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PROTEASE ACTIVITIES PRESENT IN WHEAT GERM AND RABBIT RETICULOCYTE LYSATES

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SUMMARY: Rabbit reticulocyte lysates and wheat germ lysates were found to contain significant neutral protease activity when assayed against the highly sensitive 7-amino-4-methylcoumarin (AMC) peptide substrates Phe-AMC, succinyl-Ala-Ala-Phe-AMC and t-boc-Ala-Ala-Pro-Ala-AMC (substrates for aminopeptidase, chymotrypsin and elastase-like enzymes, respectively). Additionally, wheat germ lysates contain a trypsin-like activity when assayed against CBZ-Gly-Gly-Arg-AMC and a post-proline cleaving activity which hydrolyzed the Pro-Ala bond of t-boc-Ala-Ala-Pro-Ala-AMC.

INTRODUCTION

Rabbit reticulocyte and wheat germ lysates have been widely utilized for in vitro translation of mRNAs. The primary translation products are usually identified by immunoprecipitation, sizing on denaturing gels, peptide mapping or partial (NH_2-terminal) sequence analysis. In most cases, the completed product of translation has an initiator methionyl residue at the NH_2-terminus (1-11). However, occasionally the initiator methionyl residue is not present, as in rat preproinsulin (12), rat pregrowth hormone (13), ovalbumin (14), bovine preproparathyroid hormone (15) and satellite tobacco necrosis viral coat protein (16). Both lysates frequently give incomplete translation products which are immuno-reactive, but of much smaller molecular weight than the completed peptides. Further, different mRNAs appear to be translated with varying efficiencies and the translation of mRNAs encoding large polypeptides may be particularly poor. While several explanations for these observations have been offered, all of them could be due to proteolytic degradation of the in vitro translation products.

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MATERIALS AND METHODS

Chymostatin, leupeptin, antipain, phosphoramidon, and elastatinal were provided by the U.S.-Japan Cooperative Cancer Research Program. Bestatin was obtained from H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan. Phenylmethanesulfonyl fluoride, N-carbobenzyloxy-2-phenylethyl chloromethyl ketone and L-tosylamido-2-phenylethyl chloromethyl ketone were obtained from Chemalog, South Plainfield, N.J.

Rabbit Reticulocyte and Wheat Germ Lysate Preparation: Rabbit reticulocyte lysates, Lots 0351 and 0421 were purchased from Bethesda Research Laboratories, Bethesda, Md. or prepared in this laboratory according to the method of Pelham and Jackson (17) and contained approximately 20 mg of protein per ml. Wheat germ lysates were prepared by the method of Marcu and Dudock (18) and contained 95-120A_{260} units and 22-27 mg of protein per ml.

Assays for Protease Activities: The peptide substrates 7-(N-carbobenzyloxy-L-glycylglycylargininamido)-4-methylcoumarin trifluoroacetate salt (Cbz-Gly-Gly-Arg-AMC), 7-(succinyl-alanylalanylphenylalanyl)-4-methylcoumarin-trfluoroacetate (Suc-Ala-Ala-Phe-AMC), 7-(N-acetylalanylalanylproylalaninamido)-4-methylcoumarin (Ac-Ala-Ala-Pro-Ala-AMC) and Phe-AMC were prepared by the methods of Zimmerman et al. (19,20). Activities of the lysates (2-10 µl) against these substrates (0.2 mM) at 22°C were determined in 50 mM TES buffer, pH 7.5, containing 5% dimethylsulfoxide (Me_2SO) in a final volume of 0.5 ml. Fluorescence of the 7-amino-4-methylcoumarin (AMC) produced was determined as previously described (20).

Identification of the products of post-proline activity were performed following 2 hour incubations of enzyme extract with t-boc-Ala-Ala-Pro-Ala-AMC (0.2 mM) in a final volume of 200 µl at 25°C. An aliquot of the reaction was spotted on Silica Gel G TLC plates. Chromatograms were developed with chloroform:methanol (80:20 v/v). Reaction products were visualized under long wave uv light.

