low dosage of EDC, the cross-linking was effectively suppressed, but meanwhile the pI could not be increased to a desirable level. The modification performed at 4 °C showed no difference from the modification at room temperature (data not shown). For comparison, the EDA modification was applied to other proteins such as BSA and whey protein, which have a more defined globular structure. The modification could be

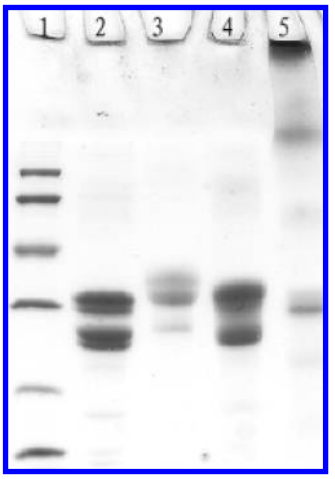



**Figure 3.** EDA modification. Lane 1, low molecular weight marker proteins of 97, 67, 45, 30, and 20 kDa; lane 2, sodium caseinate control; lane 3, succinylated caseinate by 1 g of succinic anhydride per gram of sodium caseinate (SUCA); lane 4, succinylated caseinate by 0.06 g of succinic anhydride per gram of sodium caseinate; lane 5, EDCA.

performed neatly, showing a large increase in molecular weight in MALDI-TOF MS and a single band with fairly little cross-linked byproduct (data not shown). However, caseinate had been reported as a highly susceptible substrate to the cross-linking reactions. For example, transglutaminase cross-links caseinate very easily by acting on glutamine and lysine residues (*24*); therefore, in terms of EDA modification of caseinate, cross-linking might be an inevitable side reaction.

The increase of molecular mass due to EDA modification was further confirmed by MALDI-TOF MS measurements (**Figure 2B**). As a large portion of the product was cross-linked into modified multimers whose molecular weight was beyond the measurable range, the spectrum observed in MALDI-TOF corresponded to the modified monomer fraction in the mixture. The average number of carboxyl groups that reacted with EDA was calculated to be 17.2 when 0.06 g/g succinylated caseinate was used as the starting material. It was very interesting that after EDA modification, the protein could be measured again in the positive mode using MALDI-TOF MS (**Figure 2B**, peaks C and D). However, since no peak could be obtained from the starting protein, the number of EDA modified residues could not be precisely calculated when fully succinylated caseinate was used as the starting material for the EDA modifications.

**Zeta-Potential of Modified Caseinates.** The zeta-potential of native sodium caseinate, SUCA, and EDCA was measured and plotted as a function of pH (**Figure 4**). The pH where the zeta potential is zero corresponds with the isoelectric point of the protein, and the more the pH value of a sample differs from the protein's pI, the more charged the surface of the protein is. As can be seen from **Figure 4**, after succinylation to full extent the pI of sodium caseinate decreased from 4.2 to around pH 2.7, and modification of partially succinylated caseinate (0.06 g/g of succinic anhydride to sodium caseinate) with EDA moved the pI of the caseinate mixture up to 9.4. These changes correlate well with the theoretically estimated pI of 4.98 (unmodified), 3.88 (fully succinylated), and 9.54 (EDA modified), respectively. These theoretical values were calculated using the results of the MALDI-TOF MS measurement in combination with an online protein computation tool ProtParam (http://au.expasy.org/tools/protparam.html). The amino acid sequence

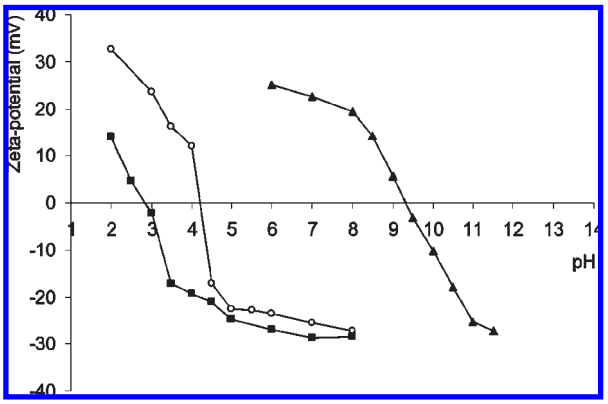



**Figure 4.** Zeta-potential of native and modified caseinates. Zeta-potential of 1 mg/mL native sodium caseinate (○), SUCA (■), and EDCA (▲) in 10 mM NaCl solutions at different pH values was measured and plotted as a function of pH.

of alfa-S1 caseinate, which is the most abundant caseinate in the mixture, was used as the input parameter of the computational tool.

