New Opportunities for Seafood Processing Waste

Appendix 5: Patagonian Toothfish (Dissostchus eleginoides) digestive enzyme purification and activity characterisation

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Project: Toothfish digestive enzyme purification and activity characterisation

Background

The Patagonian Toothfish fishery in the Southern ocean produces up to 600 tonnes of fish waste each year. This waste presents a unique utilisation opportunity through the extraction of novel functional compounds from the viscera. There is significant evidence to suggest that the digestive enzymes from cold water fish species have lower optimal temperatures for activity than those of warm water species (Carginale, Trinchella, Capasso, Scudiero, & Parisi, 2004; Feller & Gerday, 1997; Genicot, Rentier-Delrue, Edwards, VanBeeumen, & Gerday, 1996; Somero, 1978). Psychrophilic enzymes have a number of potential uses in the food and other industries.

This project was commenced after the request for an enzyme sample from Proctor & Gamble. The hypothesised lower optimal temperature of the enzymes from Patagonian Toothfish may be highly effective in cold water laundry detergents. This project will attempt to extract and determine proteinase and lipase activity of Patagonian Toothfish digestive enzymes.

Methods

<u>Enzyme extraction</u>

This method has been adapted from several sources in an attempt to extract the majority of digestive enzymes (Ketnawa, Benjakul, Martínez-Alvarez, & Rawdkuen, 2017; Pavlisko, Rial, De Vecchi, & Coppes, 1997; Zhou, Fu, Zhang, Su, & Cao, 2007).

1. Prepare 200 mM sodium phosphate buffer, 4 mM EDTA (pH 7.5 at 4°C) (4x concentrate)
2. Gently remove the stomachs from the other organs
3. Homogenise stomachs with two volumes of 50 mM sodium phosphate buffer, 1 mM EDTA (pH 7.5) for 1 min in a blender
4. Centrifuge 4 000 *g* for 20 min, 4°C, discard pellet
5. Add ammonium sulphate to final concentration of 30%
6. Centrifuge 4 000 *g* for 20 min, 4°C, discard pellet
7. Add ammonium sulphate to final concentration of 70%
8. Centrifuge 15 000 *g* for 20 min, 4°C, discard supernatant, retain pellet
9. Freeze pellet at -80˚C prior to freeze drying
    a. Freeze dry conditions: -30˚C, 0.37 mBar

Note:

- Before use dissolve freeze dried enzyme powder in suitable buffer.
- Before use activate by adding 0.1M HCl to achieve pH 2-3 (trypsin only)
- Dialysis may be required after precipitation to remove residual ammonium sulfate

Protein concentration assay

Protein concentration was determined using the microtitre plate method for the Bio-Rad Protein Assay Kit II (#5000002).

Folin & Ciocalteus Phenol reagent proteinase activity assay

The colourimetric proteinase assay utilising Folin's reagent is a commonly used method for quantitatively determining proteinase activity. The method described by Cupp-Enyard (2008) from Sigma-Aldrich was used with some modification. This method has been used successfully in a previous project and successfully adapted for microplate use. One unit of protease activity was defined as the production of 1 µmole of product per minute per millilitre at pH 7.5 and the stated temperature.

1. Prepare a 50 mM sodium phosphate buffer, pH 7.5 at 37˚C
2. Prepare a 0.65% casein solution using the previously prepared phosphate buffer. Heat solution to 85˚C with stirring to solubilise the casein
3. Prepare a 110 mM trichloroacetic acid solution
4. Prepare a 0.5 M Folin & Ciocalteus Phenol reagent on the day of use
5. Prepare a 500 mM sodium carbonate solution
6. Prepare a 10 mM sodium acetate buffer with 5 mM calcium (calcium acetate), pH 7.5 at 37˚C
7. Prepare 1.1 mM L-tyrosine standard stock solution. Gently heat and stir solution to solubilise L-tyrosine
8. Using 1.5 mL Eppendorf tubes, add 500 µL of casein solution and equilibrate to 37˚C in water bath
9. Carefully managing time (30 seconds between each test sample) add 100 µL of enzyme sample dissolved in acetate buffer to the casein solution, vortex, and allow to sit in water bath at 37˚C for exactly 10 minutes
    a. For blanks add 100 µL of acetate buffer instead of enzyme sample
10. At 10 minutes add 500 µL of TCA and vortex to stop the reaction
11. Incubate samples for 30 minutes at 37˚C
12. Prepare L-tyrosine standards:

Concentration (µM/mL)	Blank	0.0693	0.055	0.0275	0.0143	0.0066
Tyrosine solution (µL)	0	63	50	25	13	6

Water (µL)	250	187	200	225	237	244

13. Centrifuge samples at 2000 *g* for 5 minutes at 37˚C
14. Draw 250 µL of cleared solution and dispense into new Eppendorf tubes
15. Add 625 µL of sodium carbonate solution immediately followed by 125 µL of Folin's reagent to all standards, samples and blanks, vortex
16. Incubate at 37˚C for 30 minutes
17. Centrifuge samples at 2000 *g* for 5 minutes at 37˚C
18. Pipette 200 µL of sample into microplate and read absorbance at 660 nm

Lipase activity assay using DTNB

A rapid colorimetric microplate lipase assay was used with some modification (Choi, Hwang, & Kim, 2003). The thioester substrate (2,3-dimercapto-1-propanol tributyrate (DMPTB)) releases free thiol groups when hydrolysed by lipases; these products then reduce 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) releasing the chromophoric 2-nitro-5-thiobenzoate anion, which can then be measured at the wavelength 405 nm. Specific activity represents the production of 1 µmole of product per minute per milligram of protein; this definition was also used for the protease assay.

1. Prepare 0.5M Tris-Cl buffer (pH 7.5 at 37˚C)
2. Prepare 40 mM DTNB in 100% methanol
3. Prepare 50 mM Tris-Cl buffer (pH 7.2 at 20˚C), 6% TritonX-100 and 10 mM DMPTB
4. Prepare a blank solution with no DMPTB, repeat step 3 but replace the DMPTB with 50 mM Tris-Cl buffer(pH 7.2 at 20˚C)
5. Prepare 10 mM Tris-Cl buffer (pH 7.5 at 37˚C) with 10 mM KCl
6. Prepare 0.25 M EDTA solution, solubilising with 1 M NaOH

To perform the assay:

7. Prepare the standard reaction mixture using above solutions: 0.2 mM DMPTB, 0.8 mM DTNB, 1 mM EDTA, 0.17% Triton X-100, 50 mM Tris-Cl buffer, pH 7.5
   Example preparing 3 mL of standard reaction mixture:
   60 µL DMPTB solution
   60 µL DTNB solution
   12 µL 0.25 M EDTA solution
   15 µL 10% Triton X-100
   300 µL Tris-Cl buffer (pH 7.5 at 37˚C)
   2553 µL deionised water
8. Prepare a blank standard reaction mixture by repeating step 7, but replacing DMPTB solution with the prepared blank solution (Step 4)
9. Dissolve enzyme powder in KCl, Tris-Cl buffer solution

10. Load 20 µL of enzyme solution into wells including sample blank wells; add 180 µL of blank standard reaction mixture to all sample blank wells
11. Add 180 µL of standard reaction mixture to all sample wells, preferably simultaneously using a multi-channel pipette, do not re-use pipette tips to avoid bubbles
12. Immediately after pipetting, run microplate reader program:
    Temperature: 37˚C
    Shake: 10 seconds, medium
    Kinetic readout: 30 minutes
        Read: 405 nm
    End kinetic readout
13. Calculate lipase activity from linear section of the readout using a molar absorption coefficient of 13 700 $M^{-1}cm^{-1}$

Results and discussion

Extraction efficiency

The freeze dried protein extract likely contains a significant proportion of ammonium sulfate as no dialysis was performed. In addition precipitated protein collected at the top of the centrifuge tube instead of pelleting at the bottom during centrifugation; this impacted upon how effectively the precipitate could be separated from the supernatant. A final protein concentration of 0.123 mg/mg of extract powder was determined.

