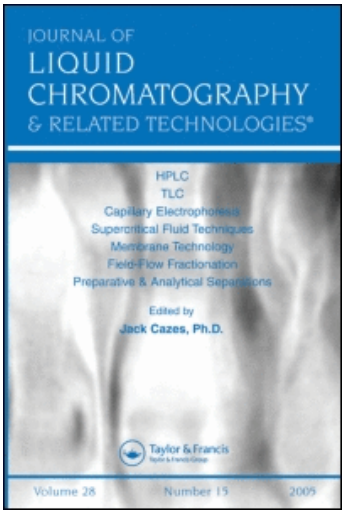

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THE ISOLATION OF PROTEINS FROM WHEY WITH A NEW STRONGLY ACIDIC SILICA-BASED ION EXCHANGER

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ABSTRACT

A new type of strongly acidic ion exchanger based on macroporous silica coated with a thin layer of hydrophilic copolymer with sulphonic acid groups has been evaluated. The coating is crosslinked and covalently linked to the silica surface via diol groups.
The performance for isolation of proteins from whey of the new ion exchanger and Spherosil S were compared using both batchwise adsorption and column operations with reconstituted whey.
Higher breakthrough capacities were observed for the new ion exchanger than for Spherosil S. Excellent column operational lifetimes were found for the new product.
A procedure is described to restore the original adsorption capacity of fouled columns.

INTRODUCTION

In recent years dairy industries have shown a growing attention to the recovery of proteins from cheese and casein whey (1,2). These proteins, being present in a concentration of about 0.6 g per 100 ml whey, possess a high human food value and interesting functional properties.

One isolation process, described by Rhône-Poulenc, is based on reversible ionic interactions between whey proteins and immobilized ions on a silica-based exchanger, i.e. the strongly acidic Sphérosil S or the strongly basic Sphérosil QMA (3 up to 8). The Sphérosil S whey protein recovery process involves passing acidic (pH4.5-4.6) whey through a Sphérosil S column to bind the proteins, rinsing with water to remove lactose and minerals and elution of the proteins with diluted ammonia. Eluted proteins are concentrated by ultrafiltration and freeze-dried. Sphérosil S itself is a strongly acidic medium consisting of rigid, macroporous silica particles (Sphérosil XOB015) coated with about 3mg/m^2 of styrene vinyltriethoxy silane copolymer to which the functional sulfonic groups ($-SO_3^- H^+$) are attached.

In the present study the operational performance of Sphérosil S is compared with that of a new type of strongly acidic silica-based ion exchanger in the isolation of proteins from acid-whey. The latter material, together with other types of ion exchangers and chromatographic materials, was developed for the isolation of a broad range of proteins from different sources (9, 10). The strongly acidic medium is prepared by first reacting large-pore silica with

3-glycidoxypropyl-trimethoxy silane to form a hydrophilic diol-silica. Next the diol-silica is reacted with N-methylolacrylamide (MAAM) and 2-acrylamido-2-methylsulphonic, acid (AMPSA) in the presence of a radical initiator to form a hydrophilic crosslinked polymer chemically coupled to the silica surface and containing sulphonic groups. First types of these new ion exchangers introduced on the market by Millipore are the strongly basic and the weakly acidic types with the respective tradenames Accell QMA and Accell CM (11).

EXPERIMENTAL

Materials

For the preparation of the new ion exchangers different types of commercial materials were applied as base silicas: Sphérosil XOB015 from Rhône-Poulenc (Paris, France), Fractosil 1000 from Merck (Darmstadt, W.-Germany) and Controlled Pore Glass CPG-10-1000 from Electro-Nucleonics (Fainfield, N.J., USA). Sphérosil S is a commercial product of Rhône-Poulenc. Whey powder, obtained by spray-drying acidic whey, containing about 4% lactose, 0.5-0.7% protein, 0.5-0.7% minerals and about 0.05% fat, was a gift from DMV-Campina (Veghel, the Netherlands). To reconstitute the acidic whey, 6 g of whey powder was dissolved in 94 ml of distilled water and subsequently, after adjusting the pH with 4N sulphuric acid, the solution was filtered through a

Schleicher and Schüll no. 595 1/2 folding-filter. Highly purified whey proteins, α-lactalbumin (product number L6010) and ß-lactoglobulin A (L-7880) from Sigma Chemical Co. (St. Louis, MO, USA) were used as reference samples for the determination of the content of those proteins in whey and effluent fractions by means of High Performance Liquid Chromatography (HPLC). To clean fouled columns, Hypafil powder and liquid from Otarés (Enschede, The Netherlands) were used. These commercial products are based on combinations of enzymes, organic sequestring and wetting agents.

