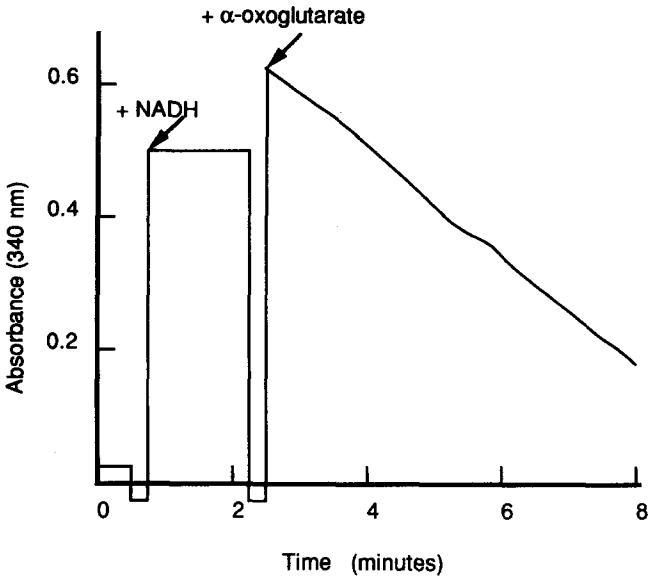

Figure 2 AST assay using rat liver extract diluted 1/10 with 0.9% NaCl

and oxaloacetate in the liver extract itself. When the liver extract is diluted by 1/10, this effect disappears (Figure 2).

Students are asked to explain the activity observed with undiluted extract prior to addition of α-oxoglutarate, and to design experiments to test their hypothesis. Students will find that reaction mixtures containing only buffer, liver extract and NADH (ie no exogenous α-oxoglutarate, aspartate or MDH) also give rise to NADH oxidation, indicating that the undiluted tissue extract contains sufficient endogenous substrate to maintain weak activity.

(d) Varying the assay conditions When students have devised a coupled assay procedure that works, they can investigate the effect of altering some of the variables in the system:
(i) measure AST activity in the presence of 5 mM, 25 mM and 50 mM aspartate
(ii) replace the buffer selected with each of the other three buffers provided and examine the effect of buffer pH on the measured AST activity
(iii) examine the effect of temperature on the measured AST activity by preincubation of the assay mixture at 25°C, 30°C and 37°C.

Questions
Each student is required to answer the following questions in their laboratory report
(1) Describe the assay procedure you devised for assaying AST activity, giving details of what you put in the assay mixture. Include the initial concentration of each substrate in the assay mixture.
(2) Work out how many μmoles of aspartate, α-oxoglutarate and NADH were present initially in your assay mixture (note that 1 mM = 1 μmole per ml). How long would it take for all the NADH in your assay system to be used up if the added tissue extract contained 0.5 IU of AST activity?
(3) Using your absorbance versus time plots, calculate the observed AST activity in: (*a*) IU per ml of rat liver extract; (*b*) IU per g of rat liver tissue. Only NADH absorbs light at 340 nm with a molar extinction coefficient of 6220 M^{-1} cm^{-1}.
(4) Why is it important to define the reaction conditions used to measure the activity of an enzyme? Briefly state the effect of buffer pH and reaction temperature on the measured activity of AST. Explain how pH could influence the enzymes involved in the assay system.

Conclusion
Students benefit from discussing an experimental problem amongst themselves, identifying problems and sharing ideas. At the completion of the class, students have developed an understanding the factors influencing the activity of an enzyme. This approach is more challenging to students than following a written protocol without any critical evaluation of the procedure, and many students expressed satisfaction at having devised something of their own which actually worked. The exercise can be completed within three hours, and requires only basic chemicals and standard laboratory equipment.

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A Rapid and Inexpensive Procedure for the Determination of Proteolytic Activity

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Introduction
Work on enzymes involves, in most cases, partial purification and determination of activity. These are important for basic and applied research as well as for industrial production and teaching purposes.

Proteases or proteinases are important enzymes from an economic viewpoint. They are used as coagulating agents in cheese manufacture, in the detergent industry, in the tenderizing of meat, in the chill-proofing of beer, the production of protein hydrolyzates, the modification of gluten, and the prevention of gelatinization of fish solubles.[1] Thus, determination of proteolytic activity is very important on the industrial scale and it is important for students to learn about it.

Proteolytic enzymes are ubiquitous in biological tissues, fluids, and bacteria, but the ease of their isolation varies considerably: bacteria are an appropriate source of proteases in the laboratory. *Bacillus subtilis* is easily maintained and grown in different media, eg SP[2] and LB[3], and strains are available that produce plentiful exoproteases[4,5] which are used in industrial and commercial processes.

