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FLUOROMETRIC ASSAY OF A CALCIUM-DEPENDENT, PAIRED-BASIC PROCESSING ENDOPEPTIDASE PRESENT IN INSULINOMA GRANULES

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SUMMARY: A novel fluorogenic substrate Cbz-Arg-Ser-Lys-Arg-AMC (RSKR-AMC) was used to characterize Ca^{++}-activated proteolytic activity present in purified insulinoma secretory granules. Secretory granules efficiently cleaved this substrate in a time- and protein-dependent manner; the hydrolysis rate was between 2 and 4 pmol/min/ug of protein, with an apparent K_m of 55 μM. Greater than 90% of the activity against this substrate was dependent on the presence of Ca^{++}, with half-maximal stimulation obtained at 100 μM Ca^{++}. The pH optimum of enzymatic activity was 5.5- 6, and the profile of inhibition by various proteinase inhibitors was similar to that previously described for the type I and II proinsulin processing enzymes. These biochemical characteristics and co-elution of the RSKR-AMC processing activity with the type II endopeptidase activity on anion-exchange chromatography suggest that the new assay selectively detects the Lys-Arg-directed, or type II, proinsulin processing endopeptidase. This fluorogenic assay is more quantitative, sensitive and rapid than methods previously used, and therefore presents a significant improvement for the study of similar Ca^{++}-activated processing endopeptidases.

The bioactive peptides contained within neuropeptide and hormone precursors are released by cleavage at pairs of basic residues. The recent discovery of mammalian subtilisin-like processing endopeptidases within neuroendocrine tissues (1-6) has supported the idea that paired-basic processing enzymes are members of a new proteinase family which are most likely Ca^{++}-dependent, as is the yeast enzyme Kex2 (reviewed in 7,8). Two candidate Ca^{++}-dependent members of this new family are the Arg-Arg-specific (type I) and Lys-Arg-directed (type II) endopeptidases in insulin secretory granules which catalyze proinsulin processing (9).

We have been interested in the development of sensitive and convenient assays for the detection of prohormone and neuropeptide precursor processing enzymes;

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Abbreviations:RSKR, Arg-Ser-Lys-Arg; AMC,aminomethylcoumarin; CBZ,carbobenzoxy; PC, prohomone converting enzyme;PMSF, phenylmethylsulfonylfluoride; TLCK, tosyllysylchloromethyl ketone.

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fluorogenic peptide substrates are often ideal in these respects. However, the use of fluorogenic tripeptide substrates that contain a simple dibasic sequence has not been widely adopted due to extremely low cleavage rates with precursor processing enzymes (10). In an effort to generate substrates which will react with the new calcium-activated subtilisin-like processing enzymes, we have tested the novel fluorogenic substrate Cbz-Arg-Ser-Lys-Arg-AMC (RSKR-AMC). This sequence, which based on homology to Kex2 (11) constitutes the predicted proteolytic site for autocatalytic cleavage of the mammalian Kex2-like protein PC1, is similar to corresponding sites present within PC2 (Arg-Lys-Lys-Arg) and furin (Arg-Thr-Lys-Arg) (1-7). We describe here the use of this substrate in the characterization of a paired-basic processing endopeptidase present in purified insulin secretory granules.

Materials and Methods

Materials: Custom synthesis of the substrate Cbz-Arg-Ser-Lys-Arg-AMC was performed by Enzyme Systems Products (Dublin, CA). AMC standard was obtained from Peninsula Laboratories (Belmont, CA), while proteinase inhibitors were obtained from Sigma Chemical Company (St. Louis, MO).

Preparation of secretory granules: Insulinoma granules were prepared from transplantable rat insulinoma tissue propagated in New England Deaconess rats (12) by a method previously described (9,13). The final insulin secretory granule preparation is essentially free of lysosomal contaminants (13). Isolated granules were stored in aliquots at a concentration of 10-15 mg/ml protein in 0.25 M sucrose/10mM Mes pH 6.0 under liquid nitrogen until use. Separation of the type I and II proinsulin endopeptidases from insulin secretory granule lysates was achieved by anion exchange chromatography as previously described (9,13).

