Evaluation of endometrial biomarkers for semi-invasive diagnosis of endometriosis

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Objective: To test the hypothesis that specific proteins and peptides are expressed differentially in eutopic endometrium of women with and without endometriosis and at specific stages of the disease (minimal, mild, moderate, or severe) during the secretory phase.

Design: Patients with endometriosis were compared with controls.

Setting: University hospital.

Patient(s): A total of 29 patients during the secretory phase were selected for this study on the basis of cycle phase and presence or absence of endometriosis.

Intervention(s): Endometriosis was confirmed laparoscopically and histologically in 19 patients with endometriosis of revised American Society for Reproductive Medicine stages (9 minimal-mild and 10 moderate-severe), and the presence of a normal pelvis was documented by laparoscopy in 10 controls.

Main Outcome Measure(s): Protein expression of endometrium was evaluated with use of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. The differential expression of protein mass peaks was analyzed with use of support vector machine algorithms and logistic regression models.

Result(s): Data preprocessing resulted in differential expression of 73, 30, and 131 mass peaks between controls and patients with endometriosis (all stages), with minimal-mild endometriosis, and with moderate-severe endometriosis, respectively. Endometriosis was diagnosed with high sensitivity (89.5%) and specificity (90%) with use of five down-regulated mass peaks (1.949 kDa, 5.183 kDa, 8.650 kDa, 8.659 kDa, and 13.910 kDa) obtained after support vector machine ranking and logistic regression classification. With use of a similar analysis, minimal-mild endometriosis was diagnosed with four mass peaks (two up-regulated: 35.956 kDa and 90.675 kDa and two down-regulated: 1.924 kDa and 2.504 kDa) with maximal sensitivity (100%) and specificity (100%). The 90.675-kDa and 35.956-kDa mass peaks were identified as T-plastin and annexin V, respectively.

Conclusion(s): Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry analysis of secretory phase endometrium combined with bioinformatics puts forward a prospective panel of potential biomarkers with sensitivity of 100% and specificity of 100% for the diagnosis of minimal to mild endometriosis. (Fertil Steril 2011;95:1338–43. ©2011 by American Society for Reproductive Medicine.)

Key Words: Endometrium, proteomics, SELDI-TOF-MS, secretory phase, endometriosis, support vector machine analysis, and presence or absence of endometriosis.

Endometriosis, defined as the ectopic presence of endometrial-like tissue, is an enigmatic, benign, estrogen-dependent disease, associated with infertility and pain. It is progressive in 40% to 50% of women (1), and recurrence of endometriosis is often observed after surgery and after cessation of medical therapy, especially in women with moderate to severe endometriosis (2). Endometriosis is associated with a high cost (2–4), estimated to be higher than the cost for Crohn’s disease (4).

Early detection of endometriosis is crucial for its timely diagnosis and treatment. Studies report an average delay of 11.7 years in the United States and 8.0 years in the United Kingdom (5) before women get a correct diagnosis after the initial onset of symptoms for endometriosis. However, endometriosis can be diagnosed only via laparoscopy, ideally combined with histologic confirmation. A noninvasive or semi-invasive diagnostic test in easily accessible fluid or tissue (i.e., plasma, serum, urine, saliva, endometrium) would be beneficial to both physicians and patients but does not exist (6–9).

Protein analysis using two-dimensional gel electrophoresis or more advanced technology may represent a promising method for developing noninvasive diagnosis of endometriosis, on the basis of...
previous reports showing differential protein expression in women with endometriosis when compared with controls in peritoneal fluid (10, 11) or in endometrium (12–15). Although two-dimensional electrophoresis has high-resolution capacity, it is labor intensive and requires large quantities of intact proteins. Protein profiling using surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) allows study of the expression of peptides and proteins that are poorly detected by other analytic methods, but precautions should be taken when designing SELDI-TOF-MS experiments to avoid bias in data interpretation (16). Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry was used initially (17) to identify biomarkers for ovarian cancer. Although this first study had some serious flaws (18), the SELDI-TOF-MS technique has been improved over the years and has been used to identify biomarkers for women with mutations of breast cancer 1, breast cancer (19), and early ovarian cancer (20). In our preliminary study, SELDI-TOF-MS protein profiling of eutopic endometrium showed that several proteins and peptides were expressed differentially in women with endometriosis when compared with controls (21), suggesting that proteomic analysis of endometrium may be a promising method for the diagnosis of endometriosis. The aim of this study was to test the hypothesis that specific proteins and peptides are differentially expressed by eutopic endometrium of women with and without endometriosis and at specific stages of the disease (minimal, mild, moderate, or severe) during the secretory phase.

