Estimating protein synthesis by measuring the amounts of active translational elongation factor-2 in tissue samples from mink*)

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Summary

Estimating protein synthesis can be very difficult and expensive. One way of circumventing some of these problems is to measure the amount of translational elongation factor-2. The rationale behind such a measure is because this enzyme is involved in the synthesis of all proteins, including itself. Elongation factor-2 can be ADP-ribosylated by the action of diphtheria toxin and this reaction is stoichiometric and very specific. Here it is shown that measuring the amount of ADP-ribosylatable eEF-2 may be used as a fast and reliable indirect measurement of protein synthesis capacity in three different tissue types from mink. The assay is repeatable with a coefficient of variation around 10% when testing the same sample repeatedly. Furthermore, the assay is very sensitive, relatively cheap and a trained person can perform several dozen tests in a working day.

Introduction

Fur producers must be interested in research methods for estimating protein synthesis, because this process is of paramount importance for the production. This is because protein synthesis is the basic process in both creating the skin and ensuring that the skin is of superior quality. However, measuring protein synthesis activities is not a simple task, and such measurements can be very expensive, technically difficult to perform and often unreliable. Therefore, many indirect measurement methods for protein synthesis have been devised, but all have drawbacks: Some are very labor consuming, some rely on externally added labile RNAs and others are sensitive to ion concentrations and very difficult to repeat (*Nygård and Hultin, 1979; Weikard et al, 1992; Lipton and Raley-Susman, 1999*).

This work was performed to test whether a measurement of the protein synthesis translational elongation factor-2 (eEF-2) is a way of circumventing some of the mentioned drawbacks in relation to indirect measurements of protein synthesis rates in mink. The logic behind measuring the amount of active eEF-2 is that this enzyme participates in the synthesis of all other proteins, including itself (*Riis et al, 1990a*). Therefore, this assay is an indirect measure of the protein synthesis capacity in a sample. The test is based on a very specific enzymatic reaction, where an ADP-ribosyl moiety from a radioactively labeled NAD^+ is transferred stoichiometrically to eEF-2 in a reaction catalyzed by diphtheria toxin. Several cellular transferases are also known to utilize NAD^+ as a co-factor for the ADP-ribosylation of various proteins, but these reactions can be inhibited by addition of histamine (*Gill and Dinius, 1973*). Because eEF-2 cannot be ADP-ribosylated while bound to the ribosomes they were dissociated, and reassociation was prevented by addition of high concentrations of NH_4Cl (*Riis, 1989*). Combined, this approach gives an accurate estimate of the total content of ADP-ribosylatable eEF-2, thus giving an indirect estimate of the protein synthesis activity. One further advantage of this method is that several dozen tests can be performed in one working day. Besides, the assay is cheap, reliable and highly reproducible. However, the hazards in handling diphtheria toxin should be investigated before any attempt to use it (*Pappenheimer, 1977 and Gill, 1982*).

Materials and methods

Animals and tissues

All tissue material was collected from Scanbrown mink kits 30-31 days old, bred and raised at the Experimental Fur Animal Farm situated at the Danish Institute of Agricultural Sciences in Foulum, Denmark. The mink were killed by injections of lethal doses of barbiturates, and all tissue material was removed from the animal shortly after its death. All work was performed in full compliance with all ethical and legal rules. The tissue samples were stored frozen at –20 or –80^{0}C, until the described manipulations were performed.

Homogenization of tissue

The different tissue types were homogenized using one part tissue to ten parts of buffer A (20 mM Tris/HCl pH: 7.6, 20 mM KCl, 0.2 mM PMSF and 0.25 M sucrose) and keeping the temperature between 0 - 4^{0}C. Liver tissue was homogenized in a glass/glass Dounce homogenizer using seven strokes of both pistil A and B. Muscle tissue was homogenized by blending in a laboratory blender followed by the use of a glass/glass homogenizer as described above. Skin tissue was homogenized as muscle tissue, after the hairs had been removed by shaving.

Total protein determination

Total soluble protein was determined using the Bradford method *(Bradford, 1976*). A commercial protein assay kit (Protein Assay™, BioRad) was used employing BSA as the standard.

