

Figure 12.4. Effect of sorbent and flow rate on the breakthrough capacity for protein A affinity chromatography. Data from Fahrner *et al.* (1999c).

specific binding since Poros 50 has the lowest value for host cell proteins. However, even the lowest clearance (for Sepharose) represents a 99.48% removal of host cell proteins, leaving a small amount of host cell proteins that can be cleared downstream.

In our work, we use Prosep A (controlled-pore glass) because it provides good purity, low protein A leakage, and good throughput. It is also easy to pack due to a fast settling rate and high permeability. We have found that it withstands re-use reasonably well, and for several antibodies we have validated its lifetime to 300–400 cycles.

Although the development of protein A affinity chromatography does not focus on purity, purity is still a concern. One way to decrease the amount of host cell proteins in the elution pool when using Prosep media is by using an intermediate wash with tetramethylammonium chloride (TMAC). Since the base material for Prosep is controlled-pore glass that is made from silica, any exposed silica surface may bind proteins. TMAC is known to elute proteins from silica (Chandha and Sulkowski, 1981; Reifsnnyder *et al.*, 1996), and in the chromatogram in *Figure 12.1*, a peak is apparent when TMAC is introduced into the column. This peak is not nearly as large when washing with other salts such as sodium chloride or sodium sulphate, which supports the theory that TMAC is eluting host cell proteins that are bound to the exposed silica.

Development of the elution stage is concerned with the elution buffer (Narhi *et al.*, 1997). For elution at pH <3, either citrate or acetate may be used. Some antibodies may partially or completely precipitate in one of these buffers, but generally either citrate or acetate will produce high recovery yield. The elution buffer should be used in an amount that will produce a pool of pH <3.8, so that no adjustment is required for viral inactivation, which requires pH <3.8 for 15–30 min.

The protein A column may typically be run in 4–10 cycles to purify a single batch. Since each cycle is only about 1 hour long, this cycling allows rapid throughput while reducing the cost of the column. The load material must be assayed for antibody concentration prior to loading, usually by an analytical protein A affinity assay. After

Table 12.3. Results from the protein A affinity chromatography re-use study

Cycle	Yield (%)	CHOP (ng/mg)	DNA (ng/mg)	Insulin (ng/mg)	Protein A (ng/mg)	Pluronic F-68 (ng/mg)
5	101	516	1.3	<1.5	6.0	<1246
150	103	525	1.5	<2.0	17.9	<1743
300	96	1455	0.5	<1.7	18.9	<1475
340	90	1604	0.1	<1.8	34.3	<1989

determining the antibody concentration, the load is split up into the minimum amount of cycles that can purify the batch. This may underload the column for each cycle. Because the measurement of the load volume may be inaccurate, it is important to have an air sensor on the load line to end loading of the last cycle if the load runs out early. During elution, the pool begins when the absorbance reaches a predetermined value. The pooling may end when either absorbance or volume reaches a predetermined value. Typically, the pH of the elution pool is adjusted to >5 before holding for an extended amount of time because the antibody may be unstable in the elution pool due to the low pH.

RE-USE VALIDATION

Because protein A affinity chromatography media is expensive, column re-use is a significant concern. For one of our antibodies, we validated the life of the column to 340 cycles. A laboratory scale study was performed to demonstrate that the quality of the product purified by the protein A affinity chromatography step was not affected by multiple re-use of the Prosep A resin. Representative cycles (5, 150, 300, and 340) over the course of the study were selected and the pools were analysed for yield and purity (host cell proteins, DNA, protein A, insulin, and Pluronic F-68). These pools were also analysed by SDS-PAGE under reducing and non-reducing conditions. Chromatograms for these selected cycles were compared to a reference chromatogram.

The yield and purity (host cell proteins, DNA, insulin, protein A and Pluronic F-68) for the four representative cycles are shown in *Table 12.3*. The yields ranged from 90% to 103%. Although there is an apparent decrease in yield with increasing cycle number, a yield of 90% is acceptable. The levels of DNA ranged from 0.1 to 1.5 ng/mg. The levels of host cell proteins ranged from 516 to 1604 ng/mg. An approximate three-fold variation in host cell protein levels in the protein A pool does not significantly affect the final product purity, given that the process has been validated to consistently clear host cell proteins by greater than one thousand-fold in subsequent steps. Insulin levels were consistently below the limit of quantitation (LOQ <2 ng/mg). Protein A levels ranged from 6.0 to 34.3 ng/mg. These levels were less than the manufacturing action limit of 50 ng/mg. The levels of Pluronic F-68 were consistently below the LOQ (<2000 ng/mg). Although the change in host cell proteins and leached protein A suggested ageing of the column, the extent of this was not sufficient to warrant reducing the column lifetime to less than 340 cycles.

Pool samples run on SDS-PAGE, under reducing and non-reducing conditions, were consistent over the course of the 340 cycles (*Figures 12.5A and 12.5B*). A small band appears in cycle 300 (lane 10, non-reduced) but does not appear in cycle 340

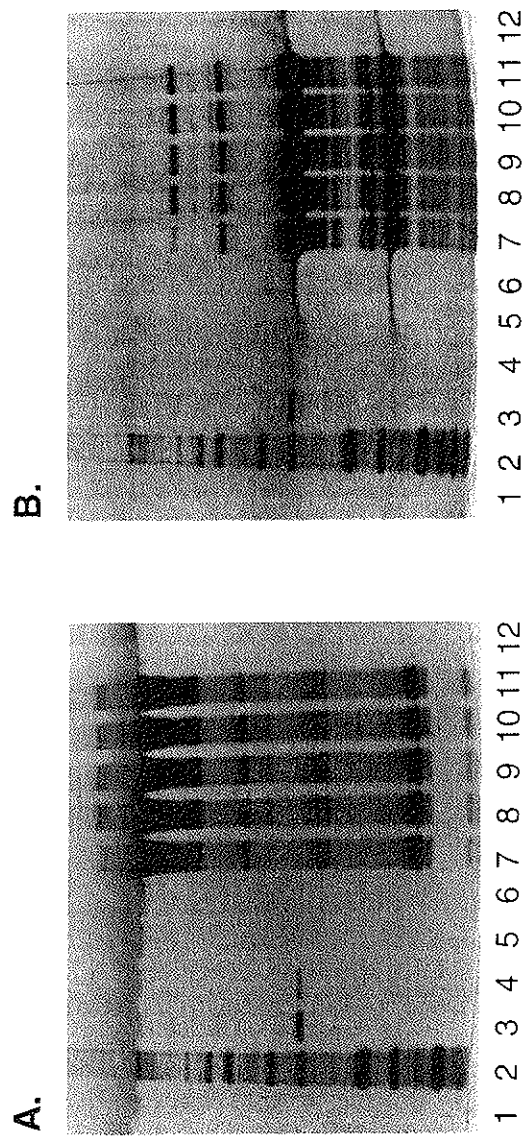


Figure 12.5. (A) Non-reduced and (B) Reduced SDS-PAGE of elution pools from the protein A affinity chromatography re-use validation study. For both gels, lane 1: buffer blank, 2: Molecular weight standards (200, 116, 97, 66, 55, 36, 31, 21, 14 kD), 3: 20 ng BSA, 4: 2 ng BSA, 5: buffer blank, 6: buffer blank, 7: antibody standard, 8: pool from cycle 5, 9: cycle 150, 10: cycle 300, 11: cycle 340.

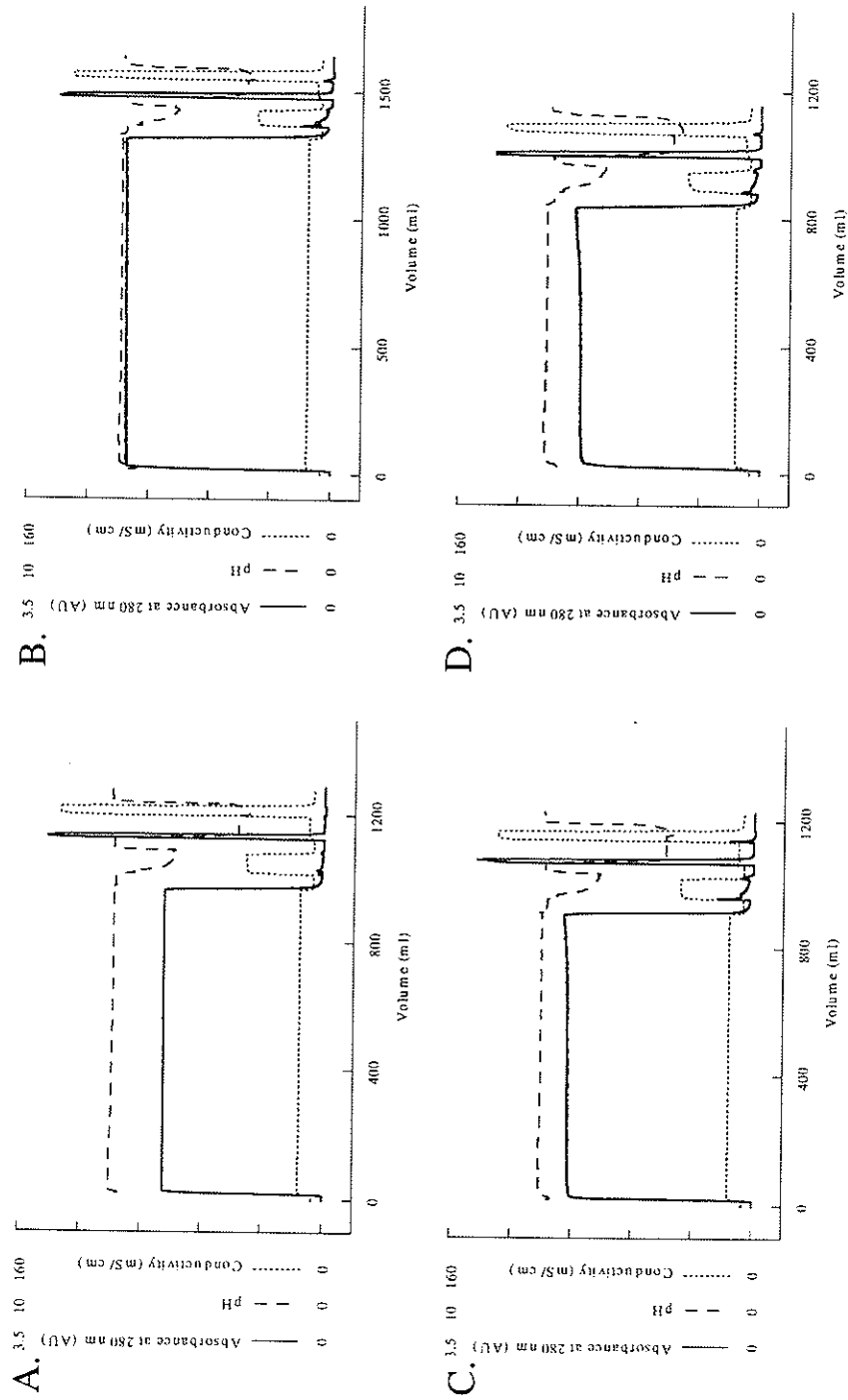


Figure 12.6. Chromatograms from the protein A affinity chromatography re-use validation. (A) Cycle 5, (B) Cycle 150, (C) Cycle 300, (D) Cycle 340.

(lane 11, non-reduced), indicating that this is not a trend. The chromatograms from the representative cycles were compared with the chromatogram from cycle 5, the reference chromatogram (*Figure 12.6*). The peak shape and the absorbance, pH, and conductivity profiles of the reference chromatogram were consistent throughout the study.

Cation exchange chromatography

DEVELOPMENT AND OPERATION

Cation exchange chromatography is used as the intermediate purification step for recombinant antibodies. Although the protein A affinity step greatly reduces the amount of host cell proteins, DNA, and endotoxin, these impurities must be further removed. In addition, the protein A affinity step does not reduce the level of aggregate, and it introduces protein A molecules into the purified antibody. Cation exchange chromatography reduces the level of host cell proteins, DNA, endotoxin, aggregate, and leached protein A. It will also reduce the level of any misformed antibody (for example, antibody with two heavy chains and one light chain). Some host cell proteins flow through the column during load, some elute with the antibody, but the majority elute during the regeneration phase. During chromatography, the leached protein A and aggregate also elute during the regeneration phase.

Development of the cation exchange step focuses on several aspects: the chromatography media, the wash and elution conditions, and the load onto the column. For large-scale use, several vendors can supply chromatography media, including Pharmacia of Uppsala, Sweden (which makes Sepharose) and PerSeptive Biosystems of Framingham, MA, U.S.A. (which makes Poros). We have found that the best media depends on the antibody. For the antibody shown in *Figure 12.2*, Poros 50 HS separated aggregate better than SP Sepharose Fast Flow, providing higher yield at equivalent purity.

There are two critical variables to investigate when developing the wash and elution conditions: the buffer pH and the amount of salt in each buffer. The pH of the separation will be determined in part by the stability of the antibody, and before finalizing the separation pH the stability of the antibody at that pH should be evaluated. We have found that a pH of 5.5 is often optimal for bind-and-elute cation exchange chromatography of antibodies. This pH is high enough that antibody stability is not a problem, and it is low enough to provide sufficient capacity. The typical column capacity at pH 5.5 and a load conductivity <8 mS/cm is about 40 g/l. The conductivity of the protein A affinity chromatography pool is low (<5 mS/cm), so capacity is not greatly affected by pH until the pH is >7 (about 2 pH units below the antibody pI), when capacity may decrease.