Protease activity

Non-specific protease activity was determined for the freeze dried powder at different temperatures and concentrations (Figure 1). Activity was lowest at 20˚C and highest at 50 ˚C. There is a large increase in activity between 40 ˚C and 50 ˚C, suggesting the optimum temperature for enzyme activity is either between these temperatures or above 50 ˚C. Samples at all temperatures showed a non-linear increase in activity as extract concentration increased; samples of 60 mg/mL at 20 ˚C showed an unexpected decrease in activity which may be an experimental error.

Protease activity from fish digestive tracts is highly variable between species. Furné et al. (2005) reported Adriatic sturgeon and Rainbow trout exhibited digestive tract protease activities of 27.39 and 57.07 U/mg of protein, respectively, when analysed at 37˚C. These values were significantly higher than those published by Hidalgo, Urea, and Sanz (1999), who reported values digestive tract protease activities between 0.46 and 3.44 U/mg for a range of fish including eel, seabream and trout. The values published for other species are

higher than those calculated in the current project. This may not necessarily be due to the differences between species. Samples for the current project were stored for an extended time (~two years) at -20˚C, during which some protease activity may be lost. The extraction method used was also different, ammonium sulfate was utilised but samples were not dialysed to remove it, residual ammonium sulfate may impact on protease activity. The feeding status of the fish samples used is not known; it may have been an extended period since last feeding resulting in lowered protease concentrations (Hidalgo et al., 1999; Xiong, Xie, Zhang, & Liu, 2011).

