Abstract

Large-cohort studies are increasingly required in multifactorial proteomics, demanding high-throughput and scalable workflows. Sample preparation automation and fast LC-MS instrumentation, including short-gradient chromatography combined with Data Independent Acquisition (DIA), have enabled rapid data generation. However, DIA data processing needs high-quality spectral libraries, which is a current bottleneck. While AI-generated libraries offer comprehensive coverage, their large size leads to time-consuming data searches. In contrast, DDA-based libraries reduce search space but limit peptide identifications to those observed in DDA runs. Here, we propose a novel strategy combining sequential protease digestion, peptide fractionation and DDA acquisitions on the timsTOF SCP. In this approach, three replicate samples are separately digested with trypsin, Arg-C, or Lys-C, followed by peptide fractionation and a final digestion with trypsin. This yields tryptic peptides distributed differently across fractions based on the initial protease, enhancing orthogonality between fractionation and LC separation.
 Compared to conventional post-trypsin fractionation, our method identifies nearly 50% more peptides. Applied to 10-minute DIA runs, libraries from sequential digestion yield 10% more protein IDs without notably increasing processing time. This strategy enhances peptide coverage and search efficiency, making it ideal for large-scale DIA proteomics requiring both speed and depth.