Final Report for a Grant from the DOE Office of Basic Energy Sciences

Project Title: Understanding and targeting a novel plant viral proteinase/substrate interaction

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The past 3 years of funding have focused our efforts on trying to understand the molecular basis of a unique substrate interaction displayed by a viral proteinase. We have made good progress and during this funding period we have made four contributions to the scientific literature and have developed the application of the proteinase in the expression and purification of recombinant fusion proteins. These advances are summarized in the statement below.

Research Summary

A comprehensive review of virus-encoded proteinases was written during the funding period. In this review, we attempted to emphasize the tremendous similarity of viral proteinases with their cellular counterparts and at the same time detail the unique characteristics which permit them to function in a cellular environment. The article was written with Dr. Bert Semler and appeared in Microbiological Reviews in December, 1993.

The focus of our research effort has been the tobacco etch virus (TEV) 27kDa NIa proteinase. This virus-encoded proteinase is a serine-type proteinase with a remarkable substrate specificity requirment. The enzyme recognizes a cleave sequence that spans a seven amino acid sequence and cleaves between a glutamine and serine or a glutamine and glycine. We were also successful in expressing the tobacco etch virus 27kDa nuclear inclusion a (NIa) proteinase in E. coli as a recombinant fusion protein containing a seven histidine tag at the amino terminus. Catalytically active and inactive (by virtue of a single amino acid change) forms of the proteinase were purified to homogeneity in a two column chromatographic procedure. The active form of the proteinase was slowly converted to a 25kDa lower molecular weight form, while the inactive form was not. This conversion was dilution independent. Isolation of the 2kDa peptide product released and the determination of its N-terminal amino acid sequence positioned the cleavage site twenty-four amino acids from the carboxy-terminus of the proteinase. A purified recombinant NIa proteinase lacking this sequence was expressed and

possessed some activity. Kinetic analyses of the cleavage of a synthetic peptide by the full length or truncated proteinase were conducted and indicated that the k_m of the truncated proteinase was approximately fourfold higher than that of the full length form. The full length proteinase was approximately twenty fold more efficient in proteolysis of the test peptide substrate than the truncated form.

We were not able to detected differences in NIa proteinase activity whether the activity was associated with the 27kDa protein or was part of the 49kDa NIa polyprotein, or a higher molecular weight polyprotein. This is not the case with other extensively studied viruses. During poliovirus polyprotein processing, the protein containing the proteinase activity is critical. Most cleavage sites can be processed by the activity associated with the 3C protein. However, a selected poliovirus P1 polyprotein cleavage requires the presence of the proteinase in a 3CD precursor form and not the 3C protein alone. Cowpea mosaic virus has also been shown to require an 'accessory protein' that modulates cleavage by the 24kDa proteinase at particular polyprotein sites. Therefore, it would appear that there is less of a constraint on the TEV NIa proteinase and the protein form it is in. This may be due to other distinguishing features of the NIa proteinase. NIa activity may be regulated by partitioning it in different cellular compartments and/or its extensive substrate requirement may preclude the need for these other contextual constraints.

The C-terminal 24 amino acid segment (=2kDa peptide) of the NIa proteinase also corresponded to a unique domain possessed by potyvirus NIa proteinases and not observed in other viral or cellular proteinases. We have speculated that this sequence may be critical in providing structure or sequences needed for the exquisite degree of substrate specificity displayed. A comparison of Km values between truncated and full length protein shows a slight decrease in substrate affinity while a larger difference is noticed when comparing Vmax. It may be with the extended substrate specificity displayed by potyviral NIa proteinases, there is not only a requirement for binding, but these proteinases also need to perform an additional step to release the cleaved products. The C-terminus, may provide this function. Other proteinases have substrate cleavage sites that are less extensive and are more like cellular proteinases in their requirements and hence lack this additional domain. This study is reported in an article that will appear in the journal Virology.

The unique specificity of this proteinase and the ability to purify a recombinant form of the proteinase to homogeneity made it an excellent candidate as a proteinase that could be used to cleave recombinant fusion proteins generated in E. coli. We developed an improved method for the production, cleavage, and purification of fusion proteins and peptides. A recombinant TEV proteinase produced with a polyhistidine tract positioned at

the amino terminus was used. The proteinase recognized a specific, extended cleavage site sequence. The peptide or protein of interest was purified as a fusion protein with a TEV proteinase cleavage site sequence located between it and an affinity carrier portion of the fusion protein. Incubation with the recombinant TEV proteinase mediated the release of the peptide or protein of interest. Use of the recombinant TEV proteinase to cleave fusion proteins was an improvement over other proteinases for several reasons: its high degree of specificity, its insensitivity to many proteinase inhibitors generally used in protein purification, and the ready separation of both the affinity tag and the proteinase from the cleaved product of interest. The system was described in an Analytical Biochemistry article. The expression system has been licensed to Gibco/Life Technologies and is the focal point around which they have developed their commercially available pPro-Ex expression system. This expression system is highlighted in a technical article that will appear in

Finally, our collaborators have been unsuccessful in obtaining crystals of the recombinant TEV proteinase. The purified protein can be kept soluble, at high concentrations (20mg/ml) and will nucleate forming small crystals. However, crystallization is terminated by the apparent incorporation of a dimerized form of the protein. We have been unable to prevent the formation of this dimer and with the termination of this project, our future attempts to crystallize this protein will not be continued.