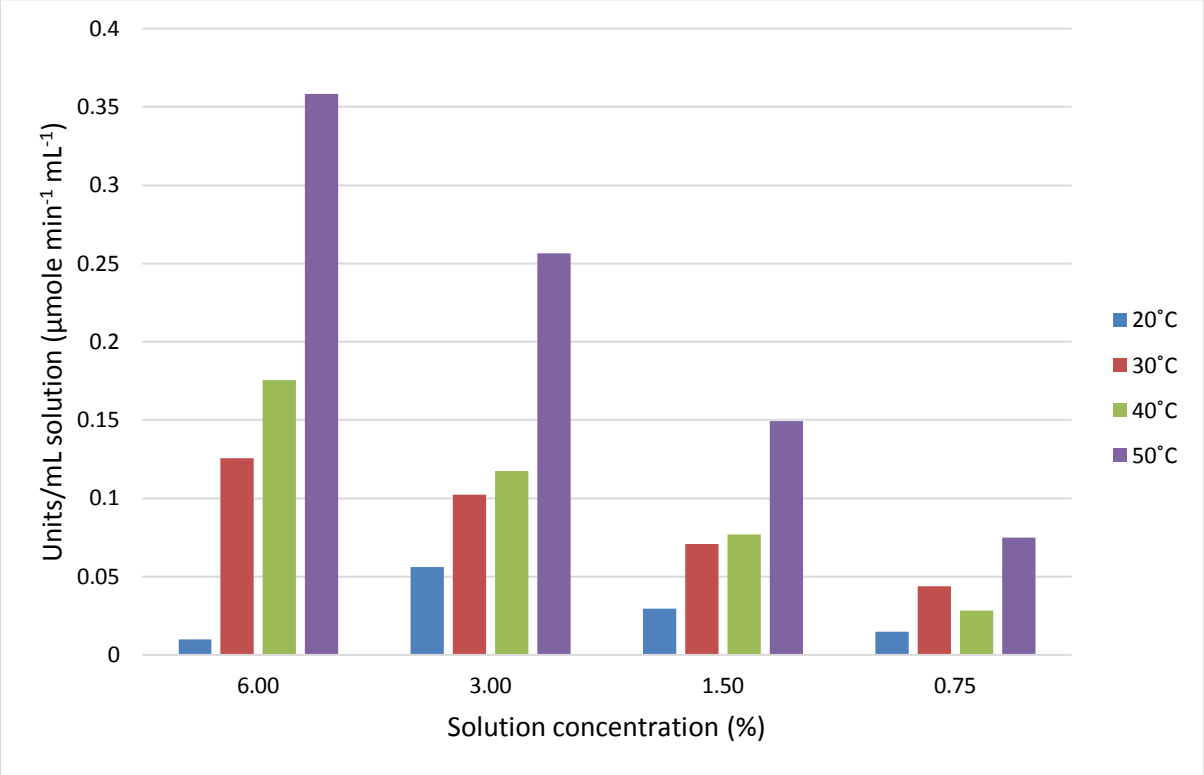

Figure 1. Protease activity of stomach extract at different temperatures and concentrations

As the protein concentration of the solution increased protease efficiency decreased (Figure 2). This likely reflects a change of the reaction's limiting factor from enzyme concentration to substrate concentration. 1.84 mg/mL of protein in solution appears to reflect a point close to maximum protease concentration, beyond this casein becomes the limiting factor.

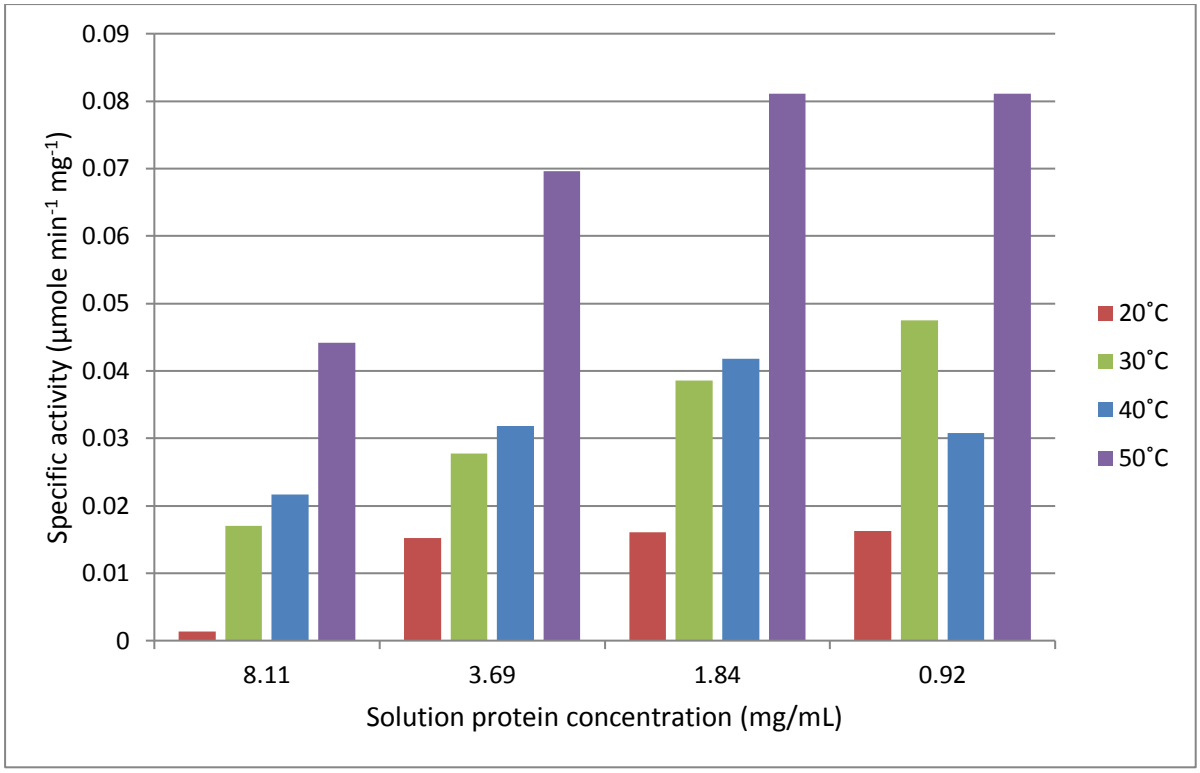

Figure 2. Protease specific activity by solution protein concentration

Lipase activity

Lipase activity was determined at several temperatures and concentrations (Figure 3). The lowest was observed at 25˚C with the most activity observed at 45˚C. Activity decreased substantially at 50˚C suggesting the optimal temperature is very close to 45˚C.