Diphtheria-Toxin-mediated eEF-2 Assay

This assay measures the content of ADP-ribosylatable eEF-2 in cell-free extracts of mink tissue samples. All materials, rotor heads, tubes, buffers etc. were pre-cooled to between 0-4^{0}C, if not otherwise specified. The cell-free extract was prepared by a method depending on the origin of the sample. The total protein content was adjusted to between 1.0 to 2.0 mg/ml by dilution with buffer B (25 mM Tris/HCl pH: 8.0, 25 mM KCl, 0.5 M NH_4Cl, 10 mM $Mg(CH_3COO)_2$, 14.4 mM β-mercaptoethanol). The extract was shaken with 4-8% (w/v) activated charcoal to remove endogenous NAD. The charcoal was removed by centrifugation (5 min at 20,000 x g). The supernatant was transferred to another tube and centrifuged again (5 min at 20,000 x g) to ensure the removal of all charcoal. The supernatant was used for protein determination and for the assay. The assay was performed in a final volume of 50 µl in buffer B containing 5 µg of diphtheria toxin, 0.18M histamine, 0.5 M NH_3Cl and 1.2 µM [^{14}C]NAD^+

(specific activity 11 GBq/mmole, Amersham, U.K.). The reaction was started by incubating the assay mixture at 37^0 C. The background was estimated using a similar protocol without the addition of diphtheria toxin. All tests were performed in triplicate. The reaction was stopped by adding 1 ml ice-cold 10% trichloroacetic acid (TCA). The mixture was filtered through a nitrocellulose filter with pores of 0.45 µm. The filter was rinsed twice with 1 ml ice-cold 10% TCA followed by one ml of H_2O under vacuum, dried and dissolved in scintillation liquid (Highsafe III, Wallac OY, Finland). The radioactivity was determined using a LKB liquid scintillation counter. Background was routinely determined and subtracted. Non-trained persons are advised against trying to perform the described assay due to the toxicity of diphtheria toxin.

Results and discussion

The present procedure measuring the amount of protein synthesis translational elongation factor-2 using diphtheria toxin catalyzed transfer of ADP-ribose moiety is a fast and reliable assay. The assay is capable of testing the amounts of active eEF-2 from samples containing less than 7 µg total soluble protein in a reliable and repeatable manner (Fig. 1). This ensures that samples obtained by skin biopsies can be tested for the content of active eEF-2. A further advantage of the assay is that it is relatively cheap. The eEF-2 assay can be performed at expenses for material of around one US dollar per sample, and several samples can be tested per hour. This ensures that many samples can be tested in one working day. The shortest practical time span for incubating the assay mixture is 20 min (Fig. 2). If the samples are incubated for a shorter time the end point of the assay is not reached, and if the incubation period is prolonged no more eEF-2 molecules are ADP-ribosylated. Prolonging the incubation time for more than one hour will result in a decreased amount of ADP-ribosylatable eEF-2 in the sample, probably due to protease degradation of the factor (data not shown). However, this situation should be avoided, and the incubation time should be standardized to 20 min in order to obtain reliable and reproducible results. Earlier work has shown that the used concentration of salt is sufficient to dissociate the eEF-2 from the ribosomes, thus assuring the measurement of all the ADP-ribosylatable eEF-2 molecules present in the sample (*Riis et al., 1989*). The amount of histamine added to the assay mixture is sufficient to ensure that the background is low by inhibiting the cellular poly(ADP-ribosyl)transferases (data not shown). Repeating the eEF-2 assay on the same sample shows that the coefficient of variation (C.V.) is 8.3 %, 8.5% and 10.7 % on samples from muscle, skin, and liver, respectively (Fig. 3).

Employing the described assay on different tissue types from three mink kits showed that the amount of eEF-2 varies in the tested tissue. The lowest content of eEF-2 was found in muscle tissue obtained from the femoral muscle of the kits. The second lowest content was detected in the skin. The highest amount was found in the liver tissue (Fig. 4). *Ad hoc* this is no surprise. Liver tissue is known to have a large capacity of protein synthesis and this tissue is also very active in creating proteins used in the steady-state maintenance of the animal. It is also of no surprise that the lowest amounts are found in muscle tissue. In tissue from large muscles from cattle no eEF-2 can be detected (data not shown), most likely because the protein turnover in such tissue is very low. The skin contained an intermediary amount of active ADP-ribosylatable eEF-2, probably reflecting an intermediary protein synthesis activity in this tissue. The protein synthesis capacity is larger in skin than in muscles. Therefore, eEF-2 can be detected both in muscle and in skin, because both tissues are undergoing fast development in young kits. Previously the assay has been used in studies of human tissue cultures (*Riis et al, 1990b*) and liver samples from rats (*Riis et al, 1993*). Furthermore, it is also known that the eEF-2 is a point where regulation of protein synthesis can be performed (*Riis et al, 1990a and Riis et al, 1993*).