MATERIALS AND METHODS

Patients and Tissue Selection

The biobank of the Leuven University Fertility Center was searched to identify 10 endometrial samples obtained during the secretory phase (day 16–26 of a 28-day menstrual cycle (22) from each of the following three groups: women with a normal pelvis (controls, n = 10), women with minimal to mild endometriosis (stages I–II, n = 9), and women with moderate to severe endometriosis (stages III–IV, n = 10). Endometriosis was staged according to the classification system of the American Society for Reproductive Medicine (23) during laparoscopy and confirmed by histopathology. Endometrial samples from patients with and without endometriosis had been collected retrospectively between April 2003 and July 2005 by endometrial biopsy during hysteroscopy or laparoscopy procedures for subfertility with or without pain (Table 1) and had been frozen at −80°C until use. All patients were white, with similar age among women with endometriosis (mean 31.7 ± 4.2 years, median 30 years, range 27–40 years) and controls (mean 31.5 ± 6.0 years, median 31.5 years, range 23–41 years). All patients had signed a written informed consent before surgery and had agreed on the collection of tissues for research. The study protocol had been approved by the institutional ethical and review board of the University Hospital Gasthuisberg for the protection of human subjects.

Patients with pelvic inflammatory disease, myomas, or urinary tract infection; patients using the oral contraceptive pill; patients taking long-term medication; and patients operated on within 6 months before the time of sample collection were excluded from this study. Only endometrial samples obtained during the secretory phase were selected for this study to rule out cycle-dependent changes in endometrial protein or peptide expression. One control patient was noted to have subacute focal endometritis. A blind screening approach was applied on these samples with use of SELDI-TOF-MS to search for potential biomarkers.

Preparation of Protein Lysate From Endometrial Samples

Homogenization of tissue Frozen endometrial tissue biopsy samples were weighed (100 mg/mL lysis buffer) and immediately thawed in phosphate-buffered saline solution while on ice. Tissues were washed five times in phosphate-buffered saline solution to rinse off any adhering hemoglobin. The tissue homogenization was realized as previously described (21), followed by hemoglobin depletion (Supplemental Materials and Methods).

Statistical analysis: data preprocessing The SELDI-TOF mass spectra were baseline corrected and normalized on the basis of total ion current with use of the Biomarker Wizard Program (Ciphergen, Fremont, CA). The same application was used for peak detection and the determination of P values. For P value calculations, peaks exceeding a peak threshold of 20% of the total ion current and exhibiting a signal-to-noise ratio of at least 3 were selected and analyzed with the nonparametric Mann-Whitney U test. All univariate analyses were carried out with use of ProteinChip Software (v3.1.1; Ciphergen) and the Prism 3 software (GraphPad, San Diego, CA). Results are expressed as mean ± SD, median, range. A differentially expressed mass peak with P value <.05 was considered to be statistically significant. Multivariate analysis was applied to evaluate and identify potential biomarkers with diagnostic value. Feature selection was accomplished through a support vector machine (SVM)–based feature ranking algorithm (24), stepwise logistic regression, and logistic ridge regression (25) to rank the selected mass peaks according to their classification power. The performance of these models was evaluated with use of leave-one-out cross validation (LOO-CV) to avoid overfitting. Subsequently, the highly ranked mass peaks overlapping between these three models (selected via simple stepwise logistic regression, with an odds ratio of at least 2 in logistic ridge regression, and highly ranked by the SVM were selected and their performance was checked by a logistic ridge regression model with 10-fold CV.

