

## **SUPPLEMENTAL MATERIAL**

### **Assessing the Reproducibility and Specificity of Pepsin and other Aspartic Proteases**

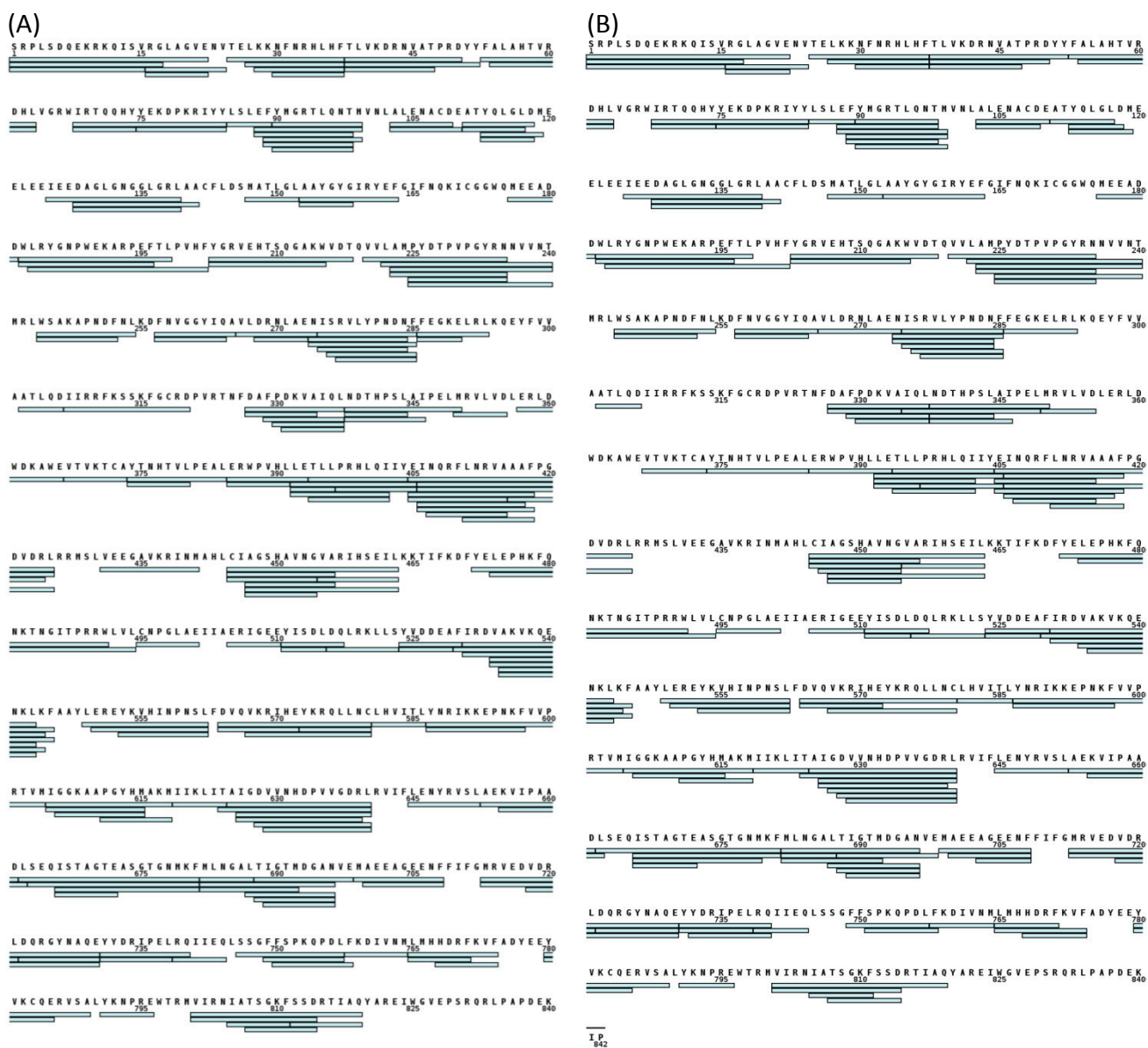
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Five supplemental figures, Figure S1-S5 and Table S1



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<b>N=5</b>	<b>% coverage</b>	<b>N=10</b>
86 %		82 %
178	<b># reproducible peptides</b>	164

Figure S1. Phosphorylase b peptide coverage map comparison by online pepsin digestion. The maps demonstrate 86% and 82% coverage using reproducible peptides from digestion replicates at N=5 (A) and N=10 (B), respectively. Each map shared mostly same peptides, which resulted in a similar number of reproducible peptides (178 peptides for N=5 and 164 peptides for N=10).