RESULTS

Aminopeptidase Activity

Rabbit reticulocyte lysates were assayed against a synthetic substrate known to be cleaved by aminopeptidase; Phe-AMC. The results show (Table 1) that various reticulocyte lysates obtained from either a commercial source or freshly prepared according to the method of Pelham and Jackson (17) all contained significant aminopeptidase activity. Cleavage of Phe-AMC was linear with respect to time and lysate concentration. This activity was inhibitable by bestatin (Figure 1) in a dose dependent manner with greater than 95% inhibition of activity at 40 µg/ml. Additionally, 1,10-phenanthroline at 5×10^{-4}M completely inhibited the aminopeptidase activity (data not shown). The wheat germ preparations contained approximately 100-300 times higher levels of aminopeptidase activity as compared with the reticulocyte lysates.

Table 1. Various protease activities[a] present in rabbit reticulocytes and wheat germ lysates.

Source	Aminopeptidase Activity[b]	Chymotrypsin Activity[c]	Elastase Activity[d]	Trypsin Activity[e]
Reticulocyte Lot 0351	40	33	8	0
Reticulocyte Lot 0421	46	30	16	0
Reticulocyte Prep I	41	15	12	0
Wheat Germ Prep I	4,500	2	87	170
Wheat Germ Prep II	11,100	19	72	475

[a]Activity defined as moles of AMC x 10^{-9} released per 10 µl of lysate per min. per ml. Aminopeptidase[c], chymotrypsin[d], elastase and [e]trypsin-like activities were measured with Phe-AMC, Suc-Ala-Ala-Phe-AMC, t-boc-Ala-Ala-Pro-Ala-AMC and CBZ-Gly-Gly-Arg-AMC respectively at a final concentration of 0.2 mM. Enzyme activity was monitored over a 30-min period by measuring the release of AMC in a spectrofluorometer with an excitation of 383 nm and emission at 455 nm.

The wheat germ aminopeptidase activity was 95% inhibited by bestatin at 40 µg/ml.

Chymotrypsin-like Activity:

Reticulocyte and wheat germ lysates were found to hydrolyze Suc-Ala-Ala-Phe-AMC, a known synthetic fluorogenic substrate for chymotrypsin-like enzymes (Table 1). This activity was linear with respect to time and enzyme concentration. Figure 1 shows that low levels of chymostatin (50 µg/ml) inhibited over 95% of the chymotrypsin-like activity present in the reticulocyte lysate. The chymotrypsin-like activity present in the wheat germ lysate was also inhibited by low levels of chymostatin (50 µg/ml).

Trypsin-like Activity:

Wheat germ lysates contained a trypsin-like activity when assayed against CBZ-Gly-Gly-Arg-AMC (Table 1). The trypsin inhibitors antipain (20 µg/ml), leupeptin (20 µg/ml) and L-tosyl-L-lysine chloromethyl-ketone

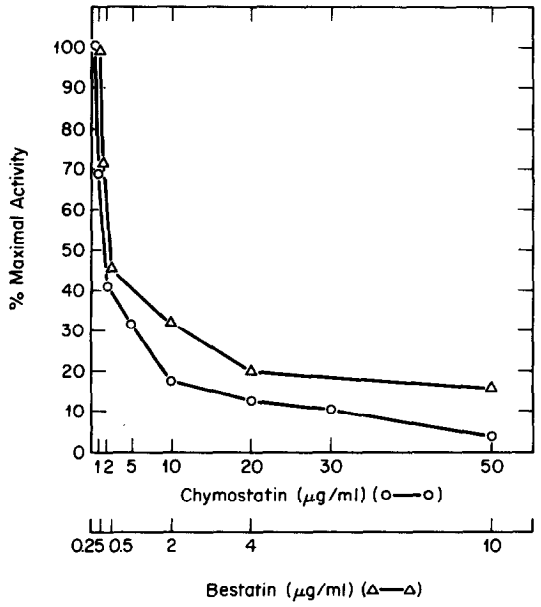

Figure 1. Inhibition of chymotrypsin-like activity and aminopeptidase activity in rabbit reticulocyte lysates by chymostatin (0-0) and bestatin (Δ - Δ), respectively. Incubations were performed 0.5 ml of Tes buffer, pH 7.5, containing either Suc-Ala-Ala-Phe-AMC (0.2 mM) or Phe-AMC (0.2 mm).