Quantitative Determination of α-Lactalbumin (α-la) and ß-Lactoglobulin (ß-lg) by HPLC

A High Performance Size Exclusion Chromatographic (HPSEC) method analogous to the one described by Humphrey (12) was selected for determination of α-la and ß-lg in the effluent fractions collected during the whey experiments with the ion exchangers. A TSK 3000SW column (length 50 cm, 7,5 mm I.D., Toya Soda Manufacturing Co, Japan) was used for separation. A precolumn (4.0 mm x 4.0 cm) regularly repacked with Waters protein I-125 (bead sizes of 37-53 micrometers) was necessary to protect the main column from deterioration. Analyses were performed on a modular HPLC system of Waters Associates (Milford, Mass, USA) equipped with two types of 6000 A pumps, the model 720 System Controller, the WISP Automatic Samples Processor, the 730 Data Module and the model 446 UV Absorbance Detector (wavelength 280 mm). With this fully automated system unattended analyses of up

to 48 samples could be carried out. Mobile phase was a 0.050 M-NaH_2PO4 buffer containing 0.1 M-NaCl at pH 7.2. A flow rate of 1.5 ml/min was applied. Volumes of the injected samples were 10 to 30 microliters.

Procedure for Batchwise Adsorption Experiments

To study the whey protein binding rate and adsorption capacity of the ion exchangers, the materials were successively equilibrated after packing in columns with 0.15 M-NaOAc buffer pH 4.5 containing 1 M-NaCl, 0.15 M-NaOAc buffer pH 4.5 and washed with distilled water. 11 ml bed volume of each ion exchanger was transferred to a flask and, after removal of the supernatant water, incubated at room temperature with gentle stirring by means of a rotavapor with 330 ml of reconstituted whey brought to pH 3.5. The adsorption was followed by taking small liquid samples (400 microliter) from the slurry and determining the content of α-la plus ß-lg by HPLC.

Procedure for Column Adsorption Experiments

To perform the comparative study under identical conditions, three C 10/20 columns (1.0 cm I.D.) assembled with two adapters (Pharmacia, Uppsala, Sweden) were simultaneously loaded with the same liquid at room temperature by means of a 3-channel peristaltic pump (type p-3 from Pharmacia). One column contained Sphérosil S, the other two contained samples of the new, strongly acidic ion exchanger. A

bed volume of 12.0 ± 1.0 ml adsorbent was used. Fractions of 20 ml effluent were collected with a fraction collector (type 2070 Ultrorac 2 from LKB, Bromma, Sweden). Columns were initially equilibrated with successively 0.05 M-Na, H-phthalate buffer pH 3.5 containing 1 M-NaCl and 0.05 M-Na, H-phthalate buffer pH 3.5 (5 bed volumes of each) to activate fully the functional groups. Then, after loading with 200 to 250 ml of reconstituted whey (adjusted to pH 3.5), the adsorbents were washed with 2 bed volumes of distilled water to remove non-protein components. Subsequently, bound proteins were quantitatively recovered in high concentrations with 5 to 8 bed volumes 1 M-NH_4OAc at pH 7.4.

RESULTS AND DISCUSSION

The New Ion Exchangers and Sphérosil S

Table 1 provides a compilation of the characteristics of the new ion exchangers, samples 1 up to 4, together with the reference sample Sphérosil S. Samples 1, 3 and 4 were made by copolymerizing equal amounts of MAAM and AMPSA onto diol-modified, macroporous inorganic materials from different suppliers according to the method described previously (9, 10). From a sample of Sphérosil S the styrene/vinyltriethoxy silane coating containing sulphonic acid groups was removed by pyrolysis resulting in a "blank" base silica. On this material the diol-modification and the MAAM/AMPSA coating was

TABLE 1

Characteristics of the New MAAM Ion Exchangers and Sphérosil S

Sample code[c]	type of base material[b]	elemental analysis			content of sulphonic acid groups (meq/kg dry material)	
		C%	N%	S%	calc. from S%	determined by microtitration
1	Sphérosil XOB015	3.48	0.58	0.86	269	275
2[a]	pyrolyzed Sphérosil S	3.85	0.63	0.88	275	249
3	Fractosil 1000	3.86	0.69	1.07	334	288
4	CPG-10-1000	3.06	0.56	0.84	263	265
Sphérosil S	Sphérosil XOB015	3.41	$\leq$0.05	1.10	334	245

(a) sample 2 is made by derivatization of blank silica (C, N and S $\leq$0.05%) obtained by pyrolysis of vacuum dried Sphérosil S at 650°C for 2 hours;
(b) all blank types are reported to possess a specific surface area somewhere in between 20 to 25 m^2/g, a mean pore diameter in the range of 100 to 125 nm and a bulk density of about 0.5 g/ml. Pore volume of Fractosil 1000 (0.5 ml/g) is lower than that of remaining types (1 ml/g);
(c) particles were sieved between 0.106 and 0.212 mm for Sphérosil -, 0.063 and 0.125 mm for Fractosil - and 0.125 and 0.180 mm for CPG - based samples, yielding approximately the original particle sizes of the blank silicas.

applied yielding sample 2 to see whether the change of the coating would influence the adsorption characteristics. Pyrolysis is not expected to alter the morphology of the silica.