The elution conditions are optimized in series of experiments where the column is loaded to capacity and eluted using varying concentrations of sodium chloride or another salt. The collected pool is analysed for yield, purity (aggregate, host cell proteins, DNA, and protein A), and peak width. Generally, at lower salt concentrations the antibody may not completely elute and yield will be low, and at higher salt concentrations the yield will be high but aggregate and host cell proteins will begin eluting with the antibody. This balance between purity and yield will then be the focus

of the development effort. The width of the eluted peak is important for process applications, since a large peak will need a large tank for collection, and the peak volume will affect the throughput of the next step since a larger peak will take a longer time to load. At higher salt concentrations the width will be narrow and at lower salt concentrations the peak will be broader.

If pH is also a factor during development of the elution conditions, the varying salt conditions are applied at varying pH values. This will produce a complex interaction between pH, salt concentration, yield, and purity.

Impurities are removed from the column by increasing the conductivity with a regeneration buffer wash. For development purposes, this wash may be 1 M NaCl or some other appropriate salt so that the regeneration fraction may be assayed for host cell proteins, DNA, and antibody to ensure mass balance. Later, in production, this high-salt wash is generally replaced with sanitization buffer such as 0.5 or 1 M NaOH. In addition to eluting proteins due to the high ionic strength, NaOH also degrades proteins and other molecules and strips them from the column.

Operation at large scale is straightforward except for two factors: column size and peak collection. Since the run time for a typical cation exchange column is about 8 hours, column cycling is not desirable. Therefore the column is usually sized to purify a batch of antibody in a single cycle. The maximum column diameter may be determined by plant size or other considerations, so often the bed height (or column length) will need to be adjusted to have a column size sufficient to purify the batch of antibody. The bed height may affect the separation, so this parameter should be defined during development or the effect of the bed height on the separation should be evaluated during development. The bed height is typically 20–30 cm.

Peak collection begins when the absorbance reaches a pre-set value. It ends when either a pre-set absorbance or volume is reached. When developing elution conditions, the balance between purity, yield and peak width may result in elution conditions where the aggregate is not baseline resolved from the antibody. In this case, rather than eluting in the regeneration, some aggregate may elute in the tail of the main antibody peak. When this occurs, special attention needs to be paid to the pooling conditions so that an antibody peak low in aggregate can be collected. Often, by ending the pool at a relatively high absorbance, a low-aggregate peak can be collected without greatly affecting yield.

PARAMETER VALIDATION

Because the salt concentration and pH of the elution buffer has a strong effect on the antibody separation, the effect of these variables was carefully validated. A laboratory scale cation exchange column was packed (6.84 ml volume, 0.66 cm diameter and 20 cm bed height). The column was packed at the maximum flow rate of the study (200 cm/h). The packed column was sanitized with 0.5 N NaOH (2 CV) and stored with 0.1 N NaOH (3 CV). The control chromatography run was performed with a load of 40 g/l and a flow rate of 100 cm/h. Values measured during the parameter validation are compiled in *Table 12.4*.

Variation in antibody loaded onto the cation exchange resin (10 to 50 g antibody per litre of resin) had no significant effects on the chromatography, recovery, or the quality of the product in the pool. Several load-dependent effects were evident in the

Table 12.4. Results from the cation exchange parameter chromatography validation study

Load (g/l)	Flow rate (cm/h)	Buffer pH	Buffer addition	Yield (%)	Pool concentration (g/l)	Aggregate (%)	DNA (log clearance)
10	-	-	-	82	1.63	0.00	>1.85
20	-	-	-	88	3.48	0.00	>2.18
30	-	-	-	91	5.44	0.57	>2.37
40*	100	5.5	-	86	6.74	0.51	>2.47
50	-	-	-	89	8.58	0.66	2.27
-	200	-	-	87	7.00	0.71	2.3
-	50	-	-	86	6.92	0.59	>2.48
-	-	5.6	-	91	7.26	1.55	>2.5
-	-	5.4	-	82	6.43	0.00	2.04
-	-	-	- 5 mM NaCl	89	6.84	0.49	>2.47
-	-	-	+ 5 mM NaCl	90	6.87	0.95	>2.48

* indicates the control run

- indicates that the baseline condition was used

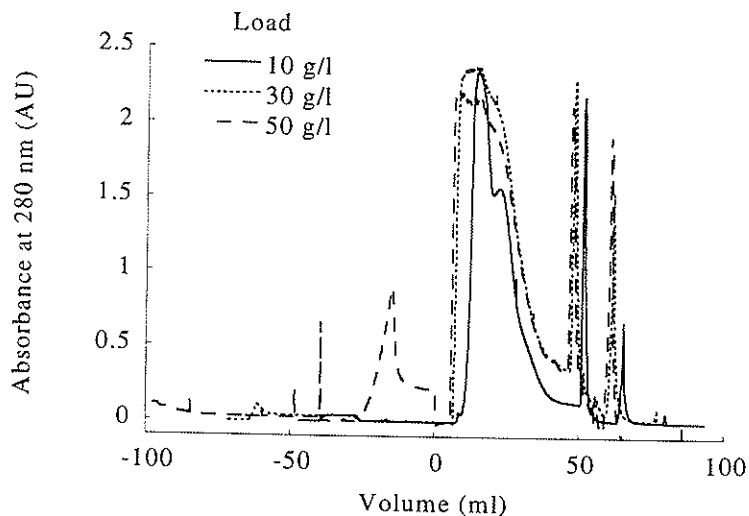


Figure 12.7. Overlaid chromatograms from the cation exchange load validation study. The x-axis is volume from the start of elution. Except for the amount of antibody loaded, conditions are the same as in *Figure 12.2*.

chromatograms (*Figure 12.7*). An increase in absorbance during loading showed some breakthrough at 50 g/l loads, but the amount of protein lost due to breakthrough was minimal (1.8% of the product loaded on the column) and had no significant effect on the recovery of product in the elution pool. Although the elution peak showed some broadening with increasing loads (initially on the tailing edge, then on the leading edge), the broadening of the elution peak does not effect the performance of the chromatography step since the defined pooling criteria (five column volumes) accommodates the largest load. As the overall mass of the load increased, the components which elute in the regeneration and sanitization peaks increased as well.

The antibody yield in the elution pool was independent of the mass of antibody loaded on the column and was approximately 85% of the protein loaded. This consistent recovery of product in the pre-set pool volume results in a concentration of antibody in the pool proportional to the antibody loaded.

The antibody quality in the pool was not affected by the amount of antibody loaded, as determined by size exclusion chromatography and DNA analysis. In addition, the relative SDS-PAGE purity of these pool samples was equivalent to the antibody produced at manufacturing scale, and a significant reduction of the highest molecular weight band seen in the non-reduced load sample was observed in each pool sample. The later finding is consistent with the SEC data of the load and pool samples. Monomeric antibody was measured at 95.4% in the load and 99.3% in the pool samples. This improvement in product quality is a result of aggregate protein being retained on the cation exchange resin during elution and is unaffected by load amount. Furthermore, the measured reduction of DNA from the load to the pool samples was a minimum of 2 logs regardless of the antibody applied to the cation exchange resin.

At the flow rate range of 50 to 200 cm/h, there was no difference in the cation exchange chromatography of antibody. There was no peak broadening either on the leading or tailing edge of the elution profile due to change in flow rate. The yield,

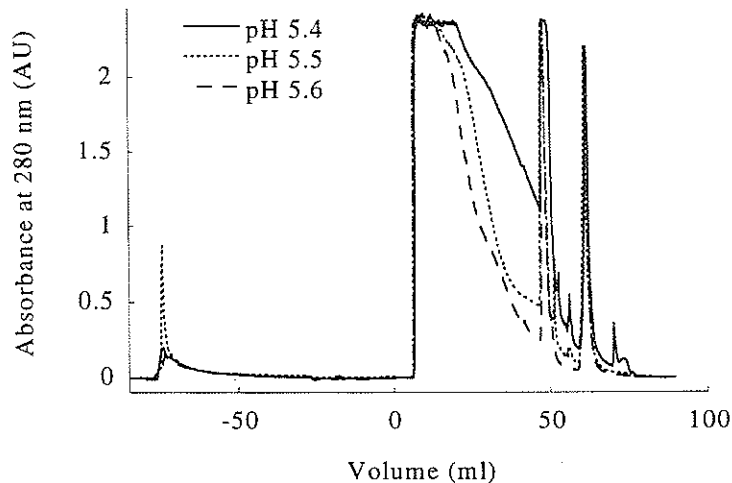


Figure 12.8. Overlaid chromatograms from the cation exchange pH validation study. The x-axis is volume from the start of elution. Except for the pH of the buffers, conditions are the same as in *Figure 12.2*.

SDS-PAGE, SEC, and DNA were consistent for all flow rates. The yield of antibody in the pools ranged from 87% to 89%. The reduced and non-reduced SDS-PAGE gel for pool samples demonstrated equivalence to the cation exchange pools produced at manufacturing scale. Finally, the DNA reduction from the load to the pool of the cation exchange chromatography was a minimum of 2.3 logs regardless of the flow rate applied to the system.

At the pH range of 5.4 to 5.6, the cation exchange chromatography and the antibody quality was not significantly affected. While buffer pH change within this range does have an effect on the elution profile (*Figure 12.8*), the recovery of product and the antibody quality in the eluate pool was acceptable. Although increasing buffer pH leads to an elution profile which approaches baseline at a faster rate, the product recovery yield of antibody in the cation exchange pool is at least 82.6%. In addition, the amount of monomeric antibody as determined by SEC is at least 98.5%. Finally, the DNA is reduced by a minimum log factor of 2.04 regardless of the buffer pH used to perform cation exchange chromatography of antibody.

With conductivity changes of ± 5 mM NaCl in each buffer used for cation exchange chromatography, the chromatography and the antibody quality was not significantly affected. With these changes in buffer conductivity, a minimal shift in the elution profile was noted. However, the resulting pool is acceptable as measured by product recovery yield, SDS-PAGE, SEC, and DNA analysis. The yield was consistent among runs, ranging from 87.1% to 89.9%. The SDS-PAGE and the SEC indicate product equivalent to the reference product. The amount of monomer is at least 99.0% in the pools produced by change of conductivity. Finally, the amount of DNA in these pools is more than 2.5 log less than the amount of DNA present in conditioned protein A pool and independent of the buffer conductivity.

The findings of this study demonstrated that this chromatography can withstand the extremes of each parameter tested to produce pools of acceptable antibody yield and purity.

Table 12.5. Results from the cation exchange chromatography re-use study

Cycle	Yield (%)	CHOP (ppm)	DNA (ppm)	Insulin (ppm)	Protein A (ppm)	Aggregate (%)
1	88.7	75	<0.006	<4.61	<3.9	0.67
11	90.4	90	0.060	<3.9	0.68	
21	90.0	85	0.012	<4.56	<3.9	0.44
31	90.6	63	0.058	<3.9	0.44	
41	95.9	35	0.015	<4.40	<3.9	0.33
50	88.3	34	0.011	<4.74	<3.9	0.30
Average	90.6	64	0.48			
Standard deviation	2.4	22	0.15			

RE-USE VALIDATION

To demonstrate that the quality of the product purified by the cation exchange chromatography step is not affected by multiple re-use of the column, a laboratory scale study was performed. A 1.6 cm inner diameter × 20 cm length column (column volume = 38 ml) was packed with cation exchange media. 50 cycles were performed on the laboratory scale column using the same operational parameters and setpoints/ranges as used in manufacturing. Because the column used at manufacturing scale is often repacked between manufacturing campaigns, the laboratory scale column was periodically repacked. The column was placed in storage buffer after each cycle. During re-use, to simulate varying process conditions encountered in production, the load of the column was changed each cycle, varying between 10–50 g/l.

Six representative pools over the course of the study were analysed for total protein and purity (SDS-PAGE, aggregate, protein A, DNA, and host cell proteins), and four representative pools were analysed for insulin (*Table 12.5*).

Yield varied from 88.7% to 95.9%, with no significant change from cycle 1 (88.7%) to cycle 50 (88.3%). DNA varied from <0.006 ppm to 0.06 ppm, with no significant change from cycle 1 (<0.006 ppm) to cycle 50 (0.011 ppm). Host cell proteins varied from 34 ppm to 90 ppm, with no significant change from cycle 1 (75 ppm) to cycle 50 (34 ppm). The level of protein A in the pools was less than detectable (<3.9 ppm). The levels of insulin were less than detectable. Aggregate varied from 0.30% to 0.67%, with no significant change from cycle 1 (0.67%) to cycle 50 (0.30%). None of the observed fluctuations are significant enough to impact the product quality or the downstream process.

The SDS-PAGE gels, both reduced and non-reduced, are shown in *Figures 12.9A* and *12.9B*. No significant new bands or change in the profile of protein distribution appeared over the course of 50 cycles, and bands on the pool samples were of comparable density to the reference material. In addition, the chromatograms were consistent with respect to absorbance, pH, and conductivity.

Yield, host cell proteins, aggregate, and DNA did not change significantly through the cycles. Insulin and protein A were reduced to non-detectable levels in all cycles assayed. The results of this study demonstrated that the cation exchange column performs consistently throughout 50 cycles. Product quality, as measured by yield, host cell proteins, DNA, insulin, and protein A, remained consistent throughout 50 cycles. Column performance, as measured by comparing chromatograms and by column integrity, remained consistent throughout 50 cycles.