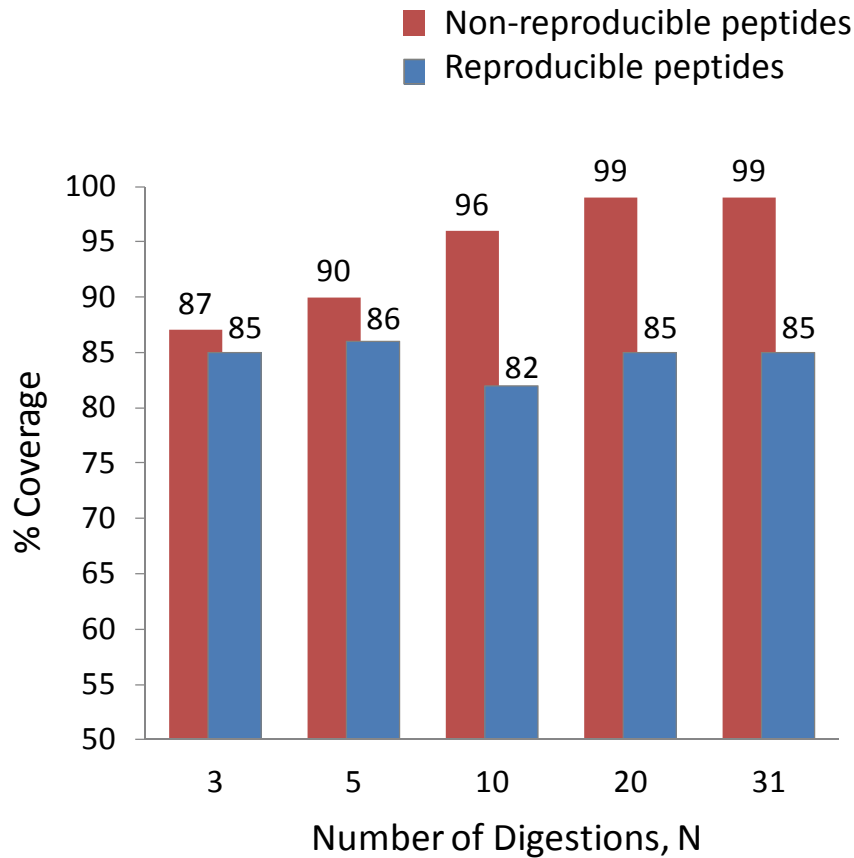


Figure S2. Percent phosphorylase b sequence coverage determined using reproducible peptides (blue) and non-reproducible peptides (red) in replicate peptide digestions (N). When only reproducible peptides were used, 82 – 86% sequence coverage was achieved. When non-reproducible peptides were included, almost completed coverage was achieved as the digestions increased.