Batchwise Adsorption Experiments with reconstituted Whey at different pH's

To establish the optimum pH for adsorption of whey proteins sample 1 and Sphérosil S (both in the sodium sulphonate, $-SO_3^-Na^+$, form) were incubated at room temperature under agitation with reconstituted casein whey, the pH of which covered the range of 3 to 6. After a fixed period of time a sample was drawn from the supernatant liquid and the amount of α-lactalbumin (α-la) and β-lactoglobulin (β-lg) was determined by HPLC. The latter two proteins constitute 60 to 80% of the total amount of protein in whey. Therefore the concentration of both proteins in whey is seen as a fairly good representation of the total protein content, i.e. α-la, β-lg, bovine serum albumin (BSA), immunoglobulins (Ig), proteose-peptones and traces of other proteins, present in whey (13). In order to be effectively adsorbed onto negatively charged, strongly acidic cation exchangers, whey proteins such as α-la and β-lg with isoelectric points (pI) in the range of 4.5 to 5.3 should carry a net positive charge, i.e. the pH of the whey solution should be about 1.5 units below their pI values (14). We thus assumed that there exists solely an ionic interaction between the adsorbent and the protein molecules. The percentages of adsorptions, i.e. the adsorbed amount

of α-la and β-lg relative to the offered amount, in relation to the pH of whey for sample 1 and Sphérosil S, are given in Figs. 1 and 2, respectively. Fig. 1 shows that the adsorption onto the MAAM-type cation exchanger (sample 1) is maximum at pH values of 3.5 and 3.0. For pH values in excess of 3.5 a sharp drop in adsorptive properties is observed. These results are indicative of a single, i.e. ionic, mechanism being involved in the adsorption process for this type of cation exchanger. On the other hand, from Fig. 2 no distinct pH-dependence for the adsorption of whey proteins onto Sphérosil S is observed. Therefore, adsorption very likely proceeds here due to the hydrophobic nature of the polystyrene coating loaded with anions according to a mixture of cooperative hydrophobic and ionic interactions forces. The different behaviour of the two materials is not surprising as polystyrene itself is known to adsorb proteins (15) and macroporous diol-silica coated with the homopolymer of MAAM does not significantly adsorb proteins (9,10).

For all further experiments carried out in this investigation the reconstituted whey was adjusted to pH 3.5 because at this pH the adsorption was maximum and no adverse effects on the stability (i.e. denaturation, insolubilization) of the proteins of interest were detected.

Batchwise Adsorption Experiments to compare Adsorption Rates and Capacities

As described in the experimental section samples 2, 3, 4 and Sphérosil S (table 1), were incubated

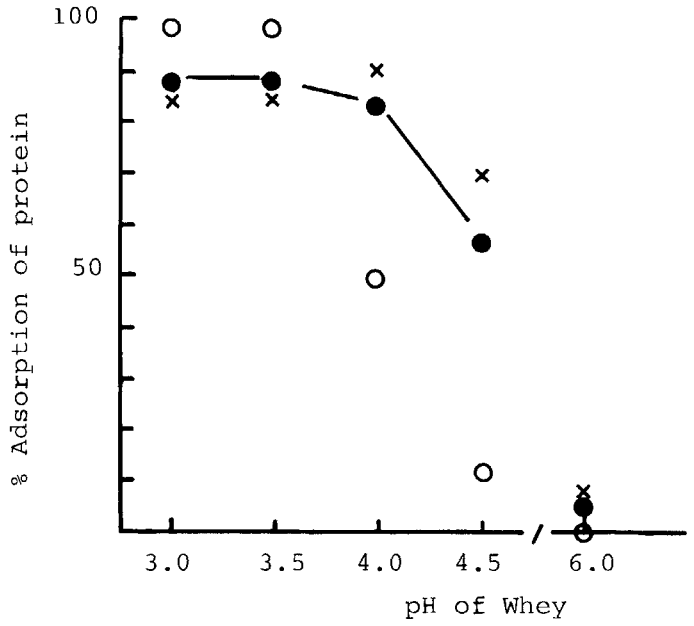

FIGURE 1

Adsorption of proteins from whey at different pH's by sample 1. 2 ml of sample 1 incubated with 15 ml of whey (2.7 mg β-lg and 0.8 mg α-la per ml) for 30 minutes; α-la (O), β-lg(×), α-la plus β-lg (●)

under identical conditions with whey at pH 3.5 reconstituted from the same batch of whey powder. In Fig. 3 percentages of adsorbed amounts of the sum of α-la and β-lg against incubation times are plotted for the four materials. Adsorption capacities i.e. final amounts of adsorbed α-la plus β-lg per ml adsorbent at equilibrium were for sample 2, 3, 4 and Sphérosil S 59, 58, 61 and 47 mg/ml respectively. The curves in Fig. 3 show two distinct stages in the rate of adsorption: a fast, initial rate within the first