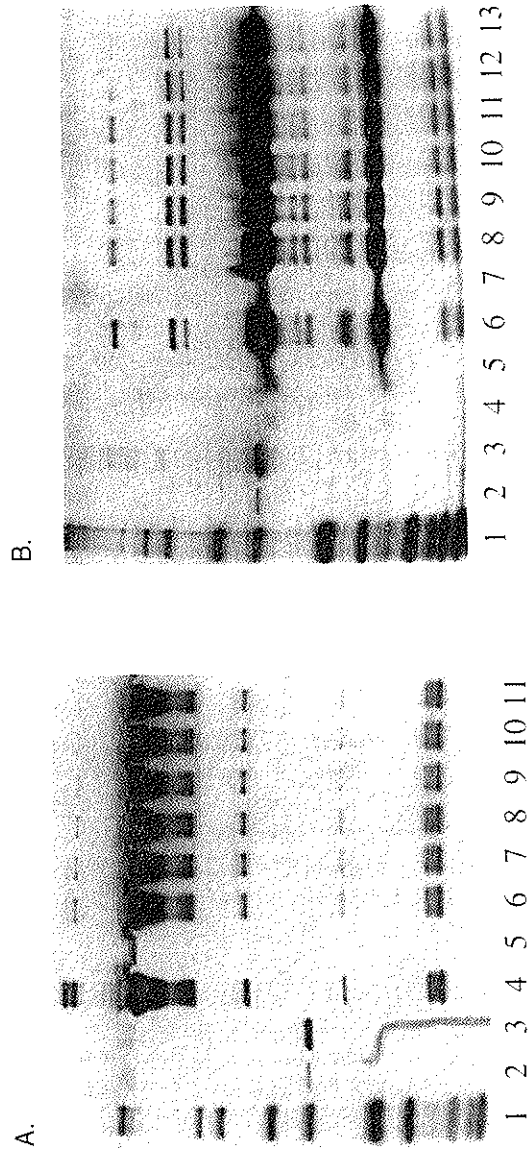


Figure 12.9. (A) Non-reduced and (B) Reduced SDS-PAGE of elution pools from the cation exchange chromatography re-use validation study. (A). Lane 1: Molecular weight standards (200, 116, 97, 66, 55, 36, 31 kD), 2: 2 ng BSA, 3: 50 ng BSA, 4: antibody standard, 5: buffer blank, 6: pool from cycle 1, 7: cycle 11, 8: cycle 21, 9: cycle 31, 10: cycle 41, 11: cycle 50. (B). Lane 1: Molecular weight standards, 2: 2 ng BSA, 3: 50 ng BSA, 4: buffer blank, 5: buffer blank, 6: antibody standard, 7: buffer blank, 8: pool from cycle 1, 9: cycle 11, 10: cycle 21, 11: cycle 31, 12: cycle 41, 13: cycle 50.

Anion exchange chromatography

DEVELOPMENT AND OPERATION

The isoelectric point of many antibodies is high (often >8 and sometimes >9), so anion exchange chromatography run in flow-through mode offers a high-yield method for final purification of antibodies with a high pI. For antibodies with a pI lower than about 8, the anion exchange chromatography step may be run in bind-and-elute mode, which may offer advantages over the flow-through mode, such as additional clearance of leached protein A.

For anion exchange chromatography run in flow-through mode, the pH of the load material is raised to about 0.5–1 pH unit below the pI of the antibody, the conductivity of the load material is adjusted to <7.5 mS/cm, and the antibody is then pumped through the column. Because the pH of the load is less than the pI of the antibody, the antibody will flow through. Since the pI of most host cell proteins is less than the pH of the load, most host cell proteins will bind to the column. Under these conditions, DNA and endotoxin will also bind strongly to the column. The purified antibody is collected after it flows through the column, and the impurities are removed from the column during the regeneration phase.

Development of the flow-through step is straightforward. The antibody is run through the column at various levels of pH and conductivity. In general, at any value of pH, decreasing the conductivity will increase the clearance of host cell proteins. Since the conductivity of the cation exchange pool may be relatively high (>12 mS/cm), the conductivity of the load onto the anion exchange column is controlled by dilution of the cation exchange pool with water. After dilution, the pH is adjusted to the appropriate value.

Since the throughput of the anion exchange column will be determined by the volume of the load, it is best to have the dilution as small as possible. Running the column pH just below antibody binding will allow the least dilution (highest conductivity), so the column is typically run at a pH that is 0.5–1 pH units below the antibody pI. When the pH is only about 0.2 units below the pI of the antibody, decreasing the conductivity below a critical value will allow the antibody to bind, resulting in yield loss. For our antibodies, we typically need a 2× dilution to reduce the conductivity to <7.5 mS/cm and the step is run at pH 8.

Like the cation exchange column, contaminants are removed from the column by increasing the conductivity with a regeneration buffer wash. For development purposes, this wash may be 1 M NaCl (or some other appropriate salt) so that the regeneration fraction may be assayed for host cell proteins, DNA, and antibody to ensure mass balance. Later, in production, this high-salt wash is generally replaced with sanitization buffer such as 1 M NaOH. In addition to eluting proteins due to the high ionic strength, it also degrades proteins and other molecules and strips them from the column.

In production, the column is sized for throughput. Because only a small amount of host cell proteins, DNA, and endotoxin bind to the column, the column dimensions could in principle be very small. However, with most commonly used anion exchange resins, the flow rate is limited. For example, when packed into a process-scale column the flow rate limit on Q Sepharose Fast Flow is about 200 cm/h. With this low flow

rate, using a column sized for binding capacity of the impurities would dramatically increase the run time. For this reason, anion exchange columns are typically about the same size as the cation exchange (about 100–200 l), and are loaded with about 100 g/l antibody.

PARAMETER VALIDATION

This study examined the effects in purification performance induced by changes in the process parameters used in the antibody anion exchange chromatography step. We examined the effect of total amount of antibody to be loaded, flow rate, buffer pH, and buffer conductivity.

A laboratory scale anion exchange column (0.66 cm diameter, 19 cm bed height and a column volume of 6.5 ml) was prepared. The column was sanitized with 0.5 N NaOH (2 CV) and stored in 0.1 N NaOH (3 CV).

Separate samples were conditioned (as performed in manufacturing) and loaded to study each parameter change. The conductivity of the load was adjusted during the conditioning by increasing or decreasing the dilution of the load with purified water. The conductivity of the load was matched to the conductivity of the elution/wash buffer and the conductivity of the elution/wash buffer was altered by changing the salt concentration in the buffer. The pH of the load and buffers was adjusted by titration with 1.5 M Tris.

A control chromatography was performed with an antibody load of 100 g/l and at a flow rate of 76 cm/h as defined for manufacturing scale. Each of the remaining chromatography experiments was performed with one variable changed from the control conditions. The results from this study are compiled in *Table 12.6*.

Analysis of the recovery yields for the different antibody loads showed no significant differences. The recovery yields for the anion exchange step were comparable and range between 95 and 100%. SDS-PAGE analysis of the anion exchange pools from the different loads showed no additional bands when the load of antibody increased from 50 g/l to 125 g/l. These results show that the capacity of the anion exchange column to remove contaminants is unchanged even at the highest load of 125 g/l. The shape of the chromatograms during the different load experiments show no unexpected discrepancies, and the level of host cell proteins was <4 ppm.

Table 12.6. Results from the anion exchange chromatography parameter validation study

Load (g/l)	Flow rate (cm/h)	Buffer pH	Buffer adjustment	Yield (%)	Host cell proteins (ng/mg)	DNA (pg/mg)
125	–	–	–	96.6	<4	<9
100*	76	8.0	(50 mM)	97.6	<4	<9
75	–	–	–	101.1	<4	<9
50	–	–	–	101.4	<4	<9
–	–	8.5	–	101.5	<4	<9
–	–	7.5	–	101.2	<4	<9
–	–	–	+ 50 mM	98.9	<4	<9
–	–	–	– 25 mM	99.2	<4	<9
–	140	–	–	100.0	<4	<9
–	40	–	–	99.1	<4	<9

* indicates the control run

The performance of the anion exchange step was evaluated at two flow rates (40 cm/h and 140 cm/h). The product recovery yields at the low and high flow rate showed no significant changes when compared to the standard anion exchange chromatography step yield. SDS-PAGE analysis of the anion exchange pools from the different flow rate experiments showed no additional bands when compared to the standard pool, and the level of host cell proteins was consistently <4 ppm. These results showed that the capacity of the anion exchange column to remove protein contaminants is not affected over the flow rate range tested. The shape of the chromatograms from the different flow rate experiments is equivalent with only variations in load volume. No discrepancies were found in the chromatograms.

The performance of the anion exchange step was evaluated at two conductivity values. The load conductivity and the equilibration/elution buffer conductivity were changed to 4.4 mS and 13.32 mS in separate experiments. The product recovery yields at the low and high conductivities showed no significant changes when compared to the control anion exchange chromatography step yield. SDS-PAGE analysis of the anion exchange pools from the different conductivity experiments showed no additional bands when compared to the standard anion exchange pool, and host cell proteins were <4 ppm. These results demonstrated that the capacity of the anion exchange column to remove protein contaminants is not affected over the conductivity range tested. The performance of the anion exchange step was evaluated at two pH ranges. The product recovery yields at the low and high pH showed no significant changes when compared to the control anion exchange chromatography step yield. SDS-PAGE analysis of the anion exchange pools from the different pH experiments showed no additional bands when the pH was changed from 8.5 to 7.5, and the level of host cell proteins was consistently <4 ppm. The results demonstrated that the anion exchange step performs adequately over the range of all the parameters tested.

RE-USE VALIDATION

A 1.6 cm inner diameter × 19 cm length column (column volume = 38 ml) was packed with Q Sepharose chromatography media. After the column was cycled 50 times, the column was unpacked and an aliquot of the used resin was packed into a 0.66 cm inner diameter × 19 cm length column (column volume = 6.5 ml), and a DNA spike challenge was performed on the column.

Several measured values for the re-use study are shown in *Table 12.7*. Yield varied from 101.0% to 109.7%, with no significant change from cycle 1 (107.6%) to cycle

Table 12.7. Results from the anion exchange chromatography re-use study

Cycle	Yield (%)	CHOP (ng/mg)	DNA (ng/mg)
1	107.6	<7	<3
11	106.4	<7	<3
21	108.5	<7	<3
31	109.7	<3	<3
41	101.0	<3	<3
50	106.6	<3	<3
Average	106.6		
Standard deviation	2.8		

50 (106.6%). The level of host cell proteins was <7 ppm for all cycles. DNA in the Q-Sepharose pool was less than detectable for all pools.

In the SDS-PAGE gels, both reduced and non-reduced, no significant new bands appeared over the course of 50 cycles, and bands on the pool samples were of comparable intensity to the reference material. The chromatograms were consistent with respect to absorbance, pH, and conductivity.

The results of this study demonstrate that the anion exchange column performs consistently throughout 50 cycles. Product quality, as measured by yield and host cell proteins, remained consistent throughout 50 cycles.

Conclusions

With the increasing use of monoclonal antibodies as pharmaceuticals, there is a need for robust, reliable, cost-effective processes. As a central part of an overall manufacturing system, the three-step recovery process we described can meet the requirements for purity, throughput, and yield. Integrating the protein A affinity, cation exchange, and anion exchange chromatography steps can provide sufficient clearance of host cell proteins, DNA, endotoxin, virus, small molecules, and aggregate. With correctly sized columns and process equipment, each batch of antibody can be recovered in less than three days. By developing and running each step correctly, an overall process yield of 70% can be achieved. The process is robust, reliable, and can be validated to operate within a range of operating parameters.

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Biotechnological Approaches to Fight Pathogens at Mucosal Sites

CHARLES G. KELLY^{1*}, DONATA MEDAGLINI², JUSTINE S. YOUNSON¹
AND GIANNI POZZI²

¹*Department of Oral Medicine and Pathology, GKT Dental Institute, King's College London at Guy's Hospital, Floor 28 Guy's Tower, London SE1 9RT, U.K. and* ²*Laboratorio di Microbiologia Molecolare e Biotecnologia, Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università degli Studi di Siena, Siena, Italy*

Introduction: pathogens at mucosal sites

Most interactions between host and pathogens occur at the host mucosal surfaces. Many pathogens, such as the human immunodeficiency virus (HIV), gain entry via the mucosa while others, including *Candida*, *Streptococcus mutans* and *Helicobacter pylori*, must become established at the mucosa to cause damage to the host. Strategies aimed at controlling pathogens at mucosal surfaces and infectious diseases in general are summarized in *Figure 13.1*. These include primarily vaccination and the use of antimicrobial chemotherapy, particularly antibiotics, which have both had an enormous impact on infectious disease (Cohen, 2000). Passive immunization has been used less with the advent of vaccines and antibiotics but is of increasing importance for treatment of immunocompromised patients (Hammarström, 1999) whilst the development and use of topical microbicides is regarded as a potentially important means of preventing infection with HIV (Lange *et al.*, 1993). Pathogens at mucosal sites, however, present particular problems for these measures, e.g. antibiotics can be very effective in clearing systemic infections while being unable to affect mucosal carriage of the pathogen. A limited number of vaccines induce protective mucosal responses and vaccines are not yet available for several microorganisms that infect mucosal surfaces. These observations, together with concern over the spread of antibiotic resistance (Hawkey, 1998; Irvin and Bautista, 1999), have stimulated investigation of additional antimicrobial strategies as well as refinement of existing approaches.

*To whom correspondence may be addressed (charles.kelly@kcl.ac.uk)

Abbreviations: CV-N, cyanovirin; ETEC, enteropathogenic *Escherichia coli*; HIV, human immunodeficiency virus; HSV, herpes simplex virus; ICAM-1, intercellular adhesion molecule-1; KT, killer toxin; LNnT, lacto-*N*-noetetraose; Mab, monoclonal antibody; NMR, nuclear magnetic resonance; SA, streptococcal antigen; ScFv, single chain variable region fragment; SIgA, secretory immunoglobulin A; SIV, simian immunodeficiency virus.