Enzyme Assay: Unless otherwise indicated, enzyme assays were carried out in duplicate or higher number of replicates using 10 ug of insulin secretory granule protein and a final substrate concentration of 250 uM RSKR-AMC (50 ul total volume). The buffer used was 50 mM Na acetate (pH 5.5), containing 5 mM $CaCl_2$, 10 uM pepstatin, 10 uM E-64, 100 uM PMSF and 100 uM TLCK. Incubations were typically carried out for 45 min at 30 C before quenching the reaction with 2.95 ml 1 N acetic acid. Fluorescence was then read at 380 nm excitation, 460 nm emission and compared to a standard curve of free aminomethylcoumarin (AMC). The K_m experiment was carried out by incubating at 37 C duplicate aliquots containing 5 ug secretory granule protein for 2.5 h with five different substrate concentrations (1.0, 0.3, 0.1, 0.03, 0.01 mM); parallel blank reactions were carried out in the absence of enzyme to correct for differential nonspecific hydrolysis of substrate. The maximum amount of substrate consumed was 6%. The processing of [^{125}I]proinsulin by the types I and II endopeptidase was assessed as previously described, with analysis by alkaline polyacrylamide electrophoresis, autoradiography and densitometric scanning (9, 14).

Other Procedures: Protein was determined by the method of Bradford (15) using bovine serum albumin as a standard. Free Ca^{++} concentrations were calculated from reference stability constants as previously described (16).

Results

Purifed insulinoma granules cleaved RSKR-AMC in a linear time and protein-dependent manner (Figure 1). This gave an average rate of RSKR-AMC hydrolysis specific activity of 3.1 $\pm$ 0.2 pmol AMC released/min/ug insulin secretory granule protein (n=6 $\pm$ SE). For optimum sensitivity and convenience, standard assay conditions of 10 ug insulin secretory granule protein/45 min at 30 C were chosen. An apparent K_m for the substrate, 55 uM, was estimated by incubating various concentrations of substrate (0.01 mM to 1.0 mM) with granule protein. Interestingly, inhibition by substrate was clearly present at concentrations of substrate above 1 mM; the hydrolysis rate at 10 mM was only one-third of that at 1 mM (data not shown).

In order to correlate the enzyme activity cleaving RSKR-AMC with type I and II proinsulin processing enzymes, granule lysate was chromatographed on a DEAE anion-exchange column at pH 6.0 and eluted with a 0- 0.4 M sodium chloride gradient (9). The fractions containing type I activity (eluting in the column void volume), and those with type II (eluting between 170- 300 mM NaCl) were desalted (as previously described; 9, 14), separately pooled, and assayed for proinsulin and RSKR-AMC processing activity and total protein content. Only fractions containing the Lys-Arg-directed type II proinsulin processing activity were able to cleave the fluorogenic substrate (Figure 2).

Calcium requirement of enzymatic activity is shown in Figure 3; half-maximal stimulation is observed at 92.1 $\pm$ 11.3 uM Ca^{++} (n=6 $\pm$ SE). The pH optimum of the

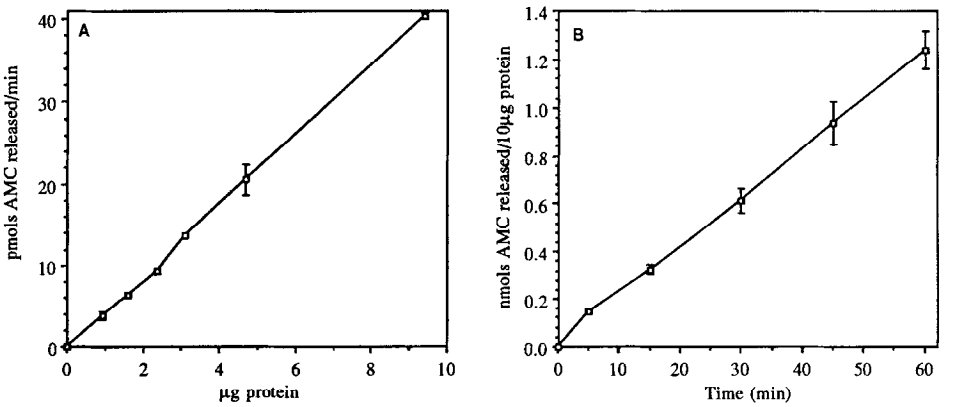


Figure 1. Protein (panel A) and time (panel B) dependence of RSKR-AMC hydrolysis by lysed secretory granules. Reactions were performed as described in Materials and Methods.

