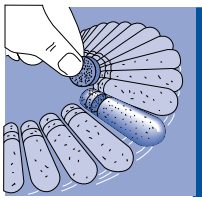

Application Information

High Performance Centrifugation

Rapid Protease Screening Using Ammonium Sulfate Precipitations and the Avanti™ J-25 High Performance Centrifuge

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One of the first steps in isolating a specific protein from a complex mixture is the selective salting out of the protein by serially increasing the concentration of ammonium sulfate. The procedure is rapid, easy to perform, and also has the advantage that the protein usually remains in its functional state.(1) Typically, the protein precipitates are collected by pelleting in a high speed centrifuge for 10-30 min. The ammonium sulfate is removed easily by dialysis, making the protein-containing fraction available in the buffer desired for the next stage of isolation. Frequently, several successive cuts are taken with increasing ammonium sulfate concentrations to crudely select the desired protein. Thus, a centrifuge that achieves shorter acceleration and deceleration times for pelleting the precipitates would have a significant impact on total preparation time.

In this paper, a porcine pancreas is screened for proteases to demonstrate ammonium sulfate precipitation techniques. A rapid screening procedure indicated that distinct protease activities were isolated in the successive precipitates.

Methods

Frozen porcine pancreas was obtained from Pel-Freeze, Rogers, AR. Trypsin (EC 3.4.21.4), α-chymotrypsin (EC 3.4.21.1), and elastase (EC 3.4.21.36) were obtained from Worthington Biochemical Corp., Freehold, NJ. Subtilisin A (EC 3.4.21.62) was obtained from Allergan, Irvine, CA. Phenylmethylsulfonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), and the peptide *p*-nitroanilides (PpNA) were obtained from Sigma Chemical Co., St. Louis, MO.

Tissue was homogenized in 0.1 M Tris, pH 8.0 using the lowest setting possible (Polytron homogenizer, Brinkman, Westbury, NY). The homogenate was then centrifuged for 6 min at 25,000 rpm, 4°C, using a JA-25.50 rotor in the Avanti J-25 High Performance Centrifuge to remove large organelles and cellular debris. The supernatant was transferred to a bottle and centrifuged in a Type 70 Ti rotor for 25 min at 60,000 rpm, 4°C, to remove the remaining membranous organelles. Using the following formula, solid ammonium sulfate was added sequentially to the supernatant of the previous step to achieve 20%, 40%, 60%, and 80% saturation solutions(1):

$$g = \frac{533(S_2 - S_1)}{100 - 0.3S_2}$$

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where g is the amount of ammonium sulfate in grams added per 1 L of solution at 20°C, S_1 is the original percent saturation, and S_2 is the desired percent saturation. The following amounts of ammonium sulfate were used to prepare the 20%, 40%, 60%, and 80% saturated solutions: 113 g added per 1 L of water to achieve 20% saturation; 121 g added per 1 L of the 20% solution to achieve 40% saturation; 130 g added per 1 L of the 40% solution to achieve 60% saturation; 140 g added per 1 L of the 60% solution to achieve 80% saturation.

After the addition of ammonium sulfate, the tube was mixed for 30 min at room temperature (20-22°C), placed in the JA-25.50 rotor and centrifuged for 6 min at 25,000 rpm, 20°C. In preliminary experiments, the 20% saturation pellet tended to be relatively soft, so the slow deceleration profile was used for this pelleting step. After the spin, the supernatant was pipetted into a new centrifuge tube. Volume was measured using the pipet gradations. Solid ammonium sulfate was added slowly to the transferred supernatant to achieve the next percentage of saturation. These steps were repeated until the 80% pellet was collected. The 80% saturation supernatant was then placed directly into dialysis tubing.

The pellets were suspended in as small a volume of 0.1 M Tris, pH 8.0, as practical. Ammonium sulfate was removed by dialysis overnight in a coldroom against 100 volumes of 0.1 M Tris, pH 8.0. Dialysis tubing with a molecular weight cutoff of 6,000-8,000 Da was used (Spectrum Medical Industries, Inc., Los Angeles); it was prepared by boiling until no sulfur odor was detected, and then boiled twice in 0.001 M EDTA. Dialysis buffer was changed a minimum of three times.