**pH Stability of the Emulsions Made with Modified Caseinates.** The emulsifying properties of the modified caseinates with altered isoelectric points were studied at pH values from 2 to 12. The emulsion made of unmodified sodium caseinate collapsed at pH of around 4.5 as can be seen from a visual inspection of **Figure 5A** and from the turbidity measurement shown in **Figure 6** (○); this value correlates well with the pI of sodium caseinate as confirmed by the zeta potential measurement in the previous section. For fully succinylated caseinate (SUCA), with a pI shifted toward a more acidic value, the emulsion lost its stability around pH 2.5−3.5 due to the introduction of additional carboxylate groups in the protein, and as a result, the emulsion was stable at pH values where the emulsion made of unmodified caseinate collapsed as can be seen from **Figure 5C**. The tubidity measurements shown in **Figure 6** (■) confirmed this. EDA modification shifted the pI of caseinate in the opposite direction to 9.4. From a visual inspection of **Figure 5B** and from the turbidity measurement shown in **Figure 6** (▲), the pH at which the emulsion lost its stability was around 8.5−10.5. It can be seen that the emulsion made from this protein mixture containing the EDA modified monomer and multimers had a very satisfactory stability in the pH 3−6 range.

**Effect of pH on Emulsification Capability and Emulsion Stability.** Besides the emulsions that were prepared at neutral pH 7, the emulsification process was also studied at three different pH values to study the effect of the modifications on emulsification capability and emulsion stability (**Table 1**). At these different pH values, the mean particle diameter was monitored for 72 h. For native sodium caseinate, as pH 5 is close to its isoelectric point (pH 4.2), most of the protein had precipitated and was filtered. The actual concentration of protein that functioned as the emulsifier was very low. Therefore, the emulsion prepared at this pH value was very coarse and unstable. The fresh emulsion already had a particle size larger than 10 $\mu$m. At pH values away from its pI, native sodium caseinate had better emulsification capability as the particle size of fresh emulsion was much smaller, and the emulsion was more stable as the mean particle size was still smaller than 1 $\mu$m after 72 h. For the modified caseinates, at pH 3 for SUCA and at pH 9 for EDCA, both pH values close to there respective pI, phase separation took place right after the pre-emulsification

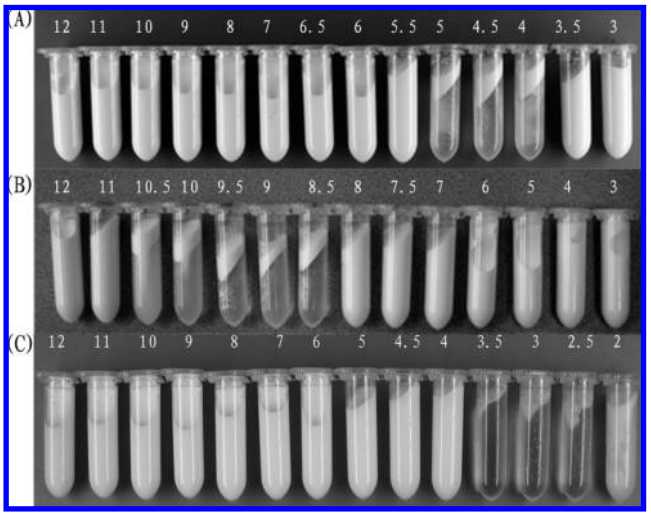



**Figure 5.** pH stability test of emulsions made of native and modified caseinates (I). Native and modified caseinates were solubilized in Milli-Q water at the concentration of 0.3%, and 90% w/w protein solutions were homogenized with 10% w/w flaxseed oil in two steps as mentioned in Materials and Methods. After adjusting the pH from 2 to12, the samples were centrifuged at 6000 rpm for 5 min. (**A**) Emulsion made of sodium caseinate; (**B**) emulsions made of EDCA; (**C**) emulsion made of SUCA.