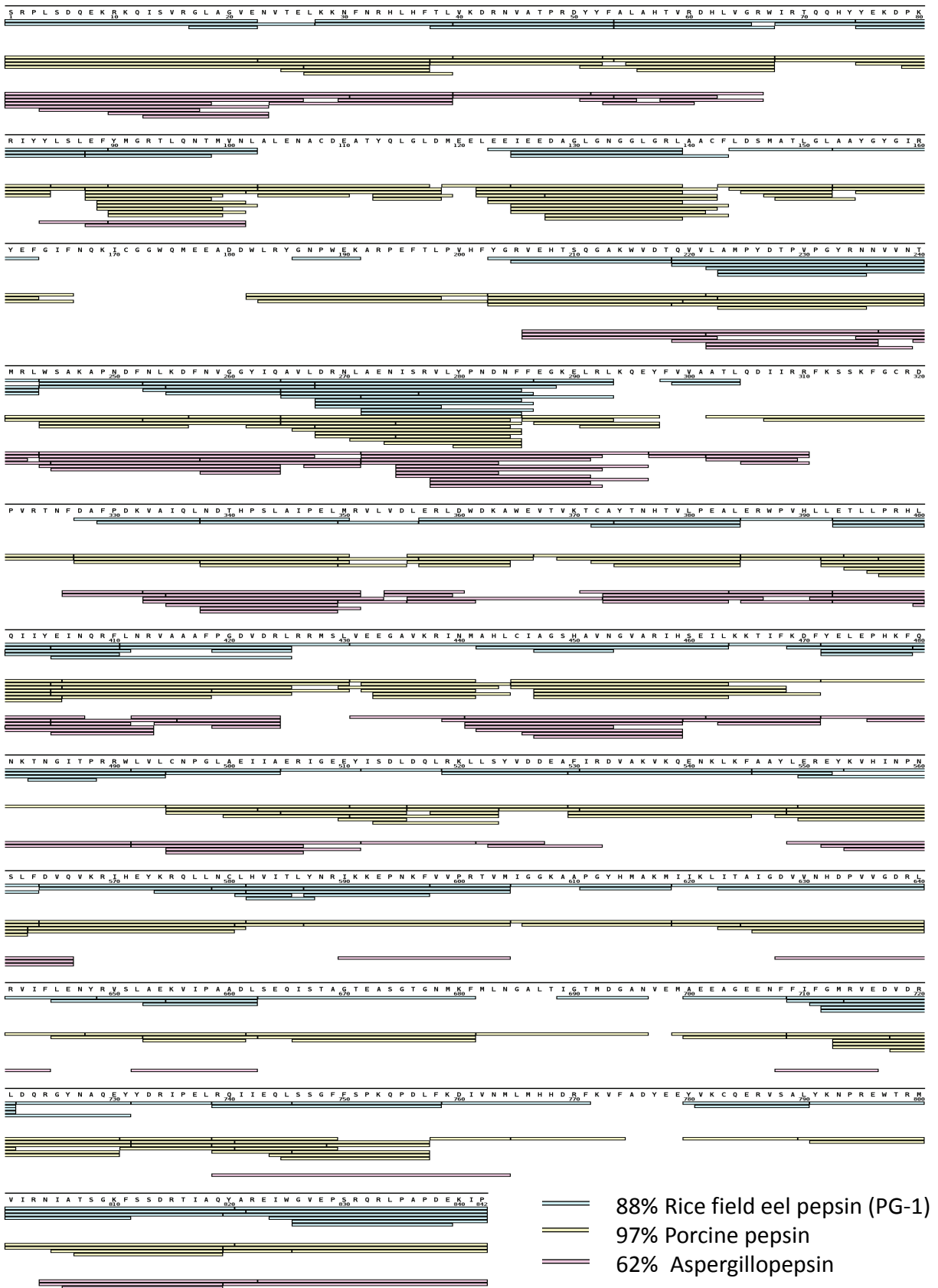


Figure S3. Full map of phosphorylase b digested by rice field eel pepsin (cyan), porcine pepsin (yellow), and aspergillopepsin (pink).