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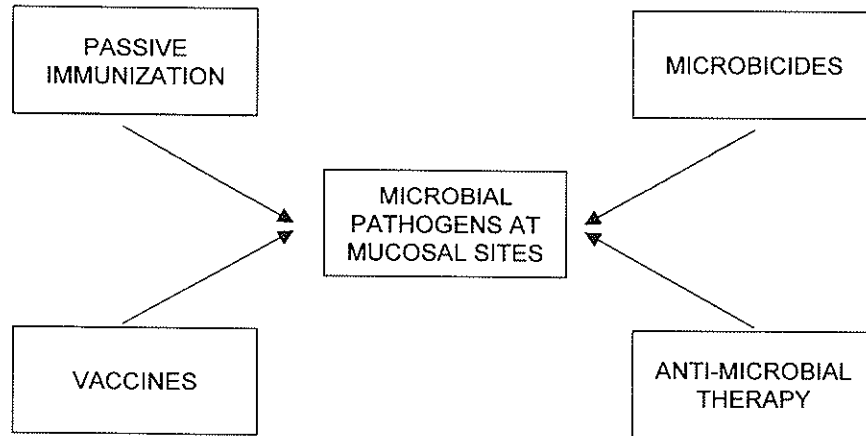


Figure 13.1. Current strategies against pathogens at mucosal sites.

The focus of many studies has been to target early events in infection and, in particular, microbial adhesion. Adhesion or attachment to mucosal surfaces is an essential step in infection or pathogenesis and is mediated by stereospecific interaction between cell surface molecules, termed adhesins, on the surface of the microorganism and receptors on host tissue. Reduced virulence *in vivo* has been demonstrated with specific adhesin-deficient microorganisms (Roberts *et al.*, 1994; Connell *et al.*, 1996) or in animal models where receptors are not expressed (Guruge *et al.*, 1998). Progress in defining the molecular basis of adhesin-receptor interactions has provided the rationale for the design of inhibitors that may selectively block adhesion of pathogens. These inhibitors include both analogues of adhesin molecules and of receptors as well as adhesion-blocking antibodies (Figure 13.2). The effectiveness of passive immunization may be improved by use of monoclonal antibodies that target adhesins of specific pathogens directly. Recent developments include use of transgenic plant technology for the expression of secretory IgA (SIgA) forms of

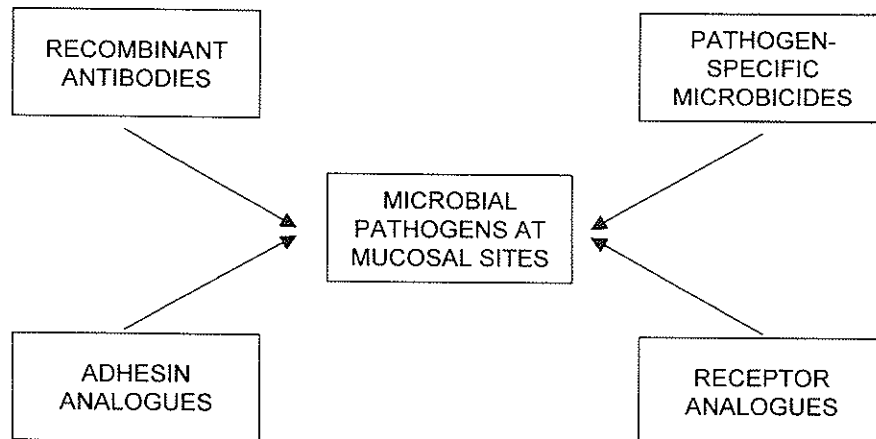


Figure 13.2. Additional targeted strategies aimed at early events in infection.

antibody, as well as combinatorial library technology for the production of novel antibodies. Similarly, targeting of microbicides either by use of improved delivery systems or by development of pathogen-specific antimicrobials has been investigated. In this article we review some of the work that underpins the new approaches to the control of infectious disease.

New strategies, new molecules: a biotechnological approach

ANTI-ADHESIN ANTIBODIES

Passive administration of antibody to mucosal surfaces has been used both prophylactically and therapeutically to prevent infection in infants and immunocompromised patients (Heikkinen *et al.*, 1998; Hammarström, 1999). The pooled immunoglobulin preparations that are most frequently used confer some degree of protection against a wide range of microorganisms but the effectiveness of the treatment is limited by the levels of antibody directed towards specific pathogens. Typically, the treatment requires relatively large and repeated doses of immunoglobulin and protection does not extend beyond the period of treatment (Heikkinen *et al.*, 1998). The use of monoclonal antibodies (Mabs) that target microbial adhesins may provide a means of considerably enhancing the effectiveness of this form of treatment with the potential to selectively remove the pathogen. Moreover, the development of technology for engineering antibodies (Winter, 1998) and for large-scale production of such antibodies has increased the availability of these novel antimicrobial agents.

Passive immunization with anti-adhesin Mabs has been shown to be effective against *Streptococcus mutans*, the main cause of dental caries, not only in animal models but also in human trials. Adhesion of *S. mutans* to the tooth surface is mediated by a streptococcal cell surface adhesin, termed streptococcal antigen I/II (SA I/II), that binds to salivary glycoproteins adsorbed to the tooth surface. Mabs raised against SA I/II were applied directly to the tooth surfaces of non-human primates and two Mabs (Guy's 1 and Guy's 13) were effective in preventing both colonization with *S. mutans* and the development of dental caries (Lehner *et al.*, 1985). Subsequently, trials with Mab Guy's 13 were performed in a human model of infection (Ma *et al.*, 1989). Subjects were treated with chlorhexidine gluconate, a broad spectrum anti-septic, to reduce *S. mutans* (and other plaque bacteria) to undetectable levels and Mab or control solution was then applied directly to the teeth on 6 occasions over a period of three weeks. Re-colonization of the oral cavity with *S. mutans* was then followed by sampling dental plaque and saliva at intervals. In control subjects treated with saline or Mab of irrelevant specificity, re-colonization was evident within 1 to 3 days following chlorhexidine treatment and *S. mutans* returned to the original levels or higher within 2–3 months. In contrast, no re-colonization was evident for at least one year in subjects treated with Mab raised against SA I/II. Protection therefore extends for a considerable period after passive immunization has stopped. The long duration of protection could not be attributed to persistence of antibody since this is no longer detectable 24 hours after application (Ma *et al.*, 1998). A model was proposed in which, following clearance with chlorhexidine, any *S. mutans* is bound specifically by anti-SA I/II Mab and prevented from adhering to the tooth surface. The bacteria

may also be opsonized and phagocytosed by neutrophils in the oral cavity. The ecological niche vacated by *S. mutans* is then occupied by other (non-pathogenic) plaque microorganisms that competitively exclude *S. mutans* and so provide long-term protection. In a further study, F(ab)₂ fragments were shown to be as effective as the intact antibody, indicating that Fc-mediated effector functions of antibody are not required for protection (Ma *et al.*, 1990).

Adhesion-blocking Mab was also shown to be effective in a human re-colonization model of infection with *Porphyromonas gingivalis* considered to be a possible cause of periodontitis (Booth *et al.*, 1996). Patients were treated with metronidazole to suppress *P. gingivalis* and saline (control) or Mab raised against the adhesin component of an arginine-specific protease, was applied subgingivally on four occasions over 10 days. Subjects treated with Mab were significantly protected against re-colonization with *P. gingivalis* for 9 months.

Protection in animal models of infection

Several studies using animal models of infection indicate that passive immunization with Mabs that block early events in infection may be effective against a variety of infectious diseases including those caused by fungal or viral infection. Vulvovaginitis, caused by infection with *Candida* spp. and most frequently by *Candida albicans*, is one of the most common diseases, with approximately 75% of women experiencing at least one episode and about 5% experiencing recurrent attacks that are not resolved by treatment (Fidel and Sobel, 1996). In the treatment with antifungal drugs, however, the emergence of drug-resistant strains (Denning, 1995) and the deficient immune response in immunocompromised patients present particular problems for therapy and have stimulated research into alternative treatments.

Adhesion of *C. albicans* to epithelial cells may involve a variety of adhesins, including the mannan component of yeast mannoprotein (Kanbe and Cutler, 1994) and secreted aspartyl proteinases or 'Saps' (Watts *et al.*, 1998). The latter may modify host surface molecules but are also thought to act directly as adhesins. In a rat model of vaginal candidiasis (De Bernardis *et al.*, 1999), intra-vaginal administration of Mab (IgM isotype) raised against a carbohydrate epitope of the mannoprotein or anti-Sap Mab (IgG1 isotype), thirty minutes before intravaginal challenge with 10⁷ yeast cells of *Candida albicans*, reduced fungal burden. In contrast, two Mabs (both IgG1) recognizing polypeptide epitopes of the mannoprotein conferred no protection (De Bernardis *et al.*, 1997). Most significantly, in the group treated with anti-Sap Mab only 1/5 rats was still infected by day 10 compared with 5/5 in the control group. These findings suggest that the epitope-specificity of Mabs is important for protection and are consistent with a mechanism that involves blocking of adhesion. The importance of antibody specificity was also suggested by separate studies using a murine model of vaginal candidiasis where two IgM Mabs and an IgG3 Mab were protective (Han *et al.*, 1998, 2000). The Mabs were again directed against epitopes within the adhesion-mediating carbohydrate moiety of the mannoprotein. As in the treatment of oral infections, protection in these models of vaginal candidiasis extended well beyond the period of antibody administration. The vagina, like the oral cavity, is a mucosal compartment that is normally colonized by a diverse flora, thus the relatively long duration of protection in this model may also be the result of competitive exclusion by other members of the vaginal flora.

Attachment of herpes simplex virus (HSV) involves initial binding of surface glycoprotein C to heparan sulphate proteoglycans followed by binding of glycoproteins B and D (Fuller and Lee, 1992). Recombinant human Fabs against glycoprotein D were selected from a phage display combinatorial antibody library generated using bone marrow lymphocyte RNA from a long-term asymptomatic HIV-1-positive individual with serum antibodies against HSV-1 and HSV-2 (Burioni *et al.*, 1994). One Fab that possessed *in vitro* virus-neutralizing activity and inhibited plaque formation was engineered in an IgG1 form of the antibody. The IgG1 Mab was compared with Fab and F(ab)₂ fragments in a murine model of vaginal transmission in which antibody or fragments were administered intravaginally immediately before inoculation with HSV-2 by the same route (Sanna *et al.*, 1996). Complete protection was achieved with both intact IgG1 and fragments, indicating that Fc-mediated effector functions are not required for protection in this model.

That anti-adhesin antibodies may also be effective in preventing bacterial infection at a mucosal site that is not normally colonized has been demonstrated in a murine cystitis model. Passive immunization with antiserum raised against the pilus-associated adhesin FimH produced a 100- to 150-fold reduction in the number of uropathogenic *Escherichia coli* recovered from the bladder (two days after challenge) compared with serum from mice immunized with adjuvant alone (Langermann *et al.*, 1997).

Transgenic plant technology in the production of recombinant antibody

Expression in transgenic plants provides a potential means of producing adhesion-blocking antibodies in the quantity that would be required for large-scale use of this form of antimicrobial. Transgenic plants are able to assemble immunoglobulin molecules efficiently (Hiatt *et al.*, 1989; Ma *et al.*, 1994) and, of particular relevance to the production of antibodies for administration at mucosal sites, can assemble SIgA (Ma *et al.*, 1995). SIgA is the main isotype associated with mucosal surfaces and because of increased avidity (due to tetra-avalency) and increased resistance to proteolysis (due to the presence of secretory component), may be more potent than IgG as a mucosal antimicrobial agent.

The effectiveness of both classes of antibody was compared in the human model of infection with *S. mutans* described above. The SIgA form of the protective Mab, Guy's 13, was expressed in transgenic tobacco plants and purified (Ma *et al.*, 1998). In a human trial, the plant-derived antibody proved to be as effective as the parent murine IgG antibody in preventing re-colonization with *S. mutans*. Persistence of the secretory antibody in the oral cavity was increased compared with IgG (three days compared with one day). The increased persistence of SIgA at mucosal surfaces should allow longer intervals between applications and this form of antibody may be particularly appropriate for application at sites that are less accessible than the oral cavity. In this study as in previous trials, no serum antibody responses were detected to the topically applied Mab.

The efficacy of transgenic plant-derived antibodies has also been demonstrated in the murine model of vaginal HSV infection. In this example, a murine Mab raised against HSV glycoprotein B was humanized (Co *et al.*, 1991) and subsequently expressed as an IgG1 antibody in soybean or a myeloma cell line (Zeitlin *et al.*, 1998).

Both forms of antibody were stable in human semen and cervical mucus and both were protective in the murine vaginal model.

ADHESIN-ANALOGUES

Soluble forms of a microbial adhesin or of an adhesin fragment may be used as competitive inhibitors to block adhesion. In several studies, adhesion epitopes have been mapped by determining the inhibitory activity of synthetic peptides or recombinant polypeptide fragments derived from the sequence of microbial adhesins, using *in vitro* adhesion assays. Filamentous haemagglutinin (FHA) and pertactin, both adhesins of *Bordetella pertussis*, bind to integrins by means of RGD sequences and binding *in vitro* is inhibited by peptides comprising the respective RGD-containing sequences (Relman *et al.*, 1990; Leininger *et al.*, 1991). Similarly, pilus-mediated adhesion of *Pseudomonas aeruginosa* is inhibited by a peptide from the semi-conserved C-terminal region of the pilin monomer (Lee *et al.*, 1994) and a recombinant 38-residue peptide from the fibronectin-binding protein of *Streptococcus pyogenes* inhibited bacterial adhesion *in vitro* (Talay *et al.*, 1992).