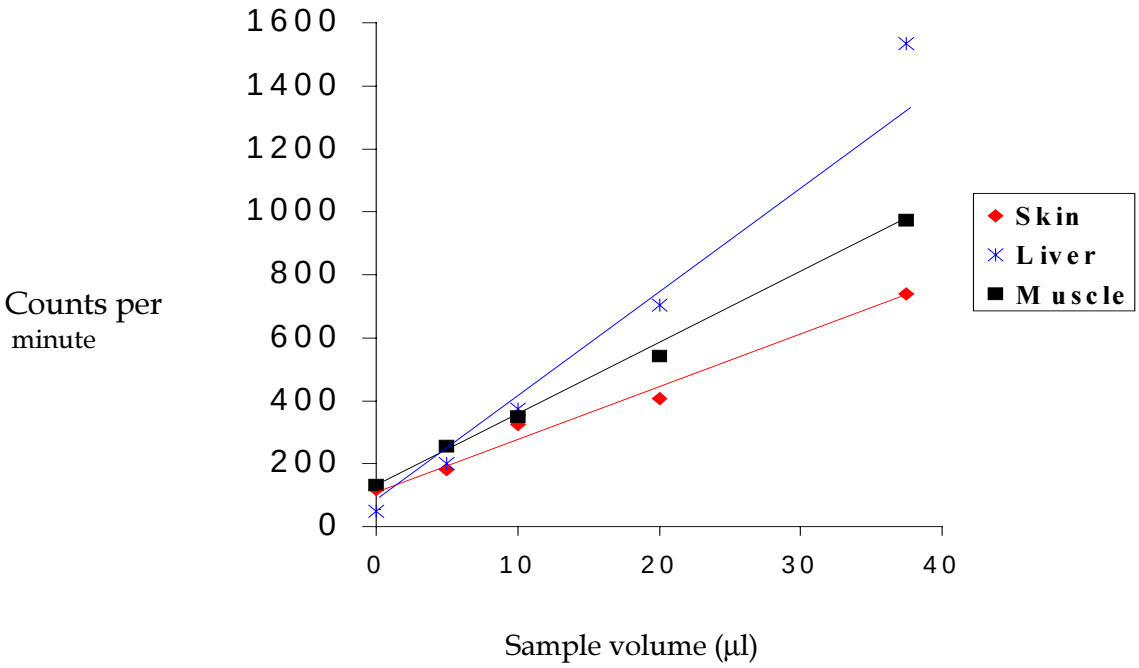

Figure 1. The assay is providing a linear response in the range used.