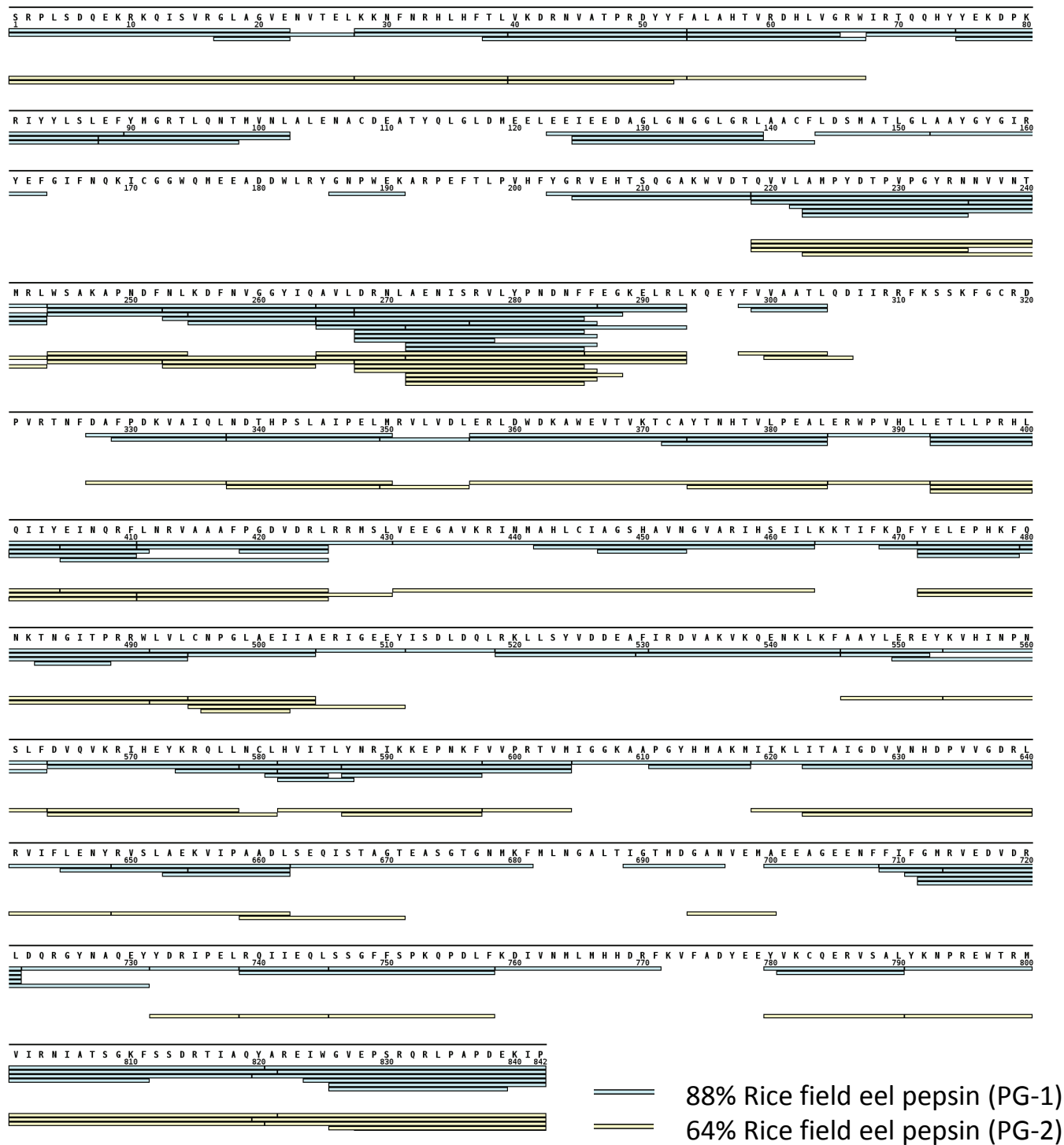


Figure S4. Within rice field eel pepsins, two different isomers [PG-1 and PG-2] were studied with phosphorylase b digestion. The cleavage specificity of isomer PG-1 (cyan) and PG-2 (yellow) was very similar, but PG-2 showed lower digestion efficiency than PG-1, as shown in the % coverage comparison (88 for PG-1 and 64% for PG-2).

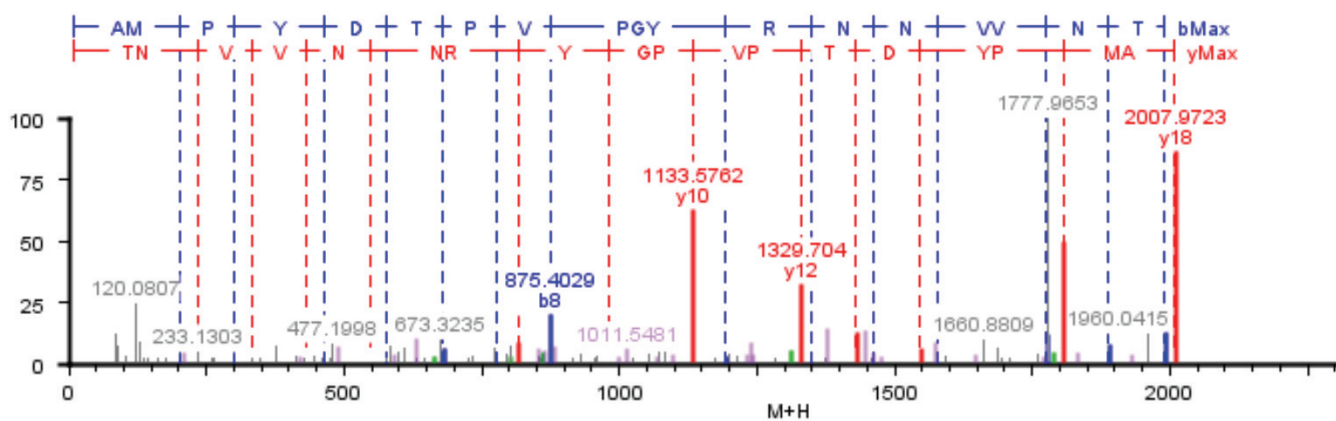


Figure S5. Example MS/MS spectrum during peptide identification. This data is for phosphorylase b peptide AMPYDTPVPGYRNNVVNT (residues 223-240) at 5.00 min retention time. A total of 26 fragment ions are shown in the 18-residue long peptide. The ratio of the number of fragment ions per amino acid is 1.44. This peptide is always selected for identification because the threshold for the number of fragment ions per amino acid was set greater than 0.3.

Phosphorylase b digestion	Peak area %RSD (N=10)
Pepsin Online	5.4
Pepsin Offline	17.0
Trypsin Online	3.9
Trypsin Offline	12.0

Online pepsin digestion	Peak area %RSD
Cytochrome C	6.4 (N=3)
Interferonalpha-2b	4.6 (N=4)

Table S1. Average %RSD peak area comparison between online vs. offline (in-solution) digestion. Online digestion provides much robust peptide generation in terms of peptide quantitation compared to offline digestion.