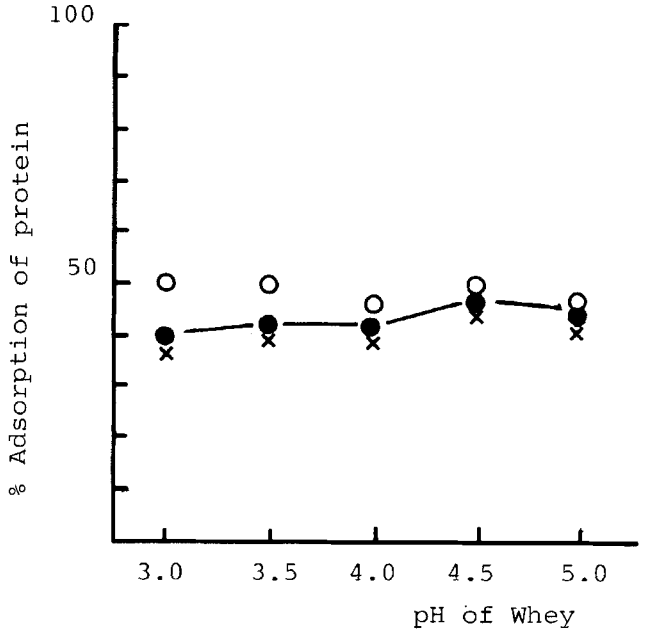

FIGURE 2

Adsorption of proteins from whey at different pH's by Sphérosil S. 2 ml of Sphérosil S were incubated with 60 ml of whey (1.63 β-lg and 0.5 ml α-la per ml) 1 hour; α-la (○), β-lg (×), α-la plus β-lg (●)

15 minutes and a consecutive, far slower rate. Of the four ion exchangers Sphérosil S showed the worst performance with respect to initial rate of adsorption and final capacity. Removal of the coating from Sphérosil S by pyrolysis and recoating the silica thus obtained according to the MAAM/AMPSA procedure yielded sample 2. This, however, showed excellent adsorption characteristics. In fact, the performance of sample 2 was comparable with that of sample 4 based on the angular shaped CPG particles.

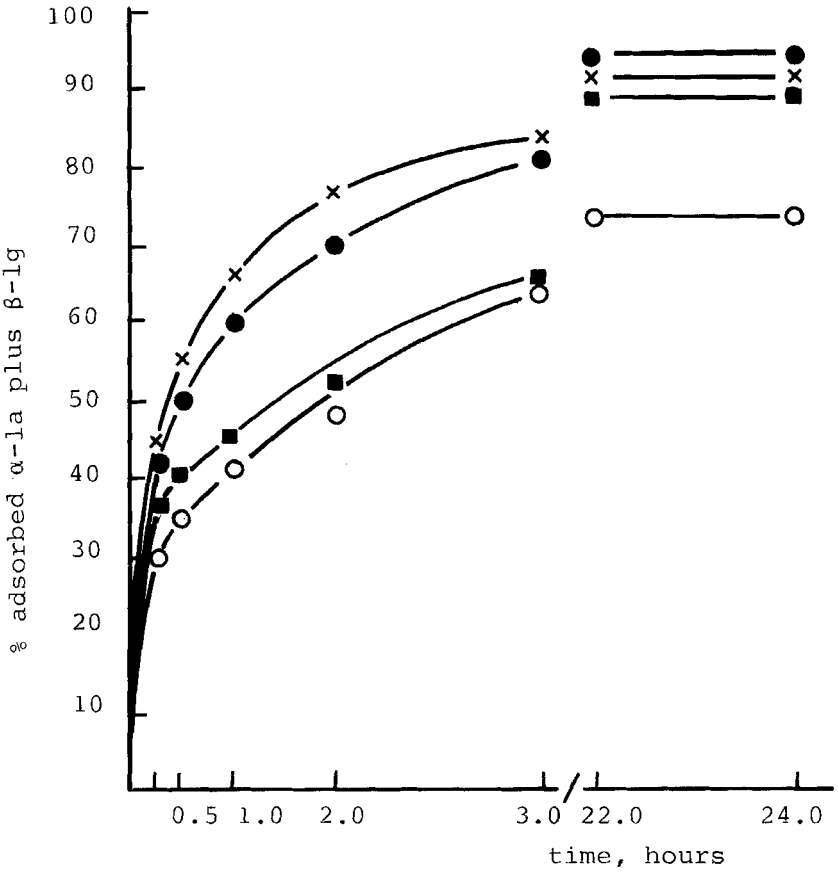

FIGURE 3

Batchwise adsorptions. Whey contained 1.63 mg β-lg and 0.47 mg α-la. Samples were Sphérosil S (○), sample 2 (×), sample 3 (■) and sample 4 (●).

Sample 3, based on Fractosil, is on a level intermediate between that of Sphérosil S and samples 2 and 4. Although it may be argued that the adsorption characteristics of sample 3 and 4 relative to Sphérosil S have been improved due to the use of other types of base materials with more accessible pore morphology and smaller particle sizes, this does not apply to sample 2. Moreover, scanning electron microscopy of Sphérosil S (see Fig. 7a) and pyrolyzed Sphérosil S used for the synthesis of sample 2 did not show any damaging effect of pyrolysis on the silica structure. Therefore, under batchwise adsorption conditions the MAAM/AMPSA coating induced better adsorption characteristics than the coating applied in Sphérosil S.

