Effect of Age and Food Restriction on Alkaline Protease Activity in Rat Liver

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The effect of age and food restriction on the hepatic alkaline protease activity of 100,000 × g supernatant has been investigated using 7-, 16-, and 26-month-old Fischer 344 rats. The proteasome, a major component of alkaline protease activity, is activated by sodium dodecyl sulfate (SDS) and this property was exploited to gain insight into the effects of age and food restriction on proteasome activity. Three alkaline protease activities, chymotrypsin-like (ChT-L), trypsin-like (T-L), and peptidylglutamyl peptide hydrolyzing (PGPH) activities were measured. These activities are also commonly used as measurement of proteasomal activities. Basal ChT-L and PGPH activities were not markedly altered by either age or food restriction. The level of T-L activity did not change with age, but was decreased by food restriction. SDS-activated ChT-L activity increased 15% between 7 and 26 months of age and this increase was blocked by food restriction. SDS-activated PGPH activity decreased 40% and the decrease was ameliorated by food restriction. In conclusion, we have shown that the alteration of alkaline protease activities by age and food restriction is not uniform and that the changes observed are likely due to alterations of proteasomal activity. The lack of uniformity in these alterations indicates that any assessment of alkaline protease activity requires the measurement of more than one of the enzymatic activities. Lastly, the first evidence suggesting that age and food restriction can modulate proteasomal activity is presented.
Preparation of cytosolic fraction. — Rat liver was homogenized in 50 mM tris[hydroxymethyl]aminomethane/HCl (Tris/HCl), pH 8.0, containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 1.0 mM 2-mercaptoethanol using a Dounce homogenizer (4 strokes with the A pestle, 4 strokes with the B pestle). The homogenate was then centrifuged at 100,000 x g for 60 min to collect the cytosolic fraction (Tanaka et al., 1986). All procedures were carried out at 4 °C. All assays were carried out in duplicate and the samples to be assayed on a given day were randomized.

Assay of alkaline protease activity. — The reaction mixture (total volume, 100 μl) contained 100,000 x g cytosolic supernatant protein, 100 mM Tris/HCl, pH 8.0, and varying concentrations of both fluorogenic substrates (Sigma, St. Louis, MO) and SDS (Sigma, St. Louis, MO). The fluorogenic substrates were used in the following concentrations: N-succinyl-Leu-Leu-Val-Tyr-4-methyl-coumaryl-7-amide (LLVY-AMC), 200 μM (ChT-L); N-t-Boc-Leu-Ser-Thr-Glu-B-naphthylamide (LLE-2NA), 200 μM (PGPH). Based on the results of pilot studies, the mixtures were incubated for 20 min (LLVY-AMC) or 30 min (LSTR-AMC and LLE-2NA) at 37 °C. The reaction was stopped by adding 0.5 ml of 1% SDS and 1.5 ml of 0.1 M sodium borate, pH 9.1 (LLVY-AMC), or 0.5 ml of 1% SDS and 1 ml of 0.1 M sodium borate, pH 9.1 (LSTR-AMC and LLE-2NA) (Tanaka et al., 1986). The peptidase activity was determined fluorometrically by measuring the release of 4-methyl-coumaryl-7-amide (AMC) (excitation 370 nm, emission 430 nm) and β-naphthylamine (excitation 323 nm, emission 400 nm). The fluorescence was measured using a Perkin Elmer LS-50 luminescence fluorometer.

SDS activation. — SDS activation curves were established by measuring alkaline protease activity in the presence of increasing concentrations of SDS ranging from 0 to 1% using 0.0025% increments. The maximal activation of ChT-L and PGPH activities was obtained as a plateau between 0.0425 and 0.0525% SDS. Two points, 0.0475% and 0.05% SDS, were chosen and the highest peptidase activity value taken as maximal activity.

Protein determination. — Protein concentration was determined by the Bradford method (Bradford, 1976) using BioRad reagent with bovine serum albumin as standard.

Statistical analysis. — The data were analyzed with SuperAnova software (ABACUS Concepts Inc., Berkeley, CA) using Duncan New Multiple Range analysis. The significance level was set at .05.

RESULTS AND DISCUSSION

As stated above, the proteasome appears to be the most important component of cellular alkaline protease activity. At the present time ChT-L, T-L, and PGPH activities are most commonly used as indices of the proteasome activity (Wilk and Orlowski, 1983; Rivett, 1989; Tanaka et al., 1989). While the relative contribution of proteasomal ChT-L, T-L, and PGPH activities to total cellular ChT-L, T-L, and PGPH activities has not been clearly defined, their common usage, in combination with the commercial availability of fluorogenic substrates, made them the alkaline protease activities of choice for this investigation.

As can be seen in the lower portion of Figure 1, basal ChT-L activity is unchanged through 16 months of age, after which there is a small but significant increase in activity at 26 months of age. Food restriction maintains a constant basal activity throughout life, lowering activities in comparison to ad libitum group as well as blocking the increase seen in the old ad libitum fed group. SDS-stimulated ChT-L activity, upper portion of Figure 1, increases with age, with the activity at 26 months of age being approximately 15% greater than that measured at 6 months of age. Food restriction completely blocks this age-related increase, maintaining a constant level of ChT-L activity throughout life. There were thus two unexpected observations arising from these data. Firstly, aging is generally associated with either unchanged or decreased levels of enzyme activity, and we have found an age-related increase in both basal and SDS activated ChT-L activity. If ChT-L activity represents an important component of the cellular alkaline protease activity, this finding would not be consistent with the concept that alkaline protease activity declines with age (Starke-Reed and Oliver, 1989). Secondly, among the enzymes that exhibit age-related changes, ChT-L appears to be the most important component of cellular alkaline protease activity.
related declines in activity, food restriction, in general, tends to maintain these enzymes at higher levels of activity. In this case, food restriction appears to block the age-related increases in ChT-L activity.

