mRNA'S FOR CHYMOSIN AND PEPsin, TWO MAIN ASPARTIC PROTEINASES OF BOVINE STOMACH AND ANALYSIS OF THEIR TRANSLATION PRODUCTS

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Introduction

Both chymosin and pepsin represent mammalian degradative enzymes whose role is to digest nutrient proteins and which may be regarded as major types of gastric proteinases. These enzymes are secreted as inactive precursors, prochymosin and pepsinogen, which are irreversibly converted into the active enzymes through limited proteolysis resulting in a final loss of the 42 amino-terminal residues of the zymogens (1). Both zymogens being secretory eukaryotic proteins are already processed primary products of gene expression (2,3) coded for as preprochymosin and prepepsinogen. For the important role of these enzymes in metabolism as well as for their uses in the food industry, namely in cheese making (chymosin as the main proteolytic enzyme in calf cheese rennet and pepsins of different origin as its frequent substitutes), and in molecular biology as a possible model for gene structure research and its expression and evolution (4), they are of great interest for those working in gene manipulation and gene engineering fields.

mRNA'S are often the only starting material for cloning of eukaryotic genes and suitable and simple analytical methods for determination of their translation products are important prerequisites of functional characterization of the isolated messengers as well as of expression products of the cloned
genes. In this paper we present comparative characteristics of mRNA's for chymosin and pepsin and of their translation products.

Results

Isolation of poly(A)$^+$RNA

The preparation of poly(A)$^+$RNA from the fundal region of the fourth calf or bovine stomach was performed by two methods giving identical results: either by hot phenol extraction (5) or using guanidine thiocyanate (6), followed in both cases by separation on a poly(U)-Sepharose column (7). Poly(A)$^+$RNA was then fractionated by sucrose gradient centrifugation and the fractions obtained were examined for their translation activity in cell-free systems (8). Maximum translation activity for preprochymosin and prepepsinogen, determined in the reticulocyte lysate, was present in fractions of the respective mRNA corresponding in both cases to 15S. Using urea-polyacrylamide gel electrophoresis, the molecular weights of both mRNA's calculated from the relative migration distances (Fig. 1) corresponded to 16S. A slight difference in the molecular weights estimated by these methods has also been described by other authors, who worked with different systems; this difference can be accounted for by various levels of secondary structure of the ribosomal RNA's used as size markers and mRNA's themselves (9). The estimated average sizes of the mRNA's for preprochymosin and prepepsinogen are in a good agreement with the chain length of the coded molecules of the correspondingzymogens and in the case of preprochymosin they have also been confirmed by others (10,11). As shown in Fig. 2, the differences between the mRNA's from two parts of the stomach (fundal and pyloric) of one individual are greater than those between the mRNA preparations from the fundal parts of the stomach of the adult animal and calf. So far, nothing is known about
Figure 1. Comparison of poly(A) RNA's from calf and bovine stomach by urea-polyacrylamide gel electrophoresis (8M urea in 3.5 % gel). 1 - mRNA from bovine stomach, 2 - fraction of 2 with maximum translation activity for prepepsinogen, 3 - mRNA from stomach of calf fed milk and hay, 4 - fraction of 5 with maximum translation activity for prechymosin, 5 - mRNA from stomach of calf fed milk only.

further products, if any, which could be coded for by mRNA fractions found on the gels (Figs. 1 and 2).

Translation products synthesized in cell-free systems and in oocytes

Cell-free systems, when programmed with mRNA for a secretory protein, synthesize precursors extended at their amino termini by the signal peptide usually without any processing (12-15). Xenopus oocytes, on the other hand, after being microinjected mRNA coding for a secretory protein perform translation, processing and secretion of the correct translation products - zymogens (16-18). Hence, the translation systems performing synthesis of proteins under direction of exogenous mRNA in
Figure 2. Urea-polyacrylamide gel electrophoresis (as in Fig. 1) of poly(A)^+mRNA from fundal (left) and pyloric (right) part of bovine stomach.

In vitro (reticulocyte lysate and wheat germ system) (19) and in vivo (Xenopus laevis oocytes) were used for further characterization of the isolated mRNA's and of their products (Fig. 3).