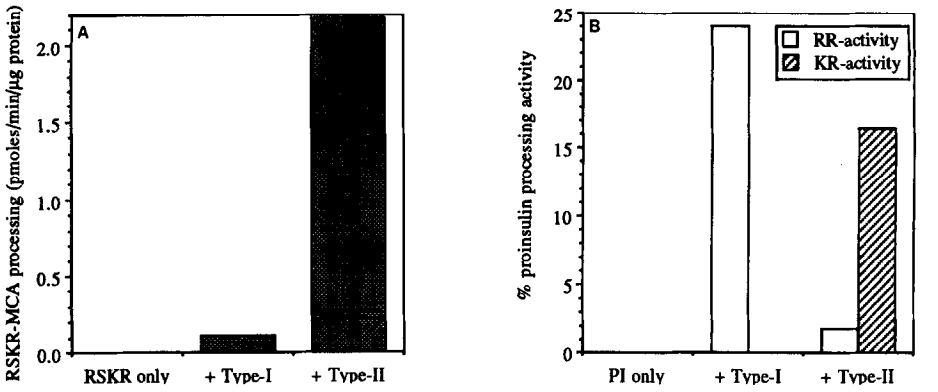
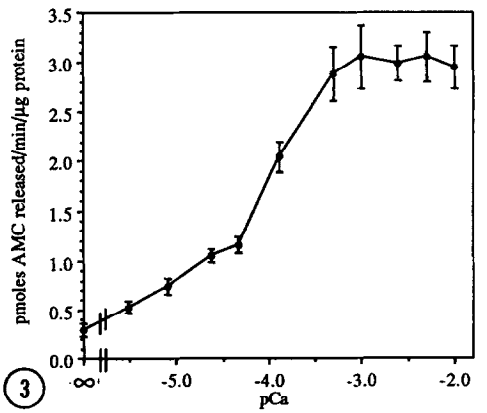


Figure 2. Correlation of RSKR-AMC hydrolysis with proinsulin conversion. Insulin secretory granule lysate was subjected to ion-exchange chromatography and the pooled fractions corresponding to the type I and type II enzyme were used as the source of enzyme for the hydrolysis of RSKR-AMC (panel A) or [^{125}I]proinsulin (panel B).

activity was between pH 5.5-6.0, with a relatively broad range between pH 4.0- 7.5; only 8.3% of the maximal activity at pH 6.0 was detected at pH 8.0 (Figure 4). The profile of inhibition by group-specific proteinase inhibitors is given in Table I. RSKR-AMC-cleaving activity was not significantly inhibited by group-specific metallo, aspartyl or cysteinyl proteinase inhibitors nor by the serine proteinase inhibitor PMSF at 1 mM. This inhibitor profile has also been observed for insulin secretory granule



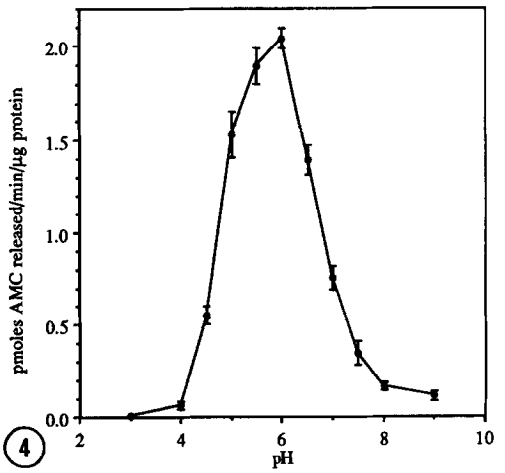


Figure 3. Calcium dependence of enzymatic activity.

Figure 4. pH optimum of enzymatic activity.

Table 1. *The effect of proteinase inhibitors on RSKR-MCA processing by an insulin secretory granule endopeptidase*

Inhibitor (mM)	% Inhibition
PMSF (1mM)	0
Pepstatin A (0.1mM)	0
TLCK (1mM)	13
TPCK (1mM)	0
E-64 (0.1mM)	0
Iodoacetamide (1mM)	8
N-ethylmaleimide (1mM)	0
EDTA (20mM)	78
EGTA (20mM)	59
CDTA (20mM)	75
1, 10 phenanthroline (1mM)	37
p-chloromercuribenzoate (1mM)	67
Hg^{++}	90
Zn^{++}	72
Mn^{++}	42
Cu^{++}	99
Co^{++}	62

RSKR-MCA processing was assessed over 45min at 30°C, as previously described (see Methods). In control samples (with no added extra proteinase inhibitor) 3.4pmoles/AMC/min/μg protein was released from the RSKR substrate. The effect of proteinase inhibitors are expressed as a percent inhibition from the control. Each value is the mean of at least 2 independent observations.

endopeptidase processing of proinsulin (17) and proalbumin (18). The metal ion chelators EDTA and CDTA, but not 1, 10-phenanthroline, did inhibit processing activity; however the EDTA inhibition was effectively overcome by the addition of excess Ca^{++} (or Sr^{++}) and not by other divalent cations (e.g. Ba^{++} and Mg^{++}; Table 2), reaffirming the Ca^{++}-dependency of this endopeptidase activity.