Screening was performed by incubating 10 µL of the dialyzate, 10 µL of 0.0044 M PpNA, and 180 µL Tris, pH 8.0, in 96-well plates (assay adapted from the elastase assay given in the Worthington Enzyme Manual[2]). Elastase, subtilisin A, trypsin, and α-chymotrypsin were used as standards. A portion of the 20% pellet was dialyzed against water, acidified and predigested with trypsin to reveal any latent zymogen activity (adapted from the chymotrypsinogen assay from the Worthington Enzyme Manual[3]) and screened as above.

When the amino acid–*p*-nitroanilide bond is split, a yellow color develops. Six color gradations, varying from intense to no color and based on visual observation of the yellow solution, were used to estimate activity. Because of the presence of color in the protein mixtures themselves, it was often difficult to tell the difference between no color and a very faint color. Likewise, at the very intense end of the range, the color choice was made by choosing the most intense color on the plate. There was little difficulty telling the difference between the three middle intensities.

The 20% fraction, 20% trypsin digest, 60% fraction, and trypsin were also tested in the presence of 0.1 M NEM, an -SH group antagonist, and 0.1 M PMSF, a weak serine protease antagonist, to obtain an indication of the type of peptidase activity in each fraction.

After the initial screening, the samples were frozen overnight, then clarified by centrifuging at 20,000 rpm in the JA-25.50 rotor for 1 h to remove the remaining particulate matter. These clarified fractions were then rescreened, and the most promising substrates were assayed using the Beckman DU® 650 spectrophotometer. Assay conditions were essentially the same as used for screening, except that the temperature was maintained at 37°C by use of the water-jacketed cuvette holder. Sample concentration was adjusted to provide useful A_{410}/min rates. Protein concentrations were determined via the Coomassie blue binding assay using bovine serum albumin as a standard.[4]

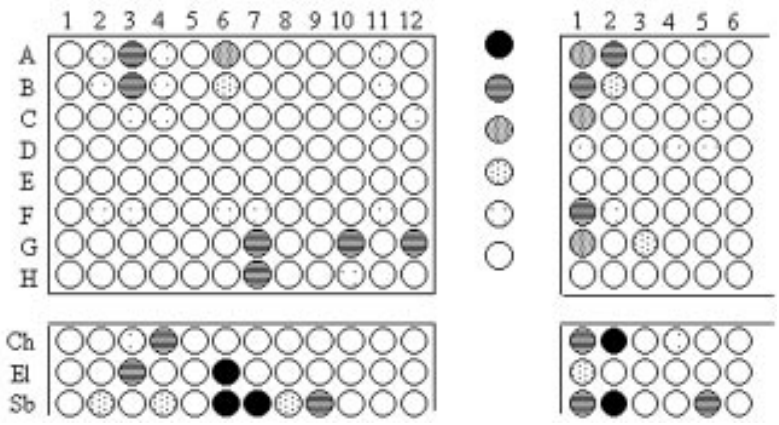
Results

The initial screening data are shown in Figure 1. The 20% and 40% pellets exhibit moderate to strong alanine and phenylalanine peptidase activity with latent phenylalanine and proline peptidase activity. The 60% pellet has moderate phenylalanine, alanine, and proline peptidase activity. The 80% pellet shows moderate alanine and phenylalanine peptidase activity and strong latent alanine peptidase activity. The 80% supernatant has virtually no peptidase activity. As shown in Figure 2, PMSF was a more effective inhibitor than NEM.