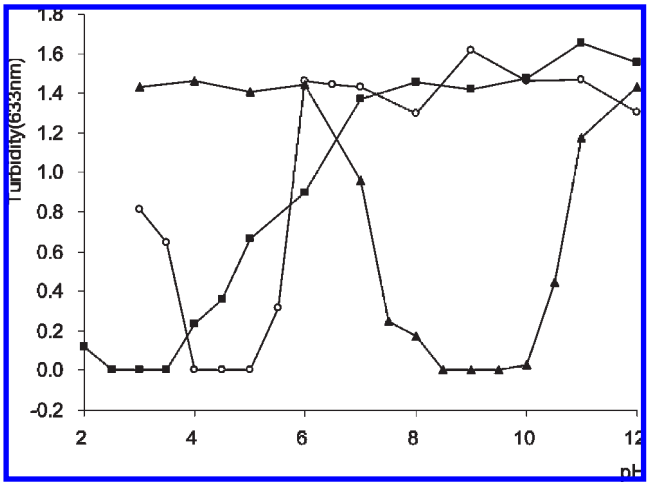



**Figure 6.** pH stability test of emulsions made of native and modified caseinates (II). The liquid phase of the emulsions made of native caseinate (○), SUCA (■), and EDCA (▲) after centrifugation was taken and diluted 100-fold into Milli-Q water. The absorbance at 633 nm was measured for each sample.

process. Therefore, the sample failed to be further homogenized as the oil phase always floated on top of the mixture and could not be drawn into the pressure homogenizer. At other pH points where emulsification is feasible, good emulsification capability and emulsion stability were observed.

**Particle Size Distribution of Freshly Made Emulsions.** The particle size distribution of freshly made emulsions using 0.3% (**Figure 7A**) and 1% (**Figure 7B**) of the differently modified caseinates and unmodified caseinate as emulsifiers was measured. Fully succinylated caseinate (SUCA) was found to have the best emulsifying efficiency as it gave the smallest particle size. A minor peak with particle size less than 0.1 $\mu$m was observed, but it was assumed to be the protein without oil. This minor peak was also observed with increased protein concentration when native sodium caseinate or EDCA were used. But in the case of SUCA, as the

**Table 1.** Mean Particle Diameter ($\mu$m) of Emulsions Made of the Different Caseins at Different pH Values[a]

| | unmodified ($\mu$m) | | | | SUCA ($\mu$m) | | | | EDCA ($\mu$m) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 h | 24 h | 48 h | 72 h | 0 h | 24 h | 48 h | 72 h | 0 h | 24 h | 48 h | 72 h |
| pH 3 | 0.68 | 0.74 | 0.86 | 1.4 | ND | ND | ND | ND | 0.46 | 0.70 | 0.65 | 0.80 |
| pH 5 | 10.6 | 13.0 | 15.9 | 34.2 | 1.4 | 1.5 | 2.1 | 3.2 | 1.4 | 1.9 | 2.2 | 2.9 |
| pH 7 | 0.39 | 0.39 | 0.41 | 0.42 | 0.31 | 0.31 | 0.32 | 0.33 | 1.23 | 8.44 | 9.22 | 10.0 |
| pH 9 | 0.26 | 0.26 | 0.33 | 0.49 | 0.15 | 0.15 | 0.16 | 0.28 | ND | ND | ND | ND |

[a] ND: Not determined, phase separation took place right after the pre-emulsification process. A protein concentration of 0.3% was used.