RESULTS

Mass Peak Expression in Women With Endometriosis Compared With Controls

After preprocessing the mass spectra with use of the Ciphergen ProteinChip Software, 73 mass peaks were expressed differentially in the secretory endometrium of all women with endometriosis (stages I–IV) compared with all controls. The mass of the differentially expressed peaks ranged between 1.923 kDa and 133.810 kDa. Peaks with a mass-over-charge <1.6 kDa were excluded to avoid interference from matrix ions. To select a set of candidate biomarkers, two models based on logistic regression were built. The first model (LOO–stepwise logistic regression model) selected 14 peaks with a performance of 76% (LOO-CV), and the second model (LOO–logistic ridge regression odds ratio >2) selected 16 peaks

<table>
<thead>
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<th>TABLE 1</th>
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<td><strong>Demographic data of women with and without endometriosis with indications for laparoscopic surgery.</strong></td>
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<tr>
<td><strong>Subjects</strong></td>
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<tr>
<td><strong>Controls</strong></td>
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<td>Endometriosis stage I–II</td>
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<td>Endometriosis stage III–IV</td>
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with a performance of 79%. There were 8 identical mass peaks between these two models including 6 mass peaks that were highly ranked by the SVM algorithm. On the basis of these 6 mass peaks a logistic ridge regression model was built that had a high sensitivity (89.5%) and specificity (90%) (Table 2) corresponding to only three misclassifications. One peak could be removed without harming the diagnostic performance. As a result, 5 down-regulated mass peaks (1.949 kDa, 5.183 kDa, 8.650 kDa, 8.659 kDa, and 13.910 kDa) were selected as potential endometrial biomarkers for the diagnosis of endometriosis (Fig. 1). Subanalysis for early, mid, and late secretory phases for these selected mass peaks showed no statistically significant difference between patients with endometriosis and controls (data not shown).

**Mass Peak Expression in Women With Minimal to Mild Endometriosis Compared With Controls**

Subgroup analysis revealed that 30 significant mass peaks were expressed differentially between secretory endometrium from women with minimal-mild endometriosis and that from the control group. To select a set of candidate biomarkers, again two models were built based on logistic regression. The first model (LOO–stepwise logistic regression model) selected three mass peaks with a LOO-CV performance of 78.9%, and the second logistic regression (LOO–logistic ridge regression with an odds ratio >2) selected four mass peaks with the similar performance of 94.7% (LOO-CV). There was one peak that overlapped between these two models and that was also highly ranked by the SVM algorithm. Additionally, there was some overlap between each logistic regression model separately and the SVM ranking. Therefore, a combined panel of four mass peaks (two up-regulated: 35.956 kDa and 90.675 kDa; two down-regulated: 1.924 kDa and 2.504 kDa) (Fig. 2) were selected as potential endometrial biomarkers for the diagnosis of minimal-mild endometriosis with a maximal sensitivity of 100% and specificity of 100% (Table 2). Subanalysis for early, mid, and late secretory phases for these selected mass peaks showed no statistically significant difference between patients with endometriosis and controls (data not shown).

The highest level of difference in expression of the up-regulated 35.956-kDa and 90.675-kDa proteins was observed at Q10 and immobilized metal affinity capture (IMAC-30-Cu) binding surfaces respectively. The 90.675-kDa and the 35.956-kDa protein bands excised after tryptic in-gel digestion were analyzed with tandem mass spectrometry analysis and use of the matrix-assisted laser desorption/ionization–TOF/TOF instrument and identified as T-plastin protein and annexin V, respectively.

**Mass Peak Expression in Women With Moderate-Severe Endometriosis Compared With Controls**

Subgroup analysis revealed that 131 significant mass peaks were expressed differentially between secretory endometrium from women with moderate-severe endometriosis and that from the control group. To select the set of candidate biomarkers, two models based on logistic regression were built. The first model (LOO–stepwise logistic regression model) selected five peaks, and the second logistic regression (LOO–logistic ridge regression with an odds ratio >2) was excluded because of poor performance. However, there was some overlap between the first logistic regression model and the SVM ranking. This resulted in four mass peaks (one up-regulated: 10.109 kDa and three down-regulated: 4.279 kDa, 5.828 kDa, and 12.172 kDa) (Supplemental Fig. 1) that were selected as potential endometrial biomarkers for the diagnosis of moderate to severe endometriosis with a sensitivity of 80% and specificity of 70% (Table 2).
DISCUSSION