Column Experiments

To allow a comparison of the performance of our ion exchangers, three columns were simultaneously loaded with the same batch of reconstituted whey adjusted to pH 3.5. Fig. 4 presents a typical set of HPLC-chromatograms taken from fractions collected at various intervals during a chromatographic run with ion exchanger. It is obvious that from the first fractions of whey passed through the column both α-la and ß-lg were totally adsorbed. In addition, some of the BSA and Ig, only present in small quantities in whey, appear to have been removed. Proteose-peptones, as may be expected from their low pI (≤ 3.7), are hardly adsorbed on the ion exchanger. Fraction 7 of Fig. 4 shows a breakthrough of the α-la and ß-lg meaning that the column starts to be saturated. Elution of the adsorbed proteins leads to a

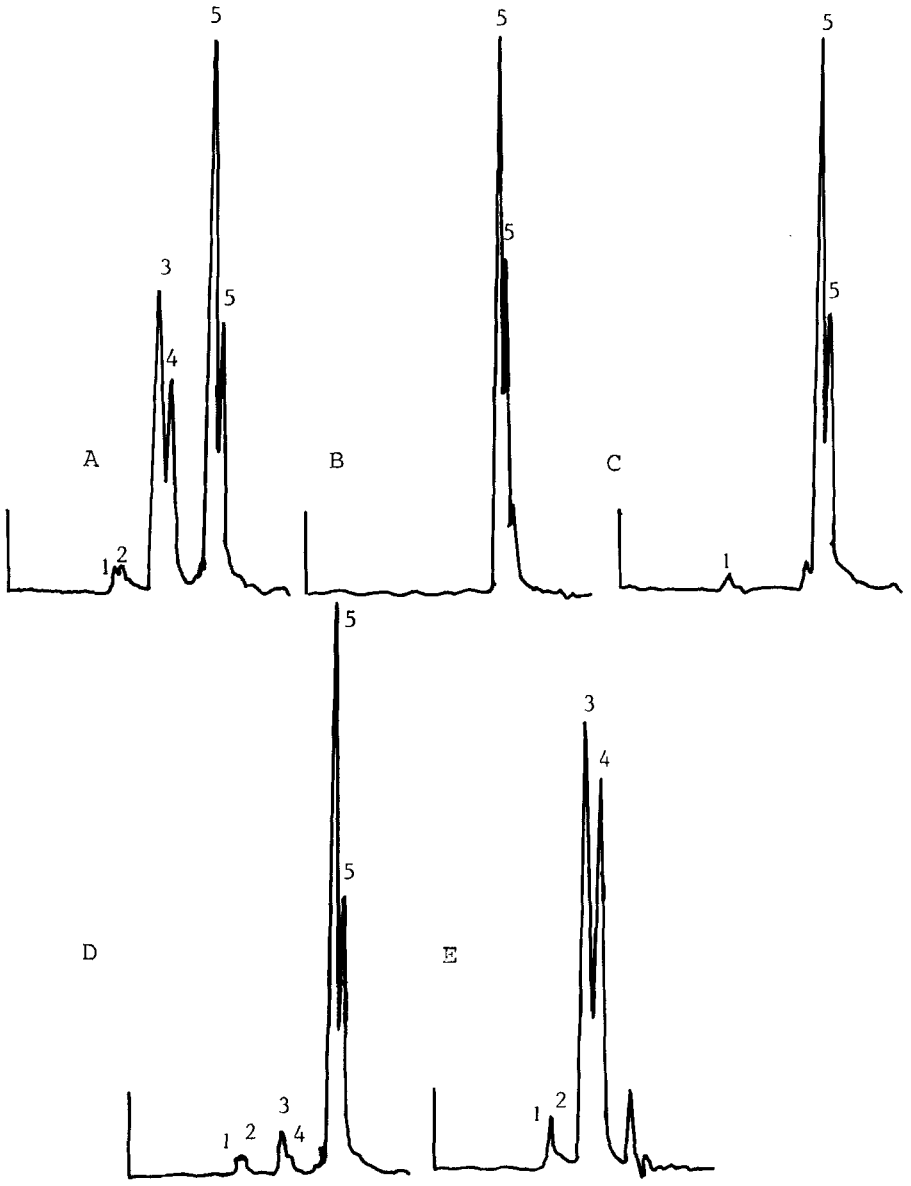

FIGURE 4
HPLC-chromatograms of reconstituted whey at pH 3.5 (Fig. 4A), first, third and seventh effluent fraction of whey passed through column (Figs. 4B, C, D, respectively); of collected elution fractions (Fig. 4E). Peaks 1 = immunoglobulin, 2 = BSA, 3 = β-lg, 4 = α-la 5 = proteose-peptones.

quantitative recovery. The HPLC chromatogram of the collected elution fractions (Fig. 4F) shows, besides α-la and β-lg, minor quantities of BSA, Ig and proteose-peptones. At a lower total adsorption percentage, a lower ratio of β-lg to α-la was detected in the eluate indicating a higher affinity of the adsorbent for α-la. Also when columns were excessively loaded with whey β-lg was displaced from the adsorbent by α-la. So far, chromatographic processes with either Sphérosil S or our new ion exchanger have qualitatively looked alike.