A synthetic peptide (p1025) corresponding to residues 1025–1044 of SA I/II, which inhibited adhesion *in vitro* of *S. mutans* to salivary receptor (Kelly *et al.*, 1995), was also shown to prevent infection with *S. mutans* in a human clinical trial (Kelly *et al.*, 1999) using the re-colonization model described above. Following treatment with chlorhexidine to eliminate *S. mutans*, p1025 (synthesized as an acetylated peptide amide) was applied directly to the teeth surfaces of subjects on six occasions over three weeks. In addition, a mouthwash containing p1025 was used daily for two weeks. Control subjects received buffer only or a non-inhibitory peptide, p1125 (spanning residues 1125–1144 of SA I/II). Re-colonization with *S. mutans* was then followed by sampling plaque and saliva as before. In control groups, re-colonization was evident by the end of the three weeks of administration of buffer or non-inhibitory peptide. By the end of the experiment (120 days), all members of the group receiving p1125 had re-colonized, as had three of the four receiving buffer. In contrast, none of the subjects treated with p1025 had re-colonized by day 88 and only one of four had re-colonized by the end of the experiment. The effect of administration of p1025 on *Actinomyces naeslundii*, a non-pathogenic member of the oral flora, was also investigated. Both the control group receiving p1125 and the group receiving p1025 had re-colonized by the end of peptide administration (day 21) suggesting that p1025 specifically targeted *S. mutans*. The peptide was not toxic to *S. mutans* and was detectable in the oral cavity for only 6 hours after application, thus the extended protection cannot be explained by continuing action of the peptide. As proposed for the protective Mab, initial inhibition of *S. mutans* adhesion to the tooth surface (by p1025) may be followed by competitive exclusion by other members of the oral flora to provide long-term protection.

That synthetic peptides may be used to prevent infection at other mucosal sites was demonstrated in a murine model of rotavirus infection (Ijaz *et al.*, 1998). Rotavirus is the main cause of viral enteritis. Viral attachment requires proteolytic cleavage of virus protein 4 (VP4) into subunits VP5 and VP8, the latter component being the adhesion domain. A synthetic peptide (residues 232–255) corresponding to the conserved sequence spanning the cleavage site inhibited virus attachment to cells *in*

vitro and was investigated as an antimicrobial *in vivo*. Peptide was administered orally to mice one hour before challenge with virus and mice were then monitored for clinical symptoms. At 72 hours, mice were killed and intestines were removed to determine viral load. Mice receiving the highest dose of peptide (1 mg) were completely protected from rotavirus infection and showed no symptoms compared with buffer-treated controls. The protease cleavage site was not previously identified as an adhesion epitope and it was suggested that inhibition of rotavirus binding may either be the result of competitive inhibition of binding to epithelial cells or of competition with rotavirus VP4 for proteolytic enzymes required for generation of the VP8 subunit. Irrespective of the mechanism, this study demonstrates that effective levels of inhibitory synthetic peptide can be delivered to the intestine.

Synthetic adhesion-blocking peptides: a new type of antimicrobial?

Synthetic peptides corresponding to adhesion epitopes can therefore be used to prevent infection at mucosal sites. These observations provide a basis for the rational design of adhesion-blocking peptides that may be applied to a variety of mucosal infections. The finding that administration of a single peptide confers protection is of interest. For *S. mutans*, at least one other adhesion epitope has been identified within SA I/II (Crowley *et al.*, 1993; Kelly *et al.*, 1999) and genome sequencing suggests there may be at least six further adhesin molecules (<http://www.genome.ou.edu/smutans.html>). Thus, in an environment such as the oral cavity where there may be competition for a limited niche, even partial inhibition of adhesion may be sufficient to prevent establishment of *S. mutans*.

In these 'proof of principle', *in vivo* studies, the effect of varying dose of inhibitor was examined either not at all or only to a limited extent. However, the affinity of a synthetic peptide for the specific host receptor is likely to be considerably lower than that of the intact adhesin and even lower than that of the microorganism which may be effectively polyvalent. There may be exceptions to this. Values for the dissociation constant (K_d) of the pilin adhesion peptide of *P. aeruginosa* were in the μM range and were similar to those of the pilin monomer (Lee *et al.*, 1994). The presence of a disulphide bond may stabilize conformation of this peptide and NMR spectroscopic analyses indicate the presence of a type I and type II β -turn in the solution structure (Campbell *et al.*, 1997). In contrast, NMR spectroscopy indicated that p1025 has no defined solution structure, an observation that may be consistent with much lower affinity of the p1025 interaction with salivary receptor than that of intact SA I/II (Kelly *et al.*, 1999). Delivery and maintenance of a sufficiently high dose of peptide to compete with the pathogen is therefore an important issue if this form of treatment is to be successful. In addition, binding of peptides to host receptors may induce some degree of pathology by the same signalling processes that occur on binding of the pathogen. It may, however, be possible to modify adhesion-blocking peptides so that they bind to receptor but do not induce signalling, as for altered peptide ligands that are used to downregulate T-cell responses (Sloan-Lancaster and Allen, 1996).

RECEPTOR ANALOGUES

The corollary to use of adhesin-analogues is the use of soluble forms of host receptor

molecules as inhibitors of microbial adhesion. Many studies have investigated soluble carbohydrate receptor analogues reflecting the frequency with which these structures are recognized by microbial adhesins. The development of technology for large-scale synthesis of oligosaccharides has contributed significantly to this approach (Zopf and Roth, 1996). Protein receptors have also been identified and treatment with soluble forms of intercellular adhesion molecule 1 (ICAM-1) has provided promising results against rhinovirus infection.

Clinical trial

Infection with rhinoviruses is the most frequent cause of the common cold. The cellular receptor for rhinovirus was identified as ICAM-1 (Greve *et al.*, 1989; Staunton *et al.*, 1989) and soluble ICAM-1 prevented infection of cultured cells with rhinovirus *in vitro* (Marlin *et al.*, 1990). In a chimpanzee model, soluble ICAM-1 was administered intranasally and prevented infection with rhinovirus (Huguenel *et al.*, 1997). Soluble ICAM-1 was then used in a four-centred clinical trial (Turner *et al.*, 1999) where it was applied intranasally to subjects who were given six doses/day (total 4.4 mg/day) at three hour intervals for a total of seven days. Experimental infection with rhinovirus was either 7 hours before the initial application of soluble ICAM-1 or 12 hours after initial application. In either case, there were significant reductions in disease severity and viral shedding compared with placebo controls.

Carbohydrate analogues in animal models of respiratory and gastrointestinal infection

Streptococcus pneumoniae is a major cause of pneumonia, otitis media, meningitis and sepsis. The bacterium can colonize the nasopharynx without causing disease but may subsequently spread and cause disease. Adhesion of *S. pneumoniae* to human cell lines and to primary respiratory epithelial cells *in vitro* was inhibited by sialylated oligosaccharides terminating in NeuAc α 2-3(or -6)Gal β 1 (Barthelsson *et al.*, 1998). Concentrations of oligosaccharide required for 50% inhibition were approximately 2 mM. Much lower concentrations (approximately 500-fold less) were required with inhibitors in polyvalent form, prepared by covalent linkage of the oligosaccharides to human serum albumin (approximately 20 molecules per molecule of protein).

Sialylated forms of lacto-*N*-neotetraose (LNnT, Gal β 1-4 GlcNac β 1-3 Gal β 1-4 Glc) as well as non-sialylated LNnT were subsequently investigated as inhibitors of pneumococcal infection in a rabbit model of pneumonia (Idänpään-Heikkilä *et al.*, 1997). To demonstrate whether the oligosaccharides could prevent adhesion *in vivo*, *S. pneumoniae* was incubated with the sugars (100 μ M) for 15 minutes before intratracheal administration. Co-administration of bacteria with either LNnT or the α 2-6-sialylated derivative (LSTc) significantly reduced the bacterial load in the lungs (determined in bronchoalveolar lavage fluid) compared with saline-treated controls and by 96 hours after challenge, infection was cleared in rabbits receiving LNnT. Significant reductions in lung pathology and in the number of animals with bacteraemia were also evident. To determine therapeutic potential of the inhibitors, a single dose of oligosaccharide was administered intratracheally at 6 hours or 24 hours after pneumococcal challenge. Although less effective than co-administration with

bacteria, treatment with LNT produced a significant reduction in bacterial load or bacteraemia.

A protective effect was also observed if the oligosaccharides were administered 24 hours before pneumococcal challenge. These results cannot be simply explained by inhibition of adhesion since persistence of the oligosaccharides for 96 or even 24 hours is unlikely. The possibility that the oligosaccharides may directly affect cells was investigated. Short-term (5–30 minutes) exposure of a human lung cell line to LNT transiently reduced the binding capacity for *S. pneumoniae*. It was suggested that the protective effect may therefore be due to both adhesion-inhibition and a direct effect on epithelial cells, such that they are made transiently refractory to pneumococcal adhesion. Neoglycoproteins, i.e. oligosaccharides coupled to human serum albumin, were also investigated in this model but, despite their increased molar potency *in vitro*, were no more effective on a molar basis than the monomeric oligosaccharides.

Helicobacter pylori, a human specific gastric pathogen, is the causative agent in chronic active and peptic ulcer disease and a risk factor in the development of gastric adenocarcinoma, one of the most common forms of cancer in humans. In a transgenic mouse model of infection, development of pathology was associated with adhesion to the gastric epithelium (Guruge *et al.*, 1998). Several different adhesins may be involved in adhesion of *H. pylori* to gastric epithelial cells, including HpaA which is specific for sialoglyconjugates (Evans *et al.*, 1993). To investigate the potential of these oligosaccharides to prevent or treat infection, the oligosaccharide 3'-sialyllactose (NeuNAc α 2-3Gal β 1-4Glc), which inhibits *in vitro* adhesion of *H. pylori*, was given to rhesus monkeys that had been persistently colonized with the bacterium for at least 12 months (Mysore *et al.*, 1999). Oligosaccharide mixed with food was given 3 times daily for a period of 94 days. Infection was cleared permanently in 2 out of 6 monkeys and transiently in one further animal. This study therefore showed some effect but the limited degree of protection may reflect a need for other receptor analogues to be used in combination. In particular, the blood-group antigen-binding adhesin (BabA) that binds to Lewis^b antigen may play an important role in adhesion of *H. pylori* to gastric epithelium and expression of the gene in clinical isolates is significantly correlated with disease (Gerhard *et al.*, 1999).

Carbohydrate receptor analogues were also investigated in a model of diarrhoea resulting from infection with enterotoxigenic *E. coli* (ETEC) in calves (Mouricout *et al.*, 1990). A mixture of oligosaccharides prepared from the non-immunoglobulin fraction of bovine plasma inhibited adhesion *in vitro* and was administered orally in water as soon as symptoms were evident following challenge with a lethal dose of ETEC. Treatment was repeated 3–5 times over 2–3 days. Treated calves were protected (11 out of 13) whereas untreated calves were not (0 out of 7) and numbers of adherent ETEC were reduced by 2 orders of magnitude in treated calves.

MICROBICIDES

Conventional microbicides are broad-spectrum agents, such as the spermicide nonoxynol-9 that has a detergent-type mode of action by which it disrupts the membranes of microorganisms, and other surface-active agents, including benzalkonium chloride and bile salts (Herold *et al.*, 1999). Other broad-spectrum antibiotics include sulphated compounds such as dextran sulphate and sodium lauryl sulphate

which block microbial adhesion (Herold *et al.*, 1997; Piret *et al.*, 2000). A major drawback of these broad-spectrum agents is interference with the normal microbial flora which, in the long run, may favour the onset of infections at mucosal sites (Rosenstein *et al.*, 1998; Stafford *et al.*, 1998).

Development of more targeted or pathogen-specific microbicides could overcome some of these difficulties. Two approaches that have been adopted to produce microbicides that may be more specifically targeted are the use of anti-idiotypic antibodies that mimic microbial toxins and screening of natural compounds to identify novel microbicides.

A Mab that neutralizes the activity of a killer toxin (KT) from the yeast *Pichia anomala* has been used in vaccination studies to induce anti-idiotypic antibody that mimics the activity of the original KT (Polonelli *et al.*, 1993). Such KT-like antibodies were considered to have potential as agents that could be applied topically. Using a phage display library from splenic lymphocytes of mice immunized with the KT-neutralizing Mab, an anti-idiotypic scFv, H6, was isolated that possessed candidacidal activity (Magliani *et al.*, 1997). In the rat model of candidal vaginitis, described above, intravaginal administration of the H6 scFv significantly decreased the time required for resolution of infection. Fourteen days after challenge with *C. albicans*, none of the mice receiving H6 (Mab or scFv) were still infected, whereas all mice in the control groups remained infected. H6 scFv was also effective *in vitro* against a multidrug-resistant isolate of *Mycobacterium tuberculosis* (Conti *et al.*, 1998).

Cyanovirin-N, CV-N, a low M_r protein, was identified from screening extracts of the blue-green alga *Nostoc ellipsosporum* for compounds that inactivated diverse strains of HIV-1, HIV-2 and SIV (Boyd *et al.*, 1997). At nanomolar concentrations, CV-N irreversibly inactivates primary isolates as well as laboratory-adapted strains and also inhibits cell-cell fusion and transmission between infected and non-infected cells. CV-N binds with high affinity to HIV gp120, although this does not prevent binding of CD4 nor attachment of intact HIV virus to target T-cell lines (Mariner *et al.*, 1998). The virucidal activity may be due to inhibition of post-attachment fusion events. Treatment of CV-N with denaturants or high temperature (boiling for 15 minutes) did not result in significant loss of anti-HIV activity. Structural analyses provide a rationale for this remarkable stability which augurs well for use of CV-N *in vivo*. The primary sequence of CV-N, 101 amino acid residues, comprises two internal repeats of 50 and 51 residues that are well conserved (Gustafson *et al.*, 1997) and the solution structure determined by NMR is that of a largely β -sheet protein comprising two symmetrically related domains formed by strand exchange between the sequence repeats (Bewley *et al.*, 1998). Each domain is further stabilized by a disulphide bond.

That these novel microbicides are proteins has also allowed development of a novel means of delivery to the mucosa as discussed below.

