Abstract

There has been a long standing question as to the level of proteolytic activity naturally occurring in collected human serum and plasma samples. Researchers have commonly added protease inhibitors to serum or plasma samples in order to renaturallize proteolytic activity present in such samples. Additions of covalently modifying protease inhibitors (i.e., AEBSF) have been shown to change isolecule properties, confounding proteolytic activity of such sample complexes. In order to better understand the effects of protease inhibitors on the study of human serum or plasma, naturally occurring or exogenous protease activity was evaluated using a fluorescing protease detection technology. Protease activity was monitored overnight or without the addition of proteases in order to elucidate the effects of endogenous protease inhibitors in regards to the stability of human serum and plasma during sample preparation and handling.

Introduction

Proteases are commonly used in proteolytic assays to preserve proteins from endogenous and exogenous proteolytic cleavage. A multitude of protease inhibitors are available which act to inhibit various protease classes, including serine and cysteine proteases, aminopeptidases, acid proteases and metalloproteases. Protease inhibitors are also commonly combined into cocktails for the purpose of concurrently inhibiting several classes of protease during protein sample preparation and purification activities. Some of these inhibitors are modified peptides and amino acids (e.g., pepstatin, bestatin, soybean and papain A-1), or native peptides (e.g., apasin) and serve as competitive, irreversible inhibitors which bind to the active site of proteases but are not cleaved. Other inhibitors (e.g., AEBSF and Phenylmethyl Sulfonyl Fluoride (PMSF)) are competitive, irreversible inhibitors, which covalently attack important amino acids in the active site of the protease. The use of AEBSF has been negatively impacted by the analysis of the protease by 2-DE.[1]

Plasma is known to contain a number of naturally occurring, proteolytic active proteins including alpha-1 protease inhibitor (alpha-1 anti-trypsin), antithrombin III, alpha-2-antiplasmin, alpha-1 antichymotrypsin, C1 inhibitor, alpha-2 macroglobulin, inter-alpha trypsin inhibitor, beta-1 antithrombin, anticoagulant and alpha-synuclein protease inhibitors. This effectiveness of these inhibitors for protecting plasma proteins from proteolytic digestion was investigated. A Protease Detection Kit was used to examine the effectiveness of fresh human plasma and in the activity of trypsin, papain, collagenase and pepsin.

Results

Sample Preparation

Lysates from HeLa cells were plated at 20,000 cells/mL in 100-cm dishes and allowed to grow for 24 h in Dulbecco’s Modified Eagle’s Medium (high glucose) Sigma (D5795) supplemented with 5% Fetal Bovine Serum (Sigma F8758) and 1% fetal calf serum (Sigma F1227). The same day the sample was centrifuged for 15 min at 3400 rpm at 5 °C. Additional adjustments to the serum or plasma samples were added to challenge endogenous (naturally occurring) protease inhibitors. At a high level, the current technology. Protease activity was monitored overnight with or without the addition of proteases in order to renaturallize proteolytic activities. Some of these inhibitors are modified peptides and amino acids (e.g., pepstatin, bestatin, soybean and papain A-1), or native peptides (e.g., apasin) and serve as competitive, irreversible inhibitors which bind to the active site of proteases but are not cleaved. Other inhibitors (e.g., AEBSF and Phenylmethyl Sulfonyl Fluoride (PMSF)) are competitive, irreversible inhibitors, which covalently attack important amino acids in the active site of the protease. The use of AEBSF has been negatively impacted by the analysis of the protease by 2-DE.[1]

Plasma was added to a number of naturally occurring, proteolytic active proteins including alpha-1 protease inhibitor (alpha-1 anti-trypsin), antithrombin III, alpha-2-antiplasmin, alpha-1 antichymotrypsin, C1 inhibitor, alpha-2 macroglobulin, inter-alpha trypsin inhibitor, beta-1 antithrombin, anticoagulant and alpha-synuclein protease inhibitors. This effectiveness of these inhibitors for protecting plasma proteins from proteolytic digestion was investigated. A Protease Detection Kit was used to examine the effectiveness of fresh human plasma and in the activity of trypsin, papain, collagenase and pepsin.

Methods

Proteolytic Activity Quantitation and Protease Inhibition in Human Serum and Plasma Samples for Proteomic Analysis

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Conclusions

• Serum and plasma samples appear to be relatively stable against endogenous proteolytic digestion for at least 20 h, as shown by SDS-PAGE (Figure 3).
• Serum and plasma samples were most effective at inhibiting trypsin (a serine protease) and also inhibited pepsin (a cysteine protease) or collagenase (e.g., trypsinogen). To a lesser extent serum and plasma were also somewhat effective at inhibiting recombinant human alpha-2 anti-trypsin.

• The inhibition of pepsin is likely to be due to the presence of alpha-2 macroglobulin, since its currently the only known aspartic protease inhibitor in human serum and plasma [Figure 4, B and E].

• Proteolytic digestion of the citrated plasma, using high protease levels, mirrored the importance of alpha-2 macroglobulin as an endogenous protease inhibitor. [Figure 5B]. Loss of this inhibitor may allow other plasma proteases to cooperate in proteolytic degradation.

References


References