Several methods are used to determine proteolytic activity, eg hydrolysis of casein,[6] hemoglobin[7] or azocasein.[8] The casein hydrolysis method is not expensive, but casein is difficult to dissolve and the pH must be controlled, because the substrate precipitates at pH 4. The hemoglobin hydrolysis method is relatively expensive, and the substrate must be denatured and standard curves prepared. The azocasein hydrolysis method avoids this but again is relatively expensive. Our proposal here is to use skim milk as a substrate. The method is inexpensive, rapid, involves little equipment, and can be used in teaching courses as well as in industrial training courses. Essentially all that is required is skim milk and a water bath!

In order to validate the method it is compared with azocasein hydrolysis. Both are followed in a purification procedure that involves salting-out, depigmentation, gel-permeation, and ion-exchange chromatography. All of these procedures are typically included in biochemistry laboratory courses.

Experimental procedure

Materials The source of the proteolytic enzymes we used was a culture of *Bacillus subtilis* used in a national industry. The broth is made from industrial waste products. DEAE-Cellulose A-50 and Sephadex G-25 (PD-10) were from Pharmacia (Uppsala, Sweden) or a column of Sephadex G-25 with the following dimensions: 9.1 ml bed volume and 5 cm bed height was used. The Bradford reagent was from Bio-Rad GmbH (München, Germany). Skim milk was purchased from a local market.

Purification The enzyme is obtained from a fermented *Bacillus subtilis* broth as follows:

(i) Salt fractionation. The broth is centrifuged at 5000 × *g* for 10 min at 4°C and 2000 ml of supernatant is precipitated using ammonium sulfate up to 30% saturation, kept at 5°C for 16 hours and centrifuged at 2500 × *g* for 15 min at 4°C. 500 ml of the supernatant (Fraction A) is precipitated using ammonium sulfate up to 60% saturation. The pellet is resuspended in water to final concentration 0.2 M in ammonium sulfate. This solution is called Fraction B.

(ii) Deodorization and depigmentation, using our own modification of Fellig's method.[9] After salt fractionation, Fraction B is further deodorized and depigmented. It is added to 0.5 M barium acetate solution pH 7.0 (1 part of Fraction B: 2 parts of barium acetate solution) and kept at 5°C for 1 hour. It is then centrifuged at 5500 × *g* during 20 min, at 4°C. The supernatant is called Fraction C.

(iii) Desalting by gel-permeation. Fraction C is desalted by gel-permeation using Sephadex G-25 with 0.1% calcium acetate, pH 6.4, as the elution solution. 2.5 ml of Fraction C is applied to a PD-10 column and 3.5 ml of eluant is obtained.

(iv) Ion-exchange chromatography.[10] DEAE-cellulose is added at the rate of 1 g for each gram of enzyme extract used. 3.5 ml of desalted fraction are stirred batchwise for 30 min at 5°C and then filtered under vacuum. The gel is washed with 0.1% acetate solution at pH 6.4. The washings and passthrough are pooled and this is called Fraction D.

Proteolytic activity

Milk coagulation method The activity of the enzyme preparation is determined by our own modification of the Guntelberg method.[11] Two ml of buffer (1 M sodium acetate, pH 5.0, containing 40 mM $CaCl_2$) is added to 3 ml of skim milk. This mixture is kept at 30°C in a water bath for 10 min. One ml of an appropriate dilution of enzyme preparation is added to a glass tube with a screw cap, incubated in a water bath at 30°C, and spun horizontally with the fingers.

In order to help the visualization of the coagulated milk we use a light source. With a light passing through the tube, one determines the number of seconds needed for the production of coagulated milk particles the size of the head of a pin on the glass surface. An adequate period for accuracy is between one and two minutes.

The Enzyme Unit (EU) is defined as the amount of enzyme needed to form the first detectable coagulated milk in one minute under the experimental conditions chosen, ie EU = 1/t (min).

Andrews and Asenjo method (azocasein) Proteolytic activity is also determined[8] using azocasein as substrate and measuring the amount of colour released by proteolysis. The Enzyme Unit (EU) is defined as the amount of enzyme needed to produce an increase of one absorbance unit per second at 337 nm under the experimental conditions chosen.

Protein concentration is measured by the Bradford method.[12]

Results and Discussion

The raw material (fermented broth) used in this purification scheme was obtained from a national industry. It is made with industrial wastes which makes it highly coloured.

The salt fractionation increases the specific activity from 0.18 in the crude material to 66.80 in Fraction B (Table 1), and 67.6% activity is recovered.

Fraction B is an undesirable brown colour and so deodorization and depigmentation are required. Fraction B is added to barium acetate solution which permits the formation of the barium sulfate depigmentation reagent *in situ*. This precipitates and removes pigments and proteins, thus purifying the preparation. The protein content is decreased from 190 mg to 61.3 mg, but the specific proteolytic activity increases 2.6-fold from 66.8 (Fraction B) to 105.3 (Fraction C). In addition, 56.3% of the activity is recovered.

Purification using ion-exchange chromatography increase the specific proteolytic activity to 265.8 with a recovery of 37.9% (Table 1).

