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PIXE-PAGE Analysis by Scanning Proton Microprobe

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Abstract. Metal content of metalloproteins can be detected and even quantified by the PIXE-PAGE method. In this technique the proteins are separated by thin layer electrophoresis (by polyacrylamide gel electrophoresis (PAGE) in most cases) and the properly dried gel sections are analyzed by PIXE using "band-shaped" proton milli-beam. This PIXE-PAGE method was adapted for our scanning proton microprobe. The microPIXE-PAGE version provides two-dimensional elemental mapping of the protein bands. In addition, the fast continuous scanning reduces the risk of the thermal deterioration of the sample and the X-ray contribution from dust-impurities can be filtered out in the data evaluation process.

Keywords: PIXE-PAGE, scanning proton microprobe, metalloproteins

INTRODUCTION

The PIXE-PAGE technique, i.e. the combination of PIXE spectroscopy and polyacrylamide gel electrophoresis, was originally developed for the use of standard millimeter-sized proton beam. In a gel electrophoretogram the proteins and protein fragments are separated in narrow bands, by direct scanning of the gel by properly focused proton beam the metal ions bound to the proteins can be detected [1]. This method has substantially increased the information available from gel electrophoresis. The micro-beam version of PIXE-PAGE analysis can provide two dimensional mapping of trace metals in bands with a micrometer scale lateral distribution. Higher sensitivity and lower background are also expected. Some technical details of the PIXE-PAGE setup are given and results of the measurements on test gel electrophoretograms of myoglobin and cytochrome c are presented. Efforts to eliminate the disturbing artifacts from dust-like impurities are also described.

EXPERIMENTAL

The Scanning Proton Microprobe

The micro-beam adaptation of the PIXE-PAGE technique was demonstrated on the Budapest-Hamburg scanning proton microprobe [2]. The drawing of the PIXE chamber is shown in Fig.1. In order to maximize the sensitivity of the system the unique property of the microprobe was exploited: a large area Si(Li) detector of 80 mm^2 was installed just behind the gel sample at 0^o position. The solid angle of the detector was as large as 220 mSr in this manner. The sample is positioned by a computer controlled stage with an accuracy of 1 μm. Taking into account that the beam size was not critical in these measurements, no special efforts were done in focusing, beam size of about 3 x 3 μm^2 was used at both 2.5 and 2.0 MeV energies. The beam current varied between 0.5 and 1 nA.

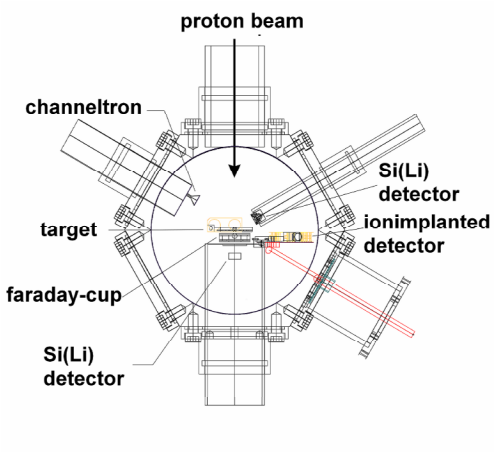

FIGURE 1. The PIXE target chamber

Sample Preparation

The gel samples were prepared in vertical slab gel apparatuses using discontinuous buffer system, the thickness of the dried gels were less than 1 mm. Taking into account that the length of the electrophoretograms are much longer than the openings of the standard Al sample holder frame, the gels were cut in smaller fitting parts and only the relevant sections were fixed on the holder (Fig. 2). (The fragile, sometimes unevenly curved gels were not possible to mount as a whole on a holder with larger opening.)

FIGURE 2. Section of the gel electrophoretogram containing 30 µg cytochrome c protein. The band is visible due to the high protein concentration.