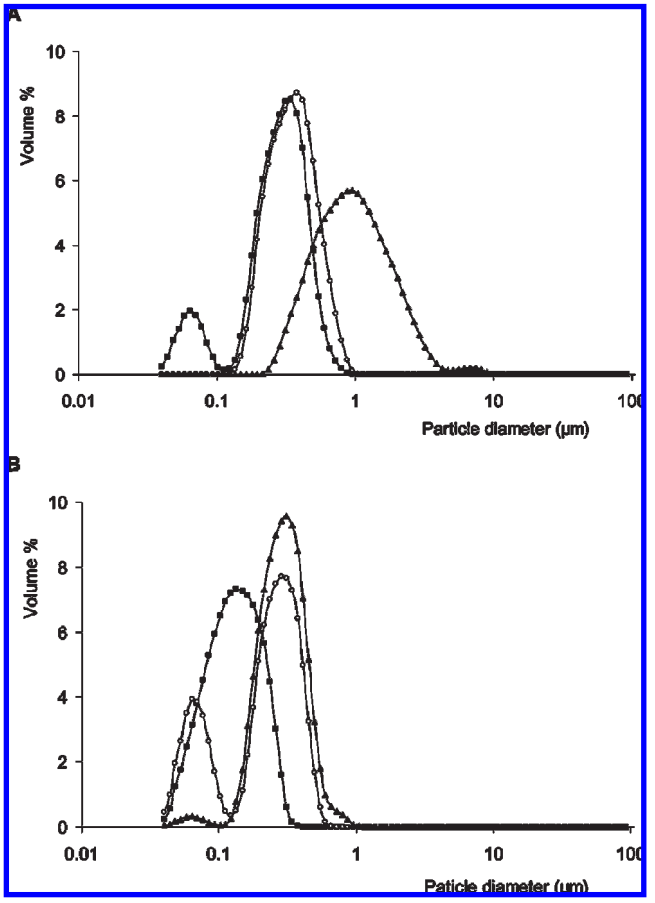



**Figure 7.** Particle size distribution of freshly made emulsions. The volume distribution curves of freshly made emulsions made of native sodium casseinate (○), SUCA (■), and EDCA (▲) were measured at pH 7 by putting 1−2 drops (~100 $\mu$L) of each sample by light scattering using laser diffraction instrumentation. (**A**) Emulsions made of a 0.3% protein solution; (**B**) emulsions made of a 1% protein solution.

amount of excessive protein apparently increased, leading to an increased height of the minor peak and a smaller particle size of the emulsified portion, moving the major peak toward smaller particles size, the two peaks merged into a single one as a result.

**Effect of Protein Concentration on Emulsion Stability.** The storage stability of the emulsions of modified caseinates was studied by measuring the particle size distribution of the freshly made emulsions compared to emulsions stored at ambient temperature for 24, 48, and 72 h. When the mean particle diameter of emulsions made of 0.3% native caseinate and SUCA (1 g/g succinic anhydride/sodium caseinate) was followed in time, the starting mean particle diameter of fresh emulsions was 0.40 and 0.31 $\mu$m, respectively, and no distinct