Local delivery systems

Delivery of the adhesion-blocking antimicrobials to mucosal surfaces and maintenance of sufficiently high concentrations of antimicrobial at mucosal surfaces has generally been achieved by repeated doses with relatively large quantities of inhibitor. Development of delivery systems that allow prolonged local release of the

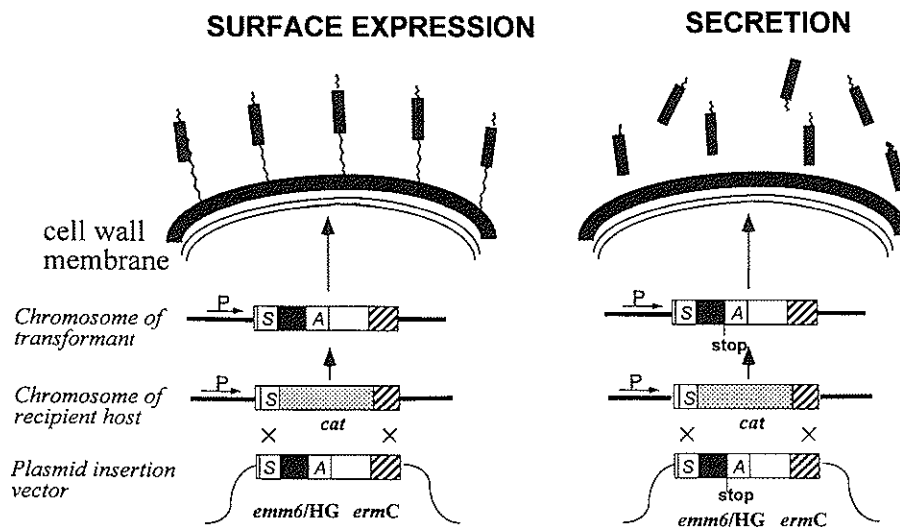


Figure 13.3. Schematic representation of the strategy used for surface expression or secretion of heterologous antigens in *Streptococcus gordonii*. The recombinant insertion vector contains a resistance marker (*ermC*), and an *emm6*-based gene fusion (*emm6*-HG). The regions of *emm6* encoding for the signal sequence (S) and for the C-terminal anchor (A) of the M6 protein are indicated. In the chromosome of the recipient, two regions of homology with the insertion vector are flanking a different resistance marker (*cat*). The DNA of the insertion vector is linearized during the transformation process, and recombination occurs at both the homologous segments. In the chromosome of the transformants, the DNA fragment containing both *emm6*-HG and *ermC* is integrated into the chromosome, *cat* is deleted, and *emm6*-HG is downstream a strong resident promoter (P). When the region encoding the anchor is part of the fusion, the recombinant protein is expressed on the bacterial surface (left panel) while, when a stop codon (stop) is inserted after the heterologous gene, the anchor is not part of the fusion and the recombinant protein is secreted in the supernatant (right panel).

antimicrobial molecule in a bioactive form at mucosal surfaces could reduce the number of repeat doses and might allow more effective targeting to less accessible mucosal sites within the gastrointestinal tract. One strategy that has been exploited successfully is the use of non-pathogenic commensal bacteria capable of colonizing mucosal surfaces as live vectors for the delivery of microbicidal molecules (Beninati *et al.*, 2000; Oggioni *et al.*, 2000; Pozzi *et al.*, 2000).

THE *STREPTOCOCCUS GORDONII* HOST-VECTOR SYSTEM

S. gordonii is a human commensal which was developed as a model system for expression of heterologous proteins and mucosal delivery of heterologous antigens for vaccine purposes (Medaglini *et al.*, 1997a; Pozzi *et al.*, 1997). *S. gordonii* is naturally competent for transformation and therefore easily amenable to genetic manipulation (Pozzi *et al.*, 1990). In this system, the heterologous DNA encoding the recombinant protein is integrated into the bacterial chromosome downstream from strong resident promoters, yielding stable genetic constructs and avoiding the problems connected with the cloning in *E. coli* of promoters from Gram-positive bacteria. To deliver heterologous antigens to the bacterial surface, the M6 protein, a fibrillar surface protein of *Streptococcus pyogenes*, was chosen as the vector

molecule due to its conserved signal and anchoring sequences (Fischetti, 1989). The signal sequence allows export of the M6-based fusion proteins, whereas the presence of the anchor determines the cell-surface display of the recombinant proteins. When the anchor is not part of the fusion, the recombinant proteins are not attached to the cell surface but instead are secreted in the supernatant (Medaglini *et al.*, 1993). The system devised for heterologous gene expression in *S. gordonii* uses a two step procedure (Oggioni and Pozzi, 1996; Oggioni *et al.*, 1999a) (Figure 13.3): (i) construction of translational fusions of the heterologous gene to the M6 protein in *E. coli* plasmid vectors, and (ii) transformation of the recombinant vectors into specially engineered recipient strains of *S. gordonii* where regions of homology allow for integration into the chromosome downstream of a resident promoter. Using this system, a wide variety of antigens, ranging in size from 15 to over 400 amino acids, were expressed in *S. gordonii* and were efficiently recognized by monoclonal and polyclonal antibodies as well as human T-cell clones (Pozzi *et al.*, 1997). Furthermore, the recombinant antigens expressed on the bacterial surfaces were also immunogenic in different animal models following both local or systemic immunizations (Oggioni *et al.*, 1995, 1999b; Medaglini *et al.*, 1997b, 2000; Di Fabio *et al.*, 1998; Ricci *et al.*, 2000).

Recently, the system was extended to allow the expression of two different fusion molecules (fusion-proteins) on the surface of *S. gordonii*. The integration of the two different gene-fusions downstream of two different chromosomal promoters ensures stable expression of the two fusion proteins. Using this strategy, it could be possible to express on the same bacterium two different microbicidal molecules with potentially a stronger chance of success in fighting the infectious agent.

MUCOSAL COLONIZATION AND *IN VIVO* EXPRESSION OF HETEROLOGOUS PROTEINS BY RECOMBINANT BACTERIA

Although *S. gordonii* is a commensal bacterium commonly present in the human oral cavity, it was also shown to stably colonize the oral mucosa of mice (Medaglini *et al.*, 1995; Oggioni *et al.*, 1995), the vaginal mucosa of both mice (Medaglini *et al.*, 1997b, 1998) and rats (Beninati *et al.*, 2000) and to persist in the vagina of cynomolgous monkeys (Di Fabio *et al.*, 1998). *S. gordonii*, after a single inoculum, consistently colonize the vaginal or oral mucosa for 8 and 10 weeks respectively, with recombinant and wild-type strains being equally effective in colonization (Medaglini *et al.*, 1995, 1997b, 1998; Oggioni *et al.*, 1995). Importantly, the recombinant strains stably express the heterologous antigen *in vivo* during all the period of colonization (Medaglini *et al.*, 1997b, 1998), a crucial issue when constructing genetically engineered bacteria to be used as delivery system. Colonization, therefore, allows prolonged exposure to the bacterial-produced molecule after a single administration and is essential for stimulation of the host immune system since killed recombinant bacteria were not effective in inducing an immune response (Medaglini *et al.*, 1995, 1997b). Use of colonizing commensal bacteria for the delivery of microbicidal molecules may, therefore, offer the strong advantage of prolonged exposure to the microbicide with a single administration.

EXPRESSION AND DELIVERY OF MICROBICIDES BY RECOMBINANT *S. GORDONII*

In order to exploit the possibility of a prolonged local delivery of microbicidal antibodies by colonizing commensal bacteria, *S. gordonii* was engineered to secrete or display on the surface the H6 anti-idiotypic single-chain antibody mimicking a yeast killer toxin, described above (Beninati *et al.*, 2000). Intact bacterial cells expressing the cell surface H6 or the culture supernatant of the H6-secreting bacteria were shown to exert a strong dose-dependent candidacidal activity *in vitro*. *In vivo* candidacidal activity of the two H6-producing strains was assayed in the model of oestrogen-dependent rat vaginal candidiasis (De Bernardis *et al.*, 1999). Colonization of the rat vagina with recombinant *S. gordonii* expressing H6 cleared *Candida* infection in 75% of animals treated with the H6-secreting strain and 37% of animals treated with the strain expressing H6 on the surface while 100% of animals treated with control strain were still infected at day 21 (Beninati *et al.*, 2000). The observed candidacidal effect was comparable with that of the antimycotic drug fluconazole. *In vivo* production by human commensal bacteria of bioactive antimicrobial molecules may therefore circumvent the problem of short half-life of polypeptide-based microbicides.

To further investigate the feasibility of using recombinant commensal bacteria for vaginal delivery of microbicides, the potent HIV-inactivating protein CV-N was expressed in *S. gordonii* (Pozzi *et al.*, 2000). Recombinant *S. gordonii* strains expressing CV-N on the surface or secreting it in the supernatant were constructed. The *in vitro* HIV-inactivating capability and the *in vivo* microbicidal activity in animal models of CV-N-producing commensals are currently under investigation.

Perspective

Human clinical trials have demonstrated that administration at mucosal surfaces of adhesin analogues, receptor analogues or antibodies, directed against microbial adhesins, can prevent or reduce severity of infectious disease. Furthermore, several studies using animal models of infection indicate that these approaches may be applied to a variety of infections at mucosal surfaces. As such, these approaches may provide a useful alternative to more established therapies or preventive measures. Targeting of treatment such that pathogens are selectively removed or prevented from colonizing a mucosal surface has the advantage over more broad-spectrum antimicrobial approaches that the remaining normal non-pathogenic microorganisms may contribute to long-term protection by competitive exclusion. However, the success of passive immunization against uropathogenic *E. coli* suggests that treatment may also be applied to mucosal surfaces that are not normally colonized, although it does not seem likely that the protective effect would be extended significantly beyond the time of administration of the antimicrobial in such cases. We have also argued above that strategies aimed at this initial stage in the process of infection may be less likely to select for resistant strains of microorganism.

A common feature of many of the studies discussed in this review is that targeting of a single microbial adhesin is effective in preventing or reducing infection. This is somewhat unexpected since many bacteria possess several adhesins and suggests that becoming established in a mucosal niche can be a precarious process. Turnover of

mucous and epithelial cells, competition with indigenous flora, peristalsis and/or fluid flow may present major obstacles to pathogens at mucosal sites. Addition of a further obstacle by blocking an important adhesin–receptor interaction may be sufficient to prevent the microorganism from becoming established. Experimentally, these treatments are effective when given prophylactically or shortly after challenge infection. It remains to be seen whether they can be effective therapeutically, although the use of recombinant *S. gordonii* expressing candidacidal antibody suggests that therapeutic treatment may be possible.

A number of potential problems with treatments that target adhesins or host receptors have been identified. Concerns that mucosal administration of receptor analogues will induce auto-reactive antibody have not been substantiated to date. In several of the studies described above, serum and, in some cases, saliva have been analysed for antibodies against the applied antimicrobial. No such antibodies were detected. Of particular interest, intranasal application of soluble ICAM-1 (often regarded as an effective route of mucosal immunization) induced no detectable serum antibodies in any of 90 subjects (Turner *et al.*, 1999). The possibility that adhesin analogues may bind to host receptors and by signalling induce pathology similar to that induced by the pathogen has been discussed above. Conversely, receptor analogues may bind to specific microbial adhesins and induce expression of alternative adhesins since most microorganisms possess multiple adhesins. Although this does not appear to be a major problem in the studies reviewed here, the effectiveness of the treatment may be increased by use of more than one receptor analogue to guard against the possibility. Use of monovalent inhibitors may reduce the likelihood of signalling and, in this context, the finding that monomeric carbohydrate receptor analogues were as effective as polymeric forms in preventing experimental infection with *S. pneumoniae* (Idänpään-Heikkilä *et al.*, 1997) is significant.

For administration of antimicrobials at mucosal surfaces, repeated doses of relatively large amounts are typically used so as to maintain effective levels of the agent. This in turn creates a requirement for large-scale production. Use of transgenic plant technology offers one potential solution to this problem. Moreover, this is currently the most effective means of producing SIgA forms of antibody, so providing a further means of increasing persistence of anti-adhesin antibodies at mucosal surfaces. The use of recombinant *S. gordonii* to deliver ScFv *in vivo* (Beninati *et al.*, 2000) is an important development. Engineering of commensal bacteria to secrete antimicrobials offers a potential solution to the problems of producing large amounts of material as well as delivering and maintaining effective concentrations of antimicrobial at mucosal surfaces.

Mucosally administered adhesion-blocking molecules appear to be generally well-tolerated and effective. Given the rapid progress in this field and the expected enormous increase of potential targets that have been or will be identified as the result of genomic sequencing, several such compounds are likely to emerge as a new class of antimicrobial.

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PART 6

Plant Biotechnology

Genetic Improvement of Iron Content and Stress Adaptation in Plants Using Ferritin Gene

FUMIYUKI GOTO¹*, TOSHIHIRO YOSHIHARA¹, TARO MASUDA¹ AND FUMIO TAKAIWA²

¹*Bio-Science Department, Central Research Institute of Electric Power Industry, 1646 Abiko, Chiba 270-1194, Japan and* ²*Department of Biotechnology, National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305-8602, Japan*

Introduction

Iron deficiency resulting from an inadequate diet is a serious nutritional problem. Anaemia derived from iron deficiency causes a host of illnesses, including abortion, brain damage in infants, increased susceptibility to infection, and chronic exhaustion (Baynes and Bothwell, 1990). An estimated 30% of the world's population suffer from some level of iron deficiency, with the highest prevalence found in the developing countries. On the contrary, iron intake by people in developed countries is adequate, and the prevalence of iron deficiency is decreasing. However, anaemia derived from iron deficiency in Japanese females is a concern, and its incidence has remained constant in recent years. There are two approaches to overcome the iron deficiency: one is supplementation of iron to dairy diets, and another is fortification using biological methods. Although supplements added to food or taken in tablet form are effective in preventing and controlling iron deficiency, such treatments are difficult to implement in developing countries because of the associated high costs and lack of primary health care programmes. The other approach is the fortification using biological methods, and there are two ways. The first way is to increase the iron concentration of the hydroponic culture media or soil. This method is costly and cannot accumulate iron to a desirable part of the plant. The second way is to improve the iron content in crops genetically. This way seems better than the first one. This is

*To whom correspondence may be addressed (gotoh@criepi.denken.or.jp)

Abbreviations: AO, active oxygen; CaMV, cauliflower mosaic virus; EP, extension peptide; IRE, iron responsive element; MA, mugineic acid; MDA, malondialdehyde; NA, nicotianamine; NAAT, nicotianamine aminotransferase; PCR, polymerase chain reaction; QTL, quantitative trait locus; RT-PCR, reverse transcriptase PCR; SAM, S-adenosylmethionine; TP, transit peptide.