Milk-clotting activity of translation products

It has been reported that mRNA's injected into oocytes were translated into biologically active products (17,23-25). We tried, therefore, to demonstrate the enzymatic activity of chymosin by using the milk-clotting assay. We tested the milk-clotting activity of translation products accumulated in the incubation medium using the simple microplate assay of Emtage (26). As shown in Fig. 4 the activated incubation medium (acidification to pH 3.0 followed by neutralization to pH 6.3), surrounding oocytes microinjected with preprochymosin mRNA, contained proteins with milk-clotting activity (Fig. 4A, lanes
Figure 3. Fluorograms (20) of translation products synthesized in reticulocyte lysate, wheat germ system, and in oocytes, immunoprecipitated (21) and separated by SDS-PAGE in gradient gel (7-15%). A - translation of preprochymosin mRNA: 1 - reticulocyte lysate control, 2 and 3 - reticulocyte lysate, 4 and 5 - oocyte extract, 6 - oocyte incubation medium, 7 - wheat germ system (no immunoprecipitation), 8 and 9 - wheat germ system, 10 - marker proteins of M_r 90 000, 69 000, 46 000, 30 000 and 14 000, respectively (from top to bottom); B - translation of prepepsinogen mRNA: 1 - reticulocyte lysate, 2 - oocyte extract, 3 and 4 - oocyte incubation medium, 5 - oocyte extract control.

4 and 5), whereas no such activity was detected in the medium after incubation of oocytes injected with water only (Fig. 4A, lane 3). Similarly, oocytes injected with prepepsinogen mRNA exported proteins clotting milk (Fig. 4B, lane 4) and oocytes...
Figure 4. Milk-clotting assay of prochymosin and pepsinogen. A microplate with clotted milk in the wells is shown. From left to right the samples in the wells were diluted gradually 1:1 with water. A - prochymosin: 1 - calf chymosin, 2 - calf prochymosin after activation, 3 - oocyte incubation medium control (48 h), 4 - oocyte incubation medium 48 h after injection of mRNA, 5 - oocyte incubation medium 96 h after injection of mRNA; B - pepsinogen: 1 - oocyte incubation medium control (48 h), 2 - chymosin, 3 - swine pepsinogen (after activation), 4 - oocyte incubation medium 48 h after injection of mRNA.

Injected with water were deficient in this activity (Fig. 4B, lane 1).

Proteolytic activity of products synthesized in oocytes
Another experimental approach to the detection of enzymatic activity of proteinases synthesized in oocytes is the demon-
stration of their proteolytic activity directly on the separating gel using our specific modification of the Miskin and Soreq procedure (27). Casein (0.1 %) is embedded as the specific protein substrate in the polyacrylamide gel which is used for SDS-PAGE separation of the oocyte translation products. After renaturation of the separated proteins by washing off SDS from the gel (27) the latter is incubated at pH 3.0 for specific activation of acid proteinases and proteolysis of the present substrate. Staining of the treated gel with Coomassie brilliant blue then visualizes white spots in every place of the gel where the acidic proteinase has split the embedded casein. As demonstrated in Fig. 5 the in situ estimated proteolytic activity corresponds to enzymatic functions of the protein activated products which were synthesized in oocytes under specific direction by the microinjected preprochymosin mRNA isolated from calf stomach. This analytical method could similarly be used for the detection of the proteolytic activity of the product whose synthesis is dependent on the corresponding gene cloned in a heterologous host.

All the data presented in this paper show that the isolated poly(A)^+ RNA's represent fully functional mRNA's for preprochymosin and prepepsinogen and can be translated and processed into the biologically active enzymes.
Figure 5. Detection of proteolytic activity of chymosin in gels after SDS-PAGE of oocyte translation products. The procedure is explained in the text. 1 - prochymosin standard, 2 - oocyte incubation medium 48 h after injection of mRNA, 3 - oocyte incubation medium 96 h after injection of mRNA, 4 - oocyte extract 48 h after injection of mRNA, 5 - oocyte incubation medium control (48 h), 6 - prochymosin standard.

References