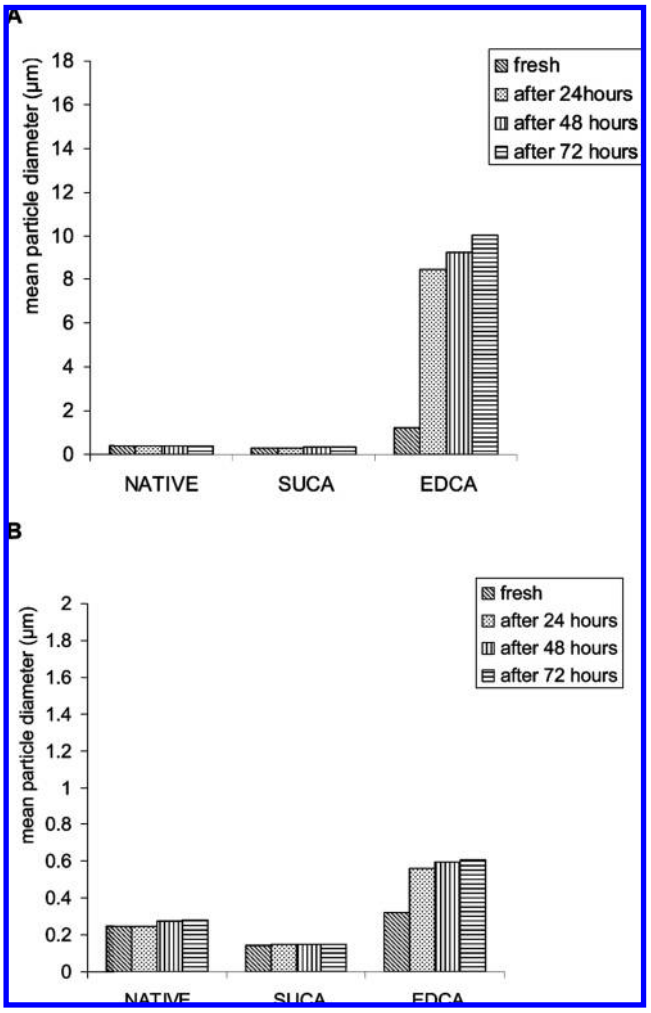



**Figure 8.** Shelf life of emulsions made of modified caseinates. The particle size distribution of freshly made emulsions at pH 7 and these emulsions stored at room temperature was measured after 24, 48, and 72 h by laser diffraction. The volume-weighted geometric mean particle diameter was calculated from the size distribution and was plotted against time. (**A**) Emulsions made of a 0.3% protein solution; (**B**) emulsions made of a 1% protein solution.

increase in particle size was observed after 72 h. The initial mean particle size of the emulsion made of 0.3% EDCA was 1.23 $\mu$m, and within 24 h, a sharp increase in size took place as can be seen from **Figure 8A**. The increase of the protein concentration from 0.3% to 1% shifted the particle size distribution of the fresh emulsions, especially of the emulsions made from EDCA modified caseinate as can be seen in **Figure 8B**. At 1% protein concentration, all particles of the emulsion made from EDCA modified caseinate were smaller than 1 $\mu$m, and the storage stability was much better than in the emulsion made at a protein concentration of 0.3%.

## DISCUSSION

Succinylation of caseinates was easier to perform compared to the EDA type of protein modification. This reaction took place almost instantaneously when protein and succinic anhydride were mixed, without the need of a catalyst, and no byproducts such as cross-linked caseinates were formed. Another advantage was, as could be shown by MALDI-TOF MS, that the extent of the modification could be subtly controlled using different amounts of the modifier. As expected, the heterogeneity of the protein sample after

modification seemed to be slightly increased as can be judged both qualitatively from the fuzziness of bands in the SDS−PAGE gels and quantitatively from the width of the peaks in the MS spectra. Since the modified protein was used as an emulsifier, this was of less relevance, but it shows the power of mass spectrometry to analyze protein based emulsifiers. The extent of succinylation as a result of different amounts of succinic anhydride used was in agreement with what was reported in the literature: 86% succinylation for beta-casein at 0.2:1 weight ratios of succinic anhydride to casein (*25*). Also, our results support the observation that in order to succinylate caseinate in high levels, large excesses of succinic anhydride are required (*26*, *27*). Interestingly, for the EDA modification, it turned out that the low solubility at the optimum pH for the reaction could be overcome using partially succinylated caseinate as the starting material, having the additional advantage that at same time there are more carboxyl groups available to be modified into amino groups. The use of succinylated caseinate as starting material opens interesting opportunities for further casein modifications.

Zeta potential measurements do not only measure the isoelectric point of modified proteins, but also give an indication of the charge of the modified caseinates at different pH values. A high zeta potential value means that the protein particles are intensively charged on the surface and repel each other. This helps to predict the protein's emulsifying efficiency at a certain pH value, as highly charged caseinate on the oil/water interface can provide emulsion droplets with sufficient repulsive forces against flocculation and coalescence. Therefore, according to the zeta potential measurement, EDCA was expected be a good emulsifier that forms droplets with a highly positive charge at pH values below pH 8. Fully succinylated caseinate, however, is suitable as an emulsifier at pH values above pH 3.5. This prediction based on zeta potential was supported by the results of the pH stability test and the emulsification studies at different pH values. Turbidity above 1.4 was observed in overlapping pH regions for the three differently modified proteins, meaning that the pH range of the protein emulsifier can be modulated by the use of different types of casein modifications.

From pH 2 to 6, EDA modified caseinate has a good pH stability performance, and above pH 6, either succinylated or native caseinate have satisfactory pH stability. When the emulsification efficiency of the different caseinate variants was studied at different pH values, a good correlation with the altered charge behavior of the proteins was also observed. At their isoelectric points, the caseinates could not be solublized and therefore lose their capability to generate and stabilize the emulsion system. Under other pH conditions, a pattern can be found that the further the pH of emulsification is away from emulsifier's pI, the better the achievement of emulsification capability and emulsion stability. This result correlates well with the protein solubility and pH stability pattern observed for the different emulsions.