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because once a plant variety with high iron content has been produced, people can obtain iron from new crops at a low cost.

We have worked on transgenic plants expressing the ferritin gene with a view to overcome the nutritional problem of iron. The iron storage protein ferritin is common to living organisms, including humans and higher plants, and consists of 24 subunits, which can store up to 4000 iron atoms. Thus, enrichment of plants with ferritin using a transgenic technology could prove of benefit for increasing the iron levels in crops. Since Hyde and co-workers' first report in 1963 (Hyde *et al.*, 1963), plant ferritin has now been identified in many species, such as soybean, French bean, cowbean, pea, maize, alfalfa, cocklebur, and whole or parts of the ferritin cDNAs have been cloned from more than ten plant species in recent years.

On the other hand, the production of stress resistant plants is also an important goal, because growth and yield of plants are always restricted by stress, whatever its cause. Such stresses in plants can be classified into two categories: biotic (pathogen, insect, weed) and abiotic (temperature, light, salinity, drought, flooding, freezing nutrition), although both categories are common in the mechanism that induces damage to inner membranes and DNA by the production of active oxygen (AO) species. Recent developments in gene transfer technology into plants has made it possible to produce transgenic plants containing genes coding anti-stress enzymes such as peroxidase and APX, with the result that these plants have obtained some resistance to stresses. Plant ferritin is thought to have an important function besides iron storage. In a plant cell, free iron is very hazardous because of its ability to generate AO species. Ferritin functions as a scavenger of AO species due to binding to free iron in the cell.

In this review we would like to consider such new ideas for the creation of novel crops expressing the ferritin gene. The structure, role, and cloned genes of plant ferritin will be explained briefly at first, followed by a summary of the iron fortification of crops by ectopic ferritin gene expression. Finally, the possibility of breeding anti-stress plants using the ferritin gene is discussed.

Overview of ferritin

STRUCTURE

Plant ferritin is synthesized as the precursor containing a unique amino acid sequence made up over 70 residues on the N-terminal, followed by the relatively conserved region among other ferritins (*Figure 14.5*). The first part of this region is called the transit peptide (TP), which is responsible for plastid targeting and processed on entry into the plastid. The second part is called the extension peptide (EP), which may be lost in the germination process (Ragland *et al.*, 1990). The TP and EP consist of about 40 and 30 amino acid residues, respectively. The conserved region neighbouring the extension peptide has been shown to have 40–50% sequence identity with mammalian ferritins.

The three-dimensional structure of almost all ferritins is highly conserved, although their primary structures vary among organisms (Harrison and Arosio, 1996). The three-dimensional structure of plant ferritin has not been determined yet, but from the computational analysis and the amino acid sequence alignment with mammalian ferritin, it is suggested that the higher-order structure of the plant ferritin is similar to

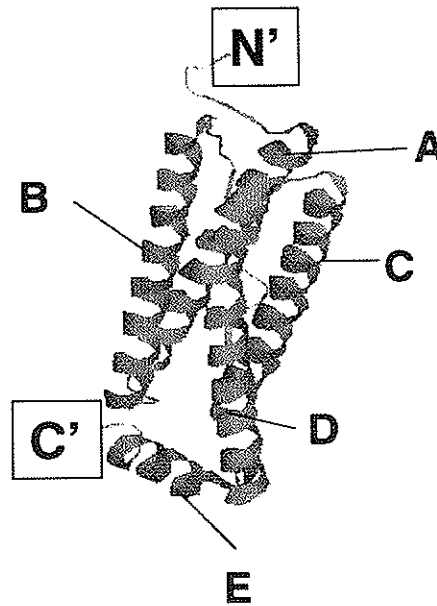


Figure 14.1. Ribbon diagram of human H ferritin (Lawson *et al.*, 1991). A–E indicates helix. N' and C' indicates an N-terminal and a C-terminal, respectively.

the animal one (Lobréaux *et al.*, 1992b). Therefore, for explaining the higher-order structure of the plant ferritin, we review here mainly the structure of mammalian H-(heavy) chain and L-(light) chain ferritins, whose crystal structure has been established (Clegg *et al.*, 1980; Lawson *et al.*, 1991; Trikha *et al.*, 1994, 1995; Hempstead *et al.*, 1997). The H-chain of ferritin possesses ferroxidase activity and is responsible for the uptake of the iron atoms into the cell (Lawson *et al.*, 1989), whilst the L-chain has no ferroxidase activity and is responsible for iron mineralization (Levi *et al.*, 1989). The subunit of ferritin is a four-helix bundle, consisting of four long helices A–D, the fifth short helix E, and a long loop connecting B and C helices (Figure 14.1). The A, B, C, D, and E helices of human H-chain consist of 30, 30, 31, 34, and 12 amino acid residues, respectively. In the case of the plant ferritin, the EP is supposed to form a unique helix called P-helix by computational analysis (Lobréaux *et al.*, 1992b).

Plant ferritins are huge oligomeric proteins, of oligomeric molecular weight estimated to be 540,000 (Laulhère *et al.*, 1988), and assembled from 24 subunits into a spherical shell, i.e. the same as for the other ferritins (Van der Mark *et al.*, 1983b). The external and internal diameters of the ferritin are about 130Å and 80Å, respectively (Trikha *et al.*, 1994). Iron atoms are stored in the inner cavity of the protein shell. In the assembled protein shell, subunits can be related by 4-fold, 3-fold, and 2-fold symmetry (Figure 14.2), and there are channels around the 3-fold and 4-fold axes, individually. The channel around the 3-fold axis is composed of relatively hydrophilic residues and it is supposed that iron atoms are passed through this channel to enter the cavity (Yablonski and Theil, 1992). This channel is thought to be well conserved among plant and mammalian ferritins. The other channels existing around the 4-fold inter-subunit symmetry axes are narrower than the 3-fold channel. The

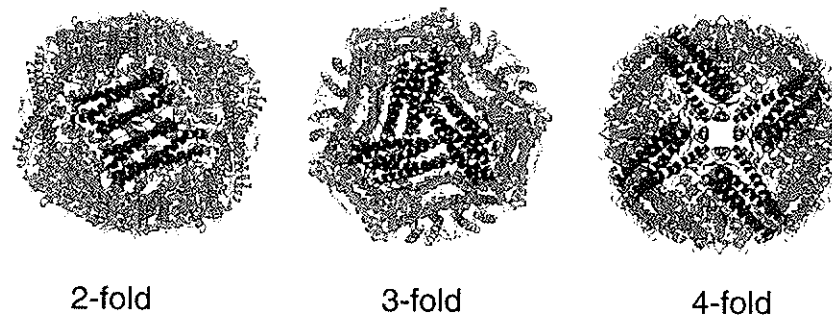


Figure 14.2. Subunit packing in the protein shell of human H ferritin. (a) 2-fold, (b) 3-fold, and (c) 4-fold inter-subunit symmetry axes exist on the assembled protein shell (Lawson *et al.*, 1991).

4-fold channel is also predicted to be hydrophilic (Lobréaux *et al.*, 1992b), although, in the case of human and other mammalian ferritins, side-chains of hydrophobic residues, such as leucine, are positioned around this channel (Hempstead *et al.*, 1997).

In general, the sequence identity of residues forming the inter-subunit interface is remarkably high, e.g. the sequence identity of interface residues is 71%, while the identity of overall sequence of the subunits is 53% between human H-chain and horse L-chain (Hempstead *et al.*, 1997). In the sequence of plant ferritin, the residues forming the inter-subunit interface across the 4-fold symmetry axes are less conserved than that of 2-fold and 3-fold axes. In particular, the apolar residues in the E-helix, which contacts with the neighbouring 4-fold axis-related subunit by hydrophobic interaction, are substituted by polar residues such as histidine in the plant ferritin. In short, there are two unique structural points of plant ferritin, first the existence of the N-terminal extension peptide, second the hydrophilicity of the channel around the 4-fold inter-subunit symmetry axes. However, the elucidation of the crystal structure of plant ferritin is needed for further understanding of iron metabolism in plants.

ROLE

Ferritin is thought to play at least two important roles in plants. The first is storage of iron, an element required by metal enzymes involved in photosynthesis and other respiratory processes. Ferritin can store up to 4000 iron atoms in its central cavity (Korc and Twardowski, 1992) and is usually observed in seeds during maturation or in nodule development in legumes (Kimata and Theil, 1994; Lucas *et al.*, 1998). However, under normal nutritional conditions, ferritin is hardly detectable in vegetative organs. Iron in soil usually exists as a part of water-insoluble compounds and is thus difficult for plants to absorb at rates required for growth. Storage of iron by ferritin alleviates this problem but a small amount of ferritin seems to be enough to store iron for this role. Therefore, the plant ferritin mainly functions as an iron reservoir for early development of juvenile plants and for the formation of legume nodules, relating to nitrogenase and leghaemoglobin (Huang and Barker, 1983; Ko *et al.*, 1987). On the other hand, ferritin is induced by iron overload in soybean, French bean and maize plantlets (Lobréaux *et al.*, 1992a; Proudhon *et al.*, 1989; Van der Mark

et al., 1983a). The second role of ferritin is thus thought to be protection of cells against the toxic effects of iron (or other metals) overload. Fe (II) in the presence of dioxygen can catalyze the production of active oxygen species, known as the Fenton reaction, which accelerates the peroxidation of lipids, inactivation of enzymes, and DNA damage. Ferritin protects the cell from such chemistry derived from free iron (Theil, 1987).

FERRITIN GENES AND THEIR EXPRESSION

In 1990, a part of a plant ferritin gene was first cloned from soybean hypocotyl by Ragland *et al.* using a frog ferritin cDNA as a probe. Her group completed the cloning of the whole cDNA of soybean ferritin from iron-induced cell suspensions (Lescure *et al.*, 1991). Since this work, other ferritin genes of beans have been cloned from French bean (Spence *et al.*, 1991), pea (Lobréaux *et al.*, 1992b), cowpea (Wicks and Entsch, 1993), and *Acacia mangium* (Hoya *et al.*, 1997). More recently, many cDNA sequences of ferritin genes have been registered on the databases, for example, watermelon (Shin, 1999), the common ice plant (Cushman, 1997), sugar beet (de los Reyes *et al.*, 2000) and rice (Uchimiyu, 1993). Buchanan-Wollaston and Ainsworth (1997) have cloned a ferritin gene as a senescence related gene from *Brassica napus*. The gene was expressed in young green leaves, transcript levels falling in mature leaves. However, the expression increased significantly at senescence stage. Hortensteiner *et al.* (2000) reported a putative ferritin gene of *Chlorella protothecoides* during the degreening process. In addition to the genes mentioned above, ferritin genes have been reported in *Medicago truncatula* (Gyorgyey *et al.*, 2000), loblolly pine (Li *et al.*, 1998) and tobacco (Yoshihara *et al.*, 2000). At first, the ferritin gene was thought to be a single copy gene, however recent observations have indicated some evidence indicating the ferritin gene exists as a gene family. For instance, Wicks and Entsch (1993) have indicated that some different ferritin genes exist in cowpea. Recently, they showed four members of the ferritin gene family in cowpea (Wardrop *et al.*, 1999). In *Arabidopsis*, Gaymard *et al.* (1996) have mentioned the existence of some ferritin genes apart from the gene they cloned in comparison with the expressed sequence tags (ESTs). Lobréaux *et al.* (1992a) have isolated two ferritin cDNAs from maize (this was the first clone from monocot plants).

The expression of the animal ferritin gene which has an iron responsive element (IRE) is regulated at the translational (post transcriptional) level in the cells. IRE has a stem loop structure and is located within the 5' untranslated region of ferritin mRNA (Theil, 1990). When the cellular iron level is low, iron regulatory protein binds to the stem loop of the IRE, resulting in inhibition of the translation of ferritin. In contrast, there is no IRE in the plant ferritin genes and the expression of plant ferritin is just controlled at the transcriptional level (Van der Mark *et al.*, 1983a; Proudhon *et al.*, 1989; Lobréaux *et al.*, 1992a). However, Lobréaux *et al.* (1993) have indicated that exogenous plant hormone abscisic acid (ABA) induces ferritin mRNA in leaves of iron-starved maize even though ferritin abundance is much lower in contrast to the situation of iron overloading. The implication of this finding was that a possibility exists for translational control in plant ferritin. In fact, soon after, Loisy *et al.* (1996) showed the translational regulation of plant ferritin gene in a maize mutant in response to iron overloading. The mechanism(s) without IRE to

regulate the expression of plant ferritin is still unclear, however Wei and Theil (2000) have reported that a novel iron regulatory element (FRE) controls iron-mediated depression of the ferritin gene.

Development of iron-rich crops

STRATEGY FOR INCREASING IRON CONTENT IN PLANTS

Two strategies have been considered for increasing the iron content in plants or to avoid the suppression of plant growth derived from iron deficiency. The first is an *agricultural method*. Iron in the soil exists as part of water-insoluble compounds, especially in alkaline soils, and it is difficult to absorb at the rates required for growth of plants. Therefore to solve this problem, spraying iron onto the leaf surface is effective for reducing chlorosis and restoring growth. Inoue *et al.* (1995) have demonstrated that iron-rich vegetables without injury could be guaranteed by controlling the iron concentration in the treatment solution and also the soaking time. However, to increase the iron content of plants without injury is difficult in general.