The skim milk method for determination of the proteolytic activity was compared with the Andrews and Asenjo method[8] (Table 2). The results showed similar recovery values (data not shown). The ratios of specific activity using the milk method and azocasein hydrolysis (CMM/AAM) in each fraction were estimated. The ratios were similar in all fractions (Table 2).

This purification scheme can be performed in three days.

Table 1 Purification of proteases. Specific Activity is given as EU mg prot^{-1}

Fraction	*Protein (mg/ml)*	*Volume (ml)*	*EU total*	*EU/ml*	*Sp Activity*	*Recovery %*
Crude	210.0	2000	75050	37.5	0.18	
A	0.6	500	15000	30.0	50.00	79.9
B	5.0	38	12692	334.0	66.80	67.6
C	1.1	55.7	10557	189.5	172.27	56.3
D	0.3	4.0	319	79.7	265.80	37.9

Table 2 Comparison of specific activity measured by the milk method (CMM) and the Andrews and Asenjo method (AAM). Specific activities are reported as EU mg prot^{-1}

Fraction	*CMM*	*AAM*	*CMM/AAM*
Crude	0.18	0.28	0.64
A	50.00	73.70	0.68
B	66.80	98.50	0.68
C	172.27	256.96	0.67
D	265.80	399.57	0.67

Conclusions

The determination of proteolytic activity proposed is fast, easy, and inexpensive. Comparing specific activity between the methods (CMM and AAM) in each fraction, we conclude that the method is validated and may be used for teaching and industrial training courses.

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Videotape Reviews

From Chance to Choice

A 23 minute programme produced by J Chomet and E Anionwu, Institute of Child Health, University of London, 30 Guildford Street, London WC1N 1EH, UK. With accompanying booklet. 1993. Format reviewed: VHS/PAL

The tape is aimed to inform and support a multi-ethnic approach to community genetics and emphasises the role of the primary health care team. Although it deals with cystic fibrosis, thalassaemia, sickle cell disease, and Tay-Sachs disease, almost no clinical or biochemical details of these are given. Similarly, although the various methods of screening (*a*) the population at large, and (*b*) fetuses possibly at risk: blood electrophoresis for haemoglobinopathies and an enzyme test for Tay-Sachs (ie hexosaminidase A), chorionic villus sampling, amniocentesis, ultrasound, the main emphasis is on how the population at large or at risk may be screened, how clinical genetics should be explained to the patients/clients ("so that they can choose from a range of reproductive options"), and on the important role of the primary health care team. The tape is basically aimed at the members of this team who for the most part do not need medical or biochemical details. The tape would be suitable as background for medical students, and might also be of interest in high schools and medical schools when questions of the ethics connected with medical genetic screening are discussed.

Controlling Your Risks: HIV in the Research Laboratory

Produced by Schumann Productions for the Howard Hughes Medical Institute Office of Laboratory Safety. 28 minute tape available on VHS format. Gratis from the Howard Hughes Medical Institute, 4000 Jones Bridge Road, Chevy Chase, MD 20815–6789, USA

In my position as Laboratory Safety Officer in a Medical School Department, with both clinical and research laboratories, I have witnessed the multiplication of rules and regulations dealing with safety and the protection of laboratory workers from hazards. These include the use of potentially toxic solvents, such as benzene, carcinogenic chemicals, such as formaldehyde, radioactive reagents and tracers, flammables, such as ether, etc. In the past decade the list has expanded to embrace biological dangers lurking in samples of urine, blood and other tissues derived from experimental animals and human subjects and patients. Of paramount importance is protection against accidental contamination by virulent viruses. These include hepatitis and HIV. How can laboratory personnel be instructed in the application of precautions to minimize and hopefully prevent infection from these viruses?

Periodic in-service training sessions provide an opportunity for gathering laboratory personnel in groups to learn about safety rules and become aware of the dangers. Such instructional meetings are often reinforced by other instructional media, such as pamphlets and videotapes, that can be reviewed at leisure. This videotape is representative of such media.

While the videotape is an excellent source of information on AIDS, it cannot serve as the sole laboratory training tool. The tape illustrates the proper use of Personal Protective Equipment, such as face shields, latex gloves, impervious gowns, and illustrates some of the engineering controls (eg fume hoods) dedicated to protection against infection, but it fails to tell the viewer when and how to implement these controls. The tape mentions BSL II and BSL III, but does not define these terms. Because the tape will be used with newcomers to the laboratory, all terms must be defined and illustrated. For example, even obvious precautions, such as no eating, drinking or mouth pipetting, may not be obvious to new personnel from other laboratory environments. The Universal Precautions must be clearly explained.

The 28 minute tape is too long to maintain interest. It is best used in conjunction with in-service presentations by laboratory safety officers, who would be available to elaborate on topics not given in sufficient detail in the tape. One attractive feature of the tape is the price. It is free.

Albert Geha