MEASUREMENTS AND RESULTS

Demonstration of the MicroPIXE-PAGE Technique

One of the main limiting factors of the sensitivity achieved in „standard milli-beam" PIXE-PAGE analyses is the deteriorating deformation of the gel caused by the energy loss of the bombarding protons. Because the current density could be 2-3 orders of magnitude larger if micro-beam is used, higher damage could be expected on the one hand, but the continous beam scanning, on the other hand, could offset this effect. For lack of previous experience in bombarding polyacrylamide gel sheets by micro-focused proton beam, our first aim was to demonstrate the possibility to locate a metalloprotein band in an electrophoretogram by measuring the characteristic X-rays. To do it a test sample of Fe-containing myoglobin was prepared. The amount of myoglobin was so large, that the position of its band was visible to the naked eye. Protein bands are usually visualised by staining, but in this process there is a serious chance of washing out of the bound metal ions. Therefore the PIXE scans have to be taken „blindly" on non-stained samples. The direct visibility of the band facilitated the exploratory measurements to a high extent. An area of 3.4 x 1.7mm^2 was scanned by covering it with 10 region of interests (ROI) stripes, x=100 and y=513 pixels for each. The ROI stripes were parallel to the protein band. The X-ray energy spectrum was calculated for each stripe, the background measured on a ROI stripe positioned far from the protein band was subtracted. The Fe Kα peak areas are calculated and normalized to the beam current. The overlapping scans were averaged. The Fe distribution along the myoglobin in the gel is shown in Figure 3. No visible deterioration of the gel was observed.

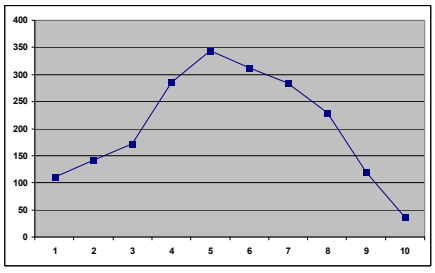

FIGURE 3. The Fe distribution in the myoglobin band.

Improvement of Sensitivity

In order to prepare usable narrow-banded, high resolution electrophoretograms the total amount of proteins supplied for gel electrophoresis is seriously limited. This limitation is even more important when mixtures of proteins have to be analysed. There are only two ways of increasing the sensitivity, i., to increase the detection efficiency and ii., to reduce the background. The first possibility was realized by inserting the X-ray detector directly behind the sample. For background reductions more measures were taken. The inside of the chamber was covered with ultraclean carbon foil to minimize the contribution from X-rays. induced by scattered protons in the target chamber. To prevent the bombarding protons from entering into the X-ray detector a properly chosen stopping layer should be inserted in front of it. A series of measurements has yielded that a sandwich from polycarbonat, carbon and Be gave the best solution to stop the beam and simultaneously to minimize the associated secondary electron brehmstrahlung. From the careful analysis of the background shape and height it was found that 1.5 ng of Fe in a band can certainly be detected. In the case of a protein of 55 kD molecular mass this value corresponds to about 1.5 μg of protein. This estimation was verified by microPIXE-PAGE runs on real gel electrophoretograms containing 30, 10 and 3 μg cytochrom c, respectively. The Fe map of the „10 μg gel" is shown in Fig. 4.

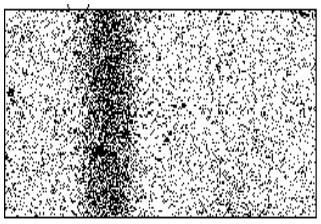

FIGURE 4. Two dimensional Fe map of the band containing 10μg cytochrome c. Image size is 3.2mm x 1.6mm. The total amount of Fe detected is ~ 7.6 ng.

All of these estimations are valid only, if no contaminations are present. The beam pipes and the target chamber itself, however, can not be considered as "clean rooms", dust or dust-like particles can be randomly deposited on the surface of the gel. Especially forepumping is a critical phase in this respect. The possible metal content of these impurities can disturbingly interfere with those of the metalloproteins. In contrast to the milli-beam case where practically nothing can be done against it, the high spatial resolution of the microprobe and the data processing system, as well, allows to eliminate, or substantially reduce the artefacts caused by these impurities. The significant dust particles can be recognized on the two dimensional map and they can be "wiped out" manually. Instead of this rather fatiguing method a computer code was written to automatize this task. The filtering algorythm calculates the number of neighbours of a pixel having counts bigger than a prefixed value and the contribution of the recognized impurities will be disregarded in the evaluation

Preliminary Analysis of Plasma Membrane Proteins

In addition to the measurements of methodological importance microPIXE scans were also made on gels containing protein bands isolated from the root plasma membrane of maize (*Zea mays* L.) On account of unexpected difficulties in the sample preparation and because of the lowering of the proton energy down to 2 MeV compelled by technical problems of the accelerator, the experimental conditions were rather far from the estimated optimal ones. In spite of these troubles a Fe containing band was finally localized (Fig. 6), but the unusually large width of the band and the absence of other band clearly show that there are a lot of refinements to do in order to develop the method for routine job.

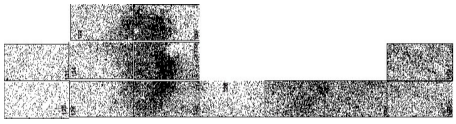

FIGURE 6. Reconstructed Fe map of maize PM protein electrophoretogram.

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