Our data strongly support the concept that endometrial profiling using SELDI-TOF-MS and selecting mass peaks with stepwise logistic regression combined with SVM provides a clinically relevant panel of biomarkers that demonstrate high sensitivity (100%) and specificity (100%) for the diagnosis of minimal to mild endometriosis. Leave-one-out cross validation was applied to rank and select the peaks according to their discriminatory power from women without endometriosis and those with early (minimal-mild) and advanced (moderate-severe) stages of the disease. When compared with our results, other investigators (26) reported a different set of 20 mass peaks with lower sensitivity (87%) and specificity (80%) for the diagnosis of minimal to mild endometriosis. Leave-one-out cross validation was applied to rank and select the peaks according to their discriminatory power from women without endometriosis and those with early (minimal-mild) and advanced (moderate-severe) stages of the disease. When compared with our results, other investigators (26) reported a different set of 20 mass peaks with lower sensitivity (87%) and specificity (80%) for the diagnosis of minimal to mild endometriosis, using another learning algorithm with a combination of three selected mass peaks (mass-to-charge ratio values of 3.957 kDa, 11.711 kDa, and 6.987 kDa). However, their study was fundamentally different in design and methodology: they studied plasma samples (26) instead of endometrium, their study population was not defined clearly with respect to menstrual cycle phase and stages of endometriosis (26), and they used only one type of surface chemistry (26) whereas we used four different types of surface chemistries. The set of mass peaks selected in our study may be a fingerprint associated with endometrial tissue and may be absent in plasma or only present in low concentrations hampering their direct detection.

A possible limitation of our study is that our controls were infertile women including some with pelvic pain (30%), instead of women of proven fertility without pain at the moment of laparoscopic sterilization. However, such a well-defined control group was unavailable in our hospital in sufficient number because of the decline in the number of laparoscopic sterilizations and the fact that a significant proportion of women undergoing laparoscopic sterilization also may have some degree of pelvic pain.

In our study, T-plastin, a cytoplasmic protein regulating actin assembly and cellular motility, was up-regulated in the secretory phase endometrium from women with minimal to mild endometriosis compared with controls. T-plastin protein functions in the formation of
actin bundles that are required for cell locomotion and maintenance of the cellular architecture (27), and studies have shown that cultured fibroblasts overexpressing T-plastin demonstrated increased mobility and altered cellular architecture (28). It has been reported that the surface mesothelium of pelvic peritoneum adjacent to active endometriosis is marked by a sequence of morphologic changes from flat cell to cuboidal or columnar epithelium (29, 30). We hypothesize that T-plastin may play a role in the development of these structural abnormalities. It is not clear whether T-plastin is present in peripheral blood, because we could not find any data in the literature showing the concentration of T-plastin in serum or plasma.

Additionally, annexin V, a calcium phospholipid-binding protein belonging to the annexin family, was also up-regulated in secretory phase endometrium from women with minimal to mild endometriosis when compared with controls. The role of annexin V in the pathogenesis of endometriosis is unclear, but, in cancer studies, annexin V is expressed exclusively in the periphery of invasively growing tumor areas, suggesting that it may play a role in proliferation and/or cell mobility and may have metastatic potential (31). Endometriosis can be considered as benign metastatic disease with characteristics similar to malignancy, including aggressive growth and localized invasion. We hypothesize that annexin V plays a role in the early invasion of endometrial cells into the mesothelium after initial attachment to the peritoneal wall. Annexin V is detectable in plasma, with higher levels in patients with acute myocardial infarction (11.0 ± 4.9 ng/mL) compared with controls (1.9 ± 0.7 ng/mL) (32).

Because of the pure exploratory nature of the present study, the identification of proteins was limited to the two proteins mentioned earlier, because they have a high molecular weight and were up-regulated in the primary group of interest to us, women with minimal to mild endometriosis, when compared with controls. We were unable to identify other potentially relevant mass peaks because of the low amounts of protein lysates that were available in the samples.