The second strategy is a *breeding method*. Researchers have selected crops grown in alkaline soils or high iron content crops. Gregorio *et al.* (2000), for example, report the selection of high iron content rice to reduce anaemia derived from iron deficiency and have mapped three QTLs for high iron trait on three chromosomes. Variety breeding biotechnology, including genetic modification of plants, has become a strong tool for breeding iron-fortified crops, and we consider below the target gene(s) to be used for these crops.

Plants can be classified according to iron uptake strategies (Romheld and Marschner, 1986) (*Figure 14.3a,b*). Most plants, except for grasses, use 'strategy I': Under iron-deficient conditions, plants of this type secrete H^+ to acidify the soil using a H^+ -ATPase (Harper *et al.*, 1989, 1990; Campos *et al.*, 1996), reduce Fe(III) to the more soluble Fe(II) form with a Fe(III) chelate reductase bound to the plasma membrane, and take up the Fe(II) form by an Fe(II) transporter. The iron form of translocation via the xylem is known as the *ferric citrate complex*. By contrast, minerals including iron may be transported at least in a chelated form in the phloem. Interestingly, the non-proteinaceous amino acid, nicotianamine (NA) – which is ubiquitous in the plant kingdom – could be a candidate for a mineral chelator in the phloem (Scholz *et al.*, 1992). NA is a precursor of mugineic acid, which is a key compound for iron uptake system in the 'strategy II' plants. The iron taken up from the soil is transported to tissues and excess iron is then isolated in the ferritin as the Fe(III) form. The structure, role and gene of ferritin are explained in detail in the section titled 'overview of ferritin'.

We have at least four target proteins to increase the iron content in plants so far. However, it may be difficult to use a modified gene of the H^+ -ATPase alone because the ability to acidify the rhizosphere depends not only on iron deficiency but to some extent on the ion uptake balance. Yi and Guerinot (1996) have confirmed that iron must be reduced before it can be transported into the cell and have concluded that Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency: this conclusion derives from the analysis of *Arabidopsis* mutants (*frd1*) which did not show induction of Fe(III) chelate reductase activity under iron-deficient conditions.

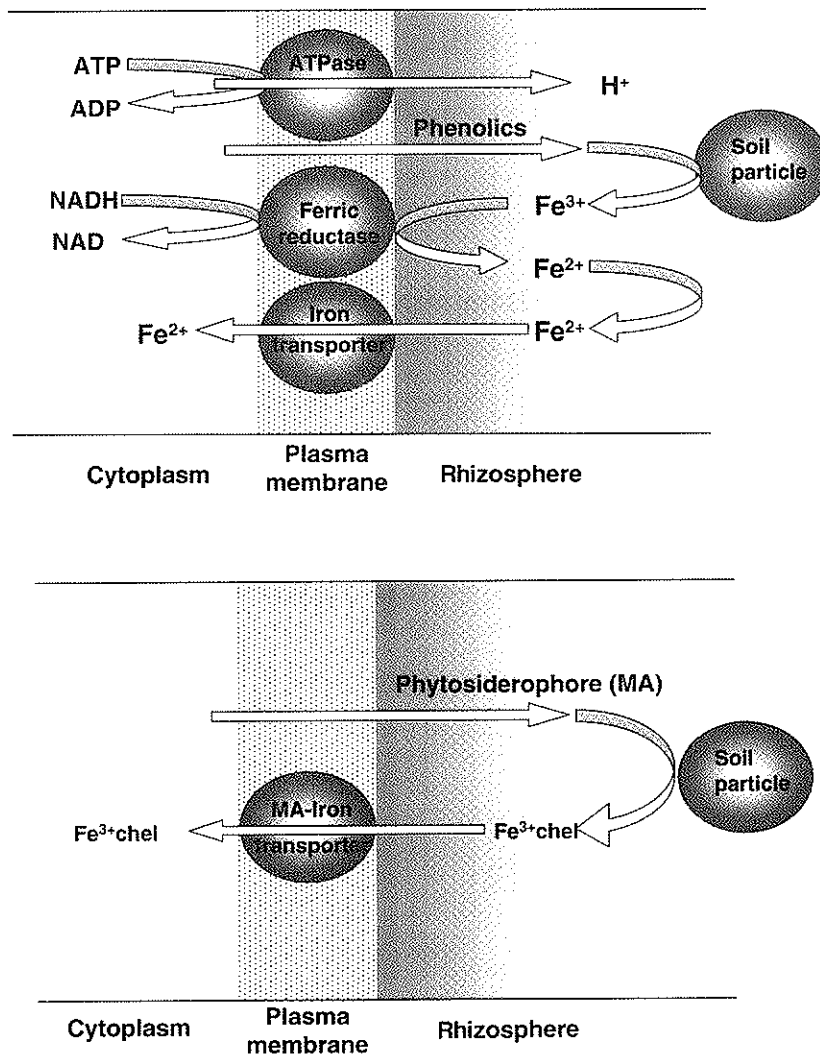


Figure 14.3. Iron absorption strategies of plants. (a) Model for iron acquisition of strategy 1 in higher plants except *Gramineae*. (b) Model for iron acquisition of strategy 2 in *Gramineae*.

In 1999 their group (Robinson *et al.*, 1999) isolated the iron reductase gene (*FRO2*) which was allelic to the *frd1* mutation. Transgenic *frd1* mutant containing *FRO2* showed that *FRO2* complemented the *frd1* mutant phenotype. Namely, the low-iron-inducible ferric chelate reductase activity was recovered by gene transformation. These results indicate that *FRO2* must be one of the useful genes for the iron uptake system in transgenic plants. Eide *et al.* (1996) identified an iron transporter gene, called iron-regulated transporter (*IRT1*), in *Arabidopsis* using functional expression in yeast. The *IRT1* was a member of a gene family, expressed in roots and induced by iron deficiency. Recently, the *IRT1* protein has been understood to be a broad-range metal ion transporter in plants because it mediated uptake of manganese, zinc, cobalt,

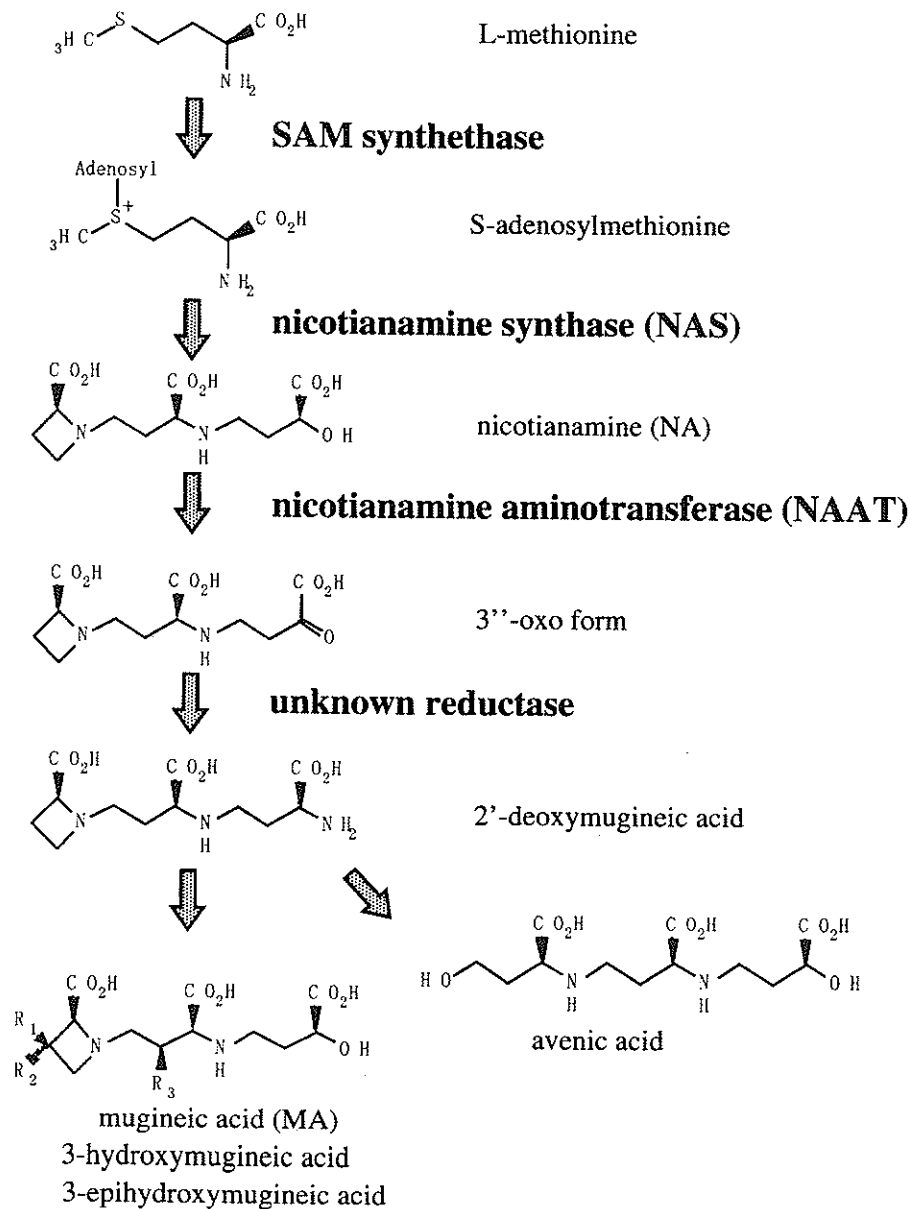


Figure 14.4. Biosynthetic pathway of the MAs of phytosiderophores (Higuchi *et al.*, 1999).

in addition to iron under iron-deficient conditions (Korshunova *et al.*, 1999). If we were to use the *IRT1* for iron fortification of crops, there would be a danger of over-uptake of metals hazardous for human health.

Strategy II plants, including major cereal crops such as rice, wheat, barley and oat, secrete low-molecular-weight compounds called phytosiderophores, e.g. mugineic acid (MA), which binds to Fe(III) (Figure 14.3b). The conjugate of Fe(III)-MA is probably taken up through an as yet unknown transport system. Another probable

system of iron uptake is that the conjugate of Fe(III)-MA splits at the outer face of the plasma membrane and ferric iron could be separately absorbed into cytoplasm. The biosynthetic pathway of the MA family has now been determined and the related genes have been cloned (Figure 14.4). *S*-Adenosylmethionine (SAM) is synthesized from methionine by SAM synthetase. Subsequently, one molecule of NA is formed with three molecule of SAM by NA synthase. NA is then converted to [3'-keto acid] by nicotianamine aminotransferase (NAAT). The MA family is produced from deoxymugineic acid which is formed from [3'-keto acid] by an as yet unknown reductase. The genes of SAM synthetase, NA synthase, NAAT have all been cloned (Mori and Takizawa, 1996; Higuchi *et al.*, 1999; Takahashi *et al.*, 1999). These genes, including the gene for the unknown reductase that catalyzes [3'-keto acid] to deoxymugineic acid, should be effective for adaptation to alkaline soils rather than for breeding of iron-fortified crops.

We have noticed that the ferritin gene offers a great advantage for increasing iron in plants among the candidate genes for the following reasons: (a) Ferritin can markedly store iron atoms in contrast with other iron binding proteins, which bind to few irons. (b) Ferritin is formed from a single subunit. Therefore there is no need to introduce more than one gene for inducing the function of the translated production. (c) Since ferritin exists in both monocot and dicot plants, the ferritin gene is useful for both types of plants. (d) Iron stored in ferritin is bioavailable. Iron form and iron absorption in the human body are important factors whatever strategy we choose for increasing iron content of plants (Schümann *et al.*, 1998). The various dietary iron sources have been examined by Beard *et al.* (1996): the bioavailability of extrinsic ferritin as an iron supplement was almost the same as that of FeSO₄ in rats which had been made anaemic by dietary iron deficiency. (e) Even if the other genes can introduce the uptake of iron into the cell, such iron has to be isolated in order to avoid the generation of oxidative stress mediated by free iron. (f) Transgenic plants

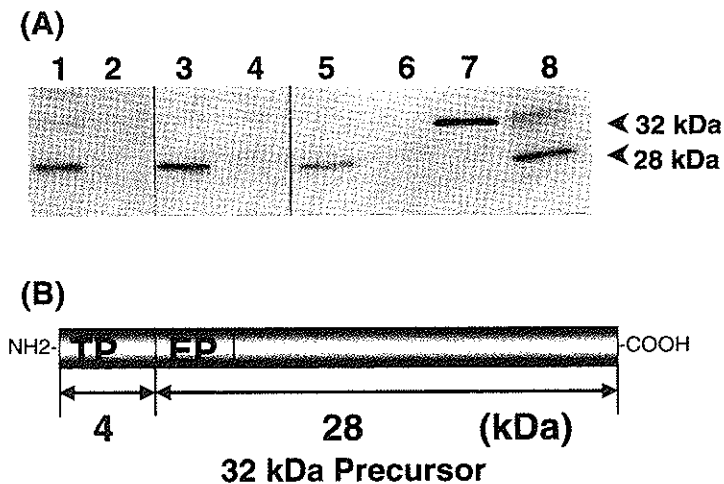


Figure 14.5. (A) Western blot analysis of transformants reproducing the soybean ferritin subunit. Rice (lane 1, 2), Tobacco (lane 3, 4), Lettuce (lane 5, 6), Soybean ferritin subunit produced by *E. coli* (lane 7), Soybean (lane 8). Transgenic plants (lane 1, 3, 5), Control plants (lane 2, 4, 6). (B) Schematic representation of the ferritin subunit. TP: transit peptide, EP: extension peptide.