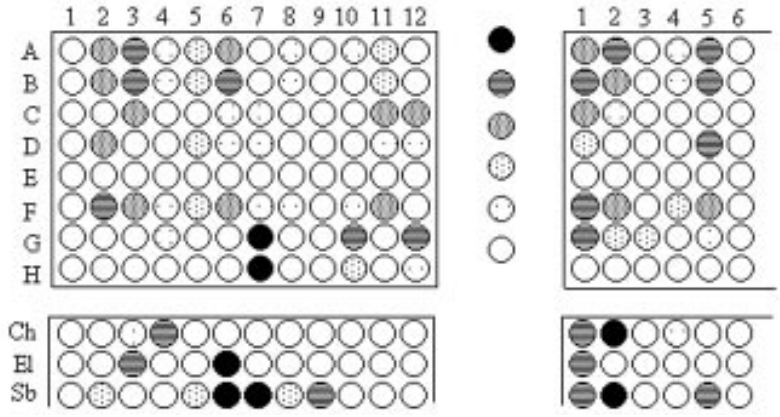
Trypsin activation of the 20% pellet appeared to digest active proteases rather than uncover any latent zymogen activity.

The screening data for frozen dialyzates, clarified by further centrifugation, are shown in Figure 3.

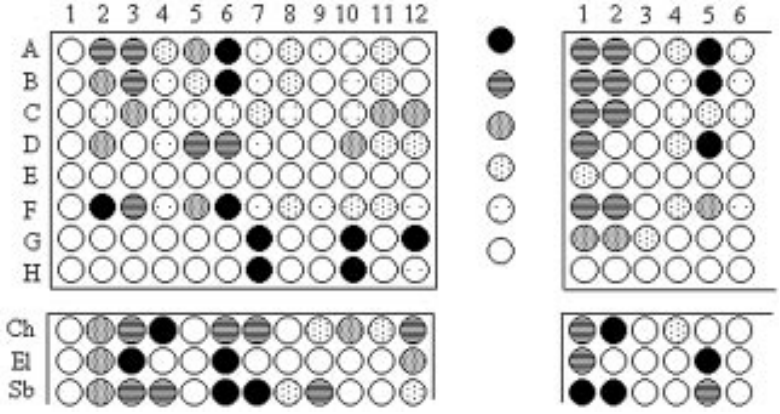
1-h results



3-h results



Overnight results



First plate:

1. H_2O
2. Ala-Ala-Ala-*p*-NA
3. *N*-Succinyl-Ala-Ala-Ala-*p*-NA
4. Ala-Ala-Phe-*p*-NA
5. Val-Ala-*p*-NA
6. Ala-Ala-Val-Ala-*p*-NA
7. D-Val-Leu-Lys-*p*-NA
8. *p*-Glu-Phe-Leu-*p*-NA
9. *N*-CBZ-Gly-Gly-Leu-*p*-NA
10. Gly-Arg-*p*-NA
11. Arg-Pro-*p*-NA
12. D-Phe-L-pipecoyl-Arg-*p*-NA

Second plate:

1. *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-NA
2. *N*-Succinyl-Gly-Gly-Phe-*p*-NA
3. Na-Benzoyl-L-Arg-*p*-NA
4. Gly-Phe-*p*-NA
5. Ala-Ala-*p*-NA
6. *N*-Acetyl-Ala-Ala-Ala-*p*-NA

Rows:

A: 20% saturated ammonium sulfate pellet
B: 40% saturated ammonium sulfate pellet
C: 60% saturated ammonium sulfate pellet
D: 80% saturated ammonium sulfate pellet
E: 80% saturated ammonium sulfate supernatant
F: Homogenate
G: Trypsin
H: Trypsin-treated 20% saturated ammonium sulfate pellet

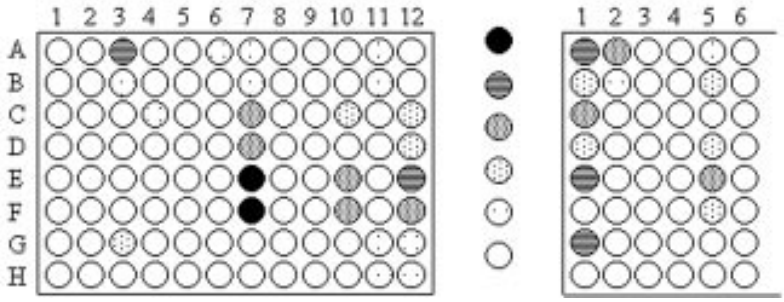
Ch: Chymotrypsin
El: Elastase
Sb: Subtilisin A

Key:

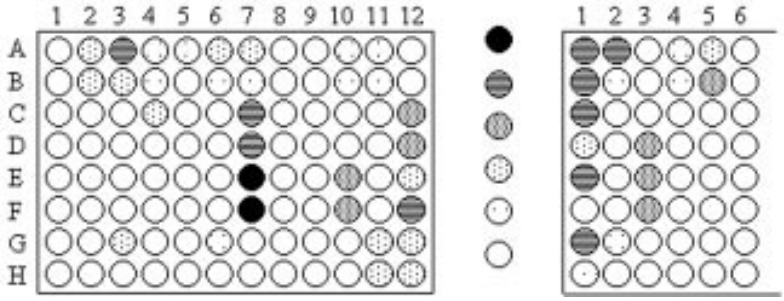
- Intense yellow
- Strong yellow
- Yellow
- Light yellow
- Very faint yellow
- No discernible color

Figure 1. *Initial quick screen.*

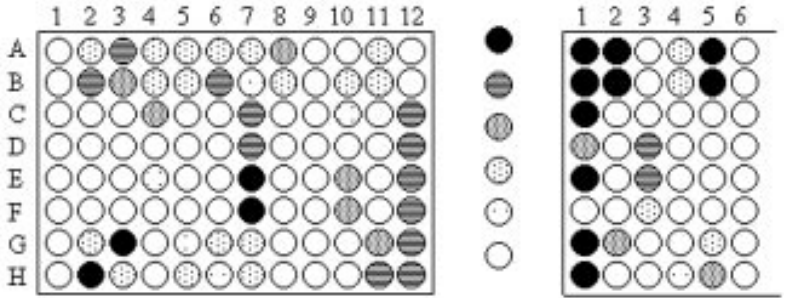
1-h results



3-h results



Overnight results



Symbol key, first, and second plates as in Figure 1.

Rows:

A: 20% saturated ammonium sulfate pellet, 100 mM NEM

B: 20% saturated ammonium sulfate pellet, 100 mM PMSF

C: Trypsin-treated 20% saturated ammonium sulfate pellet, 100 mM NEM

D: Trypsin-treated 20% saturated ammonium sulfate pellet, 100 mM PMSF

E: Trypsin, 100 mM NEM

F: Trypsin, 100 mM PMSF

G: 60% saturated ammonium sulfate pellet, 100 mM NEM

H: 60% saturated ammonium sulfate pellet, 100 mM PMSF

Figure 2. *Inhibitor plates.*

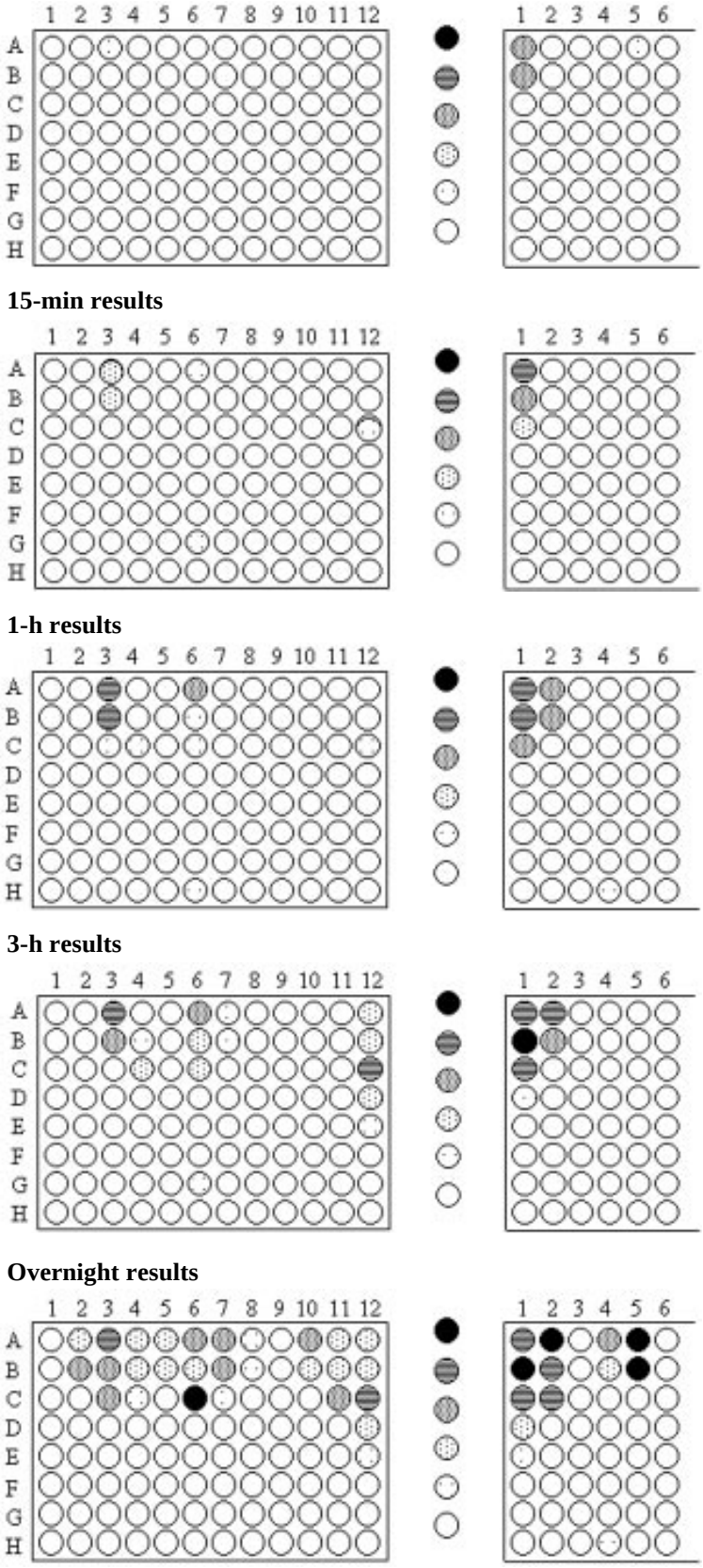
5-min results

15-min results

1-h results

3-h results

Overnight results



Symbol key, first, and second plates as in Figure 1.

Rows:

A: 20% saturated ammonium sulfate pellet

B: 40% saturated ammonium sulfate pellet

C: 60% saturated ammonium sulfate pellet

D: 80% saturated ammonium sulfate pellet

E: 80% saturated ammonium sulfate supernatant

Figure 3. *Clarified dialyzate plates.*

The 5- and 15-minute data were used to select the substrates to assay spectrophotometrically, and the specific activity and protein concentration data are shown in Table 1. The 96-well plate data show the highest activities in the phenylalanine and alanine peptidase wells. The specific activities, as determined by spectrophotometry, confirm the 96-well plate data. The highest specific activities for *N*-succinyl-Ala-Ala-Ala-*p*-NA are approximately equal in the 20% and 40% pellets. The highest activities for *N*-succinyl-Ala-Ala-Pro-Phe-*p*-NA and *N*-succinyl-Gly-Gly-Phe-*p*-NA were found in the 40% pellet fraction. The 20% pellet fraction had the highest protein concentration.

Results for the 5-min and 15-min assays are most likely to give an accurate indication of the dominant enzymatic activity present. The 3-h data are appropriate for revealing lower levels of activity or latent activities. The overnight plate results are a combination of very low levels of activity, decreased specificity of activity, and the sum of the enzymatic activity that has occurred at the previous times.

PMSF was a more effective inhibitor than NEM. This is consistent with the predominance of serine over thiol proteases in the pancreas (EC-Enzyme Database). Apparent deactivation of PMSF has been seen with time.[5] Since the *N*-succinyl-Ala-Ala-Ala-*p*-NA activities are greater than the Ala-Ala-*p*-NA activities, and the *N*-succinyl-Ala-Ala-Pro-Phe-*p*-NA and *N*-succinyl-Gly-Gly-Phe-*p*-NA activities are greater than the Gly-Phe-*p*-NA activities, it appears that the dominant proteases have greater activity with increased chain length. Peptidase patterns for the controls were consistent with the specificities given in the EC-Enzyme Database.