The basal T-L activity did not change with age in the ad libitum fed group (Figure 2). Food restriction, in this case, resulted in a lower T-L activity at each age, and there also appears to be an additional, further decline related to age (Figure 2). T-L activity was not activated by SDS; in fact, SDS, in concentrations ranging from 0 to 0.1%, produces a progressive inhibition of the enzyme (data not shown). This inhibitory effect has also been reported by other laboratories (Ozaki et al., 1992; Ugai et al., 1993). While the data suggest that cytosolic T-L activity is modulated by food restriction, the inability to demonstrate an activation by SDS prohibits drawing any conclusions concerning proteasomal T-L activity under these experimental conditions.

The basal levels of PGPH activity were not affected by either age or food restriction (lower portion of Figure 3). In contrast to the data obtained on SDS activation of ChT-L activity, SDS-stimulated PGPH activity exhibits an age-related decline, approaching a 40% decrease between 7 and 26 months of age (upper portion of Figure 3). With food restriction, the rates are approximately 30% higher at 7 months of age increasing to 60% greater at 26 months of age. Thus, food restriction enhances the SDS-stimulated PGPH activity and modulates the age-related decline such that the level of activity in the 26-month-old restricted animals is nearly equivalent to that of the 7-month-old ad libitum animals. These responses are more in keeping with the generally expected actions of age and food restriction on enzymatic processes.

We began the investigation with the assumption that all of the alkaline protease activities would respond in the same manner, i.e., a general decrease with age. This assumption was based on the reports that the age-related accumulation of oxidatively damaged proteins appears to be due to a decline in alkaline protease activity (Starke-Reed and Oliver, 1989; Stadtman, 1992). The rationale for measuring proteasome associated alkaline protease activities was based on the observations that (a) the proteasome is the major component of cellular alkaline protease activity (Wilk and Orłowski, 1983; Rivett, 1989; Ugai et al., 1993) and (b) it has been reported that the degradation of oxidatively damaged proteins is mediated by the proteasome (Rivett, 1985; Pacifici et al., 1989).

While there were relatively minor alterations in basal activities, in the presence of SDS there were marked changes in ChT-L and PGPH activities in response to both age and food restriction. SDS-stimulated activities are most likely associated with the proteasome since the property of SDS activation appears to be unique to the peptidase activity of this complex (Wilk and Orłowski, 1983; Ugai et al., 1993). Consistent with the reported decline in alkaline protease activity, SDS-stimulated PGPH activity declines with age. Contrary to expectations, SDS-stimulated ChT-L activity increased with age. This finding demonstrates that the alkaline protease system is more complex than initially expected and that the assessment of one activity cannot be considered to represent the system as a whole. In addition, if the age-related accumulation of oxidatively damaged proteins is due to a decline in proteasomal activity, it would raise the possibility that PGPH activity may be a key or rate-limiting activity for proteasomal protein degradation. In this regard,
it should be noted that in yeast PGPH activity appears to be relatively insignificant in comparison to ChT-L activity (Heinemeyer et al., 1991; Hilt et al., 1993). Whether direct differences for mammalian cells can be drawn from observations in yeast is not clear at the present time and resolution of the question awaits the elucidation of the sequential pathway of protein degradation in the mammalian proteasome.

The observations on the effects of food restriction on alkaline protease activities are of particular interest. Food restriction, while not preventing the age-related decline in SDS-stimulated PGPH activity, maintains higher levels of activity throughout life. This is the type of effect that would be generally expected in response to food restriction. In contrast, the age-related increase in SDS activated ChT-L activity was blocked by food restriction. While no mechanistic explanation is currently available, it raises interesting questions about the mechanism of action of food restriction by which it can simultaneously bring about an increase in one enzymatic activity while blocking the increase of another. Furthermore, there is evidence suggesting that the expression of proteasomal peptidase activities requires the interaction of proteasomal subunits (Orlowski, 1990). This raises additional questions with regard to the effects of both age and food restriction on the structure of the proteasome as well as the regulation of its function.

The data presented here represent the first report that age, as well as food restriction, may have effects on the proteasome. These observations have significance because of the growing appreciation of the importance of the proteasome as a major non-lysosomal protein degradation pathway. In addition to its role in degradation of post-translationally modified proteins, which includes oxidatively damaged proteins, the proteasome has been implicated in antigen presentation (Goldberg and Rock, 1992), regulation of the cell cycle (Rechsteiner, 1991), activation of transcription factors (Palombella et al., 1994), and tumor development (Rechsteiner, 1991). Ideally, future studies should focus on the direct measurement of the proteolytic activity of the proteasome. However, this will likely depend on the development of an approach for measuring proteasomal activity in a crude, unpurified preparation since proteasomal activity in a crude, unpurified preparation is associated with both structural and functional alterations of the complex (Ma et al., 1992; Kopp et al., 1993).

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References


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