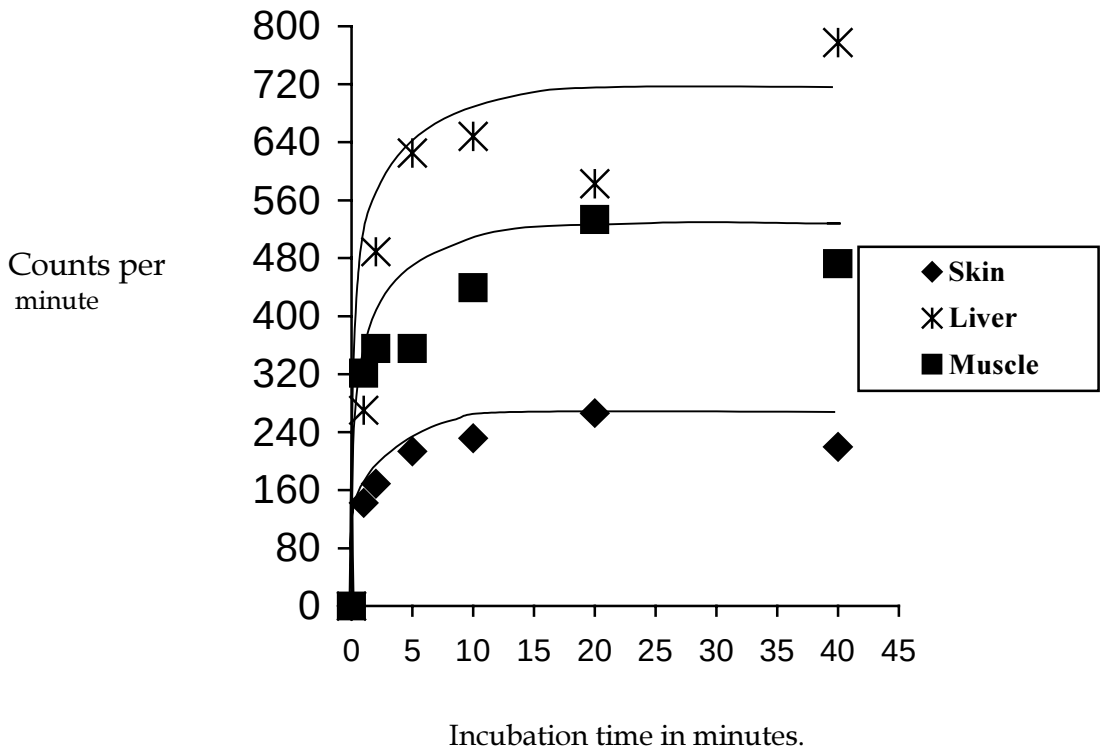

Figure 2. Time response to the incubation time.

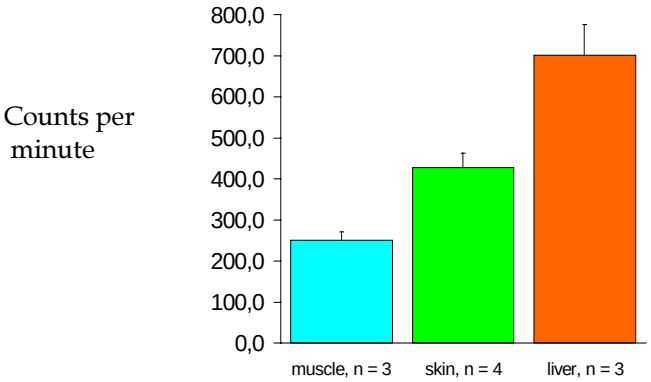

Figure 3. Averages and standard deviations of repeats on the same sample.

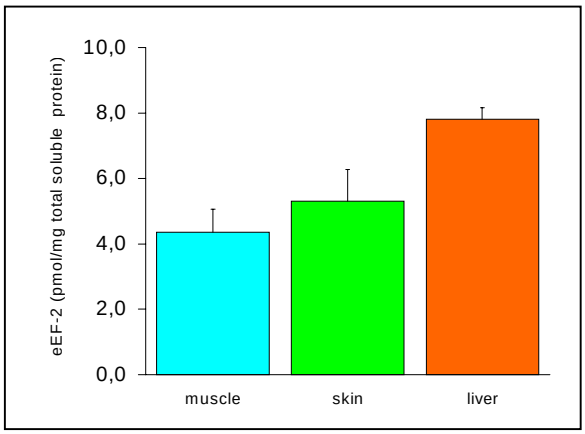

Figure 4. Content of eEF-2 in muscle, skin and liver tissue from three mink kits. Averages and standard deviation are shown (picomol pr. mg soluble protein).

Conclusions and perspectives

The present work shows that the diphtheria toxin-mediated eEF-2 assay is a reliable way to make an indirect estimate of protein synthesis rates in various tissue types from mink. It is shown that the method is linear in the ranges used, and that an incubation time of 20 min is sufficient to ensure that the end point of the assay has been reached. Furthermore, very small samples containing less than 10 µg of total soluble protein can be tested. Samples obtained by skin biopsies are therefore sufficient to perform the eEF-2 assay. Potentially, this will allow the present test to be used in feeding experiments or in breeding programs, if the purpose is to select animals with efficient turnover of nutrients or a high protein synthesis capacity.

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