Table 2 and Fig. 5 show, however, considerable differences in amounts of α-la plus β-lg adsorbed by these ion exchangers. Percentages of α-la plus β-lg adsorbed per subsequent volume of 20 ml against total volume of "de-proteinised" whey collected after passage of whey, are plotted for three adsorbents in Fig. 5. The adsorption capacity increases in the sequence Sphérosil S, sample 1 and sample 3. The point of breakthrough of α-la plus β-lg in the sigmoid curves is relevant. From this breakthrough point the breakthrough capacity of the ion exchanger can be determined. This is the amount of quantitatively adsorbed α-la plus β-lg per ml adsorbent. Numerical data on the experiments mentioned in Fig. 5 are compiled in Table 2. Breakthrough capacities are for Sphérosil S, sample 1 and 3 respectively 22, 19 and 38 mg indicating sample 3 adsorbs almost double the amount of proteins than Sphérosil S at a flow rate of 50 ml/h. Although under the comparable test conditions the total amount of adsorbed α-la plus β-lg per ml of sample 1 slightly exceeded that of Sphérosil S, the breakthrough capacity was the lowest for sample 1. This is

TABLE 2

Adsorption and Elution Values for Column Experiments with Sphérosil S and MAAM samples 1 and 3.

Code of sample	ml of whey offered	total amount of α-la + β-lg adsorbed (mg)	% of adsorption	total mg α-la +β-lg adsorbed per ml bed volume	breakthrough capacity (mg/ml)
Sphérosil S	260	377	46	34	22
sample 1	247	494	64	38	19
sample 3	234	565	77	47	38

For respective adsorption patterns, see Fig. 5

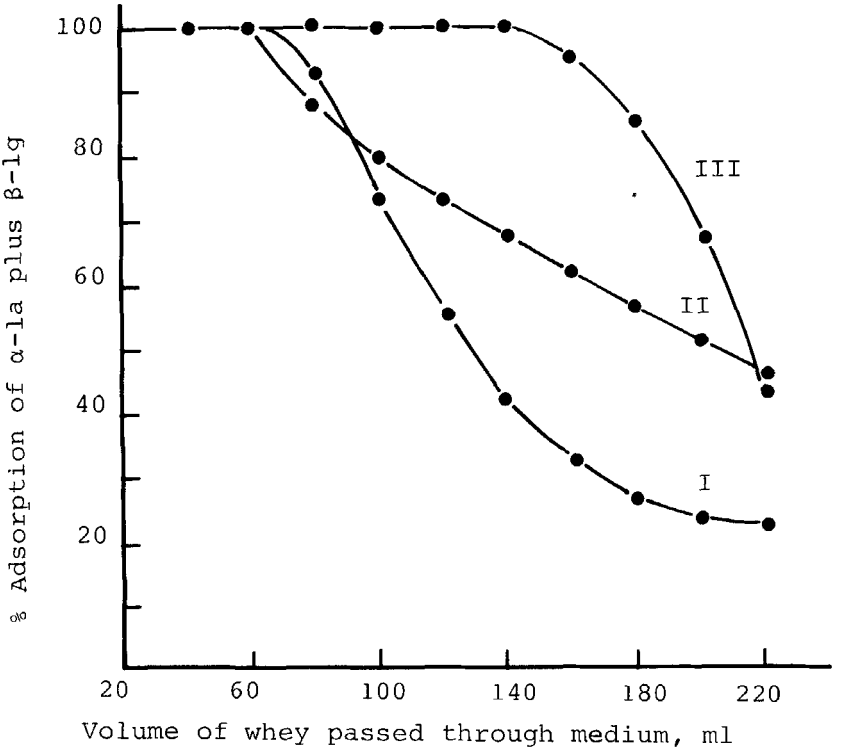

FIGURE 5

Column adsorption patterns (α-la + β-lg) for Sphérosil S and MAAM samples 1 and 3. Whey (2.60 mg of β-lg and 0.53 mg of α-la per ml) pumped with a flow rate of 50 ml/hour through the columns. Bed volumes of Sphérosil S (I), sample 1 (II) and 3 (III) were 11.0, 13.0 and 12.0 ml, respectively.

surprising as the base silica, namely Sphérosil XOB015 (16), applied for synthesis of sample 1 and Sphérosil S was identical and also because batchwise adsorption had earlier shown a higher adsorption rate for sample 2 (based on pyrolyzed Sphérosil S) than for Sphérosil S due to the superior behaviour of the MAAM/AMPSA coating.