Table 2. *Cation specificity of RSKR-MCA processing by an insulin secretory granule endopeptidase*

Cation (mM)	% Control Activity
Control	100
+ EDTA (5mM)	8
+ EDTA (5mM) + Cu^{++} (10mM)	0
+ EDTA (5mM) + Co^{++} (10mM)	11
+ EDTA (5mM) + Mg^{++} (10mM)	10
+ EDTA (5mM) + Ni^{++} (10mM)	10
+ EDTA (5mM) + Sr^{++} (10mM)	204
+ EDTA (5mM) + Mn^{++} (10mM)	6
+ EDTA (5mM) + Zn^{++} (10mM)	4
+ EDTA (5mM) + Cd^{++} (10mM)	9
+ EDTA (5mM) + Ba^{++} (10mM)	12
+ EDTA (5mM) + Ca^{++} (10mM)	114

RSKR-MCA processing was assessed over 45min at 30°C, as previously described (see Methods). In control samples (in the presence of 5mM Ca^{++}) 3.3pmoles/AMC/min/μg protein was released from the RSKR-MCA substrate. The effect of various cations on regaining RSKR-MCA processing activity after inhibition by 5mM EDTA is expressed as a percent of the control. Each assay is the mean of at least 2 independent observations.

Discussion

At least two Ca^{++}-activated, soluble endopeptidases that cleave radiolabelled proinsulin at paired basic sites are present in insulin secretory granules (9,13,14,17). Type I has a millimolar Ca^{++}-requirement, specifically cleaves C-terminally to Arg_{31}-Arg_{32} of proinsulin, and is not retained on a DEAE-cellulose column at pH 6.0. Type II is half-maximally activated at 100 uM Ca^{++} and is retained on DEAE but can be eluted between 150- 250 mM NaCl (9). Our data indicate that an enzyme with very similar characteristics to the insulin secretory granule type II endopeptidase is responsible for the processing of RSKR-AMC. The insulinoma enzyme cleaved the RSKR-AMC substrate carboxyl-terminally to the Lys-Arg, liberating the fluorescent AMC moiety; the type II enzyme preferentially cleaves carboxyl-terminally to Lys_{64}-Arg_{65} within proinsulin (9). The strongest evidence that the insulinoma enzyme measured with the RSKR-AMC assay corresponds to the type II enzyme are the calcium activation and pH sensitivity curves, which are almost identical to the curves previously published for this enzyme (17). Other data presented here- such as the profile of inhibition by various enzyme inhibitors, the ion dependence, and the fact that the enzyme is retained on DEAE at pH 6- also closely match the characteristics of the type II enzyme (9,13,14,17), clearly establishing the RSKR-AMC-cleaving activity as the type II enzyme. While active type II enzyme has not yet been purified to homogeneity, preliminary data suggest that the type II proinsulin-cleaving enzyme may correspond to the Kex2-like protein PC2 (19).

Analysis of [^{125}I]proinsulin conversion in earlier studies of the type II enzyme was accomplished either by alkaline-urea gel electrophoresis with subsequent densitometric measurements of autoradiographs; or by quantitative HPLC (9,13,14,17). Although useful information was derived from these studies, the assay methods used were technically difficult and inconvenient. A more rapid, convenient and sensitive assay is essential for the further characterization and purification of proprotein endopeptidase activities. We have found that the commercially available fluorogenic substrates Cbz-Leu-Arg-Arg-AMC, Cbz-Leu-Lys-Arg-AMC, and Cbz-Gln-Arg-Arg-AMC (purchased from Peninsula Laboratories, Belmont CA) are not significantly hydrolyzed by lysed insulin secretory granules under conditions similar to those employed here (Rhodes, unpublished observations). It seems likely that both the dibasic sequence as well as additional features of a potential substrate are involved in efficient cleavage by processing endopeptidases. A soluble form of Kex2 has recently been shown to exhibit micromolar K_ms toward several different dibasic fluorogenic substrates, with the best substrate tested corresponding to the natural pentapeptide containing the dibasic cleavage site within the alpha mating factor precursor (11). The substrate used in our study does not correspond to natural cleavage sites within proinsulin or within proenkephalin, precursors known to be cleaved by the type II enzyme (Rhodes and Lindberg, unpublished observations). However, the type II enzyme has not yet been studied using protein substrates containing the RSKR sequence, and this enzyme may

ultimately be shown to prefer- though not require- substrates containing an arginine four amino acids amino terminal to the cleavage site.

In contrast to the previously used densitometric assay of the type II enzyme (radiolabelled proinsulin conversion) the present fluorometric assay is extremely simple and rapid, requiring less than 1 hour incubation before results are obtained; these properties should make the assay useful in the purification of labile enzyme. In addition, the fluorometric assay is more sensitive than the densitometric assay, requiring less protein of the endopeptidase preparation. The apparent K_m of the enzyme for the substrate is reasonably good, enabling routine use of the substrate at low expense. Our findings suggest that the RSKR-AMC fluorometric assay may find wide application in the study of calcium-activated Lys-Arg-directed precursor processing enzymes.

Acknowledgments

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