Although our preliminary data are interesting, it can be questioned how statistical significance can be translated into clinical usefulness. Therefore, our next step is to plan a validation study in a larger number of patients with and without endometriosis to confirm our findings and directly identify the up- and down-regulated proteins and peptides. Obviously, an endometrial biopsy is still a semi-invasive procedure, and we also plan to use proteomics technology to develop a less-invasive test based on blood, urine, or saliva that would be better applicable for adolescents with pelvic pain or in women at risk for endometriosis.

In conclusion, SELDI-TOF proteomics analysis revealed a prospective panel of potential endometrial biomarkers including T-plastin and annexin V with a sensitivity of 100% and specificity of 100% for the diagnosis of minimal to mild endometriosis. Confirmation of these data in a larger and independent patient population, together with identification of all up- and down-regulated proteins and peptides, is needed and planned as the next phase of our research.

Acknowledgments: The authors acknowledge the important technical assistance of Ms. Karin Schildermans.

**FIGURE 2**

Women with minimal-mild endometriosis versus controls during luteal phase. Endometrial protein expression of 90.675 kDa (fold difference [n-fold] = 2.0) and 35.95 kDa (n-fold = 1.7) was significantly higher in women with minimal-mild endometriosis compared with controls. Decreased endometrial peptide levels for 1.924 kDa (n-fold = 1.8) and 2.504 kDa (n-fold = 3.0) in women with minimal-mild endometriosis were observed compared with controls. *P < .05; **P < .01. Stg = stage.
REFERENCES


SUPPLEMENTAL MATERIALS AND METHODS

Hemoglobin Depletion Using Spin-Column IMAC-ZnSO₄ Resin

In brief, 100 μL per well of immobilized metal affinity capture (IMAC)–resin (Biosepra, Cergy, France) was added onto a 0.45-μm filter plate of 96 wells (Millipore Corporation, Bedford, MA) and spun down at 4,000 rpm for 1 minute. The IMAC resin was saturated with 200 μL of 100 mmol/L ZnSO₄ per well and incubated for 15 minutes at room temperature, with frequent shaking. The resin solution was centrifuged at 4,000 rpm for approximately 30 seconds and the supernatant discarded. The wells were washed twice with 200 μL of Milli-Q water per well. Further saturation was done by an additional 200 μL of 100 mmol/L ZnSO₄ to 100 μL of IMAC-resin and incubated for 15 minutes at room temperature. The wells were washed again twice with 200 μL Milli-Q water. Further washing of the wells was done with use of 200 μL of 10% U9 lysis buffer in phosphate-buffered saline solution two times. Protein lysate per sample (300 μL) was applied to each well, rotated in a cold room (4°C) for 30 minutes, and centrifuged, and the eluate was transferred to an Eppendorf tube. The wells were washed with 200 μL of 100 mmol/L NH₄OAC, rotated for 30 minutes in the cold room (4°C), and spun down, and the second eluate was reconstituted with the first eluate in an Eppendorf tube. The two eluates were pooled together, and the protein concentration was estimated at 280 nm absorbance with use of a spectrophotometer (Isogen-Life Sciences, Wilmington, DE). The supernatant was aliquoted in 50-μL fractions in Eppendorf tubes and used immediately or stored at –80°C. Pooled samples from controls (n = 10) and from minimal endometriosis were used as reference to control for variation between experiments run at different times.