To study the influence of flow rate on the amount of adsorbed protein per ml of adsorbent, simultaneous experiments were set up with columns loaded with reconstituted whey at flow rates ranging from 10 to 100 ml per hour. The excellent flow characteristics of the ion exchanging materials permits the use of high flow rates without noticeable column compaction or back-pressure. As will be seen in Fig. 6 the breakthrough capacities decrease at higher flow rates for all types of adsorbents studied, suggesting that mass transport of proteins into the particles of the ion exchanger is limited. The sequence of amounts of adsorbed protein per ml of adsorbent at low flow rates (10 to 20 ml/h) reflects very well the one observed for the adsorption capacities found under batchwise conditions for Sphérosil S and for samples 2 and 3. Even sample 1 performs at these low flow rates on a level comparable with that of the other new samples. While the curves drawn in Fig. 6 for Sphérosil S, sample 2 and sample 3 do not intersect, the curve for sample 1 exhibits a sharp drop when the flow rate increases and cuts the line belonging to Sphérosil S. The breakthrough capacity is reported to depend on several factors: the dimensions of the column, the size of the particles, the temperature of the system, the composition of the whey, the flow characteristics and the nature of the ion exchanger (14). Under the test conditions selected, however, the most important factor explaining the odd behaviour of sample 1 relative to that of Sphérosil S and sample 2 had to be the nature of the ion exchanger. Therefore, by means of scanning electron microscopy (SEM) a study was made of the structure of the exterior surface of Sphérosil S (Fig. 7a), which

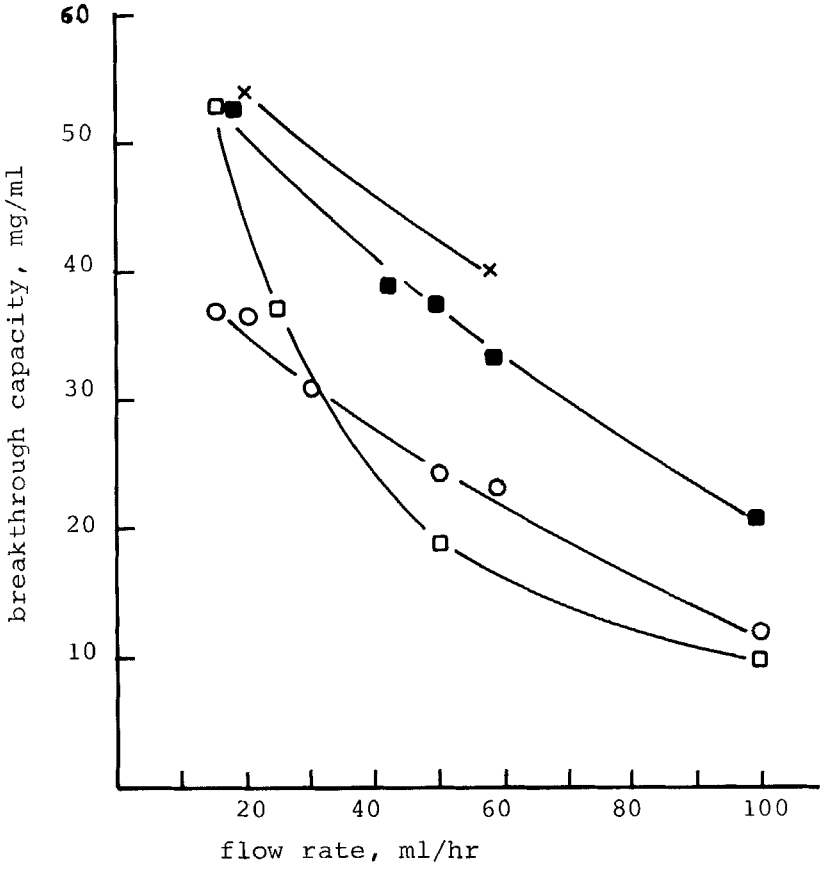

FIGURE 6

Influence of flow rates or breakthrough capacities (α-la plus β-lg). Whey contained 3 to 4 mg of α-la plus β-lg. Samples: sample 1(□), sample 2 (×), sample 3 (■), Sphérosil S (0)