(100 μg/ml) completely inhibited this trypsin-like activity (Table II). N-carbobenzyloxy-L-phenylethylchloromethyl ketone (50 μg/ml), L-tosyl-amido-2-phenylethyl-chloromethyl ketone (50 μg/ml) and phenylmethanesulfonyl fluoride (50 μg/ml) inhibited the trypsin activity 75%, 49% and 18%, respectively, while the other inhibitors listed displayed no activity. Reticulocyte lysates had no measurable activity against this substrate.

Elastase-like Activity:

Activity against t-boc-Ala-Ala-Pro-Ala-AMC, a good substrate for both pancreatic and polymorphonuclear leukocyte elastases (19) was found in both lysates (Table 1). In the reticulocyte lysates the activity was linear with respect to lysate concentration and time. Elastatinal inhibited this activity in a dose-dependent manner (90% inhibition at 50 μg/ml). However, when the wheat germ lysate was assayed against the elastase substrate, the hydrolysis was not linear with respect to either enzyme concentration or time. When bestatin (40 μg/ml) was included in the assay a linear hydrolysis rate was

Table II. Inhibition of trypsin-like activity[a] present in wheat germ lysates by various protease inhibitors

Inhibitor	Activity	Con. (μg/ml)	% Inhibition
None	3.3	-	-
Antipain	0	20	100
Leupeptin	0	20	100
TLCK[c]	0	50[b]	100
ZPCK[d]	0.8	50[b]	75
TPCK[e]	1.7	50[b]	49
PMSF[f]	2.7	50[b]	18
Bestatin	3.2	50	3.1
Trasylol	3.3	60	9
Chymostatin	3.4	50	+3
Phosphoramidon	3.8	2	+13
Soybean Trypsin Inh.	4.5	20	+26
PCMB[g]	3.3	50	0

[a]Activity defined as nmoles of AMC x 10^{-9} released per 2 μl of wheat germ lysate per min. per ml. using CBZ-Gly-Gly-Arg-AMC (0.2 mM final concentration) as substrate. [b]Wheat germ lysate and inhibitor were preincubated 30 min prior to the addition of substrate. [c]L-tosyl-L-lysine chloromethyl ketone, [d]N-carbobenzyloxy-L-phenylethylchloromethyl ketone, [e]L-tosylamido-2-phenylethyl-chloromethyl ketone, [f]Phenylmethanesulfonyl fluoride, [g]p-Chloromecuriphenyl sulfonic acid.

obtained (data not shown). Because this substrate was blocked at the NH_2-terminus, an endoproteolytic event was required. Since high levels of aminopeptidase activity were present in the extract, its action on a partially cleaved intermediate might then release free AMC.

To determine whether such a compound(s) was produced, t-boc-Ala-Ala-Pro-Ala-AMC (0.2 mM) and wheat germ lysate (5 μl) were incubated in the presence or absence of bestatin in a final volume of 200 μl and the products analyzed by thin layer chromatography. In the absence of bestatin most of the fluorescence migrated with the same R_f as free AMC (Figure 2, lane 1). However, in the presence of bestatin, the major fluorescent product comigrated with Ala-AMC (Figure 2, lane 2). It should be noted that free AMC has a fluorescent intensity at least 300 times greater than its peptide derivatives, and this should be considered in viewing all photographic figures. The fluorogenic material co-chromatographing on TLC with Ala-AMC was eluted from the plates with methanol, and applied to a C_{18} μ Bondapak