Table 1. Protein Concentrations and Specific Activities of the Clarified Fractions *

Clarified Fraction	*μg/μL Protein*	*U/mg A*	*U/mg B*	*U/mg C*
20% pellet	16.6	0.0137	0.00226	0.0975
40% pellet	4.89	0.0121	0.0143	0.228
60% pellet	5.64	0.000403	0.0	0.09
80% pellet	0.737	0.0	0.0	0.0023
80% supernatant	0.00959	0.0	0.0	0.0

* Protein concentrations were determined by Coomassie blue binding; specific activities were determined by spectrophotometry; A = *N*-succinyl-Ala-Ala-Ala-*p*-NA; B = Ala-Ala-Val-Ala-*p*-NA; C = *N*-succinyl-Ala-Ala-Pro-Phe *p*-NA; one unit (U) is one micromole of substrate hydrolyzed per minute at 37°C, pH 8.0; 0.0 denotes that no change in A_{410} was detected.

Table 2. PpNA's Used for Testing for Various Enzymes

PpNA	*Enzyme*	*Reference*
N-succinyl-Ala-Ala-Ala-*p*-NA	elastase	6
D-Val-Leu-Lys-*p*-NA	human plasmin	7, 8
Glu-Phe-Leu-*p*-NA	thiol peptidases	9
N-CBZ-Gly-Gly-Leu-*p*-NA	subtilisin, other neutral endopeptidases	10, 11
N-α-benzoyl-L-Arg-*p*-NA	trypsin, others	12, 13
D-Phe-L-pipecoyl-Arg-*p*-NA	thrombin	7, 14
N-succinyl-Ala-Ala-Pro-Phe-*p*-NA	chymotrypsin and human cathepsin G	15-18
N-succinyl-Gly-Gly-Phe-*p*-NA	chymotrypsin and *S. griseus* protease B	19
Gly-Phe-*p*-NA	cathepsin C	20

Initial Screening

The 20% fraction appears to have both elastase- and chymotrypsin-like activity present, both of which are inhibited by PMSF, but not significantly by NEM. The 40% pellet appears to have both elastase- and chymotrypsin-like activity, with a stronger elastase component. The remaining fractions do not have such clearly identifiable proteases, although the 60% fraction does appear to have more weak or latent activity than the 80% fraction. The supernatant from the 80% fraction is virtually devoid of peptidase activity.

Clarified Dialyzate Screening

The predominant early activities suggest the presence of both chymotrypsin and elastase. Elastase-specific activity was approximately equal in both 20% and 40% pellet fractions. Chymotrypsin activity was highest in the 40% fraction.

The higher relative apparent activities of the 20% fraction in the 96-well plates could be explained by the higher protein content.

Conclusion

Since the purpose of this study was to demonstrate a quick screening technique, not to isolate a specific enzyme, no attempt was made to optimize the pH, storage conditions, or assay conditions. The rapid acceleration and deceleration of the Avanti J centrifuge, which provides a higher accumulated *g*-force, allows shorter run times to be used in comparison to conventional protocols.[21] These shorter times become especially significant when repeated centrifugations are required. The typical total centrifugation times for these experiments using conventional methodology would be:

Homogenate preparation:
one 10-min spin + one 1-h spin = 1 h 10 min
Protein precipitation[1]:
four 10- to 30-min spins = 40 min to 2 h
Total centrifugation time:
1 h 50 min to 3 h 10 min

Taking advantage of the Avanti J and faster, shorter ultracentrifugation runs results in the following total time:

Homogenate preparation:
one 6-min spin + one 25-min spin = 31 min
Protein precipitation:
four 6-min spins = 24 min
Total centrifugation time:
55 min

For these experiments, the total preparation time from homogenizing the tissue to starting dialysis of the reconstituted pellets was 4-5 h.

The Avanti J centrifuge with the JA-25.50 rotor allows rapid screening of ammonium sulfate-precipitated proteins for peptidase activity. A visual determination of peptidase activity could be made from the reconstituted material. Collection of functional enzymatic activity was rapid and easy. The screening activity measurements should be easy to perform with any enzyme that has chromatogenic substrates, or any other binding reaction that produces a visible color change.

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