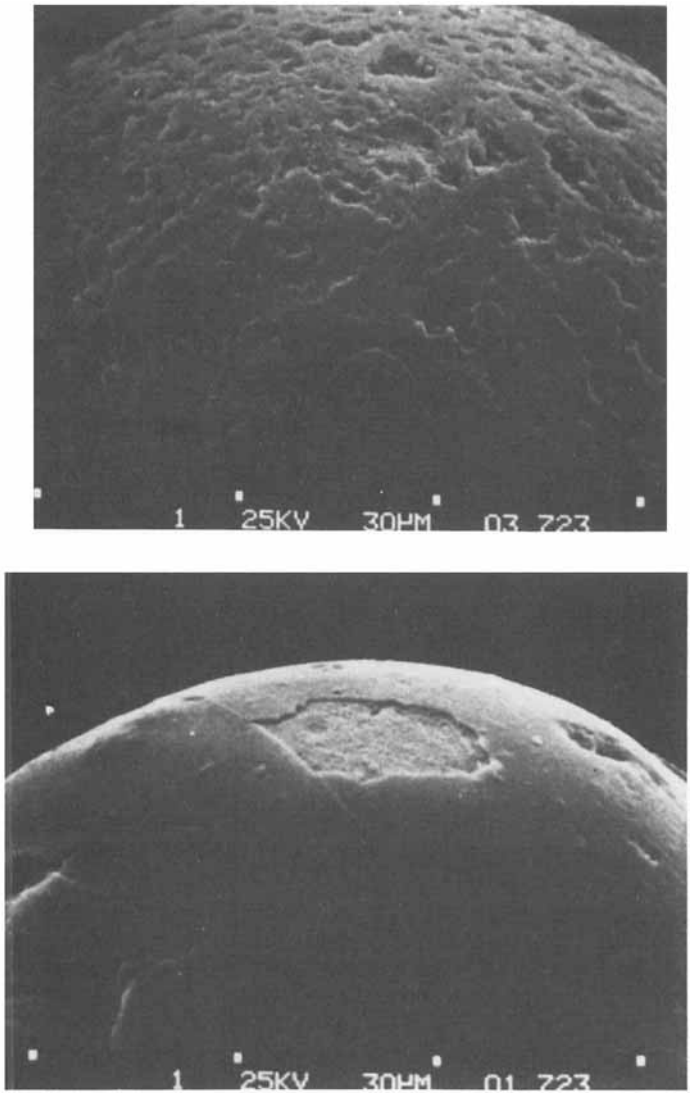

FIGURE 7

Scanning electron micrograph of Sphérosil S (A) and blank Sphérosil X0B015 (B)

is comparable with that of pyrolyzed Sphérosil S, and of Sphérosil X0B015 (Fig. 7b); the latter two are the base silicas for the preparation of samples 2 and 1, respectively. In the case of the blank Sphérosil X0B015 a major part, about 70%, of the surface of most beads is found to be covered with a dense skin of silica having a thickness of about 500 nm; the remaining part is porous and will be easily be accessible to proteins. The same observation for this material was also recently described in the literature (17). The outer surfaces of Sphérosil S and pyrolyzed Sphérosil S beads on the other hand closely resemble each other but are quite different from those of Sphérosil X0B015. Sphérosil S has a perforated, loosely bound silica skin probably obtained by etching the blank Sphérosil X0B015 before modification with the hydrophobic coating as the presence of cavities in the surface may suggest. It is obvious that the dense skin of silica on the outer surface of blank Sphérosil X0B015 used for sample 1 will obstruct a facile and fast penetration of the whey proteins at high flow rates. As batchwise experiments have already shown, sample 3 and notably sample 2 are superior to Sphérosil S during column operations (Fig. 6). At flow rates between 10 up to 100 ml/h the breakthrough capacities of sample 3 were about 50% higher than those of Sphérosil S. Quantitative recovery of adsorbed proteins was in most cases possible by eluting with 1 M-NH_4OAc in water (pH 7.4). The use of aqueous ammonia, applied in the Rhône-Poulenc process as elution medium, was discarded by us because of the corrosive effect of highly basic solutions on the silica matrix and because of possible denaturation effects on proteins.

Preliminary Investigation into the Performance of Ion Exchangers during prolonged Operational Periods

In a preliminary investigation the operational lifetimes of Sphérosil S and sample 1 were determined over a long period. The column operational cycles were simultaneously repeated many times for both samples under the conditions given in the experimental section. No cleaning or special regeneration steps were applied between the cycles. Breakthrough capacities i.e. quantities of completely adsorbed α-la plus β-lg per ml of adsorbent at a flow rate of 15 ml/hr were taken as criterion to measure the operational decline. For Sphérosil S the capacities were found to be unaltered during the first nine cycles, next a progressive decrease occurred, resulting in a loss of 25 and 50% of its original capacity (37 mg/ml) at the 15th and 25th cycle, respectively. Sample 1 was unaffected during the first 26 cycles, subsequently reduction of the original capacity (55 mg/ml) took place. A loss of 25 and 50% was observed for cycle 37 and 42, respectively. The shorter operational lifetime found for Sphérosil S might be caused by competitive adsorption of fat from whey. Owing to its nature this fat may indeed be expected to be adsorbed more effectively by Sphérosil S containing a hydrophobic polystyrene coating than by our new hydrophilic coated ion exchangers. As well as fouling of the column, adsorption of fat reduces the accessibility of ion-exchanging groups to whey proteins. To improve the operational lifetime of the ion exchangers a suitable cleaning procedure had to be developed to restore in due time the original adsorption capacity.

In preliminary trials fouled columns were flushed with aqueous solutions of ethylene diaminotetraacetic acid, disodium salt or citric acid. This had no or very little effect. Passage at room temperature of a few bed volumes of 1% Hypafil^{R} powder in water at pH 7.8 and Hypafil liquid in water at pH 9.2, however, effectively yielded 100% of the original breakthrough capacity of severely fouled ion-exchanging media. Hypafil products are applied in the food industry for the cleaning of membranes. They did not degrade even after soaking for 24 h at 60°C, our new ion exchangers as indicated by the nitrogen, carbon and sulfur content of fresh and Hypafil treated samples.

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