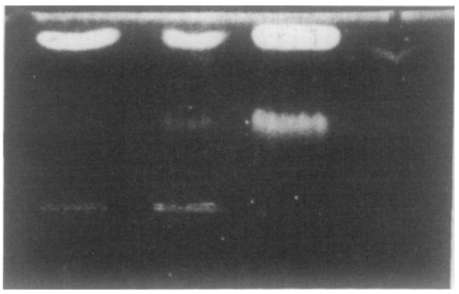

Figure 2. Chromatographic identification of the products obtained after incubation of wheat germ lysate with t-boc-Ala-Ala-Pro-Ala-AMC. Lane 3 (from top to bottom); a standard mixture of AMC and Ala-AMC. Standards were spotted at 5 x 10^{-4}M; lane 1, wheat germ lysate and t-boc-Ala-Ala-Pro-Ala-AMC were incubated in the absence of bestatin and the products were chromatographed; lane 2, wheat germ lysate, substrate, and bestatin (40 µg/ml) were incubated and the reaction products were analyzed (note decreased amount of AMC produced when compared with sample incubated without bestatin); lane 4, boiled enzyme control. Two hour incubation were performed in 1.0 ml of Tes buffer, pH 7.5 containing t-boc-Ala-Ala-Pro-Ala-AMC (0.2 mM) and wheat germ lysate (2 µl).

(Waters Assoc.) high pressure liquid chromatographic column previously equilibrated with methanol/chloroform/H_2O, 10:10:1 (v/v/v). This material had a retention time identical to that of authentic Ala-AMC (data not shown). Together these results showed that at least two peptidases were present in the wheat germ lysates: an aminopeptidase, which was inhibited by bestatin, and an endopeptidase, which cleaved the substrate between the Pro and the Ala residue.

DISCUSSION

In this study we have demonstrated that both wheat germ and rabbit reticulocyte lysates contain significant levels of a variety of protease activities when assayed against synthetic fluorogenic substrates. The wheat germ lysate had considerably higher levels of aminopeptidase and elastase activities than the rabbit reticulocyte lysate. In addition, the wheat germ lysate possessed a trypsin-like activity which was absent in the reticulocyte lysate.

The high protease activities present in the wheat germ lysate compared to those found in rabbit reticulocyte lysates may partially account for the

relatively inefficient translation of various proteins by wheat germ lysates. This might apply particularly to large molecular weight proteins or to any protein which may be unusually susceptible to proteolysis.

Both lysates also contain significant amounts of aminopeptidase(s). These enzymes are likely responsible for the removal of the initiator methionyl residue which occurs during in vitro translation of some pre-proteins (12-16). Since cleavage of the initiator is not universal, it is clear that the subsequent amino acid sequence of the nascent chains determines the ability of the aminopeptidase to cleave the substrate. From sequence data, it appears that amino acids with charged or highly polar side chains in the second position inhibit the methionyl aminopeptidase (13).

Three problems caused by amino peptidase activity in cell-free lysates might be overcome by the inclusion during translation of appropriate inhibitors such as bestatin or 1,10-phenanthroline. First, heterogeneity of NH_2-terminal sequence of the in vitro translation products might be eliminated. Second, the complete products of translation might be more easily identified with certainty (13). Third, co-translational acetylation of amino acids, which presents a problem in determining NH_2-terminal sequence (14), might be easily prevented. These considerations would also apply to in vitro reconstitution of pre-protein processing with microsomal membranes. Since these preparations also contain significant aminopeptidase activity (21,22).

One important consideration that should be taken into account when evaluating the effect of these protease activities concerns the quantitation of mRNA levels by cell-free translation. If the protease activities present in the lysate result in any significant degradation of the in vitro translation products, the measurement of specific mRNA levels by cell-free translation might severely underestimate the absolute level of functional mRNA. Additionally, inefficient translation may be due to proteolytic

degradation of some factor necessary for translation either during lysate preparation or during translation.

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