Full succinylation introduces many carboxyl groups into the protein, resulting in a negative charge when dissolved in distilled water with a pH value around 7. It has been reported that the negatively charged phosphate groups have an unfavorable effect on the ionization process of the MALDI-TOF MS measurement, as the presence of negatively charged carboxyl groups may lead to low ionization efficiency (*28*). This might explain why it was not possible to obtain spectra from highly succinylated casein samples. However, substituting a negatively charged group with a positively charged one has been described as an effective way to improve the MS signal intensity (*29*), and thus, the modification with EDA has been applied to enhance the ionization efficiency in MALDI-TOF MS (*30*). This could explain why the EDA modified samples again showed a sound peak.

The size of emulsion droplets is an important parameter that greatly affects the stability of an emulsion. Measuring the change in the particle size distribution of an emulsion with time can be utilized as a quantitative method of determining emulsifier efficiency and stability. An efficient emulsifier produces emulsions in which the particle size is small ($< 1$ $\mu$m) and the distribution does not change over time, whereas a poor emulsifier produces emulsions in which the particle size increases due to coalescence and/or flocculation. Two factors that influence the droplet size are the emulsifier concentration and the speed at which the emulsifier adsorbs in the interface (*3*). In this work, it was apparent that using a higher concentration of protein enabled the protein to cover more interfacial area and generate smaller droplets. At the same caseinate concentration, the particle size of emulsions differed because of the characteristics of each modified caseinate. After succinylation, caseinate was more soluble than the native caseinate at the pH at which the emulsions were prepared. This improved solubility seemed to allow succinylated caseinate to adsorb faster to the interface than the native caseinate. For EDCA, the modified protein was more soluble as well, but it existed in the form of multimers due to the cross-linking reaction. This may have affected the diffusion of EDCA from bulk to interface and made the reorientation process of the protein at the interface slower.

To sum up, succinylation and EDA modification of sodium caseinate resulted in caseinates with altered isoelectric points, and a pH-dependent improvement in solubility and emulsifying properties were achieved. MALDI-TOF MS was shown to be a powerful tool to study these types of modifications. It can very precisely unveil the extent of the modification and help to estimate the pI of the modified emulsifier. An idea about the level of heterogeneity in the products can also be obtained from the peak width. The two different modification methods shifted the pI of caseinate in the acidic or alkaline direction. For proteins, such as sodium caseinate, whose isoelectric point is slightly acidic, the EDA modification is very interesting since it gives the opportunity to study caseinates as emulsifiers in a different pH range. The modified protein had good solubility and emulsifying properties throughout acidic and neutral regions up till pH 7, showing that introducing additional positive charges in caseinate is an efficient way to improve its functionality in this pH range where most of the food applications of emulsion are applied.

The cross-linking reaction that multiplies the molecular mass of caseinate leads to a higher demand on the amount of EDCA to stabilize the emulsion. This side reaction was not observed when the EDA modification was applied to whey protein and BSA. It would be worth studying further the properties of EDA modified whey protein and BSA as emulsifiers as well. EDA itself is not the most ideal compound to produce modified positively charged caseinates for food applications, but this study shows that it would be worthwhile to look for food grade analogues to achieve similar properties. Most interesting would be a protein modification procedure using a biocatalytic approach to reach this goal. Transglutaminase catalyzes the deamination and amine fixation of proteins and has been widely used in the food industry. It would be worth studying whether this type of catalysts or others can be used as tools to modulate protein charge through amino acid modifications.

## ABBREVIATIONS USED

EDA, ethylene diamine; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; MALDI-TOF, matrix assisted laser desorption ionization-time-of-flight; MES, 2-(*N*-morpholino) ethanesulfonic acid; MS, mass spectrometry; O/W emulsion, oil-in-water emulsion; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; pI, isoelectric point; SDS, sodium dodecyl sulfate; TNBSA, 2,4,6-trinitrobenzene sulfonic acid; TFA, trifluoroacetic acid.

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