Lipase activity varies between species and also with the component of the digestive tract analysed. de la Parra, Rosas, Lazo, and Viana (2007) analysed Pacific Bluefin tuna (*Thunnus orientalis*) digestive enzymes and reported lipase specific activities of 3.9, 2.1, 1.5 U/mg in the proximal, middle and distal regions of the intestines, respectively. Much lower lipase activity was observed in the crude extract of Chinnok salmon (*Oncorhynchus tshawytscha*) pyloric ceca, by Kurtovic, Marshall, Zhao, and Simpson (2010). The crude extract produced a lipase specific activity of 0.36 U/mg. Kurtovic, Marshall, Miller, and Zhao (2011) also reported a lipase specific activity of 0.11 U/mg for pyloric ceca extract from New Zealand hoki (*Macruronus novaezealandiae*). These values while lower than those reported for Pacific Bluefin tuna are still greater 40 times higher than those reported here (Figure 3). Reasons for this may be the same as those described for the low protease activity levels. It should also be noted that the substrate, DMPTB, can be hydrolysed by non-specific esterases; as such the reported specific activities should be considered as a contribution of lipases and esterases.

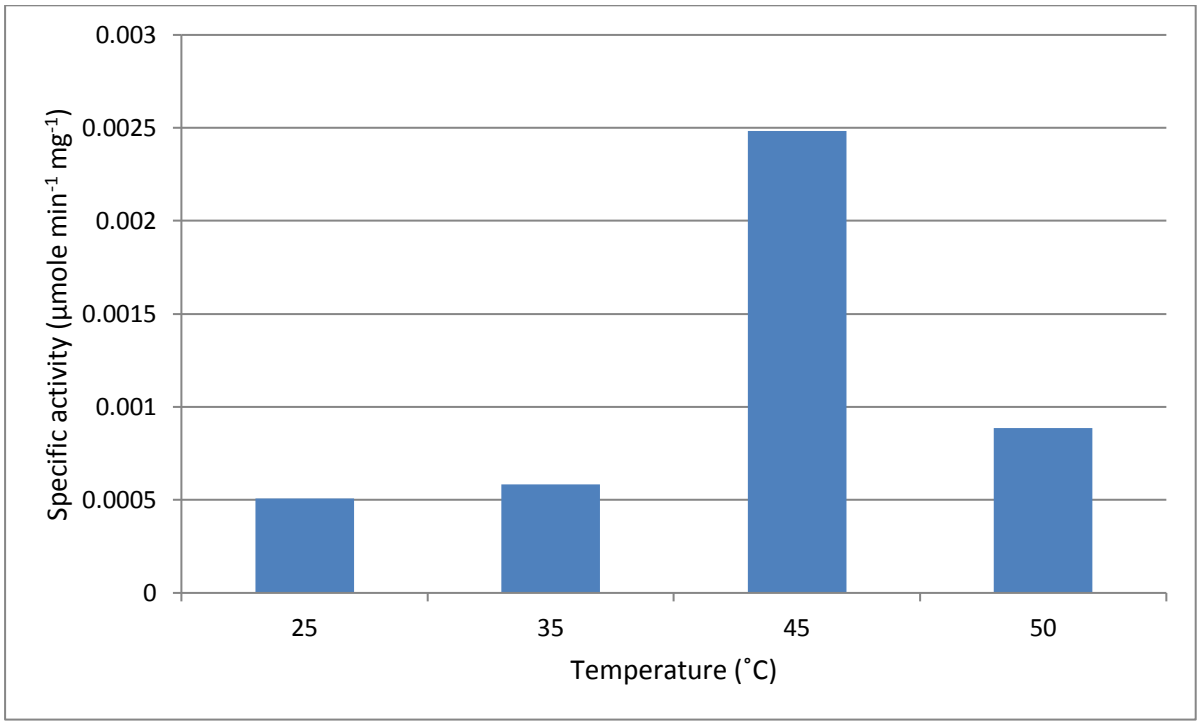

Figure 3. Lipase specific activity at different incubation temperatures

In contrast to the data presented in Figure 2, lipase efficiency did not decrease substantially with increasing solution concentration (Figure 4). Slight decreases in specific activity were observed beyond 20 minutes of incubation for the 6% solution samples. Comparing these results highlights the benefits of continuous initial rate assays (lipase assay) over one-point discontinuous assays (protease assay); enzyme activity calculated by the latter may be inaccurate due to the inclusion of data outside the period of steady-state kinetics, when the rate of reaction begins to slow. The unusually large specific activity value for the 7.371 mg/mL sample at 45˚C (Figure 4) is unexpected and is thought to be the result of experimental error.

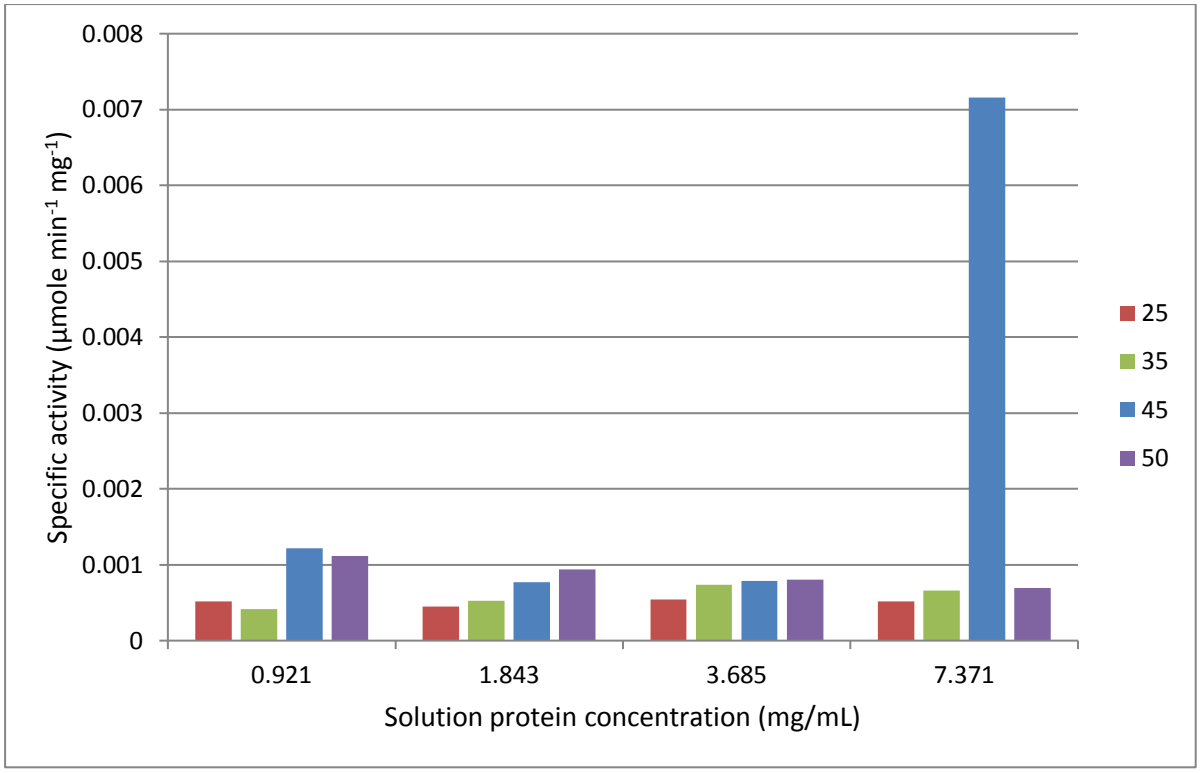

Figure 4. Lipase specific activity by solution concentration

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