Profiling of Tissue Lysates on ProteinChip Arrays

To increase the scale of detectable proteins, four different chip surfaces with distinct chromatographic properties and binding affinities were used (Supplemental Table 1). In brief, ProteinChip array spots were equilibrated with 150 μL of respective binding buffer (Ciphergen, Fremont, CA) while shaking for 5 minutes at room temperature to preactivate binding surfaces. Then, 20 μL of sample lysates (10 μg per spot) diluted (1:5 vol/vol) with surface-type dependent binding buffer (21) was loaded onto each spot in duplicate and incubated for 60 minutes at room temperature while being shaken (MicroMix5, form 20, amplitude 5; Diagnostics Product Corporation, Gwynedd, Wales, United Kingdom). The unbound proteins and peptides on the ProteinChip array surfaces were washed away with appropriate buffer twice for 5 minutes, rinsed in 150 μL of Milli-Q water, and air-dried. Mass spectra of the retained proteins were obtained by ionizing the proteins with use of two types of energy-absorbing molecules as previously described (21). Analysis of the retained proteins was performed with a Protein Biological System–IIIC linear SELDI-TOF-MS instrument (Ciphergen). Mass accuracy was calibrated externally with the all-in-one peptide molecular mass standard for the mass range of 1.6 to 20 kDa and with the all-in-one protein molecular mass standard (Ciphergen) for the mass range of 8 to 150 kDa.

Preparation of Tissue Lysate for Protein Identification

Samples with the strongest discernable differences in peak signal intensity were selected. Strong anion exchange (Q10) and immobilized metal affinity capture (IMAC-30-Cu) surfaces were used in the sample preparation procedure, because two potential biomarkers (35.956 kDa and 90.675 kDa) of high molecular weight had been observed in those binding surfaces. Therefore, to mimic the binding conditions we used 100 μL of Q ceramic HyperD F resin and IMAC HyperCel resins (Biosepra, Cergy, France) to enrich the high molecular weight had been observed in those binding surfaces. Therefore, to mimic the binding conditions we used 100 μL of Q ceramic HyperD F resin and IMAC HyperCel resins (Biosepra, Cergy, France) to enrich the protein concentration in the sample. The selected protein lysate of 200 μL was loaded onto resin (mimicking either the Q10 or IMAC-30-Cu binding surface), and the bound fraction was eluted with 200 μL of respectively 50 mmol/L NH₄OAC, pH 4, or NH₄HCO₃, pH 8, elution buffers. The eluted fractions were concentrated through vacuum and reconstituted in an appropriate buffer. These lysates were resolved in an sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel 4% to 12% NuPage (Invitrogen, Carlsbad, CA). Subsequently, the protein bands were visualized by means of SimplyBlue colloidal Coomassie staining (Sigma-Aldrich, St. Louis, MO).

Protein Identification After Tryptic Digestion and MS Analysis

Protein bands of interest were excised, destained, and subjected to in-gel trypsin digestion at room temperature. Peptide sequencing and protein identification were performed with use of an ABI 4800 matrix-assisted laser desorption/ionization–TOF/TOF mass spectrometer (Applied Biosystems Inc., Foster City, CA).
SUPPLEMENTARY FIGURE 1

Women with moderate-severe endometriosis versus controls during luteal phase. Increased endometrial protein levels for 10.1094 kDa (fold difference [n-fold] = 1.53) in women with moderate-severe endometriosis when compared with controls. Protein and peptide levels of 5.828 kDa (n-fold = 1.9), 12.172 kDa (n-fold = 2.33), and 4.279 kDa (n-fold = 2.9) were significantly lower in endometrium of women with moderate-severe endometriosis when compared with controls. *P < .05; **P < .01; ***P < .005.

## SUPPLEMENTARY TABLE 1

Different ProteinChip surfaces with their respective binding buffer that were used in the study.

<table>
<thead>
<tr>
<th>ProteinChip surfaces</th>
<th>Binding buffers</th>
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<tr>
<td>Weak cation exchange surface (CM10)</td>
<td>Low stringency binding buffer (50 mmol/L NaOAC, pH 4.0)</td>
</tr>
<tr>
<td>Immobilized metallic affinity capture surface</td>
<td>0.1 mol/L phosphate, 0.5 mol/L NaCl, pH 7.0</td>
</tr>
<tr>
<td>(IMAC-30-Cu) loaded with CuSO₄</td>
<td>10% acetonitrile, 0.1% trifluoroacetic acid</td>
</tr>
<tr>
<td>Hydrophobic surface (H50)</td>
<td>50 mmol/L Tris-HCl, pH 8.0.</td>
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<tr>
<td>Strong anion exchange surface (Q10)</td>
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Note: Tris = tris(hydroxymethyl)aminomethane.